# A study of the split fraction of 50S ribosomal subunits from Halobacterium cutirubrum.

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Sadiq Hasnain

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Department of Biochemistry
University of Ottawa

## Abstract

The 50S ribosomal subunits from Halobacterium cutirubrum, when exposed to low ionic strength buffer containing 50 mM $K^+$ and 0.3 mM $Mg^{2+}$, dissociate into soluble protein and core particles. Ultracentrifugation of this solution forms a supernatant ($S_1$) containing about 65% of the total 50S subunit protein and all the 5S RNA and pellet ($P_1$) containing the remainder of the 50S subunit protein and all the 23S RNA.

The requirements for the binding of $S_1$ proteins and 5S RNA to 50S subunits has been studied by treating these particles with varying concentrations of $K^+$ and $Mg^{2+}$.

Eighteen proteins have been isolated from the $S_1$ fraction by ion exchange chromatography and gel filtration. The molecular weight of these proteins is in the range of 9,000 to 57,000 daltons. The amino acid composition of the purified $S_1$ proteins reveals a high concentration of aspartic and glutamic acid and a low concentration of lysine in comparison to proteins of E. coli 50S ribosomal subunits.

Evidence is presented indicating that a specific 5S RNA protein complex exists under the conditions employed for the removal of 5S RNA from 50S subunits.

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## 1. GENERAL INTRODUCTION

Ribosomes are sub-microscopic ribonucleoprotein particles which have a universal distribution in animals, plants, microorganisms and even in sub-cellular organelles such as mitochondria and chloroplasts (1). Concurrent with their universal distribution is their universal function in protein biosynthesis.

An insight into the problem of protein biosynthesis was initiated by Casperson (2). He suggested that the abundance of ribonucleic acid (RNA) observed during the rapid growth phase of cells might be involved in protein synthesis. In 1943 Claude (3) obtained from vertebrate cells a fraction, which he termed "microsomal", containing most of the cytoplasmic RNA observed earlier by Casperson. Electron microscopy of this fraction revealed electron dense particles about 200 Å in diameter associated with a network of membranes (endoplasmic reticulum) (4). Later, Zamecnick (5) developed a true cell-free *in vitro* amino acid (aa) incorporation system which allowed Keller *et al.* (6) and Littlefield *et al.* (7) to associate protein

biosynthesis with the microsomal fraction. Peterman and Hamilton (8) separated the ribonucleoprotein particles from the membranes and showed particles having sedimentation coefficients of 20 to 100S. Similar ribonucleoprotein particles were isolated from bacteria (9), yeast (10), and plant cells (11). In 1957, Dintzis (12) suggested the term "ribosome" to distinguish the electron dense particles from the rest of the microsomal fraction.

On the basis of their sedimentation properties, ribosomes have been classified into three groups. In eukaryotic cells such as yeast (10), plants (13), and animals (14), the ribosomes have sedimentation coefficients of 80S. Ribosomes from prokaryotic cells have sedimentation coefficients of 70S (15). Recently 55S ribosomes have been isolated from mammalian mitochondria (16). All ribosomes are made up of two subunits: 60 and 40S particles in the 80S ribosomes, 50 and 30S particles in the 70S ribosomes and 39 and 28S particles in the 55S ribosomes. The dissociation of ribosomes into their respective subunits is governed mainly by the magnesium ion ($Mg^{2+}$) concentration. For example a decrease in the $Mg^{2+}$ concentration from 10mM to 0.1mM results in the complete dissociation of the 70S ribosomes of Escherichia coli (E. coli) into their subunits (15).

T A B L E 1

General Properties of 50S and 30S Subunits of *E. coli* Ribosomes

| Subunit | 50S | 30S |
|---|---|---|
| Molecular weight | $1.80 \times 10^6$ (15) | $0.85 \times 10^6$ (15) |
| No. of r-Protein Molecules | 30-35 (17,18) | 20 (17,18) |
| No. of rRNA Molecules | 2 | 1 |
| Sedimentation Coefficients of rRNA | 23S (19) 5S (20) | 16S (19) |

A further decrease in $Mg^{2+}$ concentration decreases the sedimentation coefficients of the two subunits due to the unfolding of the particles (15). When the $Mg^{2+}$ concentration is increased the unfolded particles again form a tight compact structure and reassociate to form 70S ribosomes.

Ribosomes are complex units consisting of RNA, protein and tightly bound cations. For example, the 70S ribosome of E. coli contains 65% RNA and 35% protein (21, 15). The main divalent cations bound to ribosomes are $Mg^{2+}$ and $Ca^{2+}$ with $K^{+}$ the main monovalent ion (1). Similar to the ions mentioned above, polyamines such as spermidine and putrescine (22, 23) are also found associated with E. coli ribosomes. The important function of the tightly bound cations and polyamines is to stabilize ribosomes by maintaining the ribosomal components in the proper conformation (1). Table 1 summarizes the general properties of 50S and 30S subunits of E. coli ribosomes. The 70S ribosomes contain 3 different molecules of ribosomal RNA (rRNA) and approximately 55 different species of ribosomal proteins (r-proteins).

## 2. PROTEIN SYNTHESIS

During the last decade great strides have been made in our understanding of the events involved in protein biosynthesis. A brief summary of the events in bacterial protein synthesis related to the functions of the 50 and 30S subunits of E. coli ribosomes will be discussed in the following section. Comprehensive reviews on this topic are available (1, 24, 25) hence only those aspects which will elucidate further discussions in this thesis will be mentioned.

The first step in protein biosynthesis involves the dissociation of the 70S ribosomes to form 50 and 30S subunits. The 30S subunit binds with messenger RNA (mRNA) to form an initiating complex, a process requiring GTP (guanosine triphosphate) and several protein factors. An amino acyl transfer RNA (aa-tRNA) binding site(site A), containing the codon AUG, is formed in the complex and this site and codon are specific for the binding of an initiator N-formyl-methionyl-transfer RNA (fmet-$tRNA_F^{met}$). In the binding of the tRNA a direct interaction occurs between the codon of the mRNA and the anticodon of the tRNA.

