ISOLATION AND ANALYSIS OF RIBONUCLEIC ACIDS

by

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This thesis is based on research conducted by the author in the Pneumoconiosis Research Unit, National Chemical Research Laboratory, South African Council for Scientific and Industrial Research, and is presented for the degree of Master of Science in the Faculty of Science at the University of Cape Town.

Pretoria, 1967.

ACKNOWLEDGEMENTS

I wish to thank Dr. P.C. Carman, Director of the National Chemical Research Laboratory, for kind permission to utilise work done in NCRL for this thesis.

I also wish to thank

Professor J.E. Kench, for guidance in the research and the writing of this thesis;

Dr. Helen M. Schwartz, for supervising this research and for generous assistance in the writing up of results for publication and for this thesis;

Dr. W.H.E. Fitschen, for instigating much of this research, and for his interest in, and encouragement thereof;

Dr. L. Novellie, for kindly allowing me the use of photographic equipment and the darkroom of the Bantu Beer Unit;

Dr. A.C. Meyer, for kind permission to use the Canalco Model E microdensitometer;

Dr. T.N. van der Walt, for kindly determining the sedimentation constant of yeast sRNA on the analytical ultracentrifuge;

and my wife, for many things, but in particular, for typing this thesis.

Alas! what boots it with uncessant care
To tend the homely, slighted shepherd's trade
And strictly meditate the thankless muse?
Were it not better done, as others use,
To sport with Amaryllis in the shade
Or with the tangles of Neaera's hair?

Fame is the spur that the clear spirit does raise
(That last infirmity of noble mind)
To scorn delights, and live laborious days.

Lycidas, John Milton.

No comment.

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CHAPTER I - INTRODUCTION

Ribonucleic acids are concentrated in certain parts of the cell. Separation of these parts usually precedes isolation of the ribonucleic acids they contain. This separation is accomplished by a process of cell fractionation (Mathias, 1966).

Cell fractionation

Tissues such as rat liver are homogenised and then separated in the ultracentrifuge into several subfractions corresponding to the various parts of the cell, namely, the nucleus, mitochondria, microsomes and cell sap. The latter two subfractions are a major source of ribonucleic acid (RNA) and justify a brief description:

The microsomes sediment in the ultracentrifuge after the mitochondria within 2 hours at 105,000g. This subfraction includes rough membranes (fragmented endoplasmic reticulum, E.R., bearing ribosomes), smooth membranes (fragmented E.R. without ribosomes), and free ribosomes. Separation of these structures from each other is readily achieved. Thus the rough membranes sediment at 78,000g after 1 hour. Smooth membranes and free ribosomes in the supernatant are separated by prolonged centrifugation in a sucrose solution of density intermediate between that of the smooth membranes and the ribosomes (Chauveau, Moule, Rouiller and Schneebeli,

1962). The smooth membranes float to the top while the ribosomes form a pellet at the bottom. The isolation of ribosomes from rough membranes is also feasible. A detergent serves to disperse the E.R. while leaving the ribosomes intact and free to sediment at 105,000g. Such ribosomes are usually a cluster of several ribosomes tied together by an extended molecule of RNA called messenger RNA (mRNA). This cluster is called a polyribosome or polysome.

The cell sap is defined as the supernatant remaining after sedimentation of the microsomes at 105,000g. Acidification of the cell sap to pH 5 causes a part to precipitate which contains so-called soluble RNA and several enzymes.

Nature of RNA

An account of the distribution of RNA in the cell must be prefaced by a description of RNA.

It is a polymer obtainable by the condensation of many nucleotides in a strict linear order. These nucleotides comprise a purine or pyrimidine base (usually adenine, guanine, cytosine or uracil, abbreviated as A, G, C, U), ribose and phosphoric acid. The base is linked by a glycoside bond to the 1-carbon of ribose while the phosphoric acid esterifies the hydroxyl group on the 5-carbon. In the polynucleotide, therefore, successive ribose units are linked together by phosphodiester bonds between their 3 and 5-carbons. The two ends of the polymer are distinguished as the 5' end (at

which a terminal nucleotide is linked to the rest of the molecule via its 3'-carbon), and the 3' end (at which a terminal nucleoside is attached to the rest of the molecule via its 5'-carbon). The 5' end is phosphorylated on the 5' OH while the 3' OH of the 3' end is unesterified. The polynucleotide so constituted is a single unbranched strand varying in the degree of tertiary structure.

It is customary to name many ribonucleic acids according to their sedimentation constants. By this token a particular acid occurring in ribosomes is called 28S RNA. As this nomenclature can be somewhat confusing, a brief explanation (and apology) is due:

28S RNA means that RNA whose sedimentation constant s, measured at infinite dilution in distilled water at 20^{o}, evaluates to 28 Svedberg units, where 1 Svedberg = $S = 1 \times 10^{-13}$ seconds. The sedimentation constant of a substance is its rate of sedimentation in a unit centrifugal field of force.

Several ribonucleic acids exist characterised by size and by their location mainly in one or other part of the cell (Watson, 1963). The least tangible of these acids is messenger RNA which is continually being synthesised in the nucleus and transferred to the polysomes. It is a replica of the gene bearing the same four bases A, G, C and U arranged in the same sequence as in the parent DNA, and is the link between the gene and its corresponding protein. A second kind of RNA occurs in ribosomes called ribosomal RNA (rRNA). It comprises three ribo-

nucleic acids characterised by sedimentation constants of 28, 18 and 5S. A third kind of RNA occurs in the cell sap called soluble or transfer RNA (sRNA or tRNA). It has a sedimentation constant of 4S.

Protein biosynthesis

The nature of the various kinds of RNA is well illustrated by their role in protein biosynthesis (Arnstein, 1965).

Essentially, this involves the condensation of many amino acids in a precise sequence. The first step towards this end is the reaction of the amino acid with ATP to form a reactive AMP-derivative. An enzyme catalyses this step called aminoacyl-tRNA-transferase, and may be prepared from the pH 5 fraction described earlier. This same enzyme causes the "activated" amino acid to react with the appropriate tRNA molecule so as to esterify the free 3' OH group of the terminal adenosine. The resultant aminoacyl-tRNA attaches itself to the polysome such that a part of the tRNA chain complexes with a specific part of the ribosome surface, while another part complexes with the appropriate trinucleotide sequence (triplet) occurring in the mRNA molecule. For every such triplet there is only one responsive tRNA which, in turn, reacts with only one amino acid. Thus, as the mRNA message is "translated" from one end to the other in steps of one triplet at a time, only the correct aminoacyl-tRNA will complex with the messenger at each step. While any one

aminoacyl-tRNA is in position, a second attaches itself to the same ribosome and complexes with the next triplet. The amino group of the newly-arrived aminoacyl-tRNA reacts with the (aminoacyl-)carboxyl group of its neighbour so as to expel the tRNA esterifying this group and form a peptide bond. The resultant dipeptidyl-tRNA is free to react with the next entering aminoacyl-tRNA. With each peptide-condensing step the ribosome moves on to the next triplet until the polypeptide-tRNA.. ribosome complex has reached the end of the messenger. Cleavage of the ester bond to tRNA occurs and the nascent protein is released from the ribosome.

This account of protein synthesis reveals the functions of the three kinds of RNA. Thus mRNA serves to organise the amino acids of the future protein into the correct sequence; rRNA provides a surface of contact for the various reactants, and tRNA acts as the adaptor between the triplet of the mRNA and the corresponding amino acid. No cognisance is, however, taken of the endoplasmic reticulum to which the polysome is attached *in vivo* and which undoubtedly plays a part.

The various ribonucleic acids to be studied in this thesis may now be considered more fully.

Transfer RNA

This name, like the name "amino acid", describes a group of substances. All have a sedimentation constant of 4S, a molecular weight about 25,000 and some 70-80

nucleotides comprise the polynucleotide (Brown & Lee, 1965).

Isolation originally proceeded from the pH 5 enzyme fraction (Hoagland, Stephenson, Scott, Hecht & Zamecnik, 1958; Holley and Goldstein, 1959; Moldave, 1963), but an alternative preparation from unfractionated material does exist for sRNA from yeast (Holley, Apgar, Doctor, Marini & Merrill, 1961), which has subsequently been adapted for preparation from rat liver (Brunngraber, 1962). The latter method is said to yield 3-4 times as much sRNA (of equivalent quality) as the previous method. The liver is not fractionated, but is homogenised directly in phenol (which denatures proteins and removes ribonucleases). The RNA in the aqueous phase is removed by ion-exchange on a DEAE-cellulose column, where it is washed before being eluted with 1M NaCl, which is fairly specific for 4S RNA. It has since been shown, however, that sRNA prepared from yeast by this method is heterogeneous and contains up to 30% of RNA both heavier and lighter than 4S. The lighter material is degraded 4S RNA, but the identity of the heavier material is unknown (Richards, Coll & Gratzer, 1965).

In view of this heterogeneity the name sRNA will be used throughout this thesis to describe material prepared in the laboratory comprising mainly 4S RNA, but which also contains other ribonucleic acids. By contrast, the name tRNA will refer only to 4S RNA, able to transfer amino acids to protein.

5S RNA

During the course of this work a new RNA was in the process of being discovered, namely 5S RNA. It was independently but belatedly "discovered" in the author's laboratory and, because of the bearing it has on this thesis, a brief historical account will be given:

Rosset & Monier (1963) analysed RNA from Escherichia coli ribosomes on methylated albumen-kieselguhr columns and managed to split the 4S peak into two peaks of sedimentation constants 4 and 5S. Using similar techniques Galibert, Larsen, Lelong and Boiron (1965) verified the existence of a 5S RNA in ribosomes from rat liver. They were unable to find it in cell sap. In 1966 Schleich & Goldstein found 5S RNA in soluble RNA prepared from E. coli and separated it by gel filtration on Sephadex. In the same year, Bachvaroff & Tongur isolated 5S RNA from rat liver ribosomes and showed that one molecule occurred per ribosome. They found that it was absent from cell sap. A sedimentation constant of 4.6 was measured. In 1967 Comb & Zehavi-Willner isolated 5S RNA from Blastocladiella emersonii. One molecule per ribosome was found. It appeared to lie between the two subunits of the ribosome and could be released from the ribosome by EDTA. A structural role was suggested. Brownlee & Sanger (1967) have begun sequence studies on the 5S RNA from E. coli.

Summarising, the following picture of 5S RNA emerges: it has a sedimentation constant close to 5S, occurs only in the ribosome and seems to hold the ribosome together.

Uncertainty about its location in the cell prevalent during the course of much of my own work, has been dispelled; and earlier assertions that it was a precursor of tRNA have been disproved.

Membrane RNA

RNA has been found in the smooth membranes of the endoplasmic reticulum (Moule, Rouiller & Chauveau, 1960; Hallinan & Munro, 1964). The nature of this material is controversial as may be inferred from the following account. That it resembles ribosomal RNA is evident from sucrose gradient centrifugation (Petrovic, Becarevic & Petrovic, 1965). However, Rodionova & Shapot (1966) find it has a sedimentation constant of 12-14S in a sucrose gradient (but cannot rule out a possible origin by degradation of ribosomal RNA). Shapot & Pitot (1966) find several components between 4 and 10S using Sephadex and DEAE-cellulose chromatography. Bergeron-Bouvet & Moule (1966) find a close resemblance to ribosomal RNA on a sucrose gradient, but the 28S peaks of ribosomal and membrane RNA are not identical.

To summarise, it has been found that smooth membranes almost certainly contain RNA, but on the whole this material turns out to be similar to ribosomal RNA. Evidence for the existence of a unique RNA in smooth membranes is inadequate. In particular, the much sought after messenger RNA has not been identified in membrane RNA.

CHAPTER II - OBJECTIVES

A preliminary aim was the isolation of yeast sRNA by the method of Holley et al. (1961) with a view to acquiring familiarity with the method and the material.

It was then my intention to prepare sRNA from rat liver by the methods of Brunngraber (1962) and Moldave (1963); to compare the products with respect to yield and aminoacyl acceptor activity as Brunngraber had done, and, in addition, with respect to a further criterion, namely, heterogeneity as revealed by disc electrophoresis (according to Richards et al., 1965). It was expected that the heterogeneity already shown for yeast sRNA would also obtain for rat liver sRNA, while its extent and nature for the two products might conceivably differ in view of the radically different preparative procedures involved.

A further objective was to devise a satisfactory method for the isolation of membrane RNA and to analyse this material by a suitable technique. It was expected that membrane RNA would contain mainly ribosomal RNA.

As the latter task required a knowledge of ribosomal RNA, a simultaneous examination of rRNA was also to be undertaken.

CHAPTER III - RESUMÉ OF RESULTS

1. While the superior yield granted by Brunngraber's method was readily demonstrable, equivalence of the two products was not found.

2. Contrary to Brunngraber's results the aminoacyl acceptor activities differed. Brunngraber-sRNA was more active and contained a higher percentage of tRNA.

3. Calculation of this activity on the basis of tRNA content again revealed that Brunngraber-tRNA was more active. It was inferred that Moldave-sRNA was inferior in quantity and in quality.

4. Both Moldave and Brunngraber-sRNA proved to be heterogeneous on electrophoresis.

5. This was accounted for mainly by the presence of 5S RNA in Brunngraber-sRNA (which was found to be absent from Moldave-sRNA), and by the presence in Moldave-sRNA of ribonucleic acids <4S, presumably arising by degradation of 4S RNA.

To summarise, Brunngraber-sRNA was shown to be heterogeneous and to contain 5s RNA, by the technique of disc electrophoresis. Moldave-sRNA did not contain 5S RNA and was appreciably degraded. These results were original and were accordingly submitted for publication (King, 1967).

6. The preparation of RNA from smooth membranes initially resulted in degraded material.

7. Precautions had to be taken against degradation and the undegraded material thus obtained was electrophoresed on polyacrylamide.

8. The composition of membrane RNA in terms of its constitutent ribonucleic acids was determined and compared with ribosomal RNA.

9. Similarities in composition elicited the conclusion that contaimination of smooth membranes with free ribosomes was largely responsible for so-called membrane RNA.

10. However, a ribonucleic acid was found only in smooth membranes that has not hitherto been described. No clues to the nature of this material have been provided by this research.

To summarise, relatively undegraded RNA was obtained from smooth membranes, but was composed mainly of ribosomal RNA. A novel RNA was, however, also present.

CHAPTER IV - MATERIALS AND METHODS

Several abbreviations have been resorted to:

Abbreviations

ATP, adenosine 5'triphosphate

DEAE, diethylaminoethyl

EDTA, ethylene diamine tetraacetic acid

γ, gamma or microgram

H_2O, distilled water

isooctane, 2,2,4 trimethylpentane

M.W., molecular weight

POPOP, 1,4 bis 2-(4 methyl 5 Phenyloxazolyl) benzene

PPO, 2,5 diphenyloxazole

TCA, trichloroacetic acid

TEMED, tetramethylethylenediamine

tris, trishydroxymethyl amino methane

Materials were obtained from the following sources:

Materials

ATP (disodium, trihydrate) was obtained from Boehringer & Soehne, Mannheim

DEAE-cellulose, from Serva, Heidelberg

Isooctane, from Eastman organic chemicals, New York

PPO and POPOP, from Packard Instrument Company, Illinois

Radioactive aminoacids, from Schwarz BioResearch, New York

Sephadex, from Pharmacia, Uppsala

TEMED, from Canalco, Maryland.

Yeast, from Compressed Yeast (Pty) Ltd, Johannesburg

Other common reagents (invariably analytical reagent grade) came from Merck or British Drug Houses.

METHODS

M.1. The preparation of ribosomes from rat liver

About 9 rats (unstarved young white females, weighing about 160 g each) are beheaded, and 50 g liver is removed, washed and drained and homogenised with an MSE homogeniser in 100 ml of Wettstein's Medium A (.25M sucrose,.05M tris, .025M KCl, .005M $MgCl_2$). Nuclei and mitochondria are centrifuged down at 20,000 g for 15 minutes. One g of sodium desoxycholate (DOC) is dissolved in 20 ml Medium A and added to the 20,000g supernatant.

