THE UNIVERSITY OF MANITOBA

RIBOSOMAL RNA INSTABILITY: AN INDICATION OF RIBONUCLEASE ACTIVITY ASSOCIATED WITH THE 50S SUBUNIT OF *E. coli* RIBOSOMES.

by

HOWARD CERI

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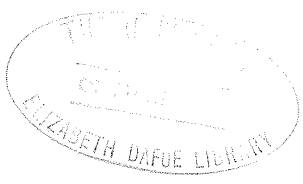

ABSTRACT

STABILITY OF RIBOSOMAL RNA: AN INDICATION OF RIBONUCLEASE ACTIVITY ASSOCIATED WITH THE 50S SUBUNIT OF E. COLI RIBOSOMES

by Howard Ceri

Under the supervision of Dr. P. Y. Maeba

The specific instability of 23S RNA of ribosomes isolated from Escherichia coli MRE 600, an RNase 1^- strain was studied. Although the instability was first observed in intact ribosomes at elevated temperatures, rapid degradation of 23S RNA could be shown at lower temperatures after separation of ribosomal subunits. The nuclease activity remained associated with the 23S RNA after proteins were extracted from 50S subunits by phenol or urea-lithium chloride, and after centrifugation of subunits in cesium chloride. Temperature and a series of effectors govern the rate of nuclease activity. A protein fraction was dissociated from urea-lithium chloride extracted ribosomal RNA on DEAE-cellulose. The fraction displayed nuclease activity on stabilized 23S RNA. The protein co-electrophoresed with one of the 50S ribosomal proteins on urea-polyacrylamide split gels.

T O M Y P A R E N T S

ACKNOWLEDGEMENT

The author wishes to express his sincere appreciation to Dr. P. Y. Maeba for his guidance and encouragement throughout the course of this work and during the preparation of this manuscript.

ABREVIATIONS

ATP - adenosine-5'-triphosphate

DEAE - diethylaminoethyl cellulose

GTP - guanosine-5'-triphosphate

RNA - ribonucleic acid

rRNA - ribosomal ribonucleic acid

RNase - ribonuclease

SDS - sodium doedecyl sulfate

TCA - tricarboxylic acid

Tris - Tris (hydroxymethyl) aminoethane

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I. INTRODUCTION

In attempting to obtain stable 23S RNA to serve as a component for the reconstitution of 50S subunits of *E. coli* ribosomes, we have consistently observed the degradation of 23S RNA. Both large molecular weight species of RNA were intact after isolation of ribosomes from *E. coli* MRE 600, an RNase 1^- strain. However, upon separation of the ribosomes into subunits, the RNA of the 50S subunit was degraded, while the 16S RNA remained intact. This specific instability of the 23S RNA has been reported previously by several authors (see Historical).

This study was undertaken to gain a better understanding of the problems entailed in obtaining stable 23S RNA. By characterization of the degradation process, we hoped to determine whether degradation of 23S RNA was due to enzymatic nuclease activity and whether the activity could be removed or controlled. If this nuclease activity could be traced to one of the 50S ribosomal proteins, another facet in the mystery of the structure and function of the ribosome would be revealed.

II. HISTORICAL

A staggering amount of literature devoted to the ribosome has been produced since 1954, the year Zamecnik(1960) first showed the involvement of ribosomes of the microsomal fraction in protein synthesis. These studies have been reviewed extensively (Zamecnik,1960; Osawa, 1968; Schlessinger and Apirion, 1969; Spirin and Gavrilova, 1969; Kurland, 1970; Möller and Garrett, 1970; Nomura, 1970).

It has been shown that ribosomes of prokaryotes have a sedimentation value of 70S (Taylor and Storch, 1964), and that they can be reversibly separated into two unequal subunits in low magnesium (1 m M) buffers (Tissières and Watson, 1963). The larger subunit has a molecular weight 1.8×10^6 and a sedimentation constant of 50S, and the smaller subunit a molecular weight of $0.7 - 1.0 \times 10^6$ and sediments at 30S (Tissieres et al., 1959). Tissieres (Tissieres et al., 1959) also determined that ribosomes from E. coli consist solely of RNA and protein, with 66% of the ribosomal mass being made up of RNA. It has since been shown that traces of polyamines are also present in the ribosome (Cohen and Lichtenstein, 1960; Spahr, 1962).

It was first thought that ribosomal proteins were a minor component of ribosomes, because of the high percentage of RNA associated with their structure. It was suggested that the proteins formed an outer skeleton around the RNA, much like the protein coats of viruses. However, electron microscopy using positive staining of RNA with uranyl acetate (Huxley and Zubay, 1960), showed that the ribosomal proteins were dispersed throughout the ribosome.

The first studies on ribosomal proteins were carried out by Waller and Harris (Waller and Harris, 1961; Waller, 1964). They identified 12 separate proteins associated with the 30S subunit after extraction with 66% acetic acid. Since this initial study, several groups have characterized the proteins of the 70S ribosome (Spitnik-Elson, 1964; Traut, 1966; Hosokawa et al., 1966; Staehelin and Meselson, 1966; Traut et al., 1967; Kaltschmidt et al., 1967; Möller and Chrambach, 1967; Moore et al., 1968; Fogel and Sypherd, 1968; Otaka et al., 1968; Craven et al., 1969; Hardy et al., 1969; Traut et al., 1969; Nomura et al., 1969; Traut et al., 1969; Staehelin et al., 1969; Sypherd et al., 1969; Kaltschmidt and Wittman, 1970; Allet et al., 1972).

Studies showed that the 30S subunit is made up of 19-22 proteins (Craven et al., 1969), while the 50S subunit contains 21-35 proteins (Kurland et al., 1969; Kaltschmidt and Wittman, 1970). Moore et al., (1968) reported that proteins of the 30S subunit were all present in a ratio of one protein per subunit. However, subsequent work by Kurland (Kurland et al., 1969) showed that the 30S ribosomal proteins were heterogenous, that is, some proteins were present in a lower ratio than one copy per subunit. Whether this work indicated different species of 30S subunits containing different protein combinations, or whether there is exchange of proteins dependent upon ribosomal function has yet to be resolved. On the other hand, Kurland's group (Traut et al., 1969) showed that the proteins of the 50S subunit were present in a 1:1 ratio. Both chemical (Kurland, 1960; Strand and Syphand, 1969; Traut et al., 1969), and immunological studies (Traut et al., 1969) revealed that no proteins were shared between the two subunits.

Studies on three-dimensional organization of ribosomes have to this point been limited to determining which proteins are on the surface of the ribosome and exposed to the environment. Three general approaches have been taken in these studies. The first involves dissociation of ribosomal surface proteins into a split protein fraction by cesium chloride centrifugation. The method was first applied by Staehelin and Meselson (1966), with a very complete study recently published by Allet (1972). The second method involved mild protease treatment of ribosomal subunits, such that only surface proteins are affected (Chang and Flaks, 1970, 1971; Craven and Gupta, 1970). The third method involved competitive labelling with labelled lysine, to find exposed proteins (Kaplan et al., 1972; Visentin et al., 1972). From these studies it has been determined that several proteins lie partially or totally exposed at the ribosome surface, while several others are found buried in the RNA core. Interestingly, Flaks (Chang and Flaks, 1970) showed an inverse relationship between exposure of a protein to protease treatment, and the order of binding in Nomura's 30S reconstitution system (Mizushima and Nomura, 1970), which will be discussed later.

The first successful attempt at reconstitution, that is, the formation of active ribosomal subunits from constituent parts, was performed by Nomura (Hosokawa et al., 1966; Staehelin and Meselson, 1966; Nomura and Traut, 1968) by adding back the split protein fraction to the core particles obtained by cesium chloride centrifugation. Complete reconstitution of the 30S subunit was not achieved until 1968 by Nomura (Traut and Nomura, 1968a). His group was able to show that temperature-specific steps gave rise to intermediate particles during reconstitution.

They were also able to show the order of protein binding and that binding was co-operative in nature. Some proteins played a role in the structural formation of the ribosome while others were required for protein-synthesizing activity (Traut and Nomura, 1968b; Mizushima and Nomura, 1970). The reconstitution of the 50S subunit has proven to be much more difficult. Nomura and Erdmann, (1970), reconstituted active 50S subunits of B. stearothermophilus from components which presumably were able to withstand the high temperatures employed in their system. Maruta and co-workers, (1971), successfully reconstituted E. coli 50S subunits, in a reaction mixture that required 16S RNA from 30S subunits along with components of the 50S subunit. Reconstitution showed temperature-dependent steps and specific sequential binding of proteins (Chu and Maeba).

As previously mentioned, RNA is the major component of the ribosome. Three species of RNA have been isolated. The 50S subunit contains one molecule with a molecular weight of 1.1×10^6 daltons which sediments at 23S (Stanley and Bock, 1965), and another strand of 120 nucleotides, and a molecular weight of 40,000 daltons, sedimenting at 5S (Brownlee and Sanger, 1967; Brownlee and Barrel, 1967). The 30S subunit contains only one strand with a molecular weight of 0.55×10^6 daltons, sedimenting at 16S (Kurland, 1960).

The instability of the 23S RNA fraction of E. coli ribosomes led early workers to suspect that the RNA was not a continuous strand, but rather a series of short fragments (Hall and Doty, 1959; Takanami, 1960; Osawa, 1960; Tashiro et al., 1960). It was also suggested that the 23S RNA was made up of two 16S RNA sections. This was supported by several lines of evidence. Firstly, fragments sedimenting at 16S were

isolated from 50S subunits (Kurland, 1960). Also Midgely (1965) was able to show specific conversion of 23S RNA to 16S RNA. Finally Fellner and Sanger (1968) using T_1 ribonuclease showed that there are two moles of methylated oligonucleotides produced from 23S RNA for every mole produced from 16S RNA. However, these suggestions have been excluded on the following grounds. There are differences in the base composition of 23S and 16S RNA (Stanley and Bock, 1965), in oligonucleotide patterns after nuclease digestion (Aronson, 1962), and in their 5' terminal sequences (Takanami, 1967). The short fragment theory was eliminated by the demonstration of intact 23S RNA by several workers (Stanley and Bock, 1965). This suggested that the fragments reported earlier may have been due to contaminating nuclease activity.

The first reports of nuclease activity associated with ribosomes were made by Elson (Elson, 1958, 1959). Since this initial work, the presence of several nucleases loosely associated with ribosomes have been reported.

Spahr and Hollingworth, (1961), isolated and characterized the first of these nucleases known as Ribonuclease I. This heat-stable enzyme displayed a pH optimum of 8.1 and was activated by monovalent cations K^+ and Na^+, but inhibited by Mg^{+2}. The enzyme displayed high activity toward low molecular weight RNA, and was much more active in excising adenine nucleotides than guanine, such that guanine-rich cores accumulated in the later stages of digestion. Neu and Heppel (1964) have since shown that RNase 1 is actually found in the periplasmic space between the cell wall and membrane, and that it becomes specifically associated with the 30S subunit upon rupture of the cell. RNase 1^- strains have been isolated by Cammack and Wade (1965) and by Gesteland (1966).

Ribonuclease II was first reported by Wade (Wade, 1961; Wade and Lovett, 1961) and later purified by Spahr (1964). The enzyme showed requirements for both a monovalent cation, either K^+ or NH_4^+, and a divalent cation, either Mg^{+2} or Mn^{+2}. Optimum ionic concentrations of 5×10^{-2}M and $10^{-4} - 10^{-3}$M respectively were reported. RNase II was inhibited by urea, sodium ions, and ATP (Venkov et al., 1971).

Zinder (Robertson et al., 1967) isolated Ribonuclease III which was unique in that it was specific for double stranded RNA.

Ribonuclease IV an endonuclease was reported by Spahr and Gesteland (1967). Very loosely associated to ribosomes, it was removed in the first ammonium sulfate wash in Kurland's (1960) purification procedure.

Ribonuclease V, so named because it releases 5'-nucleotides in a 5' to 3' direction, was isolated by Kurvano et al., (1969, 1970). They showed the enzyme required: ribosomes, G and T factors, tRNA, K^+ or NH_4^+ ions, Mg^{+2}, GTP and a sulfhydryl compound to degrade poly U, poly A and T_4 phage or E. coli in RNA.

Kurland (1960) found that 23S RNA of RNase 1^- mutants, prepared by ammonium sulfate precipitation and washing, freeing them of RNase II and RNase IV, was still unstable. Szer (1969) has shown that RNA degradation takes place under conditions in which RNase I, II and V are not active.

There has been some evidence presented which suggests that this nuclease activity may be associated with a protein that is ribosomal in origin. Szer (1969) working with E. coli Q_{13} demonstrated endonucleolytic activity associated with the split protein fraction of 50S ribosomal subunits. Furthermore, RNA in the resulting core particle became stable

after removal of split proteins. In E. coli MRE 600, Staehelin (1969) showed that a nuclease activity was tightly bound to the subunit and was present after formation of core particles.

It is at this point that our knowledge of ribosomal RNA instability stands except to point out that instability of ribosomal RNA is not limited to the ribosomes of E. coli. Leaver and Ingle (1971) have shown that the 23S RNA of chloroplast ribosomes from a great many plant species, was specifically degraded. In some cases the instability was specifically associated with the 16S RNA species. Rosenthal and Iandola (1970) demonstrated that it was the 16S RNA of S. aureus that was specifically degraded. The 28S RNA of 80S ribosomes also demonstrated specific instability. Ravson et al. (1971) have shown this with Euglena gracilis ribosomes, and recently Ishikawa and Newburgh (1972) working with Galleria mellonella have added further support for a ribosomal nuclease activity. They were able to demonstrate the instability of 28S RNA under conditions in which the precursor RNA remained stable. This would suggest that the RNA became unstable after the binding of ribosomal proteins.

III. MATERIALS AND METHODS

Organism: Escherichia coli MRE 600, a RNase 1^- mutant (Cammack and Wade, 1965) served as the source for all ribosomes used in this study. The cells were grown in Casamino acid-enriched basic salt media of Kjelgaard and Kurland (1963) to 3/4 stationary phase in 15 liter carboys with forced aeration at 37°C. The cells were harvested with a Sharples centrifuge, dispensed into 50 g lots, and stored at -76°C.

Isolation of Ribosomes: Ribosomes were routinely isolated from 50 g cells following the method of Kurland (1960). The cells suspended in TM buffer (0.01 M Tris pH 7.6 + 0.01 M magnesium acetate) were ruptured in an Aminco french pressure cell at 10,000 p.s.i. The lysate was centrifuged at 27,000 x g for 30 minutes in a Sorvall RC2-B. The supernatant was subjected to 3 successive ammonium sulfate fractionations. The resulting pellet was solubilized in TM buffer containing 0.6 M ammonium sulfate and pelleted at 100,000 x g for 180 minutes. The last step was repeated twice. The final pellet was dissolved in TM buffer to a concentration of 500-800 A_{260} units and dialysed against the same buffer. The dialysed ribosome fraction was divided to 1 ml portions, frozen in liquid nitrogen and stored at -76°C.

Isolation of Ribosomal Subunits: Ribosomal subunits were isolated by sucrose gradient centrifugation in either (a) a Beckman SW25.2 rotor, or (b) a Ti-15 Zonal rotor.

(a) SW25.2 Gradient Centrifugation: 70S ribosomes in TM buffer were either dialysed against TKM buffer (0.01 M Tris + 0.05 M KCl + 0.001 M $Mg(Acet)_2$) for 8 hours prior to being applied to gradients, or they were pelleted at 100,000 x g for 3 hours in a Beckman 50.1 rotor and then

dissolved in the same low magnesium buffer. Between 250 and 270 A_{260} units of ribosomes in a volume of 1 ml were applied to a 60 ml 5-20% sucrose gradient prepared in the same buffer. The gradients were centrifuged in a Beckman SW25.2 rotor at 25,000 r.p.m. for 12 hrs. at 4°C in a Beckman L2-65B ultracentrifuge. The gradients were collected by piercing the bottom of the tube using a peristaltic pump to collect 1.6 ml fractions in an Isco model 272 fraction collector. Aliquots of each fraction were appropriately diluted and assayed spectrophotometrically at 260 nm. The 50S and 30S peaks were pooled as in Figure 1, and precipitated with 0.6 volumes of ethanol (Staehelin et al., 1969). The precipitate was collected by centrifugation at 10,000 x g for 15 minutes, dissolved and dialysed against TM buffer, at a concentration of 300-400 A_{260} units/ml, then stored in 1 ml portions at -76°C until used.