After the binding of the fMet-tRNA to the 30S initiation complex the 50S ribosomal subunit joins to form a 70S complex. Then, by a process referred to as translocation (a step requiring GTP hydrolysis) the ribosome shifts along the mRNA so that fMet-tRNA is now situated at site P on the 70S ribosome leaving site A open for the binding of the next aa-tRNA. The binding of this aa-tRNA is directed by the codon next to AUG and requires GTP and additional specific protein factors.

The next step in protein synthesis, the formation of a peptide bond, is catalysed by the enzyme peptidyl transferase which is associated with the 50S subunit. The fMet group on fMet-$tRNA_F^{met}$ (site P) is transferred to the aa-tRNA in site A to form the dipeptide fMet-aa-tRNA. The uncharged $tRNA_F^{met}$ is then released from site P and fMet-aa-tRNA moves from site A to site P by a translocation step. Site A is exposed for the binding of the next aa-tRNA directed by the third codon of mRNA. This process repeats itself until the ribosome reaches one of the terminating codons (UAG, UAA or UGA). The termination process requires specific protein factors and results in the release of the polypeptide chain. There is some evidence to suggest that peptidyl transferase mediates the

release of the polypeptide chain. 70S ribosomes are released from mRNA, then dissociate into 30 and 50S subunits and the whole process repeats itself.

## 3. RIBOSOMAL PROTEINS

### A. General Introduction

It is evident from the data on protein biosynthesis that ribosomes are intricately involved in a very complex sequence of reactions as the protein molecule is synthesized. In order to determine the role of the individual components in the ribosome a vast amount of work has been done on the purification of r-proteins and rRNA. Using these purified components Nomura and his group (26) have been able to reconstitute biologically active particles and these studies in turn have proved useful in determining the roles of the individual r-proteins and rRNA (26).

### B. Heterogeneity of r-Proteins

Ribosomal proteins are hydrophobic in nature and require low pH, urea or high ionic strength or a combination of these factors to extract the proteins from the ribosomes. Waller and Harris (27) were the first to extract the ribosomal proteins from E. coli. Their method of extraction

involved suspending of ribosomes in a solution of 66% acetic acid which resulted in the solubilization of r-proteins and precipitation of rRNA. Since then other methods such as treatment of ribosomes with urea-LiCl (28), CsCl (29, 30), 2-chloroethanol (31) or the digestion by ribonucleases (RNase) in the presence of urea (32) have been used.

Waller and Harris (27) showed that E. coli r-proteins gave several distinct basic protein bands on starch gels. This data suggested that r-proteins were heterogeneous. Waller and Harris also demonstrated in a comparison of r-proteins extracted separately from 30 and 50S subunits that the proteins from each subunit were also mutually exclusive. This work, however, was criticized on the basis that the multiplicity of bands observed could be due to aggregation of protein.

Definite proof for the heterogeneity of r-proteins was provided by Kurland's group (34). They isolated and purified 21 r-proteins, with a molecular weight range of $1 \times 10^3$ to $65 \times 10^3$, from 30S subunits of E. coli ribosomes. Amino acid analysis, tryptic peptides and molecular weight analysis of purified proteins show evidence that the multiple bands visualized on polyacrylamide gels represent different

proteins and not artefacts of isolation (34). They have also shown that these multiple protein bands do not arise from the formation of disulphide bridges, enzymatic degradation or contamination from the supernatant. Traut et al. (35) have separated 34 proteins from the larger ribosomal subunit (50S) of E. coli. These proteins have a molecular weight range of $8 \times 10^3$ to $50 \times 10^3$. Immunological data suggests that these proteins are also mutually exclusive. Kaltschmidt et al. (17) and Dzionara et al. (18) using different isolation techniques have also purified E. coli ribosomal proteins. The agreement of their data with that of Kurland et al. (33) and Traut et al. (35) is further evidence that r-protein heterogeneity is not dependent on the isolation procedure.

C. Stoichiometry of r-Proteins

An interesting and important concept about r-proteins has recently been postulated by Kurland et al. (34). They have demonstrated that there exists on ribosomes two stoichiometric classes of r-proteins. In the 30S subunit of E. coli they found that 12 of the proteins exist as 1 copy per ribosome (unit proteins), whereas, the other 8 proteins are represented less than 1 copy per ribosome (fractional proteins). Kurland and co-workers found that

no protein was present more than one copy per ribosome (36, 34). In the 50S subunit only 4 proteins do not occur in unimolar amounts (34). There appears, however, to be one exception to the unit and fractional concept. Möller et al. (37) have determined that two molecules of the most acidic protein (A-protein) of E. coli 50S subunit are found per subunit.

D. Functions of r-Proteins in Protein Synthesis

Having established the heterogeneity of the large number of ribosomal proteins, it is of interest to determine whether these proteins are also functionally heterogeneous. Are all these different proteins involved in individual roles, do they function as groups of proteins or do all the proteins contribute in all the functions? These questions have been answered to a large degree by the reconstitution studies of several groups (38, 36, 39, 26, 40). Initial reconstitution work involved reconstituting particles active in protein synthesis from CsCl solubilized proteins (split proteins) and inactive sedimentable ribonucleoprotein cores. Using this technique Raskas and Staehlin (38) showed that 30S-mRNA binding was a function of the 30S split proteins and not the core particles formed in CsCl equillibrium gradients. Similarly they demonstrated that

T A B L E 2

Functions of the acidic and basic fractions of the CsCl split proteins from E. coli ribosomal subunits(41).

| Function | Ribosomal Subunit | | | |
|---|---|---|---|---|
| | 50S | | 30S | |
| | Acidic | Basic | Acidic | Basic |
| polypeptide synthesis | + + | + - | + - | + + |
| aa-tRNA binding | - | + - | - | - |
| poly-U binding | - | - | - | + + |
| mRNA directed tRNA binding | - | - | + - | + + |

legend:

+ + indispensible
+ - stimulatory but dispensible
\- dispensible

aa-tRNA binding to the 50S subunit was a function of the 50S split proteins. These protein fractions obtained from 30 and 50S subunits were observed to be specific groups of proteins found only in the split fraction and not in the core particles (39, 42, 43). Traub and Nomura (41) further fractionated the CsCl split fractions of E. coli 30 and 50S ribosomal subunits into acidic fractions (those proteins that bind to a DEAE cellulose column at neutral pH) and basic fractions (those proteins that do not bind to a DEAE cellulose column but instead bind to a phosphocellulose column at neutral pH) (Table 2). They observed that the acidic group of proteins from the 50S subunit split proteins was indispensible in polypeptide synthesis but not for aa-tRNA binding activity. On the other hand the basic group of proteins from the 50S split proteins was found to be stimulatory but not indispensible in polypeptide synthesis and aa-tRNA binding. In the 30S subunits, however, the basic split-protein fraction was indispensible in amino acid incorporation and mRNA directed tRNA binding as well as poly-U binding, whereas the acidic fraction was dispensible but stimulatory in both amino acid incorporation and mRNA directed tRNA binding. Recently Möller et al. (44) have isolated and characterized the most acidic protein of E. coli 50S subunits. Antibodies to A-protein strongly inhibited

poly-U induced poly-phenylalanine synthesis (45) suggesting that peptidyl transferase activity may be a function of A-protein on the 50S subunit. However, some controversy still persists in regard to the functions of E. coli 50S subunit acidic split-protein fraction (39) and hence the data of Möller (45) and Traub and Nomura (41) must be interpreted with caution.

The functions of the individual components of the 30S subunits have been examined more fully in the elaborate reconstitution studies of Nomura et al. (26, 46). They purified 20 r-proteins and 16S RNA from E. coli 30S ribosomal subunits and under specific temperature and ionic conditions they reconstituted compact 30S particles which fulfilled all the assigned biological roles of normal 30S subunits. By omitting a certain protein or a group of proteins they examined the functions of all the different components. They observed that some 30S r-proteins were essential for assembly of compact 30S functional particles and others were important for several or all the functions assayed but not required for assembly. Based on their results they established the following 4 classes of r-proteins.

(a) Proteins that are compulsory for maintaining protein synthesis activity.

(b) Proteins that have only a stimulatory effect in protein synthesis.

(c) Proteins that are necessary in the assembly of reconstituted ribosomal subunits.

(d) Other proteins whose role is not known.

## 4. RIBOSOMAL RNA

The bacterial 50S ribosomal subunit contains two molecules of r-RNA; a 23S polynucleotide chain (19) which has a molecular weight of $1.1 \times 10^6$ and a 5S chain (20) with a molecular weight of $4 \times 10^4$, whereas the 30S subunit contains a 16S RNA molecule of molecular weight $5.5 \times 10^5$ (19). Base composition, sequence analysis and functional tests have shown that 16S and 23S RNA's are two uniquely distinct species (47, 49, 50, 36). The principal function so far determined for 16 and 23S RNA's is the specific binding of r-proteins to form ribosomal units (26, 46). Doty et al. (48) have observed that 60-65% of ribosomal RNA structure is in the form of double stranded helix. Conformational stability in the polynucleotide chain arises from the interaction of regions which form single stranded hairpin loops (48). It has also been suggested that r-proteins may attach in the vicinity of these hairpin loops (51).

It is now known that E. coli 5S RNA is also a unique species, different from 23S and 16S RNA's (52, 53). The complete base sequence of E. coli 5S RNA has been determined and it has been shown that this species of rRNA has a high degree of base pairing (52).

Aubert et al. (54) observed that E. coli 5S RNA was released from 50S subunits under specific conditions of low $Mg^{2+}$ concentration. They concluded that binding of 5S RNA to 50S subunits occurs with the participation of a ribosomal protein fraction in the presence of $Mg^{2+}$. Nomura and Erdman (40) have further shown that 5S RNA was necessary for restoring the protein synthesis activity of reconstituted Bacillus stearothermophilus 50S subunits.

## 5. RNA-PROTEIN INTERACTIONS

It is now evident that the great variety of r-proteins are required to restrict the overall conformation of 16 and 23S RNA's in the bacterial ribosome (55). Sypherd et al. (55) have shown by chromatography on methylated albumin Kieselghur that prior to reconstitution of active 30S particles, rRNA must undergo a conformational change. This conformational change is induced by elevated temperature and

in the presence of r-proteins. They also suggested that each specific protein recognizes a particular oligonucleotide sequence and binds to it. The multiple RNA-protein binding sites concept has been further substantiated by Santer and Szekely (56). By treating E. coli ribosomes with nuclease they found many RNase resistent fragments which were tightly bound to protein. This concept of multiple binding sites for r-protein and rRNA was originally postulated from the observation that 30 and 50S subunits could gradually be unfolded without a loss of r-proteins (57, 58). This effect can be achieved by gradually lowering the $Mg^{2+}$ concentration which results in a particle with a lower sedimentation coefficient.

Mizushima and Nomura's (46) reconstitution experiments from 20 r-proteins and 16S RNA of E. coli 30S subunits demonstrated that not all the 30S subunit proteins have an equal binding strength for 16S RNA. They observed that certain proteins were required to bind to the 16S RNA before other proteins could be bound. They concluded that the binding of r-proteins to rRNA is highly cooperative and occurs in a specific order during the assembly process.

Aubert et al. (54) have demonstrated that 5S RNA binds specifically to a site formed by multiple protein

participation and that tRNA cannot compete for this site. They also demonstrated that a reversible modification of 5S RNA prevented binding to the 50S subunit. Their studies have not determined whether 5S-23S interactions are involved in the binding of 5S RNA to the 50S subunit. It is clear, however, that cations play a significant part in maintaining normal RNA-protein interactions.

The actual chemical nature of RNA-protein binding is not yet clear. Since proteins can be washed off ribosomes under conditions of high ionic strength this binding may partly be due to electrostatic interactions. However, the evidence for high specificity of binding indicates that electrostatic attraction is not the only method of interaction and that proteins actually recognize certain base sequences on the RNA (26).

## 6. RIBOSOMAL CONSERVATION

Since the overall mechanism of protein synthesis has been conserved to a large degree in all forms of life it should not be surprising to observe conservation of some of the elements involved in the critical mechanisms in this process. The reconstitution studies of Nomura *et al.* (59) in

which they formed hybrid 30S subunits from the components of distantly related bacterial species have indicated a conservation of regions of 16S RNA. They suggested that these conserved regions may be specific binding sites for individual r-proteins. If this is so it would also suggest that there are regions of conservation of polypeptide sequences in certain protein molecules of ribosomes from different bacterial species. Conservation has also been observed in the protein moiety of ribosomes of different bacterial species. Certain electrophoretic, chromatographic and amino acid composition homologies have been reported for various proteins of both 50 and 30S subunits (32) but unless primary structure is determined these results are very speculative.

