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DOCTOR OF PHILOSOPHY

OF

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STUDIES ON YEAST SOLUBLE-RIBONUCLEIC ACID

ABSTRACT

Part I of the thesis describes attempts to control the extent of hydrolysis of yeast s-RNA with pancreatic ribonuclease. The extent of hydrolysis was shown to be a function of temperature, concentration of magnesium ions and the s-RNA : ribonuclease ratio (w/w). Digestion of s-RNA with pancreatic ribonuclease (ratio, 2000:1, w/w) in the presence of 0.2 M magnesium and at 0°, followed by chromatographic analysis, indicated that about 75% of the ultraviolet absorbing material was of medium to high molecular weight. Analysis of the nucleotide composition of the fraction containing the intermediate size oligonucleotides showed that fraction to be enriched in the odd bases, Y, T, 1-methylG, N,N-dimethylG and several other unidentified nucleotides. This result was incompatible with the 'hairpin' models for s-RNA, proposed by McCully and Cantoni (1962). The presence of spermine in the digestion mixture clearly masked the catalytic action of pancreatic ribonuclease, albeit to a lesser extent than magnesium ions, contrary to a report by Thomas and Hübst (1963) in their study of the affect of spermine on the catalytic activity of E. coli ribonuclease toward E. coli ribosomal RNA.

Part II of the thesis is concerned with the fractionation of mixed s-RNA from yeast.

Part II-A describes attempts to retain $^{14}$C-cysteinyl-s-RNA on an organomercurial-cellulose (Material I) derived from aminomethyl-cellulose. It appeared that the salt concentration required to overcome the ion-exchange properties of Material I precluded any interaction between the mercury of Material I and the sulfhydryl group of $^{14}$C-cysteinyl-s-RNA. This difficulty was overcome by preparing an organomercurial-cellulose devoid of ion-exchange properties (Material II). Preliminary studies showed that Material II can retain the radioactivity associated with $^{14}$C-cysteinyl-s-RNA. It is suggested that Material II might fractionate nucleic acids according to their base composition.
Part II-B describes some of the attempts to fractionate yeast s-RNA by column partition chromatography. In general, resolution of acceptor activities of s-RNA, as well as the recovery of biological activity, was poor.

Part II-C describes some of the author's contributions to the studies carried out in this laboratory on the fractionation of yeast s-RNA on another new chromatographic material, benzoylated-DEAE-cellulose (Material III). Certain variables (such as, magnesium ion concentration and pH), which can be manipulated during rechromatography of s-RNA on Material III, are discussed in light of the chromatographic behaviour of glycine and other acceptor RNA's. The forces responsible for this fractionation are also discussed.

Part II-D describes a chemical procedure for the isolation of glycine s-RNA which, when combined with the chromatographic procedures described in Part II-C, afforded glycine-specific s-RNA; in high purity. Observations on the effect of magnesium ion during enzymatic synthesis of glycyl-s-RNA are discussed. The advantages and possible consequences of using purified aminoacyl-s-RNA synthetase enzymes for the preparation of the aminoacyl-s-RNA's are also discussed.

Part III describes a simple procedure for the preparation and partial purification of quantities of yeast aminoacyl-s-RNA synthetase enzymes, suitable for charging gram quantities of s-RNA with a given amino acid. Two procedures are described for lysing yeast cells. When yeast cells were lysed with toluene at 37°, most of the synthetase enzymes were preserved by controlling the pH of the toluene-yeast mixture. When yeast cells were lysed in toluene containing excess dry ice, followed by an incubation period at 3-5°, all of the aminoacyl-s-RNA synthetase enzymes were detected in the cell-free preparation. Partial purification of the aminoacyl-s-RNA synthetases was achieved by chromatographing the cell-free preparation on hydroxyapatite at 3-5° and in the presence of 40% glycerol (v/v).
GRADUATE STUDIES

Field of Study: Biochemistry

Structure and Function of Proteins

Biochemistry of Nucleic Acids

Biochemistry of Amino Acids and Proteins

Biochemistry of Carbohydrates

Biochemistry of Lipids

Biochemistry of Steroids and Hormones

THESES AND PUBLICATIONS


STUDIES ON YEAST SOLUBLE-RIBONUCLEIC ACID

by

STEWART MILLWARD

B.Sc. (Zoology/Biochemistry), University of British Columbia, 1960
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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in the Department
of
Biochemistry

We accept this thesis as conforming to the
required standard

THE UNIVERSITY OF BRITISH COLUMBIA

June, 1967
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Department of BIOCHEMISTRY

The University of British Columbia
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Date June 28th, 1967
Abstract

Part I of this thesis describes the digestion of bakers' yeast s-RNA at 0° and at room temperature in the presence of various concentrations of magnesium ions and at several different s-RNA : enzyme ratios (w/w). Digestion of s-RNA with pancreatic ribonuclease (ratio, 2000:1, (w/w) ) in the presence of 0.2 M magnesium and at 0°, followed by chromatographic analysis, indicated that about 75% of the ultraviolet absorbing material was of medium to high molecular weight. Analysis of the nucleotide composition of the fraction containing the intermediate size oligonucleotides showed that fraction to be enriched in the odd bases ψ, T, 1-methylG, N, N-dimethylG and several other unidentified nucleotides. This result was incompatible with the 'hairpin' models for s-RNA, proposed by McCully and Cantoni (1962). The presence of spermine in the digestion mixture clearly masked the catalytic action of pancreatic ribonuclease, albeit to a lesser extent than magnesium ions, contrary to a report by Thomas and Hubst (1963) in their study of the affect of spermine on the catalytic activity of E. coli ribonuclease toward E. coli ribosomal RNA.

The studies described in Part II of this thesis are concerned with the fractionation of mixed s-RNA from yeast.
Part II-A describes a new chromatographic material consisting of a bifunctional mercuri-dioxane derivative attached through a thiol group to the cellulose matrix. A previous report (Eldjarn and Jellum, 1963) had shown that HS-proteins could be fractionated on an organomercurial-cellulose (Material I) derived from aminoethyl-cellulose. The studies reported here suggest that $^{14}$C-cysteiny1-s-RNA cannot be retained on Material I because the salt concentration required to overcome its ion-exchange properties preclude any interaction between the mercury of Material I and the sulfhydryl group of $^{14}$C-cysteiny1-s-RNA. The present study describes how this disadvantage was overcome by preparing an organomercurial-cellulose devoid of ion-exchange properties (Material II). Preliminary studies showed that Material II can retain the radioactivity associated with $^{14}$C-cysteiny1-s-RNA. These studies suggested that Material II might fractionate nucleic acids according to their base composition.

Part II-B describes some of the attempts to fractionate yeast s-RNA by column partition chromatography. In general, resolution of acceptor activities of s-RNA, as well as the recovery of biological activity, was poor.

Part II-C describes some of the author's contributions to the studies carried out in this laboratory on the fractionation of yeast s-RNA on another new chromatographic material, benzoylated-DEAE-cellulose
(Material III). Certain variables (such as, magnesium ion concentration and pH), which can be manipulated during rechromatography of s-RNA on Material III, are discussed in light of the chromatographic behaviour of glycine and other acceptor RNA's. The forces responsible for this fractionation are also discussed.

Part II-D describes a chemical procedure for the isolation of glycine s-RNA which, when combined with the chromatographic procedures described in Part II-C, afforded glycine-specific s-RNA, in high purity. Observations on the effect of magnesium ion during enzymatic synthesis of glycyl-s-RNA are discussed. The advantages and possible consequences of using purified aminoacyl-s-RNA synthetase enzymes for the preparation of the aminoacyl-s-RNA's are also discussed.

The need to prepare large quantities of aminoacyl-s-RNA synthetases derives from their indispensable role in the development of chemical methods for purifying amino acid-specific s-RNA's (see Part II-D).

Grinding of yeast cells with glass beads, which is the usual method employed for disrupting yeast cells, was found to be inadequate. Lysis of yeast cells with toluene at 37° was found to be a partial solution to this problem. Although previous studies showed that most of the synthetase enzymes were destroyed by this treatment (von Tigerstrom
and Tener, 1967), investigations described in Part III of this thesis, show that most of the synthetase activities can be preserved by controlling the pH of the toluene-yeast mixture. However, there were still several synthetase activities missing in the cell-free extracts prepared by the warm toluene method.

Preparation of large quantities of yeast cell-free extract containing all of the aminoacyl-s-RNA synthetases was finally achieved by treatment of the yeast cells with toluene containing excess dry ice followed by an incubation period at 3-5°C.

Partial purification of the aminoacyl-s-RNA synthetases was achieved by chromatographing the cell-free preparation on hydroxyapatite. The presence of 40% glycerol in the eluting buffers was found to be essential for the preservation of most of the synthetase activities. When the cell-free preparations were chromatographed on hydroxyapatite, most of the protein, but only a few of the synthetases were eluted by a linear gradient of phosphate buffer to 0.20 M. Most of the synthetases were eluted only when the column was washed with ammonium sulfate. The significance of this observation is discussed.
INDEX

PART I. DEGRADATION OF YEAST SOLUBLE-RIBONUCLEIC ACID WITH PANCREATIC RIBONUCLEASE

Table of Contents 2
List of Figures 4
List of Tables 6
Introduction 7
Experimental 11
Discussion 36
Bibliography 52

PART II. FRACTIONATION OF YEAST s-RNA

Table of Contents 56
List of Figures 61
List of Charts 63
Introduction 64
Experimental 76

A. Attempts to Isolate Cysteiny1-s-RNA by Chromatography on Mercuri-Cellulose 78
B. Partition Chromatography of s-RNA on Biogel 97
C. Fractionation of s-RNA on Benzoylated-DEAE-Cellulose 103
D. A Combined Chemical and Chromatographic Method for the Isolation of Amino Acid-Specific s-RNA's

Discussion

Bibliography

PART III. PREPARATION AND PARTIAL PURIFICATION OF AMINOACYL-s-RNA SYNTHETASES FROM BAKERS' YEAST

Table of Contents

List of Figures

List of Tables and Charts

Introduction

Experimental

Discussion

Bibliography
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PART I

DEGRADATION OF YEAST SOLUBLE-RIBONUCLEIC ACID

WITH PANCREATIC RIBONUCLEASE
# TABLE OF CONTENTS

| LIST OF FIGURES                                  | 4 |
| LIST OF TABLES                                   | 6 |
| INTRODUCTION                                    | 7 |
| EXPERIMENTAL                                    | 11 |
| Abbreviations Used                              | 11 |
| Materials                                       | 11 |
| Methods                                         | 12 |
| 1. Preparation of the DEAE-Cellulose Column     | 12 |
| 2. Purification of Crude s-RNA                  | 13 |
| 3. Digestion of Yeast s-RNA by Ribonuclease at Various Concentrations of Magnesium Ion | 15 |
| 4. Quantitative Precipitation of s-RNA          | 15 |
| 5. Release of Acid-Soluble Nucleotides During Ribonuclease Digestion at Room Temperature | 17 |
| 6. Effect of Pronase on the Digestion of s-RNA by Pancreatic Ribonuclease | 18 |
| 7. Removal of Pancreatic Ribonuclease with CM-Cellulose | 20 |
| A. Behaviour of Pancreatic Ribonuclease on CM-Cellulose | 20 |
| B. Removal of Ribonuclease from a Digestion Mixture | 20 |
8. Release of Acid-Soluble Nucleotides During Ribonuclease Digestion at $0^\circ$ in the Presence of Magnesium Ions .......................... 21

9. Release of Acid-Soluble Nucleotides During Ribonuclease Digestion at Room Temperature in the Presence of Spermine .................. 22

10. Controlled, Large Scale Digestion of s-RNA with Ribonuclease at Room Temperature ................................................................. 22

11. Large Scale Digestion of s-RNA with Ribonuclease at $0^\circ$ in the Presence of 0.2 M MgCl$_2$ .......................................................... 26

12. Base Analysis of Fraction A ................................................................. 28


   A. G-100 Sephadex Chromatography .................................................. 31

   B. Digestion with Pancreatic Ribonuclease ....................................... 34

DISCUSSION ......................................................................................... 36

BIBLIOGRAPHY .................................................................................. 52
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>19</td>
</tr>
<tr>
<td>4</td>
<td>23</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
</tr>
<tr>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td>7</td>
<td>27</td>
</tr>
</tbody>
</table>

1. **DEAE-cellulose chromatography of s-RNA from bakers' yeast**
2. Increase in optical density at 260 μm during digestion of s-RNA with ribonuclease at room temperature in the presence and absence of Mg. s-RNA : ribonuclease ratio, 2.3:1 (w/w)
3. Release of acid-soluble ultraviolet absorbing material during digestion of s-RNA with pancreatic ribonuclease at room temperature in the presence and absence of Mg. s-RNA : ribonuclease ratio, 16:1 (w/w)
4. Release of acid-soluble ultraviolet absorbing material during digestion of s-RNA with pancreatic ribonuclease at 0° in the presence and absence of Mg. s-RNA : ribonuclease ratio, 50:1 (w/w)
5. Release of acid-soluble ultraviolet absorbing material during digestion of s-RNA with ribonuclease at 0° in the presence and absence of Mg. s-RNA : ribonuclease ratio, 500:1 (w/w)
6. Increase in acid-soluble ultraviolet absorbing material during digestion of s-RNA with pancreatic ribonuclease in the presence and absence of spermine. s-RNA : ribonuclease ratio, 500:1 (w/w)
7. **DEAE-cellulose chromatography of s-RNA after digestion with ribonuclease at room temperature in the presence of 0.2 M MgCl₂. s-RNA : ribonuclease ratio, 500:1 (w/w)**
FIGURE

8. DEAE-cellulose chromatography of s-RNA after digestion with ribonuclease at 0° in the presence of 0.2 M MgCl₂. s-RNA: ribonuclease ratio, 2000:1 (w/w) 29

9. Rechromatography of Fraction II (Fig. 8) on DEAE-cellulose 30

10. A. Chromatography of an alkaline digest of Fraction A (Fig. 9) on DEAE-cellulose.

B. Composite diagram of chromatograms obtained after paper chromatography of Fractions A to I in Figure 10-A. 32

11. G-100 Sephadex chromatography of:
   A, mixed s-RNA;
   B, partially purified glycine s-RNA;
   C, rechromatography of peak II from Figure 11-B. 33

12. Digestion of mixed s-RNA (---) and partially purified glycine s-RNA (-----) in the presence and absence of 0.2 M MgCl₂ 35
## LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>Description</th>
<th>PAGE NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Comparison of the Extent of Hydrolysis of s-RNA with Pancreatic Ribonuclease under Various Conditions</td>
<td>42</td>
</tr>
<tr>
<td>II</td>
<td>Base Composition of Fraction A</td>
<td>46</td>
</tr>
</tbody>
</table>
Evidence that the s-RNA fractions obtained from organisms is composed of a population of polynucleotide chains, each specific for a particular amino acid, obtains from a variety of biological experiments (Cohen and Gros, 1960; Berg, 1961; Simpson, 1962) as well as the physical separation of s-RNA preparations with acceptor specificity toward particular amino acids (Tanaka, 1966).

Attempts to elucidate the molecular basis of the unique function and specificity of s-RNA require a knowledge of the complete nucleotide sequence of the specific s-RNA molecules. Until recently, (1965), the only approach to determining nucleotide sequences of large oligoribonucleotides was to split them with enzymes into fragments which are amenable to sequence analysis. Only two endonucleases of well defined specificity are available for this purpose.

One of these, pancreatic ribonuclease, splits polyribonucleotides at the pyrimidine-3' ester linkage giving rise to blocks of purines terminated in a pyrimidine-3' phosphate (uridine- or cytidine-3' phosphate). However, because mixed s-RNA contains 47% uridine plus cytidine (Bell et al., 1964), complete digestion of s-RNA with pancreatic ribonuclease gives a large number of small fragments (Ingram and Pierce, 1962). The work reported in this part of the thesis was carried out in 1963 to determine if the action of pancreatic ribo-
nuclease could be restricted to a small number of specific sites, yielding
large, well-defined oligonucleotide fragments.

Previous work (Penniston and Doty, 1963; Nishimura and Novelli, 1963; Zubay and Marciello, 1963) established that magnesium ions
stabilized the secondary structure of s-RNA. These workers also
showed that magnesium ions inhibit the degradation of s-RNA by a
number of ribonucleases and phosphodiesterases.

Fresco et al., (1960) attempted to describe the structure of s-RNA
in terms of a base sequence model. These authors assumed a random
base sequence and in order to reconcile the relatively high double-
helical content of s-RNA with a random base sequence they proposed a
"looping-out" of non-paired bases between double-stranded regions. A
less detailed model for s-RNA, consisting of a chain folding back upon
itself, was proposed by Brown and Zubay (1960), to explain X-ray
diffraction patterns and the effect of rapid cooling on the sedimentation
characteristics of s-RNA. The latter model was further refined by
McCully and Cantoni (1962) and received the most favour until the
complete sequence of alanine-specific s-RNA was reported by Holley
et al. (1965).

The additional or odd bases were confined, in both models, to the
loop regions. It would follow from the foregoing discussion that
digestion of s-RNA by pancreatic ribonuclease in the presence of a
concentration of magnesium ions sufficient to stabilize the base-paired, double-helical regions should give rise to small fragments enriched in the odd bases and large oligonucleotide fragments, consisting of the double-helical regions, with a reduced content of odd bases. The initial study reported here was designed to test this hypothesis.

In addition to magnesium, the effect of other multivalent cations on nucleic acids has been studied. The aliphatic polyamines have been known for many years (Ackerman, 1952, 1955) as normal biological constituents although their specific function is still unknown. Numerous studies have been published on their binding to nucleic acids (Tabor et al., 1961). Felsenfeld and Huang (1960) showed that there was a strong interaction between single stranded polyuridylic acid and polyamines; spermine having a particularly high affinity. These workers also demonstrated a hypochromic effect when the diamines, 1, 4-diaminobutane, 1, 5-diaminopentane or 1, 10-diaminodecane were added to a solution of polyadenylic and polyuridylic acid. All the diamines were equally effective and only slightly less effective than magnesium. Cantoni (1960) demonstrated that a partial fractionation of s-RNA could be obtained by differential precipitation with spermine under carefully controlled conditions of pH and ionic strength. It was shown by Zillig et al. (1959), that polyamines present in E. coli ribosomes competitively inhibited a latent ribonuclease also present in the ribosomes, although Thomas and Hubst (1963) showed that these polyamines maintained the
integrity of the ribosomes but were unable to protect the RNA from
degradation by the purified ribonuclease.

The study reported here demonstrates that spermine can inhibit
the action of pancreatic ribonuclease on s-RNA.
EXPERIMENTAL

ABBREVIATIONS USED

s-RNA: soluble ribonucleic acid and is equivalent to transfer or adapter ribonucleic acid.

DEAE-cellulose: diethylaminoethyl cellulose.

CM-cellulose: carboxymethyl cellulose.

O. D.: optical density.

The following abbreviations were used for the nucleoside-2'(3') phosphates and their derivatives:

A: adenosine-2'(3') phosphate,
C: cytidine-2'(3') phosphate,
G: guanosine-2'(3') phosphate,
U: uridine-2'(3') phosphate,
Ψ: pseudouridine-2'(3') phosphate,
T: thymine riboside-2'(3') phosphate.

MATERIALS

The s-RNA used throughout this study was prepared from freshly grown bakers' yeast obtained from Standard Brands Limited.

DEAE-cellulose and CM-cellulose were purchased from Brown Co., Berlin, N. H.
Pancreatic ribonuclease was obtained from Worthington Biochemical Corp., Freehold, N. J.

Tris(chloride) buffer was prepared by titrating a solution of Tris(hydroxymethyl)aminomethane to the desired pH with hydrochloric acid.

Pronase (Research Grade) was purchased from the California Corporation for Biochemical Research.

Spermine was purchased from Aldrich Chemical Co. Inc., Milwaukee.

Optical density measurements were made using a Cary Model II spectrophotometer. One optical density (O. D.) unit is defined as that amount of material per ml which in a 1-cm light path, at 260 m\( \mu \) gives a spectrophotometric reading of one.

METHODS

1. Preparation of the DEAE-Cellulose Column

DEAE-cellulose was freed of fines by repeated settling and decantation from a large volume of water. The settled material was made into a thin slurry by the addition of 2 M NaCl and poured into a chromatographic column (90 x 3.5 cm diam.), half filled with 2 M NaCl. Air pressure of about 3 psi was used to pack the column to the desired height of 75 cm. The column was washed with 2 M NaCl until the O. D. at 260 m\( \mu \) was zero, then with distilled water until the eluate was free
from chloride ion (AgNO₃ test) and finally with 1 litre of 0.10 M NaCl, 0.005 M Tris(chloride), pH 7.8.

2. Purification of Crude s-RNA

Crude s-RNA (14.5 g), prepared from freshly grown bakers' yeast by Dr. G. M. Tener according to the method of Bell et al. (1964), was suspended in 400 ml of 0.10 M NaCl for 1.5 hr. The insoluble residue was removed by centrifugation at 12,000 rpm for 30 min in a Sorvall, Type SS-1 centrifuge. The supernate was applied to a DEAE-cellulose column (75 x 3.5 cm diam.) prepared as described above. The column was developed using an 8 litre linear gradient from 0.10 M to 1.5 M NaCl, both vessels buffered at pH 7.8 with 0.005 M Tris(chloride). The flow rate was 3.1 ml per min and 20 ml fractions were collected. Fractions 70-180 were combined (2200 ml) and concentrated under reduced pressure at 30° to a volume of 500 ml. The solution was adjusted to pH 5 with glacial acetic acid, cooled to 0° and the s-RNA precipitated with 2.5 volumes of ice-cold ethanol and allowed to settle at 0° for 4 hr. The precipitate was collected by decantation and centrifugation. The resulting pellet was dissolved in 80 ml of 0.10 M NaCl, 0.005 M Tris(chloride), pH 7.8, and the solution distilled under reduced pressure at 30° to remove traces of ethanol. The solution was then chromatographed under the conditions described above. The elution profile is shown in Figure 1. Fractions 145-220 were combined, the pH adjusted to 7 with acetic acid
FIGURE 1: DEAE-cellulose chromatography of s-RNA from bakers' yeast.
and concentrated under reduced pressure at 30° to 500 ml. The concentra-
tion was adjusted to pH 5 with acetic acid and the solution cooled to 0°.
Ice-cold ethanol (2.5 volumes) was added and the solution left overnight
at 0°. The pellet, obtained by decantation and centrifugation, was
washed with cold ethanol, then with ether and dried in a vacuum
desiccator over P₂O₅ to yield 1.005 g of yeast s-RNA.

3. Digestion of Yeast s-RNA by Ribonuclease at Various Concentrations
   of Magnesium Ion

The reaction mixture (final volume 3.0 ml) contained per ml of
final volume, 10 μmoles Tris(chloride), pH 7.3, 35 μg yeast s-RNA,
MgCl₂ at concentrations between 0 and 100 μmoles and pancreatic ribo-
nuclease. The Tris buffer, s-RNA and MgCl₂ (or distilled water in the
case of the control) were mixed in a 1-cm cuvette and the optical
density measured at 260 mμ. The pancreatic ribonuclease, contained
in 0.10 ml, was added at zero time. The O. D. was corrected for this
dilution and the reaction was followed by the hyperchromic shift
measured at 260 mμ. Digestions were carried out at 0.0 M, 0.02 M,
0.05 M and 0.10 M MgCl₂ in the presence of 15 μg per ml of pancreatic
ribonuclease at room temperature (Fig. 2).

4. Quantitative Precipitation of s-RNA

To 0.50 ml of a solution at room temperature containing 240 μg
FIGURE 2: Increase in optical density at 260 mμ during digestion of s-RNA with ribonuclease at room temperature in the presence and absence of Mg. s-RNA : ribonuclease ratio, 2.3:1 (w/w).
of s-RNA, 4 μmoles of Tris(chloride), pH 7.3, and 10 μmoles of MgCl₂ was added 0.50 ml of an ice-cold solution consisting of 50% aqueous ethanol, 2 N in HCl. The solution was mixed thoroughly, cooled in ice for 10 min then centrifuged at 11,500 rpm in a Misco Model 5500 centrifuge. About 2% of the O. D. remained in the supernate.

5. Release of Acid-Soluble Nucleotides During Ribonuclease Digestion at Room Temperature

A 4.00 ml solution containing 2.32 mg of yeast s-RNA (46.4 O. D. units), 40 μmoles of Tris(chloride), pH 7.3 and MgCl₂ (except experiment 1, which contained no MgCl₂) was prepared for each experiment. To a 0.40 ml aliquot was added 0.10 ml of Tris(chloride), pH 7.3, and 0.50 ml of ice-cold 50% aqueous ethanol, 2 N in HCl. After cooling in ice and centrifuging (as described in Section 4), a 0.15 ml aliquot of the supernate was diluted to 1.0 ml with 0.01 M Tris(chloride) buffer pH 7.3, and the O. D. measured at 260 μm. This afforded a value for the residual, non-precipitable O. D. which varied depending on the concentration of magnesium ions present. To the remaining 3.60 ml of solution was added 0.90 ml of pancreatic ribonuclease (150 μg per ml) at zero time. Thus the reaction mixture (final volume 4.5 ml) contained, per ml final volume, 480 μg s-RNA, 10 μmoles Tris(chloride), pH 7.3, 30 μg pancreatic ribonuclease and MgCl₂ at concentrations of 0.00 M, 0.05 M, 0.10 M and 0.20 M. Aliquots of 0.50 ml were removed at various time
intervals, precipitated and centrifuged as described in Section 4. A 0.15 ml aliquot of the supernate was diluted to 1.00 ml with 0.01 M Tris-(chloride) buffer, pH 7.3 and the O. D. at 260 μm was recorded. The readings obtained were corrected for the residual, non-precipitable O. D. previously determined. Each corrected reading was then expressed in terms of percent by comparing it to the reading obtained when the s-RNA was completely digested (100% value).

Values for 100% digestion were obtained by carrying out the digestion for 30 min at room temperature in the absence of MgCl₂, then adding MgCl₂ to the desired concentration followed by precipitation and centrifugation in the usual way.

The results of five experiments carried out at different concentrations of MgCl₂ are shown in Figure 3.

6. Effect of Pronase on the Digestion of s-RNA by Pancreatic Ribonuclease

The reaction mixture (final volume 1.00 ml) contained per ml of final volume 10 μmoles Tris(chloride), pH 7.3, 50 μmoles MgCl₂, 85 μg s-RNA (1.70 O. D. units), 0.5 μg pancreatic ribonuclease and Pronase at 10⁻² μg and 10⁻⁵ μg. The control contained distilled water in place of Pronase. The Tris buffer, MgCl₂ and s-RNA were mixed together in a micro-cuvette and ribonuclease was added at zero time. The reaction
FIGURE 3: Release of acid-soluble ultraviolet absorbing material during digestion of s-RNA with pancreatic ribonuclease at room temperature in the presence and absence of Mg. s-RNA : ribonuclease ratio, 16:1 (w/w).
was followed by the hyperchromic shift at 260 μm and was allowed to
proceed for several min before adding the Pronase (contained in a volume
of 0.10 ml). When Pronase alone (10⁻² μg) was added to the s-RNA in the
absence of ribonuclease no hyperchromicity was observed over 25 min at
room temperature. No inhibition of ribonuclease was observed when the
concentration of Pronase was increased to 10 μg per ml.

7. Removal of Pancreatic Ribonuclease with CM-Cellulose

A. Behaviour of Pancreatic Ribonuclease on CM-Cellulose

A 0.10 ml aliquot of a solution containing 0.5 μg of ribonuclease
was added to a CM-cellulose (ammonium) column (4.8 x 0.5 cm diam.)
previously equilibrated with 0.01 M Tris(chloride), pH 7.3 and the column
was washed with several column volumes of the same buffer. An aliquot
of the eluate was incubated with a known amount of s-RNA and the O. D.
at 260 μm recorded. No ribonuclease was detected in the Tris eluate.
Ribonuclease was eluted by washing the column with 0.2 M MgCl₂, 0.01
M Tris(chloride), pH 7.3.

B. Removal of Ribonuclease from a Digestion Mixture

The digestion mixture (final volume 3.00 ml) containing per ml
final volume, 10 μmoles Tris(chloride), pH 7.3, 75 μg s-RNA (1.5 O. D.
units), 200 μmoles MgCl₂, and 0.5 μg pancreatic ribonuclease was made
up as follows: a 2.70 ml solution containing the Tris buffer, s-RNA and
MgCl₂ was cooled to 0° then 1.5 μg of ribonuclease (in a volume of 0.30 ml) was added. The incubation was carried out at 0° for 25 min. No hyperchromicity was detected during this time. The mixture was then allowed to warm to room temperature during 25 min then quenched in an ice bath. The expected increase in O. D. was observed. The mixture was diluted to 0.02 M MgCl₂ by the addition of 27 ml of ice-cold 0.01 M Tris(chloride), pH 7.3, buffer and immediately applied to a CM-cellulose (ammonium) column (15 x 1.7 cm diam.). The column was washed with 0.01 M Tris(chloride), pH 7.3 and the eluate collected in 5.0 ml fractions. Recovery of the applied material was quantitative. An aliquot of the eluate was heated to 70° for a few minutes then cooled rapidly. The observed hyperchromicity at 260 μm was 19.5%. Another aliquot, treated with ribonuclease at room temperature, showed a 10% increase in O. D. within one minute. Both tests suggested that the RNA had not been degraded by ribonuclease during the chromatography on CM-cellulose.

8. **Release of Acid-Soluble Nucleotides During Ribonuclease Digestion at 0° in the Presence of Magnesium Ions**

The reaction conditions were similar to those described in Section 5 except that the digestion (final volume 4.5 ml) was carried out at 0° in the presence of 9.6 μg per ml of pancreatic ribonuclease and MgCl₂ at concentrations of 0.00 M, 0.01 M, 0.05 M, 0.10 M and 0.20 M.
The results are shown in Figure 4. The digestions were repeated using 0.96 µg per ml of ribonuclease at MgCl₂ concentrations of 0.00 M, 0.05 M and 0.20 M. These results are shown in Figure 5.

9. Release of Acid-Soluble Nucleotides During Ribonuclease Digestion at Room Temperature in the Presence of Spermine

This experiment was carried out in a manner analogous to that described in Section 5 except that, in place of MgCl₂, spermine hydrochloride, pH 7.3, was added to each reaction mixture at concentrations of 0.00 M, 0.10 mM, 0.50 mM, 1.0 mM, 5.0 mM and 10.0 mM. Digestions carried out at spermine concentrations of 1.0 mM and above contained, in addition to the reagents listed above, 0.10 M NaCl to prevent precipitation of the s-RNA. The results are shown in Figure 6.

10. Controlled, Large Scale Digestion of s-RNA with Ribonuclease at Room Temperature

The incubation mixture contained 50 µmoles of Tris(chloride), pH 7.3, 20 mg of s-RNA (400 O. D. units), 1 mmole of MgCl₂ and 40 µg of pancreatic ribonuclease in a total volume of 5.0 ml. The digestion was carried out for 25 min at room temperature, quenched in ice and diluted to 0.02 M MgCl₂ by the addition of 45 ml of ice-cold, 0.01 M Tris(chloride), pH 7.3 and applied immediately to a CM-cellulose (ammonium) column (20 x 2.0 cm diam.) as described in Section 7-B.
FIGURE 4: Release of acid-soluble ultraviolet absorbing material during digestion of s-RNA with pancreatic ribonuclease at 0° in the presence and absence of Mg. s-RNA : ribonuclease ratio, 50:1 (w/w).
FIGURE 5: Release of acid-soluble ultraviolet absorbing material during digestion of s-RNA with ribonuclease at 0° in the presence and absence of Mg. s-RNA : ribonuclease ratio, 500:1 (w/w).
FIGURE 6: Increase in acid-soluble ultraviolet absorbing material during digestion of s-RNA with pancreatic ribonuclease in the presence and absence of spermine. s-RNA : ribonuclease ratio, 500:1 (w/w).
The expected recovery was 360 O. D. units (measured in magnesium solution) in the absence of any digestion by ribonuclease, or 430 O. D. units if the digestion was complete. The actual recovery was 380 O. D. units suggesting that the RNA was 25-30% degraded. About half of the eluate (71 ml) from the CM-cellulose column, containing 192 O. D. units was applied to a DEAE-cellulose (chloride) column (47 x 2 cm diam.), previously equilibrated with 0.01 M Tris(chloride), pH 7.8. The column was washed with about 50 ml of equilibrating buffer and then subjected to a linear gradient of sodium chloride 0.0 - 0.3 M (total volume, 8 litres) containing 0.01 M Tris(chloride), pH 7.8. The flow rate was 1 ml per min and 10 ml fractions were collected. To ensure complete removal of the O. D. the linear gradient of sodium chloride was extended to 1.0 M (total volume 1 litre). The elution profile is shown in Figure 7.

11. **Large Scale Digestion of s-RNA with Ribonuclease at 0° in the Presence of 0.2 M MgCl2**

Pancreatic ribonuclease (20 μg) was added to an ice-cold solution containing 200 μmoles of Tris(chloride), pH 7.3, 40 mg of s-RNA and 4 mmoles of MgCl2 in a final volume of 20 ml. After 30 min incubation at 0° the reaction mixture was poured into a 200 ml slurry of CM-cellulose, 0.01 M Tris(chloride), pH 7.3, at 3-5° and filtered rapidly through a CM-cellulose column (27 x 4 cm diam.). Ten ml fractions were collected. The fractions containing ultraviolet absorbing material
FIGURE 7: DEAE-cellulose chromatography of s-RNA after digestion with ribonuclease at room temperature in the presence of 0.2 M MgCl₂. s-RNA : ribonuclease ratio, 500:1 (w/w).
were combined (520 ml, 705 O.D. units) and applied to a DEAE-cellulose (chloride form) column (92 x 1.5 cm diam.), previously equilibrated with 0.01 M Tris(chloride), pH 7.3. The column was then subjected to a linear gradient of lithium chloride 0.05 - 1.5 M (total volume, 4 litres) containing 0.01 M Tris(chloride), pH 7.3. The elution profile is shown in Figure 8. Tubes 50-115 (Fraction II) were combined (1100 ml), diluted to 13 litres with distilled water and loaded onto a DEAE-cellulose column (90 x 1.5 cm diam.). The column was then subjected to a linear gradient of lithium chloride, 0.0 - 0.8 M (total volume 4 litres) containing 0.01 M Tris(chloride), pH 7.3. The elution profile is shown in Figure 9. Tubes 80-140 (Fraction A) were combined, diluted to 1 litre with distilled water and loaded on a DEAE-cellulose (carbonate) column (53.5 x 1.5 cm diam.). The column was washed successively with water (500 ml), 0.01 M (NH₄)₂CO₃ (500 ml) and 2 M (NH₄)₂CO₃. The ammonium carbonate was removed by repeated evaporation at 30° under reduced pressure.