The mixture is spun at 105,000g for 2 hours and the combined ribosome pellets are suspended in 10 ml Medium A and frozen.

M.2. Preparation of ribosomal subunits

The 20,000g supernatant is decanted and mixed with 1.3 g DOC dissolved in 5 ml warm H_2O. The clear solution is layered carefully above 3-4 ml 1M sucrose in 40 rotor (Spinco Model L) tubes. The tubes are spun at 165,000 g for 2.5 hours (or 105,000g for 4 hours). The supernatant is aspirated and discarded and the pellets are washed briefly with ice-cold H_2O and drained and stored at -15°. The ribosome pellets are transferred to a 50 ml MSE centrifuge tube by suspension in 24 ml of .001M tris pH 7.6.

Immediately before centrifugation through a sucrose density gradient, 10 ml of .1M EDTA pH 7.6 is added to dissociate the ribosomes.

Several conditions obtained for most experiments.

Conditions

Centrifugal field forces (e.g. 105,000 g) measured in g units (gravities), represent g-average values. Unless otherwise stated, all procedures with labile materials were conducted close to 0^{o}. Water when mentioned was invariably distilled water. Percent solutions were always made up as w/v i.e. weight upon volume.

M.3. Preparation of pH 5 enzyme

Reference: Moldave (1963), and Holley and Goldstein (1959)

50g liver is homogenised in 80 ml Medium A. The 20,000g supernatant is spun at 105,000g for 2 hours, to remove microsomes and ribosomes, and decanted through a filter. The filtrate is acidified to pH 5.1 with 1M acetic acid and spun 20,000g for 15 minutes. To purify the enzyme, this precipitate is resuspended in H_2O and spun down: then resuspended in .1M tris pH 7.5, again precipitated at pH 5.1 and spun down.

This precipitate is resuspended in 12 mls .1M tris pH 7.5 and clarified by spinning at 15,000g for 10 minutes.

M.4. DEAE-cellulose chromatography

Reference: Doctor et al. (1961).

The above pH 5 enzyme is mixed with 300 mg of DEAE-cellulose to remove dissolved sRNA. The slurry is layered on top of 300 mg of DEAE-cellulose in a column (the DEAE-cellulose must first be equilibrated with .1M tris pH 7.5) and allowed to flow through at 10 ml/hour.

The effluent is rapidly frozen in 1 ml aliquots and stored at -15°.

M.5. Preparation of smooth membranes

35g of liver obtained from 7 unstarved rats is homogenised in 140 ml Medium A containing 30% w/v sucrose. The 20,000g supernatant is spun 78,000g for 60 minutes to sediment rough membranes. Only the upper 60% of

the supernatant is aspirated (80 ml) and mixed with 40 ml of iso-octane and homogenised in a Dounce homogeniser (15 strokes). The emulsion is spun in one single batch at 165,000 g for 2 hours (or 105,000g for 3 hours). The smooth membranes at the interface are collected and put under several ml iso-octane. They are freeze-dried for 30 - 40 minutes to remove all the iso-octane.

The membranes are then shaken at room temperature with 15 ml of sodium dodecyl sulphate (SDS) solution (Solution S .1M tris pH 7.6, 1% SDS, .1% 8 hydroxy-quinoline, .1% naphthalene disulphonate).

M.6. Yeast sRNA

Reference: Holley et al. (1961).

The same method is used as the authors'. One lb of yeast cakes is homogenised gently in 600 ml H_2O to suspend the yeast cells. Nine hundred ml of 74% phenol is added and the mixture is stirred for 1 hour. It is allowed to cool overnight at 0° and is then centrifuged to separate the two phases. The aqueous phase is aspirated and mixed with 20% potassium acetate pH 5.0 (final concentration 2%) and 2.5 volumes of ethanol (100%). The mixture is allowed to cool overnight at 0°. The precipitate is recovered by centrifugation and suspended in 150 ml of .1M tris pH 7.5. It is shaken with 2 volumes ether and the aqueous phase is poured through DEAE-cellulose, 7½ g. The column is washed with 650 ml of tris. RNA is eluted from the column with 200 ml of 1M NaCl and ethanol is added to precipitate the RNA.

The precipitate is washed (by solution in H_2O and reprecipitation with ethanol) and dried over P_2O_5 in vacuo.

M.7. sRNA B from rat liver

Reference: Brunngraber (1962).

The method is essentially the same as the authors'. Twenty-one rats are beheaded to provide 100g of liver which is homogenised in an aqueous phenol medium (120 ml .1M tris pH 7.5, .005M EDTA and 1M NaCl plus 150 ml 74% phenol). The mixture is shaken for 1 hour at 0° and centrifuged at 20,000g for 15 minutes. The aqueous phase is decanted and spun at 78,000g for 45 minutes to sediment glycogen. The supernatant is carefully aspirated and poured into 600 ml ethanol cooled to -15°. The stringhy white precipitate is centrifuged down and dissolved in .1M tris by homogenisation. The solution is centrifuged at 78,000g for 15 minutes to sediment residual glycogen. The supernatant, a transparent lemon yellow solution, is carefully aspirated and mixed with 1g DEAE-cellulose. The slurry is poured onto 2g DEAE-cellulose in a column. A flow of 1 drop per 4 seconds is maintained by hydrostatic pressure. The column is washed with 1 litre of .1M tris pH 7.5 under pressure which takes 12 hours. The sRNA is eluted as a brown zone on the column with 200 ml of 1M NaCl. Fractions rich in RNA are pooled and shaken with phenol for 60 minutes at 0°. RNA is isolated from the aqueous phase by precipitation with ethanol, washed and dried.

M.8. Ribosomal RNA

10 ml frozen ribosomes (see M.1.) are thawed and mixed with 10 ml .2M tris pH 7.5 containing 300 mg SDS and shaken at room temperature for 5 minutes. Twenty-five ml of 74% phenol is added and the mixture is shaken for 45 minutes at room temperature. The aqueous phase is recovered and shaken with a second portion of phenol for 15 minutes. The aqueous phase is spun at 78,000g for 45 minutes to sediment glycogen. The supernatant is aspirated and mixed with ethanol and potassium acetate. The precipitate is washed, dried and weighed. About 75 mg of a dry white powder is obtained.

M.9. Ribosomal subunit RNA

Given 45 ml of frozen .01M sodium acetate solution pH 7.6 (containing sucrose) containing about 5 mg of ribosomal subunits (see M.17. for preparation), the solution is thawed, made .01M with $MgCl_2$ and brought to pH 5.0 with acetic acid. The fine precipitate is recovered by centrifugation at 78,000g for 30 minutes. It is dissolved in 15 ml of Solution S (mainly SDS, see M.5) and extracted with four successive portions of 85% phenol (85 ml redistilled phenol, 15 ml Solution S). The final aqueous phase is carefully aspirated and mixed with sodium acetate (final concentration 10% pH 6.5) and 2 volumes of acetone. After 12 hours at 0° the precipitate is recovered by centrifugation, washed in dilute EDTA pH 7.6 solution, and dried and stored at -15°.

M.10. ^{14}C aminoacyl-sRNA

Reference: Moldave (1963).

The pH 5 enzyme solution (see M.3.), supplemented with .025M KCl, .005M $MgCl_2$ is incubated with 5 ml .05M $MgCl_2$ solution (containing 120 mg ATP pH 7.5) and 25 µc of ^{14}C valine (or ^{14}C yeast protein hydrolysate) for 20 minutes at 37°, cooled in ice, acidified to pH 5.1 and centrifuged at 20,000g for 15 minutes. It is then resuspended in 12 ml .1M tris pH 7.5, 1.0M NaCl and extracted once with 74% phenol. RNA is isolated from the aqueous phase by precipitation with ethanol, washed, and dried.

M.11. sRNA M from rat liver

References: Moldave (1963) and Holley & Goldstein (1959)

The pH 5 enzyme (prepared as in M.3., but with only one precipitation at pH 5) was made 1.0M with NaCl and shaken with 74% phenol for 1 hour at 0°. The aqueous phase was ether extracted and precipitated with ethanol. The precipitate was washed, lyophylised and dried. A white fluffy material was obtained.

M.12. Membrane RNA

15 ml of smooth membranes (see M.5.) are thawed and extracted four times with 5 ml portions of 85% phenol at 0°. The deproteinised aqueous phase is mixed with sodium acetate and acetone to precipitate RNA. The precipitate is washed with EDTA solution pH 7.6 and dried and stored at -15°.

M.6-12. Resume, isolation of RNA from solutions of protein

Procedures adopted vary with the needs. Phenol used to denature protein may require SDS to ensure release of RNA into the aqueous phase. Inhibition of ribonuclease may be secured by the addition of hydroxy-quinoline and naphthalene disulphonate. RNA may be precipitated from the aqueous phase by ethanol and 1M NaCl, by ethanol and potassium acetate pH 5, or by acetone and sodium acetate pH 6.5. The latter procedure is only necessary at low concentrations of RNA.

M.13. Assay of acceptor activity of sRNA

Compare Wettstein & Noll (1965).

Activity of sRNA is measured as follows (triplicate controls, unknowns and standards are included):

< 30 µg of sRNA is incubated for 10 minutes at 37° with 0.5 µc of yeast protein hydrolysate ^{14}C amino acids (1.25 µc/µg) in 0.3 ml of tris.HCl (40 µmoles pH 7.5), KCl (10 µmoles) $MgCl_2$ (8 µmoles) and ATP (5 µmoles). Transferase enzyme (see M.4 for preparation) is added last (0.2 ml). Controls are devoid of added RNA; standards contain sRNA of known acceptor activity in amount equal to that present in the unknown. Reaction is terminated by adding 0.3 ml of cold 17% TCA, filtering and washing the precipitate on a Millipore (RAWP025) cellulose nitrate filter followed by drying and counting of ^{14}C in toluene containing phosphor in a Packard Tricarb liquid scintillation counter (see M.14). Control counts per minute are subtracted from unknown and standard. Using a freshly prepared and very active enzyme the

standard sRNA accepted 693 ± 17 cpm/µg. Because the enzyme varies in activity readings are corrected by the formula

$$\frac{\text{cpm/µg of unknown}}{\text{cpm/µg of standard}} \times 693 \text{ cpm/µg}$$

M.14. Measuring Carbon Fourteen

Millipore filter discs bearing ^{14}C precipitates are dried, immersed in 10-15 ml toluene (containing 5g PPO and 300 mg POPOP per litre) and counted for 10 minutes with a gate setting of 50-815 and gain of 5% in a Packard Tricarb Liquid Scintillation Counter. Under these conditions about 60% of the disintegrations are counted.

M.15. Measuring carbon fourteen in polyacrylamide gel

The gel containing ^{14}C is sliced logitudinally after extrusion from the electrophoresis tube; one half is stained while the other is cut into 2 mm portions, placed in vials and immersed in 0.4 ml of 0.1M KOH for 36 hours at room temperature. Then 15 ml Bray's (Bray, 1960) solution (4g PPO, 200 mg POPOP, 60g naphthalene, 100 ml methanol, 20 ml glycol in 1 litre toluene) is added and the radioactivity is counted.

M.16. Sucrose gradient centrifugation with rotor SW 25

A sucrose gradient apparatus is made, similar to that described by Stead, Nourse and Hawtrey (1964), but with a static outflow down a cotton thread resting against the inside of an SW 25.1 rotor tube. Gradients

are made using 13 mls of 20% and 14 mls of 5% sucrose, .01M with respect to tris pH 7.5, containing .001% SDS. A solution of ribosomal RNA is made in .01M tris and 2½% sucrose solution containing 2.4 mg RNA/ml. One ml of this solution is layered on the gradient and centrifuged in the cold in the Spinco Model L ultracentrifuge for 16 hours at 23,000 rpm. The tube is punctured at the base with a syringe needle and 1 ml fractions are collected. Absorbance at 260 mμ is measured in the Unicam S.P. 8PP spectrophotometer in a 5 mm cuvette.

The fractions are frozen and stored at -15°.

M.17. Separation of ribosomal subunits

Reference: Gould and Klucis (1966).

1200 ml of a linear 10-25% sucrose gradient is made in .01M sodium acetate, .001M tris, pH 7.6 above a 30% cushion of sucrose. The dissociated ribosomes (see M.2.), about 120 mg, are layered above the gradient at 5,000 rpm. The rotor is accelerated to 40,000 rpm and allowed to run for 6 hours whereupon it is decelerated to 5,000 rpm and the gradient pumped out through a 1 cm flow cell and the optical density at 290 mμ measured with a Beckman DB recording Spectrophotometer.

Fractions of 45 ml are collected and frozen.

M.18. Polyacrylamide electrophoresis of sRNA

Reference: Richards, Coll & Gratzer (1965).

A Canalco model 6 apparatus and Model 200 power supply is used.

Clean and dry ten glass tubes ($\frac{9}{16}$ x $\frac{3}{16}$"). Mark the tubes 1 and 2 cm from one end (marks B and A respectively). Close the bottom (the other end) of the tubes with parafilm or with rubber caps. Stand the tubes erect. Constitute 10% monomer solution by mixing, in a flask, 1 ml of buffer A (2.94g tris, 5 ml 1 N HCl in 12.5 ml) 1 ml of TEMED (tetramethylene ethylene diamine 0.28% $^{w}/_{v}$), 2 ml of 40% monomer (9.5g acrylamide and 0.5g of bisacrylamide in 25 ml), 4 ml of ammoniumpersulphate, $(NH_4)_2 S_2O_8$ (140 mg in 100 ml). Evacuate the flask 1 minute to remove dissolved air. Transfer portions of the 10% monomer solution to each tube up to mark A. Layer about 50 µL of H_2O above the solution to provide a flat meniscus during polymerisation. Blot the upper surface of the gel and introduce the 5% monomer solution to mark B (1 ml buffer B viz. 609 mg tris plus 5 ml 1N HCl in 12.5 ml; 1 ml H_2O, 1 ml TEMED, 1 ml 40% monomer and 4 ml persulphate). Layer water above as before. Meanwhile prepare reservoir buffer (808 mg tris and 11.052 g diethyl barbituric acid in 2 litres).

Dissolve 1 mg of sRNA in 0.5 ml reservoir buffer. Add 0.5 ml reservoir buffer containing .5M sucrose. The latter may also contain Bromophenol blue (5γ) and acridine orange (40γ). Insert up to 6 tubes in the apparatus. Pour in the reservoir buffer into both electrode vessels and dislodge bubbles which may insulate either end of the polyacrylamide gel from the reservoirs. Layer 10-100 µL of sRNA solution with a microsyringe above the gel surface. Set the current at 5 mamp/tube and electrophorese for

20-30 minutes. Then remove the tubes and with a stiff wire ease the gel away from the inner walls of the tube. Eject the gel with gentle pressure. Immerse the gel overnight in stain solution (1g of lanthanum acetate, 2g of acridine orange in 100 ml of 15% acetic acid). Remove excess stain (destain) by inserting the gels in large bore tubes plugged at the base with 10% polyacrylamide gel, and electrophoresing in 7.5% acetic acid at 10 mamps/tube for 2-3 hours and store the destained gels in 7.5% acetic acid.

All reagents must be stored at 0°. Bromophenol blue moves with the anion front and illuminates the course of the electrophoresis. When acridine orange is mixed with sRNA it renders the sRNA visible as an orange or green fluorescent material in the gel.

M.19. Polyacrylamide electrophoresis of high M.W. RNA

Reference: Loening (1967)

Reagents are:

Concentrated buffer (12.1g tris, 4.1g sodium acetate, 1.5 g EDTA in 100 ml pH 8.0);

reservoir buffer (40 mls of the above diluted to 1L);

ammonium persulphate, 1g in 100 ml;

TEMED, 3.3 ml in 100 ml;

sucrose, 40g in 100 ml;

monomer solution, 10g acrylamide + 500 mg bisacrylamide in 100 ml.

The composition of the 5% monomer solution is: water 3.3 ml, concentrated buffer .36,

TEMED .2, monomer 4.5 and persulphate .66 ml while the 2½% solution contains:

sucrose 3 ml, water .6, concentrated buffer .24,

TEMED .15, monomer 1.5 and persulphate .5 ml.