It is this interesting phenomenon of conservation which has in part stimulated research on ribosomes of unique bacterial origin such as thermophiles, moderate halophiles and extreme halophiles.

## 7. HALOBACTERIUM CUTIRUBRUM RIBOSOMES

Halobacterium cutirubrum is an extreme halophile requiring almost saturated salt solutions for growth (60). Their viability in their extreme environment is not merely

an exclusion of external ions. They have a selective internal ion concentration of $Na^+$ and $K^+$ greater than 1M and 4M respectively (61). It is evident that all their cellular structure and functions have adapted to these high internal salt concentrations (62). Bayley and Kushner (63) reported a normal ratio of RNA to protein of 1.8:1 and normal sedimentation coefficients compared to other bacterial ribosomes such as E. coli. When ribosomes of H. cutirubrum were suspended in low ionic strength buffers, commonly used for conventional bacterial ribosomes, loss of low molecular weight RNA and considerable r-proteins was observed. Starch gel electrophoresis at pH 8.0 of the split proteins demonstrated that these proteins were all acidic (63) in contrast with proteins of E. coli ribosomes which are predominantly basic. However, those proteins which remained attached to the cores were found to contain both acidic and basic proteins. (64). Bayley suggested that the negatively charged proteins on H. cutirubrum ribosomes are probably neutralized by $K^+$ which then allows normal RNA-protein interactions to occur. He also suggested that $Mg^{2+}$ may be responsible for stabilizing the ribosomal configuration.

Rauser and Bayley (65) demonstrated that the ribosomal population of H. cutirubrum was mainly in the form of poly-

somes with the occurrence of a small proportion of subunits. At concentrations of 10mM $Mg^{2+}$ most of the 70S ribosomes dissociated into 30 and 50S subunits and reversibly formed 70S ribosomes with an increase in the $Mg^{2+}$ concentration.

Visentin et al. (66) have fractionated the 50S subunit of H. cutirubrum ribosomes into three seemingly distinct fractions referred to as $S_1$, $S_2$ and $S_3$. On the basis of electrophoretic and amino acid analysis of the gross fractions it appears that each fraction is a distinct group of proteins. The proteins in the $S_1$ fraction are mainly acidic on the basis that they migrate on pH 8.7 urea-polyacrylamide gels whereas $S_2$ and $S_3$ fractions contain both acidic and basic proteins. The $S_1$ fraction also contains all of the low molecular weight RNA but contains no 23S RNA (66).

The aim of this thesis was to isolate and characterize the $S_1$ proteins of the 50S subunits as part of a program to study the overall structure of H. cutirubrum ribosomes. This involved:

a) an investigation into the selectivity of extraction of the various $S_1$ proteins, to determine whether $S_1$ could be further subfractionated.

b) purification of the individual proteins and the

determination of some of the properties of the purified proteins.

c) an investigation into the importance of certain $S_1$ proteins on 5S RNA binding to the 50S subunit and to undertake preliminary studies on the nature of the 5S RNA-r-protein interaction.

# C H A P T E R  I I

## MATERIALS AND METHODS

### 1. Growth of H. cutirubrum

H. cutirubrum cells were grown in the complex medium of Sehgal and Gibbons (67) (Table III), at 37° in a 120 litre fermenter with vigorous aeration. When the cells reached early log phase growth (absorbance of 0.18 to 0.20 units at 660 nm measured in a Coleman Jr. colorimeter with a 1 cm cell) the cells were quickly cooled and harvested in a Sharpell centrifuge at 4°. All further manipulations were done at 4° unless otherwise indicated. The cells were gently resuspended in the H. cutirubrum wash solution (63) (Table IV) and centrifuged at 6,000 rpm for 20 minutes in a Sorvall GSA rotor.

When $^{14}C$ labelled ribosomes were required H. cutirubrum was grown in the modified synthetic media of Onishi et al.(68) and labelled with $^{14}C$-lysine.

### 2. Preparation of Ribosomes

The washed cells (300 gm) were resuspended in 1500 ml of solution D (Table V) and disrupted by grinding with

alumina (300 gm) in the presence of deoxyribonuclease (5 mg). This suspension was centrifuged at 12,000 x g for 30 min to remove cell debris followed by centrifugation at 60,000 x g for 30 min to give an S-60 supernatant. For preparing 70S ribosomes the S-60 supernatant was centrifuged at 250,000 x g for 4 hours in a Spinco Ti 60 rotor. All preparative ultracentrifugation was done in the Spinco model L2-65B ultracentrifuge. If ribosomal subunits were required the S-60 supernatant was dialysed against solution D′(65) (Table V) to dissociate the 70S ribosomes into 30 and 50S subunits. The dialysed solution was concentrated in an Amicon Diaflo ultrafilter with a PM-10 membrane to a final volume of 50 ml.

3. Separation of Subunits by Zonal Ultracentrifugation

A 1400 ml gradient of 2 to 35% sucrose in solution D′was formed in a Spinco Ti-15 zonal rotor. A 50 ml ribosomal sample containing the subunits (15,000 $A_{260}$ units) was layered on the 2% sucrose of the gradient. After centrifugation at 35,000 rpm for 18 hours, 10 ml fractions were collected by pumping the gradient out of the rotor with a cushion of 60% sucrose. The linearity of the gradient was determined by measuring refractive index. The combined fractions containing the 30 and 50S subunits were dialysed against solution D and concentrated in an Amicon

TABLE III

Growth Medium for H. cutirubrum(67)

| Chemical | Concentration |
|---|---|
| NaCl (rock salt from Windsor Salt Co.) | 4.3 M |
| KCl | $2.7 \times 10^{-2}$ M |
| $MgSO_4$ | $8.0 \times 10^{-2}$ M |
| $FeSO_4$ | $1.8 \times 10^{-4}$ M |
| Casamino acids (Difco) | 7.5 gm/l |
| Yeast extract (Difco) | 10 gm/l |

Final pH 6.2 to 6.5 adjusted with concentrated HCl

TABLE IV

H. cutirubrum Wash Solution (63)

| Chemical | Concentration |
|---|---|
| NaCl (reagent grade) | 4.5 M |
| KCl | $3.0 \times 10^{-2}$ M |
| $MgCl_2$ | 0.1 M |

Final pH 7.6

Diaflo apparatus containing a PM-10 membrane.