(b) Zonal Centrifugation: Fifteen thousand A_{260} units of 70S ribosomes in TM buffer were centrifuged at 100,000 x g for 3 hours in a Beckman 50.1 rotor, and the resulting pellet was resuspended in 40 ml of TKM buffer. A 1,300 ml 5-20% sucrose gradient with a 365 ml cushion of 30% sucrose, all in TKM buffer, was generated in a Beckman Ti 15 rotor with the use of an Isco Dialagrad Pump. The pump was adjusted as in Table A so that the gradient was made automatically by mixing 30% sucrose in TKM with the buffer itself. The sample was applied to the gradient with a syringe followed by 50 ml of TKM buffer as an overlay. The rotor was accelerated from 3,000 r.p.m. to 34,000 r.p.m. and maintained at this speed for 12 hours at 4°C. After centrifugation the rotor was slowed to 3,000 r.p.m. and unloaded by expelling the gradient with 30% sucrose at a rate of 10 ml/min. The fractions were collected in a refrigerated fraction

FIGURE 1. Sedimentation profile of 50S and 30S ribosomal subunits isolated by centrifugation in a 5-20% sucrose gradient prepared in TKM with a Beckman SW25.2 rotor at 25,000 r.p.m. for 12 hours.

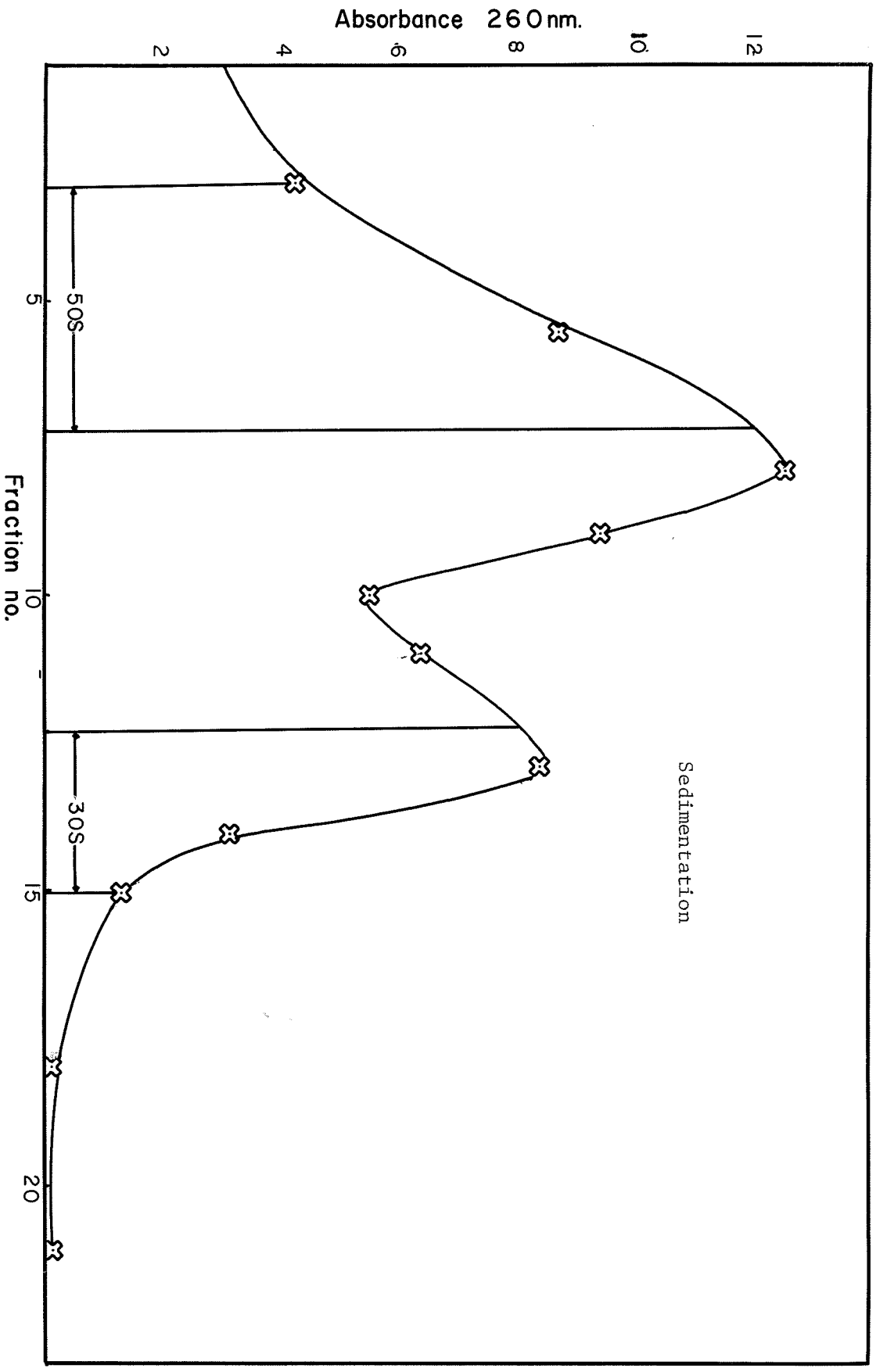

Absorbance 260 nm.
2
4
6
8
10
12
Fraction no.
5
10
15
20
50S
30S
Sedimentation

TABLE 1

ZONAL PROGRAM 5-20% NON-LINEAR GRADIENT

PROGRAM SETTING	"B" SETTING	ACTUAL % SUCROSE
0	16.6%	5.0%
1	24.6%	7.4%
2	32.6%	9.8%
3	40.6%	12.2%
4	48.6%	14.6%
5	56.6%	17.0%
6	60.0%	18.0%
7	63.3%	19.0%
8	66.6%	20.0%
9	100.0%	30.0%
10	100.0%	30.0%

collector, and assayed spectrophotometrically at 260 nm after samples were appropriately diluted. The 50S and 30S peaks were pooled as in Figure 2. The pooled fractions were precipitated with 0.6 volumes of ethanol, collected by centrifugation at 10,000 x g for 15 minutes, dissolved in and dialysed against TM buffer, then stored in 1 ml portions (300-400 A_{260} units) at -76°C.

The level of contamination between 50S and 30S subunits was determined by analytical sucrose gradient centrifugation in a Beckman SW50.1 rotor. A total of 1 A_{260} unit of ribosomal subunits in 0.1 ml TKM was layered on a 5-20% sucrose gradient of 5 ml prepared in the same buffer. After centrifugation at 50,000 r.p.m. for 1 3/4 hours, the gradients were analysed at 260 nm using a flow-through cell adapted to a Gilford Model 2400 spectrophotometer (Figure 3).

Cesium Chloride Core Particles: Staehelin's method (Staehelin and Meselson, 1966) for separating surface proteins of the 50S subunit into a split protein fraction, and a core fraction composed of 23S RNA and the remaining 50S ribosomal proteins was used. Two hundred A_{260} units of 50S subunits in a 5 ml gradient composed of 4.2 M cesium chloride, 0.01 M magnesium acetate, 0.015 M Tris (pH 8.0) and 6 x 10^{-3} M, β-mercaptoethanol were centrifuged at 50,000 r.p.m. for 48 hours at 4°C in a Beckman SW50.1 rotor. After piercing the bottom of the gradient 0.5 ml fractions were collected and assayed at 260 nm at the appropriate dilution (Figure 4).

Isolation and Electrophoresis of Ribosomal Protein: Proteins were isolated by the method of Spitnik-Elson (1964). Ribosomal subunits in TM buffer (300-400 A_{260} units/ml) were mixed with an equal volume of 8M urea-4 M

FIGURE 2. Sedimentation profile of 50S and 30S ribosomal subunits isolated by zonal centrifugation in a 5-20% sucrose gradient prepared in TKM.

⊡ — Concentration of sucrose.

⊙ — Absorbance at 260 nm.

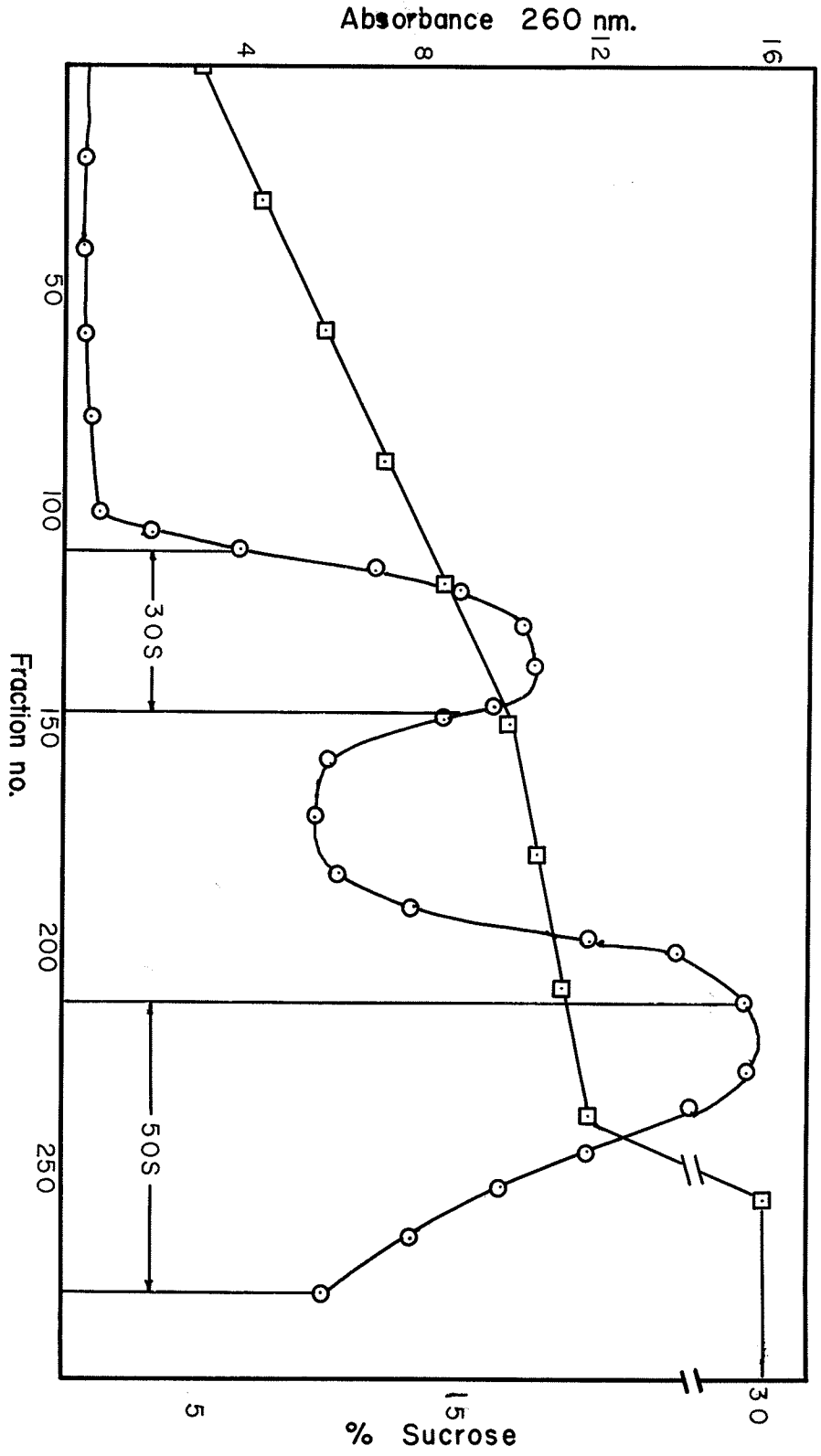

Absorbance 260 nm.
4
8
12
16
50
100
150
200
250
Fraction no.
30S
50S
5
15
30
% Sucrose

FIGURE 3. Sedimentation profile of purified 50S ribosomal subunits after analytical centrifugation in a 5-20% sucrose gradient prepared in TKM buffer at 50,000 r.p.m. for 1 3/4 hours in a Beckman SW50.1 rotor.

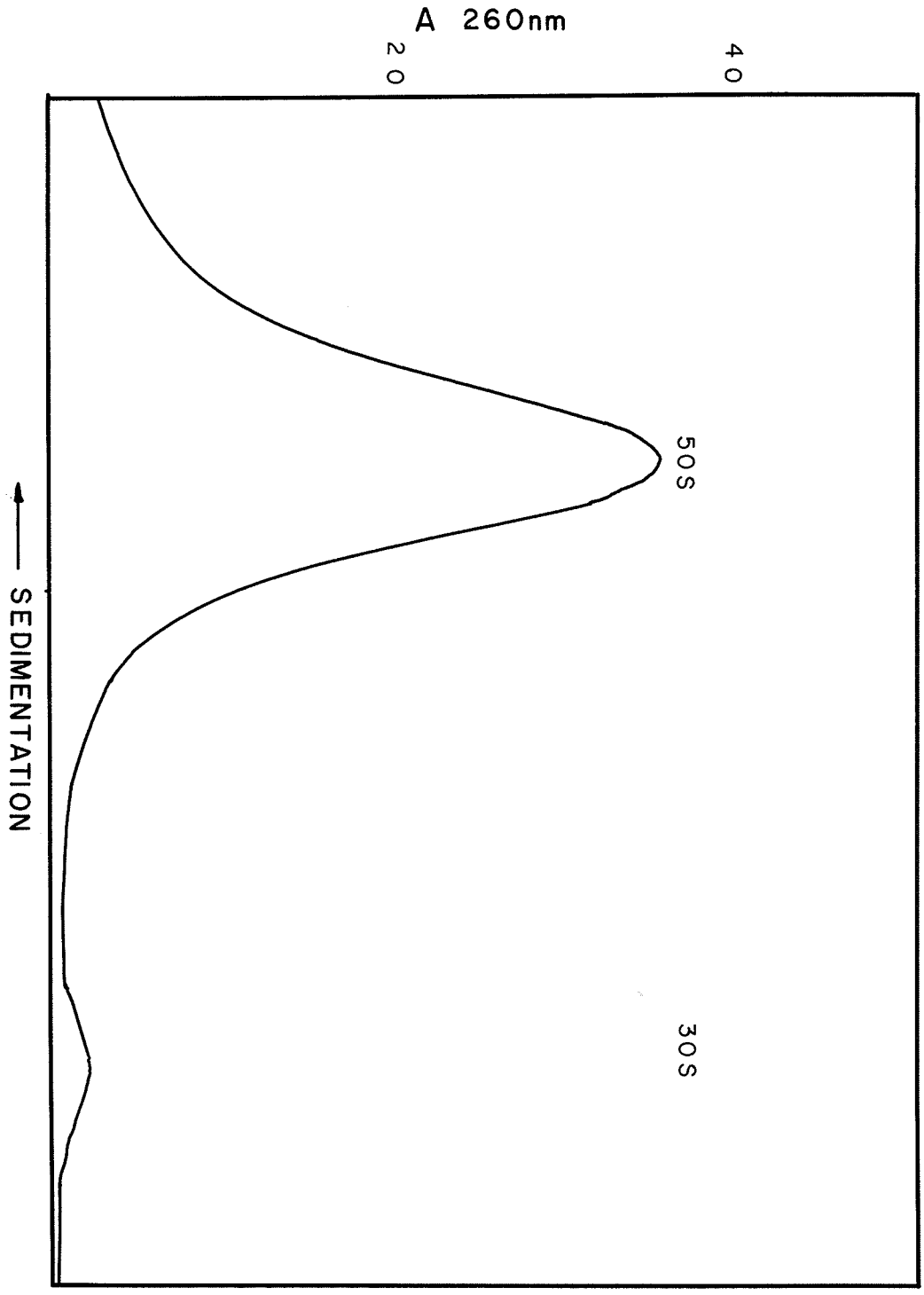
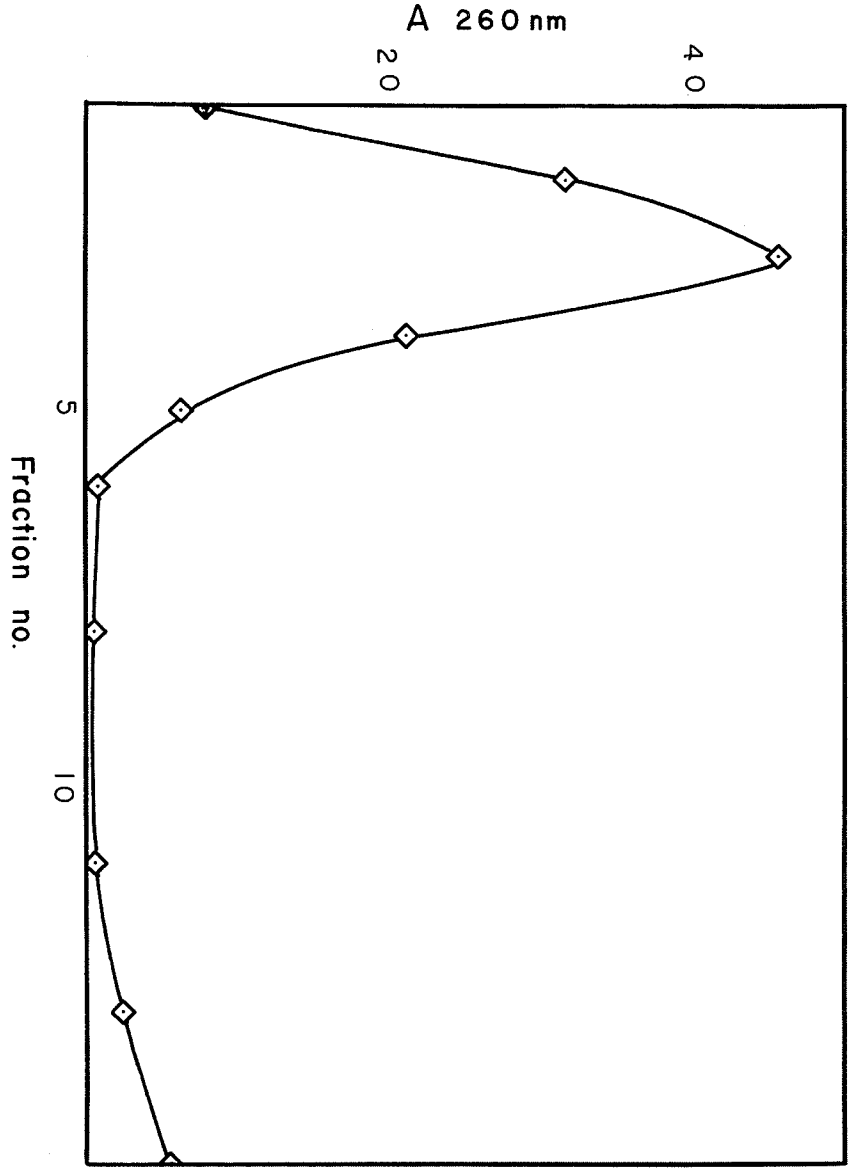