Only minor changes have been made in the method devised by Loening. Thus he prefers a gel of only one concentration, but as the concentration required here, 2.5% yields an exceedingly fragile gel, it is sometimes better to make the gel in two parts, viz. a lower 5 and upper (10-20 mm long) 2.5% gel. The lower gel greatly facilitates the manipulation of the gel. RNA is generally dissolved in reservoir buffer containing 5% sucrose. 10-50 µL samples containing <60γ RNA are electrophoresed below room temperature at 5 mamps/gel for 50 minutes. Reservoir solution is used fresh for each electrophoresis.

M.20. Densitometry of gels

Gels are scanned in 7½% acetic acid in a Canalco Model E microdensitometer using a blue filter, gain usually 8, chart speed 8 and integration counts of 20 mm^2.

M.21. Calibration of 2.5% polyacrylamide

A total of about 10 mg RNA is centrifuged on sucrose gradients (see M.16). Corresponding 2 ml fractions from each gradient are pooled. RNA is recovered from each fraction by acetone precipitation in the presence of sodium acetate pH 6.5. Precipitates are washed and dried and stored at -15°. The precipitates are dissolved in 0.3 - 0.6 ml of diluted reservoir buffer containing sucrose.

About 20γ aliquots are electrophoresed on polyacrylamide gel (as described in M.19).

M.22. Photography

The object, a transparent gel in a test tube, is placed (erect) a short distance away from the lens of the camera. Light is provided only from behind the gel by a 100 W pearl bulb shielded by a square of opal perspex. An aperture of about 8 and shutterspeed of about 1/30th is used. Adox KB 14 (ASA 20) film is used. D 76 diluted 1 : 1, for 9 minutes is used for developing. Printing is on Brovira BH 1 using D163 (1+3).

M.23. Sephadex gel filtration

Reference: Schleich & Goldstein (1966).

20g of Sephadex G 100 is equilibrated with 1M NaCl and poured into a 140 x 1.6 cm column. 4 mg samples of sRNA are washed through the column with 1M NaCl and 2 ml fractions are collected using a Central (Central Ignition Co., London) fraction collector. Absorbance at 260 mμ of each fraction is measured in a 5 mm cuvette using a Unicam S.P. 800 Spectrophotometer.

M.24. Phenol distillation

A.R. phenol is mixed with zinc dust and distilled in vacuo. The distilled phenol is stored in the dark at 0°.

CHAPTER V - RESULTS, SOLUBLE RNA

1. YEAST sRNA

Yeast offers a ready source of sRNA during the preparation and subsequent manipulation of which, some preliminary experience of the techniques and the substance itself, might be gained.

1.1 Preparation

The method of Holley et al. (1961) was used as described in Matarials and Methods, method M.6. From about 200 g of yeast cakes some 100 mg of a brown dry powder was obtained.

1.2 Absorption spectra

The ultraviolet absorption spectrum of yeast sRNA was determined and is shown in figure 1.

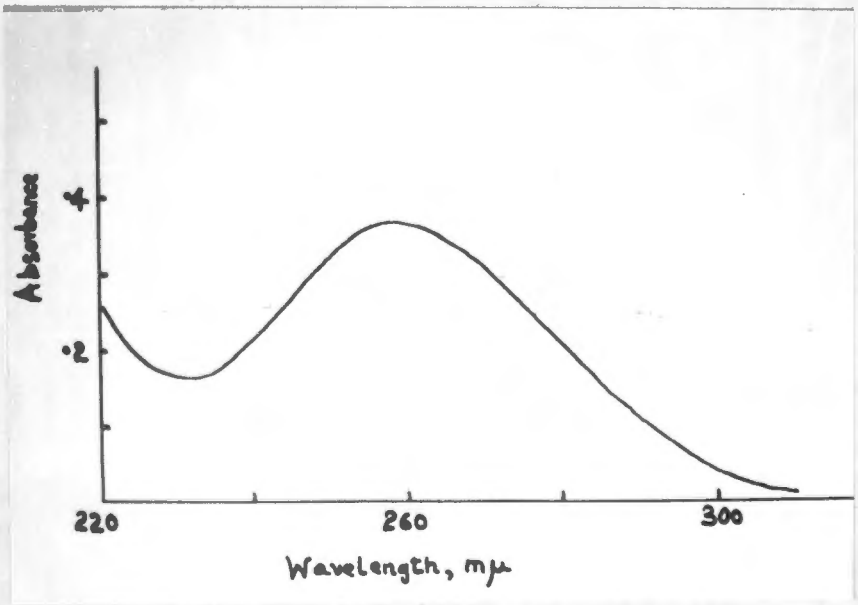


Fig. 1. Ultraviolet absorption spectrum of yeast sRNA.

The spectrum on comparison with that obtained by other authors for sRNA (e.g. Tissieres, 1959) is seen to be typical and has the following features:

(a) maximum optical density at 258 mμ

(b) minimum at 230 mμ

(c) optical density ratios at 260:280 mμ, and 260: 230 equal to 1.8 and 2.0 respectively.

(d) 1 mg/ml had an optical density at 260 mμ of 18.0.

This may be compared with the figure given by Holley et al. (1961) of 19.0. Hence the product comprises mainly RNA.

Some information on the condition of this RNA may be obtained by comparing the absorption curve before and after hydrolysis. Evidently not all the nucleotides in the RNA chain are able to absorb light owing to their proximity to one another. Hydrolysis releases them and permits them to absorb light freely. Salt causes a tighter coiling of the RNA chain and hence diminishes its absorption relative to water as solvent. Extensive hydrolysis of the sRNA preparation will be apparent if these effects are only slight. The relative optical densities measured at 260 mμ are given in Table 1 and may be deduced from figure 2. (See overleaf)

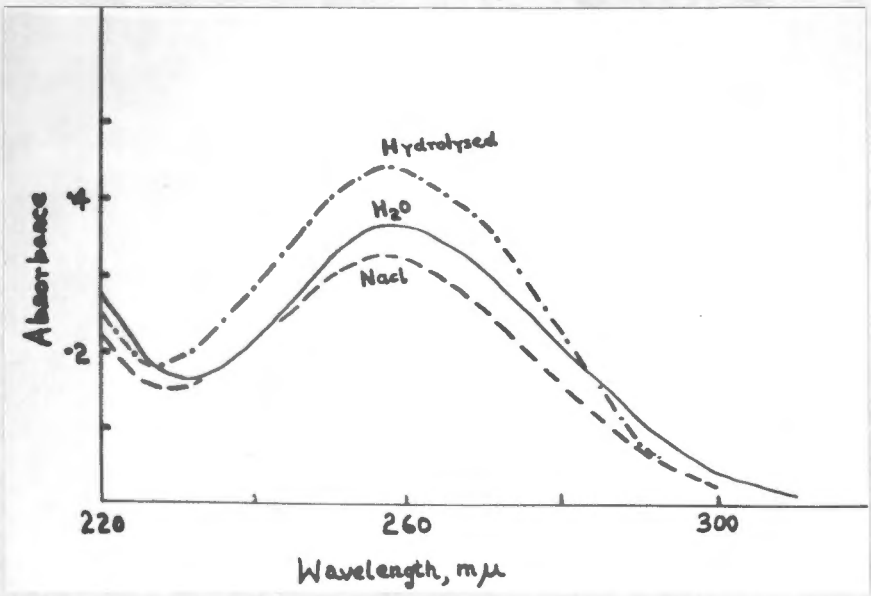


Fig. 2. Effect of salt and hydrolysis on the absorption spectrum of yeast sRNA.

Table 1.

RNA	Solvent	Relative Optical Density
-	.1M NaCl	100
-	.05M KCl	104
-	H_2O	116
Hydrolysed sRNA	-	139

The hydrolysis of 400γ of sRNA was conducted in 3 ml of .3N KOH for 17 hours at 37°. The solution was then neutralised with HCl and diluted to 20.0 ml.

Clearly these effects are present and the sRNA prepared is therefore essentially an undegraded polynucleotide.

1.3 Sedimentation constant

A value of 4.0 is reported for yeast sRNA (Osawa, 1960) and serves very largely to identify this material. Accordingly the sedimentation constant, s, was measured at 20° in a Spinco Model E analytical ultracentrifuge equipped with Schlieren optics (by Mr. N. van der Walt, National Chemical Research Laboratories, Council for Scientific and Industrial Research) on three samples of sRNA dissolved in .1M NaCl, .05M $NaPO_4$ at pH 6.8 at a maximum concentration of 13 mg RNA per ml. s was calculated and plotted against concentration as shown in figure 3.

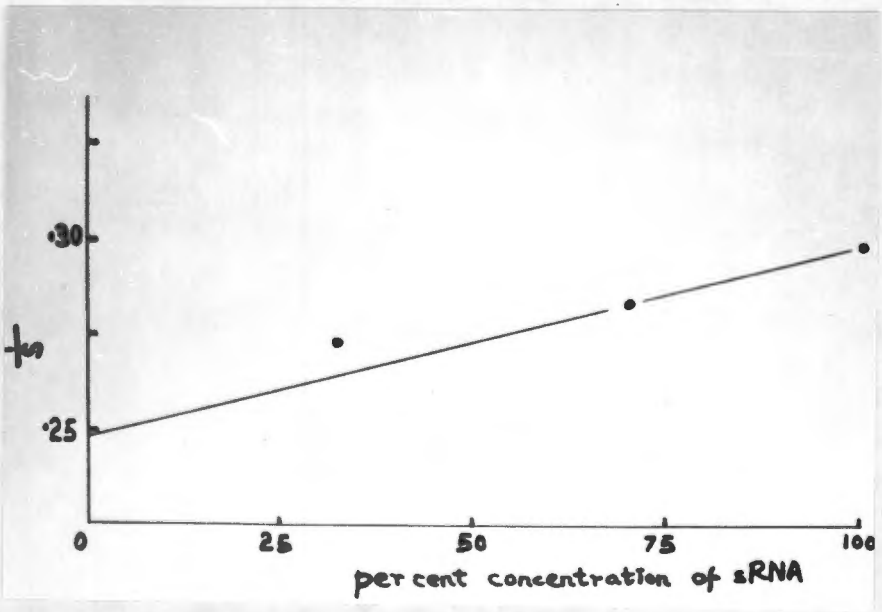


Fig. 3. Extrapolation of the sedimentation constant to infinite dilution.

At zero concentration a value s = 4.05 was obtained in close agreement with the literature.

From plate 1 the homogeneity of the material is apparent:

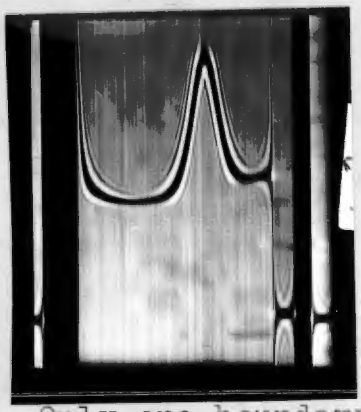


Plate 1. Sedimentation of yeast sRNA in the analytical ultracentrifuge.

Only one boundary is present suggesting that the sample contains only 4S RNA.

1.4 Electrophoresis

Polyacrylamide gel offers a sensitive display of sRNA as demonstrated by Richards, Coll & Gratzer (1965), and also permits an assessment of the homogeneity of the material. At the same time the rate of migration of the zone in the gel is in proportion to the sedimentation constant of the RNA. Thus RNA of s = 4.0S should migrate such that R_f (distance moved by a given zone relative to the distance moved by the anion front in the same time) is 5.4 (Richards, et al., 1965). Using this technique, a sample of yeast sRNA was electrophoresed. The result is shown in plate 2, wherein the bulk of the material is seen to occupy a broad, slightly irregular zone in the centre of the electrophoretogram.

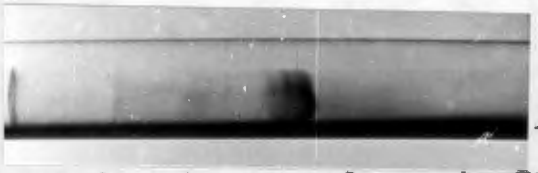
Cathode  Anode

Plate 2. Electrophoretogram of yeast sRNA.

The R_f value of this zone was measured and came to .52 in close agreement with the figure given earlier. From plate 2 may also be seen that faster moving zones are essentially absent, hence degradation is slight and the bulk of the sample is tRNA.

1.5 Conclusion

The sRNA isolated from yeast has therefore been characterised as a nucleic acid by its absorption spectrum. Some idea of its size has been obtained, which is the size expected of yeast sRNA. Essentially the same behaviour and characteristics may be expected of rat liver sRNA. In particular, the method of preparation devised by Brunngraber is similar (and in fact is based on the method used by Holley to prepare yeast sRNA), and the experience gained here should prove useful.

2. RAT LIVER sRNA

The methods of Brunngraber (1962) and Moldave (1963) were applied with slight changes to the preparation of the respective soluble ribonucleic acids from rat liver. During the course of this work I came upon an article by Richards et al. (1965) which claimed that sRNA preparations were not homogeneous, and offered an easy test of homogeneity. This information greatly facilitated the comparison of the two sRNA preparations which I had undertaken to make.

2.1 Preparation of sRNA (Moldave)

The method is described by Moldave (1963) and is based on the method of Holley & Goldstein (1959). It was applied as described in the Methods , methods M.3 & M.11, and may be summarised as follows: Several rat livers are homogenised and the nuclei, mitochondria and microsomes are centrifuged down. The supernatant is acidified and a precipitate is obtained which is denatured with aqueous phenol. RNA is recovered from the deproteinised aqueous phase by precipitation with ethanol.

By this procedure 12 mg of a fluffy white material was obtained from 100 g liver, and designated sRNA, M.

2.2 Preperation of sRNA (Brunngraber)

The method of Brunngraber (1962) was used as described (see Methods M.7). 100 g liver yielded

60 mg of a white material, sRNA, B. In this method the liver is homogenised in aqueous phenol. RNA is removed from the aqueous phase by DEAE-cellulose from which it is eluted by 1M NaCl.

The distinction between the two procedures is that the first separates nuclei, mitochondria and ribosomes from the sRNA at an early stage by centrifugation whereas the second releases all the low molecular weight RNA from the liver homogenate by phenol. The subsequent elution of RNA retained by DEAE-cellulose with 1M NaCl serves to release only low molecular weight RNA such as sRNA from the column.

2.3 Absorption spectra

1 mg per ml of .01M tris, pH 7, solution had an O.D. at 260 mμ of 21.0 ± 0.5 which compares well with published figures (Brunngraber, 1962; Wettstein & Noll, 1965). Absorption spectra typical for RNA were obtained and are reproduced in figures 4 and 5.

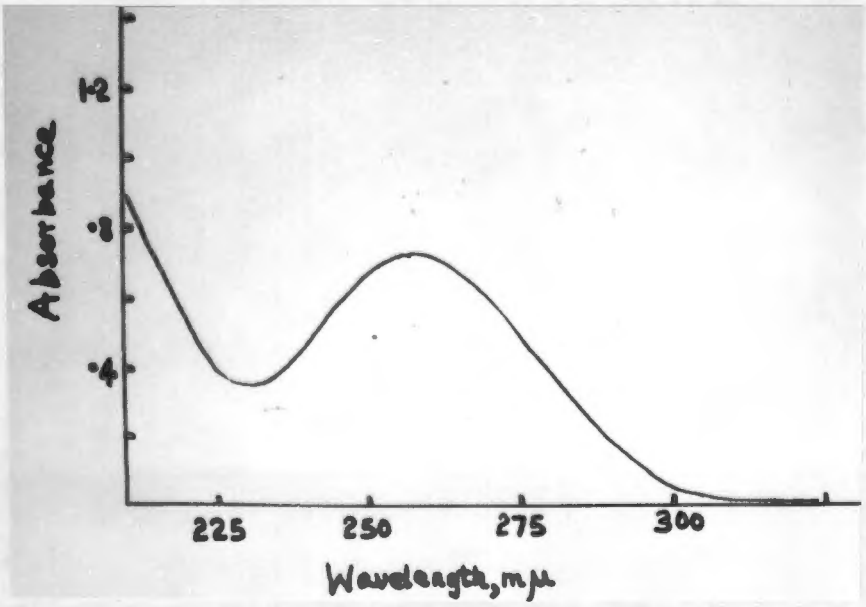


Fig. 4. Absorption spectrum of sRNA, M.