The purity of 50S subunits was checked by sucrose gradient centrifugation in a SW 25 rotor. A 30 ml linear gradient of 2 to 35% sucrose in solution D was prepared. A sample (30 $A_{260}$ units) in a volume of 0.1 ml was applied to the top of the gradient. The gradient was centrifuged at 20,000 rpm for 18 hours. After centrifugation the tubes were removed from the rotor buckets and the contents were removed by piercing the bottom of the tube and pumping in a cushion of 60% sucrose. The sample was thus forced out from the top of the tube into a flow cell in a Gilford model 240 spectrophotometer attached to a Texas Servorite recorder.

4. Fractionation of 50S Subunits into $S_1$ and $P_1$

H. cutirubrum 50S ribosomal subunits were dialysed overnight against TMK buffer (Table V). The ribosome suspension was then centrifuged for 24 hours at 165,000 x g in a Spinco Ti-50 rotor to give a supernatant $S_1$ and a pellet $P_1$ (66). The supernatant was decanted immediately after centrifugation. The amount of protein was measured by the method of Lowry et al. (69) using bovine serum albumin as standard.

TABLE V

Buffer Solutions for Ribosome Studies

| Solution | Reagents | | | | | pH |
|---|---|---|---|---|---|---|
| | KCl (M) | MgAc (M) | Tris-Cl (M) | *BME (M) | $NH_4Cl$ (M) | |
| D (75) | 3.4 | 0.1 | 0.01 | ——— | ——— | 7.6 |
| D'(65) | 3.0 | 0.01 | 0.02 | ——— | 0.5 | 8.05 |
| E | 3.4 | 0.01 | 0.01 | 0.006 | ——— | 7.6 |
| F | 3.4 | 0.001 | 0.01 | 0.006 | ——— | 7.6 |
| G | 3.4 | 0.0003 | 0.01 | 0.006 | ——— | 7.6 |
| H | 0.05 | 0.1 | 0.01 | 0.006 | ——— | 7.6 |
| TMK (66) | 0.05 | 0.003 | 0.01 | 0.006 | ——— | 7.6 |

* 2-Mercaptoethanol

5. Acrylamide Gel Electrophoresis

Urea-acrylamide gel electrophoresis on pH 8.7 (7.5% acrylamide) and pH 4.5 (10% acrylamide) gels was done according to the method of Leboy et al. (70).

Molecular weights of proteins were determined by the SDS-acrylamide gel electrophoresis method of Weber and Osborn (71).

Ribosomal RNA was qualitatively analysed by SDS-acrylamide gel electrophoresis according to the method of Loening (72). E. coli Q 13 rRNA was used as a marker. After electrophoresis gels were scanned for rRNA at 260 nm in a Gilford spectrophotometer equipped with a model 2410 linear transporter.

Acrylamide gels were stained for protein according to the procedure of Ortec Inc (73). Briefly, gels were fixed in 12.5% trichloroacetic acid for 30 min at 65°, stained with 0.2% coomassie brilliant blue (0.2% coomassie blue in 45% ethanol and 10% acetic acid w/v/v) for 30 min at 65° and destained in ethanol-acetic (25% ethanol and 10% acetic acid v/v) for two intervals of 20 and 30 min at 65°. The gels were then passed through several changes of

10% acetic acid at 65°. In some experiments the stained gels were scanned at 570 nm in a Gilford spectrophotometer.

6. Urea Purification

When urea solutions with a low absorbance were required the purification method of Hardy et al. (74) was used. Urea solutions (8M) were treated with granular activated charcoal (50 gm/l) and filtered through a millipore filter with a pore size of 0.8 u. Methylamine (MA) (0.012M) was added to scavenge cyanate ions and this solution was diluted to prepare a 6M solution for column chromatography and gel filtration. The $A_{230}$ of this urea solution was usually in the range of 0.1 to 0.25 units.

7. Equilibration of DEAE Cellulose for Column Chromatography

DEAE cellulose (type DE 52; microgranular series from Whatman) was equilibrated with Tris buffer (0.01M; pH 8.0) for 24 hours. Fines were decanted until all the exchanger sedimented within 15 minutes. The ion exchanger was then filtered on Whatman #1 filter paper in a Buchner funnel and washed twice with equal volumes of Tris-urea buffer (6M urea, 0.009M MA, 0.01M Tris-basic, 0.1 mM dithiothreitol; final pH was adjusted to 8.0 by addition of

concentrated HCl). The exchanger was then suspended in Tris-urea buffer in a ratio of 1:2 (v/v) and the preparation was deaerated under vacuum.

8. Fractionation of H. cutirubrum $S_1$ Proteins

Several methods for the isolation of $S_1$ proteins have been attempted including gel filtration, Shandon preparative electrophoresis separation and finally ion exchange column chromatography. The first two techniques were found to be unsuitable.

A preliminary fractionation of a $S_1$ sample labelled with $^{14}C$-lysine was carried out on a DEAE cellulose column with dimensions 0.5 cm x 15 cm. The column was equilibrated with Tris-urea buffer (pH 8.0) and the sample ($7 \times 10^4$ cpm) in TMK buffer was applied to the top of the exchanger. The sample was eluted first with Tris-urea buffer, followed by step-wise addition of Tris-urea buffer containing the following concentrations of KCl: 0.1M, 0.2M, 0.3M, 0.4M, 0.5M, and 3.4M. One ml fractions were collected and 0.2 ml aliquot of each fraction was mixed with 15 ml of Aquasol fluid (Nuclear Chicago). Radioactivity was determined in a Beckman model LS-250 liquid scintillation counter.

For a large scale fractionation of $S_1$ proteins a larger column (1.5 cm x 90 cm) was used. The column was packed with DEAE cellulose in the following manner. The outlet was occluded and the column filled with deaerated Tris-urea buffer. A reservoir attached to the top of the column was filled with the DEAE suspension in Tris-urea buffer and the suspension was slowly stirred in the reservoir to ensure uniform packing. When about 5 cm of DEAE had settled to the bottom of the column the outlet was opened and the packing continued. The packed column was washed with 2 bed volumes of Tris-urea buffer using a Beckman Acuflu pump adjusted to a flow rate of 20 ml/hr.