A 260nm
20
40
50S
30S
SEDIMENTATION

FIGURE 4. Cesium cloride core particles prepared by centrifugation of 200 A_{260} units of 50S ribosomes at 50,000 r.p.m. for 48 hours through a gradient of 4.2 M cesium chloride in 0.01 M Tris, 0.01 M magnesium acetate, and 6×10^{-3} M β-mercaptoethanol in an SW50.1 rotor.


A 260 nm
20
40
5
10
Fraction no.

lithium chloride and left in ice for 48 hours. The precipitated RNA was removed by centrifugation at 10,000 x g for 10 minutes. The supernatant containing ribosomal proteins was dialysed against 0.02 M Tris (pH 8.0), 6 M urea, and 6 x 10^{-3} M β-mercaptoethanol.

Electrophoresis was carried out by the method of Leboy, Cox and Flaks (1964) at pH 4.5. Fifty to 100 micrograms of protein was applied to each gel. After electrophoresis at 3 ma/tube the gels were fixed for 2 hours in 12.5% TCA and stained overnight in 0.05% Coomasie Brilliant Blue in 12.5% TCA. Gels were transferred to 10% TCA for destaining.

Isolation of Ribosomal Ribonucleic Acid: Ribosomal RNA was isolated from 70S particles after dialysis against 0.1 M sodium acetate, 1 m M EDTA (pH 6.5). The samples were mixed with an equal volume of 8 M urea-4 M lithium chloride and 0.6 volumes of 10 M lithium chloride. The mixture was allowed to stand at 4°C for 48 hours before the rRNA was pelleted by centrifugation at 10,000 x g for 10 minutes. The supernatant was removed with an aspirator and the pellet washed in 4 M urea-2 M lithium chloride. The rRNA was either dissolved in the sodium acetate-EDTA buffer and stored at -76°C, or dissolved in TKM buffer and separated into 23S and 16S RNA fractions by sucrose gradient centrifugation in a Beckman SW 25.2 rotor (Methods), for 24 hours at 25,000 r.p.m. The pooled fractions (Figure 5) were precipitated with 2 volumes of ethanol and stored in this form at -20°C. Analytical sucrose gradient centrifugation (Fig. 6) revealed little free 16S RNA contamination of the 23S RNA.

FIGURE 5. Sedimentation profile of 23S and 16S ribosomal RNA in 5-20% sucrose gradients prepared in TKM and centrifuged at 25,000 r.p.m. for 24 hours in a Beckman SW25.2 rotor.

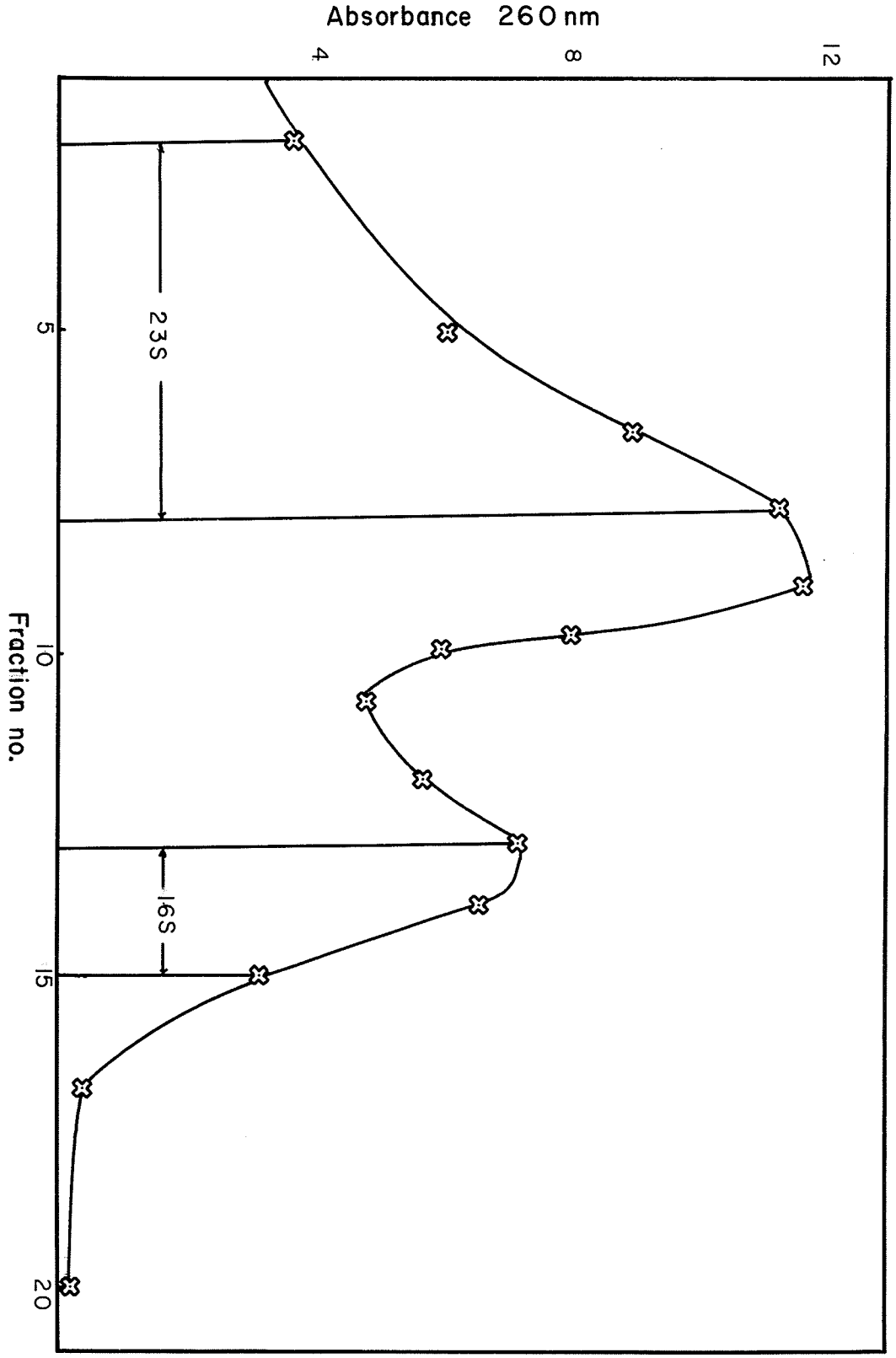
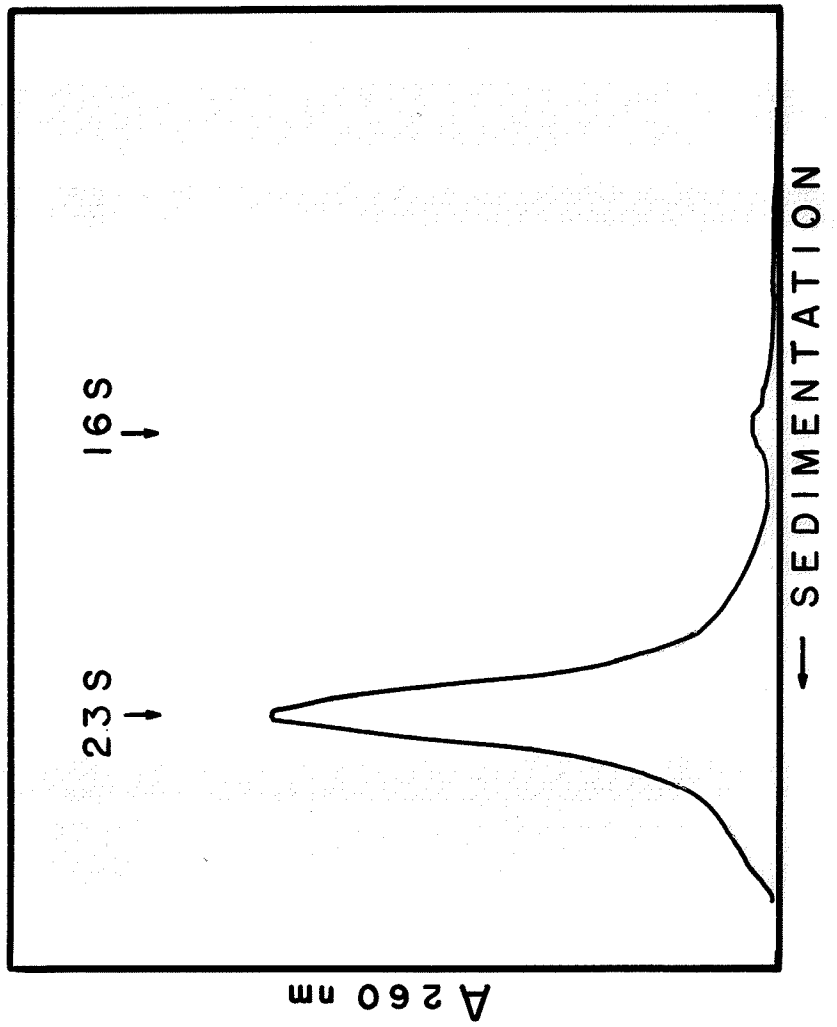

Absorbance 260 nm
4
8
12
5
10
15
20
Fraction no.
23S
16S

FIGURE 6. Analytical Sucrose Gradient Centrifugation Sedimentation profile of isolated 23S RNA on a 5 - 20% sucrose gradient spun for 1 3/4 hrs at 50 K in a Beckman SW 50.1 Rotor.


A 260 nm
23S
16S
SEDIMENTATION

Ribonuclease Assay: Ribonuclease activity was assayed by examination of electrophoretic migration patterns of rRNA in 0.2% SDS - 2 1/2% polyacrylamide gels after appropriate incubation. Assays were carried out in final volumes of 0.1 ml of 0.01 M Tris (pH 7.8) containing 170-350 µg rRNA. The reaction was stopped by making the assay mixture 0.2% SDS and cooling in ice. Samples were heat-denatured at 72°C for 1 minute to disrupt secondary structure (Stanley and Bock, 1965) prior to electrophoresis. Electrophoresis was carried out by the method of Peacock and Dingman (1967) except the system contained 0.2% SDS in both gels and buffer. Electrophoresis was carried out at pH 4.5 at 3 ma/tube. RNA migration was analysed by staining gels or spectophotometrically. When stained 35-44 µg of RNA was applied to the gels. The gels were fixed in solution A (40% ethanol, 4% acetic acid) for 1-2 hours at 37°C, and stained in 0.09% Toluidene Blue O solution A. Destaining was carried out at room temperature in solution B (30% ethanol, 1% acetic acid). Gels were also analysed by scanning at 260 nm in a Gilford Model 2400 or Shimadzu MPS-500L spectrophotometer equipped with a linear transport accessory.

For spectrophotometric analysis with the Gilford 35-44 µg of RNA was applied to gels whereas with the Shimadzu only 17-22 µg of sample was required. The amount of RNA left intact after incubation is expressed as a percentage of the 23S RNA in the control calculated by weighing the 23S peaks.

Protein Determinations: Protein was assayed by the method of Lowry et al. (1951).

IV. RESULTS

Ribosomal Components: Proteins of ribosomal subunits, isolated by sucrose gradient centrifugation (Methods), were extracted from 200 A_{260} units of subunits in urea-lithium chloride (Methods). Fifty to 100 µg of ribosomal proteins were electrophoresed into urea-polyacrylamide gels then stained with Coomassie Blue (Methods). The electrophoretic patterns of the 30S and 50S proteins (Plate 1) were identical to those obtained by Kurland, (1971), using the same procedures. This indicates that the ribosomal preparation was pure containing no extraneous protein.

The integrity and purity of ribosomal RNA was examined by electrophoresis in SDS-polyacrylamide gels at pH 8.6 (Methods). Five A_{260} units of ribosomes in 0.1 ml of 0.01M Tris (pH 7.8), were made 0.2% with SDS, heat denatured at 72°C for 1 min., and 10 micro-liters were applied to gels. After electrophoresis, gels were scanned at 260 nm in a recording spectrophotometer (Methods). The RNA appeared as 2 distinct peaks representing 23S and 16S RNA, and was free of other species of RNA and degradation products (Plate 2). Generally the 5S RNA could not be clearly distinguished, since the gel was too porous to retard this species, and the actual content of the 5S RNA in the samples was very small, less than 1 µg.