12. **Base Analysis of Fraction A**

The non-volatile residue (5 mg) remaining after removal of the ammonium carbonate from fraction A was digested with 0.3 N KOH for 18 hr at 37° (Markham and Smith, 1952). The digest was neutralized with Dowex 50 (hydrogen) resin and added to a DEAE-cellulose (carbonate) column (14 x 1.7 cm diam.) (Bell et al., 1964). The column was subjected
FIGURE 8: DEAE-cellulose chromatography of s-RNA after digestion with ribonuclease at 0° in the presence of 0.2 M MgCl₂. s-RNA : ribonuclease ratio, 2000:1 (w/w).
FIGURE 9: Rechromatography of Fraction II (Fig. 8) on DEAE-cellulose.
to a linear gradient of ammonium carbonate, 0.0 - 0.3 M (total volume, 2 litres), pH 7.3. The elution profile is shown in Figure 10-A. The flow rate was 0.5 ml per min, and fractions of 2.3 - 2.5 ml each were collected. Fractions were combined (A - I) as shown in Figure 10-A, lyophilized, applied to two 20 cm sheets of Whatman 40 paper previously washed with a solvent composed of isobutyric acid : H₂O : conc. NH₃, (66:33:1), and developed for 18 hr in the same solvent (Fig. 10-B). The ultraviolet-absorbing areas were cut out, eluted quantitatively and made up to 1.0 ml with water. The spectra of the eluates were recorded under neutral, acidic and alkaline conditions. The resulting nucleotide composition (mole percent) was as follows: A, 14.0; G, 10.7; C, 21.0; U, 27.7; Ψ, 8.8; T, 4.8; 1-methyl-G, 1.7; 2-N, N-dimethyl-G, 2.0; and unidentified ultraviolet absorbing compounds, 9.3.


A. G-100 Sephadex Chromatography

Mixed yeast s-RNA (50 mg - Calbiochem) was dissolved in 3.0 ml of 1.0 M NaCl and added to the top of a G-100 Sephadex column (115 x 1.5 cm diam.), previously equilibrated with 1.0 M NaCl. The flow rate was 0.45 ml per min and 5.4 ml fractions were collected (Fig. 11-A). The major peak (II) contained 81.3% of the O. D. and was collected by precipitation with ethanol at 0°.
FIGURE 10: A - Chromatography of an alkaline digest of Fraction A (Fig. 9) on DEAE-cellulose.

B - Composite diagram of chromatograms obtained after paper chromatography of Fractions A to I in Figure 10-A.
FIGURE 11: G-100 Sephadex chromatography of: A - mixed s-RNA, B - partially purified glycine s-RNA, C - rechromatography of peak II from Figure 11-B.
Glycine s-RNA (50 mg), which was purified 12-fold by chromatography on benzoylated DEAE-cellulose (Tener et al., 1966), was chromatographed on G-100 Sephadex as described above (Fig. 11-B). The major peak (II) contained 93.5% of the O. D. and was collected by ethanol precipitation at 0°. Rechromatography of peak II (10 mg) under the same conditions gave a symmetrical elution profile (Fig. 11-C).

B. Digestion with Pancreatic Ribonuclease

Solutions (final volume 1.5 ml) of the mixed and partially purified s-RNA from peak II (Figs 11-A and 11-B), contained per ml of final volume, 10 μmoles Tris(chloride), pH 7.5, 0.5 O. D. units of s-RNA, 0 or 10 μmoles MgCl₂ and 0.33 μg pancreatic ribonuclease. The Tris buffer, s-RNA and MgCl₂ (or distilled water in the case of the controls) were mixed in a 1-cm micro-cuvette and the O. D. measured at 260 μm. The pancreatic ribonuclease, contained in 0.10 ml, was added at zero time. The absorbance at 260 μm was corrected for this dilution and the hyperchromic shift was recorded. The results are shown in Figure 12 as percent increase in absorbance over the absorbance at zero time in the presence and absence of magnesium.
FIGURE 12: Digestion of mixed s-RNA (---) and partially purified glycine s-RNA (-----) in the presence and absence of 0.2 M MgCl$_2$. 

- % INCREASE IN O.D. (260nm) 
- TIME (min) 

- Mg 
- Mg$^+$
DISCUSSION

Bovine pancreatic ribonuclease is not markedly inhibited by divalent cations such as magnesium ion when enzymatic activity is determined by measuring the increase in acid-soluble material using high molecular weight yeast RNA as substrate (Dickman et al., 1956; Nishimura, 1960). However when s-RNA was used as substrate a marked inhibition of pancreatic ribonuclease was observed, as measured by the hyperchromic shift in absorbance at 260 m\textmu; (Fig. 2).

In the absence of magnesium the hydrolysis was essentially complete in 3 min while in the presence of 0.05 M magnesium the hydrolysis was only 55% complete in 20 min and in the presence of 0.10 M magnesium, 27% of the maximum hyperchromicity was observed in 20 min.

To ensure that the hyperchromic shift at 260 m\textmu; was a reasonable indication of the extent of hydrolysis, s-RNA was digested with pancreatic ribonuclease and the release of acid-soluble, ultraviolet absorbing material was measured. Quantitative precipitation of the s-RNA was achieved by adding to the solution, an equal volume of 50% aqueous ethanol, 2 N in HCl at 0° followed by centrifugation. In the absence of pancreatic ribonuclease, less than 2% of the ultraviolet absorbing material remained in the supernate.
Figure 3 shows the release of acid-soluble ultraviolet absorbing material when s-RNA is digested with pancreatic ribonuclease at room temperature in the presence of different concentrations of magnesium ions. The results are expressed as percent of the acid-soluble material released by ribonuclease during 30 min at room temperature in the absence of magnesium ions.

In the absence of magnesium, the hydrolysis again appears complete in three min, but in the presence of 0.05 M and 0.10 M magnesium the hydrolysis was only 34% and 23% complete, respectively. However, when the hyperchromic shift at 260 m\(\mu\) was used to measure the extent of hydrolysis, values of 55% and 27% (in 0.05 M and 0.10 M magnesium, respectively) were obtained.

Attack by the enzyme on the loop regions of s-RNA would cause a hyperchromic shift due to cleavage of the phosphodiester linkage which could be at least partially offset by an increase in the strength of the interactions in the base paired regions adjacent to the loop. This effect would lower the hyperchromic shift. On the other hand, if large fragments were released these would be precipitated under the conditions used to measure the extent of hydrolysis by the release of acid-soluble material since magnesium is known to enhance the precipitation of polynucleotides by ethanol (Razzell, 1963). Since both effects would tend to give less than the true value for the extent of hydrolysis it seems
probable that the hyperchromic shift at 260 m\(\mu\) is closest to the true expression for the extent of hydrolysis.

Both methods for measuring the extent of hydrolysis show an initially rapid rate which decreases exponentially as the magnesium ion concentration increases. The most probable explanation for this observation is that the magnesium ions stabilize the secondary structure of s-RNA and thus protect the ribonuclease sensitive sites which are present in helical regions. In this connection it should be pointed out that complementary RNA becomes resistant to pancreatic ribonuclease after it assumes a double-stranded structure (Geiduschek et al., 1962). Binding of magnesium ions to the phosphate groups would tend to neutralize or shield the repulsive forces between phosphate groups thus decreasing any tendency toward strand separation. If this is true for s-RNA then the secondary structure of complementary regions would remain intact, even in molecules which have suffered a few enzymatic cleavages.

When magnesium is absent from the reaction mixture the sites in the non-helical regions which are susceptible to attack by pancreatic ribonuclease might still be cleaved first. Following these cleavages however, the secondary structure might be sufficiently unstable to render other bonds susceptible, particularly if the ribonuclease itself still retains some affinity for the double-stranded structure. Relative
to this postulate is the study carried out by Felsenfeld et al. (1963) in which they showed that ribonuclease had a pronounced destabilizing effect on the helical structure of DNA.

In light of the above observations that s-RNA, in the absence of magnesium, is susceptible to pancreatic ribonuclease to the extent that a large number of small oligonucleotide fragments are released, (Ingram and Pierce, 1962) while complementary RNA is not susceptible, it is unlikely that long complementary regions exist in s-RNA as suggested by Cantoni's model (McCully and Cantoni, 1962). It seems rather more probable that the complementary regions are interrupted by loops of non-helical sequences along the length of the molecule as suggested by Fresco et al. (1960). The complete sequences of five s-RNA molecules have been elucidated since this study was carried out and their most probable structure seems to conform to a cloverleaf arrangement consisting of three major loops and a minor loop (Holley et al., 1965; Madison et al., 1966; Zachau et al., 1966; RajBhandary et al., 1967).

When loss of amino acid acceptor function of s-RNA was taken as a measure of hydrolysis, Nishimura and Novelli (1963), showed that magnesium was unable to protect leucine s-RNA. They suggested that the -CCA amino acid acceptor end did not participate in helix formation and was therefore easily hydrolyzed by pancreatic ribonuclease. In support of this hypothesis these workers showed that B. subtilis
ribonuclease, which produces mainly 3'-purine nucleotides and therefore cannot attack the -CCA end, was markedly inhibited by magnesium ions. In addition these workers showed a striking shift in the melting curve of s-RNA in the presence of magnesium ions, which was not parallel to the original curve as expected by increasing the salt concentration (Tissières, 1959). The results reported in this thesis are consistent with other evidence in support of the hypothesis that magnesium ions affect the physical state of s-RNA, causing it to assume a more rigid secondary structure and thereby rendering it more resistant to attack by pancreatic ribonuclease.

Having established the feasibility of controlling the extent of hydrolysis of s-RNA with pancreatic ribonuclease by carrying out the digestion in the presence of magnesium ions, it became necessary to remove the enzyme at the end of the specified reaction time. Initially, enzymatic digestion of ribonuclease was attempted using Pronase, a broad spectrum protease isolated from *Streptomyces griseus* (Nomoto and Narahashi, 1959; Nomoto et al., 1960). Pronase failed to suppress the catalytic activity of pancreatic ribonuclease under the conditions studied. A recent report on the suppression of ribonuclease activity by Pronase (Huppert and Semmel, 1965) showed that a twenty fold excess of Pronase and an incubation period of 2 hr at 37° was required to suppress 99.8% of ribonuclease activity. In the present work, the removal of ribonuclease from digestion mixtures was readily achieved
by diluting the digest with ice-cold buffer then filtering it through a column of CM-cellulose. The filtrate contained no detectable ribonuclease activity. No significant degradation of s-RNA occurred during the dilution and subsequent filtration steps as determined by (i) the hyperchromicity at 260 m\(\mu\) when an aliquot of the filtrate was heated and rapidly cooled and (ii) the observed hyperchromicity at 260 m\(\mu\) when ribonuclease was added to an aliquot of the filtrate.

During attempts to remove the ribonuclease from reaction mixtures a solution containing s-RNA and magnesium was prepared and cooled to 0\(^\circ\) prior to the addition of ribonuclease to determine if the catalytic activity could be quenched. When ribonuclease was added (in a ratio of 5:1 (w/w), s-RNA : RNase) at 0\(^\circ\) and the digestion mixture maintained at 0\(^\circ\), no hyperchromicity at 260 m\(\mu\) was observed. Studies at room temperature, in which the ratio of s-RNA to ribonuclease was 7:1, showed a readily detectable hyperchromic shift at 260 m\(\mu\). This suggested that besides the ratio of s-RNA to ribonuclease and the magnesium ion concentration, a third parameter namely temperature, could be introduced to control the extent of hydrolysis.

Accordingly, a study of the digestion of s-RNA by pancreatic ribonuclease in the presence of magnesium ions was carried out at 0\(^\circ\). Although the study shown in Figure 3, which was carried out at room temperature, contained three times the amount of ribonuclease used in
the study shown in Figure 4, the decrease in the extent of hydrolysis when the digestion was carried out at the lower temperature was too large to attribute only to the decreased ribonuclease concentration. The extent of hydrolysis in 20 min at room temperature (about 23°) and at 0°, at several concentrations of magnesium is compared in Table I.

**TABLE I**

*Comparison of the Extent of Hydrolysis of s-RNA with Pancreatic Ribonuclease under Various Conditions*

<table>
<thead>
<tr>
<th>Concentration of Magnesium Ions</th>
<th>EXTENT OF HYDROLYSIS (%) IN 20 MINUTES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Room Temperature</td>
</tr>
<tr>
<td></td>
<td>16:1 (^a)</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>0.01</td>
<td>71</td>
</tr>
<tr>
<td>0.05</td>
<td>34</td>
</tr>
<tr>
<td>0.10</td>
<td>23</td>
</tr>
<tr>
<td>0.20</td>
<td>13</td>
</tr>
</tbody>
</table>

\(^a\) Ratio of s-RNA : RNase (w/w)

It is immediately apparent that the ratio of s-RNA to ribonuclease, the concentration of magnesium ions and the temperature, are all important parameters which can be controlled for the limited hydrolysis of s-RNA.
The exponential decrease in the extent of hydrolysis with increasing concentration of magnesium at room temperature, as shown in the second column of Table I, has been discussed previously. A similar relationship exists for the digestions carried out at 0°C (columns 3 and 4 of Table I). Also apparent from columns 3 and 4 is that a ten-fold decrease in the relative concentration of pancreatic ribonuclease approximately doubles the protective influence at a given magnesium concentration (Figs 4 and 5).

In the presence of 0.20 M magnesium, a 500:1 ratio (w/w) of s-RNA to ribonuclease and 0°C, the extent of hydrolysis was 2.5%. The cleavage of two nucleotides from the -CCA end of a molecule consisting of 80 nucleotides would correspond to 2.5% release of acid-soluble material.

The hyperchromic shift at 260 mμ, when s-RNA is digested by pancreatic ribonuclease in the presence of spermine is shown in Figure 6. The presence of spermine clearly masks the catalytic action of ribonuclease although a previous report showed that spermine did not inhibit the catalytic activity of E. coli ribonuclease on E. coli ribosomal RNA (Thomas and Hubst, 1963). Thus spermine probably acts on the s-RNA substrate rather than the ribonuclease molecule. Complexing between nucleic acids and polyamines has been the subject of a large number of studies (Tabor et al., 1961).
Spermine concentrations of 1.0 mM and higher caused the s-RNA to precipitate unless the ionic strength of the solution was increased. This interdependence between ionic strength of the solution and the solubility of the spermine-s-RNA complex was described by Cantoni (1960).

Comparison of Figures 5 and 6 in which the enzyme to s-RNA ratios are both 1:500 (w/w) clearly shows that spermine is less effective than magnesium as an inhibitor of pancreatic ribonuclease. Although the mechanism of the inhibition of pancreatic ribonuclease by spermine is probably attributable to neutralization of the phosphate groups in the s-RNA with a consequent increase in the effectiveness of hydrogen bonding, other explanations such as cross linkage, cannot be completely excluded. It was in fact hoped that complexing between the s-RNA and spermine would render susceptible sites in the s-RNA chain unavailable to ribonuclease. This expectation was not realized and because of the more pronounced effects of magnesium, the large scale digestions were carried out in the presence of 0.2 M magnesium.

When s-RNA was digested at room temperature for 25 min in 0.2 M magnesium with an s-RNA to ribonuclease ratio of 500:1, the recovery of O. D. units suggested that the s-RNA was 25-30% degraded. This was the expected order of magnitude. However when the digest was chromatographed on DEAE-cellulose more than 75% of the O. D. units were eluted before the salt gradient had reached 0.13 M NaCl (Fig. 7).
Thus a large number of small oligonucleotide fragments were released even in the presence of 0.2 M magnesium.

This result suggests that under the conditions used in this experiment the ribonuclease had cleaved many more linkages than was indicated by the hyperchromic shift. The easiest explanation for this observation is that when the linkages along the chain are cleaved the s-RNA molecule still retains some semblance of order due to the stabilizing influence of magnesium.

Lowering the temperature to 0° and increasing the s-RNA : ribonuclease ratio to 2000:1 (w/w) in the presence of 0.20 M magnesium gave the desired selective hydrolysis. Figure 8 shows the elution profile of the digestion products from DEAE-cellulose chromatography. About 75% of the total digestion products were of medium to high molecular weight (fraction II). This is in close agreement with the value of about 65% obtained by Litt and Ingram (1964) for the digestion of s-RNA in the presence of 0.10 M magnesium at 0°. Rechromatography of fraction II on DEAE-cellulose (Fig. 9) showed that 71% of the O. D. in fraction II consisted of intermediate molecular weight material (fractions A and B) while the remaining 29% was eluted by a salt concentration corresponding to undegraded s-RNA (fraction C). It must be emphasized at this point, that until the specificity of this reaction has been established it cannot be stated unequivocally that the hydrolysis has been
completely selective. The effect of temperature on the rate of hydrolysis and the decreased amount of enzyme used in this experiment have not been excluded as alternative explanations for the observed, limited hydrolysis.

The distribution of major and minor bases in Fraction A (30% of the O. D. before digestion) was determined by the method of Bell et al. (1964). The results are listed in Table II.

**TABLE II**

Base Composition of Fraction A (Fig. 10)

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Fraction A</th>
<th>Undegraded a</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>14.0</td>
<td>16.7</td>
</tr>
<tr>
<td>U</td>
<td>27.7</td>
<td>18.3</td>
</tr>
<tr>
<td>G</td>
<td>10.7</td>
<td>26.9</td>
</tr>
<tr>
<td>C</td>
<td>21.0</td>
<td>28.5</td>
</tr>
<tr>
<td>ψ</td>
<td>8.8</td>
<td>3.15</td>
</tr>
<tr>
<td>T</td>
<td>4.8</td>
<td>0.9</td>
</tr>
<tr>
<td>1-MeG</td>
<td>1.7</td>
<td>1.15</td>
</tr>
<tr>
<td>N, N-diMeG</td>
<td>2.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Unidentified</td>
<td>9.3</td>
<td></td>
</tr>
</tbody>
</table>

a Values for undegraded s-RNA from Bell et al. (1964)
The protective influence of magnesium is particularly evident from the enrichment of U in the intermediate molecular weight material. Fraction A is also enriched in the odd bases, \( \Psi \) (2.8x), T(5.3x), N, N-dimethylG (3.3x), 1-methylG (1.5x).

Since this study was carried out, reports have been published in considerable detail by Ingram and co-workers (Litt and Ingram, 1964; Armstrong et al., 1964; Wagner and Ingram, 1966; Armstrong et al., 1966), whose results are in qualitative agreement with those reported here.

Further study on mixed s-RNA was discontinued when Litt and Ingram (1964), published the results of their study on the controlled degradation of s-RNA with pancreatic ribonuclease in the presence of magnesium at 0°. Efforts were directed instead toward the fractionation of mixed s-RNA since only on purified s-RNA could the specificity of these reactions be established. If some species of s-RNA were more susceptible to ribonuclease than others, either because of differences in their secondary structure and/or base composition, then it is possible that certain s-RNA molecules would be largely degraded while others remain largely intact. Such a situation has been suggested by Nishimura and Novelli (1963) based on the results of a study of the resistance of s-RNA to ribonucleases in the presence of magnesium as measured by the loss of amino acid acceptor functions. Thus the s-RNA's with the most
odd bases might have a less stable secondary structure and as a result, be more susceptible to ribonuclease. Optical rotatory dispersion studies revealed evidence pertaining to differences in the secondary structure among purified samples of yeast s-RNA (Sarin et al., 1966). Differences in the base composition of fractionated s-RNA have also been reported (Brown, 1963).

Evidence relating to differences in the secondary structure between mixed s-RNA and a partially purified fraction was obtained in the present work by a study of the ability of magnesium to inhibit the action of pancreatic ribonuclease.

Mixed s-RNA, and a fraction enriched 12-fold in glycine acceptor activity by chromatography on benzoylated DEAE-cellulose (Tener et al., 1966), were checked for homogeneity with respect to molecular size (Figs 11-A, B and C), then digested with pancreatic ribonuclease in the presence and absence of magnesium (Fig. 12).

In the presence of magnesium the partially purified sample showed an 8.1% hyperchromic shift compared to 12.4% hyperchromicity for mixed s-RNA over a period of 30 min. Thus the protective influence of magnesium was greater for the partially purified glycine s-RNA than for the mixed s-RNA. The different susceptibilities of the two s-RNA fractions to ribonuclease in the presence of magnesium was probably not due to contamination of the mixed s-RNA sample by non-s-RNA (which
might not be protected by magnesium against ribonuclease attack) since the mixed s-RNA fraction had been freed of other components by passage through a G-100 Sephadex column prior to digestion.

In the absence of magnesium, the partially purified sample (from peak I, Fig. II-C) showed 54% more hyperchromicity than the mixed s-RNA sample (from peak II, Fig. II-A). This is a significant difference which can be attributed either to a greater number of susceptible sites, and therefore a greater pyrimidine content in the partially purified glycine s-RNA compared to the average s-RNA molecule, or to a more rigid secondary structure than the average s-RNA molecule, which upon disruption would lead to a larger hyperchromic effect.

The larger hyperchromicity associated with digestion of the partially purified fraction is believed to be due to its more rigid secondary structure for the following reasons: 1) if the odd bases in s-RNA are to be considered indispensible, then an increase in pyrimidine content above that of the average s-RNA molecule, must be at the expense of the purine content; 2) since the U and C of mixed s-RNA (18.3% and 28.5% respectively) already exceed the A and G content (16.7% and 26.9% respectively) (Bell et al., 1964), it is difficult to reconcile an increased pyrimidine content with increased double-helical regions; 3) it follows from (2) that an increased pyrimidine content leads to a decreased double-helical content (compared to the average in mixed s-RNA) and therefore,
the protection afforded by magnesium against ribonuclease attack should be diminished. However the results in Figure 12 clearly show this not to be the case.

It must be emphasized here that in the above discussion the concept of secondary structure has been viewed in the classical terms of hydrogen bonding and base stacking in those regions along the length of the molecule which are capable of forming a double helix.

On the basis of hydrogen exchange studies, Englander and Englander (1965) have concluded that about 80% of the nucleotides in a typical s-RNA molecule are hydrogen bonded. Thermal denaturation studies (Fresco et al., 1963; Felsenfeld and Cantoni, 1964) have led to the conclusion that as much as 75% of the bases in unfractionated s-RNA could be paired in hydrogen bonded double helices of the DNA type.

Keselev et al. (1964) studied the rate of liberation of acid-soluble nucleotides from mixed yeast s-RNA by attack with pancreatic ribonuclease at 20° in the absence of magnesium; they concluded that about one-quarter of the mixed s-RNA 'molecule' was completely free of hydrogen bonding, about one-half was fully hydrogen bonded and the remainder was 'imperfectly' hydrogen bonded and slowly liberated by the action of the enzymes. The proposed conformations of the s-RNA species whose primary structures have been elucidated show that about 50% of the constituent nucleotides could form base pairs of the Watson-
Crick type. It therefore seems likely that we shall have to revise our thinking in relation to the secondary structure of s-RNA.

In this regard, it is not unlikely that the protective influence of magnesium ions described in this study, is due to the ability of magnesium (and spermine) to stabilize a folded conformation of s-RNA in which two of the major loops are in juxtaposition, such that hydrogen bonding and/or hydrophobic interactions could occur between the bases in the loops.

Masking of the repulsive, negative charges on the phosphate groups would clearly tend to enhance the strength of these interactions, particularly if the interactions are primarily of the short range, hydrophobic type.

Such a conformation could account for the observations made in this study as well as those of Fresco et al., (1963); Felsenfeld and Cantoni, (1964); Keselev et al., (1964); and Englander and Englander, (1965).


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PART II

FRACTIONATION OF YEAST s-RNA
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>61</td>
</tr>
<tr>
<td>LIST OF CHARTS</td>
<td>63</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>64</td>
</tr>
<tr>
<td>EXPERIMENTAL</td>
<td>76</td>
</tr>
<tr>
<td>Abbreviations Used</td>
<td>76</td>
</tr>
<tr>
<td>Materials</td>
<td>77</td>
</tr>
<tr>
<td>Methods</td>
<td>78</td>
</tr>
<tr>
<td>PART II-A. ATTEMPTS TO ISOLATE CYSTEINYL-s-RNA BY CHROMATOGRAPHY ON MERCURY-CELLULOSE</td>
<td>78</td>
</tr>
<tr>
<td>1. Titration of AE-Cellulose with Alkali</td>
<td>78</td>
</tr>
<tr>
<td>2. Thiolation of AE-Cellulose with N-Acetylhomocysteine Thiolactone (HTL)</td>
<td>79</td>
</tr>
<tr>
<td>A. Thiolation of AE-Cellulose with HTL in Dioxane</td>
<td>79</td>
</tr>
<tr>
<td>B. Thiolation of AE-Cellulose with HTL in Aqueous Medium in the Presence of Silver Nitrate</td>
<td>79</td>
</tr>
<tr>
<td>3. Titration of HSAE-Cellulose with Iodoacetic Acid (IAA)</td>
<td>80</td>
</tr>
<tr>
<td>4. Large Scale Thiolation of AE-Cellulose with N-Acetylhomocysteine Thiolactone</td>
<td>81</td>
</tr>
<tr>
<td>5. Synthesis of bis-3,6-(Acetatomercurimethyl)-Dioxane</td>
<td>84</td>
</tr>
</tbody>
</table>
6. Preparation of the Mercuri-Cellulose (HgSAE-Cellulose) Column 85

7. Behaviour of s-RNA on HgSAE-Cellulose 86

8. Preparation of Aminoacyl-s-RNA Synthetases from Yeast 87

9. Preparation of $^{14}$C-Cysteinyl-s-RNA 87

10. Chromatography of $^{14}$C-Cysteinyl-s-RNA on HgSAE-Cellulose 88

11. Acetylation and Deacetylation of Cellulose 88

12. Preparation of Tosyl-Cellulose 89
   A. Recrystallization of Tosylchloride 89
   B. Tosylation of Deacetylated Cellulose Acetate 90

13. Characterization of Tosyl-Cellulose 91

14. Preparation of S-Acetyl-Cellulose 92

15. Hydrolysis of S-Acetyl-Cellulose 93
   A. Hydrolysis in 0.02 N Sodium Methoxide 93
   B. Hydrolysis in 0.10 N Sodium Hydroxide 93

16. Large Scale Preparation of HS-Cellulose 94

17. Chromatography of s-RNA on HS-Cellulose 95

18. Conversion of HS-Cellulose to Mercuri-Cellulose 96

19. Chromatography of $^{14}$C-Cysteinyl-s-RNA on HgS-Cellulose 96
PART II-B. PARTITION CHROMATOGRAPHY OF s-RNA ON BIOGEL

1. Partition Chromatography of s-RNA on Biogel P-10 using the System of Warner and Vaimberg (1958)
   A. Determination of the Distribution Coefficient
   B. Partition Chromatography of s-RNA: Experiment 1
   C. Partition Chromatography of s-RNA: Experiment 2

2. Partition Chromatography of s-RNA on Biogel P-10 Using the System of Zachau et al. (1961)
   A. Preparation of the Tributylammonium Salt of s-RNA
   B. Preparation of the Biogel P-10 for Partition Chromatography
   C. Partition Chromatography of s-RNA
   D. Assay of the Samples Obtained from Partition Chromatography

PART II-C. FRACTIONATION OF s-RNA ON BENZOYLATED-DEAE-CELLULOSE

1. Preparation of Benzoylated-DEAE-Cellulose
2. Preparation of the BDEAE-Cellulose Column
3. Chromatography of Mononucleotides on BDEAE-Cellulose
4. Recovery of s-RNA from Column Eluates
5. Preparation of Yeast Aminoacyl-s-RNA Synthetase Enzymes
6. Assay of Column Fractions for Specific Amino Acid Acceptor Activity
7. Chromatography of Yeast s-RNA on BDEAE-Cellulose under Conditions of Varying NaCl and/or MgCl$_2$

A. NaCl Gradient in the Presence of 0.10 M MgCl$_2$, pH 5.0

B. NaCl Gradient in the Presence of 0.02 M MgCl$_2$, pH 5.0

C. NaCl Gradient in the Presence of 0.005 M MgCl$_2$, pH 5.0

D. NaCl Gradient in the Presence of 10$^{-3}$ M EDTA, pH 5.0

E. MgCl$_2$ Gradient in the Presence of 0.10 M NaCl, pH 5.0

F. Pulse Gradient of NaCl in the Presence of 0.005 M MgCl$_2$, pH 5.0

(i) Chromatographic Method

(ii) Determination of the Melting Curves

(iii) G-100 Sephadex Chromatography of Fractions XV and XVI

G. Preparative Column Chromatography of s-RNA on BDEAE-Cellulose

H. Rechromatography of the Glycine-I Fraction

(i) Chromatography at pH 3.5 in the Presence of 0.05 M MgCl$_2$

(ii) Chromatography at pH 4.0 in the Presence of 10$^{-3}$ M EDTA

J. Rechromatography of the Glycine-II Fraction

(i) Chromatography at pH 5 in the Presence of 0.01 M MgCl$_2$

(ii) Chromatography at pH 3.5 in the Presence of 10$^{-3}$ M EDTA
PART II-D. A COMBINED CHEMICAL AND CHROMATOGRAPHIC METHOD FOR THE ISOLATION OF AMINO ACID-SPECIFIC s-RNA's

1. Preparation of the Acylating Agent

2. Experiment I

   A. Conditions for Maximum Charging of s-RNA with Glycine
   B. Preparation of $^{14}$C-Glycyl-s-RNA
   C. Derivatization of $^{14}$C-Glycyl-s-RNA
   D. Chromatography of the N-(Phenoxyacetyl)-$^{14}$C-glycyl-s-RNA on BDEAE-Cellulose

3. Experiment II

   A. Conditions for Maximum Charging of s-RNA with Glycine using a Partially Purified Glycyl-s-RNA Synthetase
   B. Isolation of Glycine-Specific s-RNA
      - Step 1. Removal of Phenylalanine Acceptor s-RNA from mixed s-RNA
      - Step 2. "Sham" Phenoxyacetylation
      - Step 3. Preparation of $^{14}$C-Glycyl-s-RNA
      - Step 4. Phenoxyacetylation of $^{14}$C-Glycyl-s-RNA
      - Step 5. Isolation of the N-(Phenoxyacetyl)-$^{14}$C-glycyl-s-RNA from the Mixture
      - Step 6. Chromatography of Glycine-s-RNA on BDEAE-Cellulose

DISCUSSION

BIBLIOGRAPHY
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-A, B, C</td>
<td>Titration curves for HSAE-cellulose</td>
</tr>
<tr>
<td>2-A, B</td>
<td>Corrected titration curves for HSAE-cellulose</td>
</tr>
<tr>
<td>2-c</td>
<td>A plot of pH vs alkali consumed for the titration of HSAE-cellulose with IAA</td>
</tr>
<tr>
<td>3</td>
<td>Chromatography of $^{14}$C-cysteinyl-s-RNA on HgS-cellulose</td>
</tr>
<tr>
<td>4</td>
<td>Chromatography of monoribonucleotides on BDEAE-cellulose</td>
</tr>
<tr>
<td>5</td>
<td>Chromatography of monoribonucleotides on BDEAE-cellulose</td>
</tr>
<tr>
<td>6</td>
<td>Fractionation of bakers' yeast s-RNA on BDEAE-cellulose using a NaCl gradient in the presence of 0.10 M MgCl$_2$, pH 5.0</td>
</tr>
<tr>
<td>7</td>
<td>Fractionation of bakers' yeast s-RNA on BDEAE-Cellulose using a NaCl gradient in the presence of 0.02 M MgCl$_2$, pH 5.0</td>
</tr>
<tr>
<td>8</td>
<td>Fractionation of bakers' yeast s-RNA on BDEAE-cellulose using a NaCl gradient in the presence of 0.005 M MgCl$_2$, pH 5.0</td>
</tr>
<tr>
<td>9</td>
<td>Fractionation of bakers' yeast s-RNA on BDEAE-cellulose using a NaCl gradient in the presence of 10$^{-3}$ M EDTA, pH 5.0</td>
</tr>
<tr>
<td>10</td>
<td>Fractionation of bakers' yeast s-RNA on BDEAE-cellulose using a MgCl$_2$ gradient in the presence of 0.10 M NaCl, pH 5.0</td>
</tr>
<tr>
<td>11</td>
<td>Apparatus for supplying pulse gradients during column chromatography</td>
</tr>
</tbody>
</table>
FIGURE PAGE NUMBER

12 Pulse gradient elution of bakers' yeast s-RNA from BDEAE-cellulose. NaCl gradient in the presence of 0.005 M MgCl₂, pH 5.0 125

13 Gel filtration of Fractions XV (Fig. 13-A) and XVI (Fig. 13-B) on Sephadex G-100 127

14 Preparative column chromatography of 5 g of brewers' yeast s-RNA on BDEAE-cellulose 129

15 Chromatography of Glycine-I fraction on BDEAE-cellulose in the presence of 0.05 M MgCl₂, pH 3.5 131

16 Chromatography of Fraction I (Fig. 15) on BDEAE-cellulose in the presence of 10⁻³ M EDTA, pH 4.0 132

17 Chromatography of Glycine-II fraction on BDEAE-cellulose in the presence of 0.01 M MgCl₂, pH 5.0 134

18 Chromatography of Fraction I (Fig. 17) on BDEAE-cellulose in the presence of 10⁻³ M EDTA, pH 3.5 135

19 Chromatography of N-(Phenoxyacetyl)-¹⁴C-glycyl-s-RNA on BDEAE-cellulose 140

20 Effect of magnesium ions on the synthesis of ¹⁴C-glycyl-s-RNA (Range, 3.2 to 10³ μ-moles MgCl₂) 144

21 Effect of magnesium ions on the synthesis of ¹⁴C-glycyl-s-RNA (Range, 0 to 28 μ-moles MgCl₂) 145

22 Chromatography of N-(phenoxyacetyl)-¹⁴C-glycyl-s-RNA on BDEAE-cellulose 151

23 Chromatography of glycine acceptor RNA on BDEAE-cellulose 153
# LIST OF CHARTS

<table>
<thead>
<tr>
<th>CHART</th>
<th>PAGE NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Fractionation of Glycine-I Acceptor RNA on BDEAE-Cellulose</td>
</tr>
<tr>
<td>II</td>
<td>Fractionation of Glycine-II Acceptor RNA's on BDEAE-Cellulose</td>
</tr>
<tr>
<td>III</td>
<td>Synthesis of N-(Phenoxyacetyl)-aminoacyl-s-RNA</td>
</tr>
<tr>
<td>IV</td>
<td>Isolation of Glycine-I s-RNA from Whole s-RNA (Part II-D, Section 3-B)</td>
</tr>
</tbody>
</table>
INTRODUCTION

Since Hoagland et al. (1958) first observed the essential role of s-RNA in protein synthesis, considerable effort has been expended in attempts to elucidate the unique function and specificity of this relatively low molecular weight RNA.

The term soluble RNA (s-RNA), derives from the fact that this family of ribonucleic acids exists in the supernate of a cell-free extract following ultracentrifugation of a cellular homogenate. Terms equivalent to s-RNA, which are in common use, are transfer RNA (t-RNA), acceptor RNA and adaptor RNA, and these derive from the biological function of this family of ribonucleic acids.