A sample containing 200 $A_{230}$ units of $S_1$ protein (about 100 mg in 5 ml) was added to the column followed by an equal volume of Tris-urea buffer to wash in the sample. The column was washed with 40 ml of Tris-urea buffer to remove protein that did not adsorb to the DEAE. The sample was then eluted with a 2 litre gradient of 0-0.35 M KCl (in Tris-urea buffer) at a flow rate of 20 ml/hr. Ten ml fractions were collected and the fractions read at 230 and 260 nm in a Gilford spectrophotometer. Gradient linearity was determined by measuring the conductivity of some of the fractions with a Radiometer.

9. Gel Filtration

Sephadex G-100, used in gel filtration experiments, was equilibrated in the following manner. A quantity of Sephadex G-100 (Pharmacia) was added to an equivalent amount of Tris-urea buffer and allowed to sit overnight at 4°. The Sephadex was then placed in a bath at 65° for 4 hours for equilibration after which it was deaerated under vacuum. A column similar to the one used for DEAE chromatography was packed with Sephadex. A pressure head of less than 20 cm resulted in a flow rate of about 5 ml/hr. Proteins were eluted with Tris-urea buffer and either 1 or 2 ml fractions were collected and read in a Gilford spectrophotometer at 230 nm.

10. Amino Acid Analysis of Purified $S_1$ Proteins

Amino acid analysis was done on purified proteins with the assistance of Dr. M. Yaguchi. The protein samples were dialysed against cold distilled water and freeze dried. Each sample (about 50 ug) was dissolved in 0.5 ml of a concentrated (5.7 N) HCl solution and frozen in a heavy wall pyrex glass tube. The tube was constricted, evacuated for 15 min, sealed and then placed at 110° for 20 hr. The hydrolysate was taken rapidly to dryness under vacuum, resuspended in Li-citrate buffer (pH 2.0) and applied to

a cartridge containing C-4 chromo bead resin. The analyser used was a Technicon single column (75 x 0.63 cm) sequential multisample amino acid analyser equipped with an automatic integrator. The column temperature was maintained at 44°. Total analysis time including regeneration and equilibration was 210 min (66).

# C H A P T E R III

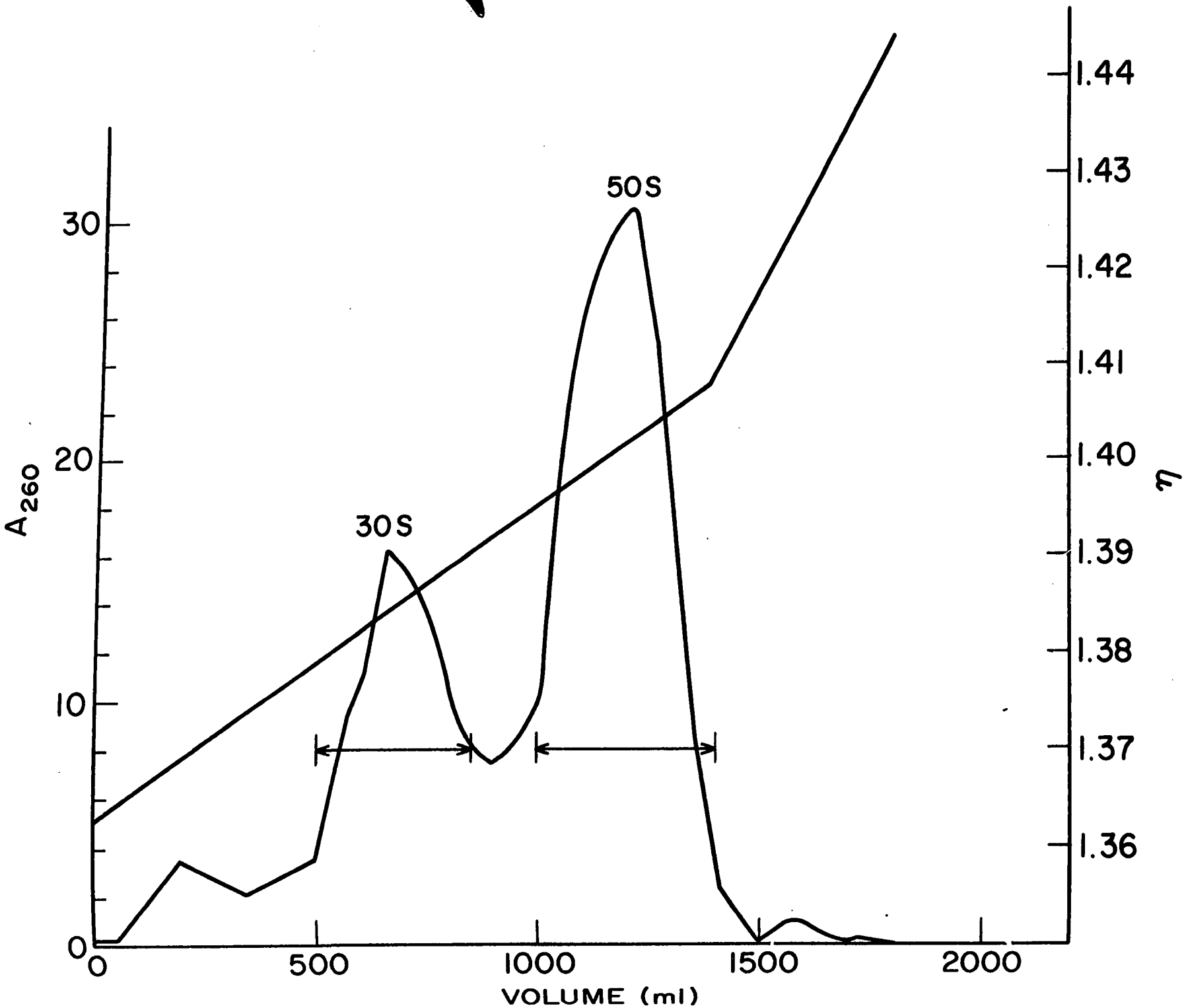
## RESULTS AND DISCUSSION

### 1. Preparation of H. cutirubrum 50 and 30S Ribosomal Subunits

H. cutirubrum 50 and 30S ribosomal subunits were prepared in large quantities by zonal ultracentrifugation on linear gradients of 2-35% sucrose (Fig. 1). About twice as much $A_{260}$ material was recovered with the 50S subunits as with 30S subunits. The fractions marked within the arrows were pooled, dialysed against solution D (Table V) and concentrated.