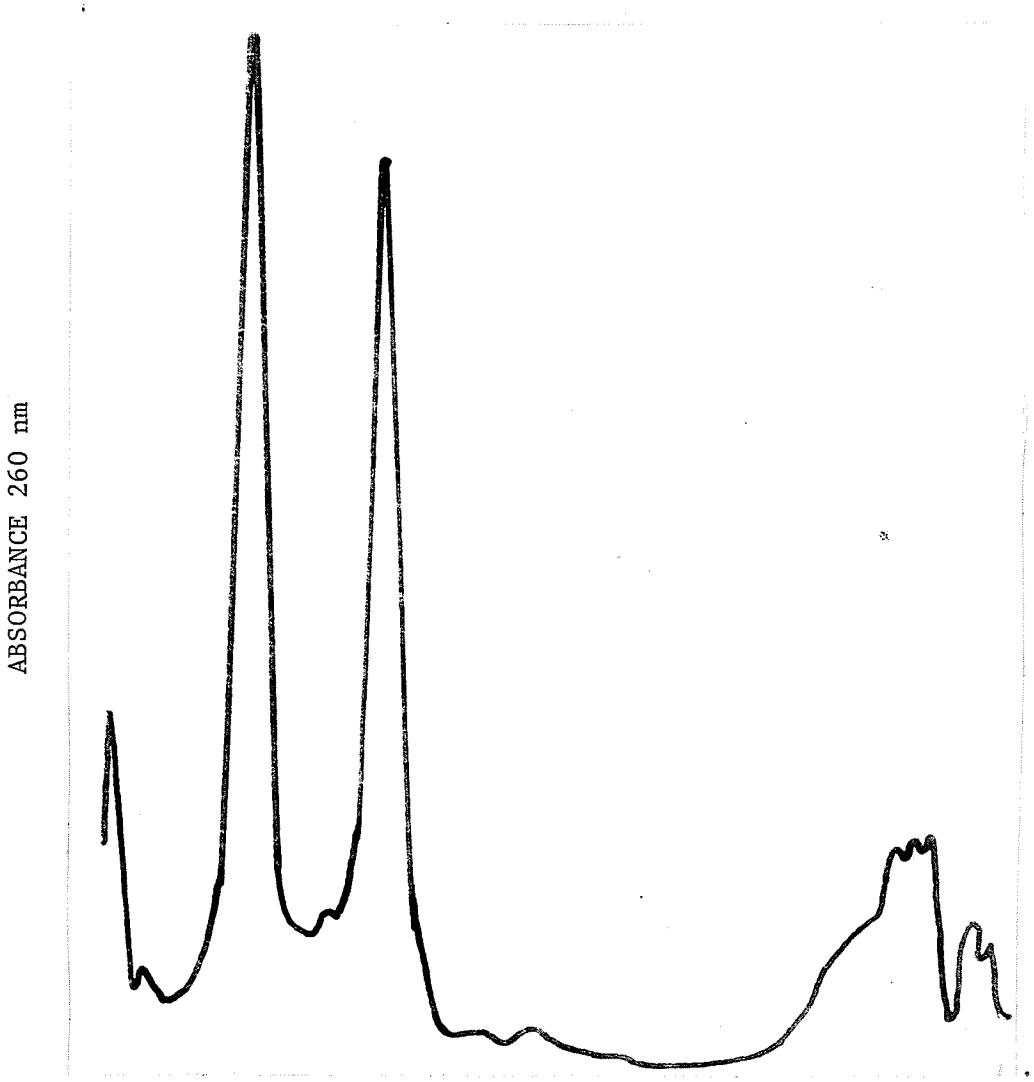
Stability of Ribosomal RNA in Intact Ribosomes: The results shown in Plate 2 demonstrated that both 23 and 16S RNA species were intact when examined *in situ* in 70S ribosomes. Storage of ribosomes at 0°C did not alter the results. The stability of RNA after incubation at 37°C, 54°C, and 60°C is shown in Figure 7. The standard assay mixture (Methods)

PLATE 1

50S RIBOSOMAL PROTEINS

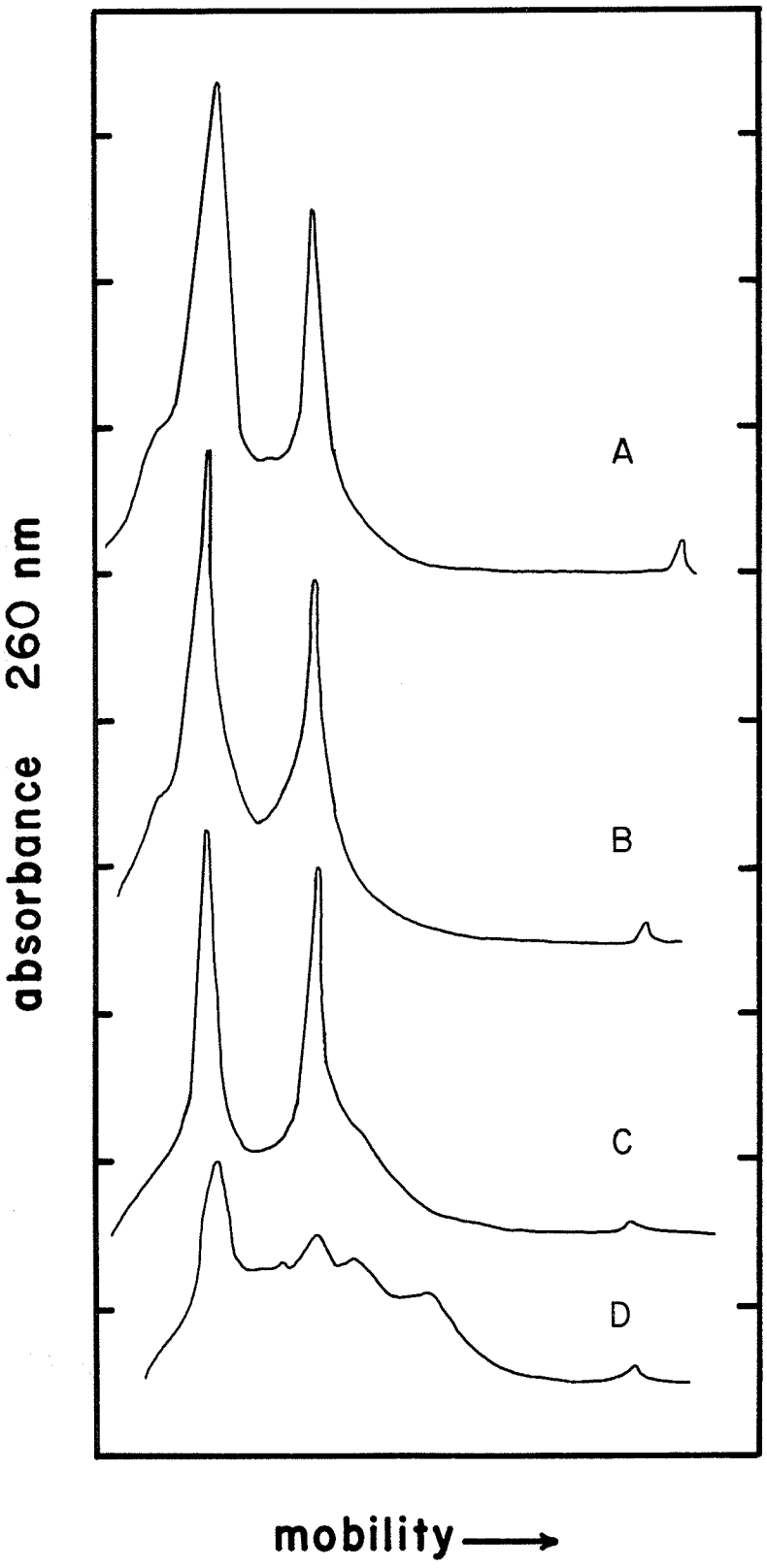
IN UREA-POLYACRYLAMIDE ELECTROPHORESIS

PLATE 2



70S RIBOSOMAL RNA

FIGURE 7 - Stability of Ribosomal RNA. Reaction mixtures contained 8.7 A_{260} units of RNA were incubated at desired temperatures. Samples were made 0.2% with SDS, heat denatured and electropheresed (Methods) A. Control B. 3 hrs 37°C C. 1 hr 54°C D. 1 hr 60°C.


absorbance 260 nm
A
B
C
D
mobility ⟶

containing 8.7 A_{260} units of ribosomes was incubated at the stated temperatures. Samples were removed at 30 min. intervals for 3 hrs., made 0.2% with SDS and placed in ice to stop the reaction. After heat denaturation at 72°C for 1 min., electrophoresis was carried out and gels scanned at 260 nm. Figure 7, showed that rRNA remained stable over a 3 hr. period at 37°C, but that degradation occurred after 1 hr. at 54°C, as judged by the decrease in area under the peaks. The rate of degradation (Figure 8) was approximated by weighing the 23S peaks cut from tracings of gels scanned at 260 nm (Methods). The percentage of intact 23S RNA was calculated and plotted against time. The results showed that RNA was autodegraded in situ by a process that was dependent on temperature and time.

Monovalent Cations: The effect of monovalent cations on RNA degradation was assayed by adding KCl, NaCl, or NH_4Cl at concentrations of 0.05M to 0.15M to the standard assay mixtures (Methods) containing 8.7 A_{260} units of ribosomes in the presence or absence of 1mM Magnesium. Auto-degradation was measured at 37 and 60°C for 1 hr. The reactions were terminated with SDS (final concentration 0.2%,w/v) and 10 micro-liter samples were electrophoresed in 2 1/2% polyacrylamide gels (Methods). The gels were scanned and recorded at 260 nm and the 23S peak was cut from tracings, weighed and compared to control gels. The results summarized in Table 2 show that degradation of RNA in intact ribosomes was only marginally affected by monovalent cations in the presence or absence of low magnesium concentrations. When high concentrations of magnesium were used, RNA did not penetrate the gels, presumably due to aggregation.

FIGURE 8. Time Course Degridation of 23S RNA of Intact Ribosomes. Electrophoresis of Ribosomes incubated at the desired temperatures was carried out. The 23S RNA peaks of the scanned gels were cut out and weighed. The % 23S RNA intact was plotted against time.

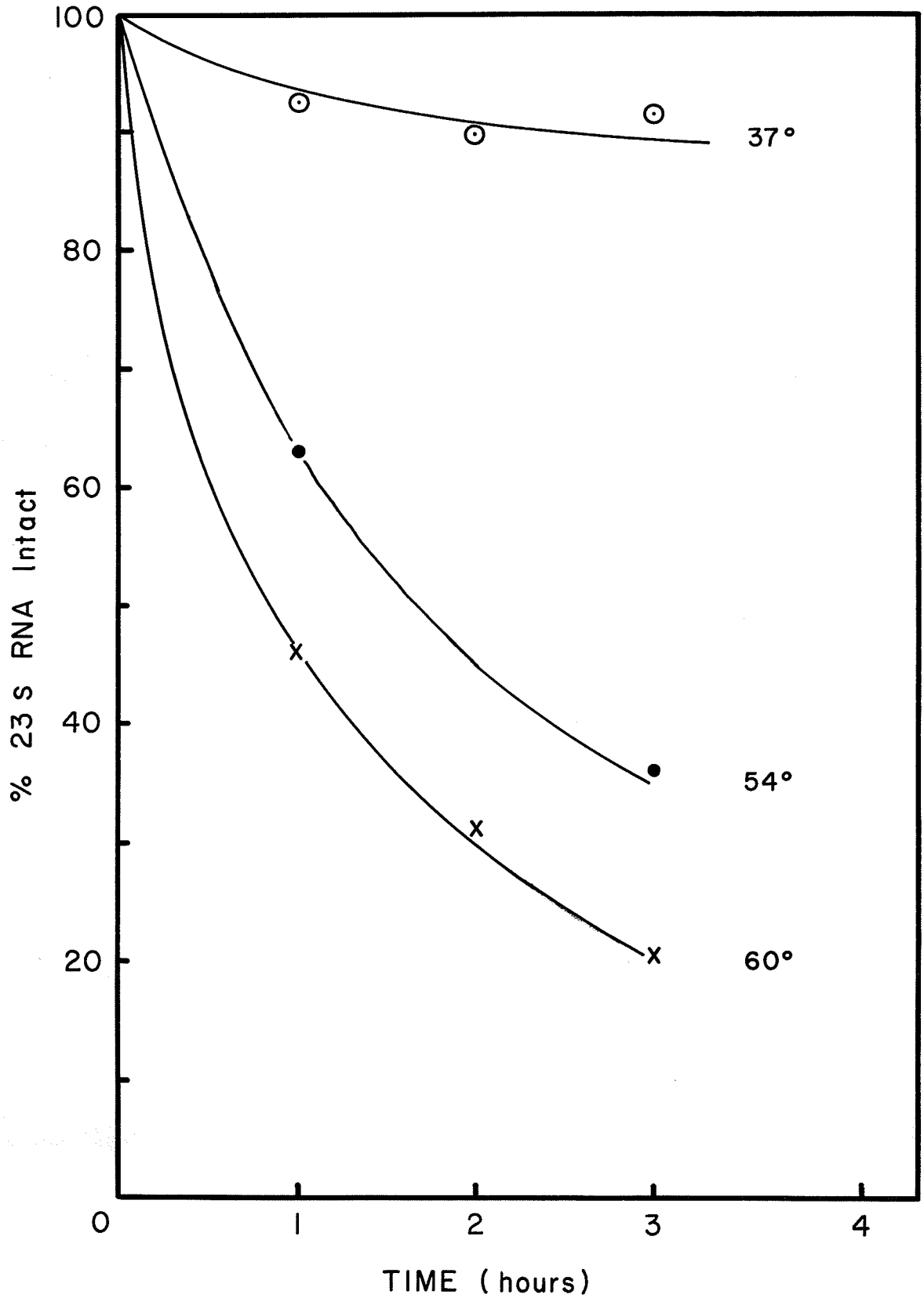

100
80
60
40
20
0
1
2
3
4
% 23 S RNA Intact
TIME (hours)
37°
54°
60°

TABLE 2

Intact Ribosomes

Assay Mixture*	% 23S RNA Intact at 37°C for 1 hr.	% 23S RNA Intact at 60°C for 1 hr.
Standard Assay (0.01 M Tris pH 7.8)	97%	48%
Standard Assay + 1m M $Mg(Acet)_2$	97%	54%
Standard Assay + 0.05 M NaCl	96%	47%
Standard Assay + 0.05 M NaCl + 1m M Mg_{Acet}	97%	42%
Standard Assay + 0.05 M NH_4Cl	100%	49%
Standard Assay + 0.05 M KCl	96%	48%
Standard Assay + .05 M KCl + 1M M $Mg(Acet)_2$	98%	49%

* Standard Assay Mixtures contained 5-10 A_{260} units of ribosomes in 0.1 ml of 0.01 M Tris (pH 7.8). Following incubation mixtures were made 0.2% with SDS and heat denatured at 72°C for 1 min. One A_{260} unit of ribosomes was electrophoresed (Methods) at 3 ma/tube, which were then scanned at 260 nm. The 23S RNA peaks were cut from the tracings, weighed and the percent RNA intact calculated from non-incubated controls.

Stability of Isolated Ribosomal RNA: The stability of ribosomal RNA following extraction of ribosomal protein in urea-lithium chloride (Methods) was assayed. The protein content of the extracted RNA was assayed by the method of Lowry (Lowry et al., 1951), using lysozyme as a standard and showed 98.6% of the ribosomal protein had been removed. The RNA was dissolved in and dialysed against 0.01M Tris (pH 7.8), and incubated at 37° and 60°C to see whether extracted RNA could undergo autodegradation. The degradation of the extracted RNA can be seen in Figure 9 which when compared to Figure 7 reveals a greater degree of instability in the extracted form. In these experiments 1 A_{260} units of rRNA was electrophoresed and scanned at 260 nm. The results show that the degradative activity remains associated with the rRNA even after removal of most of the ribosomal proteins.

Dialysis of Ribosomes in Low Magnesium Buffers: The stability of rRNA under the conditions used in isolation of ribosomal subunits was tested, that is, dialysis against low magnesium buffers (1mM). Purified 70S ribosomes were dialysed against TKM buffer (0.01M Tris, 0.05M KCl, 1.0mM Mg Acct (pH 7.8)) for 12 hrs. at 4°C. Samples were made 0.2% with SDS and heat denatured at 72°C for 1 min., after which 0.8 A_{260} units of RNA was applied to gels for electrophoresis (Methods). As seen in Figure 10, both species of RNA remained intact after dialysis.

Integrity of RNA in Isolated Subunits: Ribosomes were dissociated into subunits by dialysis in TKM buffer, and isolated by centrifugation in a 5-20% sucrose gradient prepared in the same buffer (Methods). Integrity of RNA in 30S and 50S subunits was assayed by electrophoresis of 1 A_{260} unit of heat-denatured subunits in 10 micro-liters of 0.01M Tris (pH 7.8)

FIGURE 9. Stability of RNA isolated from ribosomes in urea-lithium chloride determined by electrophoresis (Methods) of samples incubated in 0.01 M Tris (pH 7.8) after incubation at temperatures indicated below and scanned at 260 nm.