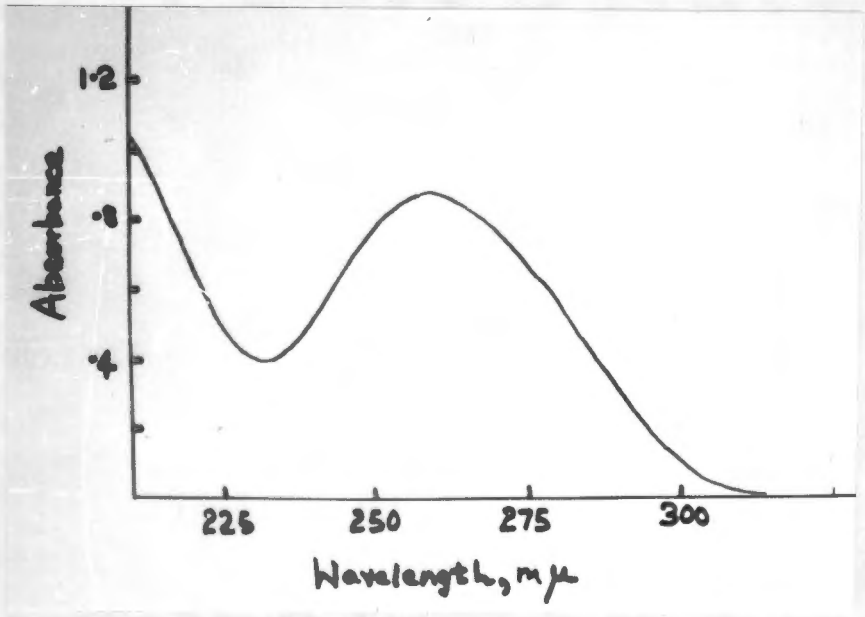


Fig. 5. Absorption spectrum of sRNA, B.

The effect of hydrolysis on the O.D. is shown in figure 6, namely a 40% increase in the O.D. at 260 mμ.

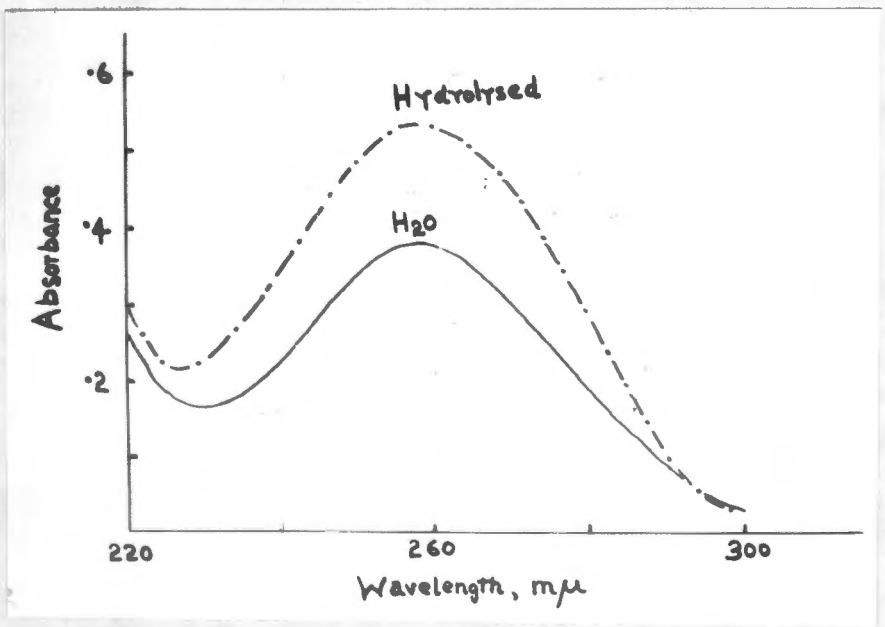


Fig. 6. Alkaline hydrolysis of sRNA.

NaCl at 0.1M had no effect, relative to distilled water; it is presumed that NaCl contaminated the preparation and hence a strict absence of NaCl from the distilled water solution may not have been realised. In any event the elevation of absorbance on hydrolysis is substantial proof of the polynucleotide condition of the RNA. Typical figures for absorbance increases on hydrolysis are provided by Sarkar (1962). Thus KOH elevates absorbance by 41% (compare 40%) and ribonuclease by 37%. The latter figure may be compared with the 31% increase obtained in this laboratory by the experiment depicted in figure 7.

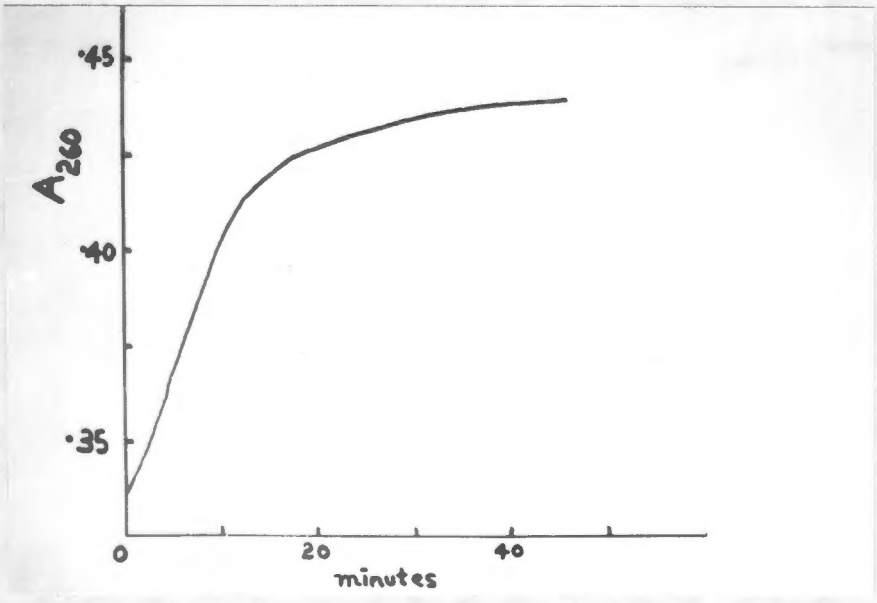


Fig. 7. Rate of hydrolysis of sRNA by ribonuclease.

2.4 Aminoacyl acceptor activity

Brunngraber claimed that the products of both methods had equal acceptor activity. By acceptor activity is meant the ability of tRNA to be esterified at the 3'

terminal adenosine by amino acids in the presence of the aminoacyl-tRNA-transferase enzymes. Decreased activity is essentially a measure of the extent to which the preparation has been degraded by ribonuclease during isolation.

Accordingly samples of sRNA M and B were assayed for activity as described in the Methods , M.13. Yeast protein hydrolysate was used as a source of ^{14}C amino acids. TCA was used to precipitate protein and RNA and thereby terminate the reaction. Controls did not contain added sRNA and the radioactivity measured in their precipitates was subtracted.

The following results were obtained:

Table 2

sRNA	M	B
Preparation number	1666	25366
Acceptor activity cpm/μg	700 ± 10	1260 ± 40

It is premature to discuss these results, but they suggest that sRNA prepared by the two methods may not in fact have equal acceptor activity. Reasons why such equality is unlikely will be apparent later.

2.5 Disc electrophoresis

The method of Richards et al. (1965), (described in M.18) was used with minor changes. In this technique a sample of 50-100γ of sRNA is electrophoresed through a porous material which selectively retards migration

according to size. After a suitable interval RNA occupying zones in the gel is fixed by lanthanum acetate and stained by acridine orange dye.

Various preparations of sRNA M and B were electrophoresed. Typical electrophoretograms are shown in the photograph, plate 3.

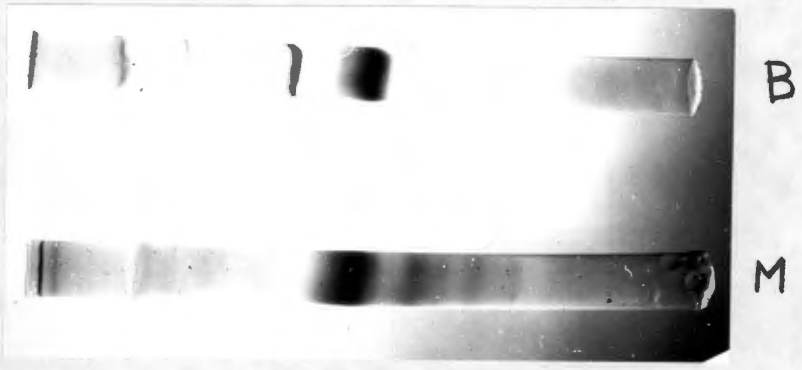


Plate 3. Electrophoretograms of sRNA M & B.

Several differences between M and B are apparent. The relative importance of each zone may be assessed from the curves drawn by the densitometer, figure 8.

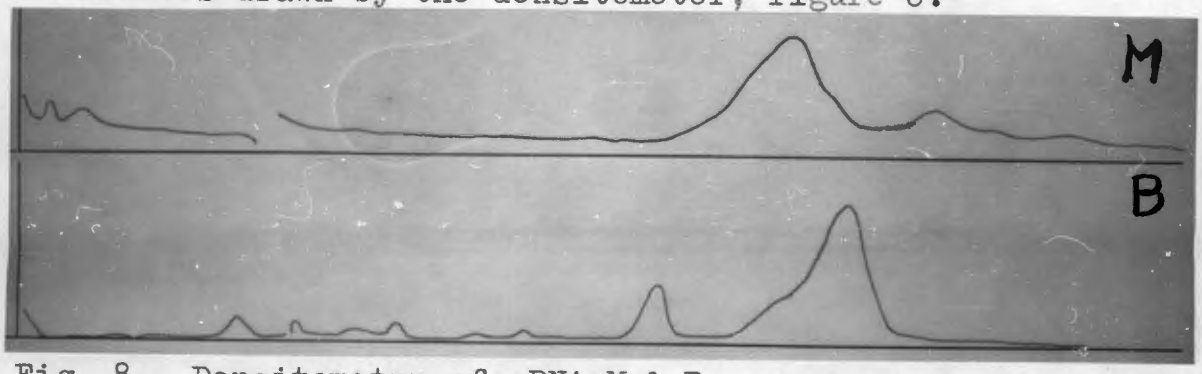
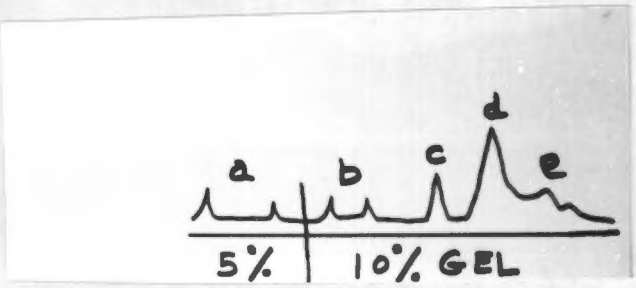


Fig. 8. Densitometry of sRNA M & B.

The peaks have been lettered as shown below, to facilitate reference: (See overleaf)



Integration of the curves provides the following data for the two major peaks, c and d:

Table 3

sRNA	peak c	peak d
1666 M	0	59
YPH, M	0	57
Val, M	2	66
25366 B	11	78
4366 B	9	63
266 B	10	52
1965 B	6.4	36

It is apparent from these results that peak c is a prominent component in sRNA B whereas it is virtually absent from sRNA M. At the same time, peak d which is tRNA (as will be shown later) is seldom above 60% of sRNA M. The residual 40% is accounted for mainly by degraded 4S RNA!

Owing to difficulties experienced in the method of preparation of sRNA B, early samples were appreciably degraded and this is reflected in low percentages of peak d material. These difficulties were finally surmounted with the production of sample 25366 B, which contained about 80% of peak d material.

The identity of peak d is revealed by the R_f value of 0.57, calculable from the electrophoretogram, plate 3. Richards et al. (1965) give a figure of 0.54 for yeast tRNA (c.f. my value 0.52 for yeast tRNA). Hence peak d is probably (rat liver) tRNA.

Further evidence of the identity of peak d is provided by the electrophoresis of ^{14}C aminoacyl-sRNA made as described in M.10, followed by scintillation counting of successive portions of the gel as described in M.15. The results are presented in figures 9 and 10.

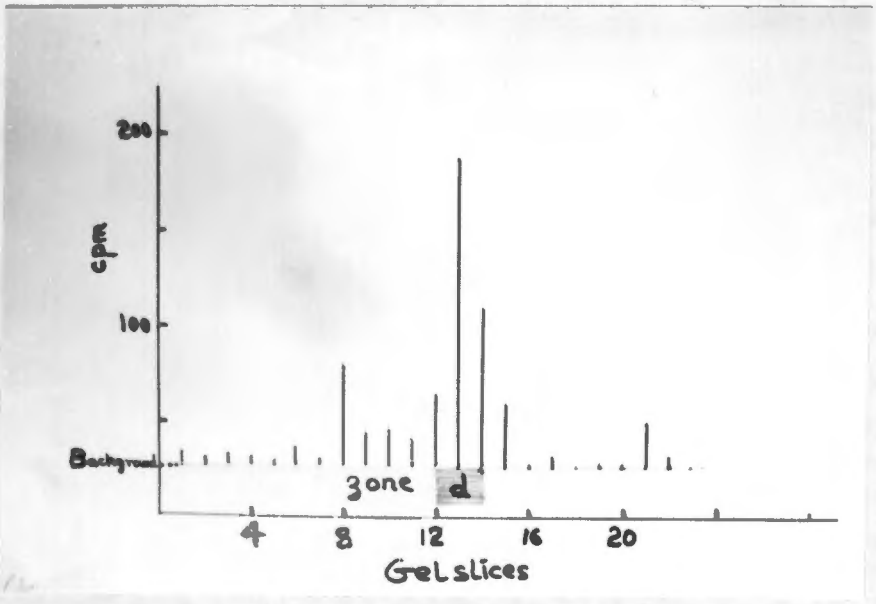


Fig. 9. Location of tRNA in polyacrylamide gel using ^{14}C valine -sRNA.

"Zone d" in figure 9 indicates the position of this zone in the gel relative to the gel slices taken for ^{14}carbon counting.

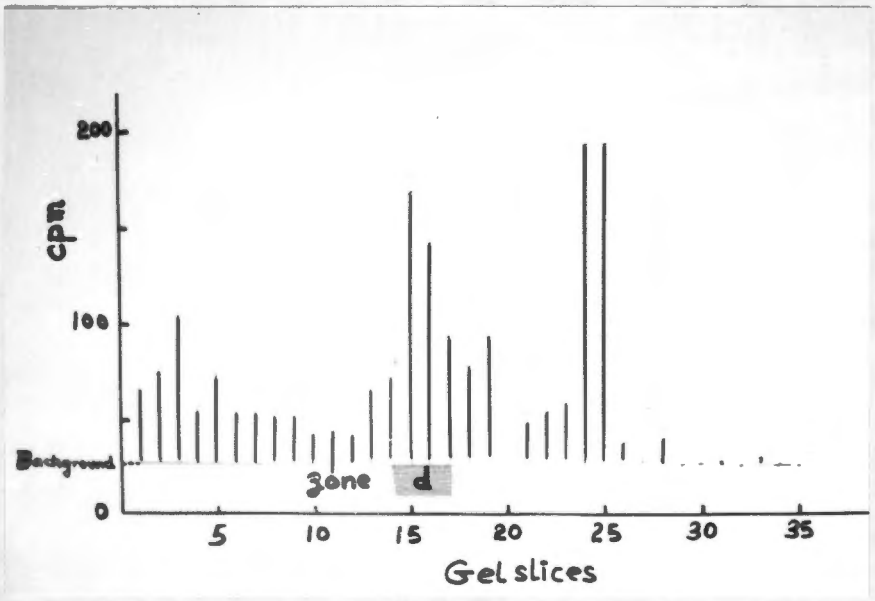


Fig. 10. Location of tRNA in polyacrylamide gel using ^{14}C yeast protein hydrolysate-sRNA.