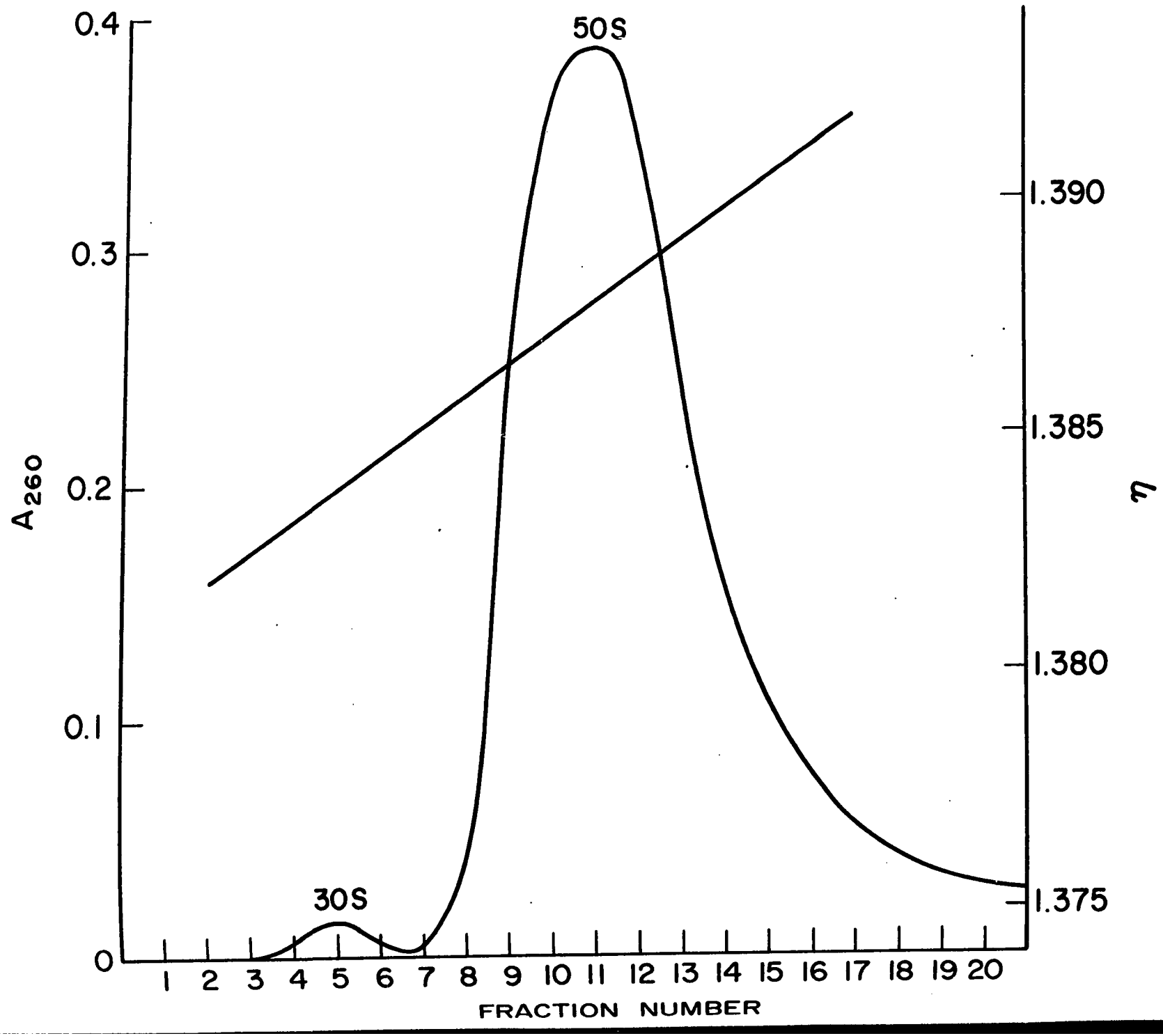
The purity of the 50S subunits was determined on linear sucrose density gradients of 2-35% sucrose (Fig. 2). It has been determined by analytical centrifugation analysis that ribosomes migrating to a point in the gradient with a refractive index of about 1.388 have a sedimentation coefficient of 50S ( 66 ). The contamination by 30S subunits is negligible and hence these subunits could be used without further purification.

The protein to RNA ratio for purified 50S subunits was calculated to be about 1.7 to 1.


50S
30S
30
20
10
0
$A_{260}$
0
500
1000
1500
2000
VOLUME (ml)
1.44
1.43
1.42
1.41
1.40
1.39
1.38
1.37
1.36
η

F I G 1

Isolation of H. cutirubrum 30 and 50S ribosomal subunits on a sucrose density gradient by zonal ultracentrifugation in a Ti-15 rotor. A 50 ml sample (15,000 $A_{260}$ units) was layered on a gradient of 2-35% sucrose in solution D'. Centrifugation was done at 35,000 rpm for 18 hours.

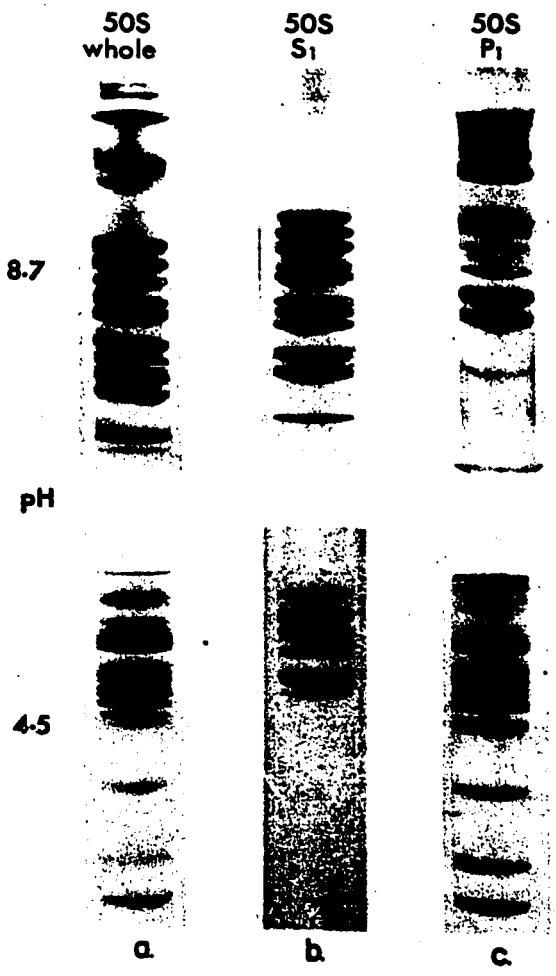

0.4
0.3
0.2
0.1
0
$A_{260}$
50S
30S
1.390
1.385
1.380
1.375
η
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
FRACTION NUMBER