A. Control

B. 1 Hr. at 37°C

C. 1 Hr. at 60°C

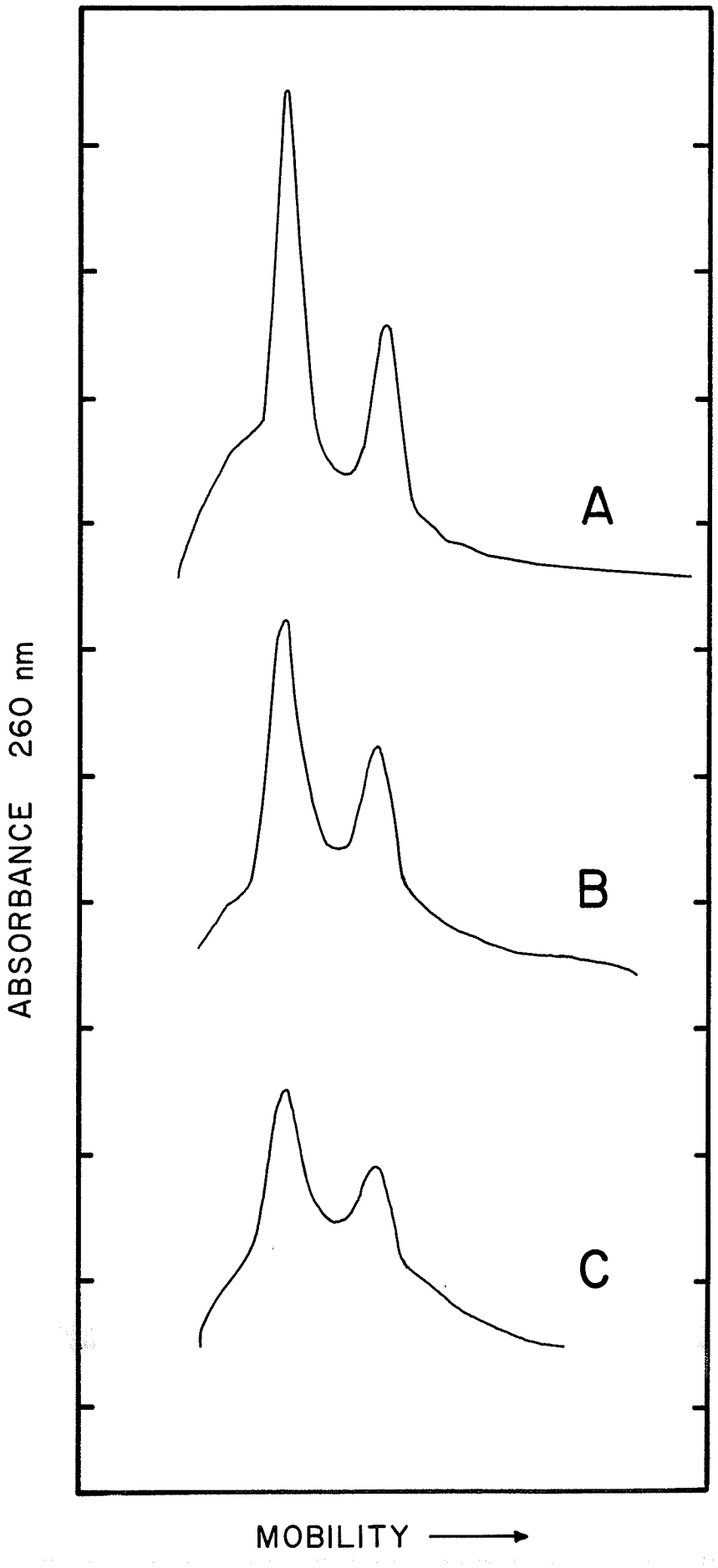
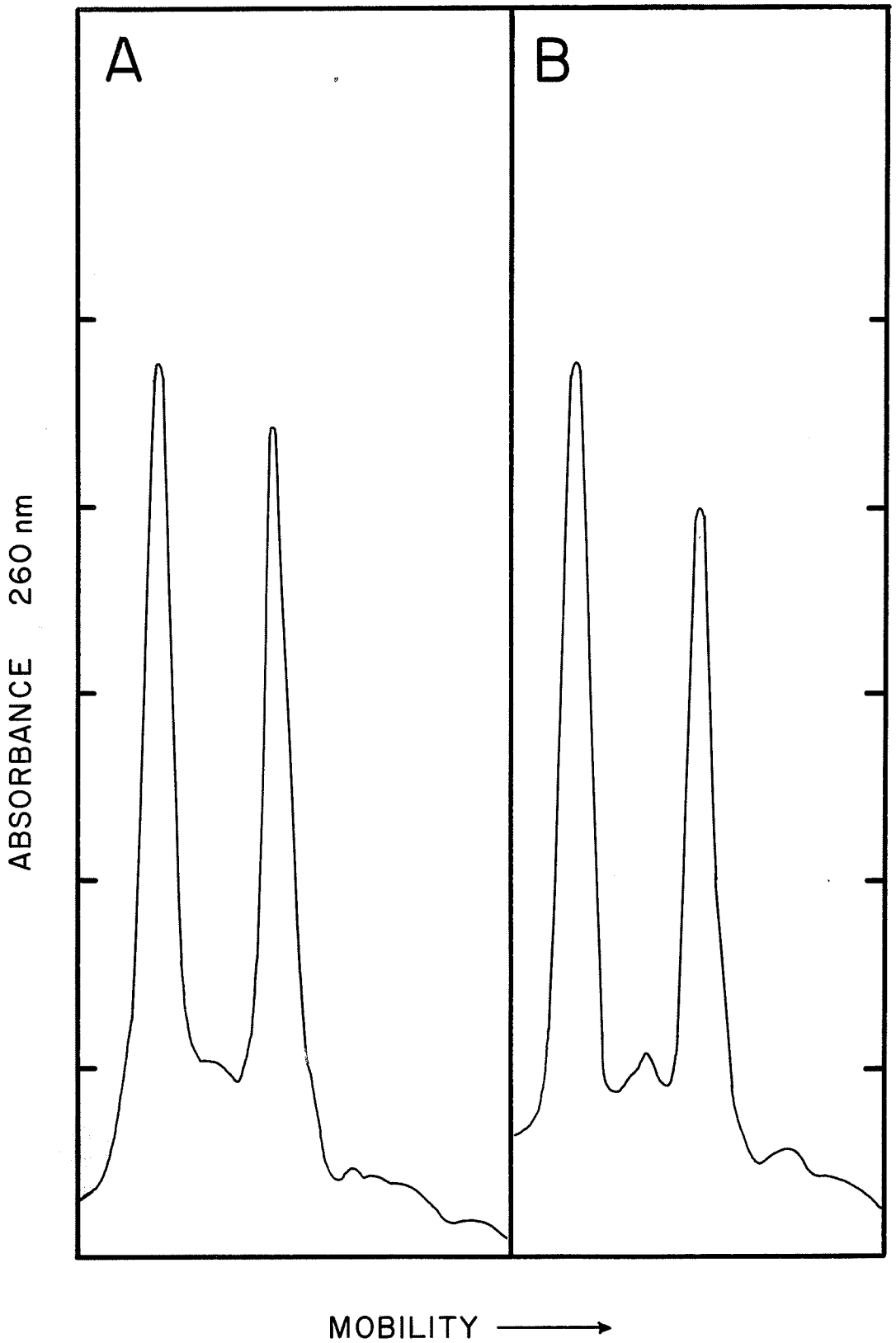

A
B
C
ABSORBANCE 260 nm
MOBILITY

FIGURE 10. The stability of ribosomal RNA following dialysis against low magnesium buffer (TKM). Samples were dialysed overnight and a portion made 0.2% with SDS. 1 A_{260} unit was applied to gels for electrophoresis and scanned at 260 nm (Methods).


A
B
ABSORBANCE 260 nm
MOBILITY

and 0.2% SDS (Methods). The 16S RNA of the 30S subunit was intact (Plate 3A), however the 23S RNA of the 50S subunit showed marked degradation (Plate 3B).

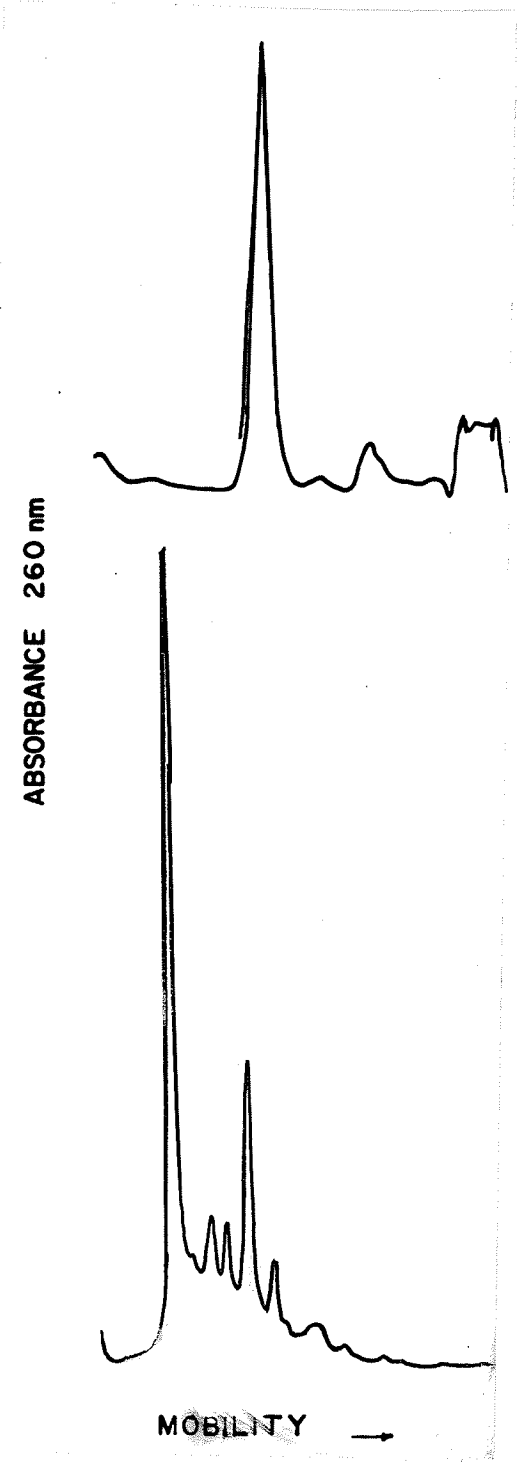
Fifteen to 20% of the RNA of the 50S subunit banded where 16S RNA normally migrates. However, analytical sucrose gradients (Methods) showed the 50S were less than 5% contaminated with 30S subunits, indicating that this material was not 16S RNA from 30S subunits. Since dialysis against low magnesium buffers does not induce degradation of 23S RNA (Figure 10), the observed breakdown must have occurred during or following the physical separation of the ribosomal subunits by centrifugation.

Instability of RNA in Ribosomal Subunits: Incubation of 50S subunits in 0.01M Tris (pH 7.8) at temperatures between 20 and 70°C showed degradation of 23S RNA could continue *in situ* in the subunits. Gels scanned at 260 nm following electrophoresis of 1 A_{260} unit of 50S subunits incubated as described above for 1 hr. at 37 or 60°C are shown in Plate 4. The characteristic pattern of degradation and temperature dependence of the reaction can be seen.

The 16S RNA of 30S subunits did not degrade when incubated at temperatures up to 60°C, however at temperatures greater than 60°C, degradation of 16S RNA was observed. Although this observation was not investigated further, it may be that the RNase activity of the contaminating 50S subunits (<5%) was responsible.

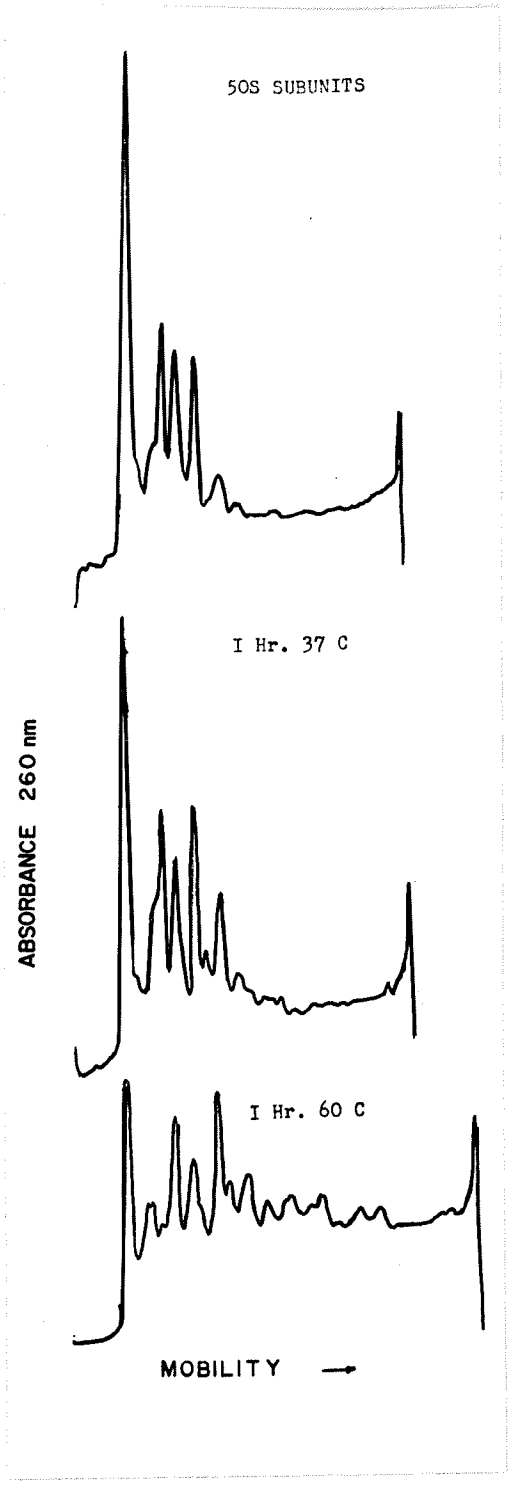
The addition of monovalent cations to the reaction mixture containing 1 A_{260} unit of 50S subunits in 0.01M Tris (pH 7.8) had little effect on RNA degradation (Table 3). Sodium, potassium, and ammonium

Plate 3



Isolated 30S and 50S ribosomal subunits were made 0.2% with SDS and IOD was applied to gels for electrophoresis and scanned (Methods).

Plate 4



50S Ribosomal subunits were incubated at temperatures indicated in Tris (pH 7.8), made 0.2% with SOS and electrophoresed and scanned (Methods).

TABLE 3

50 S Ribosomal Subunits

Assay Mixture*	% 23S Ribosomal RNA Intact 37°C
Standard Assay Mixture	44%
Standard + 0.05 M KCl	42%
Standard + 0.05 M NaCl	46%
Standard + 0.05 M NH_4Cl	43%

* Standard Assay Mixtures contained 5-10 A_{260} units of ribosomes in 0.1 ml of 0.01 M Tris (pH 7.8). Following incubation mixtures were made 0.2% with SDS and heat denatured at 72°C for 1 min. One A_{260} unit of ribosomes was electrophoresed (Methods) at 3 ma/tube, which were then scanned at 260 nm. The 23S RNA peaks were cut from the tracings, weighed and the percent RNA intact calculated from non-incubated controls.

chlorides were tested in concentrations from 0.05M to 0.1M.

The results show that the degradative ability remained active in isolated subunits and was specifically associated with the 50S subunit.

Effectors of RNA Degradation: The effect of polyvalent anions, (phosphate, or sulfate) on the rate of RNA degradation was measured at 37°C in standard assay mixtures containing 0.5M anions ($PO_4^{\equiv}$ or $SO_4^{=}$) as the potassium salt. Samples were removed at 2 min. intervals, made to 0.2% SDS, placed in ice, then electrophoresed and scanned at 260 nm (Methods). The 23S RNA peaks from the scanned tracings of RNA gels were cut out and weighed. The percentage of RNA remaining intact was compared to a control without anions. The rate of RNA degradation was found to be enhanced several fold by the addition of polyvalent anions. Figure 11 indicates that the addition of trivalent anions has a greater enhancement than temperature as degradation in $PO_4^{\equiv}$ at 37°C was more rapid than at 60°C in the standard assay mixture. Optimal activity was seen at concentrations of 0.05 to 0.15M anions. Urea had a similar effect between the concentrations of 2-6M as did 1mM EDTA. Since these reagents affect the secondary structure of RNA, their effect may be the result of opening sections of RNA to nucleolytic attack. The increase in activity due to temperature may occur by the same mechanism.

The incubation of 50S subunits at 60°C for 1 hr. resulted in the recovery of >90% of the 23S RNA when reaction mixtures (5 A_{260} units of 50S subunits in 0.1 ml of 0.01M Tris (pH 7.8), were made 10% (v/v) with methanol, ethanol, or isopropanol (Table 4). Again it was not possible to determine whether the effect of alcohols was due to alterations of secondary structure of RNA or a direct effect on nucleolytic activity.

FIGURE 11. Time course degradation of 50 S Ribosomal Subunits. Subunits in Tris buffer were incubated at 60°C, those in 0.05 M $PO_4^{\equiv}$ buffer were incubated at 37°C. Samples were removed 2 min intervals from $PO_4^{\equiv}$ treated subunits and at 15 min for subunits in Tris buffer. Samples were made 0.2% with SDS, electrophoresed and scanned (Methods). The 23 S RNA peaks were cut out weighed and the % RNA left intact calculated.