The results are somewhat approximate, but zone d and the peak of ^{14}C activity coincide in the manner expected if d is in fact equivalent to tRNA. (The second peak, gel slices 24 and 25, figure 10, corresponds to the anion front and represents free ^{14}C amino acids in the gel.)

Hence when measuring the acceptor activity of preparations of sRNA only a portion of the sample (60% of sRNA M 1666 and 78% of sRNA B 25366) is able to be acylated by amino acids. The results obtained earlier are more correctly expressed in terms of this portion, the tRNA fraction, as follows:

Table 4

sRNA	M (1666)	B (25366)
activity of sRNA, cpm/μg	700	1260
percent tRNA	59	78
activity of tRNA, cpm/μg	1190	1620

The discrepancy between the two materials has diminished, but it remains appreciable.

While peaks c and d are the most dramatic features of these electrophoretograms of sRNA several minor features are worth noticing: (See figure 8 and the diagram below figure 8 for lettering.)

(a) Peaks a are common to both preparations of sRNA. Ribosomal (28 and 18S) RNA does not enter 5% gel and these bands may therefore derive from degraded ribosomal RNA.

(b) Peaks b are numerous and fine in B, but few and faint in M. Perhaps the exclusion of nuclei, mitochondria and microsomes from M contributes to this distinction.

(c) Peak c. It is of interest here merely to observe that the R_f measures about .33. A similar zone can be observed in the photographs published by Richards et al. (1965) with an R_f = .31 (approximately). This comparison serves to indicate that peak c may not be unique to rat liver sRNA B.

(d) Peak d is broad in both M and B; however the densitometer generally describes a flatter peak in M and

a sharper peak in B. While a symmetrical peak is not expected of tRNA, which is a mixture of about 20 similar molecules, the greater breadth of peak d in M suggests the presence within the peak of other material (possibly degraded tRNA).

(e) Peaks e are always prominent in M, but usually almost absent in B. Richards believes these bands to represent degraded tRNA. If this is so, and the breadth of these bands suggests as much, then contamination of peak d (in sRNA M) is also probable.

2.6 Sephadex gel filtration

Schleich & Goldstein (1966) showed that sRNA prepared from E. coli by phenol extraction of whole cells was heterogeneous by gel filtration on sephadex G100. This technique offered promise and was therefore applied to the analysis of sRNA M and B as described in M.23. A simple correlation exists between disc electrophoresis and sephadex gel filtration as shown by Richards & Gratzer (1964) and hence it was expected that the latter technique should confirm the results already apparent after electrophoresis on polyacrylamide gel. This expectation was realised as may be inferred from figures 11 and 12 wherein the absence or presence of a single peak may be discerned.

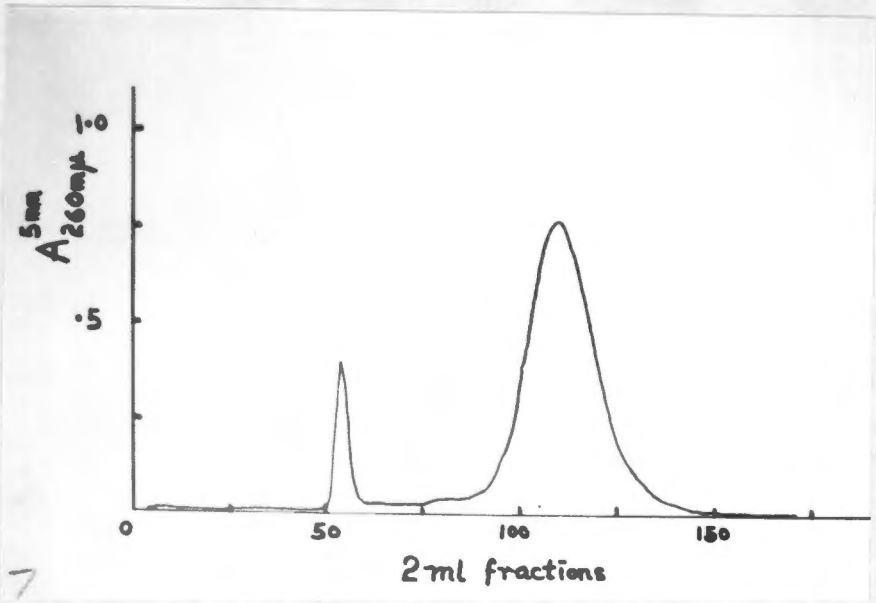


Fig. 11. Sephadex gel filtration of sRNA M.

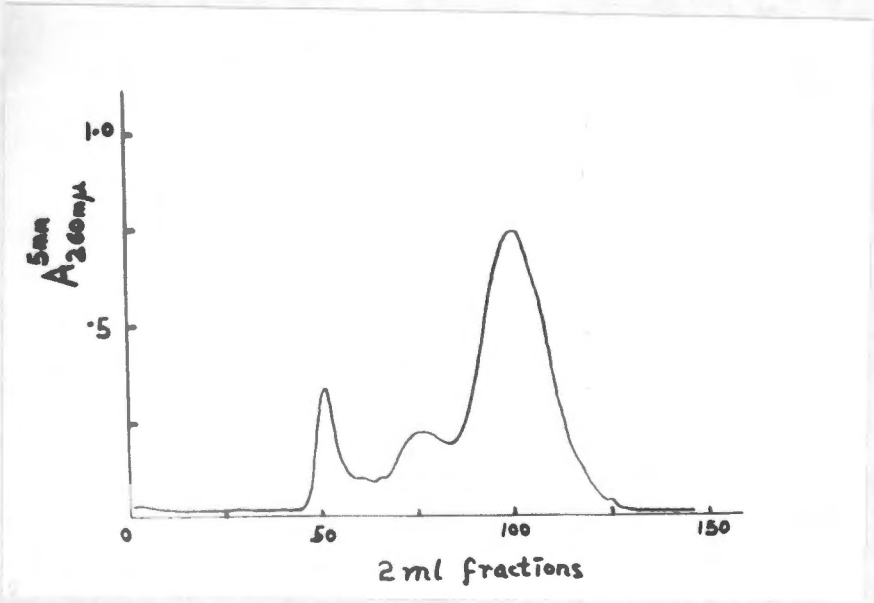
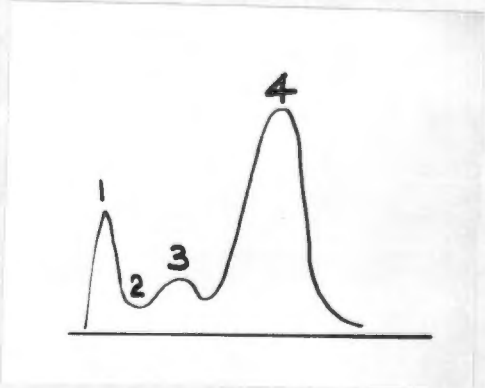


Fig. 12. Sephadex gel filtration of sRNA B.

The peaks may be numbered as in Schleich & Goldstein (1966):



3. pH 5 AND RELATED ENZYMES

The nature of the enzyme used to assay the acceptor activity of sRNA warrants a few remarks.

3.1 Preparation of pH 5 enzyme

This is described in the Methods under M.3. As the name implies the "enzyme", actually a mixture of several enzymes, is characterised by a propensity to precipitate at pH 5, together with soluble RNA. The constituent enzymes are concerned with the activation of amino acids and their subsequent transfer to tRNA.

3.2 Composition

The two major constituents are protein and sRNA. Protein may be calculated from Warburg's formula (Layne, 1957):

$$\text{Protein, mg/ml} = 1.55\ A_{280} - 0.76\ A_{260}.$$

By analogy with this formula a second formula can be derived, RNA mg/ml = $.0714\ A_{260} - .0429\ A_{280}$, based on the following data:

(1) The O.D. of 1 mg/ml of RNA; $A_{260} = 20$;
of protein $A_{280} = .895$.

(2) The O.D. ratio A_{260}/A_{280} for RNA is 2.0,
for protein 0.59.

Using these formulae, typical preparations of pH 5 enzyme are found to contain 10 mg protein and 0.75 mg RNA/ml.

3.3 Assay of enzyme activity

A good measure of the quality of the enzyme may be obtained by determining the ability of the enzyme to esterify endogenous sRNA. This ability is measured experimentally by the following reaction:

$tRNA_1$ + ^{14}C amino acid ——> ^{14}C aminoacyl$_1$-$tRNA_1$

Transfer RNA_1 and its product are precipitated and the radioactivity in the precipitate is measured. This radioactivity is directly proportional to the amount of product. The result, in cpm of radioactive precipitate per µg of total sRNA measures the extent to which the enzyme has catalysed this reaction (in 10 minutes at 37°).

The result is a composite of several variables namely

(1) The transferase activity per mg of enzyme protein.
(2) The concentration of ^{12}C amino acids.
(3) The presence of ribonuclease.
(4) The extent of degradation of endogenous sRNA.

These factors must be taken into account in any evaluation of a preparation of pH 5 enzyme. The assay can be performed as described under M.13. for the assay of acceptor activity of (added) sRNA. However, no sRNA is added during the assay and control counts are obtained hy hydrolysing the ^{14}C precipitate with hot TCA (20 minutes at 90°) or with ribonuclease. Both methods give similar control values (see figure 13) and specifically release amino acids covalently attached to sRNA into the

supernatant. Hence such results are a reliable measure of the esterification of endogenous sRNA, expressed in cpm/μg endogenous sRNA.

Early preparations had low activity:

Table 5.

Date of preparation of enzyme	cpm/μg
11.11.65	20
4.1.66	38
but compare 19.1.66	408.

The reasons for this will be discussed later.

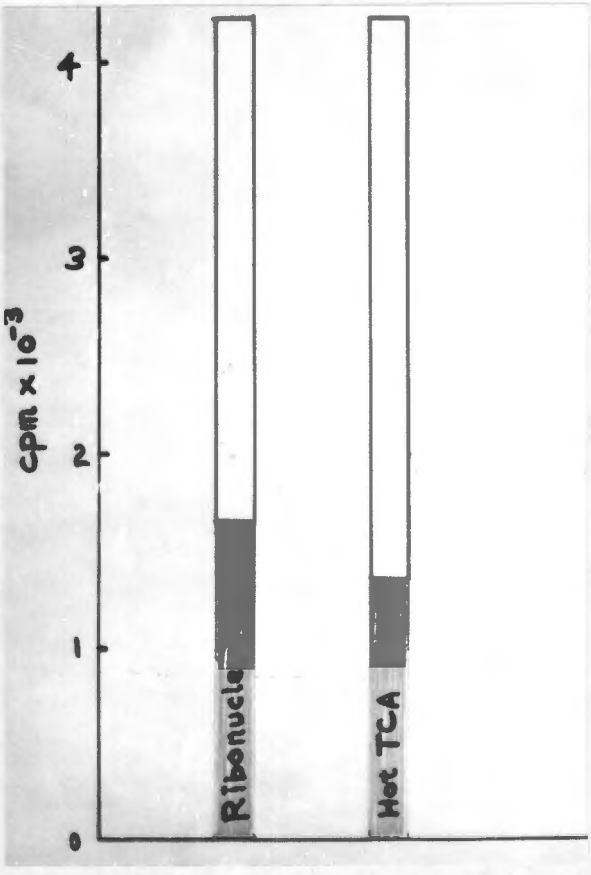


Fig. 13. Equivalence of controls obtained by ribonuclease and hot TCA.

The heights of the lower rectangles denote the respective

counts after treatment with ribonuclease or hot TCA. The combined upper and lower rectangles denote the cold TCA (i.e. untreated) counts/minute. The difference (upper rectangles only) represents the ^{14}C aminoacyl-tRNA in the precipitate.

3.4 Alternative assay of enzyme activity

Alternatively a given sample of sRNA may be assayed for acceptor activity by various enzyme preparations and these results taken as a relative measure of enzyme activity. Results for three enzymes are depicted in figure 14 where activity has been assayed at several concentrations of added sRNA.

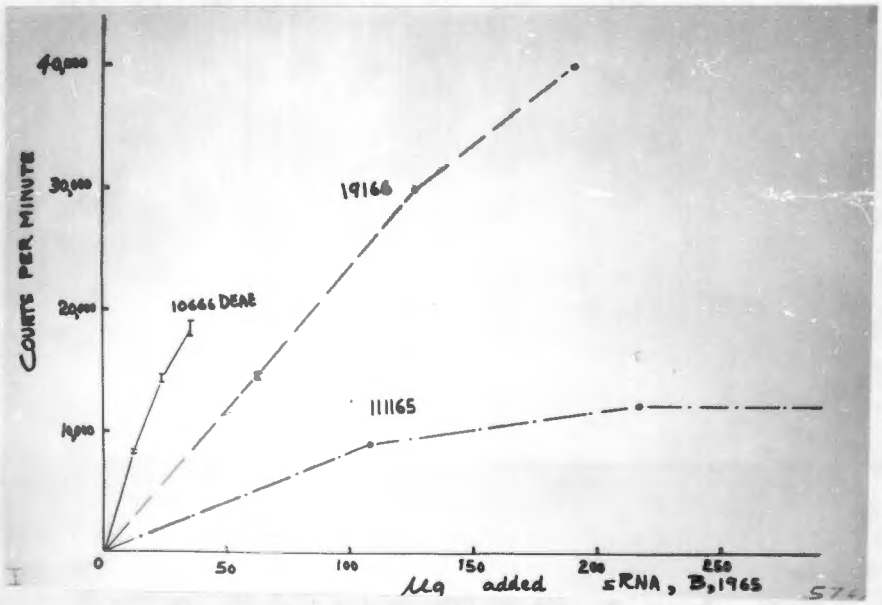


Fig. 14. Assay of activity of transferase enzyme preparations. Numbers describe date of preparation. "DEAE" means treated with DEAE-cellulose. Only one preparation of sRNA was used throughout, viz., sRNA, B 1965.

From the initial slope of each curve the following enzyme activities are calculable:

Table 6.

Date of preparation of enzyme	cpm/µg	µc
11.11.65	80	1.3
19.1.66	228	.52
10.6.66 DEAE	693	.52

The relatively low activity of early preparations is again evident. ("µc" indicates the amount of ^{14}C amino acids present during each assay. Since an excess is added, this should not, ideally make any difference to the result.)

The latter assay is slightly more accurate in so far as it is not influenced by the quality of the endogenous sRNA (whose amount need not be calculated).

3.5 Improvements in the preparation of enzymes

A major factor determining the quality of such preparations is the concentration of ^{12}C amino acids. If this is high then added ^{14}C amino acids are diluted during the assay of activity. The result is a less sensitive assay. Evidence of this effect is provided, when the amount of ^{14}C added, already in excess, is increased:

Table 7.

enzyme	microcuries	cpm/µg
31165	.65	25
31165	1.00	65

Further evidence is provided by Sephadex G25 filtration of the enzyme:

Table 8.

enzyme	before	after
4166	38	297
but compare 19166	408	347

Clearly "19166" is devoid of ^{12}C amino acids.

Several changes were necessiatated in the original method described by Holley & Goldstein (1959), before adequate levels of activity were attainable. Briefly there were as follows:

(1) The use of medium A during homogenisation of liver. Alternatives, such as .05M KCl or .25M sucrose (in the presence of tris buffer) seemed detrimental to the enzyme.

(2) Two precipitations at pH 5 were needed to decrease the level of endogenous ^{12}C amino acids.

(3) Dialysis and Sephadex G25 were unnecessary and the former probably permitted ribonuclease to degrade endogenous sRNA.

The method as finally described in M.3 made possible the preparation of an enzyme of high activity.

3.6 DEAE-cellulose chromatography

If pH 5 enzyme contains 75γ of sRNA in 0.1 ml of solution then it will not prove very sensitive to the addition of 10γ of sRNA for the assay of acceptor activity of sRNA. Greater sensitivity is achieved by removing endogenous sRNA with DEAE-cellulose (Doctor et al., 1961). Figure 15 illustrates the effect this has on the absorp-

tion spectrum of the enzyme.