Aminoacyl-s-RNA synthetases catalyze the formation of an enzyme bound aminoacyl-adenylate complex which interacts with a specific s-RNA molecule resulting in the transfer of the amino acid from AMP to the adenosine terminal of the s-RNA molecule. The aminoacyl-s-RNA synthetases are essentially specific for a given amino acid as well as for a given s-RNA molecule. The reactions are as follows:

\[
\text{amino acid} + \text{ATP} + \text{enzyme} \rightleftharpoons \text{aminoacyl-AMP-enzyme} + \text{PP}_i
\]

\[
\text{aminoacyl-AMP-enzyme} + \text{s-RNA} \rightleftharpoons \text{aminoacyl-s-RNA} + \text{AMP} + \text{enzyme}
\]

sum:

\[
\text{amino acid} + \text{ATP} + \text{s-RNA} \rightleftharpoons \text{aminoacyl-s-RNA} + \text{AMP} + \text{PP}_i
\]
Having accepted the amino acid, the s-RNA leaves the enzyme surface and transfers the amino acid to the protein-synthesizing polysome complex (Brown, 1963).

The unique role played by s-RNA in the protein synthesizing machinery of the cell has attracted the attention of a large number of investigators. Although studies on unfractionated s-RNA have resulted in the accumulation of considerable information, the isolation of s-RNA molecules specific for a given amino acid is essential for the elucidation of the primary structure as well as the unique function and specificity of s-RNA's.

Preparations of s-RNA consist of populations of s-RNA molecules each specific for a particular amino acid. In addition, a given amino acid can have more than one s-RNA. There may in fact be as many as fifty different s-RNA species. All s-RNA species have similar molecular weights ranging around 25,000, and are of similar chain length. Such a complex mixture of closely related molecules presents an unusual challenge to those attempting to isolate a single unique s-RNA.

Early attempts, and until recently the only successful approach, to prepare s-RNA specific for single amino acids utilized the differences in partition coefficients of s-RNA molecular species in two-phase solvent systems.
Warner and Vaimberg (1958) first reported the effectiveness of the countercurrent distribution technique for the fractionation of ribonucleic acids using a solvent system composed of phosphate buffer (pH 8) - formamide - isopropanol. Holley and Merrill (1959) applied this method with minor modifications, to the fractionation of rat liver s-RNA. Subsequent reports by Holley and co-workers (Holley et al., 1961; Doctor et al., 1961; Apgar et al., 1962) clearly demonstrated the effectiveness of this technique for the purification of several s-RNA's from yeast. These workers also showed that the loss of amino acid acceptor activity could be minimized by lowering the pH of the phosphate buffer from 8 to 6. The phosphate-formamide-isopropanol system has also been used by Goldstein et al., (1964) for the fractionation of E. coli s-RNA's and by Hoskinson and Khorana (1965) for the purification of phenylalanine-specific s-RNA from yeast. The elucidation of the primary sequences of yeast alanine (Holley et al., 1965) and phenylalanine (RajBhandary et al., 1967) s-RNA's attests to the effectiveness of this method for the isolation of certain specific s-RNA molecules.

The two phase solvent system devised by Kirby (1960a, 1960b) was employed, with some modifications, for the countercurrent distribution of yeast s-RNA (Doctor and Connelly, 1961). The modified Kirby system afforded a distribution pattern similar to that obtained by Apgar et al., (1962).
Another two-phase solvent system consisting of phosphate buffer-polyethylene glycol (Albertsson, 1961) was adapted by Wiesmeyer et al., (1962) for the countercurrent distribution of $E. \text{coli}$ s-RNA. Some separation of acceptor activities was observed but the overall result was not too encouraging.

Zachau et al. (1961), demonstrated the separation of the tri-n-butylammonium salts of amino acid-specific s-RNA's in a solvent system composed of n-butanol-water-tri-n-butylamine-acetic acid-di-n-butylether.

The major drawback to the countercurrent distribution technique lies in the fact that the majority of specific acceptor RNA species are not clearly separated from one another when the fractionation is carried out in a 200 tube transfer apparatus. A thousand plate machine gave extensive fractionation of $E. \text{coli}$ s-RNA and revealed 29 s-RNA species (Goldstein et al., 1964), but such machines represent a considerable investment and are not widely used. Another disadvantage of the countercurrent distribution method lies in the sensitivity of the system to small variations in temperature.

In an attempt to combine the resolution and capacity of countercurrent distribution with the operational simplicity of column chromatography, the two phase solvent systems used in the countercurrent distribution methods have been employed for column partition
chromatography of s-RNA. Everett et al., (1960) fractionated yeast s-RNA by partition chromatography on silicic acid columns using a modified phosphate buffer-formamide-isopropanol solvent system. Although some fractionation of amino acid-specific s-RNA's was achieved, only 40% of the applied s-RNA was recovered. Assuming that this disadvantage was due to the silicic acid, Tanaka et al., (1962) used Sephadex G-25 as the supporting medium for the partition chromatography of yeast s-RNA using the two phase system of Zachau et al. (1961). The separation of amino acid-specific s-RNA's was comparable to the 200 tube transfer procedure of countercurrent distribution. By similar procedures, Bergquist and Scott (1964), were able to fractionate s-RNA from yeast and from E. coli. One distinction between column partition chromatography and countercurrent distribution is the opportunity in the former for gradient elution by means of an increasing partition coefficient in the mobile phase. This technique was exploited by Muench and Berg (1966b). These workers fractionated E. coli s-RNA on partition columns composed of G-25 Sephadex beads and a biphasic solvent mixture of phosphate buffer (pH 6.88) - ethoxyethanol - butoxyethanol - mercaptoethanol - triethylamine. An increase in the concentration of triethylamine was used to elute the s-RNA. One distinct disadvantage of the partition columns is the time required for isolating and assaying each of the fractions for the different acceptor RNA's as relatively large volumes of solvent are used to develop the column.
A simple extraction process employing liquid-liquid ion-exchange (Khym, 1965) offered a potentially useful modification of the partition process. In this process, alkylammonium salts of s-RNA are partitioned in a biphasic solvent system comprised of tricaprylylmethylammonium chloride-trichlorotrifluoroethane - aqueous sodium chloride. This technique has been exploited, with some modifications, for the reversed phase column chromatographic fractionation of *E. coli* s-RNA (Kelmers et al., 1965). A large sample of highly purified phenylalanine s-RNA was prepared by this method (Kelmers, 1966).

Another adsorbant used for the column chromatographic fractionation of s-RNA is hydroxyapatite. These columns are eluted with increasing concentrations of phosphate buffer. This method was originally reported by Hartmann and Coy (1961), for the fractionation of *E. coli* s-RNA. Muench and Berg (1966a) have further refined this method and claim it to be suitable on both analytical and preparative scales.

Methods for the fractionation of s-RNA by ion exchange chromatography have been developed by a number of investigators. Nishiyama et al. (1961), reported the partial resolution of rat liver leucyl-s-RNA on DEAE cellulose columns. These workers also studied the fractionation of s-RNA on DEAE-Sephadex columns and reported Sephadex to be somewhat more effective than DEAE-cellulose (Kawade
et al., 1963). Much more impressive fractionation of s-RNA on DEAEcellulose and DEAE-Sephadex was reported by Cherayil and Bock (1964, 1965) who employed increasing concentrations of sodium chloride, in the presence of 7 M urea, to elute the s-RNA. This method offered the advantages of good recovery of amino acid acceptor activity, good flow rates and simple, inexpensive chromatographic equipment. In this method it would appear that urea opens the s-RNA structure allowing increased interaction between the constituent nucleotides and the exchanger.

A similar opening of the s-RNA structure may be achieved by elevating the temperature during chromatography. This idea has been exploited recently by Baguley et al., (1965a, 1965b) for the fractionation of yeast s-RNA. These workers fractionated s-RNA on DEAE-cellulose at 65° untilizing a non-linear salt gradient to elute the s-RNA. They also showed that s-RNA could be fractionated on DEAE-Sephadex by employing a decreasing temperature gradient, at constant salt concentration, to elute the s-RNA. As the temperature is lowered, the s-RNA molecules presumably renature, thus exposing fewer of the constituent nucleotides to the ion exchanger.

More recently, Smith (1966) has reported the fractionation of s-RNA from Salmonella typhimurium on DEAE-Sephadex columns by elution with pH and salt gradients. The author suggests that resolution
of the s-RNA depends on a change in the s-RNA from a partially denatured state at the beginning of elution to the native state at the end.

In addition to the column chromatographic techniques already discussed, there is the methylated albumin column of Mandell and Hershey (1960), which was used by Sueoka and Yamane (1962) for resolving the amino acid acceptor activities of \textit{E. coli} s-RNA. Melchers and Zachau (1965) reported the fractionation of yeast s-RNA on columns of methylated albumin-kieselguhr. The major disadvantage of methylated serum albumin-kieselguhr columns is their small capacity, although they are still very useful in analytical procedures. Okamoto and Kawade (1963), improved the capacity by using silicic acid as a support, which adsorbs much more methylated serum albumin than does kieselguhr.

A survey of existing methods for preparing large amounts of specific s-RNA's for structural and biological studies revealed the necessity for additional fractionation procedures. A number of approaches were tried, most of which met with little or no success. Of the four methods described in this part of the thesis, two involve the isolation of families of amino acid-specific s-RNA's, (Parts II-A and -D), and two involve the fractionation of mixed s-RNA (Parts II-B and -C).

Part II-A describes a new chromatographic material consisting of a bifunctional mercuri-dioxane derivative attached through a thiol
group to the cellulose matrix. The use of mercuri-cellulose (HgS-Cellulose) for fractionating nucleic acids and their derivatives evolved from the studies of several groups on the interaction of various heavy metals (including mercury) with DNA, RNA, etc. (Thomas, 1954; Yamane and Davidson, 1961; Davidson et al. 1965) and also from a report by Eldjarn and Jellum (1963), in which they described the fractionation of HS-proteins on a bifunctional mercury derivative of aminoethyl cellulose. In the present study, the use of HgS-cellulose as a chromatographic material has not been fully investigated. However, preliminary experiments showed that HgS-cellulose could retain the radioactivity associated with $^{14}C$-cysteinyl-s-RNA.

The procedures described in Parts II-B and -C for the fractionation of s-RNA involve partition chromatography on a highly cross-linked polyacrylamide matrix (Part II-B), and adsorption-ion-exchange chromatography on a new chromatographic material, benzoylated-DEAE-cellulose (BDEAE-cellulose) (Part II-C). The latter procedure turned out to be a particularly useful method and may be considered to be the method of choice for the fractionation of acceptor-RNA's.

It must be emphasized that the development of BDEAE-cellulose was a cooperative project involving several people working in the laboratory of Dr. G. M. Tener. These were, in addition to Dr. G. M. Tener, Dr. I. C. Gillam, Dr. E. Wimmer, Dr. I. Maxwell, M. von
Tigerstrom, D. Blew and the author. The development of BDEAE-cellulose was based on an attempt to determine what properties of methylated serum albumin might be responsible for the fractionation of s-RNA's on columns of methylated albumin-kieselguhr (Sueoka and Yamane, 1962). When the negatively charged carboxyl groups of albumin are methylated, the albumin becomes an anion-exchanger as a result of the positive charges on the lysine (12%) and arginine (6%) residues. However, in addition to the basic amino acids, albumin also contains 13% aromatic amino acids and 30% of other amino acids with lipophilic side chains. One of the functions of serum albumin is the transport of lipids. Thus methylated serum albumin is a lipophilic anion-exchanger.

When DEAE-cellulose is benzoylated, its properties are changed from that of a hydrophilic anion-exchanger to a lipophilic anion-exchanger.

Part II-C describes the chromatographic behaviour of the glycine s-RNA's on BDEAE-cellulose under different conditions of elution.

Numerous reports have appeared describing combined chemical and chromatographic techniques for the isolation of families of amino acid-specific s-RNA's. Most involve charging the mixture of s-RNA's with a given amino acid followed by oxidation of the uncharged species with periodate to form the RNA dialdehydes.
Using this procedure, Zamecnik et al. (1960) converted the oxidized RNA to a dye-complex and removed the dye-bound RNA by fractional precipitation. More efficient methods for removing the dialdehydes have been developed by way of a coupling reaction between the dialdehydes and solid polyacrylic acid hydrazide (Zachau et al., 1961), phenylhydrazine-cellulose (Saponara and Bock, 1961) or aminoethyl-cellulose Zubay, 1962). These methods however, have the economic disadvantage of degrading all s-RNA's except that charged with the amino acid.

More economical chemical procedures have been reported but do not appear to have been very productive. For example a method developed by Mehler and Bank (1963) involved a reaction between s-RNA, previously charged with an amino acid, and ε-trifluoroacetyllysine N-carboxyanhydride. The Leuch's anhydride forms a growing polytrifluoroacetyllysine (polyTFAlysine) chain on the free α-amino group of the bound amino acid of aminoacyl-s-RNA.

The growing polyTFAlysyl side chain eventually effects precipitation of the aminoacyl-s-RNA. The recovery of amino acid acceptor activity after hydrolysis of the ester linkage between the amino acid and s-RNA was quite low. A similar method which utilized β-benzyl-N-carboxyl-L-aspartate anhydride in place of TFAlysine N-carboxy anhydride was reported by Simon et al. (1964). A more recent
report by the same workers utilized the same reaction for derivatizing and precipitating aminoacyl-s-RNA (Katchalski et al., 1966). They removed the polyaspartyl moiety with the aid of the proteolytic enzyme, Pronase, thus improving on the recovery of biological activity.

Methods utilizing aminoacyl-s-RNA synthesis must be combined with a chromatographic fractionation procedure because it is known that many amino acid-specific s-RNA's consist of more than one molecular component. In addition it should be pointed out that large amounts of purified aminoacyl-s-RNA synthetases are required to prepare sufficient amounts of s-RNA for structural studies.

Part II-D describes a combined chemical and chromatographic procedure for the isolation of the glycine acceptor-RNA. The method described in this thesis is an adaptation of a procedure developed by Drs G. M. Tener and I. C. Gillam. It involves the chemical attachment of a lipophilic 'handle' to an aminoacyl-s-RNA followed by stepwise elution of the mixture from BDEAE-cellulose. Intrafamily s-RNA species may then be separated by gradient elution chromatography on BDEAE-cellulose.
ABBREVIATIONS USED

**s-RNA**: soluble ribonucleic acid

**HTL**: N-acetyl homocysteine thiolactone

**IAA**: iodoacetic acid

**TCA**: trichloroacetic acid

**Ci**: curie

**EDTA**: ethylenediaminetetraacetic acid

**Tosyl**: p-toluenesulfonic acid

**DMF**: N,N-dimethylformamide

**TLC**: thin layer chromatography

**DCC**: di-cyclohexylcarbodiimide

**THF**: tetrahydrofuran

**AE-cellulose**: aminoethyl-cellulose

**HSAE-cellulose**: aminoethyl-cellulose which has been thiolated with HTL

**HgSAE-cellulose**: HSAE-cellulose which has been complexed with bis-3,6-(acetatomercurimethyl)-dioxane

**HS-cellulose**: 6-mercapto-cellulose

**HgS-cellulose**: HS-cellulose complexed with bis-3,6-(acetatomercurimethyl)-dioxane

**DEAE-cellulose**: diethylaminoethyl-cellulose
**BDEAE-cellulose**: benzoylated-DEAE-cellulose

**pC, pU, pG and pA**: the nucleoside-5' phosphates of cytidine, uridine, guanosine and adenosine, respectively

**Up, Gp, and Ap**: the nucleoside-2'(3') phosphates of uridine, guanosine and adenosine, respectively

**PPO**: 2, 5-diphenyloxazole

**dimethyl POPOP**: 1, 4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene

**T_M**: mid-point in the thermal denaturation curve defined as the 'melting' temperature

The standard abbreviations for the **amino acids** are used.

**MATERIALS**

**s-RNA**. Bakers' yeast s-RNA was purchased from Calbiochem Corporation, Los Angeles. Brewers' yeast s-RNA was purchased from Boehringer and Soehne, Mannheim.

**14_C-amino acids** were obtained from New England Nuclear Corporation, Boston, Mass.

**PPO** (Scintillation Grade), was obtained from Kent Chemicals Ltd., Vancouver, Canada.

**dimethyl POPOP** (Scintillation Grade), was obtained from the Packard Instrument Company Inc., La Grange, Ill.
Optical density measurements were made using a Cary Model 11 spectrophotometer. One optical density (O. D.) unit is defined as that amount of material per ml which, in a 1-cm light path at 260 μm, gives a spectrophotometric reading of one.

The mg of protein per ml of solution were calculated from the ratio of absorbances at 280 μm and 260 μm (280:260 ratio) (Warburg and Christian, 1941).

Nitroprusside test. When a few drops of sodium nitroprusside solution were added to the test solution, a deep magenta colour formed immediately in the presence of thiols. The reaction with thioesters was much slower.

METHODS

PART II-A. ATTEMPTS TO ISOLATE CYSTEINYL-s-RNA BY CHROMATOGRAPHY ON MERCURI-CELLULOSE

1. Titration of AE-Cellulose with Alkali

AE-Cellulose (1.00 g) was suspended in 20.0 ml of 1 M NaCl for 2 hr at room temperature, adjusted to pH 1.4 with 2 N HCl, then titrated with 0.100 N NaOH using a pH meter equipped with a glass electrode. A value of 0.6 meq per gram of dry AE-cellulose was obtained.
2. **Thiolation of AE-Cellulose with N-Acetylhomocysteine Thiolactone (HTL)**

**A. Thiolation of AE-Cellulose with HTL in Dioxane**

Attempts to thiolate AE-cellulose with HTL in refluxing dioxane were unsuccessful. When catalytic amounts of imidazole were included in the reaction mixture described above, a product was obtained which reacted weakly with nitroprusside.

**B. Thiolation of AE-cellulose with HTL in Aqueous Medium in the Presence of Silver Nitrate (Eldjarn and Jellum, 1963)**

Washed and dried AE-cellulose (1 g, 0.6 meq) was suspended in 50 ml of distilled water, stirred for 1 min at low speed in a Waring blender and allowed to swell in water for 24 hr. Then 286 mg of HTL (1.8 meq) dissolved in a few ml of distilled water, was added, followed by 3.0 ml of AgNO₃ solution (1.8 meq) which was added in increments of 0.10 ml per 2 min. During and after the addition, the pH was maintained between 7.5 and 8.0 with 1 N NaOH. The reaction mixture was stirred slowly. Samples of the cellulose were removed at 1, 2 and 3 hrs (samples I, II, and III) after all the AgNO₃ had been added, and mixed immediately with a solution made up of equal volumes of 1 N HNO₃ and saturated thiourea solution. (The colour of the product changes from bright yellow to very pale yellow). The samples were centrifuged and
the supernates decanted. The nitric acid-thiourea wash was repeated, then the products were washed repeatedly with distilled water until neutral. All three samples of thiolated AE-cellulose (HSAE-cellulose) reacted strongly with nitroprusside. Samples I, II and III were titrated with iodoacetic acid at pH 8.0 (see Section 3) and consumed 0.225, 0.226 and 0.235 meq of 0.100 N NaOH per dry gram of HSAE-cellulose respectively, indicating that the maximum degree of thiolation (Sample III) was 39% of theoretical based on a value of 0.6 meq per dry gram of AE-cellulose.

3. Titration of HSAE-Cellulose with Iodoacetic Acid (IAA)

Titrations were carried out using a Radiometer Titrigraph (Copenhagen).

Samples of the HSAE-cellulose (100 mg) were each suspended in 2.0 ml of 0.3 M KCl, 0.005 M Tris(chloride), pH 6.8, for several hours before each titration. A stock solution of IAA, containing 0.27 meq of IAA per ml, was prepared by dissolving 3.0 g of IAA in 50 ml distilled water, titrating to pH 6.5 with 1 N NaOH and extracting the solution with CHCl₃ until the organic layer was colourless. Solid KCl (0.7 g) was added and the volume made up to 60 ml. A hydrated sample of HSAE-cellulose was transferred quantitatively to the Titrigraph reaction vessel at 25°, and the volume was adjusted to 4.0 ml with 0.3 M KCl, 0.005 M Tris-(chloride), pH 6.8. The pH was adjusted to the desired value by the
addition of 1 N NaOH and a 0.37 ml aliquot of the standard IAA solution was added to the reaction vessel with the Titrigraph statted at the desired pH. Titrations were carried out at pH 7.0, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5 and 11.0. Each titration was followed for 50 min at 25°C. The meq of 0.100 N NaOH consumed per dry gram of HSAE-cellulose is shown in Figures 1A, B and C. A straight line of slope greater than 0 was obtained after about 10 min reaction time (except for the pH 7.0 titration, Fig. 1-A), which, when extrapolated back to zero time, made possible the calculation of a correction factor for the carboxymethylation of amino groups. The corrected titration plots are shown in Figures 2-A and B. From these plots are obtained the meq of 0.100 N NaOH consumed per gram of thiolated cellulose at various pH's which when plotted against pH (Fig. 2-C) permit the calculation of the pK$_{SH}$ of the thiol group by locating the midpoint in the slope of the curve. A value of 9.75 was obtained. Titrations of samples were routinely carried out at pH 8.0.

4. Large Scale Thiolation of AE-Cellulose with N-Acetylhomocysteine Thiolactone

AE-Cellulose (100 g) was left under water for about 12 hr after which the excess water was decanted and 18.5 g (116 meq) of HTL, dissolved in 1,200 ml of distilled water, was added. A 1 N AgNO$_3$ solution was added slowly, with rapid stirring and with the simultaneous
FIGURE 1. Titration curves for HSAE-cellulose.
FIGURE 2. A, B - Corrected titration curves for HSAE-cellulose. C - A plot of pH vs alkali consumed, for the titration of HSAE-cellulose with IAA.
addition of 2 N NaOH, to maintain the pH at 7.5. A total of 116 meq of Ag⁺ ion was added. The reaction mixture was then stirred slowly until the pH remained constant at 7.5 for a period of 15 min. Total reaction time was 4.5 hr. The brownish-yellow product was poured into a chromatographic column and washed with a solution of thiourea and HNO₃ (saturated thiourea solution: 1 N HNO₃, 1:1 (v/v)), until the effluent gave a negative nitroprusside test, and then washed neutral with distilled water.

5. **Synthesis of bis-3, 6-(Acetatomercurimethyl)-Dioxane** (Chatt, 1951)

Mercuric nitrate (16.5 g), was dissolved in a minimum of dilute HNO₃ and warmed to 50°. Allyl alcohol (3.5 ml) was added and the mixture allowed to stand at room temperature for 1 hr. The resulting white precipitate was collected on a Buchner funnel, washed with ice-cold water, redissolved in a minimum of 0.5 N KOH and reprecipitated by the careful addition of dilute HNO₃. The supernate was decanted and the white product pressed to dryness between filter papers and stored under vacuum in a desiccator.

The nitrate form of the mercuri-dioxane derivative was converted to a 1% solution of the acetate form by dissolving 6.2 g of the above product in a minimum of 0.5 N NaOH, diluting to 310 ml with distilled water and passing the solution through a Dowex-1 (acetate) column (38 x 1.4 cm diam.). The effluent containing the organomercury
compound was collected in 310 ml of ice-cold 0.02 M sodium phosphate buffer, pH 7.4. The container was protected from light by an aluminum foil wrapper and stored at 3-5°C until ready for use.

6. Preparation of the Mercuri-Cellulose (HgSAE-Cellulose) Column

HSAE-cellulose (containing about 0.23 meq of HS per dry gram) was suspended in 0.01 M sodium phosphate buffer, pH 7.4, stirred for about 1 min at low speed in a Waring blender then allowed to swell overnight at 3-5°C. A 1% solution of the bis-mercuri-dioxane derivative was added to the suspension. An excess of the organomercuri compound was maintained (NaI test) during 2 hr and then the suspension was poured into a chromatographic column of the desired dimensions. Alternatively, the suspension was poured directly into the chromatographic column which was then washed with a 1% solution of the organomercuri derivative until the latter was detected in the effluent (NaI test). In either case the column was then washed with distilled water until a negative test with NaI was obtained.

An analytical portion of the HgSAE-cellulose was suspended in 0.01 M NaOAc, pH 5.2 and centrifuged. The supernate gave negative NaI and nitroprusside tests. The organomercuri-cellulose also gave a negative nitroprusside test. However, when it was resuspended in very dilute mercaptoethanol and washed repeatedly with distilled water, the resulting HSAE-cellulose then gave a strong positive reaction to
nitroprusside.

7. **Behaviour of s-RNA on HgSAE-Cellulose**

To a small column (7 x 1 cm diam.) of HgSAE-cellulose, previously equilibrated with 0.01 M NaOAc, pH 5.2, was added 20 O. D. units of s-RNA dissolved in 1.0 ml of distilled water. The column was washed successively with 50 ml of 0.01 M NaOAc, pH 5.2, 100 ml 0.01 M NH4OAc, pH 5.2, then with 150 ml 0.01 M NaOAc, pH 5.2, 0.10 M NaCl. No ultraviolet absorbing material was eluted. The s-RNA was finally eluted by washing with 0.01 M NaOAc, pH 5.2, 1.0 M NaCl. The effluent gave a negative test with NaI indicating that the Hg had not been eluted.

After washing the column free of chloride ions with 0.01 M NaOAc, pH 5.2, an additional 10 O. D. units of s-RNA were applied and the column developed successively with 50 ml of 0.01 M NaOAc, pH 5.2, 75 ml of 0.10 M NaCl, 0.01 M NaOAc, pH 5.2, then with 0.01 M NaOAc, pH 5.2, 0.01 M mercaptoethanol. The O. D. observed at 260 μm was due to mercaptoethanol and to the Hg II ion. Finally the column was washed with 1 M NaCl which eluted the s-RNA originally applied. That the O. D. observed was due to s-RNA was confirmed by adding a small crystal of ribonuclease to an aliquot and observing the hyperchromicity. The aliquot showed 18.4% hyperchromicity in 6 min.
8. **Preparation of Aminoacyl-s-RNA Synthetases from Yeast**

The aminoacyl-s-RNA synthetases used in this part of the experimental were prepared from freshly grown bakers' yeast by grinding the cells with glass beads in a Virtis "45" homogenizer followed by centrifugation and dialysis. The procedure is described in detail in Part III Section 1-A.

9. **Preparation of $^{14}$C-Cysteinyl-s-RNA**

The reaction mixture (final volume 12.0 ml) contained 3.7 mmoles of Tris(chloride), pH 7.4, 1.6 μmoles of $L$-$^{14}$C-cysteine (3.74 μCi), 130 μmoles of MgCl₂, 250 μmoles of KCl, 53 μmoles of ATP, 24 μmoles of CTP, 30 μmoles of EDTA, 370 μmoles of mercaptoethanol, 20 mg of s-RNA (about 40 μmoles) and aminoacyl-s-RNA synthetase enzymes (containing about 10 mg of protein). The reaction mixture was incubated for 60 min at room temperature then cooled in ice. All subsequent operations were carried out between 0 and 5°C. To the cold reaction mixture was added 1.0 ml of 5 M NaCl and 6.0 ml of cold ethanol with thorough mixing. The mixture was left in ice for 10 min before centrifuging. The supernate was decanted and the pellet washed by suspending it in an ethanol : 1.5 M NaCl (2:1) solution (1 ml) followed by centrifugation. The washing was repeated twice more. The final pellet was dissolved in cold 0.5 M NaCl, 0.01 M NaOAc, pH 5.2.
A 20 μl aliquot was spotted on a planchet, dried and counted in a windowless gas-flow counter (Nuclear Chicago). A total of 500 cpm were incorporated.

10. **Chromatography of \(^{14}\text{C}\)-Cysteinyl-s-RNA on HgSAE-Cellulose**

The solution of \(^{14}\text{C}\)-cysteinyl-s-RNA was applied immediately to a column of HgSAE-cellulose (prepared as described in Section 6 and equilibrated with 0.5 M NaCl, 0.01 M NaOAc, pH 5.2) and allowed to incubate in contact with the column for 20 min before eluting with 3 column volumes of 0.5 M NaCl, 0.01 M NaOAc, pH 5.2, followed by 3 column volumes of the same buffer containing 10\(^{-3}\) M cysteine. Fractions of 1.0 ml each were collected. The absorbance at 260 μ was recorded and radioactivity was determined by applying 50 μl from each fraction to a planchet, drying under a heat lamp and counting as described previously. All the applied radioactivity and nearly all the applied ultraviolet absorbing material appeared at the elution front of the column. No radioactivity was detected in the cysteine wash.

11. **Acetylation and Deacetylation of Cellulose**

Whatman coarse grade cellulose powder (50 g) was mixed with 1 litre of glacial acetic acid in a Waring blender at low speed. To the suspension contained in a 2 litre flask was added 0.6 ml of conc. H\(_2\)SO\(_4\) then the mixture was left 3 hr at room temperature. Then 250 ml of
acetic anhydride was added and the mixture agitated for 5 hr at room temperature. The viscous liquid was divided among two 10 litre vessels of cold tap water in the form of a thin stream with constant stirring. The resulting filamentous precipitate was poured into a large Buchner funnel lined with cheesecloth and washed with cold tap water until the filtrate no longer frothed. Excess water was removed by suction and the cellulose acetate suspended in 2 litres of ethanol, 0.5 N NaOH. After 20 hr the suspension was stirred in a Waring blender at low speed for a few min then left a further 40 hr. The suspension was then poured into a chromatographic column and washed with 12 litres of distilled water. The cellulose was extruded, resuspended in 1600 ml of distilled water, stirred in a Waring blender at low speed then poured back into the chromatographic column. The column was washed with an additional 2 litres of distilled water. The eluate now showed a neutral reaction to pH paper. The washed cellulose was spread on a glass plate and dried in a vacuum oven at 60° in the presence of silica gel. The resulting white powder (43 g) was stored under vacuum until ready for use.

12. Preparation of Tosyl-Cellulose

A. Recrystallization of Tosylchloride

The following procedure was suggested by Dr. K. N. Slessor. Equal volumes of water and tosylchloride were warmed until the latter
formed an oil in the bottom of the flask. Upon cooling, the oil solidified and the water was decanted. The solid was dissolved in boiling petroleum ether (b. p. 30-60°), treated with charcoal and filtered by gravity. The white crystals, which resulted on cooling of the filtrate, were collected by decanting the supernate and spreading the crystals on a filter paper to dry at room temperature.

B. Tosylation of Deacetylated Cellulose Acetate

Deacetylated cellulose acetate (16 g) was "activated" by shaking overnight in a flask containing 100 ml of distilled water and 200 ml of pyridine (Malm et al., 1948). The cellulose was then sucked dry on a Buchner funnel with the aid of a rubber sheet, washed 3 times with anhydrous pyridine and transferred to a flask containing 38 g of recrystallized tosyl chloride in 250 ml of anhydrous pyridine. After shaking for 31 hr at room temperature, the gel was stirred with 3 litres of methanol. The granular, almost white, product was suction-filtered on a Buchner funnel, stirred in a Waring blender containing methanol, filtered again and washed free of chloride ions with methanol (AgNO₃ test). The product was then spread on glass plates and dried in a vacuum oven at 50° to yield 33.3 g. Theoretical yield for 6-O-tosylation was 31.2 g.
13. Characterization of Tosyl-Cellulose

Sulfur analysis was carried out according to the method of Lysyj and Zarembo (1958). To a 250 ml iodine flask was added 25 ml of a 6% solution of $\text{H}_2\text{O}_2$, two drops of methyl red indicator and the contents titrated to the end point with 0.02 N NaOH. A sample of the tosylated cellulose (about 5 mg) was weighed on an ashless filter paper (Whatman 41), 2 cm x 2 cm square with a 5 cm x 0.3 cm wick projecting from one corner. The paper was folded twice lengthways, rolled into a tight cylinder and inserted into the centre of a spiral platinum wire with the wick projecting upwards and the platinum wire suspended from the glass hook below the stopper of the iodine flask. After flushing the flask with oxygen for one min, the wick was lighted and the stopper held down firmly until the combustion was complete. The flask was then shaken vigorously to absorb all the gases, opened and the walls rinsed with 100 ml of distilled water. The volume was reduced to about 25 ml on a hot plate, then another 100 ml of distilled water was added and the volume reduced again to about 25 ml. After cooling the flask, 2 drops of methyl red indicator were added and the contents of the flask titrated to the end point with 0.02 N NaOH. The percent S in the sample was calculated from the formula:

$$\% S = \frac{(\text{ml})(N)(16.03)(100)}{\text{wt. of sample}}$$
where: \( N \) = normality of NaOH used in the titration
\( ml \) = ml of NaOH used in the titration.

The average value for duplicate analyses was 10.9% S. Theoretical for a DS = 1 was 10.14% S.

14. **Preparation of S-Acetyl-Cellulose**

The potassium thioacetate was a gift from Dr. K. N. Slessor and was prepared according to Bannister and Kagan (1960).

Tosyl-cellulose (1.0 g, D.S. = 1.07) and 0.8 g of potassium thioacetate (KSAc) were dissolved in 20 ml of N,N-dimethylformamide (DMF) and heated on a steam bath for 2 hr with occasional stirring. The solution became very viscous. Then 100 ml of methanol was added slowly to effect the precipitation of the cellulose, followed by the consecutive addition of 50 ml of 80% aqueous methanol and 50 ml of distilled water. The precipitate was collected by centrifugation and washed with 80% methanol until the supernate gave a negative nitroprusside reaction. The product was then washed with 25 ml of ethanol followed by 25 ml of ethyl ether and dried in a vacuum oven at 45°. Yield was 0.63 g and a slow reaction was obtained with the nitroprusside reagent, as expected for thiol esters.
15. **Hydrolysis of S-Acetyl-Cellulose**

   **A. Hydrolysis in 0.02 N Sodium Methoxide**

   To the S-acetyl-cellulose (0.60 g), suspended in 8 ml of methanol, was added 2.0 ml of 0.1 N NaOCH₃ in methanol and the solution stirred at room temperature for 21 hr. After acidifying to pH 5.0 with acetic acid, the product was filtered, washed until neutral with methanol, then washed with 80% aqueous methanol and finally with methanol. The product was then dried in a vacuum oven at 40° to yield 0.486 g, dry weight. It reacted slowly with the nitroprusside reagent but did not react with iodoacetic acid under the conditions described in Section 3.

   **B. Hydrolysis in 0.10 N Sodium Hydroxide**

   100 mg of S-acetyl-cellulose were suspended in 10 ml of 0.10 N NaOH and heated at 100° for 20 min. A gel formed upon heating and the cellulose could not be precipitated by cooling or by dilution with an equal volume of water. Precipitation was effected by the addition of 1 volume of distilled water and 10 volumes of acetone under rapid stirring. Precipitation was completed by the addition of ethyl ether to the acetone-water mixture. The supernate was decanted and the granular, light yellow product collected by centrifugation from methanol. It was washed with methanol, then with ethyl ether and dried in vacuum oven at 50°. The product reacted rapidly and strongly with nitroprusside.
and reacted with IAA to the extent of 0.5 meq per dry gram of product during 80 min under the conditions described in Section 3.

When 1 g of S-acetyl-cellulose was hydrolyzed in 35 ml of 0.1 N NaOH, i.e., increased ratio of S-acetyl-cellulose : 0.1 N NaOH (w/v) the HS-cellulose could be recovered by neutralizing the alkaline solution with glacial acetic acid, centrifugation and washing the product with water.