50 S subunits in tris buffer

50 S subunits in phosphate buffer

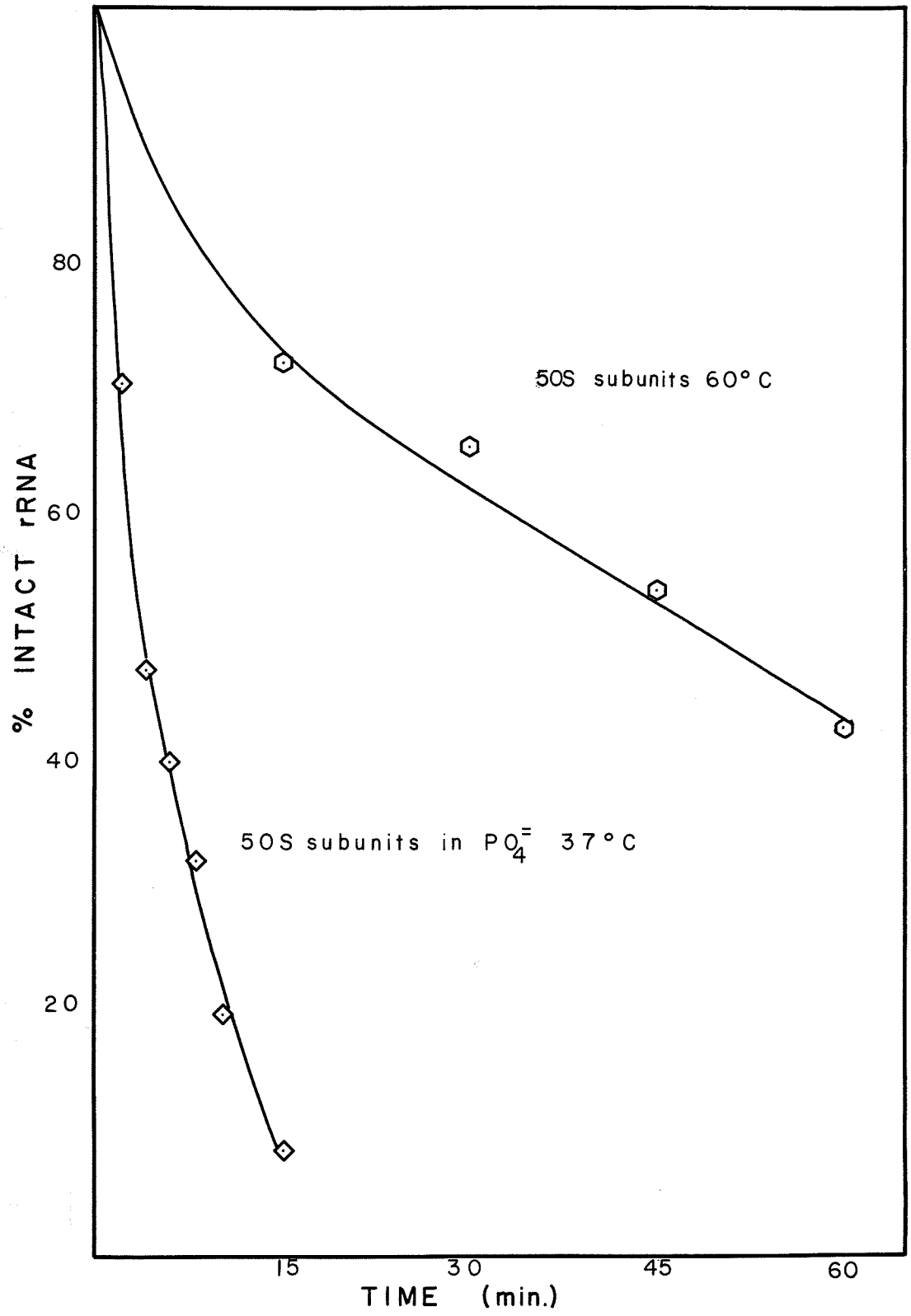

80
60
40
20
% INTACT rRNA
50S subunits 60° C
50S subunits in $PO_4^=$ 37° C
15
30
45
60
TIME (min.)

TABLE 4

50S Ribosomal Subunits

Assay Mixture*	% 23S RNA Intact at 60°C for 1 hr.
Standard Assay	44%
+10% Alcohol (methanol, ethanol, isopropanol)	97%
+ 0.02% SDS	99%
+ 1m M Mercuric Chloride	99%

* Standard assays containing 10 A_{260} units of 50S subunits in 0.01 ml of 0.01 M Tris (pH 7.8) $\pm$ effectors were incubated at 60°C for 1 hr. These were made 0.2% SDS and heat denatured at 72°C for 1 hr. One A_{260} unit was electrophoresed at 3 ma/tube. The 23S RNA peak was cut from the tracings of the gels scanned at 260 nm followed by electrophoresis, and weighed. The percentage of the 23S RNA peak intact following incubation was then calculated from non-incubated controls.

The presence of 1mM mercuric chloride or 0.2% SDS (w/v) in the standard assay mixture (Methods), completely inhibited RNA breakdown (Table 4). As both reagents are known enzyme inhibitors, this result may indicate involvement of a ribonuclease in the degradation of 23S RNA. Mercury inhibition may indicate the participation of sulfhydryl groups in this process. Inhibition could not be achieved with iodoacetamide, and may indicate the inability of this reagent to react with the active side due possibly to the size of the molecule.

RNA Stability in Core Particles: Core particles were prepared in CsCl from 50S ribosomal subunits (Methods). The process removes some of the ribosomal proteins from the subunit. Staehelin (Staehelin et al., 1969) has shown the number of proteins removed is dependent or the concentration of Cesium chloride. The core prepared in 4.2M Cesium chloride in urea-polyacrylamide gels (Plate 5) resembled the γ cores prepared by Staehelin at the same cesium concentration. The core particles were incubated in the standard assay system at 37°C for 1 hr., made 0.2% with SDS, and 1 A_{260} unit electrophoresed following heat denaturation (Methods). Considerable degradation of RNA was observed on incubation (Plate 6) indicating that the nucleolytic acitivity is tightly bound to the 23S RNA. The results argue against the occurrence of nonspecific binding of cytoplasmic RNase to ribosomes during preparation.

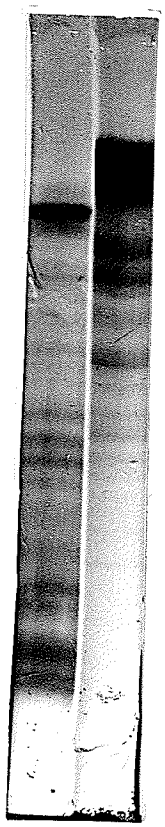
PLATE 5

A B

A. 50 S Ribosomal Proteins

B. Core Proteins

Plate 6



incubated control

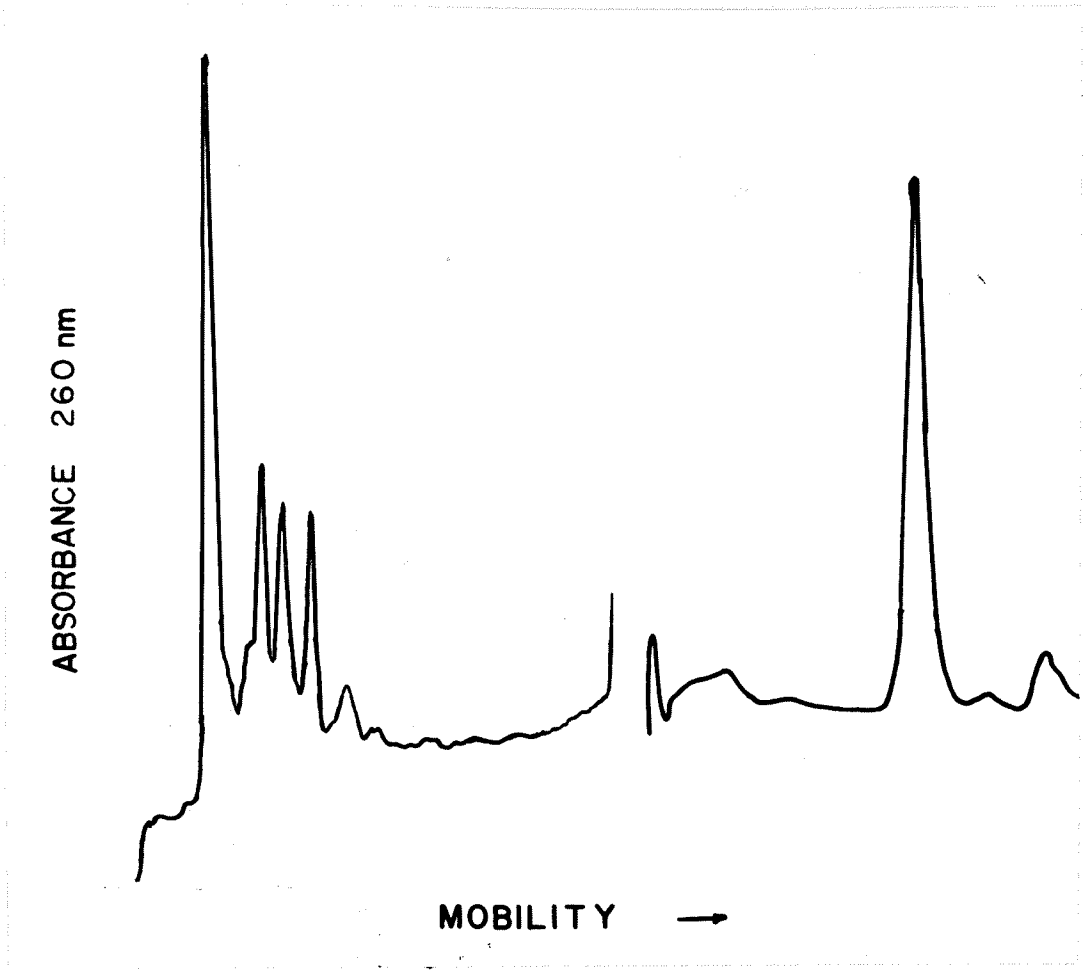
Cesium chloride core particles were isolated and suspended in Tris (pH 7.8) in which they were incubated at 37°C for 1 hr. Samples were made 0.2% with SDS and electrophoresised and scanned (Methods).

Degradation of Isolated 23S RNA: As shown previously (Fig. 9A), both 16 and 23S RNA's appear intact when extracted from 70S ribosomes. However, 23S RNA purified by sucrose gradient centrifugation (Methods) from the same RNA was shown to be degraded. The integrity of rRNA fractionated by this procedure was assayed by electrophoresis in polyacrylamide gels (Methods). The gels scanned at 260 nm (Plate 7) revealed the purified 23S RNA was considerably degraded while the 16S RNA migrated as a single peak. Lowry protein determinations revealed 0.52 µg of protein remained bound per A_{260} unit of 23S RNA, i.e., approximately 1% of the original 44 µg of protein of the 50S subunit remained attached during the isolation procedure. The data shows that degradation is specific for 23S RNA and indicates that the presence of 16 and/or 5S RNA prevents degradation.

Isolated 23S RNA was tested for stability by incubating 5 A_{260} units of RNA in 0.1 ml of 0.01 M Tris (pH 7.8) at temperatures ranging from 4-60°C. The time course study of 23S RNA degradation is shown in Plates 8 and 9. The gels showed the progressive breakdown of 23S RNA took place resulting in the accumulation of material migrating at approximately 5S and the formation of numerous bands between 5S and 23S. The rate of 23S RNA degradation was calculated as previously described, was again found to be temperature-dependent (Fig. 12). The degradation was again independent of monovalent cation concentration. The 16S RNA remained intact when incubated under similar conditions (Fig. 12).

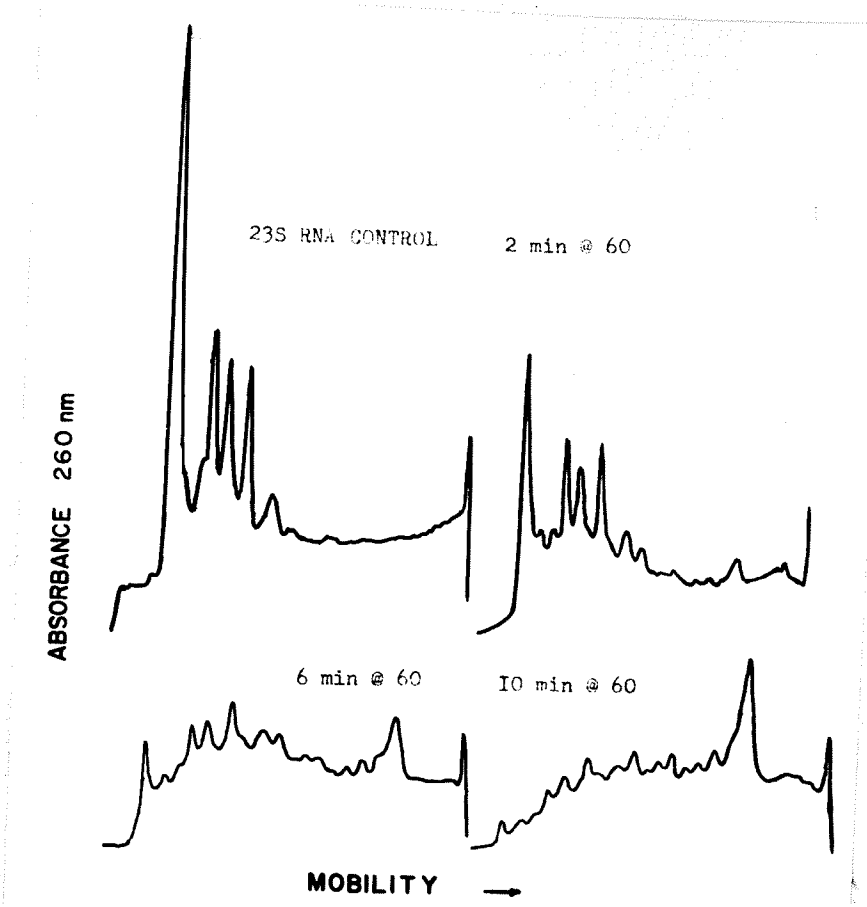
Effectors of 23S RNA Degradation: The addition of phosphate or sulfate salts to the standard assay mixtures, increased the rate of 23S RNA

Plate 7



RNA isolated by urea-lithium chloride extraction from intact Ribosomes was separated into 23S and 16S fractions by gradient centrifugation (Methods). Samples were made 0.2% with SDS and electrophoresed and scanned (Methods).

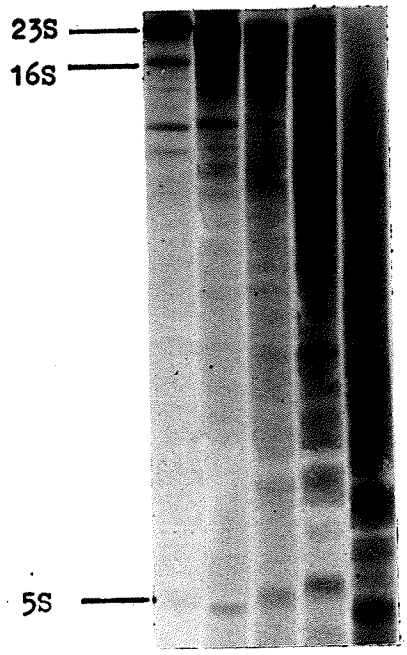
Plate 8



Time course degradation of 23S RNA. Samples were incubated at 60°C in Tris (pH 7.8). They were removed at 2 min. intervals and made 0.2% SDS and electrophoresed and scanned (Methods).