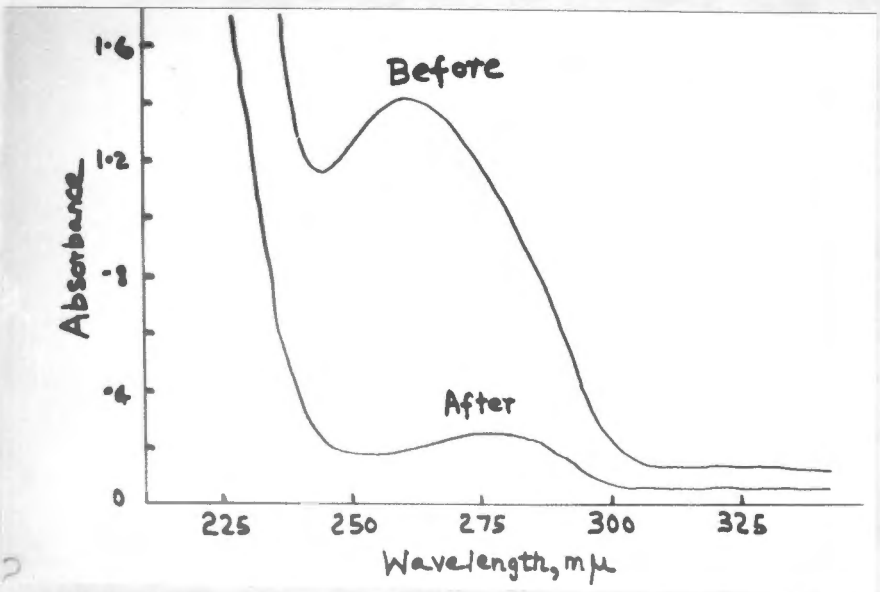


Fig. 15. Spectra of pH 5 enzyme (before) and of resultant DEAE-cellulose treated enzyme (after).

A substantial decrease in material absorbing at 260 mμ, and hence of sRNA, is evident. The resultant enzyme has however, one drawback namely a greater lability on storage.

3.7 Accuracy of assays with enzymes

Early results were inaccurate presumably because TCA precipitates bearing ^{14}C aminoacyl-tRNA may occlude free ^{14}C aminoacids unless thoroughly washed. Such washing is facilitated if the precipitates are fine and uniform. By taking these factors into account duplicate results need not differ more than 5% (see e.g. curve 10666 DEAE, figure 14).

3.8 Conclusion

Some of the experience underlying the preparation of an enzyme suitable for the assay of the acceptor activity of sRNA has been presented. Only due attention to these considerations can ensure the preparation of an enzyme of adequate activity, and a reliable assay.

4. SUMMARY AND CONCLUSIONS, sRNA

(1) From 100 g of liver can be obtained about 12 mg of sRNA M or about 60 mg of sRNA B.

(2) Rat liver sRNA is heterogeneous.

(3) sRNA M contains about 60% of tRNA while sRNA B contains about 80% of tRNA.

(4) At the same time B contains 10% of an unknown, apparently homogeneous RNA

(5) This unknown RNA distinguishes M, which is devoid of it, from B. This distinction is revealed both by disc gel electrophoresis and by sephadex gel filtration.

(6) sRNA M is only 56% as active as sRNA B while tRNA M is 73% as active as tRNA B. Brunngraber measures only the activities of soluble RNA M and B which he finds are equal. This result is not readily compatible with the results obtained in this laboratory.

(7) This data makes it clear that preparation of sRNA by the method of Brunngraber is preferable. Ribonuclease degradation is not a hazard in sRNA B, but is appreciable in sRNA M. Both in amount and in quality, B is superior.

5. DISCUSSION

The identity of peak c RNA

Schleich & Goldstein, 1966, have identified peak 3 and peak 4 on their graph (see diagram below figure 12) page 44, as 5S and 4S RNA respectively. Peak d obtained on polyacrylamide electrophoretograms in this laboratory has been proved to be 4S RNA and hence is identical with peak 4. As peak c on polyacrylamide and peak 3 on Sephadex both occur in sRNA B, but not in sRNA M their mutual identity is apparent. My peak 3 is the same as that of Schleich & Goldstein, as a comparison of our graphs reveals. Hence the distinction between sRNA M and B is that B contains 5S RNA, but M does not.

Numerous articles on 5S RNA have appeared both during and after completion of this work, as detailed in the introduction. Briefly, they affirm the existance of an RNA peculiar to ribosomes with a sedimentation constant close to 5.0, absent elsewhere in the cell.

Hence the distinction between sRNA M and B is readily explained. In M the RNA is recovered from the high-speed supernatant after removal of nuclei, mitochondria and microsomes. In particular the removal of the microsomes (which contain the ribosomes) and together with them, the free ribosomes which sediment during 2 hours at 105,000 g means that 4S RNA left in the supernatant is devoid of contaminating 5S RNA. On the other hand direct phenol extraction of the liver homogenate

releases all low molecular weight RNA from protein into the aqueous phase. Elution of DEAE-cellulose with 1M NaCl does not discriminate between 4 and 5S RNA, but only between low and high molecular weight RNA.

It may be inferred that the proportion of 4 and 5S RNA found in sRNA B reflects the proportion of tRNA and ribosomes occurring in the liver. Thus in sRNA B, the ratio of peak c to peak d is 1:8; the approximate molecular weight of 5S RNA is 35,000 (Bachvaroff & Tongur, 1966) whereas for sRNA it is about 25,000; hence the molar ratio of 5S to 4S is 1:11 and thus for every ribosome in the cell there are about 11 tRNA molecules, a figure closely in line with corresponding data for E. coli and Blastocladiella emersonii (Comb & Zehavi-Willner, 1967).

CHAPTER VI - RESULTS, RIBOSOMAL AND MEMBRANE RNA

1. RIBOSOMAL RNA

There are two main reasons why ribosomal RNA is relevant to this work. Thus membrane RNA bears a close resemblance to ribosomal RNA, as mentioned in the introduction. Hence it is necessary to compare membrane RNA with ribosomal RNA in order to determine what differences, if any, exist.

Ribosomal RNA is well-characterised and hence is useful for the purpose of "calibrating" new separation techniques such as electrophoresis in polyacrylamide gel. The application of the latter facility will be evident from the following account.

1.1 Preparation of ribosomal RNA

RNA is readily isolated from rat liver ribosomes as described (see M.1 and M.8). A white material is obtained in excellent yield. It is characterised as RNA by its absorption spectrum, figure 16.

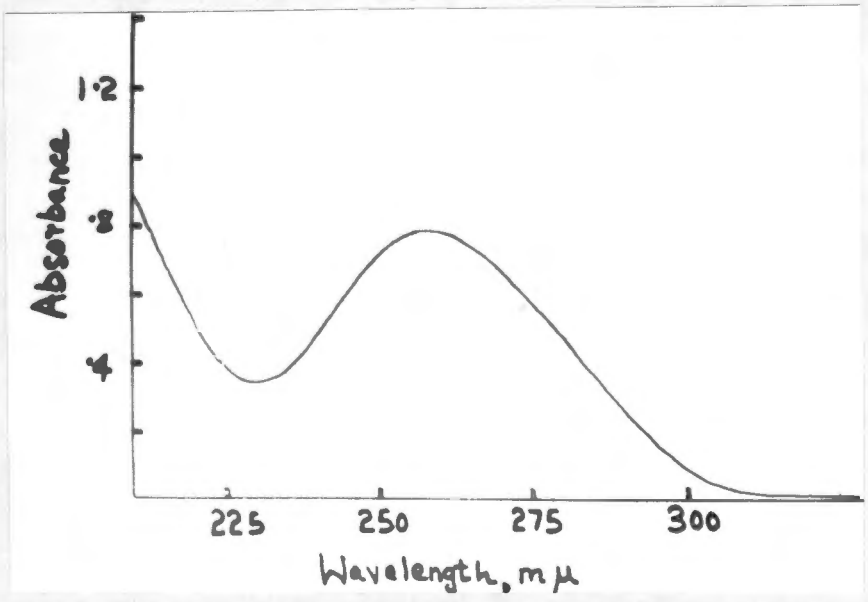


Fig. 16. Absorption spectrum of ribosomal RNA.

1.2 Sucrose gradient centrifugation

About 2.4 mg RNA was centrifuged through a linear sucrose gradient as described (see M.16) in the Spinco SW 25.1 rotor. The fractionation obtained was characteristic of ribosomal RNA (see e.g. Hastings & Kirby, 1966) and is shown in figure 17.

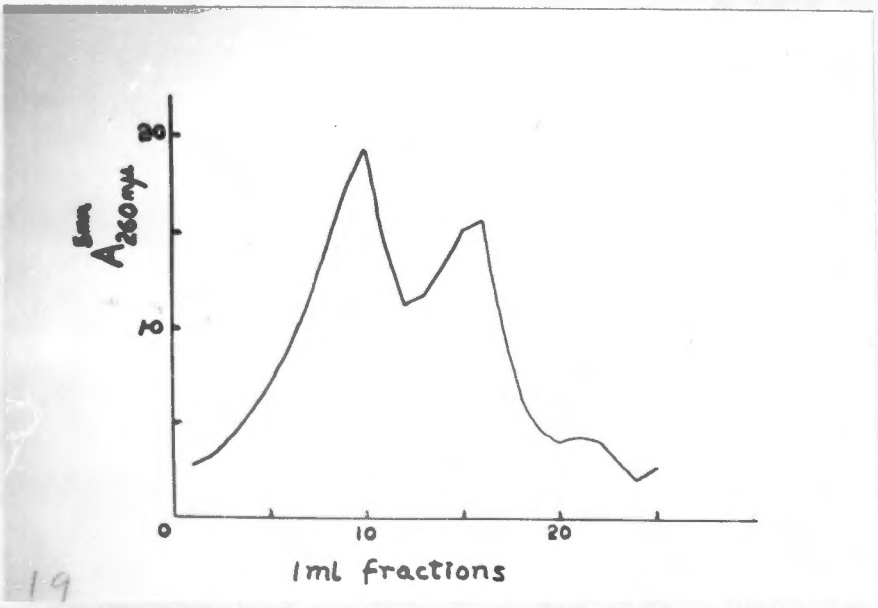
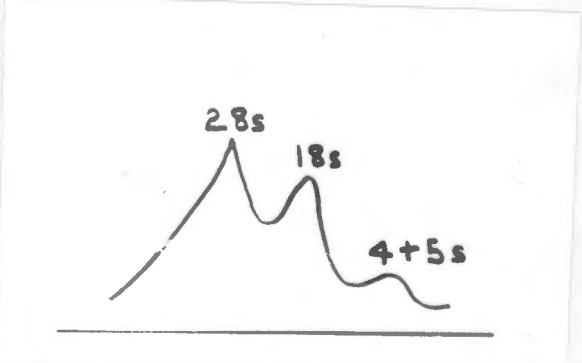


Fig. 17. Fractionation of ribosomal RNA.

There is no doubt of the identity of the three peaks which are, from left to right, 28, 18 and (4 + 5)S RNA as shown in the diagram below.



Names of peaks in figure 17

A somewhat more sensitive analysis may be conducted by centrifuging a smaller amount of RNA. Thus figure 18 was obtained with 150γ RNA using the SW 39 rotor. (The gradient was allowed to flow through a flow-cuvette, to facilitate measurement of optical density).

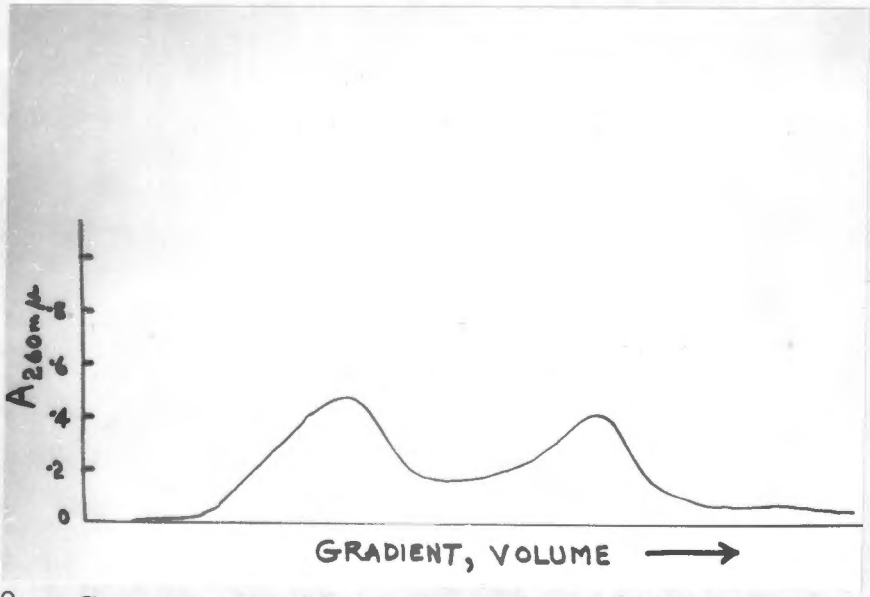


Fig. 18. Sucrose gradient analysis of ribosomal RNA.

A better separation of the two major peaks results than that depicted in figure 17.

1.3 Polyacrylamide gel electrophoresis

The method devised by Richards et al. (1965) for the analysis of soluble RNA should be applicable to ribosomal RNA if the gel concentration is lowered from 10% to 2.5%. This was in fact done in this laboratory, but the gel did not appear porous enough for 28S RNA, which penetrated poorly. That the gel is in fact sufficiently porous has since been shown by Loening

(1967), who first successfully resolved ribosomal RNA on polyacrylamide gel. He made possible the penetration of the gel by 28S RNA by using EDTA and claimed that both heavy metal cations and traces of protein were the cause of the problem, termed "sticking" (non-penetration). With the appearance of this publication the need to study alternative media for the electrophoresis of RNA such as agar or starch gel, no longer arises; polyacrylamide has been shown to be more than adequate.

Confident interpretation of polyacrylamide electrophoretograms may be assisted by comparisons with Loening's results, but is best assured by isolating ribosomal RNA fractions from sucrose gradients and then electrophoresing them through polyacrylamide gel. By this approach the unambiguous identity of each zone found in the gel may be established.

For this purpose ribosomal RNA was fractionated on sucrose gradients as described (see M.16). From successive fractions RNA was recovered by precipitation and then electrophoresed on polyacrylamide gel as described in M.19. The original gradient is shown in figure 17 and the electrophoretic analysis in plate 4. (See overleaf.) The expected inverse correlation between sedimentation constant and electrophoretic mobility is seen: the larger the RNA molecules are, the less readily do they penetrate the gel. Fractions 1-10 (figure 17) derive from the 28S peak and constitute the slowest zone as shown in tubes 1-5 (plate 4) (some sticking is

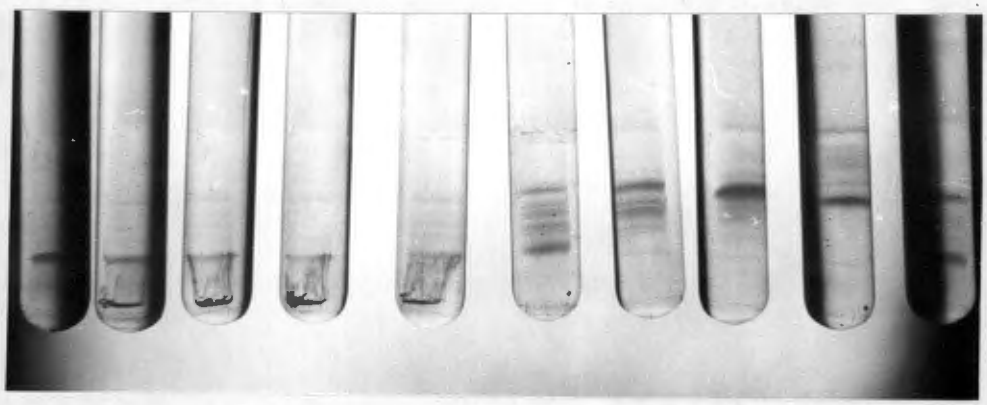


Plate 4. Electrophoretic analysis of sucrose gradient fractions of ribosomal RNA.