16. Large Scale Preparation of HS-Cellulose

To 11.4 g of tosyl-cellulose (36 mmole of glucose) and 8.2 g of KSAc (72 mmole), was added 100 ml of DMF and the mixture heated on a steam bath for 2 hr. The tosyl-cellulose dissolved in the hot solvent. After 2 hr on the steam bath a portion (sample I) was removed and an additional 0.3 g of KSAc was added to the remaining mixture and heating continued for another hour (sample II). After cooling, one volume of DMF in 5 volumes of methanol was added slowly with stirring, to each sample. The yellow supernate was decanted and 2 volumes of methanol : DMF (9:1, v/v) were added to the residue. The solution was stirred for a few minutes, then 4 volumes of methanol were added and the product allowed to settle. The product was collected by centrifugation, washed with methanol then with ethyl ether and dried in a vacuum oven at 50°. The yield was 1.7 g and 5.1 g, for samples I and II respectively. Portions of samples I and II (100 mg each) were hydrolyzed as
described in Section 15-B. Both reacted rapidly and strongly with nitroprusside and both reacted with IAA under the conditions described in Section 3. The results of the titrations showed 1.2 meq and 1.7 meq per gram of dry Samples I and II respectively when the titration reaction was followed for 3 hr. However, both samples still reacted with nitroprusside indicating that the carboxymethylation of HS-groups was incomplete. When the titration of Sample II was followed for 6 hr a value of 1.9 meq of HS-groups per gram of dry HS-cellulose was obtained. Again the reaction was incomplete as shown by the titration curve and the nitroprusside test. That the reaction was indeed incomplete was shown by titrating tosyl-cellulose under the same conditions. No alkali was consumed during 5 hr.

17. Chromatography of s-RNA on HS-Cellulose

s-RNA (10 mg) was dissolved in a few mls of 0.10 M NaOAc, pH 5.2 and applied to an HS-cellulose column (10 x 1 cm diam.) previously equilibrated with 0.10 M NaOAc, pH 5.2 and developed with a linear gradient of NaCl (0 to 0.3 M NaCl), total volume 600 ml, with both vessels buffered at pH 5.2 with 0.10 M NaOAc. About 75% of the O. D. was eluted at the solvent front with the remainder being eluted at about 0.03 M NaCl. Both peaks had similar ultraviolet spectra and hyperchromic effects on digestion with pancreatic ribonuclease.
18. **Conversion of HS-Cellulose to Mercuri-Cellulose (HgS-Cellulose)**

The HS-cellulose was suspended in water overnight then packed in a jacketed, water-cooled (about 4°) column (14 x 1 cm diam.) and washed with distilled water until the odor of thioacetic acid was no longer detected in the effluent. The column was then washed with a 1% solution of bis-3, 6-(acetatomercurimethyl)-dioxane in 0.01 M sodium phosphate, pH 7.6 until a positive NaI test (white precipitate) was obtained in the effluent, then with distilled water until a negative NaI test was obtained. The column was then equilibrated with 0.01 M NaOAc, pH 5.2.

19. **Chromatography of $^{14}$C-Cysteinyl-s-RNA on HgS-Cellulose**

$^{14}$C-Cysteinyl-s-RNA was prepared as described in Section 9 and the pellet dissolved in 6.0 ml of 0.01 M NaOAc, pH 5.2. A 3.5 ml aliquot of the solution, containing per ml, 66 O. D. units and 1600 cpm, was applied to the HgS-cellulose column (14 x 1 cm diam.) and allowed to incubate in contact with the HgS-cellulose for 10 min at about 4°. The column was then washed with a 1 litre linear gradient from 0 to 0.6 M NaCl, both vessels buffered at pH 5.2 with 0.01 M NaOAc. The flow rate was 28 ml per hr and 2.8 ml fractions were collected. The gradient was terminated at 0.4 M NaCl and elution continued with 0.4 M NaCl, 0.01 M NaOAc, pH 5.2, 0.01 M mercaptoethanol. The absorbance at 260 nm was recorded and 0.10 ml aliquots of the fractions were applied.
to planchets, dried and counted in a windowless gas-flow counter. The results are shown in Figure 3. When the $^{14}$C-cysteinyl-s-RNA was applied to a HgS-cellulose column equilibrated with 0.10 M NaCl, 0.01 M NaOAc, pH 5.2 the bulk of the applied material and all the radioactivity was eluted after about one column volume of the linear gradient (0.1 to 0.4 M NaCl) had passed through the column. Neither applied material nor radioactivity was detectable in the mercaptoethanol wash.

PART II-B. PARTITION CHROMATOGRAPHY OF s-RNA ON BIOGEL

1. Partition Chromatography of s-RNA on Biogel P-10 using the System of Warner and Vaimberg (1958)

A two phase system described by Warner and Vaimberg (1958) was prepared according to Apgar et al. (1962). The biphasic system consisted of 131 g of K$_2$HPO$_4$·3H$_2$O, 156 g of NaH$_2$PO$_4$·H$_2$O, 1360 ml of distilled water, 130 ml of formamide and 700 ml of isopropanol. The mixture was shaken and allowed to equilibrate overnight. Attempts to chromatograph s-RNA in this system using Biogel P-300, P-150, P-60 and P-30 were technically impractical due to the slow flow rates. Thus Biogel P-10 was used as supporting material for the stationary phase.
FIGURE 3. Chromatography of $^{14}$C-cysteinyl-s-RNA on HgS-cellulose.
A. Determination of the Distribution Coefficient (K)

A 1 mg sample of s-RNA was dissolved in 2 ml of the upper phase then 2 ml of the lower phase was added and the mixture shaken vigorously. After allowing 4 hr for the system to reach equilibrium the O. D. at 260 μm of the upper and lower phases was determined. The ratio of the O. D. in the upper phase to the O. D. in the lower phase (K) was 0.93. The procedure described above was repeated except this time the s-RNA sample was dissolved in the lower phase, then an equal volume of the upper phase was added. A value of K = 1.01 was obtained indicating that the system had almost reached equilibrium after 4 hr.

B. Partition Chromatography of s-RNA: Experiment 1

A 6 mg sample of s-RNA was dissolved in 5 ml of upper phase and applied to a Biogel P-10 column (22 x 1.8 cm diam.) which had been packed and washed with the lower phase and equilibrated with several column volumes of the upper phase. The column was washed with the upper phase but no s-RNA was eluted. When the column was washed with a solution of upper phase : distilled water, in a ratio of 3:1 (v/v) the s-RNA was eluted.

C. Partition Chromatography of s-RNA: Experiment 2

The P-10 Biogel column (22 x 1.8 cm diam.) was repacked in lower phase and equilibrated with several column volumes of upper
phase. To a 10 mg sample of s-RNA dissolved in 0.5 ml of distilled water was added 0.45 ml of upper phase and applied to the column. The column was developed with a linear gradient made up of 200 ml of upper phase in the mixing vessel and 200 ml of upper phase and distilled water, in a ratio of 3:2 (v/v), in the reservoir. The flow rate was 16 ml per hour and 4.0 ml fractions were collected. The s-RNA was eluted between fractions 22 and 50. The O. D. profile was divided into 3 sections and the s-RNA in each recovered by exhaustive dialysis followed by lyophilization. Recovery of the applied sample was quantitative. Fractions I and III were combined and rechromatographed under the conditions described above. The s-RNA was eluted as a broad peak and no resolution of the applied samples could be detected.

2. **Partition Chromatography of s-RNA on Biogel P-10 Using the System of Zachau et al. (1961)**

   **A. Preparation of the Tributylammonium Salt of s-RNA**

   **Method 1:** The sodium salt of s-RNA was dissolved in water and passed through a Dowex 50 (tributylammonium) column. The effluent containing the s-RNA was collected and lyophilized.

   **Method 2:** The sodium salt of s-RNA was dissolved in distilled water, cooled to 0° and 2.5 volumes of cold 0.2 N HCl was added. The mixture was left 10 min in ice and then centrifuged. The supernate was decanted
and the pellet washed twice with a small volume of 0.05 N HCl, then with ethyl ether and dried at room temperature. The dry pellet, which was now in the form of the free acid, was dissolved in the upper phase as the tributylammonium salt.

B. Preparation of the Biogel P-10 for Partition Chromatography

The partition system used was that described by Zachau et al. (1961) for the countercurrent distribution of s-RNA and consisted of n-butanol, H₂O, tributylamine (freshly distilled) acetic acid and butyl ether, (100:130:10:2.5:27). The Biogel P-10 was packed in a column using the lower phase, then washed with several column volumes of lower phase and finally equilibrated with several column volumes of upper phase.

C. Partition Chromatography of s-RNA

A 250 mg sample of sodium s-RNA was converted to the free acid as described in Section 2-A (Method 2) and dissolved in 7.0 ml of upper phase. This was applied to a Biogel P-10 column (88 x 3.0 cm diam.) prepared as described in Section 2-B with the temperature maintained at 24° by means of thermostatted water circulating through a glass jacket. The column was washed with upper phase. Fractions of 8 ml each were collected at a flow rate of 32 ml per hr.
To each fraction was added 0.60 ml of 2 M NaOAc, 0.001 M Mg(OAc)$_2$ and 0.60 ml of dibutyl ether. Each fraction was thoroughly mixed on a Vortex mixer and transferred to the cold room (3-5°C) overnight. The upper phase was removed by suction and to the lower phase was added 2 volumes of cold ethanol. After a minimum of 20 min in ice, each fraction was centrifuged, and the pellet washed with 0.50 ml of ethanol : ethyl ether : 1 M NaOAc: 0.5 mM Mg(OAc)$_2$, (3:1:1:1). To each odd numbered fraction was added 1.0 ml of distilled water. A 0.10 ml aliquot was then diluted to 2.0 ml with distilled water and the absorbance at 260 nm was recorded. The column effluent, containing the s-RNA, was then combined into 8 fractions which were dialyzed against distilled water and assayed for acceptor activity for glycine and histidine.

D. Assay of the Samples Obtained from Partition Chromatography

Each assay mixture (final volume 0.50 ml) contained 90 μmoles of Tris(acetate), pH 7.4, 1.5 μmoles of sodium ATP, 7.7 μmoles of MgCl$_2$, 15.4 μmoles of KCl, 0.4 μmole of EDTA, 0.37 μmole of mercaptoethanol, 4 O. D. units of s-RNA, 0.5 μCi of $^{14}$C-L-amino acid, 43 μmoles of glycine or 52 μmoles of L-histidine, 0.6 μmole of the amino acids not being assayed and 0.12 ml of the aminoacyl-s-RNA synthetase enzymes, prepared as described in Part III, Section 1. The mixture was incubated for 60 min at room temperature then 0.15 ml of cold, 5 M NaCl and 1.5 ml of cold ethanol were added with mixing.
After 20 min in ice the solution was centrifuged and the supernate decanted. The pellet was washed three times with 0.3 ml of a cold ethanol - NaCl solution (2:1, ethanol : 1.5 M NaCl) then the pellet was dissolved in 0.15 ml of 1.5 N NH₄OH. A 25 µl aliquot was applied to a planchet, dried and counted in a windowless gas-flow counter.

PART II-C. FRACTIONATION OF s-RNA ON BENZOYLATED-DEAE-CELLULOSE

1. Preparation of Benzoylated-DEAE-Cellulose

107 g of DEAE-cellulose (Schleicher and Schuell, containing 0.91 meq per gram and about 7% water (w/w)) were dried at 80° in a vacuum oven for 20 hr. The dry powder was suspended in 2.5 litres of pyridine, then 200 ml of benzoyl chloride (1.74 moles, i.e., about 3 moles of benzoyl chloride per mole of glucose moiety), were added. The mixture was heated until refluxing was observed and maintained at reflux temperature for 15 min, after which the mixture was allowed to cool until refluxing ceased. A further 150 ml of benzoyl chloride (1.3 moles) was then added with thorough mixing. The mixture was again heated and refluxing continued for 30 min. The mixture was then allowed to cool below reflux temperature (but not below about 90° or else the product began to come out of solution) and poured, as a thin stream, into 20 litres of tap water, with vigorous stirring, to form long tubular
strands. The centres of the strands had trapped unreacted benzoyl chloride and pyridine solvent. The water was decanted and the strands suspended overnight in 40 litres of tap water. After decanting the water, the strands were disrupted in a Waring blender, containing 75% aqueous ethanol. The blender was operated intermittently at high speed until a fine slurry was formed. The slurry was poured into a large chromatographic column, and washed with 75% aqueous ethanol until the effluent cleared. The product was treated again in the Waring blender then graded between 50 and 200 mesh screens. The particles which collect in the 50 mesh screen were treated again in the Waring blender as described above. The 50-200 mesh benzoylated-DEAE-cellulose (BDEAE-cellulose) was packed in a chromatographic column and washed with 50% aqueous ethanol, 1 M NaCl until the absorbance (260 nm) of the effluent approached zero. The column was then washed free of chloride ions with distilled water and stored in 10-15% aqueous ethanol until required.

2. Preparation of the BDEAE-Cellulose Column

BDEAE-cellulose, prepared as described above, was suspended in 2 M NaCl and packed in a chromatographic column of the desired dimensions. The column was washed with 2 M NaCl until the absorbance (at 260 nm) of the effluent was zero then thoroughly equilibrated with the starting buffer. The s-RNA, dissolved in starting buffer, was applied
to the column, washed on with a few mls of the starting buffer, then an increasing linear gradient of salt was applied. The flow rate was maintained at about one ml per min with the aid of an Accu-Flo pump (Beckman Instruments).

3. Chromatography of Mononucleotides on BDEAE-Cellulose

5 mg Samples of Ap, Gp, Up and pC were dissolved in 20 ml of starting buffer and the pH of the solution adjusted to 7.4 with 0.2 N NaOH. The solution was then applied to a BDEAE-cellulose column (95 x 1.5 cm diam.), previously equilibrated with 1500 ml of starting buffer. The column was developed with a 3 litre linear gradient of NaCl, from 0.01 to 0.05 M. Both vessels were buffered at pH 7.4 with 0.005 M sodium phosphate. The flow rate was 1 ml per min and 10.4 ml fractions were collected (Fig. 4). The peaks eluted by the salt gradient were identified by ultraviolet spectral analysis at pH 1, 7 and 12. Peaks 1 to 4 consisted of pC, Up, Gp and Ap, respectively. Peak 5 was mostly Ap. Aliquots of 2.0 ml, from test tubes 30, 48, 58, 78, 100, 115 and a 4.0 ml aliquot from test tube 90 were lyophilized, redissolved in a minimum of distilled water and chromatographed on thin layer cellulose plates in saturated ammonium sulphate, isopropanol, 0.10 M NaOAc, pH 6.0, (80:2:18). The 2'- and 3'-isomers of Up were not clearly separated. Peak 3a (tube 78) was G-3' phosphate and peak 3b (tube 90) was G-2' phosphate. Peak 4a (tube 100) was A-3' phosphate and peak 4b
FIGURE 4. Chromatography of monoribonucleotides on BDEAE-cellulose.

OPTICAL DENSITY (260 μm)

-901-
(tube #115) contained A-3' phosphate and A-2' phosphate. The purine-2' phosphate isomers ran ahead of the purine-3' phosphate isomers. When 5 mg samples of the 5' isomers of the four common ribomononucleotides were chromatographed under the conditions described above (except the linear gradient was 0.01 to 0.04 M NaCl) the elution profile shown in Figure 5 was obtained.

4. Recovery of s-RNA from Column Eluates

Method 1: In the early stages of this study, the desired fractions from the column chromatography of mixed s-RNA were consolidated, reduced in volume by evaporation at about 30°, under reduced pressure, and precipitated in the cold with 2 to 3 volumes of ethanol. Fractions obtained from the upper end of the gradient (i.e., high salt concentrations) were concentrated, dialyzed and concentrated further before precipitating with cold ethanol. Magnesium chloride was routinely added to a concentration of about 0.01 M to those fractions obtained from columns run in the presence of EDTA. Precipitations were usually carried out at pH 5. The precipitates were collected by centrifugation, redissolved in a small volume of 1 M NaCl, 0.01 M MgCl₂ and precipitated again with cold ethanol. The resulting pellets were washed with cold ethanol then with ethyl ether, dried at room temperature and stored in the cold as a dry powder. Portions were dissolved in distilled water for the purpose of assaying and these were stored in the frozen state at about -18°.
FIGURE 5. Chromatography of monoribonucleotides on BDEAE-cellulose.
Method 2: Fractions from the column chromatography of mixed s-RNA containing the s-RNA acceptor activity of interest, were consolidated and concentrated to a small volume by ultrafiltration (Blatt et al., 1965) on an Amicon Diaflo apparatus (Amicon Corp., Cambridge, Mass.) equipped with a UM-1 membrane, at 0-5°C. The cold concentrate was precipitated as described above, with attention to the presence of magnesium and a sufficiently high ionic strength to effect quantitative precipitation of the t-RNA (Razzell, 1963). The precipitate was collected by centrifugation, washed twice with cold ethanol then with ethyl ether and dried at room temperature.

5. Preparation of Yeast Aminoacyl-s-RNA Synthetase Enzymes

Method 1: Preparation of the activating enzymes by grinding the yeast cells with glass beads in a Virtis "45" homogenizer, followed by centrifugation and dialysis, is described in Part III, Section 1.

Method 2: Preparation of the aminoacyl-s-RNA synthetase enzymes by toluene lysis at 37°C under conditions of controlled pH, followed by centrifugation and gel filtration is described in Part III, Section 2.

Method 3: This preparation, involving the lysis of yeast cells at low temperature and partial purification by chromatography on hydroxyapatite is described in Part III, Section 3.
6. **Assay of Column Fractions for Specific Amino Acid Acceptor Activity**

**Method 1:** Stock solutions of the s-RNA fractions to be assayed were prepared as described in Section 4 (Method 1) and contained between 50 and 100 O. D. units of s-RNA per ml. The incubation mixture (final volume 0.5 ml) contained 120 μmoles of Tris(chloride), pH 7.4, 2 μmoles of sodium ATP, 1 μmole of sodium CTP, 7.7 μmoles of MgCl₂, 15 μmoles of KCl, 0.4 μmole of EDTA, 0.37 μmole of mercaptoethanol, 0.5 μCi and 40-50 nμmoles of ^14C-L-amino acid (unless otherwise noted), about 4 O. D. units of s-RNA and about 0.10 ml of aminoacyl-s-RNA synthetase enzymes prepared in the Virtis "45" homogenizer (see Section 5, Method 1). The blank contained distilled water in place of s-RNA. The controls, assayed simultaneously, contained 4 and 2 O. D. units of mixed s-RNA. After incubating at room temperature for 60 min the incubation mixture was placed in ice and treated according to one of the two following procedures:

a) **Ethanol precipitation** - to the incubation mixture was added 0.15 ml of cold 5 M NaCl, 0.05 ml of carrier-RNA (30 mg/ml), and 1.5 ml of cold ethanol with thorough mixing. After 10 min in ice, the mixture was centrifuged, the supernate decanted and the pellet washed three times with 0.3 ml of cold ethanol-NaCl solution (2:1, ethanol : 1.5 M NaCl). Finally the pellet was dissolved in 0.20 ml of 1.5 N NH₄OH and a 25 μl aliquot applied to a planchet, dried and counted in a windowless gas-flow counter.
b) **TCA precipitation** - to the incubation mixture was added 0.15 ml of cold 5 M NaCl and 1.5 ml of cold ethanol. After leaving in ice at least 10 min, the mixture was poured into a Millipore apparatus containing a few mls of cold 10% TCA and equipped with a 2.5 cm (diam.) Whatman 1 filter disk. The incubation vessel was rinsed twice with cold, 10% TCA and the rinses added to the Millipore apparatus. Suction was applied, and the filter disk washed twice with 10% TCA. Total washing volume was 50 to 60 ml of cold, 10% TCA. The filter disk was removed, glued to a planchet, dried and counted as in (a) above.

**Method 2**: The following method was based on the assay procedure developed by Cherayil and Bock (1965).

The amino acid acceptor assays were performed on Whatman 3MM, 2.3 cm diam. paper disks. Aliquots of 100 µl each from the column fractions were added to the numbered paper disks. The disks were allowed to dry until the wet sheen was gone and then transferred to a vessel containing cold 75% ethanol, 0.03 M KCl (about 10 ml of wash solvent per paper disk) for about 10 min with occasional stirring. The disks were washed a second time in the same solvent then the excess solvent was removed by suction filtration and the disks dried at room temperature. The dry disks were suspended on stainless steel pins anchored in a block of styrofoam. A 100 µl aliquot of an incubation mixture, described below, was added to the suspended disks. The
styrofoam block was then placed in a polyethylene box lined with wet paper towels or in a glass box containing water and the box sealed to maintain a humid atmosphere during the incubation period. The incubation mixture contained per 100 µl, 50 µl of the appropriate activating enzyme solution and 50 µl of a solution containing 3.7 µmoles of MgCl₂, 6.7 µmoles of KCl, 27 µmoles of Na cacodylate, pH 7.3, 0.5 µmole of sodium ATP, 0.1 µmole of sodium CTP, 0.12 µmole of mercaptoethanol, 0.1 µmole of EDTA, 75 mmol of glycine and 2.68 µCi per µM of ¹⁴C-glycine. The solution of activating enzymes which was stored at about -18° in 40% glycerol, was diluted 1:1 with distilled water before adding it to the incubation mixture.

After incubating for 30 min the disks were removed and washed at 0° for a minimum of 10 min, with occasional stirring, in each of the following baths, two consecutive baths of 10% TCA, one ethanol bath and finally an ethyl ether bath. Each bath contained 10 ml of solvent per paper disk except the ether bath which contained 3 to 5 ml of solvent per disk. The disks were then spread on paper towels and dried with the aid of a heat lamp. The radioactivity was determined in a scintillation spectrometer by suspending the disks in 5 ml of scintillation fluid containing 3 g of PPO and 0.3 g of dimethyl POPOP per litre of reagent toluene.
7. Chromatography of Yeast s-RNA on BDEAE-Cellulose under Conditions of Varying NaCl and/or MgCl₂

A. NaCl Gradient in the Presence of 0.10 M MgCl₂, pH 5.0

The BDEAE-cellulose column (75.5 x 2.5 cm diam.) was equilibrated with 2 litres of 0.10 M MgCl₂, 0.005 M NaOAc, pH 5.0, then 600 mg of s-RNA (Calbiochem.) dissolved in 35 ml of the starting buffer was added. The column was developed with an 8 litre linear gradient of NaCl from 0 to 0.45 M, both vessels containing 0.10 M MgCl₂ and 0.005 M NaOAc, pH 5.0. The flow rate was about 1 ml per min and 15.2 ml fractions were collected. The elution profile was divided into fractions (I - XI), as shown in Figure 6, and assayed for acceptor activity toward glycine and histidine according to Method 1-a (Section 6). The radioactivity data are expressed in Figure 6 as cpm per O. D. unit of s-RNA. Fractions I to V were 1.4, 1.8, 3.9, 2.7, and 1.7 fold enriched, respectively, for glycine acceptor activity while Fractions VIII to X were 3.3, 2.2 and 1.3 fold enriched respectively, for histidine acceptor activity.

B. NaCl Gradient in the Presence of 0.02 M MgCl₂, pH 5.0

The BDEAE-cellulose column (91 x 3.5 cm diam.) was equilibrated with several column volumes of 0.10 M NaCl, 0.02 M MgCl₂, 0.005 M NaOAc, pH 5.0, then 1.00 g of s-RNA (Calbiochem.), dissolved
in the presence of 0.10 M MgCl2, pH 5.0.

FIGURE 6. Fractionation of baker's yeast s-RNA on DEAE-cellulose using a NaCl gradient.
in 150 ml of starting buffer, was added. The column was developed with an 8 litre linear gradient of NaCl between 0.10 and 1.0 M, both vessels containing 0.02 M MgCl₂ and 0.005 M NaOAc, pH 5.0. The flow rate was about 1 ml per min and 18.5 ml fractions were collected. The elution profile was divided into Fractions I to XV, as shown in Figure 7, and the fractions assayed for glycine, histidine and alanine acceptor activity according to Method 1-a. The radioactivity data are expressed in Figure 7 as cpm per O. D. unit of s-RNA. The specific activity of the ¹⁴C-amino acids in the assays were: alanine and glycine, 1.1 μCi per μM; histidine, 10 μCi per μM. Fractions I, II, III, V and VI were 9.1, 11, 1.7, 3.3 and 1.6-fold enriched respectively in glycine acceptor activity. Fractions X, XI, XII, and XIII were 1.2, 4.6, 4.0 and 1.9 fold enriched respectively in histidine acceptor activity. Fractions III, IV, V, VI and VII were 4.3, 2.6, 6.2, 2.2 and 1.3 fold enriched respectively in alanine acceptor activity.

C. NaCl Gradient in the Presence of 0.005 M MgCl₂, pH 5.0

The BDEAE-cellulose column (89.2 x 3.5 cm diam.) was equilibrated with several column volumes of 0.10 M NaCl, 0.005 M MgCl₂, 0.005 M NaOAc, pH 5.0, then 1.00 g of s-RNA (Calbiochem., 14, 250 O. D. units) dissolved in 150 ml of starting buffer was added. The column was developed with an 8 litre linear gradient of NaCl between 0.10 and 1.0 M, both vessels containing 0.005 M MgCl₂, and 0.005 M
FIGURE 7. Fractionation of baker's yeast s-RNA on BDEAE-cellulose using a NaCl gradient in the presence of 0.02 M MgCl₂, pH 5.0.

OPTICAL DENSITY (260μm)
cpm per O.D. unit x10⁻²
NaOAc, \( \text{pH} \) 5.0. The flow rate was 88 ml per hr and fractions of 18.6 ml each were collected. The elution profile was divided into Fractions I to XVI, as shown in Figure 8. Fractions I to XVI were assayed for acceptor activity toward glycine, valine and phenylalanine. Glycine and phenylalanine acceptor activities were assayed by Method 1-a and valine acceptor activity was assayed by Method 1-b. The specific activities and concentrations of the \(^{14}\text{C}-\text{amino acids} in the assays were: glycine, 8.25 \( \mu \text{Ci} \) per \( \mu \text{M} \) and 77 \( \mu \text{moles} \); phenylalanine, 11.1 \( \mu \text{Ci} \) per \( \mu \text{M} \) and 47.4 \( \mu \text{moles} \); valine, 5.56 \( \mu \text{Ci} \) per \( \mu \text{M} \), and 48 \( \mu \text{moles} \). Fractions I, II, III, IV and V were enriched in glycine acceptor activity 11.2, 3.3, 3.3, 4.1, and 2.2 fold respectively. Fractions II, III, IV, V and VI were enriched in valine acceptor activity 3.6, 5.4, 4.1, 1.7 and 2.0 fold respectively. The only fraction exhibiting significant acceptor activity toward phenylalanine was fraction XII (0.8 fold). Fractions I to XVI represented 83\% of the applied O. D.

D. \( \text{NaCl} \) Gradient in the Presence of \( 10^{-3} \) M EDTA, \( \text{pH} \) 5.0

The BDEAE-cellulose column (90 x 3.5 cm diam.) was equilibrated with several column volumes of 0.3 M NaCl, \( 10^{-3} \) M EDTA, 0.005 M NaOAc, \( \text{pH} \) 5.0, then 1.00 g of s-RNA (Calbiochem., 16, 500 O. D. units), dissolved in 150 ml of starting buffer, was added. The column was developed with an 8 litre linear gradient of NaCl from 0.3 to 1.1 M, both vessels containing \( 10^{-3} \) M EDTA and 0.005 M NaOAc, \( \text{pH} \) 5.0.
FIGURE 8: Fractionation of baker's yeast 3'-RNA on DEAE-cellulose using a NaCl gradient in the presence of 0.005 M MgCl$_2$, pH 5.0.

Fraction No. (18.5 ml)

cpm per O.D. unit: gly and phe $\times 10^{-3}$, val $\times 10^2$
The flow rate was about 1 ml per min, and fractions of 18.6 ml each were collected. The elution profile was divided into Fractions I to XIV, as shown in Figure 9, and assayed for acceptor activity toward glycine, according to Method 1-a. The specific activity of the $^{14}$C-glycine in each assay was 1.1 $\mu$Ci per $\mu$M. Fractions I, II, III and IV were enriched in glycine acceptor activity 5.5, 2.6, 2.9 and 2.6 fold, respectively. Fractions I to XIV represented 72% of the applied material.

E. MgCl$_2$ Gradient in the Presence of 0.10 M NaCl, pH 5.0

The BDEAE-cellulose column (90 x 2 cm diam.) was equilibrated with 2 litres of 0.02 M MgCl$_2$, 0.10 M NaCl, 0.005 M NaOAc, pH 5.0, then 300 mg of s-RNA (Calbiochem.), dissolved in 50 ml of starting buffer, was added. The column was developed with a 6 litre linear gradient of MgCl$_2$ between 0.02 and 0.45 M, both vessels containing 0.10 M NaCl and 0.005 M NaOAc, pH 5.0. The flow rate was 1 ml per min and fractions of 19 ml each were collected. The elution profile was divided into Fractions I to XII, as shown in Figure 10, and assayed for acceptor activity toward glycine and histidine according to Method 1-a. Fractions I to VII were enriched in glycine acceptor activity 1.2, 1.5, 1.4, 2.6, 2.7, 2.9 and 2.3 fold, respectively. Fractions X to XII were enriched in histidine acceptor activity 1.2, 4.8 and 3.0 fold, respectively.
FIGURE 9. Fractionation of bakers' yeast s-RNA on BDAE-cellulose using a NaCl gradient in the presence of 10-3 M EDTA, pH 5.0.

OPTICAL DENSITY (260 nm)
and cpm per OD unit x 10^{-2} (gly)
Figure 10. Fractionation of baker's yeast s-RNA on DEAE-cellulose using a MEGA2 gradient in the presence of 0.1 M NaCl, pH 5.0.

Fraction No. (19ml)

Optical Density (260nm)

cpm per O.D. unit x 10^3

Control gradient

end of gradient
F. Pulse Gradient of NaCl in the Presence of 0.005 M MgCl$_2$, pH 5.0

(i) Chromatographic Method

The apparatus used to supply pulses of increasing concentrations of NaCl is shown in Figure 11. The reservoir and mixing vessel were arranged in the usual way for a linear gradient of increasing NaCl concentration. The solution from the mixing vessel passed through an Accu-Flo pump (Beckman Instruments), which controlled the flow rate, into a vessel equipped with a removable syphon (Syphon vessel). The syphon used in the following experiment delivered 200 ml volumes to the column reservoir vessel. The air spaces in the syphon vessel and column reservoir vessel were joined by means of Tygon tubing. The closed system was necessary so the pump could control the flow rate of the column. Prior to applying the pulse gradient to the column, the syphon vessel was allowed to fill and flow into the column reservoir, then valve 'A' was closed and the system connected to the chromatographic column. The main reservoir contained 4 litres of 1.0 M NaCl and the mixer contained 4 litres of 0.10 M NaCl. Both vessels contained 0.005 M MgCl$_2$ and 0.005 M NaOAc, pH 5.0.

To the BDEAE-cellulose column (114 x 3.0 cm diam.), previously equilibrated with 0.10 M NaCl, 0.005 M MgCl$_2$, 0.005 M NaOAc, pH 5.0, was added 1.00 g of s-RNA (Calbiochem., 14,300 O. D. units), dissolved
FIGURE 11. Apparatus for supplying pulse gradients during column chromatography.
in 150 ml of starting buffer. After washing the column with 50 ml of starting buffer, the pulse gradient was applied. The flow rate was about 1 ml per min and 14.3 ml fractions were collected, except for the methoxyethanol eluate (see Fig. 12) which was collected as 11.2 ml fractions.

The elution profile was divided into Fractions I to XVI as shown in Figure 12 and assayed for amino acid acceptor activity toward glycine and phenylalanine by Method 1-a. Specific activities and concentrations of the $^{14}$C-amino acids in the assays were: glycine, 4.45 $\mu$Ci per $\mu$M and 120 $\mu$moles; phenylalanine, 11.1 $\mu$Ci per $\mu$M and 47.4 $\mu$moles. Fractions I, II, III, IV and V were enriched in glycine acceptor activity, 9.3, 9.0, 4.1, 2.7 and 1.3 fold respectively. Fractions XV and XVI were similarly enriched 2.8 and 4.3 fold, respectively in phenylalanine acceptor activity. Over 95% of the applied material was recovered, while about 12% of the recovered material was eluted in the methoxyethanol Fractions XV and XVI.

(ii) Determination of the Melting Curves

The melting profiles ($T_M$'s) were determined on Fractions I, V, IX, XIII, XVI and on mixed s-RNA in a Gilford, Model 2000 spectrophotometer. A cuvette containing 3.0 ml of 0.005 M Tris(chloride), pH 7.4, 0.005 M EDTA, and 0.006 M MgCl$_2$ was degassed under vacuum, then about 1 O. D. unit of s-RNA was added and the cuvette placed in the
FIGURE 12. Pulse gradient elution of bakers' yeast s-RNA from BDEAE-cellulose. NaCl gradient in the presence of 0.005 M MgCl₂, pH 5.0.
heating chamber of the Gilford spectrophotometer. The temperature was increased at the rate of $1^\circ \text{C}$ per min. The $T_M$ curves of Fractions I, V, IX and XIII showed clear inflections with melting occurring between 55 and 85$^\circ$. Fraction XVI and the mixed s-RNA sample showed early melting starting at about 35 - 40$^\circ$. The $T_M$'s of all the samples were 68 - 70$^\circ$.

(iii) G-100 Sephadex Chromatography of Fractions XV and XVI

A G-100 Sephadex column (40 x 2.5 cm diam.) was equilibrated with 1.0 M NaCl. Fractions XV and XVI were each dissolved in 1.0 ml of 1.0 M NaCl and chromatographed, separately, on the column. The flow rate was 0.35 ml per min. The elution profiles are shown in Figure 13-A and B (Fractions XV and XVI respectively). The elution profile of Fraction XV showed that 34% of the O. D. (test tubes 19 to 36) was not s-RNA, while that of Fraction XVI showed that 27% of the O. D. (test tubes 18 to 34) was not s-RNA. The phenylalanine acceptor activity of the major peak from Fractions XV and XVI (peak a in Figures 13-A and B) was doubled. Peak b from Fraction XVI showed no acceptor activity toward phenylalanine.

G. Preparative Column Chromatography of s-RNA on BDEAE-Cellulose

Chromatography of 5 g of brewers' yeast s-RNA, as described below, was carried out by Dr. I. C. Gillam.
Figure 13. Gel filtration of fractions Xv (Fig. 13-A) and XvI (Fig. 13-B) on Sephadex G-100.