PLATE 9

Time Course of 23S Degradation (60 C)



A. 0 time - not heat denatured
B. 0 time - heat denatured
C. 5 minutes - "
D. 10 " - "
E. 15 " - "

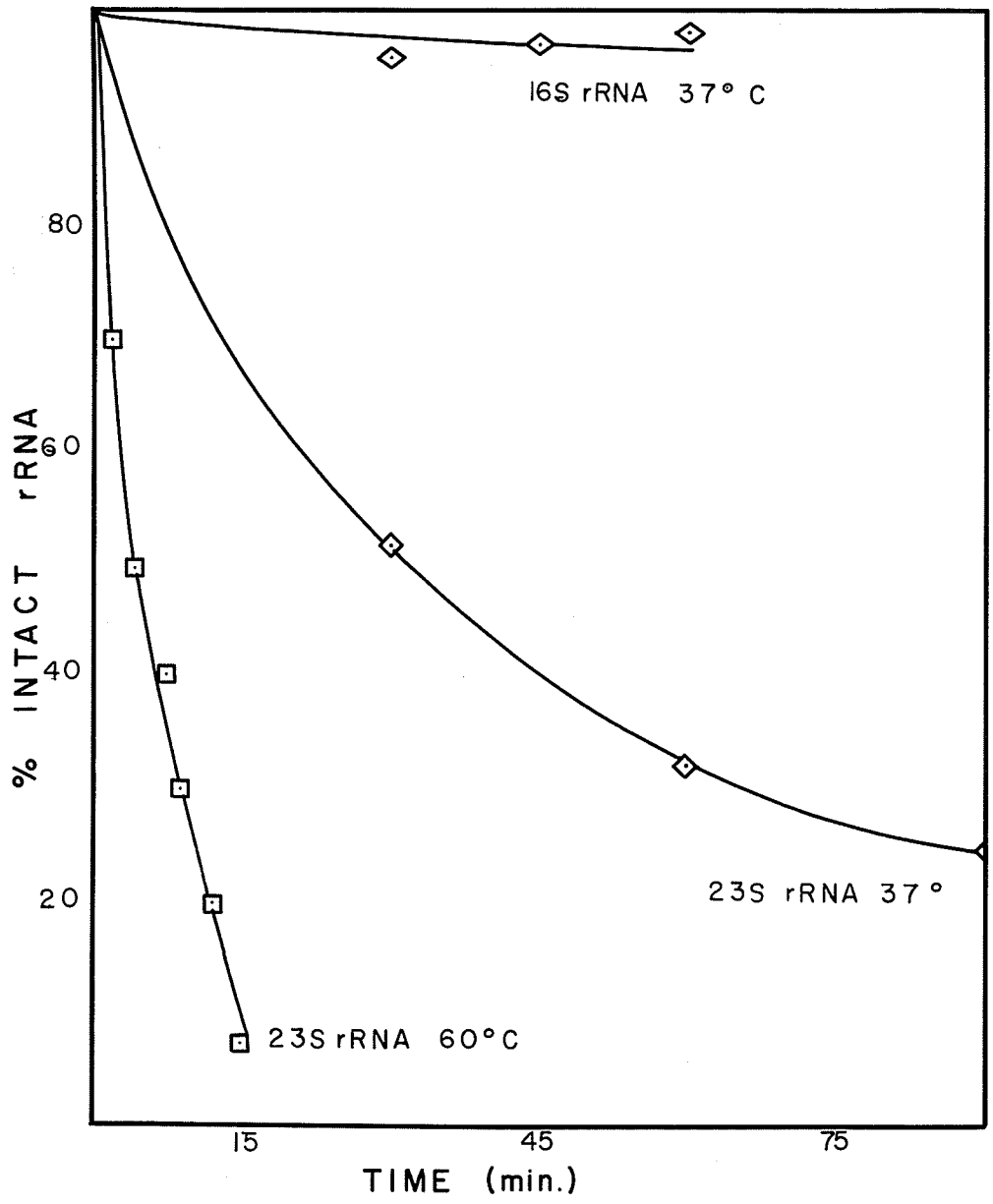

16S rRNA 37° C
23S rRNA 37°
23S rRNA 60°C
% INTACT rRNA
80
60
40
20
15
45
75
TIME (min.)

FIGURE 12. Time course degradation of 23 S and 16 S RNA. Samples of 23 S and 16 S RNA were incubated in Tris buffer at 60 and 37°C. Samples were removed at 2 min. intervals at 60°C and at 15 min intervals at 37°C. These were made 0.2% with SDS and 1 A_{260} units of RNA was electrophoresised and scanned (Methods). The 23 S or 16 S RNA peaks were cut out weighed and the % RNA remaining intact was calculated.

16 S RNA at 37°C

23 S RNA at 37°C

23 S RNA at 60°C

degradation at 37°C equal to that seen at 60°C in 0.01 M Tris (pH 7.8). Optimal rates were reached with divalent anion concentrations ranging from 0.05 M to 0.15 M. Urea at concentrations of 2 to 6 M also increased the rate of RNA degradation and approximately the same degree (Table 5).

Addition of alcohols (methanol, ethanol, isopropanol) to a final 10% (v/v) concentration completely inhibited RNA degradation in standard assay mixtures incubated at 60°C for 1 hr. (Table 5). Total inhibition was also observed with 0.2% SDS and 1m M mercuric chloride.

Isolation of Ribonuclease Activity: As stated previously approximately 1% of the ribosomal protein remained bound to the 23S RNA following extraction in urea-lithium chloride (Methods). This RNA was dissolved in Tris-EDTA buffer (0.01 M Tris-HCl, pH 7.8, 1m M EDTA), dialysed against this buffer and applied to a DEAE column equilibrated in the same buffer. The column was eluted with a 1,200 ml, 0-1.5 M linear KCl gradient in Tris-EDTA. Eight ml fractions were collected and read at 260 and 280 nm. The absorbance profile obtained (Fig. 13) shows only one peak in which the 280/260 ratio is low (approximately 0.2) indicating the presence of RNA free protein. This peak labelled Fraction II was pooled. After lyophilization fraction II was dissolved in Tris-EDTA buffer to a concentration of 240 µg protein/ml. Fraction II protein was electrophoresed in urea-polyacrylamide gels at pH 4.5 (Methods). The sample gel was partitioned vertically by a thin plastic sheet such that one half contained 50 µg of 50S ribosomal protein, while the other half contained the fraction II sample. The "split gels" were run at 3ma/tube and

TABLE 5

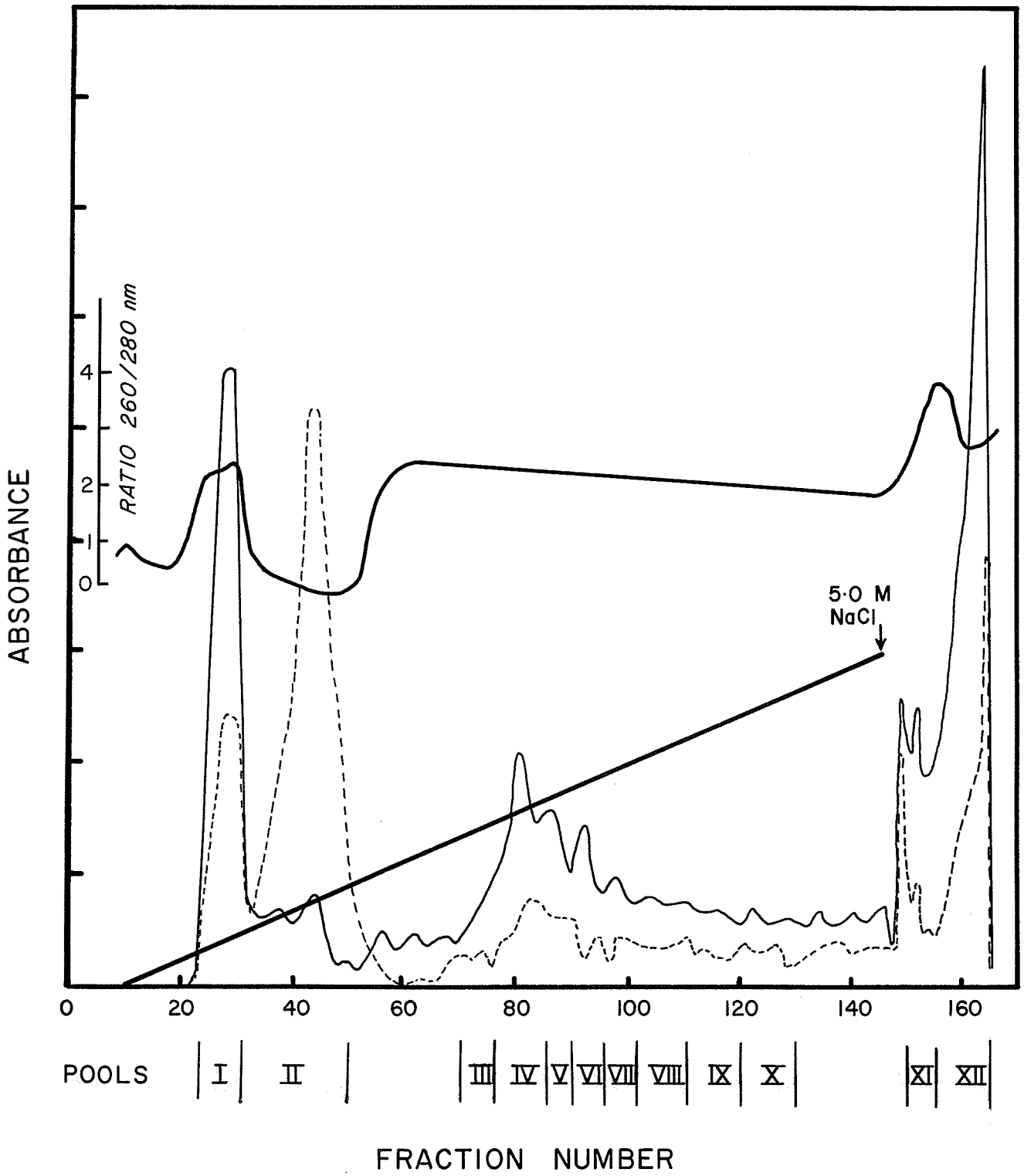
Isolated 23S RNA

Assay Mixture	% 23S RNA Intact	Conditions of Incubation
Standard Assay	36% <5%	36°C for 1 hr. 60°C for 15 min.
+ 10% Ethanol	96%	60°C for 1 hr.
+ 10% Methanol	93%	60°C for 1 hr.
+ 10% Isopropanol	94%	60°C for 1 hr.
+ 0.2% SDS	98%	60°C for 1 hr.
+ 1m M $Hg(Cl)_2$	98%	60°C for 1 hr.
+ 0.05 M K_2HPO_4	<5%	37°C for 15 min.
+ 0.05 M Na_2HPO_4	<5%	37°C for 15 min.
+ 0.05 M K_2SO_4	<5%	37°C for 15 min.
+ 2 - 6 M Urea	<5%	37°C for 15 min.

FIGURE 13. Elution profile of isolated 23 S RNA from a DEAE cellulose column using a linear gradient of KCl. Fractions were read at 260 and 280 nm.

———————— Absorbance at 260

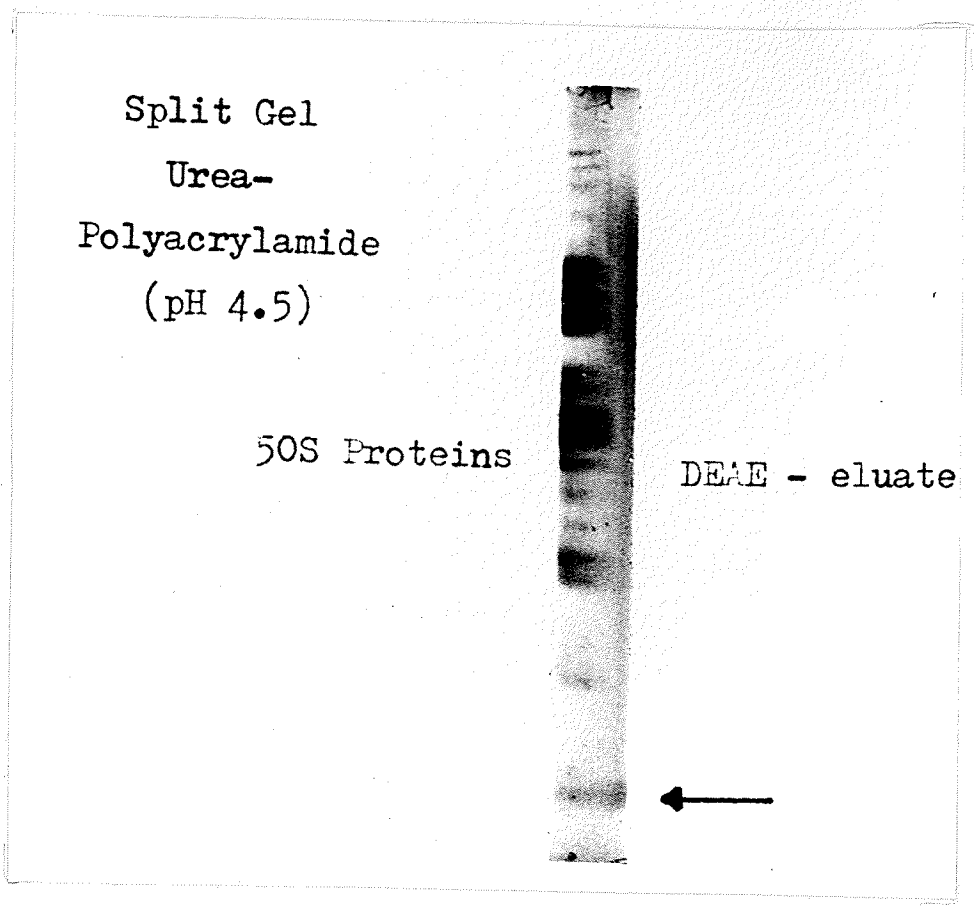
---------- Absorbance at 280


ABSORBANCE
RATIO 260/280 nm
4
2
1
0
5·0 M
NaCl
0
20
40
60
80
100
120
140
160
POOLS
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
FRACTION NUMBER

stained in Coomssie Blue (Methods). As seen in Plate 10 only one protein band was visible in the fraction II sample which co-electrophoresed with one of the 50S ribosomal proteins. Judging from its electrophoretic mobility, the protein was one of the most basic proteins and probably corresponds to protein 32, 33 or 34 of Kaltschmidt and Wittman, (1970). It is hoped that 2 dimensional electrophoresis of the protein will show its exact identity.

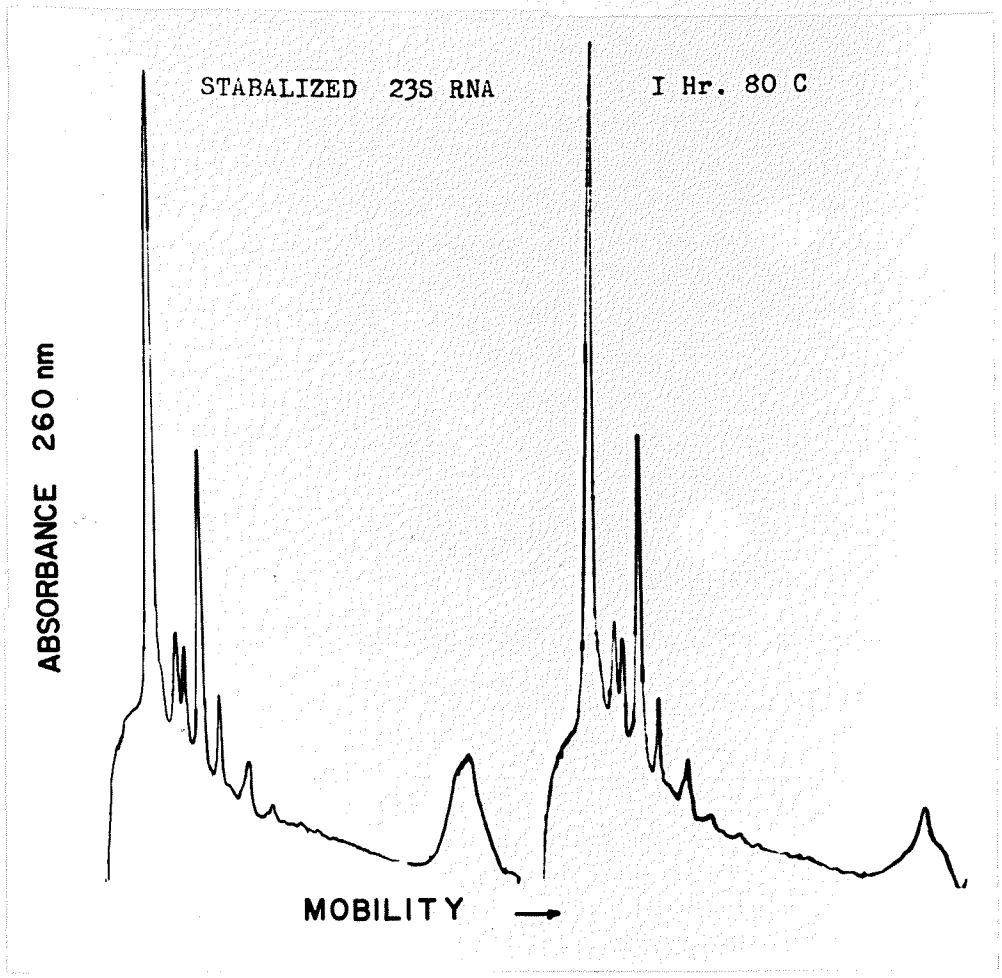
Ribonuclease Activity of Fraction II: Nuclease activity of the fraction II protein was assayed using stable 23S RNA as a substrate. The RNA was made stable by repeated precipitation in alcohol. This RNA remained stable at temperatures up to 80°C for 1 hr. (Plate 11). For this assay 5 A_{260} units of stable 23S RNA in 0.1 ml of 0.01 M Tris (pH 7.8) was incubated with fraction II protein (1.8 µg or 3.6 µg). Equal volumes of Tris-EDTA were added to controls. The assays were incubated at 37°C for 1 hr., then made 0.2% with SDS and heat denatured at 72°C for 1 min. One A_{260} unit of RNA was electrophoresed and scanned at 260 nm (Methods). From Plate 12 it can be seen that control samples remained stable following incubation but that those containing Fraction II protein exhibited marked degradation.