On the extreme right is a sample of unfractionated ribosomal RNA. The 9 tubes shown to the left of this sample are 2 ml samples corresponding to the fractions recovered from the gradient in figure 17. Thus tube 1 contains fractions 1 and 2, tube 2 fractions 3 and 4 ..... tube 9 fractions 17 and 18.

evident, unfortunately). Fractions 11-12 derive from the trough and are revealed as several fine zones in tube 6; clearly there exists RNA of s, 18-28S present in ribosomal RNA, of unknown identity! Fractions 13-18 derive from the 18S peak and constitute a faster, prominent zone in tubes 7-9. Tube 10 contains the corresponding, unseparated 28 and 18S zones.

The identity of faster bands in the gel may be ascertained by comparison with the behaviour of sRNA, B on 10, 5 and 2.5% gel. 4 and 5S RNA may as a result be easily recognised in polyacrylamide electrophoretograms when present. Furthermore the characteristic broad tRNA zone next to the fine 5s RNA zone presents an

unmistakable appearance.

With this information it should now be possible to proceed to the analysis of ribosomal RNA on polyacrylamide.

Accordingly, ribosomal RNA was electrophoresed in 2.5% polyacrylamide gel (see M.19). The gel was photographed, and scanned by a densitometer:

Plate 5. Electrophoresis of ribosomal RNA.

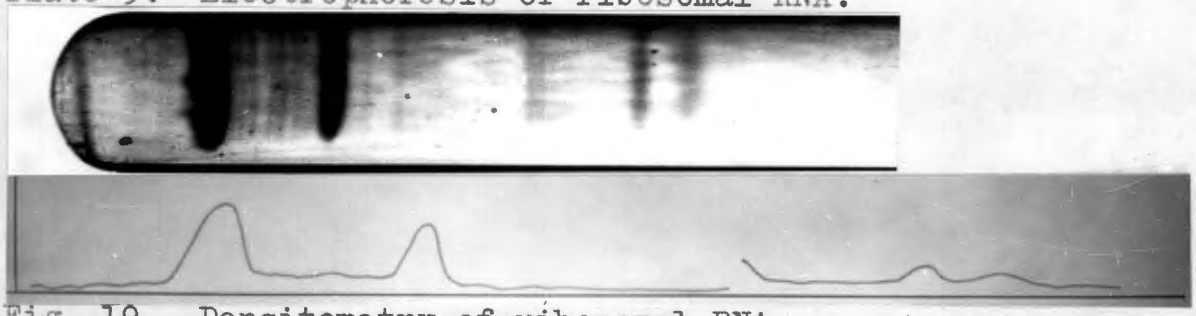
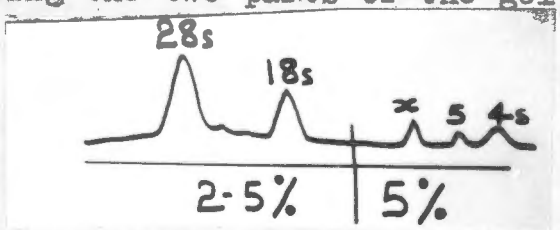


Fig. 19. Densitometry of ribosomal RNA.

The peaks from left to right are readily identified as 28, 18, 5 and 4S RNA as shown in the diagram (note the line separating the two parts of the gel):



(Peak x is absent from ribosomal RNA, but will be referred to later. The spurious peak in figure 19 midway between 18S and 5S RNA is the junction between the two parts of the gel). The curve drawn by the densitometer closely resembles that obtained by Loening. However, also present are several fine bands, between the 28 and 18S peaks. The latter are not apparent from Loening's results and have not been seen elsewhere.

Integration of the curve drawn by the densitometer reveals the following composition:

Table 9.

RNA, s =	28	18-28	18	5-18	5	4
percent	43	13	23	10	6	7.5

Some 30 percent is not ribosomal RNA. The three ribosomal components show approximately the correct proportionality; however 5S RNA somewhat exceeds the figure of 1% given in the literature, (Bachvaroff & Tongur, 1966) and is closer to 8% of the strictly ribosomal RNA.

The above analysis assumes proportionality between acridine orange staining of zones and the amount of RNA in these zones. Accordingly this assumption was tested by electrophoresis and densitometry of amounts of RNA varying from 20 to 100γ. plate 6.

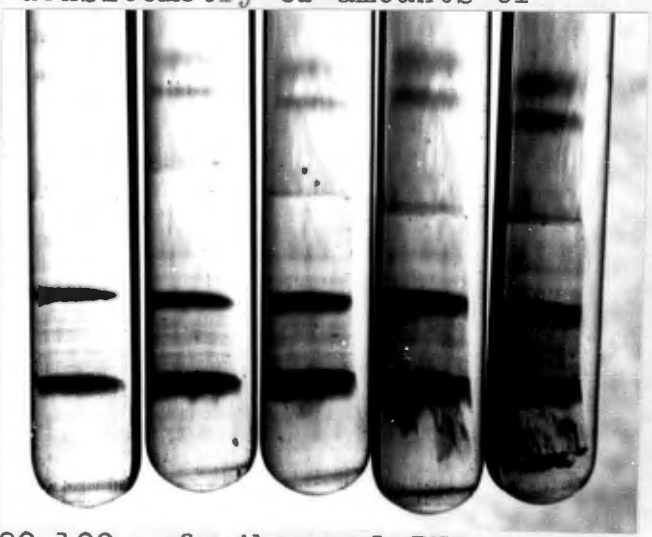


Plate 6. Electrophoresis of 20-100γ of ribosomal RNA.

In particular it is seen that excessive amounts of RNA (gels on the right hand side) manifest the behaviour termed "sticking".

Stained bands in the 2.5% gel only have been measured by densitometry and plotted against total mass of RNA on each gel as shown in figure 20. Proportionality is seen to apply. A more accurate relation

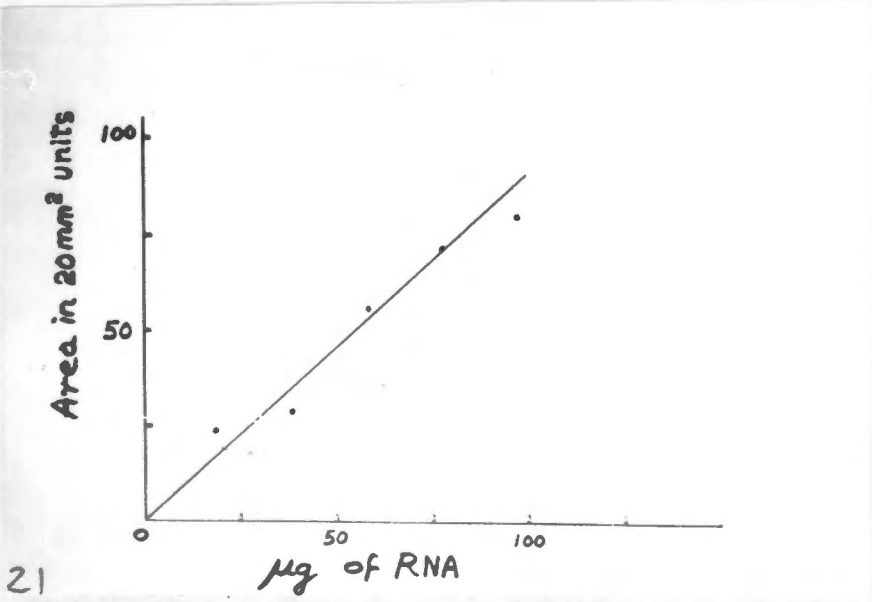


Fig. 20. Standard curve relating area of acridine orange peaks to RNA content.

between peak area and mass is obtainable with RNA of one kind only. Under these circumstances the ratio of total peak area (in 20 mm^2 units) to mass (in γ) is about 2.0.

Because "sticking" is not cured completely by Loening's method, and may be attributable to heavy metal cations, RNA showing this tendency, e.g. in tubes 2-5 (plate 4) was washed by solution in .05M tris pH 8.0, .01M EDTA and precipitation with ethanol. RNA analysed in tube 5 was chosen for this experiment. While sticking seemed to disappear, degradation set in. When degradation was avoided, sticking persisted. Subsequently it was noticed that increasing the phenol extractions

from 2 to 4 did reduce sticking. It was concluded that protein impurities may be more important than heavy metal cations. If this is correct, it should be possible to use the electrophoresis buffers of Richards et al. (1965), which do not contain EDTA, instead of Loening's. EDTA may not be essential.

The need for a more effective removal of protein is evident; Loening has mentioned this and recommends triisopropyl naphthalene sulphonate.

1.4 Ribosomal subunits

While ribosomes are an excellent source of RNA for various purposes such as I have mentioned earlier, a single drawback is the heterogeneous nature of this RNA. Sometimes a single pure RNA is preferable, when e.g. a defined "carrier" is needed to facilitate the isolation of small amounts of a particular RNA. It was reasoned that ribosomes on dissociation into subunits would release most extraneous RNA such as tRNA, and that either subunit might prove a rich source of a pure RNA.

At the same time such an experiment promised to confirm the release of 5S RNA from the ribosome as a result of dissociation caused by EDTA (Bachvaroff & Tongur, 1966; Comb & Zehavi-Willner, 1967).

Ribosomes were prepared (see M.2) and dissociated and separated into subunits on a sucrose gradient in a B IV zonal rotor (see M.17). A satisfactory separation was achieved as shown in figure 21, (see overleaf) and two fractions namely fraction 15 (of the smaller

subunit) and fraction 22 (the larger subunit) were treated to isolate RNA as described (see M.9).

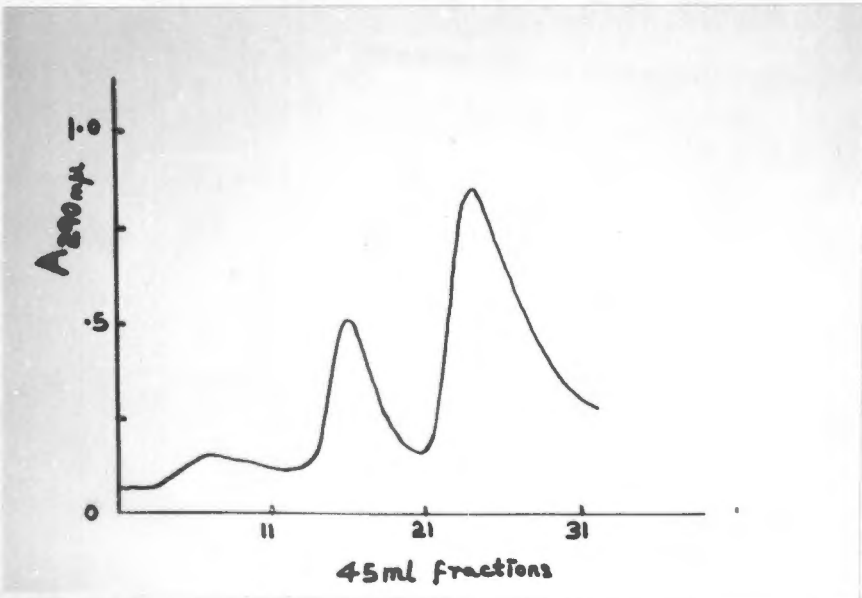


Fig. 21. Separation of ribosomal subunits.

The small peak on the left is mainly 5S RNA; the peak in the centre is the smaller subunit of the ribosome, and the tall peak on the right is the larger subunit. The sucrose gradient increases in density from left to right.

The RNA was electrophoresed (see plate 7) and the composition of each fraction was determined by densitometry of the electrophoretograms.

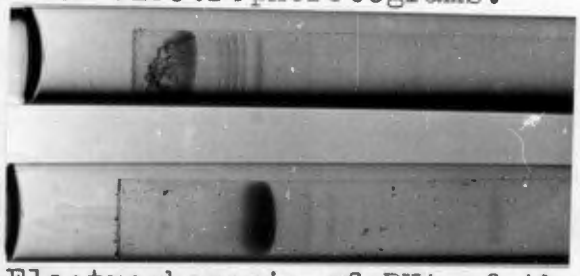


Plate 7. Electrophoresis of RNA of the ribosomal subunits. The upper gel is fraction 22; the lower gel is fraction 15. The two major zones are 28S RNA on the left and 18S RNA on the right.

Fraction 15 was found to contain 91% of 18S RNA and only traces of 28S RNA. 5S RNA made up only 0.9% which clearly is due to contamination of the 18S subunit peak by the neighbouring (5 + 4)S peak during separation on the sucrose gradient.

Fraction 22 contained 82% of 28S RNA, 14% of 18-28S RNA and 4.7% of 18S RNA.

The smaller and larger subunit therefore contain mainly 18 and 28S RNA respectively. EDTA causes release of 5 and 4S RNA from the ribosome. The smaller subunit is clearly an excellent source of fairly pure 18S RNA.

Of interest is the 18-28S RNA clearly seen in fraction 22, plate 7 as four fine zones. Several zones have been observed before (see plate 4) in this region. Their present association with the larger subunit may merely reflect their distribution in a sucrose gradient. Their occurrence has also been noted in smooth membranes (see later).

2. MEMBRANE RNA

2.1 A method of Preparation

Hallinan & Munro have made an important contribution to the preparation of smooth membranes (which contain RNA) by adapting the iso-octane method introduced by Hawtrey and Shirren (1962). Hawtrey's method fails, in so far as he attempts to separate the endoplasmic reticulum of the microsomes from adhering polysomes. Iso-octane is unable to do this. Hence Hallinan & Munro first remove the rough membranes by sedimentation at 78,000g for 60 minutes whereupon a supernatant is left containing free ribosomes and smooth membranes. The latter is emulsified with iso-octane and centrifuged to break the emulsion. Owing to the hydrophobic nature of iso-octane the smooth membranes are attracted to the interface between the iso-octane and lower aqueous phase while the free ribosomes sediment to the bottom.

The latter method offers the advantage of being rapid and effective and accordingly has been used in this laboratory.

Polyacrylamide electrophoresis was chosen for the analysis because it offered the best resolution of membrane RNA although it had not, prior to Loening, been used for the analysis of high M.W. RNA.

2.2 Preparation of membrane RNA

The original method used in this laboratory, based on the method of Hallinan & Munro (1965), yielded (in my hands) degraded RNA. Several changes had to be

made before undegraded RNA became obtainable. Such changes were the replacement of 30% sucrose for homogenisation by Medium A containing 30% sucrose; the use of distilled phenol at 0° in the presence of 8 hydroxyquinoline and naphthalene 1, 5 disulphonate as used by Bergeron-Bouvet & Moule (1966) instead of phenol only: the recovery of RNA from the aqueous phase by precipitation with acetone and sodium acetate pH 6.5, instead of dialysis and freeze-drying. Perhaps the factor most responsible for degradation was however, the following: in the original method as applied in this laboratory the mixture of iso-octane and incipient smooth membranes was centrifuged in two portions because its volume exceeded the capacity of the 40 rotor. The second portion was kept frozen while the first was being centrifuged. This process of freezing and thawing may have released ribonucleases which hydrolysed the RNA in the second portion. This danger was accordingly avoided by appropriate modification of the method.

The method finally devised, (see M.5 and M.12) has been shown to produce a more or less undegraded RNA. The important features of this method are: the mechanical homogenisation of liver; the careful exclusion of rough membranes; the rapid careful transfer of smooth membranes to the collecting flask. Freezing and thawing is avoided and temperatures throughout are close to 0°.

2.3 Polyacrylamide gel electrophoresis

A uniform 2.5% polyacrylamide gel is sometimes preferred to the 2.5 and 5% gel described (M.19) despite its fragility. This is because the "interface" between the 2.5 and 5% polyacrylamide, when present, causes some RNA to accumulate there, presenting the appearance of a single zone whereas in fact no such zone exists. Polyacrylamide of a single concentration is without this complication. Sample volumes of 10-20 μL are desireable, containing about 30γ. Duplicate or triplicate samples are advisable. " "Sticking" is prevalent whenever 28S RNA is present in rhe sample, but 4 phenol extractions instead of 2 or 3 may eliminate it.