OPTICAL DENSITY (260m\textmu )

FRACTION NO. (2.8ml)

0 10 20 30 40 50 60 70 80

Blue Dextran
The BDEAE-cellulose column (110 x 3.2 cm diam.) was equilibrated with 0.45 M NaCl, 0.01 M MgSO₄, then 5.0 g of s-RNA (70,000 O.D. units) dissolved in 200 ml of 0.45 M NaCl, 0.01 M MgSO₄ was added. The column was developed with a 10 litre linear gradient of NaCl from 0.45 to 1.0 M, both vessels containing 0.01 M MgSO₄. When the linear NaCl gradient had finished, the column was washed with 1 M NaCl, 0.01 M MgSO₄ 10% methoxyethanol (v/v). The flow rate was maintained at 80 ml per hour with the aid of an Accu-Flo pump and fractions of about 20 ml each were collected. The elution profile is shown in Figure 14. The column fractions were assayed for acceptor activity toward 20 amino acids, as described by Gillam et al., (1967). The position of elution of the glycine acceptor activity is also shown in Figure 14.

H. Rechromatography of the Glycine-I Fraction

(i) Chromatography at pH 3.5 in the Presence of 0.05 M MgCl₂

The BDEAE-cellulose column (119 x 1.2 cm diam.) was prepared and equilibrated as usual, then the glycine-I fraction from the 5 g preparative column was dissolved in 40 ml of starting buffer (2880 O.D. units) and added to the column. A 2 litre linear gradient of NaCl from 0.40 to 1.0 M was applied. Both vessels contained 0.05 M MgCl₂ and 0.005 M sodium formate, pH 3.5. Fractions of 11.4 ml each were
FIGURE 14: Preparative column chromatography of 5 6 of brewers' yeast s-RNA on DEAE-cellulose.

Tube Number

Nanomoles of C-glycine Esterified per ml of Eluate vs. NaCl (M)

Optical Density (260nm) vs. OD to 68
collected at a flow rate of 1.1 ml per min (Fig. 15). The column fractions were assayed for glycine acceptor activity according to Method 2 (Section 6) and the results are shown in Figure 15. Fractions 28 to 60, (378 ml, 1930 O. D. units) were combined (Fraction I, Fig. 15), and the s-RNA isolated by Method 2 (Section 4).

(ii) Chromatography at pH 4.0 in the Presence of $10^{-3}$ M EDTA

Fraction I (Fig. 15) was dissolved in 10.6 ml of starting buffer (1880 O. D. units) and applied to a BDEAE-cellulose column (119 x 1.2 cm diam.), previously equilibrated with 0.45 M NaCl, $10^{-3}$ M EDTA and 0.005 M sodium formate, pH 4.0. A 2 litre linear gradient of NaCl from 0.45 to 1.2 M was applied. Both vessels contained $10^{-3}$ M EDTA and 0.005 M sodium formate pH 4.0. The flow rate was 1.1 ml per min and 11 ml fractions were collected (Fig. 16). The column fractions were assayed for glycine and methionine acceptor activity according to Method 2 (Section 6) and the results are shown in Figure 16. Fractions 86 to 98 were 95 to 100% glycine-I s-RNA. Fractions 78 to 100 were combined (253 ml, 970 O. D. units) and the s-RNA was isolated by Method 2 (Section 4) to yield 45.7 mg of white powder.

J. Rechromatography of the Glycine-II Fraction

(i) Chromatography at pH 5 in the Presence of 0.01 M MgCl$_2$

The BDEAE-cellulose column (119 x 1.2 cm diam.) was prepared
FIGURE 15. Chromatography of Glycine-1 fraction on BDEAE-cellulose in the presence of 0.05 M MgCl$_2$, pH 3.5.
FIGURE 16. Chromatography of Fraction 1 (F16) on DEAE-cellulose in the presence of 10 mM EDTA, pH 4.0.
and equilibrated as usual, then the glycine-II fraction from the 5 g preparative column was dissolved in 30 ml of starting buffer (6870 O. D. units) and added to the column. A 2 litre linear gradient of NaCl, from 0.40 to 1.0 M, with both vessels containing 0.01 M MgCl₂ and 0.005 M NaOAc, pH 5.0, was applied. The flow rate was 1 ml per min and fractions of 10 ml each were collected and assayed for glycine acceptor activity by Method 2 (Section 6). The results are shown in Figure 17. Fractions 55 to 90 were combined (Fraction I) and the s-RNA isolated by Method 2 (Section 4) for rechromatography at pH 3.5.

(ii) Chromatography at pH 3.5 in the Presence of 10⁻³ M EDTA

The s-RNA from Fraction I (Fig. 17) was dissolved in 12 ml of starting buffer (4800 O. D. units) and applied to a BDEAE-cellulose column (119 x 1.2 cm diam.), previously equilibrated with 0.45 M NaCl, 10⁻³ M EDTA, 0.005 M sodium formate, pH 3.5. The column was developed with a 2 litre linear gradient of NaCl, from 0.45 to 1.2 M, with both vessels containing 10⁻³ M EDTA and 0.005 M sodium formate, pH 3.5. Fractions of 10.5 ml each were collected at a flow rate of 1 ml per min. The column fractions were assayed for acceptor activity towards glycine and alanine according to Method 2 (Section 6). The results are shown in Figure 18. Column fractions 125 to 205 were combined (855 ml, 1639 O. D. units) and the s-RNA isolated by Method 2 (Section 4) to yield 93.3 mg of white powder (Glycine-II-β fraction).
in the presence of 0.01 M NaCl, pH 5.0.

FIGURE 11. Chromatography of Glycine-II fraction on DEAE-cellulose
Figure 18. Chromatography of Fraction I (Fig. 17) on DEAE-cellulose in the presence of 10 mM EDTA, pH 3.5.
PART II-D. A COMBINED CHEMICAL AND CHROMATOGRAPHIC
METHOD FOR THE ISOLATION OF AMINO ACID-
SPECIFIC s-RNA'S

1. Preparation of the Acylating Agent

The N-hydroxysuccinimide ester of phenoxyacetic acid was
prepared by Dr. G. M. Tener according to a general procedure for the
preparation of the N-hydroxysuccinimide esters of acyl amino acids
described by Anderson et al. (1964).

2. Experiment I

A. Conditions for Maximum Charging of s-RNA with Glycine

Several assays were set up, each containing the same concentra-
tion of co-factors and synthetase enzymes but varying amounts of s-
RNA. In this way the conditions for maximal charging of the s-RNA
were established. Thus, the aminoacyl-s-RNA used in the following
sections was prepared under conditions in which a linear relationship
existed between the s-RNA concentration and the amount of amino acid
incorporated.

B. Preparation of $^{14}$C-Glycyl-s-RNA

A 50 mg sample of s-RNA, enriched 6 fold in glycine acceptor
activity, was obtained from the glycine-I peak following chromatography
on BDEAE-cellulose as described in Part II, Section 7-G.

The reaction mixture (final volume 6.5 ml), contained per ml of final volume, 43.5 μmoles MgCl₂, 87 μmoles KCl, 180 μmoles sodium cacodylate, pH 7.3, 2.03 μmoles sodium ATP, 0.615 μmole sodium CTP, 1.15 μmoles mercaptoethanol, 0.615 μmole EDTA, 3.85 μmoles sodium phosphate, pH 7.5, 11% glycerol (v/v), 128 O. D. units of s-RNA, 6.16 μmoles glycine, 1.23 μCi ¹⁴C-glycine and 3.6 mg protein containing aminoacyl-s-RNA synthetase enzymes prepared as described in Part III, Section 2-D. (The enzymes had been stored in 40% glycerol (v/v), 10⁻² M sodium phosphate and 10⁻³ M mercaptoethanol). The mixture was incubated at 25° for 50 min. The reaction was followed by removing 20 μl aliquots at intervals, applying them to Whatman 3MM filter disks and washing the disks twice in cold, 10% TCA, once in cold ethanol and finally in ethyl ether. The disks were dried and counted in a scintillation spectrometer. The reaction was stopped after the 50 min incubation period by placing in ice and adding 1.0 ml 0.5 N sodium formate, pH 3.0, 2.0 ml 5 M NaCl and 25 ml ethanol, all solutions pre-cooled in ice. The pellet, obtained by centrifugation, was washed three times with 2 ml of cold ethanol : salt solution (2:1, ethanol : 1.5 M NaCl). The pellet was dissolved in 15 ml of 0.3 M NaCl, 0.01 M MgCl₂, 0.005 M NaOAc, pH 4.5, at 0°, and the solution extracted with 7 ml of water-saturated phenol. The aqueous layer was removed after centrifugation and the phenol layer was extracted twice with cold buffer. The washings were
added to the aqueous layer which was then extracted with ethyl ether. The s-RNA in the aqueous layer was precipitated by the addition of two volumes of cold ethanol. The precipitate was collected by centrifugation and the resulting pellet washed three times with ethanol: salt solution.

C. Derivatization of $^{14}$C-Glycyl-s-RNA

A portion of the previously prepared $^{14}$C-glycyl-s-RNA (400 O. D. units and 114,700 cpm) was dissolved in 5.0 ml of cold, 0.10 M triethanolamine hydrochloride, 0.02 M MgCl$_2$, pH 4.0. The derivatization was carried out in an ice bath by adding 0.20 ml of tetrahydrofuran, containing 10 mg of the acylating agent, to the cold solution and adjusting the pH to 8.0 with 1 N NaOH. The reaction was allowed to proceed for 10 min, then the pH of the solution was adjusted to 4.5 with cold 2 N acetic acid. The s-RNA was precipitated by the addition of 3 volumes of cold ethanol. The white precipitate, which appeared when the acylating agent was added, dissolved in the cold, aqueous ethanol, solution. After 20 min in ice the solution was centrifuged and the resulting pellet washed first with an ethanol: salt solution (2:1, ethanol: 1.5 M NaCl) then with ethanol. The pellet was drained free of excess ethanol, then dissolved in 5 ml of 0.3 M NaCl, 0.01 M MgCl$_2$, 0.005 M NaOAc, pH 4.5. The radioactivity in a 0.10 ml aliquot was determined as described in the next section. The recovery of radioactivity was 68%.
D. Chromatography of the N-(Phenoxyacetyl)-$^{14}$C-glycyl-s-RNA on BDEAE-Cellulose

The derivatized glycyl-s-RNA, dissolved in 5.0 ml of 0.3 M NaCl, 0.01 M MgCl$_2$, 0.005 M NaOAc, pH 4.5, was applied to a BDEAE-cellulose column (16 x 2.2 cm diam.), which had been previously equilibrated with the same buffer. The column was washed consecutively with the following solutions: 50 ml of 0.3 M NaCl, 200 ml 0.8 M NaCl then with 1.5 M NaCl, 10% ethanol. All the solutions were buffered at pH 4.5 with 0.005 M NaOAc and contained 0.01 M MgCl$_2$. Fraction volumes of 4.5 ml were collected except for the fractions from the 10% ethanol wash which were 2.8 ml each. A 0.10 ml aliquot from each fraction was added to 10 ml of modified Bray's solution and the radioactivity determined in a scintillation spectrometer. The modified Bray's solution consisted of 60 g naphthalene, 4 g PPO and 0.2 g dimethyl POPOP in 1 litre of dioxane. The O. D. of each fraction was also recorded. Both data are shown in Figure 19.

Fractions I and II (Fig. 19) consisted of 63.5% and 33.8% respectively, of the O. D. applied to the column. Fraction I (64 ml) and Fraction II (50 ml) were adjusted to pH 9 with 1.5 N NH$_4$OH and left 2 hr at room temperature. Both fractions were then concentrated to about 7 ml each in a Diaflo apparatus equipped with a UM-1 membrane. The s-RNA was precipitated in the cold with ethanol and collected by
FIGURE 19: Chromatography of N-(phenoxycetamido)-14C-Bicyl-s-RNA on BioEAE cellulose.
centrifugation. The pellets were washed twice with an ethanol - salt solution (2:1, ethanol : 1.5 M NaCl) then dissolved in 0.5 ml of distilled water and stored at about -18°C.

Fractions I and II were assayed for acceptor activity toward glycine. Unfractionated s-RNA was used as control. The incubation mixture (final volume, 150 µl) contained 3.7 µ moles MgCl₂, 6.7 µ moles KCl, 27 µ moles sodium cacodylate, pH 7.3, 0.33 µ mole sodium ATP, 0.10 µ mole sodium CTP, 0.10 µ mole EDTA, 0.17 µ mole mercaptoethanol, 75 µ moles glycine and 0.2 µCi ¹⁴C-glycine (2.67 µCi per µM), 46.3 O. D. units of mixed s-RNA or 12.7 O. D. units of Fraction I or 1.64 O. D. units of Fraction II, and 50 µl of the same crude synthetase enzyme preparation used in Section 2-B. The mixture was incubated at 25°C and 15 µl aliquots were removed at 4 min intervals. All three assays showed maximum incorporation of ¹⁴C-glycine in less than 8 min. Fraction II was 9.4 fold enriched and Fraction I was still 2.6 fold enriched in glycine acceptor activity.

Fraction II was rechromatographed on BDEAE-cellulose in the stepwise manner, described above. The 0.8 M NaCl eluted 93.6% of the applied material while the remaining 6.4% was eluted by the 1.5 M NaCl 15% ethanol (v/v). The s-RNA eluted by the 0.8 M NaCl wash was recovered and assayed for acceptor activity toward glycine as described above. Fraction II was 10.0 fold enriched in glycine acceptor activity.
Thus the acceptor activity was not significantly enriched by rechromatography.

3. **Experiment II**

   **A. Conditions for Maximum Charging of s-RNA with Glycine using a Partially Purified Glycyl-s-RNA Synthetase**

   Fraction 198 (from Part III, Section 6-B, Fig. 4), containing the glycyl-s-RNA synthetase activity was passed through a G-25 Sephadex column equilibrated with $10^{-2}$ M sodium phosphate, $10^{-3}$ M mercaptoethanol, $10^{-4}$ M EDTA, 40% glycerol (v/v), pH 7.5, to remove the excess ammonium sulfate. The eluate from the Sephadex column containing the glycyl-s-RNA synthetase activity contained 0.38 mg of protein per ml and was stored at -18°.

   Experiments were carried out using constant amounts of enzyme and cofactors while varying the amount of mixed s-RNA in the incubation mixture. The incubations were carried out in 1 ml glass tubes (final volume 300 µl) containing 3.3 µmoles MgCl$_2$, 6.6 µmoles KCl, 27 µmoles sodium cacodylate, pH 7.3, 0.33 µmole sodium ATP, 0.12 µmole mercaptoethanol, 0.10 µmole EDTA, 75 µmole glycine (2.68 µCi per µM), 19 µg protein (as described above), and s-RNA from 2.8 mg to 11.2 mg. The blanks contained distilled water in place of the s-RNA.
The incubations were carried out at 25° and 50 μl aliquots were removed at intervals and applied to Whatman 3MM paper disks. The disks were washed and counted as described in Section 2-B.

The synthesis of glycyl-s-RNA was not complete during the 30 min incubation period under any of the conditions studied. In addition, it appeared that the initial rates of glycyl-s-RNA synthesis decreased with increasing concentrations of s-RNA. This observation led to an investigation of the effect of magnesium ion concentration on the reaction velocity. Thus incubations were carried out as described above except they all contained 5.6 mg mixed s-RNA and the concentration of magnesium ions varied from 3.2 μmoles to 103 μmoles. Aliquots of 50 μl each were removed at 10, 20, 30 and 40 min, and applied to Whatman 3MM paper disks. The disks were washed and counted as described previously. The results are shown in Figure 20.

Another series of incubations were carried out to study the effect of magnesium in the range 0 to 28 μmoles per assay. The incubations were carried out as described above except that EDTA was added to obtain magnesium ion concentrations below 3.3 μmoles per assay. The results are shown in Figure 21, in which the cpm per 50 μl aliquot is plotted against effective magnesium ion concentration for incubation times of 10, 20 and 30 min. The effective concentration of magnesium ion was calculated by subtracting the concentration of EDTA from the
FIGURE 20. Effect of magnesium ions on the synthesis of $^{14}$C-glycyl-s-RNA (Range, 3.2 to 103 μmoles MgCl$_2$).
FIGURE 21. Effect of magnesium ions on the synthesis of $^{14}$C-glycyl-s-RNA (Range, 0 to 28 μmoles MgCl$_2$).
concentration of magnesium ions added to each incubation mixture. The results (Fig. 21) suggest that 0.5 to 0.7 \( \mu \)mole of magnesium is required per mg of s-RNA for maximum rate under the conditions studied.

Having established an optimal concentration of magnesium ion, the minimal amount of synthetase enzyme required to fully charge the mixed s-RNA with glycine was then determined as described in Section 2-A. The conditions were such that maximum glycyl-s-RNA synthesis was obtained in a 10 to 20 min incubation period.

B. Isolation of Glycine-Specific s-RNA

Step 1. Removal of Phenylalanine Acceptor s-RNA from mixed s-RNA

The s-RNA (ca. 5 g) was dissolved in 0.3 M NaCl, 0.005 M MgSO\(_4\), 0.005 M NaOAc, pH 4.5 and applied on BDEAE-cellulose column previously equilibrated with the same buffer. The column was then washed, first with about one column volume of the same buffer, then with 0.9 M NaCl, 0.01 M MgSO\(_4\), 0.005 M NaOAc, pH 4.5, and finally with 1.5 M NaCl, 0.005 M MgSO\(_4\), 0.005 M NaOAc, pH 4.5, 15% ethanol (v/v). The s-RNA removed by the 0.9 M NaCl wash was recovered by ethanol precipitation in the usual way and used in Step 2. The material eluted by the ethanol-salt wash contains all the phenylalanine acceptor activity.
Step 2. "Sham" Phenoxyacetylation

The pellet (ca. 4.5 g) obtained from Step 1 (eluted by 0.9 M NaCl) was dissolved in 125 ml of distilled water (including washings) and added to 200 ml of 0.10 M triethanolamine·HCl, pH 4.5. The solution was cooled to 0°, then 1 g of phenoxyacetylating agent, dissolved in 25 ml of THF was added. The pH of the solution was adjusted to 8.0 with 1 N NaOH and the mixture stirred for 20 min at 0°. The pH was then adjusted to 4.5 with acetic acid and the s-RNA collected by precipitation with 3 volumes of cold ethanol followed by centrifugation. The resulting pellet was dissolved in 0.3 M NaCl, 0.005 M MgSO₄, 0.005 M NaOAc, pH 4.5 and chromatographed on BDEAE-cellulose as described in Step 1. The s-RNA eluted by the 0.9 M NaCl (about 94% of the applied material) was recovered in the usual way and used in Step 3. The ethanol-salt wash removes the material which was readily phenoxyacetylated under the conditions described.

Step 3. Preparation of ¹⁴C-Glycyl-s-RNA

The reaction mixture (final volume 21 ml) contained 0.6 mmoles MgCl₂, 1.2 mmoles KCl, 4.8 mmoles sodium cacodylate, 60 μmoles sodium ATP, 18 μmoles EDTA, 22 μmoles mercaptoethanol, 27.7 μmoles glycine (1.27 μCi per μM), 1.00 g of s-RNA previously treated as described in Steps 1 and 2 and 2.7 mg of protein prepared as described
in Section 3-A. The incubation was carried out in a 150 ml Corex centrifuge bottle at 30°.

Aliquots of 50 μl each were removed at intervals during the incubation and applied to Whatman 3MM filter disks. The disks were washed and counted as described in Section 2-B.

After 40 min, the incubation mixture was placed in ice and the pH of the solution adjusted to 4.5 with cold, 2 N acetic acid. The solution was then extracted with 20 ml of water-saturated phenol. Following centrifugation, the phenol layer was removed and washed twice with a few mls of 0.3 M NaCl, 0.01 M MgCl₂. The washings were added to the aqueous layer which was subsequently extracted with ice-cold ethyl ether.

**Step 4. Phenoxyacetylation of ¹⁴C-Glycyl-s-RNA**

To the cold aqueous layer obtained in Step 3 was added 5 ml of THF containing 1 g of phenoxyacetylation agent. The pH of the solution was adjusted to 8.0 by the careful addition of 1 N NaOH and the mixture stirred for 10 min at 0°. The pH was then adjusted to 4.5 with cold, 2 N acetic acid and, after adding 2 ml of 5 M NaCl to the solution, the s-RNA was precipitated by the addition of 2.5 volumes of cold ethanol. The mixture was left at 0° for 20 min then centrifuged. The resulting pellet was washed, first with 15 ml of cold ethanol : salt solution (2:1,
ethanol : 1.5 M NaCl), then with 30 ml of ice-cold ethanol.

**Step 5. Isolation of the N-(Phenoxyacetyl)\(^{14}\)C-glycyl-s-RNA from the Mixture**

The pellet, obtained from Step 4, was dissolved in 50 ml of 0.3 M NaCl, 0.01 M MgCl\(_2\), 0.005 M NaOAc, pH 4.0 and the solution centrifuged to remove the unreacted, sparingly soluble phenoxyacetylated agent. The supernate was added to a BDEAE-cellulose column (24 x 3.5 cm diam.), previously equilibrated with the same buffer. The column was washed, first with about 150 ml of 0.3 M NaCl, 0.01 M MgCl\(_2\), 0.005 M NaOAc, pH 4.0, then with 1200 ml of 0.8 M NaCl, 0.01 M MgCl\(_2\), 0.005 M NaOAc, pH 4.0, and finally with 1.5 M NaCl, 0.01 M MgCl\(_2\), 0.005 M NaOAc, pH 4.0, 15% ethanol (v/v) until the absorbance of the eluate was less than 0.2 (about 300 ml). The flow rate was about 4 ml per min and 19 ml fractions were collected. The absorbance at 260 m\(\mu\) was recorded and the radioactivity in the column fractions was determined by adding a 0.10 ml aliquot to 10 ml of a modified Bray's solution and counting in a scintillation spectrometer.

The first two fractions of the O. D. peak eluted by the 0.8 M NaCl wash contained some unreacted \(^{14}\)C-glycyl-s-RNA. The two fractions were therefore combined (38 ml) and cooled to 0\(^\circ\). Then 1 ml of 0.10 M triethanolamine-HCl, pH 4.5 was added (to facilitate adjustment of the pH to 8) along with 1 ml of THF containing 50 mg of
phenoxyacetylating agent. The solution was adjusted to pH 8 with 1 N NaOH, stirred for 10 min at 0°, then the pH was readjusted to 4.5 with 2 N acetic acid. The solution was then combined with the fractions containing the radioactivity eluted by the ethanol - 1.5 M NaCl wash. The s-RNA was isolated from the combined solutions by precipitation with cold ethanol followed by centrifugation. The resulting pellet was rechromatographed on BDEAE-cellulose as described above except that 12.5 ml fractions were collected at a flow rate of about 3 ml per min. The elution profile and radioactivity data are shown in Figure 22. The non-phenoxyacetylated s-RNA (eluted by the 0.8 M NaCl wash) was recovered (8,190 O. D. units) in a manner analogous to that described below.

Fractions 83 to 96 inclusive were combined (175 ml, 1275 O. D. units, 1,113,000 cpm) and the pH of the solution was adjusted to 9 with 1.5 N NH₄OH. After 2 hrs at room temperature the pH was adjusted to 5 with acetic acid and the s-RNA solution concentrated to about 45 ml in a Diaflo apparatus equipped with a UM-1 membrane. The concentrate was cooled to 0° and the s-RNA isolated by precipitation with cold ethanol followed by centrifugation. After washing once with cold ethanol, the pellet was dissolved in 15 ml of 0.40 M NaCl, 0.01 M MgCl₂. Less than 0.1% of the radioactivity was retained. The solution was then chromatographed on BDEAE-cellulose as described in Step 6.
FIGURE 22. Chromatography of N-(phenoxyacetyl)-$^1^4$C-glycyl-s-RNA on BDEAE-cellulose.
Step 6. Chromatography of Glycine-s-RNA on BDEAE-Cellulose

The 15 ml solution of glycine-s-RNA obtained from Step 5 was applied to a BDEAE-cellulose column (103 x 1.2 cm diam.), previously equilibrated with 0.40 M NaCl, 0.01 M MgCl$_2$. After washing the column with a few ml's of the same buffer, a 2 litre linear gradient of NaCl from 0.40 to 0.80 M was applied. Both vessels contained 0.01 M MgCl$_2$. When the linear gradient had finished, the column was washed with 1.5 M NaCl, 0.01 M MgCl$_2$, 15% ethanol (v/v). The flow rate was 1 ml per min and 16 ml fractions were collected. The column fractions were assayed for acceptor activity toward glycine as described in Part II-C, Section 6, Method 2. The elution profile and assay data are shown in Figure 23.

The s-RNA from Peak I in Figure 23 was recovered and rechromatographed at pH 4.0 in the presence of 0.001 M EDTA as described in Part II-C, Section 7H. The s-RNA was eluted as a single peak in the elution profile. Assay of the column fractions, as described above, gave a single peak of glycine acceptor activity which coincided with the O. D. profile.
FIGURE 23. Chromatography of glycine acceptor RNA on BDEAE-cellulose.
DISCUSSION

The past six years or so have witnessed an ever increasing effort by many investigators to fractionate the complex mixture of closely related molecules called soluble-RNA. The difficulties presented by this family of RNA's have been discussed.

We wished to isolate species of s-RNA on which no other group was working. This meant the development of unique methods for the fractionation of s-RNA. A number of approaches were tried, most of which were unsuccessful.

The method described in Part II-A involves the immobilization of mercury on a cellulose support. A report by Eldjarn and Jellum (1963) described the preparation of an organomercurial-polysaccharide which was used as a chromatographic material for the separation and isolation of HS-proteins. The separation depended on the presence of a reactive sulfhydryl group in the protein, which could interact with the immobilized organomercurial, firmly anchored to crosslinked dextran.

In the present work, aminoethyl-cellulose was thiolated with N-acetylhomocysteine thiolactone (HTL). The use of the latter compound as a thiolating agent was first introduced by Benesch and Benesch (1958). Attempts to thiolate aminoethyl-cellulose with HTL in refluxing dioxane were unsuccessful. When a catalytic amount of
imidazole was included in the reaction mixture, a product was obtained which reacted only weakly with nitroprusside. Thiolation of proteins with thiolactones is known to occur much more readily in the presence than in the absence of imidazole (Klotz and Elfbaum, 1964). When the thiolation of aminoethyl-cellulose with HTL was carried out at room temperature, and in the presence of silver nitrate (Schwyzer and Huerliman, 1954; Benesch and Benesch, 1958) a product was obtained which reacted strongly with nitroprusside. The silver nitrate was added in increments with the simultaneous addition of sodium hydroxide. The silver nitrate reacts with HTL to form a sparingly soluble complex with the concomitant release of a proton. It is the silver complex which reacts with the amino groups on the cellulose (Benesch and Benesch, 1958). The alkali (used for neutralizing the released protons) must be added cautiously so that the pH of the solution does not exceed 9. Otherwise the silver ions will be reduced by the reducing end groups of the cellulose. The silver ions were readily removed from the thiolated product by washing it with a solution consisting of nitric acid and concentrated thiourea.

The number of HS- groups in the thiolated aminoethyl-cellulose was determined by titrating samples of the product with iodoacetic acid (Part II-A, Section 3). Iodoacetic acid or iodoacetamide has been used by many investigators for determining the number of HS- groups in protein. The method depends on the formation of carboxymethylthioether
which is accompanied by the release of a proton from the HS- group. Thus by carrying out the titration in a pH stat, the number of equivalents of alkali required to neutralize the protons released correspond to the number of equivalents of HS- groups reacting with the iodoacetic acid. Obviously then, one must know the approximate pK of the thiol group. An approximate $pK_{SH}$ was obtained in the present study by carrying out a series of titrations over a wide range of pH's (Part II-A, Section 3 and Figures 1-A, B, C and 2-A, B).

When aminoethyl-cellulose was thiolated with HTL in the presence of silver nitrate, the maximum degree of thiolation obtained was about 0.23 meq of HS- groups per gram of dry product.

A column of the thiolated aminoethyl-cellulose was readily converted to the organomercurial derivative by passing a solution of bis-3, 6-(acetatomercurimethyl)-dioxane through the column. Preliminary experiments, designed to test the behaviour of s-RNA on the organomercurial-cellulose, showed that the applied s-RNA was being retarded as a result of the ion-exchange properties of the derivatized aminoethyl-cellulose. When $^{14}$C-cysteinyl-s-RNA was chromatographed on this organomercurial-cellulose in the presence of 0.5 M sodium chloride, the radioactivity was eluted at the solvent front along with the rest of the s-RNA. The easiest explanation for this is that the concentration of sodium chloride required to overcome the ionic interactions was high
enough to compete effectively for the mercury. To get around the salt requirement it was decided to prepare a thiolated cellulose which did not have ion-exchange properties.

This was achieved by the procedures described in Part II-A, Sections 11 to 16 inclusive. The principle was to prepare the 6-O-tosyl-cellulose which could be converted to 6-S-acetyl-cellulose by a nucleophilic displacement reaction with potassium thiolacetate. Alkaline hydrolysis of the 6-S-acetyl-cellulose would then give the desired 6-deoxy-6-mercapto-cellulose. The maximum degree of substitution for the preparation of 6-O-tosyl-cellulose was obtained by activating the deacetylated cellulose acetate in aqueous pyridine (Malm et al., 1948; Whistler and Shasha, 1964) prior to tosylation. Sulfur analysis by the method of Lysyj and Zarembo, (1958), indicated a D. S. of 1.07. The tosyl-cellulose was readily converted to 6-S-acetyl-cellulose by reaction with potassium thioacetate. The 6-S-acetyl-cellulose reacted weakly with nitroprusside, as expected for thioesters and was converted to 6-deoxy-6-mercapto-cellulose by alkaline hydrolysis. The number of HS-groups in the mercapto-cellulose could not be accurately determined by titration with iodoacetic acid. Thus, even after 6 hr the carboxymethylated mercapto-cellulose still reacted strongly with nitroprusside.

When s-RNA was chromatographed on a column of mercapto-cellulose in 0.10 M sodium acetate, most of the applied material (75%)
was eluted at the solvent front while the remainder was eluted by about 0.03 M sodium chloride. Both peaks showed similar hyperchromic effects when digested with pancreatic ribonuclease and both had similar ultraviolet spectra. The mercapto-cellulose therefore, appeared to have the desired characteristics in that all the applied s-RNA could be recovered at very low salt concentrations. No further studies have been carried out on the mercapto-cellulose preparations except as the organomercurial-cellulose derivative.

The mercapto-cellulose was converted to organomercurial-cellulose, as described previously. When $^{14}$C-cysteiny1-s-RNA was chromatographed on the organomercurial-cellulose, using an increasing gradient of sodium chloride, the results were encouraging (Fig. 3). The first component to be eluted had spectral properties similar to cytidine. The second, and slightly larger peak, had the spectral properties of adenosine. The ratios of the first two peaks are what would be expected for the cytidine- and adenosine-5' triphosphates used in the preparation of the $^{14}$C-cysteiny1-s-RNA. This observation suggests that adenosine interacts with mercury more strongly than does cytidine, and is in agreement with the results of other investigations on the interaction of mercury with nucleic acids (see Davidson et al., 1965, and later discussion). The s-RNA was eluted as a sharp peak at about 0.3 M sodium chloride (Peak I, Figure 3). The nature of the material preceding peak I has not been investigated although the mixed s-RNA used in this
experiment is known to be contaminated with non-s-RNA material. No radioactivity could be detected until the column was washed with 0.4 M sodium chloride containing 0.01 M 2-mercaptoethanol (Peak II). Insufficient material was recovered in Peak II for further investigation.

The use of the organomercurial-cellulose described here, for the fractionation of nucleic acids as well as sulphydryl containing proteins, has not been investigated further. Plans were to use the method for isolating cysteine-specific s-RNA in large quantities suitable for structural work. In addition attempts were to be made to make the method more general by investigating procedures for thiolating aminoacyl-s-RNA's.

Studies on the interaction between nucleic acids and metal cations have been reported by a number of investigators. Early studies by Thomas (1954) and by Yamane and Davidson (1961) showed that DNA complexed with mercury. It was concluded from the spectral properties that the metal interacted with the bases in DNA.

Singer (1964), studied the complexes formed between tobacco mosaic virus and several multivalent cations (including mercury). This investigator found that slightly less than one mole of either silver or mercury were bound independently, and competition experiments suggested that 0.5 mole of each was bound on specific independent sites. Davidson et al. (1965) demonstrated that the amount of binding at a given concentration of mercury varied with the base composition of DNA, based on
Cs$_2$SO$_4$ density gradient centrifugation. These workers utilized cesium sulfate density gradient centrifugation in the presence of mercury for the separation of crab dAT polymer from the main DNA component.

Thus it would appear that organomercurial-cellulose might be useful for fractionation of nucleic acids by exploiting the interactions between mercury and the constituent nucleotides. Having an organomercurial-cellulose devoid of ion-exchange properties makes such a study feasible.

Further investigations on the use of organomercurial-cellulose for fractionating nucleic acids was discontinued when the potential of benzoylated diethylaminoethyl-cellulose for the fractionation of s-RNA was realized (see Part II-C).

The experiments reported in Part II-B utilize the differences in partition co-efficients of s-RNA molecular species in two-phase solvent systems. The advantages and disadvantages of column partition chromatography, compared to countercurrent distribution methods, have been mentioned in the Introduction to Part II.

Comments pertaining to the experiments described in Part II-B can be summarized by stating that, in general, the resolution of acceptor activities as well as the recovery of biological activity of s-RNA was poor. In fairness however, it should be pointed out that
other investigators have had considerably greater success in fractionating s-RNA by column partition chromatography (Tanaka et al., 1962; Bergquist and Scott, 1964; Muench and Berg, 1966a).

The reasons for the poor results described in Part II-B are by no means clear but probably reflect to a large extent, the use of Biogel as a support for the lower phase. Other workers, e.g. Tanaka et al. (1962), and Muench and Berg, (1966a) used G-25 Sephadex as a supporting medium for the stationary phase.

Of the many procedures reported for the fractionation of s-RNA (see Introduction) MAK (methylated albumin-kieselguhr) columns provide reasonably good resolution of amino acid acceptor activities (Sueoka and Yamane, 1962). The low capacity of these columns is their major disadvantage for fractionating s-RNA on a preparative scale.

As mentioned in the introduction to this part of the thesis, it was concluded that the forces responsible for the fractionation of s-RNA on columns of MAK were probably of a lipoidal nature resulting from the aromatic amino acid content of the albumin. It seemed reasonable to test the validity of this conclusion by converting DEAE-cellulose from a hydrophilic anion-exchanger to a lipophilic anion-exchanger. This was easily achieved by reacting the hydroxyl groups of the cellulose with benzoyl chloride (Section 1) to yield a product referred to here as benzoylated-DEAE-cellulose (BDEAE-cellulose). The product had a
saponification equivalent of 159 (Gillam et al., 1967) (tri-O-benzoyl-cellulose has a calculated saponification number of 158). BDEAE-cellulose, when prepared as described in Section 1, can be packed in chromatographic columns without the aid of air pressure and has excellent flow characteristics.

Column chromatography of s-RNA on BDEAE-cellulose is operationally simple. The s-RNA is dissolved in a small volume of starting buffer then applied to the top of the column packing previously equilibrated with the same buffer. After washing the column with a few ml of starting buffer a linear gradient of increasing salt concentration is applied.