Plate 10



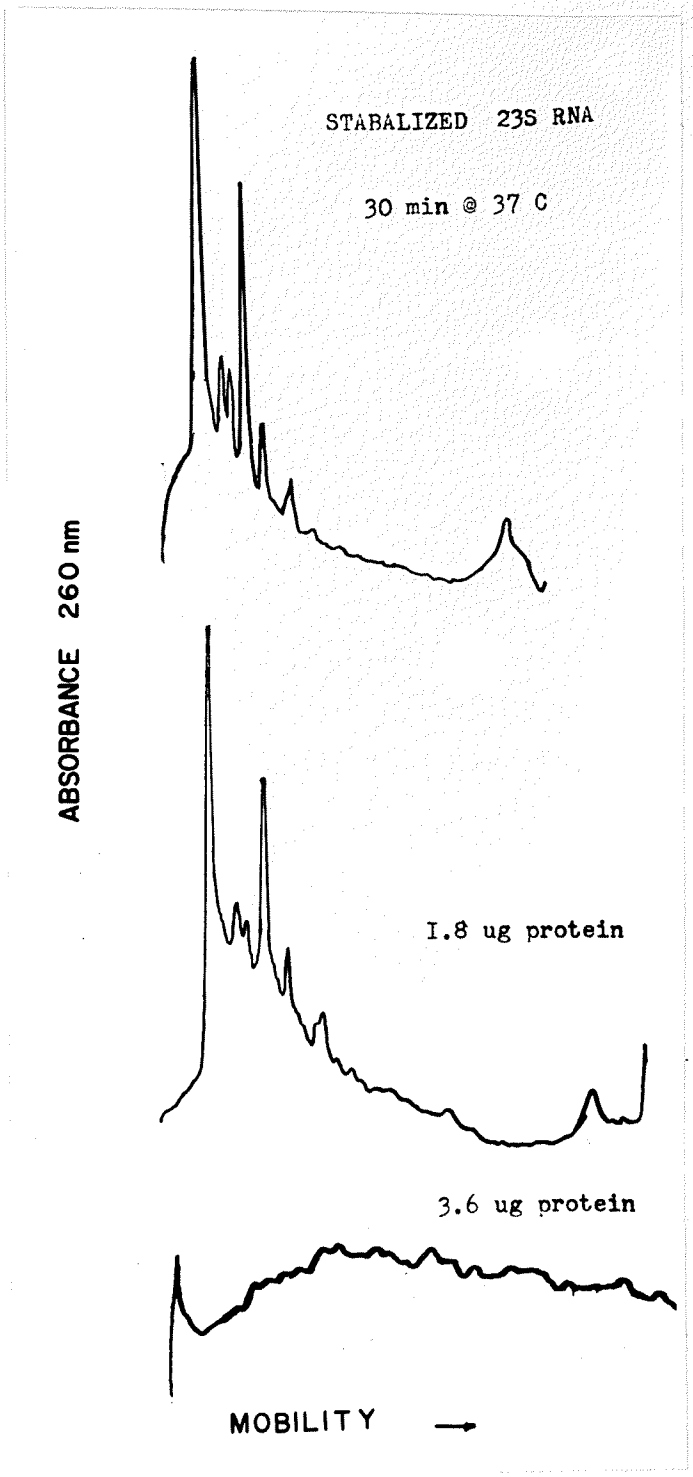
Urea-polyacrylamide gels (Methods) were prepared in such that the upper gel was divided in half by a plastic divider. Urea-lithium chloride extracted 50S proteins were run in one half the gel and the Fraction II DEAE protein on the other.

Plate 11



Purified 23S RNA was subjected to repeated precipitation in TK buffer with 0.6 V ethanol. Following treatment samples were incubated at 80°C in Tris (pH 7.8) buffer for 1 Hr and then made 0.2% with SDS. Samples were then electrophoresed and scanned (Methods).

Plate 12



Stab lized 23S RNA was incubated in Tris - EDTA buffer with 0, 1.8, or 3.6 µg of Fraction II protein at 37°C for 30 min. Samples were made 0.29 SDS and electrophoresed and scanned (Methods).

V. DISCUSSION

The instability of 23S RNA has been a confusing factor in attempts to elucidate the structure and function of the ribosome. Early beliefs that 23S RNA was composed of short RNA fragments or two strands of 16S RNA were set aside with the isolation of ribosomes with intact 23S RNA (see Historical). However, the isolation of intact, stable 23S RNA has still not been achieved. It is therefore evident that during the procedures of isolation, 23S RNA is degraded either by nuclease activity, or through some rupturing of specific weak areas in the RNA polymer.

The spontaneous breakage of RNA strands is a difficult concept to perceive in the light of the wealth of knowledge amassed on the chemical nature of nucleotide bonding. We have been able to demonstrate the loss of instability of isolated 23S RNA upon storage of the RNA as an ethanol precipitate, or by repeated reprecipitation in ethanol. This again is difficult to explain by spontaneous and specific cleavage of the RNA.

There is, however, much evidence pointing to the specific degradation of the 23S RNA by nuclease activity which seems to be associated with the 50S subunit. It was shown that degradation of rRNA followed a specific course of events with the specificity expected of an enzyme generated reaction as seen in Plate 8, and Plate 9. Secondly, although it is often difficult to determine if effectors are acting on the substrate, or enzyme, complete inhibition of RNA degradation can be brought about by 0.2% SDS or 1m M mercuric chloride. Both these reagents inhibit enzyme activity either by disrupting protein conformation, or by binding sulfhydryl groups in active sites of proteins. The stabilization of

RNA as a precipitate in ethanol would indicate the possible loss of the enzymatic activity which was associated with the RNA on isolation (Plate 7 and 8). Finally the degradation of stabilized rRNA by addition of a protein component eluted from urea-lithium chloride extracted RNA on DEAE-cellulose (Plate 14), supports the contention that an RNase is involved.

Characterization of Nuclease Degradation:

Intact Ribosomes: Szer, (1969), working with E. coli Q_{13}, an RNase 1^- strain (Gesteland, 1966), reported that both 23S and 16S RNA were unstable after storage at 4°C and that the rate of degradation was temperature dependent. In E. coli MRE 600 (Cammack and Wade, 1965), rRNA was found to be much more stable. Both 23S and 16S RNA species were intact upon isolation of ribosomes (Plate 2), and remained so after several months of storage at 0°C. The rRNA remained stable following incubation for 3 hrs. at 37°C with preferential degradation of the 23S RNA occurring after 1 hr. at 54°C (Figure 7). The apparent specific loss of 23S RNA may have been due to the formation of 16S RNA as a product of 23S RNA degradation, replacing the last 16S RNA. The addition of monovalent cations to stabilize the secondary structure of ribosomal RNA had no effect on stabilizing RNA incubated at elevated temperatures. Dialysis of ribosomes against dissociation buffer (TKM), resulted in stable RNA (Figure 9) in spite of lowering the Mg^{+2} ion concentration to 1m M.

Approximately 1% of the ribosomal protein remained bound to rRNA following extraction of intact ribosomes in urea-lithium chloride (Methods). The isolated ribosomal RNA was found to be intact upon isolation, and stable in storage. It was, however, more susceptible to nuclease degradation, showing instability at 37°C and rapid breakdown at 45°C (Figure 9).

It appears that both 23S and 16S RNA species are stable in intact ribosomes or RNA isolated from intact ribosomes of E. coli MRE 600. Differences in RNA stability between strains Q_{13} and MRE 600 may be due to the location of the nuclease. As will be shown later, the nuclease of Q_{13} was found in the split protein fraction of a CsCl gradient, while that of MRE 600, in the core. The surface position of the nuclease could lead to easier release, or conformation activation of the enzyme in Q_{13}. The RNA stability was not altered by the addition of monovalent cations to a concentration of 0.15 M or by the reduction of the magnesium ion concentration in dissociating buffer (TKM) to 1mM. This indicates that the RNA placed on sucrose gradients for dissociation was intact prior to the separation of ribosomal subunits. The greater stability of rRNA in intact ribosomes as compared to isolated ribosomal RNA was most likely due to increased conformational stability brought about by the specific protein-nucleic acid, and possibly protein-protein interactions, which confers the conformation of the ribosome. The 16S RNA moiety also seems to have a stabilizing effect, as degradation commences immediately upon separation of 16S RNA from 23S RNA (Plate 3 and 7).

RNA Stability in Isolated Subunits: On isolation of ribosomal subunits it was found that 16S RNA remained intact, although considerable degradation of the 23S species had occurred (Plate 3). As stated previously, the rRNA was intact when applied to gradients, indicating that degradation took place during or immediately following separation of ribosomal subunits. The cause for the immediate activation of nuclease activity is unknown. As isolation was carried out in TKM buffer, in which RNA remained stable previously, it doesn't appear to be an ionic factor. Infante and his

co-workers (Infante and Bairlein, 1971; Infante and Krauss, 1971), have shown that separation of subunits is due to a pressure phenomena rather than a conformational change brought about in low magnesium. Whether pressure dissociation occurs or alters conformation is yet unknown, but dissociation of subunits does seem to trigger RNA breakdown. Gels revealed a characteristic breakdown pattern (Plate 3 and 4) with the majority of the degraded RNA banding near 16S. Incubation of 50S subunits led to a further loss of 23S RNA as judged by the loss of the 23S RNA peak (Plate 4). No exonuclease activity could be found by measuring the supernatants of reaction mixtures following precipitation with acid or alcohol at 260 nm. As with intact ribosomes the degradation of RNA was independent of the monovalent cation concentration.

The rate of RNA degradation was markedly increased with the addition of phosphate or sulfate salts (sodium or potassium), at concentrations between 0.05 M to 0.15 M. The rate of degradation in 50S subunits is much slower than that seen in isolated 23S RNA. Samples of 50S subunits incubated at 37°C in 0.05 M PO_4^{3-}, degraded at the same rate as 23S RNA at 60°C. If the increase in activity was due to unfolding of RNA, it would appear that trivalent anions were more effective in opening RNA to attack than elevated temperature. The same effect was seen when reaction mixtures were made from 2-6 M with urea. EDTA also had the same effect on RNA degradation probably by opening the RNA to attack. Rodgers, (1964) has shown that bound magnesium was not lost upon dialysis against 1m M magnesium or distilled water, but that it was released in 1m M EDTA resulting in the unfolding of rRNA.

Inhibition of RNA degradation was also displayed. Again it was difficult to determine if effectors altered the nuclease activity or the substrate conformation. Alcohols (methanol, ethanol, or isopropanol) in a 10% (v/v) ratio completely inhibited RNA degradation. This may have been due to an increase in the secondary structure of the RNA or protein in the more hydrophobic environment due to the addition of the alcohol. It may also have been due to the complete denaturation of the nuclease although degradation of RNA stored as ethanol precipitation does occur after resuspension of the RNA in Tris buffer. Mercuric chloride and SDS, two strong protein inhibitors also stopped the degradation of ribosomal RNA when reaction mixtures were made 1m M or 0.2% (w/v) respectively (Table 4).

It appears that separation of ribosomal subunits activates nuclease activity, specifically against the 23S RNA of the 50S subunit. This degradation was temperature dependent and proceeded in what appears to be a stepwise manner from 23S $\rightarrow$ 16S $\rightarrow$ $\rightarrow$ 5S and 4S. RNA degradation was stimulated by the addition of trivalent anions, urea, and EDTA. Although all unfold RNA, the exact mechanism of their effect cannot be judged. A greater rate of degradation was seen in the presence of these effectors than was seen with increased temperatures. The nuclease activity was stopped by the addition of alcohols to the reaction mixture, and in the presence of mercuric chloride and SDS.

Ribosomal Core Particle: Szer, (1969) found that rRNA of E. coli Q_{13} was stabilized by the formation of core particles by cesium chloride centrifugation. He was also able to demonstrate ribonuclease activity

associated with the "split" protein fraction, which was active against stabilized ribosomal RNA and MS2 phage RNA. Staehelin et al., (1969) working with strain MRE 600, found that the RNA in the cesium chloride core particle continued to be unstable. Our results were in agreement with Staehelin in that cores (Plate 5) formed by 48 hrs. of centrifugation in 4.2 M CsCl were unstable when incubated at 37°C (Plate 6). As centrifugation of 50S subunits in cesium chloride strips the surface proteins, it would appear that the nuclease is buried deep in the RNA core, indicating that it may be ribosomal in origin. No nuclease activity was seen when the "split" protein fraction was incubated against 16S RNA at 37°C.

Isolated Ribosomal RNA: Isolated ribosomal RNA was prepared by extraction of the protein fraction of intact ribosomes in urea-lithium chloride (Methods). The RNA thus extracted was intact (Figure 9) and remained so on storage at 0°C. In assays the extracted RNA behaved in much the same way as that found in the intact ribosome except for a decrease in stability.

Purified 23S RNA was prepared from the urea-lithium chloride extracted ribosomal RNA by sucrose gradient centrifugation (Methods). The separation of the 23S RNA from the 16S moiety led to the degradation of the former while the latter remained stable. The reason for the 23S RNA stability in the complex is unknown. The addition back of the 16S RNA did not stabilize the 23S RNA during incubation at 28 or 37°C regardless of the magnesium or cation concentrations up to 1m M or 0.15 M respectively.

The isolated 23S RNA was far more unstable than that in the 50S subunit. In the 50S subunit the rate of 23S RNA degradation was much more sensitive to the divalent anion concentration than it was to temperature. Isolated 23S RNA however showed the same maximal rate of degradation at 60°C as that seen at 37°C in 0.05 M Phosphate. This is probably an indication of the structural stability conferred by the ribosomal proteins in the 50S subunit (Figures 11, 12). Although 23S RNA was more unstable at 60°C, the addition of alcohols (10% w/v methanol, or ethanol), SDS (0.2%) or mercuric chloride (1m M) completely stabilized the 23S RNA (Table 5).

Lowry protein determinations revealed that approximately 1% of the 50S ribosomal proteins remained bound to the 23S RNA following extraction in urea-lithium chloride, or in phenol (Nomura et al., 1968). As the extracted RNA was susceptible to autodegradation the RNase must be a tightly bound protein.

As stated previously the nucleolytic activity associated with 23S RNA was far more unstable than in intact ribosomes or subunits and was be lost on prolonged storage as an ethanol precipitate or by many repeated resuspensions in TK buffer.

Isolated Nuclease Activity: As the nucleolytic activity was tightly bound to 23S RNA, this was isolated and used as the source for the enzyme. The strongly acid nature of the RNA was taken advantage of in binding the 23S RNA to a DEAE-cellulose column equilibrated in Tris-EDTA (Results). Elution of the column with a linear 0-1.5 M KCl gradient yielded only one protein rich, RNA free peak (Figure 13) as judged by

the 280/260 absorbance ratios (0.2). The protein was concentrated to 240 µg/ml by lyophilization and resuspension in Tris-EDTA. "Split" protein gels (Methods), revealed only one protein band present in the Fraction II sample which co-electrophoresed with protein 32, 33 or 34 of Wittman et al., (1970). These proteins are the most basic of the ribosomal proteins and may account for the close association with the RNA. Characteristic degradation patterns were seen when stabilized 23S RNA (see above), was incubated with fraction II samples.

The results indicate that the instability of isolated 23S RNA was due to the existence of a nucleolytic enzyme in the 50S subunit. It appears that while the ribosome was stable, the nuclease was held in check to become active only when the conformation of the ribosome was altered. It is not known if the latent activity was due to the nuclease being in an inactive conformation or whether the active site was held away from the RNA skeleton. The degradation of 23S RNA on isolation of 50S subunits would indicate that present procedures for subunit isolation fall short in yielding stable subunits. It also appears that the ribosomal proteins are not required to maintain the ribosome stability in the presence of 16S to 5S RNA, but that they do increase the stability when present.

The reason for the presence of the nuclease can only be speculated upon. It may be a self-destruct mechanism turned on by altering ribosomal conformation for the purpose of ribosomal turnover or as a long term control over protein synthesis. It may play a part in 5S RNA synthesis. Or it may play a role in the maturation of 23S RNA and the eventual assembly of the 50S subunit. The enzyme does warrant further consideration as to the role it plays in the functioning of the ribosome.

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