Two early electrophoretograms are shown in plate 8 and reveal extensive background discoloration

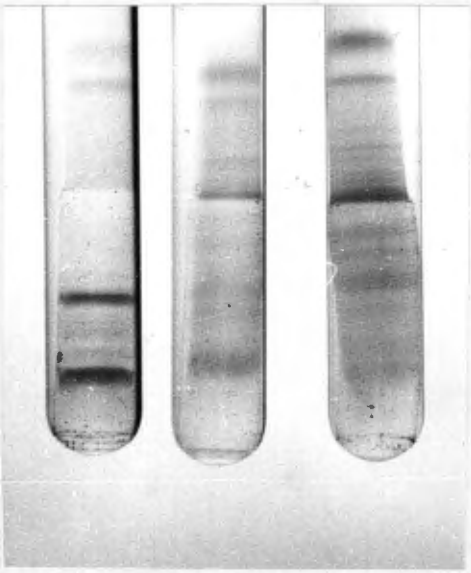


Plate 8. Electrophoresis of degraded membrane RNA shown alongside undegraded ribosomal RNA (on left).

and interfacial material. Degradation is evident. Several bands of s <18S occur, which at this stage may represent new kinds of RNA or simply, degraded 18 and 28S RNA. Despite the poor quality of the preparations, zones of 28, 18, 5 and 4S RNA are seen. This is the first domestic evidence of the presence of ribosomal RNA within membrane RNA. The 4S band is noticeably darker than the 5 band; because the tRNA is in the supernatant from which the smooth membranes are separated, it is an expected contaminant.

With the improved method of preparation the RNA was almost completely undegraded as is evident from plate 9, which portrays from left to right, membrane RNA preparations 11467, 4567 B, 4567 A and 7667. Less

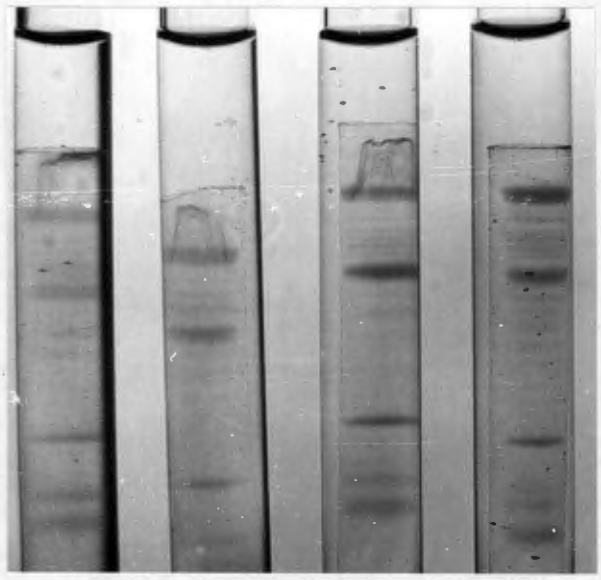


Plate 9. Electrophoresis of four samples of membrane RNA.

degradation is evident from the decreased background colour (and less interfacial material, not shown in

this photograph).

Five major zones, barely visible in plate 8 are distinguishable. Four of these may be identified (compare figure 19 and the diagram below) namely 28, 18, 5 and 4S RNA, while a fifth is also present between the zones of 18 and 5S RNA. Even several fine 18-28S zones are distinguishable in the gels on the right, plate 9.

It seems to be a feature of the degradation of RNA encountered, that even when the 28 and 18S zones are indistinct (as in plate 8) the 5S zone is sharp and prominent. This must mean that 5S RNA is less susceptible to degradation; an unexplained and interesting observation. (It does not mean that 5S RNA is a degradation product of high molecular weight RNA. (This possibility has been discounted by Comb & Zehavi-Willner, 1967. Such degradation products are characterised by 5' OH and 3' PO_4 terminals which are the opposite of those found in 5S RNA by these authors).

A striking feature shown in plate 9 is the prominent sharp band lying between 18 and 5S RNA. The same zone is evident in earlier preparations (see plates) where however it is fainter. It seems to be more susceptible to hydrolysis than is 5S RNA. It is not found in ribosomal RNA (compare plate 8). During the subsequent account it will be referred to as xRNA.

2.4 The composition of membrane RNA

Only the four most recent preparations will be considered because only these four were made by the improved method. The yields are compared below:

Table 10.

RNA, preparation	11467	4567A	4567B	7667
g of liver	52	27	30	38
mg RNA	2.4	2.4	2.9	1.9
Comment	Accident	-	-	-

Of importance is the low yield in preparation 7667 which is not due to an "accident" but to the careful exclusion of rough membranes (which contain mainly ribosomal RNA) by taking only the upper 60% of the 78,000g supernatant. (In previous preparations probably about 75% was taken).

A typical ultraviolet spectrum is shown in figure 22 which however is hardly different from that obtainable for soluble RNA! It may be inferred that absorption spectra do not permit a distinction to be made between various kinds of RNA.

A photograph and a densitometer curve for membrane RNA preparation 7667 is shown in figure 23 which is typical for membrane RNA except for a more pronounced 18-28S region: (See overleaf)

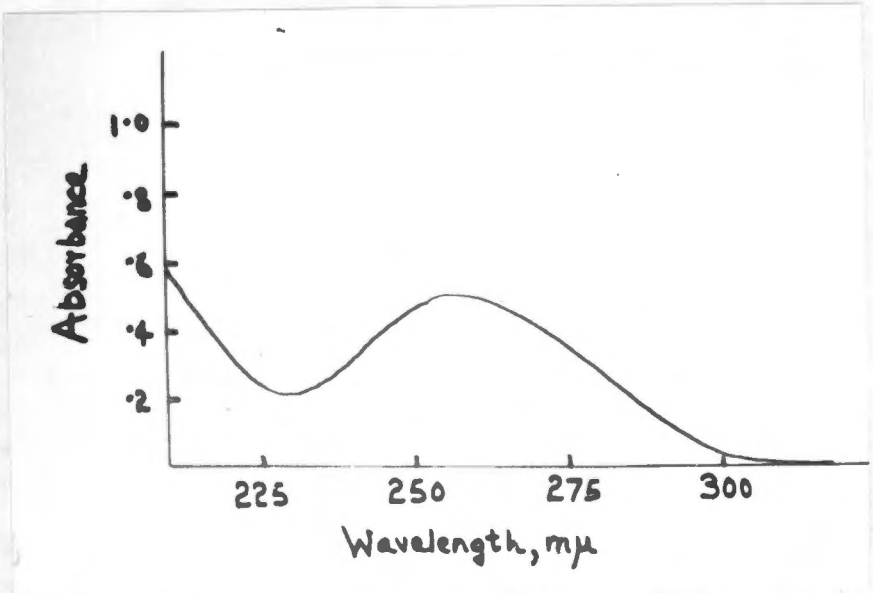


Fig. 22. Absorption spectrum of membrane RNA.

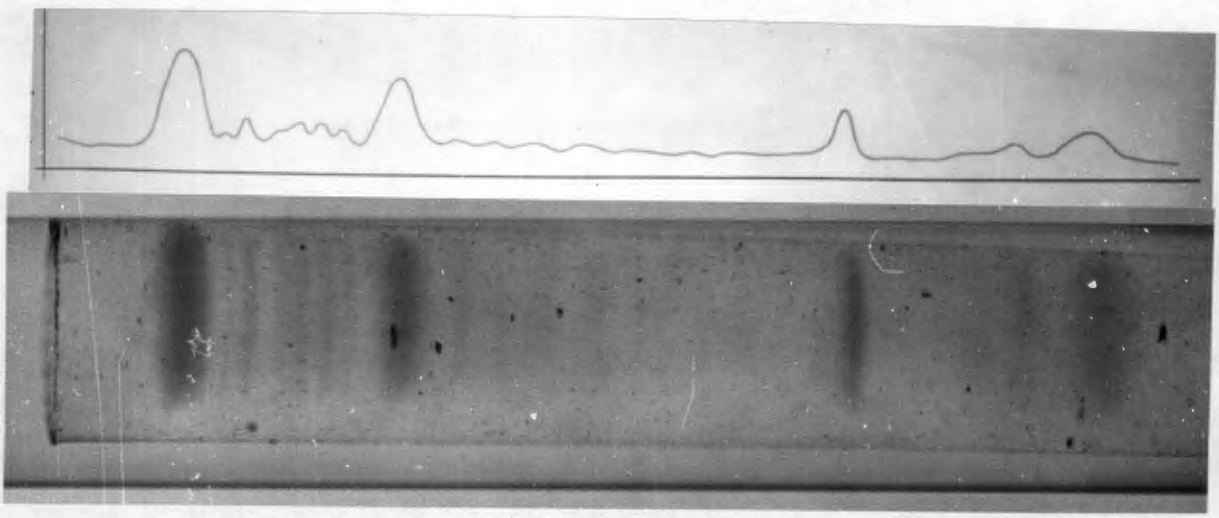


Fig. 23. Photograph and densitometry of membrane RNA 7667.

The five major peaks seen here have already been identified in plate 9.

The composition of membrane RNA has been determined by densitometry and is compared with that of ribosomal RNA in the following table:

Table 11.

Preparation	11467	4567A	4567B	7667	Ribosomal
RNA, s = 28	24	39	31	27	43
18-28	-	8	10	16	13
18	10	23	15	17	23
x-10	-	4	6	17	10
x	8	9	7	7.5	0
5- x	-	1	8	3.5	
5	10	5.5	7.5	3.5	6
4	14	10	15	9	7.5
28/18	2.4	1.7	2.1	1.5	1.9
5/5+18+28 "Index"	22	8.8	15	7.3	8.2
'Sticking'	+	+	+	-	-

The "index" calculated from these figures, which is the percentage of 5S RNA expressed relative to the total ribosomal RNA, should be 1% as mentioned previously. However, some degradation of 28 and 18S RNA is extremely difficult to avoid. The value of this index is an indication of the quality of the RNA. Values above 22 are difficult to measure and characterise electrophoretograms whose major bands are not readily distinguished against the background. The ratio of 28 and 18S RNA is always close to 2 and is the same for ribosomal and

for membrane RNA (compare Bergeron-Bouvet & Moule, 1966 who obtain 2.5 for membrane RNA. However, sucrose gradient centrifugation does not permit a distinction between 28S and 18-28S RNA).

Some slight uncertainty attaches to the figures given for 18-28S RNA (and to other areas where small peaks are measured), and is best dispelled by electrophoresing triplicate samples, by thorough destaining, and and by accurate setting of the zero of the densitometer. Care has been taken to reduce such sources of error, and the high figure obtained for 7667 is significant. This 18-28S region is best displayed in figure 24 where it is compared with several other preparations. Six

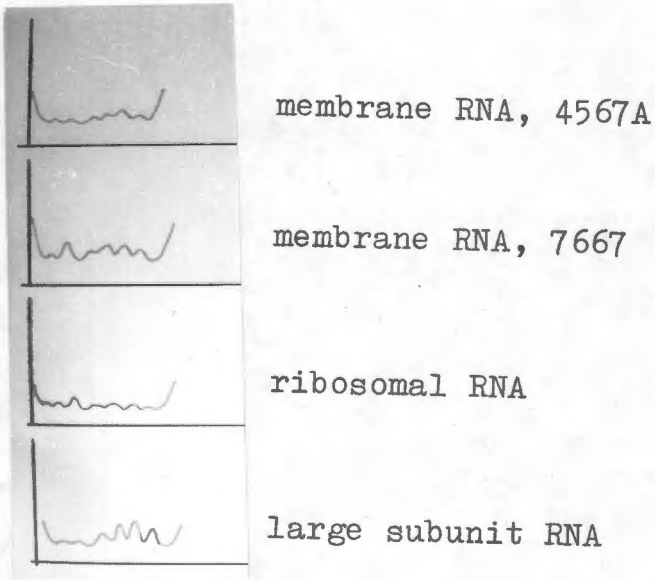


Fig. 24. Densitometry of various electrophoretograms of 18-28S RNA. (28S RNA peak lies to left of these peaks; 18S RNA peak to the right.)

and sometimes 7 peaks are seen in this region and their sharpness increases with increasingly undegraded samples

of RNA. Thus they are not degradation artefacts. Nor are they likely to represent covalent 18S messenger RNA molecules such as postulated by Gould etal. (1966), as their appearance is not attended by a diminution of the 18S peak relative to the 28S peak. It can be surmised that they are mRNA molecules of sedimentation constant between 18 and 28, but more evidence is needed.

There is more of this material in membrane RNA, 7667 than in the other preperations of membrane RNA. It is observed that this corresponds with the decreased yield of 7667 due to more complete removal of rough membranes.

If the more complete removal of rough membranes means that the proportion of ribosomes in the preparation of smooth membranes has been reduced, then there is a case for arguing that 18-28S RNA derives from the smooth membranes. The alternative belief that 18-28S RNA derives from polysomes as the messenger of the polysomes, remains to be investigated.

3. SUMMARY AND CONCLUSION, MEMBRANE RNA.

1. RNA isolated from smooth membranes is mainly ribosomal RNA.

2. Only one prominent RNA does occur in smooth branes which does not occur in ribosomes. This substance has been named xRNA (for the purposes of this account) and constitutes 8% of membrane RNA. It has not been described in the literature.

3. Some 6-7 ribonucleic acids lying between 18 and 28S occur both in membrane and in ribosomal RNA in trace amounts. No attempt has been made to ascertain their nature.

4. DISCUSSION

The identification of ribosomal RNA in membrane RNA rests on the presence therein of 28, 18 and 5S RNA and on the proportions of these three ribonucleic acids, which are the same as in ribosomes. It may be estimated that if about 3% of the total liver ribosomes contaminate smooth membranes, then the amount of ribosomal RNA found in membrane RNA can be accounted for. It remains to be seen whether EDTA present during preparation could reduce the level of contamination (by causing the ribosomes to dissociate. This presupposes that polysomes not free ribosomes, are the source of the contamination).

The so-called xRNA is <u>not</u> haemoglobin messenger RNA (the 9S RNA isolated from reticulocytes by Huez, Burny, Marbaix and Schram, 1967) because

(a) Reticulocyte polysomes contain only 2% of 9S RNA hence liver smooth membranes may be expected to contain much less.

(b) The relative electrophoretic mobilities differ:

RNA	R_f, Loening 1967	R_f, King
18S	.41	.40
x or 9S	.69	.78

(c) The 9S mRNA splits into three zones in 2.5% polyacrylamide on prolonged electrophoresis (Loening, 1967) whereas xRNA, electrophoresed in 2.5 <u>and</u> in 5% polyacrylamide remains a single sharp band. It is therefore homogeneous whereas 9S RNA is not.

Because the 6-7 ribonucleic acids of 18-28S occur in a preparation of ribosomes, which are supposed to contain only 28, 18, 5 and 4S RNA, it may be speculated that they derive from the polysome fraction of the ribosomes and hence are most likely messenger ribonucleic acids. Analysis of polysomal RNA should answer this question; it may be expected that these substances would then prove more abundant.

If xRNA is a constituent of smooth membranes, then, it may be reasoned, the method of Brunngraber, which is used to prepare soluble RNA and which at the same time yields 5S RNA, may also be expected to yield xRNA. Indeed, samples of Brunngraber-sRNA do appear to contain a zone on electrophoresis corresponding in position to xRNA. This observation needs, however, to be confirmed. Should it prove correct, then Brunngraber sRNA, rather than membrane RNA, might prove an ideal source from which to isolate xRNA for further study.

In any event a suitable method of isolation would be by preparative polyacrylamide gel electrophoresis.

Several pertinent questions regarding xRNA need to be answered:

(a) Is it a ribonucleic acid, in terms of ribonuclease susceptibility and ultraviolet absorption spectrum? While acridine orange staining is fairly specific for RNA and while DNA is an unlikely contaminant of smooth membranes (and protein contamination is expected to be negligible after four phenol extractions) - nontheless,

unequivocal demonstration of its identity as a ribonucleic acid is desireable.

(b) What is its sedimentation constant?

(c) What is its base composition, and does it contain unusual bases such as those in tRNA?

(d) Does it have a messenger function, i.e. does it stimulate protein synthesis?

All these questions may receive an answer once it has been isolated.

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