A number of experiments were carried out on the fractionation of s-RNA using linear gradients of increasing concentrations of sodium chloride and constant concentrations of magnesium chloride or EDTA. It soon became apparent that the presence of magnesium ion had a profound affect on the elution profile.

When s-RNA was chromatographed on DEAE-cellulose using an increasing linear gradient of salt (see Part I, Fig. 1), about 90% of the O. D. was eluted between 0.55 and 0.7 M chloride ion concentration. When s-RNA was chromatographed on BDEAE-cellulose in the presence of EDTA (to chelate any multivalent metal ions present), the leading edge of the elution profile appeared at about 0.68 M chloride ion
concentration and only 70% of the applied material was recovered up to 1.1 M chloride ion concentration (Fig. 9). When magnesium ions are included in the eluting solvent at concentrations of 0.02 M and 0.005 M (Figs 7 and 8, respectively) the leading edge of the elution profile appears at a similar concentration of chloride ion (0.64 and 0.65 M respectively). However when the magnesium ion concentration in the eluting solvent is increased to 0.10 M (Fig. 6), or when the column is developed with an increasing gradient of magnesium chloride (Fig. 10), the leading edge of the elution profile appears between 0.3 and 0.4 M chloride ion concentration.

To determine if the spreading of the O. D. was a general phenomenon or if it truly reflected the fractionation of individual species of s-RNA, the elution profile was divided into fractions, then the s-RNA was recovered from the fractions and assayed for acceptor activity toward several amino acids. The results of these assays are shown in Figures 6 to 10. The separation of individual acceptor activities is clearly evident. It should be pointed out here that the sharpness of the acceptor profiles shown in Figures 6 to 10 depend in part on how one groups the column fractions for assay purposes. In later studies, the column fractions were assayed individually according to the rapid method reported by Cherayil and Bock (1965).
The position of elution of the glycine acceptor activity in the presence and absence of magnesium illustrates the profound influence of magnesium on the elution characteristics of s-RNA. Thus when magnesium ions were included in the eluting solvent at concentrations between 0.005 and 0.02 M, two peaks of glycine acceptor activity were observed (Figs 8 and 7 respectively). The first peak (glycine-I) appeared at the leading edge of the elution profile in both cases. The second peak of glycine acceptor activity (glycine-II) appeared further along the elution profile and in the presence of 0.02 M magnesium (Fig. 7) was clearly separated from the first.

When a magnesium chloride gradient was employed for elution (Fig. 10), or when 0.10 M magnesium chloride was included in the elution solvent (Fig. 6), only one broad peak of glycine acceptor activity was observed, and this appeared behind the leading edge of the elution profile. In addition, the histidine acceptor activity eluted closer to the glycine acceptor activity when magnesium chloride was used to develop the column (Fig. 10).

It appeared then, that magnesium ions were influencing the interactions between the exchanger and the different species of s-RNA molecules and that this influence was greater for some species than for others. It further appeared that those s-RNA molecules eluted later from the column were affected by magnesium to a greater extent than those
eluted early from the column. The effect of magnesium ions on the secondary structure of s-RNA is, of course, well documented in the literature (for example, see Part I of this thesis). It was anticipated that this differential effect of magnesium on the elution position of the acceptor activities would be a useful variable during rechromatography of fractions from a preparative column.

To further emphasize the differences in the degree of interaction between the exchanger and the various s-RNA species it should be noted that the phenylalanine acceptor activity was not eluted from BDEAE-cellulose even by chloride ion concentrations above 1.0 M. The acceptor profile for phenylalanine, shown in Figure 8, was only a fraction of the phenylalanine acceptor activity applied to the column and either represented multiple acceptor activities for phenylalanine or degraded phenylalanine s-RNA (missing the terminal adenosine moiety). The latter interpretation is favoured, for reasons which will be discussed below. To elute the major phenylalanine acceptor RNA it was necessary to include in the eluting solvent a lipophilic bond breaker such as urea, methoxyethanol, or ethanol, (see Fig. 12). When this step was included at the end of a sodium chloride gradient, 95-100% of the applied material was recovered.

Differences in the chromatographic behaviour of s-RNA on BDEAE-cellulose, compared to DEAE-cellulose have been mentioned.
The reasons for these differences may become clear when the nature of the interactions involved between the nucleotides and DEAE- or benzoylated-DEAE-cellulose is more fully understood.

Secondary binding forces (non-ionic) between oligonucleotides and DEAE-cellulose have been attributed primarily to hydrogen bonding between the ion-exchanger and the constituent nucleotides (Tomlinson and Tener, 1963a). Separation of mixed oligonucleotides on DEAE-cellulose has also been shown to be a function of the constituent purine-pyrimidine ratio (Bartos et al., 1963). These forces can be diminished by chromatography on DEAE-cellulose in the presence of 7 M urea (Tomlinson and Tener, 1963a, 1963b) or eliminated by chromatography on DEAE-Sephadex A-25 in the presence of 7 M urea (Rushizky et al., 1964). These forces were probably responsible for the slight resolution of rat liver leucyl-s-RNA on DEAE-cellulose (Nishiyama et al., 1961).

The impressive fractionation of s-RNA on DEAE-cellulose and DEAE-Sephadex reported by Cherayil and Bock (1965) was not a consequence of secondary interactions. These investigators used high concentrations of urea in their eluting systems and urea is known to reduce the secondary forces between nucleotides and DEAE-cellulose (Tomlinson and Tener, 1963a). It seems likely that urea opens the s-RNA structure allowing increased interaction between the constituent nucleotides and the ion-exchange groups of the cellulose.
A similar opening of the s-RNA structure occurs at elevated temperatures. This has been exploited by Baguley et al., (1965a, 1965b) and by Bergquist (1966) for the fractionation of yeast s-RNA. These workers fractionated s-RNA on DEAE-cellulose at 65° utilizing an exponentially increasing salt gradient to elute the s-RNA. They also showed that s-RNA could be fractionated on DEAE-Sephadex by employing a decreasing temperature gradient at constant salt concentration. As the temperature is lowered, the s-RNA molecules presumably renature, thus exposing fewer of the constituent nucleotides to the ion-exchanger. However secondary bonding forces might well play an important role in this method.

s-RNA molecules are similar in size and have similar numbers of phosphate dissociations. Thus it would be difficult to fractionate them by utilizing only their ion-exchange properties other than at acidic pH's where cytosine is protonated. The above discussion however, strongly suggests that the constituent nucleotides of mixed s-RNA are available for interaction with ion-exchangers to variable extents. In addition it was suggested that the forces primarily responsible for fractionation of s-RNA on MAK columns were a function of the constituent bases rather than the phosphate dissociations.

The forces involved in the fractionation of s-RNA on benzoylated DEAE-cellulose are probably hydrophobic in nature, resulting from the
interaction of the exposed nucleotides and the aromatic rings of the BDEAE-cellulose. The ion-exchange forces would be relegated to holding the s-RNA molecules in close contact with the cellulose matrix so that the relatively short range hydrophobic interactions can be effective.

That the secondary bonding forces in DEAE-cellulose and benzoylated DEAE-cellulose are different, is clear from the order of elution of the monoribonucleotides from BDEAE-cellulose (Figs 4 and 5). The order of elution in Figure 4 is pC, Up, Gp and Ap. Some separation of the 2' and 3' isomers is evident in Figure 4, with the 3' isomers eluting earlier than the 2' isomers. The order of elution of the monoribonucleotides from DEAE-cellulose is Cp, Up, Ap and Gp (Staehlin, 1961). Several comments can be made about the elution of the ribonucleoside-5' phosphates from BDEAE-cellulose (Fig. 5). Firstly, the pyrimidines, as a class, are less strongly held than the purines although the order of elution is the same as for the ribonucleoside-2'(3') phosphates. Secondly, the purine-5' phosphates (Fig. 5) are not as clearly separated as the purine-2'(3') phosphates (Fig. 4). Thirdly, the cytidine nucleotide elutes as the same salt concentration in both experiments (as it should since cytosine-5' monophosphate was used in both experiments), whereas the 5' phosphates of uridine, guanosine and adenosine all eluted much earlier in the salt gradient than their 2'(3') phosphate counterparts. It is difficult to see how the difference in the
elution profiles could be explained on stereochemical grounds. It is equally difficult to explain the differences in terms of electronic interactions between the 5' phosphate and the base moiety. If such an interaction existed in the ribonucleoside-5' phosphates to an appreciable extent, it should be reflected in the ultraviolet spectra of the isomers. No correlations could be detected.

During early studies on the chromatographic behaviour of s-RNA on BDEAE-cellulose it was found by conductivity measurements (Radiometer, Copenhagen) that the early part of the salt gradient was going through the column in a stepwise manner. This phenomenon occurred only when there was a large difference in densities between the mixer and the reservoir vessel (e.g., 0.1 to 1.0 M sodium chloride) and could be prevented by using capillary tubing for the U-tube, or by drawing out one side (the mixer side) of the U-tube to a 1 to 2 mm bore.

It was suspected that this pulsing phenomenon, observed when the mixer and reservoir were connected by 6 mm glass tubing, was partly responsible for the impressive fine structure of the elution profiles reproduced in Figures 7 and 8. On the suspicion that pulse gradients might give improved resolution of acceptor activities, an apparatus was devised which would deliver 200 ml pulses from a linear gradient of increasing sodium chloride (Fig. 11). The resolution of glycine acceptor activity, when s-RNA was chromatographed on BDEAE-cellulose using
the pulse gradient (Fig. 12) was no better than that obtained using a simple linear gradient. Thus the fine structure in the elution profiles of Figures 7 and 8 can be attributed mainly to the intrinsic superiority of BDEAE-cellulose rather than to the mechanics of elution.

As mentioned previously, the phenylalanine acceptor s-RNA was not eluted from BDEAE-cellulose unless a lipophilic bond breaking substance such as ethanol, methoxyethanol, urea, etc., was incorporated in the eluting solvent. When the column, described in Figure 12 was washed with 1.0 M sodium chloride, 15% methoxyethanol (v/v), via the pulse gradient device, three peaks of O. D. were eluted. When the peaks were assayed for acceptor activity toward phenylalanine, most of the activity was contained in the second and third peaks (Fractions XVI, Fig. 12). This observation suggests that the strongly held phenylalanine acceptor s-RNA might be eluted in high purity if chromatographed by an increasing gradient of ethanol or methoxyethanol in the presence of 1.0 M sodium chloride. However, unpublished experiments by Dr. I. Maxwell indicated that the use of an ethanol gradient gave no better purification than stepwise elution with ethanol.

When Fractions XV and XVI (Fig. 12) were examined by gel filtration (Figs 13-A and 13-B, respectively), 34% of the O. D. from Fraction XV and 27% of the O. D. from Fraction XVI did not show acceptor activity toward phenylalanine. It is interesting to note that
although the major peaks from the gel filtration studies (Peak a in Figs 13-A and B) represent 60% and 70% of the O. D. from Fractions XV and XVI respectively, they now accept twice as much phenylalanine per O. D. unit as did the same fractions before gel filtration. The nature of the material excluded by the G-100 Sephadex has not been investigated.

The melting profiles of the s-RNA from representative fractions (Fractions I, V, IX, XIII, and XVI, Fig. 12) from a BDEAE-cellulose column and a sample of mixed s-RNA all had the same $T_M$'s ('melting' temperatures). Fraction XVI, which was eluted by methoxyethanol, and the mixed s-RNA sample showed more early melting than those eluted by the salt gradient. However, it was not possible to detect significant differences in the secondary structure of the s-RNA's by this method.

As pointed out earlier in this discussion, magnesium ions seemed to affect the s-RNA molecules eluted late from the column, to a greater extent than those which were eluted earlier. That is, the order of elution of s-RNA from BDEAE-cellulose seemed to be a function of the exposed bases capable of interacting with the exchanger. It is further argued, that the number of interactions per s-RNA molecule is probably small and that they are hydrophobic in nature. It might be expected that the nucleotide triplet representing the anti-codon region of s-RNA would be among those exposed. This reasoning led, in the early studies, to a correlation between the order of elution of the s-RNA's and their
proposed anti-codon composition. Results of the chromatography of the mononucleotides added more weight to the correlation. However, attempts to place this correlation on a quantitative basis were unsuccessful. In addition, a detailed analysis of the acceptor activities eluted during preparative column chromatography of s-RNA (see Gillam et al., 1967) soon revealed exceptions to this hypothesis.

It is worth noting that the s-RNA used in the studies to this point was bakers' yeast s-RNA (Calbiochem.). Brewers' yeast s-RNA (Boehringer and Soehne) was used for the remainder of the study.

Chromatography of a 5 g sample of brewers' yeast s-RNA was carried out by Dr. I. Gillam in this laboratory. The detailed results of this and other studies will be published (Gillam et al., 1967). The elution profile and the regions of the glycine-I and glycine-II acceptor activities are shown in Figure 14. The glycine-I and the glycine-II fractions from this chromatography were used as a source of material for further investigations on the chromatographic behaviour of the glycine s-RNA's. The experimental procedures used for the rechromatography of the glycine-I and glycine-II peaks are summarized in Charts I and II, respectively.

The glycine-I peak (see Fig. 14, tube numbers 73 to 92) contained, in addition to glycine-I acceptor activity, major peaks of methionine and isoleucine acceptor activities and minor shoulders of valine and proline
CHART I

Fractionation of Glycine-I Acceptor RNA on BDEAE-Cellulose

5 g mixed brewers' yeast s-RNA

Chromatography 1
(Figure 14)

0.01 M MgSO₄, NaCl gradient, no buffer

(Tube 73 to 92 contain glycine-I, methionine, isoleucine, valine and proline acceptor RNA's)

Chromatography 2
(Figure 15)

0.05 M MgCl₂, NaCl gradient, pH 3.5

<table>
<thead>
<tr>
<th>Major Peak</th>
<th>Minor Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>glycine-I, methionine, isoleucine and valine or proline acceptor RNA's</td>
<td>valine or proline acceptor RNA</td>
</tr>
</tbody>
</table>

(Fraction I, Fig. 15)

Chromatography 3
(Figure 16)

1 mM EDTA, NaCl gradient, pH 4.0

<table>
<thead>
<tr>
<th>Major Peaks</th>
<th>Minor Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>methionine acceptor RNA</td>
<td>glycine-I, isoleucine acceptor RNA</td>
</tr>
</tbody>
</table>

| valine or proline acceptor RNA |

(Part II-C, Section 7-H(ii))
acceptor activities. When the glycine-I peak was rechromatographed on BDEAE-cellulose at pH 3.5 in the presence of 0.05 M magnesium ions two peaks appeared in the elution profile (Fig. 15). The minor peak corresponded, presumably, to one of the minor, contaminating acceptor activities (valine or proline acceptor RNA). The major peak contained two regions of glycine acceptor activity. The smaller of the two, which appeared at the leading edge of the elution profile, is probably glycine-I s-RNA missing its terminal adenosine. The main glycine acceptor region was displaced from the centre of the major peak in the elution profile. This observation suggested that we were approaching the conditions required to remove the major contaminants from the glycine-I acceptor RNA.

It is known that lowering the pH tends to open the s-RNA structure thus exposing more nucleotides to the exchanger. Smith (1966), has recently exploited this for the fractionation of s-RNA on DEAE-Sephadex columns. It is not unreasonable to assume that magnesium would antagonize this action of pH on the s-RNA molecules. Thus when the major peak of s-RNA (Fraction I, Fig. 15) was rechromatographed at pH 4.0 and in the presence of EDTA, it eluted as two major and one minor peak (Fig. 16). The minor peak presumably represents the removal of the other minor contaminant referred to earlier (valine or proline acceptor RNA). Methionine acceptor activity coincided with the leading peak and the glycine acceptor activity was displaced to the
tailing edge of the second peak of the elution profile. The isoleucine acceptor activity (not assayed in detail) was concentrated between the methionine and the glycine acceptor RNA's. Thus, although the leading edge of the glycine-I acceptor peak is still contaminated with isoleucine acceptor activity, fractions 86 to 98 (Fig. 16) were 95 to 100% glycine-I s-RNA as determined by the incorporation of $^{14}$C-glycine into TCA-insoluble product.

The glycine-II peak (see Fig. 14, tube numbers 103 to 120) contained, in addition to glycine-II s-RNA, major peaks of alanine and proline acceptor activity and minor activities toward valine, threonine, glutamic acid and glutamine. Since the glycine-II fraction contained so many other activities it was considered advisable to alter the conditions only slightly during each rechromatography. The experimental procedures are summarized in Chart II.

When the glycine-II fraction, from the preparative column, was rechromatographed at pH 5 and in the presence of 0.01 M magnesium ions, two peaks appeared in the elution profile (Fig. 17). The glycine acceptor activity, which was associated with the first peak (Fraction I, Fig. 17), was uncharacteristically broad and, in retrospect at least, was suggestive of more than one glycine acceptor activity.

When Fraction I (Fig. 17) was rechromatographed at pH 3.5, in the presence of EDTA, two peaks appeared in the elution profile (Fig. 18).
CHART II

Fractionation of Glycine-II Acceptor RNA's on BDEAE-Cellulose

5 g brewers' yeast s-RNA

Chromatography 1
(Figure 14)

0.01 M MgSO₄, NaCl
gradient, no buffer

(Tube 103 to 120 contain glycine-II, alanine
proline, valine, threonine, glutamic acid
and glutamine acceptor RNA's)

Chromatography 2
(Figure 17)

0.01 M MgCl₂, NaCl
gradient, pH 5.0

Major peak (Fraction I)
contains glycine-II
acceptor activities

Minor peak
not analyzed

Chromatography 3
(Figure 18)

1 mM EDTA, NaCl
gradient, pH 3.5

glycine-II-α, glycine-II-β and
two minor peaks of glycine acceptor activity
The glycine acceptor activity appeared as two major and two minor peaks. Also shown in Figure 18 is the acceptor activity for alanine. The position of elution of the other contaminating s-RNA's was not determined. Thus the glycine-II fraction from the preparative column consisted of at least two distinct s-RNA's (glycine-II-α and glycine-II-β, Fig. 18).

The minor peak of glycine acceptor activity on the leading edge of the glycine-II-β peak might be glycine-II-β s-RNA missing its terminal adenosine. The question as to whether or not an acceptor RNA has an intact sequence of nucleotides can be answered by assaying the column fractions with the purified aminoacyl-s-RNA synthetase enzyme of interest. This test has not been applied.

These studies show that mixed s-RNA from brewers' yeast contains three major and four or five minor acceptor RNA's for glycine. Two of the minor acceptor RNA's for glycine are evident from Figure 14. Another one appeared during rechromatography of the glycine-I peak (see Fig. 15) and it was suggested that this minor peak was glycine-I s-RNA missing its terminal adenosine. Two more minor peaks of glycine acceptor activity appeared during rechromatography of the glycine-II peak (see Fig. 18). However, it should be pointed out that both of these exist by virtue of single experimental points.

Four glycyl-s-RNA's have been isolated recently from brewers' yeast by Bergquist (1966). Sequence studies on these s-RNA's have
shown that only small differences occur in their base composition. Several of these differences involved the substitution of uridylic, pseudouridylic or ribosyl thymidylic acid for each other in small guanylic acid-containing oligonucleotides. Another site of heterogeneity appeared to be at the 5'-terminus of the molecules. It is remarkable that such closely related molecules can be resolved in the chromatographic systems. Their separation, particularly when the differences involve only pyrimidine derivatives (recall the chromatography of the pyrimidine mononucleotides), may be explained by differences in their secondary structure.

The conditions for fractionating s-RNA on BDEAE-cellulose must be determined empirically. However, the general scheme described in this part of the thesis which employs an initial chromatography at neutral pH in the presence of 0.01 M magnesium ions followed by rechromatography at a different pH and in the presence or absence of magnesium ions (EDTA) seems to be the best approach. One parameter which has not been investigated in detail is the use of elevated temperature or temperature gradients.

A particularly useful feature of BDEAE-cellulose is that the chromatography can be carried out at low pH (down to pH 3.5). Thus, for analytical purposes, s-RNA can be charged with a particular labelled amino acid, then chromatographed at pH 4 to 5, on BDEAE-
cellulose. Determination of the radioactivity in the column eluate would then reveal the position of elution of the aminoacyl-s-RNA (Gillam et al., 1967). In the past, this type of analysis has been limited principally to MAK column chromatography (Sueoka and Yamane, 1962). Unsuited to this type of analysis on BDEAE-cellulose are the aromatic aminoacyl-s-RNA's (tryptophane, tyrosine, and phenylalanine). For example, tyrosine acceptor RNA is normally eluted toward the end of the salt gradient. However, Dr. E. Wimmer, while working in the laboratory of Dr. G. M. Tener, observed that when tyrosine was esterified to its acceptor RNA and subsequently chromatographed on BDEAE-cellulose, some alcohol had to be included in the eluting solvent in order to elute the tyrosyl-s-RNA. After hydrolysing the ester and rechromatography, the tyrosine acceptor RNA again eluted from the column at the end of the salt gradient.

From these observations a general method for the isolation of populations of s-RNA's specific for a given amino acid has been developed. The principle of the method involves the attachment of a 'lipophilic handle' to the desired aminoacyl-s-RNA. Thus, mixed s-RNA is charged with a particular amino acid, then reacted with the phenoxyacetyl ester of N-hydroxysuccinimide. The resulting mixture of s-RNA's and N(phenoxyacetyl)-aminoacyl-s-RNA is applied to a column of BDEAE-cellulose. The non phenoxyacetylated material is eluted by washing the column with 0.8 M sodium chloride. The phenoxyacetylated
material is then eluted by including some alcohol in the eluting solvent. The chromatography is carried out at pH 4.5 to stabilize the ester linkage between the amino acid and its corresponding s-RNA. The derivatized aminoacyl-s-RNA is hydrolyzed by incubating the solution at pH 9.0 for 2 hr at room temperature. The regenerated s-RNA is then recovered and chromatographed on BDEAE-cellulose using a linear gradient of sodium chloride. An outline of the reactions is shown in Chart III. Part II-D of this thesis demonstrates the feasibility of the method using glycine acceptor RNA as an example.

The major disadvantage to any chemical procedure involving aminoacyl-s-RNA's lies in the requirement for the aminoacyl-s-RNA synthetase enzymes which are required in quantity. In addition, the synthetases should be free from nucleases and peptidases. Part III of this thesis describes the solution to this problem for most of the aminoacyl-s-RNA synthetases.

The use of the phenoxyacetyl ester of N-hydroxysuccinimide for acylating the \( \alpha \)-amino group of aminoacyl-s-RNA's was preferred because of its greater selectivity compared to the acyl chlorides.

Glycine-\( 1^4 \) s-RNA, previously enriched 6 fold by chromatography on BDEAE-cellulose (Part II-C, Section 7-G), was charged with \( ^{14} \)C-glycine, then phenoxyacylated as shown in Chart III. When the N-(phenoxyacetyl)-\( ^{14} \)C-glycyl-s-RNA was chromatographed on BDEAE-cellulose by step-
Synthesis of N-(Phenoxyacetyl)-aminoacyl-s-RNA

混和s-RNA

氨基酸酰化

R-CH-COO⁻ (enzymatic synthesis)

<table>
<thead>
<tr>
<th>R-CH-C-O-s-RNA + s-RNA</th>
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苯氧基酰化

<table>
<thead>
<tr>
<th>R-CH-C-O-s-RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH⁺</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>R-CH-C-O-s-RNA</th>
<th>+</th>
<th>s-RNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH⁺</td>
<td>O=C</td>
<td>CH₂O-CH₂O-N</td>
</tr>
</tbody>
</table>

色谱法

| BDEAE-cellulose |

<table>
<thead>
<tr>
<th>0.8 M NaCl</th>
<th>1.0 M NaCl + alcohol</th>
</tr>
</thead>
<tbody>
<tr>
<td>s-RNA</td>
<td>N-(phenoxyacetyl)-aminoacyl-s-RNA</td>
</tr>
</tbody>
</table>
wise elution, about 66% of the applied material was eluted by 0.8 M sodium chloride (Fig. 19). The ethanol-salt wash, which removed the remaining 34% of the applied material contained essentially all of the applied radioactivity (Fraction II, Fig. 19). Thus it would appear that the reaction conditions used (10 min, pH 8.0, 0°; Section 2-C) were sufficient for complete acylation of the $^{14}$C-glycyl-s-RNA. The conditions used by de Groot et al., (1966), for the acetylation of glycyl- and phenylalanyl-s-RNA with the acetyl ester of N-hydroxysuccinimide, involved a reaction time of 15 hr at pH 5.0 and at room temperature.

The derivatized aminoacyl-s-RNA, eluted by the ethanol-salt wash (Peak II, Fig. 19), was completely stripped of N-(phenoxyacetyl)-$^{14}$C-glycine in two hr at pH 9 and at room temperature. When the s-RNA from this peak was recovered and assayed for glycine acceptor activity it showed a 9.4 fold enrichment in glycine acceptor activity compared to crude s-RNA. This represents only a 3.4 fold enrichment over the starting material. The major reason for this small increase in purity was the fact that Fraction I (eluted by 0.8 M sodium chloride) was still 2.6 fold enriched in glycine acceptor activity compared to crude s-RNA. This means either that the s-RNA was incompletely charged with glycine, or that considerable hydrolysis had occurred between the time of preparation of the $^{14}$C-glycyl-s-RNA and the phenoxyacetylation reaction. The recovery of radioactivity following phenoxyacetylation was 68% (Section 2-C). However, this does not mean that the
remainder was lost during the phenoxyacetylation reaction because the $^{14}$C-glycyl-s-RNA had been stored as a pellet for about two weeks at 
-18° prior to the derivatization. In fact, the recovery of radioactivity from the phenoxyacetylation reaction when the glycyl-s-RNA was derivatized immediately was 84% (Section 3).

The fold enrichments quoted above were calculated in comparison to the glycine acceptor activity of crude (or whole) s-RNA. It must be emphasized therefore, that it was the glycine-I s-RNA fraction that was used in the experiments discussed above and that glycine-I represents roughly 60% of the total glycine acceptor activity in whole s-RNA.

When preparing aminoacyl-s-RNA's it is important to determine beforehand the conditions for maximum charging of the s-RNA with any given amino acid. A number of the co-factors such as potassium chloride, buffer, mercaptoethanol (if required by the enzyme), and amino acid, can be present in a reasonable excess without affecting total incorporation. The addition of ATP, in excess of that required for activation of the amino acid and any repair processes, is important by virtue of its ability to chelate magnesium. EDTA was routinely included in the incubation mixtures at very low concentration to remove heavy metals. Again, EDTA cannot be added indiscriminately because of its ability to chelate magnesium. When experiments were carried out to determine the amount of magnesium required to achieve maximum
synthesis of glycyl-s-RNA, an unexpected result was obtained. It is clear from the results shown in Figures 20 and 21 that there is an optimum magnesium requirement for the formation of glycyl-s-RNA. Concentrations above or below the optimum result in a rapidly decreasing reaction velocity. Hele (1964) has described similar results for the affect of magnesium ions on the amino acid-dependent pyrophosphate exchange reaction using rat liver as a source of enzymes and rat liver or yeast as a source of s-RNA. This investigator explained her results in terms of an "allosteric control mechanism" in which the enzymes catalyzing the ATP-pyrophosphate exchange reaction aggregate in the presence of magnesium and RNA. Fraser (1963) has also reported similar observations on the effect of magnesium ions on both the ATP-pyrophosphate exchange reaction and the incorporation of $^{14}$C-glycine into rat liver s-RNA. In addition, observations similar to those reported here have been made for yeast phenylalanyl-s-RNA (Roy and Tener, 1967). Thus it is particularly important to observe the restrictions imposed on this reaction by magnesium since it is desirable to achieve maximum charging of s-RNA in the shortest period of time to avoid the action of interfering enzymes.

Starting from mixed (or whole) s-RNA it was possible to obtain glycine-I s-RNA in high purity. The experimental procedures employed are summarized in Chart IV.
CHART IV

Isolation of Glycine-I s-RNA from Whole s-RNA (Part II-D, Section 3-B)

<table>
<thead>
<tr>
<th>Step 1</th>
<th>Stepwise elution from BDEAE-cellulose</th>
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</thead>
<tbody>
<tr>
<td>0.9 M NaCl eluate</td>
<td>1.0 M NaCl, alcohol eluate</td>
</tr>
<tr>
<td>s-RNA</td>
<td>phenylalanine s-RNA + other 'RNA'</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Phenoxyacetylation</td>
</tr>
<tr>
<td>2. Stepwise elution from BDEAE-cellulose</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminoacylation (enzymatic)</td>
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<tr>
<td>aminoacyl-s-RNA + s-RNA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenoxyacetylation</td>
</tr>
<tr>
<td>N-(phenoxyacetyl)-aminoacyl-s-RNA + s-RNA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Step 5 (Fig. 22)</th>
<th>Stepwise elution from BDEAE-cellulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.8 M NaCl eluate</td>
<td>1.0 M NaCl, alcohol eluate</td>
</tr>
<tr>
<td>s-RNA</td>
<td>phenoxyacetlated aminoacyl-s-RNA</td>
</tr>
<tr>
<td>pH 9.0, 2 hr, room temperature</td>
<td></td>
</tr>
</tbody>
</table>

| Regenerated s-RNA |

<table>
<thead>
<tr>
<th>Step 6 (Fig. 23)</th>
<th>Gradient elution from BDEAE-cellulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak I</td>
<td>Peak II</td>
</tr>
<tr>
<td>(glycine I)</td>
<td>(glycine-II ?)</td>
</tr>
</tbody>
</table>
The first step removed the phenylalanine acceptor RNA and other RNA's (non s-RNA) which were only eluted when ethanol was included in the eluting solvent. This was easily and rapidly accomplished by stepwise elution of whole s-RNA from BDEAE-cellulose (see Section 3-B, Step 1).

Step 2 removed any material which was readily phenoxyacetylated by the reagent prior to charging the s-RNA with the desired amino acid. For example, the aminoacyl adenylate nucleotides described by Hall (1964) might be removed in this step. When the phenylalanine acceptor RNA is not desired, Steps 1 and 2 can be combined.

The s-RNA eluted from BDEAE-cellulose by the 0.9 M sodium chloride wash (Step 2, Section 3-B) is now free of phenylalanine acceptor activity, other strongly held non-acceptor RNA and any nucleotide material which is readily phenoxyacetylated under the conditions described in the experimental. The material obtained from Step 2 was charged with glycine (Step 3) using a purified enzyme (Section 3-A), then phenoxyacetylated (Step 4). The recovery of radioactivity following acylation was 84%. It is recommended that the amount of phenoxyacetylation agent used in Step 4 (Section 3-B) be reduced from 1 g to 100 mg, since the acylating agent is only sparingly soluble in an aqueous medium and vast excesses tend to occlude the s-RNA. The N-(phenyoxyacetyl)$^{14}$C-glycyl-s-RNA was isolated by chromatography
on BDEAE-cellulose (Step 5, Fig. 22). All of the radioactivity was recovered in the eluate of the ethanol-salt wash. The s-RNA in peak I (Fig. 22) was completely stripped of precipitable radioactivity in two hours at pH 9 and at room temperature. Chromatography of the regenerated s-RNA on BDEAE-cellulose using an increasing linear gradient of sodium chloride and 0.01 M magnesium chloride resulted in the elution of a major (Peak I) and a minor peak (Peak II) of s-RNA, (Fig. 23, Step 6). About 46% of the regenerated s-RNA was eluted by the ethanol-salt wash (Peak III). This relatively high percentage of O. D. units eluted by the ethanol-salt wash can probably be reduced by using less phenoxyacetylation agent than was used in this experiment (as recommended above) and by carrying out the phenoxyacetylation reaction in triethanolamine buffer.

Peak I, in Figure 23 represents 2.86% of the O. D. units obtained from Step 2 (prior to aminoacylation), while Peak II (fractions 78 to 90) represents 0.76% to give a combined total of 3.62%. The theoretical recovery based on the amount of glycine-I plus glycine-II s-RNA in whole s-RNA was 4.75% (with glycine-I s-RNA contributing 2.85%, and glycine-II contributing 1.90% of the O. D. units). Thus the recovery of glycine acceptor RNA from Step 6 was 76% of the glycine acceptor RNA present in whole s-RNA.
However it must be emphasized that the $^{14}$C-glycyl-s-RNA was prepared using a purified enzyme. The purification of the glycyl-s-RNA synthetase on hydroxyapatite (described in Part III, Section 6-C) clearly shows the presence of two glycyl-s-RNA synthetase activities. It is possible that the synthetase used in this experiment will only charge the glycine-I acceptor RNA and either the glycine-II-\(\alpha\) or glycine-II-\(\beta\) acceptor RNA's (see Part II-C, Section 7-G and 7J).

The validity of Peak I in Figure 23, being the glycine-I fraction has been established by rechromatographing the s-RNA from Peak I under conditions which are known to split the glycine-II acceptor activity into glycine-II-\(\alpha\) and glycine-II-\(\beta\) s-RNA peaks (see Part II-C, Section 7-J). Only one peak of O. D. was eluted and this contained all the glycine acceptor activity.

The O. D. units in Peaks I and II (Figure 23) are in the correct ratio if Peak I is glycine-I acceptor RNA and Peak II is either glycine-II-\(\alpha\) or glycine-II-\(\beta\) acceptor RNA's. This possibility is being explored by charging whole s-RNA with $^{14}$C-glycine using one or the other glycyl-s-RNA synthetases, then analyzing the products on BDEAE-cellulose using a linear gradient of increasing sodium chloride to develop the column (R. Warrington, experiments in progress).

The presence of more than one aminoacyl-s-RNA synthetase enzyme for a given amino acid is not new. Barnett and Brown (1967)
have shown that mitochondrial preparations from *N. crassa* have their own seemingly unique set of s-RNA's and also that *N. crassa* contains two synthetase enzymes for both phenylalanine and aspartic acid.

Alternative explanations for the results obtained in Figure 23 have not been excluded. It is possible, for example, that either the glycine-II-α or the glycine-II-β acceptor RNA's contains a group which is particularly reactive with the acylating agent. In this case that particular acceptor would be removed by the 'sham' phenoxy-acetylation step (Step 2). This possibility could be tested by first carrying out the 'sham' phenoxyacetylation on mixed s-RNA followed by chromatography on BDEAE-cellulose by the stepwise procedure. The s-RNA eluted in the 0.9 M sodium chloride eluate could then be charged with $^{14}$C-glycine using a crude enzyme preparation and the product subsequently chromatographed on BDEAE-cellulose by gradient elution. Determination of the radioactivity in the column eluate would reveal a change in the ratio of the two peaks corresponding to glycine acceptor RNA.
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PART III

PREPARATION AND PARTIAL PURIFICATION
OF AMINOACYL-s-RNA SYNTHESES FROM BAKERS' YEAST
# Table of Contents

<table>
<thead>
<tr>
<th>LIST OF FIGURES</th>
<th>199</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF TABLES AND CHARTS</td>
<td>200</td>
</tr>
<tr>
<td>INTRODUCTION</td>
<td>201</td>
</tr>
<tr>
<td>EXPERIMENTAL</td>
<td>205</td>
</tr>
<tr>
<td>Abbreviations Used</td>
<td>205</td>
</tr>
<tr>
<td>Materials</td>
<td>205</td>
</tr>
<tr>
<td>Methods</td>
<td>206</td>
</tr>
<tr>
<td>1. Preparation of Aminoacyl-s-RNA Synthetases from Yeast: Method 1</td>
<td>207</td>
</tr>
<tr>
<td>2. Preparation of Aminoacyl-s-RNA Synthetases from Yeast: Method 2</td>
<td>208</td>
</tr>
<tr>
<td>A. Assay Procedure</td>
<td>208</td>
</tr>
<tr>
<td>B. Lysis of Yeast Cells with Toluene at $37^\circ$</td>
<td>209</td>
</tr>
<tr>
<td>C. Ammonium Sulfate Fractionation of the Cell-Free Extract</td>
<td>210</td>
</tr>
<tr>
<td>D. Preparation of Aminoacyl-s-RNA Synthetase Enzymes Used in Part II-D, Section 2-B</td>
<td>212</td>
</tr>
<tr>
<td>E. Chromatography of Fraction B on Hydroxyapatite</td>
<td>213</td>
</tr>
<tr>
<td>F. Radiochromatographic Analysis of the Ammonium Sulfate Eluate</td>
<td>215</td>
</tr>
<tr>
<td>3. Chromatography of Yeast Cell-Free Extract on Hydroxyapatite by Stepwise Elution</td>
<td>216</td>
</tr>
<tr>
<td>A. Preparation of the Yeast Cell-Free Extract</td>
<td>216</td>
</tr>
</tbody>
</table>
B. Chromatography 1

C. Chromatography 2

D. Chromatography 3

4. Effect of Ammonium Sulfate and Di-isopropylfluorophosphate on the Aminoacyl-s-RNA Synthetase Activity

   A. Effect of Ammonium Sulfate
   
   B. Effect of Di-isopropylfluorophosphate

5. Preparation and Partial Purification of Yeast Aminoacyl-s-RNA Synthetase Enzymes: Method 3

   A. Preparation of the Cell-Free Extract
   
   B. Ammonium Sulfate Fractionation of the Cell-Free Extract
   
   C. Detection of Aminoacyl-s-RNA Synthetases in the Ammonium Sulfate Fractions
   
   D. Chromatography of Ammonium Sulfate Fraction III on Hydroxyapatite
   
   E. Detection of Aminoacyl-s-RNA Synthetases in the Hydroxyapatite Column Eluate
   
   F. Determination of Aminoacyl-s-RNA Synthetase Activities

6. Preparation and Partial Purification of Aminoacyl-s-RNA Synthetases from Yeast: Method 3 (Modified)

   A. Preparation of the Cell-Free Protein Extract
B. Partial Purification of the Aminoacyl-s-RNA Synthetases 232

C. Detection of Aminoacyl-s-RNA Synthetase in the Hydroxyapatite Column Eluate 233

DISCUSSION 238

BIBLIOGRAPHY 254
<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Elution Profile from the Chromatography of Fraction B on Hydroxyapatite</td>
</tr>
<tr>
<td>2</td>
<td>Results of the Radiochromatographic Analysis of the Ammonium Sulfate Eluate</td>
</tr>
<tr>
<td>3</td>
<td>Elution Profile from the Chromatography of Fraction III on Hydroxyapatite</td>
</tr>
<tr>
<td>4</td>
<td>Chromatography of the 0-80% Protein Fraction on Hydroxyapatite</td>
</tr>
<tr>
<td>5</td>
<td>Elution Profiles of Aminoacyl-s-RNA Synthetases from Hydroxyapatite</td>
</tr>
<tr>
<td>6</td>
<td>Elution Profiles of Aminoacyl-s-RNA Synthetases from Hydroxyapatite</td>
</tr>
</tbody>
</table>
# List of Tables & Charts

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Properties of Ammonium Sulfate Fractions A-D</td>
</tr>
<tr>
<td>II</td>
<td>Glycyl-s-RNA Synthetase Activity in Fractions A and B</td>
</tr>
<tr>
<td>III</td>
<td>Protocol for Ammonium Sulfate Fractionation</td>
</tr>
<tr>
<td>IV</td>
<td>Properties of Ammonium Sulfate Fractions II - IV</td>
</tr>
<tr>
<td>V</td>
<td>Aminoacyl-s-RNA Synthetase Activities in the Ammonium Sulfate Fractions</td>
</tr>
<tr>
<td>VI</td>
<td>Aminoacyl-s-RNA Synthetases Present in the Ammonium Sulfate Eluates</td>
</tr>
<tr>
<td>VII</td>
<td>Specific Activities of Several Aminoacyl-s-RNA Synthetases</td>
</tr>
<tr>
<td>VIII</td>
<td>Aminoacyl-s-RNA Synthetases Present in the Whole-protein Extract</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CHART</th>
<th>PAGE NUMBER</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Summary of Experimental (Method 3)</td>
</tr>
<tr>
<td>II</td>
<td>Summary of Experimental (Method 3, modified)</td>
</tr>
</tbody>
</table>
INTRODUCTION

The aminoacyl-s-RNA synthetases occupy a significant position in the pathway leading from free amino acids to proteins by virtue of their ability to recognize both a particular amino acid and the corresponding s-RNA acceptor. Thus the aminoacyl-s-RNA synthetases are proteins which can discriminate between structurally similar nucleic acid molecules. The molecular basis for this strict specificity remains one of the central problems in understanding the translation of the genetic message. It is obvious that a mistake at the aminoacyl-s-RNA synthetase level would lead to an error in the translation of the genetic message during ribosomal protein synthesis. Thus the study of the relationship between s-RNA and the aminoacyl-s-RNA synthetases has become the subject of considerable interest.

Besides their intrinsic properties and the elucidation of the molecular basis of their unique interactions with nucleic acids, interest in the aminoacyl-s-RNA synthetases derives from their indispensable role in the development of chemical methods for purifying amino acid-specific s-RNA's (discussed in Part II-D). Methods for preparing specific s-RNA's which involve the preformation of the aminoacyl-s-RNA will obviously require relatively large amounts of the synthetase enzymes. It is also important to have synthetase enzymes free from contaminating enzymes such as, ribonucleases, diesterases and peptidases.
McCorquodale (1964), described a simple procedure for the preparation and partial purification of aminoacyl-s-RNA synthetases from *E. coli*. His procedure involved grinding the cells with alumina followed by ultracentrifugation and chromatography on two columns. Another method for the preparation and partial purification of the aminoacyl-s-RNA synthetases from *E. coli* is described in the treatise of Cantoni and Davies (1966). This treatise also describes procedures for the purification of tyrosyl-, isoleucyl-, glutamyl-, glutaminyl-, lysyl- and prolyl-s-RNA synthetases from *E. coli*. Also contained in the text are procedures for the purification of seryl-, phenylalanyl-, arginyl- and leucyl-s-RNA synthetases from yeast.

In addition to *E. coli,* and yeast, some of the aminoacyl-s-RNA synthetases from a variety of other organisms have also been studied, e.g. rat liver (Bublitz, 1966a), chick embryo (Bublitz, 1966b), calf liver and spleen (Wevers, et al., 1966), beef pancreas (Lemaire et al., 1967), *N. crassa* (Barnett and Epler, 1966), *Salmonella typhimurium* (Roth and Ames, 1966), wheat germ and pea seed (Moustafa, 1966), and tobacco leaves (Anderson and Rowan, 1966).

The ready availability of yeast is a desirable feature of this organism but the difficulty encountered in disrupting yeast cells, compared with those of bacterial or animal origin, is a major disadvantage for the preparation of large quantities of yeast enzymes. The
usual method employed for disruption of yeast cells consists of grinding the cells with glass beads at high speed in a homogenizer. Makman and Cantoni (1965) have used this method for disrupting 100 g batches of yeast cells.

In this laboratory, grinding yeast cells with glass beads provided sufficient quantities of yeast cell-free extract for assaying s-RNA. However, the preparation of aminoacyl-s-RNA synthetases by this method, in amounts sufficient to charge gram quantities of s-RNA, was tedious.

In a search for alternative procedures, the use of toluene to lyse yeast cells was investigated. Kunitz (1952), (see also Heppel, 1955) had reported that toluene effectively lysed yeast cells by warming to 38 to 40° then leaving the mixture at 20 to 25° for 3 hr. A modification of this method was employed successfully for the purification of yeast histidyl-s-RNA synthetase (von Tigerstrom and Tener, 1967). However, the aminoacyl-s-RNA synthetase activities for amino acids other than histidine were either absent, or present in very low amounts in the cell-free extract. Procedures described in this thesis have significantly overcome this loss of activity by controlling the pH of the yeast suspension during lysis with toluene in the presence of buffer.

Lazarus et al. (1966) in a report on the purification of yeast hexokinase described the lysis of yeast cells in a toluene-dry ice mixture. This procedure has been successfully employed in the present
studies, for the preparation of large quantities of yeast cell-free extracts.

A partial purification of the aminoacyl-s-RNA synthetases by chromatography on hydroxyapatite is also reported.
EXPERIMENTAL

ABBREVIATIONS USED

ATP: adenosine-5'-triphosphate.
CTP: cytidine-5'-triphosphate.
EDTA: ethylenediaminetetraacetic acid.
PPO: 2, 5-diphenyloxazole.
Dimethyl POPOP: 1, 4-bis-(4-methyl-5-phenyloxazolyl)-benzene.
DFP: di-isopropylfluorophosphate.

The standard abbreviations were used for the amino acids.

MATERIALS

Bakers' yeast was obtained locally from Standard Brands Ltd.
Yeast extract was "Bacto-Yeast Extract" obtained from Difco Laboratories, Detroit, Michigan.
ATP and CTP were obtained as the disodium salts from P-L Biochemicals Inc., Milwaukee, Wisconsin.

Hydroxyapatite (Bio-Gel HTP) was purchased as the dry powder from Bio-Rad Laboratories, Richmond, California.

The Diaflo apparatus was purchased from Amicon Corporation, Cambridge, Mass.
Superbrite glass beads, Type 110-5005, were purchased from Minnesota Mining and Manufacturing Co.

The $^{14}$C-amino acids were purchased from New England Nuclear Corp., Boston, Massachusetts.

The $^{14}$C-amino acid mixture was a uniformly labelled Algal protein hydrolysate purchased from New England Nuclear Corporation, Boston, Massachusetts.

PPO (Scintillation Grade) was obtained from Kent Chemicals Ltd., Vancouver, Canada.

Dimethyl POPOP (Scintillation Grade) was obtained from the Packard Instrument Company, Inc., La Grange, Illinois.

Optical density measurements were made using a Cary Model 11 spectrophotometer. One optical density (O. D.) unit is defined as that amount of material per ml which, in a 1-cm light path at the specified wavelength, gives a spectrophotometric reading of one.

METHODS

All buffers used in this study were passed through a Type HA-0.45μ Millipore filter prior to use. This was particularly important when the buffers contained glycerol. Flow rates during chromatography on hydroxyapatite were maintained with the aid of an Accu-Flo Pump (Beckman Instruments).
1. **Preparation of Aminoacyl-s-RNA Synthetases from Yeast:**

   **Method 1**

   Crumbled yeast cake (9 g) was mixed in a 500 ml Erlenmeyer containing 200 ml of distilled water, 16 g of sucrose, 4 g of yeast extract, 3 g of (NH₄)₂HPO₄, 100 mg of Mg(OAc)₂·H₂O, and 1.5 ml of lactic acid. The pH of the medium was adjusted to 3.5 with lactic acid and incubated at 33° for 17 hr with a continuous stream of air bubbling through the medium.

   The cells were harvested by centrifugation in an International refrigerated preparative centrifuge and washed twice with 0.05 M Tris-(acetate), pH 7.4, 0.005 M Mg(OAc)₂. The cells were suspended in a small amount of the same buffer then transferred to the pre-cooled jar of a Virtis "45" homogenizer containing about 40 g of glass beads, a drop of octyl alcohol and 0.5 ml of 5% mercaptoethanol. The jar was packed in ice and the homogenizer turned to full speed for 5 min, stopped and cooled for 3 min and the process was repeated twice. The homogenate was decanted from the glass beads and centrifuged at 11,000 rpm in the Sorvall, Type SS-1 centrifuge at 3-5°. The supernate was decanted and then centrifuged in the "40" rotor of a Beckman Model L preparative ultracentrifuge at 100,000 x g for 2 hr. The middle, clear portion of the supernate was dialyzed for 12 hr against 3 litres of 0.05 M Tris(acetate), pH 7.4, 0.005 M Mg(OAc)₂, containing 0.5 ml of
mercaptoethanol. The dialysate was then transferred to ampoules, which were then sealed and stored in liquid nitrogen until ready for use.

2. Preparation of Aminoacyl-s-RNA Synthetases from Yeast:

Method 2

A. Assay Procedure

The presence of aminoacyl-s-RNA synthetase enzymes can be detected by incubating the test solution with the appropriate $^{14}$C-labelled amino acid, s-RNA, and co-factors. The aminoacyl-s-RNA synthetase enzymes convert the TCA-soluble $^{14}$C-amino acid to TCA-insoluble $^{14}$C-aminoacyl-s-RNA.

The reaction mixture (total volume, 0.15 ml) contained 27 $\mu$moles sodium cacodylate, pH 7.3, 6.7 $\mu$moles KCl, 3.7 $\mu$moles MgCl$_2$, 0.5 $\mu$ mole sodium ATP, 0.10 $\mu$ mole sodium CTP, 0.12 $\mu$ mole mercaptoethanol, 0.10 $\mu$ mole EDTA, 75 $\mu$moles glycine, 0.2 $\mu$Ci $^{14}$C-glycine, 1.4 mg s-RNA and 50 $\mu$l of enzyme solution or phosphate buffer in the case of the blank. The reaction mixture was incubated at 25° and 20 $\mu$l aliquots were removed at intervals and applied to Whatman 3MM papers (2 cm$^2$). The filter papers were allowed to dry for 30 seconds before plunging them into cold 10% TCA. The filter papers remained at least 15 min, with occasional stirring, in each of three 10% TCA baths. Each bath contained 10 ml of 10% TCA per filter paper. After the TCA washes, the filter papers were washed in cold ethanol then in ethyl ether, dried and placed
in standard counting vials. To each vial was added 5 ml of scintillation fluid (containing 3 g PPO and 0.3 g dimethyl POPOP per litre of toluene) and the radioactivity determined in a scintillation spectrometer. A unit of enzyme catalyzes the formation of 1 \textmu m mole of $^{14}$C-aminoacyl-s-RNA per min.

B. Lysis of Yeast Cells with Toluene at 37°

To a 1 litre beaker, containing 200 ml of toluene in a 45° water bath, was added 400 g of crumbled bakers' yeast followed by 120 ml of 0.65 M Tris(acetate), pH 8.0. The mixture was warmed to 37° with slow stirring then the beaker was transferred to a 38° water bath and the stirring continued. The viscosity of the mixture decreased and the evolution of gas was apparent as the temperature of the mixture approached 37°. A drop in the pH of the solution was observed concurrent with the evolution of gas. The pH was monitored with a Leeds and Northrop glass electrode and was maintained at about 7 by the addition of 1 N NaOH. The lysis was essentially complete 40 min after the mixture had reached 37°, as indicated by the consumption of alkali (50 ml). The temperature of the mixture was maintained at 37° for a total time of 60 min during which 52 ml of 1 N NaOH was consumed. The mixture was then cooled to about 5° in an ice bath and 100 ml of 0.65 M Tris(acetate), pH 8.0, was added. All subsequent operations were carried out between 0 and 5°. The mixture was centrifuged at 2000 rpm.
for 15 min in a refrigerated International Centrifuge. The aqueous layer was removed from beneath the toluene layer and centrifuged at 11,000 rpm for 20 min in a Sorvall, Type SS-1 centrifuge. The middle, aqueous layer (total volume, 340 ml) was removed with the aid of a large syringe and centrifuged at 100,000 x g in the Type-30 rotor of the Beckman, Model L ultracentrifuge for 3.5 hr. About 230 ml of supernatant fluid, containing 0.27 mg of protein per ml was recovered. The 280:260 nm ratio of the cell-free extract was 0.66.

C. Ammonium Sulfate Fractionation of the Cell-Free Extract

To 180 ml of the cold, cell-free extract obtained above, was added 68.9 g of ammonium sulfate. The mixture was stirred until all the ammonium sulfate had dissolved then left for an additional 20 min at 0°. The precipitate was collected by centrifugation (ammonium sulfate fraction A). To the supernate (192 ml) was added 12.7 g of ammonium sulfate and the mixture treated as described above (ammonium sulfate fraction B). Ammonium sulfate fractions C and D were obtained in an analogous manner by adding 13.1 g and 14.0 g of ammonium sulfate to the supernates from fractions B (190 ml) and C (195 ml), respectively. The supernate from ammonium sulfate fraction D was discarded. Pellets A to D were dissolved in 0.10 M sodium phosphate, pH 7.4 then glycerol was added to 35% (v/v) and the solutions stored at -18° until ready for use. Table I lists the data relating to the ammonium sulfate fractions A to D.
### TABLE I
Properties of Ammonium Sulfate Fractions A-D

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (^a) (ml)</th>
<th>Total O. D. Units (280 m(\mu))</th>
<th>Total protein (g)</th>
<th>280:260 (^b) ratio</th>
</tr>
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<tbody>
<tr>
<td>Crude</td>
<td></td>
<td>14,700</td>
<td>9.685</td>
<td>0.85</td>
</tr>
<tr>
<td>A</td>
<td>111</td>
<td>4,470</td>
<td>3.881</td>
<td>1.10</td>
</tr>
<tr>
<td>B</td>
<td>30</td>
<td>1,920</td>
<td>1.097</td>
<td>0.77</td>
</tr>
<tr>
<td>C</td>
<td>15.5</td>
<td>550</td>
<td>0.289</td>
<td>0.74</td>
</tr>
<tr>
<td>D</td>
<td>9.2</td>
<td>277</td>
<td>0.146</td>
<td>0.74</td>
</tr>
<tr>
<td>Supernate of D</td>
<td>195</td>
<td>7,520</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Volume of 0.10 M sodium phosphate plus glycerol added to pellets

\(^b\) 280:260 ratios were determined after passage through G-25 Sephadex as described in Section 2-C.

Portions of the crude cell-free extract and fractions A to D were desalted on G-25 Sephadex equilibrated with 0.10 M sodium phosphate, pH 7.4, prior to assaying for glycyl-s-RNA synthetase activity.

The reaction mixture (Section 2-A) for assaying the crude enzyme contained 0.018 O. D. units (280 m\(\mu\)) (12 \(\mu\)g of protein) of the Sephadex treated crude extract. The synthesis of \(^{14}\)C-glycyl-s-RNA was linear over 20 min. Ammonium sulfate fractions A and B were assayed for
glycyl-s-RNA synthetase activity using 0.020 and 0.010 O.D. units (at 280 m\(\mu\)) of enzyme respectively. Fractions C and D contained negligible glycyl-s-RNA synthetase activity. The data are summarized in Table II.

**TABLE II**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Specific Activity</th>
<th>Total Units</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>0.76</td>
<td>7.35</td>
<td>100</td>
</tr>
<tr>
<td>A</td>
<td>0.18</td>
<td>0.70</td>
<td>9.5</td>
</tr>
<tr>
<td>B</td>
<td>5.04</td>
<td>5.53</td>
<td>75.2</td>
</tr>
</tbody>
</table>

a. Specific Activity is units of enzyme activity per mg of protein

b. Total units are obtained by multiplying specific activity by the total mg of protein

D. **Preparation of Aminoacyl-s-RNA Synthetase Enzymes Used in** Part II-D, Section 2-B

A 2.0 ml portion of ammonium sulfate fraction B (Section C) was desalted on G-25 Sephadex equilibrated with 0.01 M potassium phosphate, pH 7.5, 0.001 M mercaptoethanol, 35% glycerol (v/v). The column fractions were assayed for glycyl-s-RNA synthetase activity as described in Section 3-E. The fractions containing the desired enzymatic activity were pooled and stored at -18\(^{\circ}\) until ready for use.
E. Chromatography of Fraction B on Hydroxyapatite

The hydroxyapatite powder (Bio-Gel HTP) was suspended overnight in $10^{-2}$ M potassium phosphate, $10^{-2}$ M mercaptoethanol 35% glycerol (v/v), pH 6.8 (starting buffer) then packed under pressure in a chromatographic column maintained at 3-5°C. The column (14 x 3.0 cm diam.) was equilibrated with 500 ml of starting buffer. The flow rate was controlled by means of an Accu-Flo pump (Beckman Instruments Inc.). A 10 ml portion of fraction B (366 mg protein) was diluted to 100 ml with 35% aqueous glycerol then applied to the hydroxyapatite column. After washing the column with a few mls of starting buffer, a 2 litre linear gradient of potassium phosphate, from $10^{-2}$ M to 0.20 M was applied with both vessels containing $10^{-2}$ M mercaptoethanol and 35% glycerol (v/v). The flow rate was about 1 ml per min and 15 ml fractions were collected. When the 2 litre linear gradient finished, a 400 ml linear gradient of ammonium sulfate from 0 to 10% (w/v) was applied with both vessels containing 0.20 M potassium phosphate $10^{-2}$ M mercaptoethanol, pH 6.8 and 35% glycerol (v/v). The O.D. at 280 μμ was recorded for each fraction (Fig. 1).

The fractions were assayed for glycyl-s-RNA synthetase activity according to the procedure described in Section A except the reaction mixture (total volume, 0.20 ml) contained 2.8 mg of s-RNA, and a 0.10 ml aliquot of the column fractions. The incubation period was 60 min.
Phosphate Buffer (M) and cpm (per 0.1 ml eluate) incorporated in 28 mg sRNA x 10^3

Fraction No.

Optical Density (280 mu)

Ammonium Sulfate Gradient (0-100%)

gly
F. Radiochromatographic Analysis of the Ammonium Sulfate Eluate

The reaction mixture (final volume 0.30 ml) contained 7.3 μmoles \( \text{MgCl}_2 \), 13.3 μmoles KCl, 40 μmoles sodium cacodylate, pH 7.3, 1 μmole sodium ATP, 0.2 μmole sodium CTP, 0.24 μmole mercaptoethanol, 0.2 μmole EDTA, 173 mM mixed amino acids and 0.67 μCi of mixed \(^{14}\text{C}-\text{amino acids} \), 5.6 mg of s-RNA and 100 μl of column eluate. The reaction mixture was incubated for 60 min at room temperature. A 20 μl aliquot was removed and applied to a Whatman 3MM filter paper (2 cm\(^2\)). The filter paper was washed and the radioactivity determined as described in Section A. The remainder of the reaction mixture was cooled in ice and 0.10 ml 0.5 M sodium formate, pH 3.0, 0.10 ml 5 M NaCl and 1.50 ml ethanol were added. After 10 min in ice, the mixture was centrifuged and the supernate decanted. The pellet was washed with 0.5 ml ethanol-salt solution (2:1, ethanol : 1.5 M NaCl), then centrifuged and drained free of excess wash solvent. Then the pellet was dissolved in 0.5 ml 0.10 M \( \text{MgCl}_2 \), 0.005 M NaOAc, pH 5.0 and precipitated again with 1.5 ml of cold ethanol. The pellet, obtained by centrifugation, was washed as described above, drained free of excess wash solvent and dissolved in 0.15 ml of 1.5 N NH\(_4\)OH. After incubating the mixture at 37° for 30 min, 0.60 ml of cold ethanol was added and the mixture centrifuged. Most of the supernate was applied to Whatman 1 paper and chromatographed 26 hr in n-butanol : acetic acid : H\(_2\)O (60:15:25). The chromatogram was cut into strips and scanned in the
Actigraph (Nuclear Chicago, Model 1036). The results are shown in Figure 2.

3. **Chromatography of Yeast Cell-Free Extract on Hydroxyapatite by Stepwise Elution**

All operations were carried out between 0 and 5°.

**A. Preparation of the Yeast Cell-Free Extract**

The cell-free extract was prepared from 400 g of bakers' yeast according to Method 2 (Section 2-B) except that 150 ml of Tris(acetate), pH 8.0, was added to the crumbled yeast-toluene mixture prior to lysis. Also, the addition of 100 ml of Tris(acetate), pH 8.0, following lysis was omitted. The ultracentrifugation step was also omitted. To the supernate (300 ml) obtained from the Sorvall, Type SS-1 centrifugation was added 115 g of ammonium sulfate (about 2.9 M), slowly with stirring and the mixture left in ice for 20 min after all the ammonium sulfate had dissolved. One-half of the pellets obtained by centrifugation was dissolved in 40 ml of 0.01 M potassium phosphate, pH 6.8, 10⁻³ M mercaptoethanol, 35% glycerol (v/v). The final volume was 50.0 ml and contained 2400 O. D. units (280 mÈ) (1.26 g of protein). The 280: 260 mÈ ratio of the solution was 0.74.
FIGURE 2. Results of the Radiochromatographic analysis of the ammonium sulfate eluate.
B. Chromatography

The 50 ml of crude extract were applied to a hydroxyapatite column (13.2 x 3.0 cm diam.) previously equilibrated with 0.20 M potassium phosphate, pH 6.8, 10⁻³ M mercaptoethanol, 35% glycerol (v/v) (starting buffer). After washing the column with 250 ml of the same buffer a 400 ml linear gradient of ammonium sulfate, from 0 to 10% (w/v), was applied. Both vessels contained 0.2 M potassium phosphate, 10⁻³ M mercaptoethanol and 35% glycerol (v/v). A flow rate of about 1 ml per min was maintained with the aid of an Accu-Flo pump.

The column fractions were assayed for aminoacyl-s-RNA synthetase activity using mixed ¹⁴C-amino acids. To 2.4 cm Whatman 3MM filter disks, suspended on stainless steel pins, was added 40 µl of a reaction mixture containing 8 µmoles sodium cacodylate, pH 7.3, 1.47 µmoles MgCl₂, 2.66 µmoles KCl, 0.2 µmole sodium ATP, 0.04 µmole sodium CTP, 0.05 µmole mercaptoethanol, 0.04 µmole EDTA, 34.5 µµmoles and 133 µµCi of ¹⁴C-amino acids, and 1.12 mg of s-RNA. A 20 µl aliquot of the column fractions was added and the disks then incubated for 30 min at room temperature. The blank contained a 20 µl aliquot of the starting buffer instead of column eluate. The styrofoam block supporting the stainless steel pins was contained in a glass dish containing water and covered with a tight fitting cover to maintain a moist atmosphere. After the 30 min incubation period the filter disks were
washed and the radioactivity determined as described in Section 2-A. All the synthetase activity and all the applied O. D. units were eluted by the starting buffer. The applied O. D. units were recovered in 84 ml of starting buffer.

C. **Chromatography 2**

A 20 ml portion (570 O. D. units) of the 84 ml recovered from chromatography 1 was rechromatographed and the column fraction assayed under the conditions described above (Section 3-B). All the applied O. D. units and all the synthetase activity were again eluted by the starting buffer.

D. **Chromatography 3**

A 20 ml portion (570 O. D. units) of the 84 ml recovered from chromatography 1 was dialyzed for 5 hr agains 1 litre of $10^{-2}$ M potassium phosphate, pH 7.0, $10^{-3}$ M mercaptoethanol, 35% glycerol (v/v). The dialysate (36.5 ml, 510 O. D. units) was applied to a hydroxyapatite column (13.2 x 3.0 cm diam.) previously equilibrated with $10^{-2}$ M potassium phosphate, pH 6.8, $10^{-3}$ M mercaptoethanol, 35% glycerol (v/v). The column was washed first with 180 ml $10^{-2}$ M potassium phosphate, pH 6.8, $10^{-3}$ M mercaptoethanol, 35% glycerol (v/v), then with 340 ml of 0.2 M potassium phosphate, pH 6.8, $10^{-3}$ M mercaptoethanol, 35% glycerol (v/v) glycerol and finally with 0.2 M potassium
phosphate, pH 6.8, 10^{-3} M mercaptoethanol, 35% glycerol (v/v), 10% ammonium sulfate (w/v). The column fractions were assayed as described in Section 3-B. The 10^{-2} M phosphate wash eluted 4.6% of the applied O. D. units but none of the synthetase activity. The 0.2 M phosphate step eluted 91.4% of the applied O. D. units and most of the synthetase activity. The 10% ammonium sulfate step eluted 3.9% of the applied O. D. units and a small amount of the synthetase activity.

4. Effect of Ammonium Sulfate and Di-isopropylfluorophosphate on the Aminoacyl-s-RNA Synthetase Activity

A. Effect of Ammonium Sulfate

The assays were carried out as described in Section 2-A. The reaction mixture (0.15 ml) contained 50\mu l of the ammonium sulfate fraction B (before Sephadex treatment). Assays were carried out at enzyme dilutions of 1, 2, 4, and 8 fold. The enzyme was diluted with 0.05 M potassium phosphate, 10^{-3} mercaptoethanol, 35% glycerol (v/v). The four assays each showed maximum charging in less than 20 min while the incorporation of ^{14}C-glycine into a TCA insoluble form increased with increasing dilution of the enzyme. The reaction mixtures containing the 2, 4 and 8 fold diluted enzyme showed 25%, 31% and 33% more ^{14}C-glycine incorporation than the undiluted enzyme.
B. Effect of Di-isopropylfluorophosphate

Two assays were carried out under the conditions described in Section 2-A except that one of them contained 0.15 μmole of di-isopropylfluorophosphate (DFP). The presence of DFP did not effect the synthesis of $^{14}$C-glycyl-s-RNA.

5. Preparation and Partial Purification of Yeast Aminoacyl-s-RNA Synthetase Enzymes:

Method 3

A. Preparation of the Cell-Free Extract

Two pounds of fresh, pressed bakers' yeast were thoroughly crumbled, then added to 2 litres of toluene containing excess dry ice. The mixture was left in the cold room ($-18^\circ$) for 6 hr by which time the mixture had warmed to about $-20^\circ$. The toluene was then decanted and the frozen yeast left at 3-5$^\circ$ for 44 hr. A 0.10 ml aliquot of 1 M DFP (in isopropanol) was added to the yeast paste. The paste was then centrifuged for 30 min at 14,600 x g in the GSA head of an RC-2B refrigerated ($0^\circ$) centrifuge. The supernates were combined to yield 210 ml of cell-free extract.

B. Ammonium Sulfate Fractionation of the Cell-Free Extract

To a portion of the cell-free extract (180 ml, pH 5.6) was added 50 ml of 0.65 M Tris(acetate), pH 8.0 and the resulting solution was
placed in an ice bath. The final volume of the solution was 230 ml and the pH was 7.3. The protocol for the ammonium sulfate fractionation is shown in Table III and the procedure was as follows: the required amount of solid ammonium sulfate was added slowly, with stirring, to the cold solution. After all the ammonium sulfate had dissolved, the

### TABLE III

**Protocol for Ammonium Sulfate Fractionation**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume&lt;sup&gt;a&lt;/sup&gt; (ml)</th>
<th>Ammonium Sulfate Added (g)</th>
<th>Buffer Added (ml)</th>
<th>O. D. units&lt;sup&gt;b&lt;/sup&gt; (280 myλ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>230</td>
<td>32.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>II</td>
<td>232</td>
<td>35.2</td>
<td>24</td>
<td>3600</td>
</tr>
<tr>
<td>III</td>
<td>210&lt;sup&gt;d&lt;/sup&gt;</td>
<td>16.0</td>
<td>21</td>
<td>1980</td>
</tr>
<tr>
<td>IV</td>
<td>210</td>
<td>17.8</td>
<td>24</td>
<td>2400</td>
</tr>
</tbody>
</table>

<sup>a</sup> Volume before addition of ammonium sulfate

<sup>b</sup> O. D. units were determined after passage through G-25 Sephadex as described in Section 5-B

<sup>c</sup> Fraction I was discarded

<sup>d</sup> A few mls were lost due to disturbance of the pellet

solution was left for a further 20 min in the ice bath. The solution was then centrifuged for 30 min at 11,000 rpm, in a Sorvall, Type SS-1 centrifuge at 3-5°. The pellets were dissolved in a minimum of 10<sup>-2</sup> M potassium phosphate, 10<sup>-3</sup> M mercaptoethanol, 35% glycerol (v/v), pH
7.5 and excess ammonium sulfate removed by gel filtration on G-25 Sephadex equilibrated with the same buffer. Fractions from the Sephadex column containing ultraviolet absorbing (at 280 m\textmu) material excluded by the Sephadex beads were combined and stored at -18° until ready for use.

C. Detection of Aminoacyl-\textit{s}-RNA Synthetases in the Ammonium Sulfate Fractions

The assays were carried out in 1 ml glass tubes containing (in a final volume of 0.10 ml); 5 to 10 \textmu moles sodium cacodylate, pH 7.5, 1 \textmu mole MgCl\textsubscript{2}, 0 to 1 \textmu mole KCl, 0.25 to 1.0 \textmu mole sodium ATP, 0 to 0.04 \textmu mole EDTA, 1.25 mg s-RNA, 5 \textmu moles of a given amino acid containing 20 \textmu Ci per \textmu mole except methionine (8 \textmu moles containing 12.2 \textmu Ci per \textmu mole) and phenylalanine (4.9 \textmu moles containing 37.5 \textmu Ci per \textmu mole), and 50 \textmu l of enzyme (or phosphate buffer, in the case of the blank) solution. The assay mixture for cysteinyl-\textit{s}-RNA synthetase contained, in addition, 1 \textmu mole of mercaptoethanol. Data relating to treatment of the ammonium sulfate fractions on G-25 Sephadex and subsequent dilutions made prior to assaying are summarized in Table IV. After 30 min at room temperature 25 \textmu l aliquots were removed and applied to Whatman 3MM, 2.4 cm diam. filter disks. Each disk was washed once for a minimum of 10 min in 10\% TCA, twice for 10 min in 5\% TCA, 10 min in EtOH then in ether. The disks were then dried and the radioactivity determined as described in Section 2-A.
TABLE IV

Properties of Ammonium Sulfate Fractions II - IV

<table>
<thead>
<tr>
<th>Ammonium Sulfate Fraction No.</th>
<th>280:260 (^a) ratio</th>
<th>Final mg of protein per ml</th>
<th>Assay (^b) dilution</th>
<th>mg proteins per 50 (\mu)l</th>
</tr>
</thead>
<tbody>
<tr>
<td>II</td>
<td>0.65</td>
<td>10.6</td>
<td>1:10</td>
<td>0.053</td>
</tr>
<tr>
<td>III</td>
<td>0.94</td>
<td>19.8</td>
<td>1:20</td>
<td>0.050</td>
</tr>
<tr>
<td>IV</td>
<td>0.99</td>
<td>24.4</td>
<td>1:20</td>
<td>0.061</td>
</tr>
<tr>
<td>Supernate (^c)</td>
<td>0.88</td>
<td>1.20</td>
<td>1</td>
<td>0.060</td>
</tr>
</tbody>
</table>

\(^a\) The 280:260 ratio was determined after Sephadex treatment.

\(^b\) Dilutions made with 10\(^{-2}\) M potassium phosphate, 10\(^{-3}\) M mercaptoethanol, 35% glycerol \((v/v)\), pH 7.5.

\(^c\) Supernate of ammonium sulfate Fraction IV.

Table V lists the \(\mu\)moles of the various amino acids incorporated into s-RNA under the conditions described above.

D. Chromatography of Ammonium Sulfate Fraction III on Hydroxyapatite

One-third of the ammonium sulfate Fraction III was dissolved in a minimum of 10\(^{-2}\) M potassium phosphate, 10\(^{-3}\) M mercaptoethanol, pH 7.5 and chromatographed on G-25 Sephadex equilibrated with the same buffer. The absorbance (at 280 \(m\mu\)) of the fractions from the G-25 Sephadex column was recorded and the fractions comprising the leading
TABLE V
Aminoacyl-s-RNA Synthetase Activities in the Ammonium Sulfate Fractions

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>m(\mu) moles of amino acid incorporated (^a)</th>
<th>Fraction II</th>
<th>Fraction III</th>
<th>Fraction IV</th>
<th>Supernate (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ala</td>
<td>0.05</td>
<td>0.25</td>
<td>0.22</td>
<td>0</td>
<td>0.22</td>
</tr>
<tr>
<td>arg</td>
<td>0.15</td>
<td>0.31</td>
<td>1.10</td>
<td>0</td>
<td>0.22</td>
</tr>
<tr>
<td>asp</td>
<td>0.23</td>
<td>0.96</td>
<td>0.31</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>asn</td>
<td>-0.02</td>
<td>0.07</td>
<td>0.002</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>cys</td>
<td>0.27</td>
<td>0.20</td>
<td>0.01</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>glu</td>
<td>0.01</td>
<td>0.01</td>
<td>0.002</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>gln</td>
<td>0.02</td>
<td>0.08</td>
<td>0.04</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>gly</td>
<td>0.15</td>
<td>0.57</td>
<td>0.49</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>his</td>
<td>0.10</td>
<td>0.23</td>
<td>0.39</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>ile</td>
<td>0.39</td>
<td>1.23</td>
<td>0.37</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>leu</td>
<td>0.08</td>
<td>0.34</td>
<td>0.18</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>lys</td>
<td>1.15</td>
<td>1.04</td>
<td>0.01</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>met</td>
<td>0.11</td>
<td>0.25</td>
<td>0.15</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>phe</td>
<td>0.10</td>
<td>0.56</td>
<td>0.09</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>pro</td>
<td>0.02</td>
<td>0.05</td>
<td>0.01</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>ser</td>
<td>0.17</td>
<td>0.71</td>
<td>0.74</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>thr</td>
<td>0.07</td>
<td>0.36</td>
<td>0.03</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>try</td>
<td>0.08</td>
<td>0.23</td>
<td>0.16</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>tyr</td>
<td>0.50</td>
<td>0.81</td>
<td>0.18</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>val</td>
<td>0.08</td>
<td>0.27</td>
<td>0.01</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) m\(\mu\) moles of amino acid incorporated in 1.25 mg s-RNA by 0.05 to 0.06 mg protein under the conditions described in Section 5-C.

\(^b\) supernate of Fraction IV.

peak, which were eluted after one column volume of buffer had passed through the column, were combined to yield 27 ml of solution and 817 O. D. units (280 m\(\mu\)). A 3.0 ml portion of the solution was put aside
(desalted ammonium sulfate Fraction III) and the remaining 24 ml were applied to a hydroxyapatite column (17 x 3 cm diam.) which had been previously equilibrated with $10^{-2}$ M potassium phosphate, $10^{-3}$ M mercaptoethanol, pH 7.5. The column was washed with a few mls of the equilibration buffer then a 2 litre linear gradient of potassium phosphate, from $10^{-2}$ M to 0.2 M was applied. Both vessels contained $10^{-3}$ M mercaptoethanol but no glycerol. When the linear gradient of potassium phosphate finished, the column was washed with 90 ml of 0.20 M potassium phosphate, pH 7.5, $10^{-3}$ M mercaptoethanol. Then the head of liquid above the column packing was removed and the column was washed with 0.20 M potassium phosphate, $10^{-3}$ M mercaptoethanol, 5% ammonium sulfate. The column eluate was collected as 11 ml fractions and the absorbance at 280 m$\mu$ was recorded. The elution profile is shown in Figure 3.

E. Detection of Aminoacyl-s-RNA Synthetases in the Hydroxyapatite Column Eluate

The assays were carried out as described in Section C. Aliquots of the fractions from the phosphate gradient were assayed directly. The eluate from the 5% ammonium sulfate wash (test tubes 200 to 215 inclusive, Fig. 3) was combined (174 ml, 23.5 O. D. units) and concentrated by ultrafiltration (Blatt et al., 1965) to 5.5 ml in a Diaflo apparatus (Amicon Corp.), using a UM-1 membrane, at 0°. The concentrate was
FIGURE 3. Elution profile from the Chromatography of Fraction III on hydroxyapatite.
freed from excess ammonium sulfate by passage through a G-25 Sephadex column, previously equilibrated with $10^{-2}$ M potassium phosphate, $10^{-3}$ M mercaptoethanol, 40% glycerol (v/v), pH 7.5. Fractions from the Sephadex column comprising the leading peak of absorbance (at 280 $\text{m}$) were combined and stored at -18° until ready for use (Fraction A).

Aliquots of Fraction A were diluted 1:10 with $10^{-2}$ M potassium phosphate, $10^{-3}$ M mercaptoethanol, 40% glycerol (v/v), pH 7.5 then assayed for the various aminoacyl-s-RNA synthetase activities. The results are shown in Table VI along with the corresponding synthetase activities contained in the desalted ammonium sulfate. Fraction III (see Section D) after it had been left at 3-5° during the period of chromatography. The assays were carried out at a 1:20 dilution except where noted.

The column fractions containing the arginyl-s-RNA synthetase activity (test tubes 170 to 199 inclusive, Fig. 3) were combined and concentrated from 312 ml (about 12 O. D. units at 280 $\text{m}$) to about 2 ml on a Diaflo UM-1 membrane at 0°. The concentrate was passed through a G-25 Sephadex column as described above. The Sephadex eluate containing arginyl-s-RNA synthetase activity was stored at -18° (Fraction B) prior to determination of arginyl-s-RNA synthetase activity (Section F).
### TABLE VI

**Aminoacyl-s-RNA Synthetases Present in the Ammonium Sulfate Eluates**

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>m(\mu)moles amino acid incorporated(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Desalted Fraction III</td>
</tr>
<tr>
<td>ala</td>
<td>0.18</td>
</tr>
<tr>
<td>arg</td>
<td>0.28</td>
</tr>
<tr>
<td>asp</td>
<td>0.68</td>
</tr>
<tr>
<td>asn(^c)</td>
<td>0.03</td>
</tr>
<tr>
<td>gln(^c)</td>
<td>0.01</td>
</tr>
<tr>
<td>gly</td>
<td>0.58</td>
</tr>
<tr>
<td>ile</td>
<td>0.08</td>
</tr>
<tr>
<td>leu</td>
<td>0.23</td>
</tr>
<tr>
<td>lys</td>
<td>0.67</td>
</tr>
<tr>
<td>met</td>
<td>0.23</td>
</tr>
<tr>
<td>phe</td>
<td>0.43</td>
</tr>
<tr>
<td>ser</td>
<td>0.47</td>
</tr>
<tr>
<td>thr</td>
<td>0.24</td>
</tr>
<tr>
<td>try</td>
<td>0.09</td>
</tr>
<tr>
<td>tyr</td>
<td>0.66</td>
</tr>
<tr>
<td>val</td>
<td>0.03</td>
</tr>
</tbody>
</table>

\(^a\) Values underlined indicate loss of activity.

\(^b\) m\(\mu\)moles of amino acid incorporated in 1.25 mg s-RNA by 0.05 mg protein under the conditions described in Section 5-C.

\(^c\) asn and gln assayed undiluted for Fraction A
F. Determination of Aminoacyl-s-RNA Synthetase Activities

The assays were carried out at 25° in 1 ml glass tubes containing (in a final volume of 0.20 ml) 20 μmoles sodium cacodylate, pH 7.5, 4 μmoles KCl, 2 μmoles MgCl₂, 1.5 μmoles ATP, 0.08 μmole EDTA, about 50 μmoles of amino acid with specific activity 10 μCi per μmole, 2.5 mg s-RNA, and 50 μl of enzyme solution. The enzyme, appropriately diluted, was added at zero time. Aliquots (25 μl) were removed at one min intervals, applied to Whatman 3MM filter disks and washed as described in Section C. Radioactivity was also determined as described in Section C. Enzyme activities were determined under conditions which gave linear incorporation of a given amino acid during 6 min. A unit of enzyme catalyzes the formation of 1 μmole of ¹⁴C-aminoacyl-s-RNA per min. The results for several amino acids are summarized in Table VII.

6. Preparation and Partial Purification of Aminoacyl-s-RNA Synthetases from Yeast:

Method 3 (Modified)

A. Preparation of the Cell-Free Protein Extract

Two pounds of fresh, pressed bakers' yeast were thoroughly crumbled then added to 2 litres of toluene containing excess dry ice and 0.20 ml of 1 M DFP. The mixture was left at -18° for one hour then the toluene was decanted and the frozen yeast cells left at 3-5° for 72 hr.
### TABLE VII
Specific Activities of Several Aminoacyl-s-RNA Synthetases

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Tyr</th>
<th>Asp</th>
<th>Gly</th>
<th>Lys</th>
<th>Arg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Desalted</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction III</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg protein/assay&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.005, 0.01</td>
<td>0.055, 0.11</td>
<td>0.014, 0.028</td>
<td>0.022, 0.044</td>
<td>0.028, 0.056</td>
</tr>
<tr>
<td>Specific activity &lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.4</td>
<td>2.0</td>
<td>1.8</td>
<td>2.7</td>
<td>3.4</td>
</tr>
<tr>
<td>Total units&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.4</td>
<td>1.1</td>
<td>0.94</td>
<td>1.4</td>
<td>1.8</td>
</tr>
<tr>
<td>Fraction A</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg protein/assay&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0015, 0.003</td>
<td>0.0225, 0.045</td>
<td>0.0225, 0.045</td>
<td>0.009, 0.018</td>
<td>0.011, 0.022</td>
</tr>
<tr>
<td>Specific activity &lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.0</td>
<td>3.2</td>
<td>1.0</td>
<td>6.63</td>
<td>9.1</td>
</tr>
<tr>
<td>Total units&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.67</td>
<td>0.069</td>
<td>0.023</td>
<td>0.15</td>
<td>0.205</td>
</tr>
<tr>
<td>Purification</td>
<td>4.7</td>
<td>1.6</td>
<td>0.56</td>
<td>2.5</td>
<td>2.7</td>
</tr>
<tr>
<td>Fraction B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mg protein/assay&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td>0.005, 0.0024</td>
<td></td>
</tr>
<tr>
<td>Specific activity &lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td>50.0</td>
<td>0.012</td>
</tr>
<tr>
<td>Total Units&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purification</td>
<td></td>
<td></td>
<td></td>
<td>15.</td>
<td></td>
</tr>
<tr>
<td>% Recovery</td>
<td>20</td>
<td>6.6</td>
<td>2.5</td>
<td>11</td>
<td>12</td>
</tr>
</tbody>
</table>

---

<sup>a</sup> Specific activity calculated at two enzyme concentrations

<sup>b</sup> Specific activity expressed as units of enzyme activity per mg of protein

<sup>c</sup> Total units obtained by multiplying specific activity by total mg of protein
The frozen yeast liquefied during the 72 hr at 3-5° and the pH of the paste measured about 5.8. The pH was adjusted to 7.2 by the addition of 200 ml of 1 M Tris(chloride), pH 8.0. Cell debris was removed by centrifugation at 14,600 x g for 30 min in the GSA head of an RC-2B refrigerated (0°) centrifuge. To the supernate (500 ml) were added 0.10 ml of 1 M DFP and 10 g streptomycin sulfate and the mixture left for 20 min in ice. The solution was centrifuged as described above, and the supernate decanted. Then ammonium sulfate (56.1 g per 100 ml cell-free extract) was added to the supernate and, after 20 min in ice, the precipitated material was collected by centrifugation.

B. Partial Purification of the Aminoacyl-s-RNA Synthetases

One of the six pellets obtained above (Section 6-A) was dissolved in a minimum of 10^{-2} M sodium phosphate, 10^{-3} M mercaptoethanol, 10^{-4} M EDTA and 40% glycerol (v/v), pH 7.5. Excess ammonium sulfate was removed by passing the solution through a G-25 Sephadex column equilibrated with the same buffer. The eluate from the Sephadex column containing ultraviolet absorbing (at 280 m\mu) material excluded by the Sephadex beads was combined (129 ml) and stored at -18° until ready for the chromatography.

A portion of the combined eluate (5 ml) was set aside and the remaining 124 ml was applied to a hydroxyapatite column (26 x 5 cm diam.) which had been previously equilibrated with 10^{-2} M sodium phosphate,
10^{-3} \text{ M mercaptoethanol}, 10^{-4} \text{ M EDTA}, 40\% \text{ glycerol (v/v)}, \text{pH 7.5}. The column was developed with a 3 litre linear phosphate gradient from 0.01 to 0.20 M sodium phosphate, \text{pH 7.5}, both vessels containing 10^{-3} \text{ M mercaptoethanol}, 10^{-4} \text{ M EDTA} and 40\% \text{ glycerol (v/v)}. A flow rate of about 1 ml per min was maintained with the aid of an Accu-Flo pump and 18 ml fractions were collected. When the phosphate gradient expired, a 400 ml linear gradient of ammonium sulfate from 0 to 10\% (w/v) was applied. Both vessels contained 0.2 M sodium phosphate, 10^{-3} \text{ M mercaptoethanol}, 10^{-4} \text{ M EDTA}, 40\% \text{ glycerol (v/v)}, \text{pH 7.5}. The elution profile is shown in Figure 4.

C. Detection of Aminoacyl-s-RNA Synthetase in the Hydroxyapatite Column Eluate

The assays were carried out as described in Section 5-C except that the s-RNA (1.25 mg) was added last to the cold incubation mixture and the total incubation time was 45 min. In addition, the fraction from the ammonium sulfate gradient (fractions 187 to 210, Fig. 4) were assayed individually. The blanks contained starting buffer in place of enzyme solution while the controls contained the crude ammonium sulfate precipitated and desalted cell-free extract diluted 20 fold with starting buffer. Table VIII lists the \text{m\mu}moles of 20 amino acids incorporated into 1.25 mg of s-RNA in 45 min by 0.05 mg of crude protein prior to chromatography.
FIGURE 4. Chromatography of the 0-80% Protein Fraction on Hydroxyapatite.
TABLE VIII

Aminoacyl-s-RNA Synthetases Present in the Whole-protein Extract

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>m(\mu)moles of amino acid incorporated \textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>ala</td>
<td>0.27</td>
</tr>
<tr>
<td>arg</td>
<td>0.80</td>
</tr>
<tr>
<td>asp</td>
<td>0.52</td>
</tr>
<tr>
<td>asn</td>
<td>0.09</td>
</tr>
<tr>
<td>cys</td>
<td>0.22</td>
</tr>
<tr>
<td>glu</td>
<td>0.03</td>
</tr>
<tr>
<td>gln</td>
<td>0.13</td>
</tr>
<tr>
<td>gly</td>
<td>1.13</td>
</tr>
<tr>
<td>his</td>
<td>0.72</td>
</tr>
<tr>
<td>ile</td>
<td>0.28</td>
</tr>
<tr>
<td>leu</td>
<td>1.10</td>
</tr>
<tr>
<td>lys</td>
<td>1.65</td>
</tr>
<tr>
<td>met</td>
<td>0.52</td>
</tr>
<tr>
<td>phe</td>
<td>0.52</td>
</tr>
<tr>
<td>pro</td>
<td>0.003</td>
</tr>
<tr>
<td>ser</td>
<td>0.52</td>
</tr>
<tr>
<td>thr</td>
<td>0.21</td>
</tr>
<tr>
<td>try</td>
<td>0.26</td>
</tr>
<tr>
<td>tyr</td>
<td>0.20</td>
</tr>
<tr>
<td>val</td>
<td>0.21</td>
</tr>
</tbody>
</table>

\textsuperscript{a} m\(\mu\)moles of amino acid incorporated in 1.25 mg of s-RNA by 0.05 mg of whole-protein under the conditions described in Section 6-C.

Figures 5 and 6 show the position of elution of the various aminoacyl-s-RNA synthetases from the hydroxyapatite column.
FIGURE 5. Elution profiles of aminoacyl-s-RNA synthetases from hydroxyapatite.
DISCUSSION

The need to prepare aminoacyl-s-RNA synthetases in relatively large quantities arose as a result of attempts to develop a chemical procedure for the isolation of amino acid-specific s-RNA's. Thus the procedure described in Part II-D depends on the availability of the aminoacyl-s-RNA synthetase enzymes in quantities sufficient for charging gram quantities of s-RNA with a specific amino acid. In this regard, yeast presents a particular problem because of the difficulties encountered in disrupting the cell under conditions which are sufficiently mild to preserve the desired enzyme activities.

The usual method employed to disrupt yeast cells is by grinding them with glass beads in a homogenizer. This procedure (Section 1, Method 1) was adequate for preparing the synthetase enzymes in quantities sufficient for the purpose of assaying for amino acid acceptor activity. Attempts to scale up the procedure such that 50 and 100 g batches of yeast cells could be disrupted were unsuccessful. Although the enzyme activities were present in the supernate after the ultracentrifugation step they were present in relatively low amounts. The low levels of synthetase activity probably reflected the inefficiency of the glass bead method for disrupting the yeast cells. It should be pointed out here, however, that Makman (1966) has successfully employed this method for the disruption of 100 g batches of yeast cells for the purification of seryl-, phenylalanyl-,...
arginyl- and leucyl-s-RNA synthetases.

Use of certain other procedures for disruption of yeast cells, such as treatment with toluene, incubation with bicarbonate and freezing in a dry ice-ethanol bath have been tried but were found to be unsatisfactory for preparation of the serine enzyme (Makman and Cantoni, 1965). Sonic disintegration, or freezing in liquid nitrogen followed by homogenization with glass beads (Bennett et al., 1963) resulted in crude extracts with seryl-s-RNA synthetase activity comparable to that obtained by the glass bead method.

An alternative procedure investigated for disrupting large quantities of yeast cells utilized the ability of toluene to lyse yeast cells when warmed to 38 to 40° (Kunitz, 1952; Heppel, 1955). This procedure has been employed by von Tigerstrom and Tener (1967) for the purification of histidyl-s-RNA synthetase from yeast. Although the yeast cells were effectively lysed with toluene at 37° most of the synthetase enzymes were destroyed by this treatment. Studies described in this thesis show that the inactivation of the synthetase enzymes during toluene lysis at 37° was due to a drop in the pH of the medium. As described in Section 2-B, when yeast cells were lysed with toluene at 37° a drop in the pH of the medium could be observed by monitoring the solution with a pH meter, using a glass electrode. The drop in pH of the medium was observed concurrently, with the evolution of gas and
the liquefaction of the yeast paste. The pH of the mixture was maintained at about 7 by the addition of 1 N sodium hydroxide and the consumption of alkali was linear up to about 35 min after the temperature of the mixture had reached 37°. Using this as an indication of the extent of lysis, the yeast cells were essentially all disrupted after 40 min at 37°. Centrifugation of the mixture yielded a cell-free extract containing most of the aminoacyl-s-RNA synthetase enzymes. Prolyl-, glutaminyl- and glutamyl-s-RNA synthetases were notable exceptions. The cell-free extract was then fractionated using ammonium sulfate as described in Section 2-C. Most of the glycyl-s-RNA synthetase activity was precipitated between 2, 4 and 2.9 M ammonium sulfate (Table I, Ammonium Sulfate Fraction B).

In addition to being able to prepare large quantities of yeast cell-free extract, it was also desirable to have a simple procedure for the partial purification of the synthetase enzymes. It was considered important to have preparations of the synthetase enzymes which were free from nucleases and peptidases for use in the combined chemical and chromatographic procedure described in Part II-D. The nucleases would of course degrade the s-RNA while the peptidases could supply amino acids other than the one of interest which would tend to complicate the procedure.
Muench and Berg (1966) have described the fractionation of the aminoacyl-s-RNA synthetase enzymes from *E. coli* on hydroxyapatite. A linear gradient of phosphate buffer from 0.01 M to 0.20 M was used to elute all the aminoacyl-s-RNA synthetase enzymes.

After desalting on Sephadex, a portion of the ammonium sulfate fraction B was applied to a hydroxyapatite column and the latter developed with a linear phosphate gradient up to 0.20 M. The incorporation of glycerol (35% v/v) in the developing solvents permitted storage of the column eluate at -18° without freezing. This condition greatly enhanced the stability of the synthetase enzymes. Glycerol has been used to stabilize other enzymes during storage and purification (Jarabak et al. 1962). Since the chromatography was carried out in the cold room at 3-5° in the presence of 35% glycerol, it was essential to use a pressure pump in order to maintain a reasonable flow rate of the order of 1 ml per min.

Although most of the protein was eluted by the 0.01 to 0.20 M phosphate gradient (see Fig. 1), the eluate showed no activity when assayed for glycyll-s-RNA synthetase activity. The latter was eluted when the column was washed with an increasing concentration of ammonium sulfate from 0 to 10% (w/v).

Radiochromatographic analysis was carried out on alternate fractions of the ammonium sulfate eluate to determine if aminoacyl-s-
RNA synthetases other than the glycine enzyme were present (Section 2-F). Figure 2 clearly shows the presence of at least nine synthetase activities. Thus many of the aminoacyl-s-RNA synthetases are not eluted from hydroxyapatite by up to 0.2 M phosphate buffer. Also evident from Figure 2 is the fact that the activities are eluted as relatively sharp peaks as judged by the appearance and disappearance of the different peaks of radioactivity on the chromatogram.

The reason so many of the synthetase enzymes show such a strong affinity for hydroxyapatite is far from clear. There are a few indications that the affinity may be a result of the formation of some type of nucleotide- or polynucleotide-enzyme complex. For example, Makman and Cantoni (1966) have demonstrated an increased thermal stability of certain synthetase enzymes in the presence of ATP and magnesium, which suggests the formation of a definite ATP-magnesium-enzyme complex. In addition these workers (Makman and Cantoni, 1965) have suggested that the purified seryl-s-RNA synthetase from yeast contains a nucleotide, as yet unidentified, attached to the enzyme molecule. It is also interesting to note that nucleotide polyphosphates are strongly adsorbed to the hydroxyapatite and require high molarities of phosphate buffer to be eluted (Bernardi, 1964).

Since many of the aminoacyl-s-RNA synthetase activities were not eluted by the 0.01 to 0.20 M phosphate gradient an attempt was made
to remove the bulk of the protein by washing the hydroxyapatite column in a single step with 0.20 M phosphate buffer (Section 3B, Chromatography 1). All of the applied material and all of the synthetase activity were eluted in the 0.2 M phosphate buffer. To be sure the column had not been overloaded the chromatography was repeated (Chromatography 2) by applying an amount of crude extract equivalent to that used in the gradient chromatography. Again all the applied material and all of the synthetase activity was eluted in the 0.2 M phosphate buffer. The chromatography was repeated again except the crude extract was applied in 0.01 M phosphate buffer and the column subsequently washed with the same buffer. No synthetase activity and only a small amount of the applied material was eluted. When the column was washed with 0.20 M phosphate buffer, more than 90% of the applied material was eluted and the eluate contained most of the original synthetase activity. Only a small amount of the synthetase activity was detected when the column was subsequently washed with 10% ammonium sulfate (w/v).

It became clear that the crude extract had to be applied to the column in low concentrations of phosphate buffer. In addition, the column must be developed either with small increments (increasing in 0.05 M steps) or preferably with an increasing linear gradient of phosphate buffer, followed by an ammonium sulfate (10% w/v) wash.
Lysis of yeast cells with toluene at 37° under conditions of controlled pH followed by chromatography on hydroxyapatite offered a simple and useful procedure for obtaining most of the synthetase enzymes. However there were still a few synthetases which were absent. Thus, methods were investigated for lysing the yeast cells under milder conditions of temperature.

A recent report on the preparation and purification of yeast hexokinase (Lazarus et al., 1966) described the lysis of yeast cells using toluene in excess dry ice. Section 5-A describes the preparation of a yeast cell-free extract by essentially the same procedure. A summary of the experimental procedures employed is shown in Chart I.

Although two pound lots of crumbled yeast cake were lysed by toluene containing excess dry ice, the method is easily applied to much larger quantities. In Section 5-A the yeast cells were left in the toluene-dry ice mixture for six hours prior to incubation at 3-5°. However, as described in Section 6-A, one hour in the toluene-dry ice mixture is sufficient. It should also be pointed out that the optimum time of incubation of the cell paste at 3-5° after decanting the toluene has not been rigorously established. An aliquot of the yeast paste which was removed after 12 hr incubation at 3-5° was shown to contain aminoacyl-s-RNA synthetase activity in the standard assay. In addition the 44 hr incubation period used in Section 5-A was better than the 72 hr
CHART I

Summary of Experimental (Method 3)

2 lb bakers' yeast - toluene, dry ice (6 hr)
- incubation at 3-5°, (44 hr)
- centrifugation

Cell-free extract
- ammonium sulfate fractionation

Fractions I, II, III, IV, supernate of IV. (Table III)
- G-25 Sephadex

Desalted Fractions II, III, IV, supernate of IV. (Table IV)

- assayed with 20 amino acids (Table V)

Desalted Fraction III

Hydroxyapatite Chromatography (No glycerol) (Fig. 3)

A portion left 3 days at 3-5° then assayed with 16 amino acids (Table VI)

Ammonium sulfate eluate - combined & concentrated
then G-25 Sephadex (40% glycerol)

phosphate gradient eluate
- tubes 170 to 199 combined,
concentrated then G-25 Sephadex (40% glycerol)

Fraction A
- Assayed with 16 amino acids (Table VI)

Fraction B
- Contains arginyl-s-RNA synthetase
incubation period used in Section 6-A. Thus it seems likely that
incubation periods of less than 44 hr at 3-5° would provide just as
efficient lysis of the yeast cells since the breakdown of the cell wall
probably occurs as a result of freezing at -70° in the presence of toluene.

Di-isopropylfluorophosphate (DFP) was added routinely during
preparation of the cell-free extracts to decrease degradation of the
synthetase enzymes by proteolytic enzymes during the extended
incubations at 3-5°. This precaution would also result in the formation
of fewer end groups for the peptidases, so that when the enzyme
preparations are used for charging s-RNA with a particular amino acid,
extraneous amino acids would be present in minimal amounts. The
presence of 0.15 μmole of DFP did not affect the synthesis of 14C-glycyl-
s-RNA under the conditions described in Section 4-B.

The cell-free extract, obtained after removal of the cell debris
by centrifugation, was fractionated by the addition of ammonium sulfate
(see Chart I). The resulting three fractions (Fractions II to IV) and the
supernate from Fraction IV were assayed for 20:aminoacyl-s-RNA
synthetase activities as described in Section 5-C. It was necessary to
remove the excess ammonium sulfate prior to assaying since ammonium
sulfate decreases the level of maximum incorporation of glycine into s-
RNA (Section 4-B). Removal of ammonium sulfate was readily achieved
by gel filtration on G-25 Sephadex. An aliquot of the combined Sephadex
eluate was diluted prior to assaying, to about 0.05 to 0.06 mg of protein per 50 μl. This amount of protein would not fully charge the s-RNA under the conditions described in Section 5-C. Thus a rough indication of the amount of each aminoacyl-s-RNA synthetase activity was obtained for each fraction. The results are tabulated in Table V. Essentially all the synthetase activities were precipitated between 1.0 and 3.0 M ammonium sulfate at 0°. Only arginyl-s-RNA synthetase was present to an appreciable extent in the supernate of Fraction IV. While all the synthetase activities were present in Fraction III (2.0 to 2.5 M ammonium sulfate) only cysteinyl- and lysyl-s-RNA synthetases were present to a greater extent in Fraction II (1.0 to 2.0 M ammonium sulfate) while arginyl-, histidyl- and seryl-s-RNA synthetases were present to a greater extent in Fraction IV (2.5 to 3.0 M ammonium sulfate).

A portion of Fraction III was chromatographed on hydroxyapatite in the absence of glycerol (see Figure III). The eluate from the 5% ammonium sulfate wash was combined, concentrated, and finally desalted on Sephadex (Fraction A) in the presence of 40% glycerol (v/v). The desalted ammonium sulfate Fraction III which had been set aside prior to column chromatography (Section 5-D) and which contained no glycerol, was assayed, along with Fraction A. Table VI lists the amount of the various amino acids incorporated into s-RNA by the desalted ammonium sulfate Fraction III and by Fraction A. It must be emphasized
that the desalted ammonium sulfate Fraction III was stored at the same
temperature at which the chromatography was carried out (3-5°) and also
that glycerol was not used in either case. Comparison of the values
listed in Table VI for Fraction III with those listed in Table V for
Fraction III (when stored at -18° in 40% glycerol) clearly show a large
loss of enzyme activity when the enzymes are not stored at -18° in
40% glycerol. The only activities which do not appear to have
benefitted by storage at -18° in 40% glycerol were the glycyl- and
methionyl-s-RNA synthetases.

Ten of the 16 amino acids studied were found to be enriched in
Fraction A as compared to the desalted Fraction III (see Table VI).
However comparison of the values listed for Fraction A in Table VI
with those for Fraction III in Table V show that only 8 of the 16 amino
acids studied have enriched synthetase activities in Fraction A. Also
apparent from this comparison is the suggestion that the isoleucyl-,
leucyl-, phenylalanyl-, seryl, tryptophanyl- and valyl-s-RNA
synthetases were the most labile of the 16 studied. However, it was
later discovered that the first four mentioned synthetases were eluted
by the phosphate gradient (Figs 5 and 6).

Although the arginy1-s-RNA synthetase activity was enriched in
Fraction A, considerable synthetase activity for this amino acid was also
eluted in the latter part of the phosphate gradient (see Fig. 3, fraction B).
The specific activities of several of the aminoacyl-s-RNA synthetases present as enriched activities in Fraction A were determined (Table VII). The synthetase activity for arginine eluted by the phosphate gradient (fraction B) was also determined. The recoveries of the enzyme activities in fraction A were poor and the overall purifications were not encouraging (with the possible exception of the arginyl-s-RNA synthetase activity in fraction B which was 15 fold purified). From the amount of protein eluted by the phosphate gradient the enzyme activities eluted by the ammonium sulfate wash should be much higher than observed. Even taking into account the skepticism with which one must view the determinations of specific activity values for these enzymes, it is obvious that the chromatography must be carried out in the presence of glycerol and that the column fractions must be stored at -18°C as soon as possible after being collected.

Although enriched fractions may be obtained, it is apparent from Table V that fractionation of a cell-free extract with ammonium sulfate does not cleanly separate any of the synthetases, one from the other. It was therefore decided to precipitate the protein from the cell-free extract in one step and subsequently chromatograph the ammonium sulfate precipitated protein on hydroxyapatite (see Chart II for summary of experimental procedures). This time glycerol was added to 40% (v/v) following ammonium sulfate precipitation. Also incorporated into
CHART II

Summary of Experimental (Method 3, modified)

2 lb bakers' yeast - toluene, dry ice (1 hr)

- incubation at 3-5° (72 hr)

- centrifugation

Cell-free extract

- ammonium sulfate precipitation

0 - 80% protein fraction

- G-25 Sephadex (40% glycerol)

assay with 20 amino acids (Table VIII)

Hydroxyapatite chromatography (40% glycerol)(Fig. 4)

Position of elution of the activities for 20 amino acids in Figs 5 and 6
the eluting system was $10^{-4}$ M EDTA. The latter compound has been shown to have a stabilizing effect on the seryl-s-RNA synthetase enzyme (Makman and Cantoni, 1965).

The crude protein preparation was assayed for synthetase activity toward 20 amino acids (Table VIII). Comparison of the values listed in Table V with those in Table VIII (keeping in mind that the results in Table VIII were derived from protein obtained by a single ammonium sulfate precipitation) suggest that there is little to be gained by extended incubation at 3-5°. On the other hand the 72 hr incubation period did not result in a significant loss of synthetase activity.

When the crude, whole-protein extract was chromatographed on hydroxyapatite, the usual elution pattern was obtained (Fig. 4). The column eluate was assayed in detail for all 20 aminoacyl-s-RNA synthetase activities (Figs 5 and 6). The activities detected only or largely in the ammonium sulfate eluate were alanyl-, aspartyl-, asparaginyl-, cysteinyll-, glutamyl-, glutaminyl-, lysyl-, histidyl-, methionyl-, prolyl-, tryptophanyl-, threonyl-, tyrosyl- and valyl-s-RNA synthetases. Activities detected only or largely in the eluate from the phosphate gradient were arginyl-, isoleucyl-, leucyl-, phenylalanyl- and seryl- s-RNA synthetases. Two peaks of activity were detected for the glycyl-s-RNA synthetase. The first peak was eluted towards the end of the phosphate gradient and the second peak was eluted by the ammonium
sulfate wash (Fig. 5). The significance of the shoulders on the activity profiles for several amino acids (see Figs 5 and 6) is unknown although a recent report by Barnett and Brown (1967) has shown that mitochondria might have their own unique s-RNA's and aminoacyl-s-RNA synthetases. The possible significance of the two peaks of glycyl-s-RNA synthetase activity is discussed in more detail in Part II-D, Section 3-B.

The lysis of yeast cells with toluene at low temperature has provided a method for the preparation of most of the aminoacyl-s-RNA synthetases in quantities sufficient for charging large quantities of mixed s-RNA with a given amino acid. Based on the work described in Part II-D, Section 3-B, it is estimated that two pounds of pressed bakers' yeast can provide enough enzyme to charge at least 10 g of yeast s-RNA with glycine.

Chromatography of the crude protein preparations on hydroxyapatite separates most of the synthetase enzymes from the rest of the cellular protein. In addition, low temperature lysis followed by hydroxyapatite chromatography of the cell-free extract appears to be a suitable approach for the purification of individual aminoacyl-s-RNA synthetases by further chromatographies. Shorter incubation periods at 3-5°C are suggested. It might also be advisable to add glycerol and/or neutral buffer after the yeast paste warms to 3-5°C. However, it should be kept in mind that the addition of glycerol at this stage can
complicate the fractionation of the cell-free preparation with ammonium sulfate.

The major limiting factor for the preparation of even larger quantities of the synthetase enzymes is the relatively low capacity of the hydroxyapatite columns. More protein could probably be applied to these columns if the nucleic acid content of the cell-free extract were diminished. This could be achieved by passing the cell-free extract through a DEAE-cellulose column under conditions which retard nucleic acids but not the synthetase enzymes (Holley and Goldstein, 1959) and in the presence of glycerol.

Finally it can be pointed out that better resolution of the synthetase activities in the ammonium sulfate eluate might be obtained by using an extended ammonium sulfate gradient. Alternatively the column could be developed with an exponential gradient of phosphate buffer.


McCorquodale, D. J. (1964), Biochim. Biophys. Acta 91, 541.