F I G 2

Centrifugation of H. cutirubrum 50S ribosomal subunits (30 $A_{260}$ units in 0.1 ml) on a linear density gradient of 2-35% sucrose in solution D'. Centrifugation was for 18 hours at 20,000 rpm in a SW-25 rotor.

2. Fractionation of 50S Subunits into $S_1$ and $P_1$

The selective dissociation of 50S subunits of H. cutirubrum is particularly interesting since these ribosomes require high ionic strength for stability. In comparison, other bacterial ribosomes, such as E. coli, are unstable in these high salt concentrations. Bayley (63) found that exposing H. cutirubrum ribosomes to low ionic strength buffers removed considerable amounts of r-protein. Visentin et al. (66) have shown that by suspending H. cutirubrum 50S subunits in TMK buffer (Table V) 65% of the ribosomal protein is solubilized and found in the supernatant ($S_1$) after centrifugation at 50,000 rpm for 24 hours in a Spinco Ti-50 rotor. The remaining protein is present in the pellet ($P_1$). On the basis of electrophoretic mobility on pH 8.7 and pH 4.5 urea-acrylamide gels all the $S_1$ proteins are considered to be mainly acidic whereas $P_1$ contains both acidic and basic proteins (Fig. 3). The electrophoretic evidence suggested that $S_1$ proteins may be distinct from $P_1$ proteins (66). The $S_1$ fraction contains 12 electrophoretically distinct protein bands (Fig. 3). However, there may be more than 12 proteins in the $S_1$ fraction since two or more proteins may migrate to the same position in the gel.


50S
whole
50S
S1
50S
P1
8.7
pH
4.5
a.
b.
c.

F I G 3

Urea-acrylamide gel electrophoresis of H. cutirubrum 50S ribosomal subunit proteins on basic (pH 8.7) and acidic (pH 4.5) gels.

(a) Total 50S protein was prepared for electrophoresis by extracting with 2M LiCl - 4M urea for 18 hr. at 4° (76).

(b) $S_1$ supernatant proteins obtained after centrifugation of 50S subunits suspended in TMK buffer. Centrifugation was 50,000 rpm for 24 hours.

(c) $P_1$ proteins were obtained by Li-urea treatment (76) of the pellet formed during the above centrifugation.

3. Subfractionation of $S_1$ Proteins

Since the evidence, as shown in Fig 3, indicated that the $S_1$ fraction was quite complex it was decided to attempt a fractionation which would facilitate an isolation of individual proteins. Several ionic conditions (Table V) were examined for their suitability for a subfractionation of $S_1$ proteins.

Table VI describes the fractionation scheme used in stripping the r-proteins in buffers E, F, G, and H and also summarizes the results of these experiments. Treatment of 50S ribosomes with TMK buffer removed 65% of the total protein and all of the 5S RNA (66) as shown in column 1 of Table VI. A control is present (column 2) to show that the protein solubilized in low ionic strength buffers is not due to the removal of proteins from ribosomes during centrifugation. Columns 2, 3, 4, and 5 (buffers D, E, F, and G) show the effect of gradually decreasing $Mg^{2+}$ concentration from 100 mM to 0.3 mM while maintaining $K^+$ concentration at 3.4 M. As the $Mg^{2+}$ concentration decreases more protein and 5S RNA are solubilized and found in the supernatant $S_1^A$. Columns 3, 4, and 5 show that the critical $Mg^{2+}$ concentration for

T A B L E VI

Subfractionation of $S_1$ proteins.

Extraction Scheme

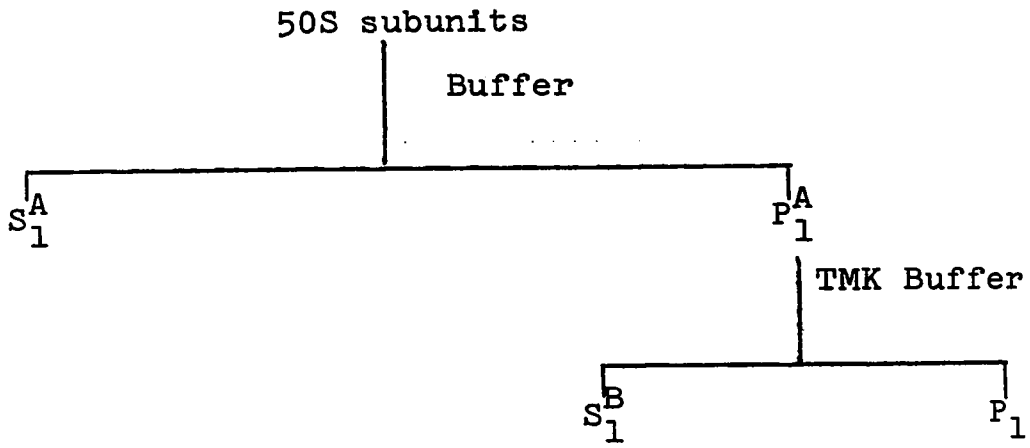



Table VI presents the per cent total protein and per cent total RNA of each of the fractions obtained by treating H. cutirubrum 50S subunits with different ionic strength buffers. In each case 470 $A_{260}$ units of 50S subunits were used. Centrifugation to obtain supernatants $S_1^A$ and $S_1^B$ was done at 50,000 rpm for 24 hours. For a complete description of the buffers see Table V in chapter II.

* % total protein and % total rRNA are a per centage of the total 50S r-protein and rRNA.

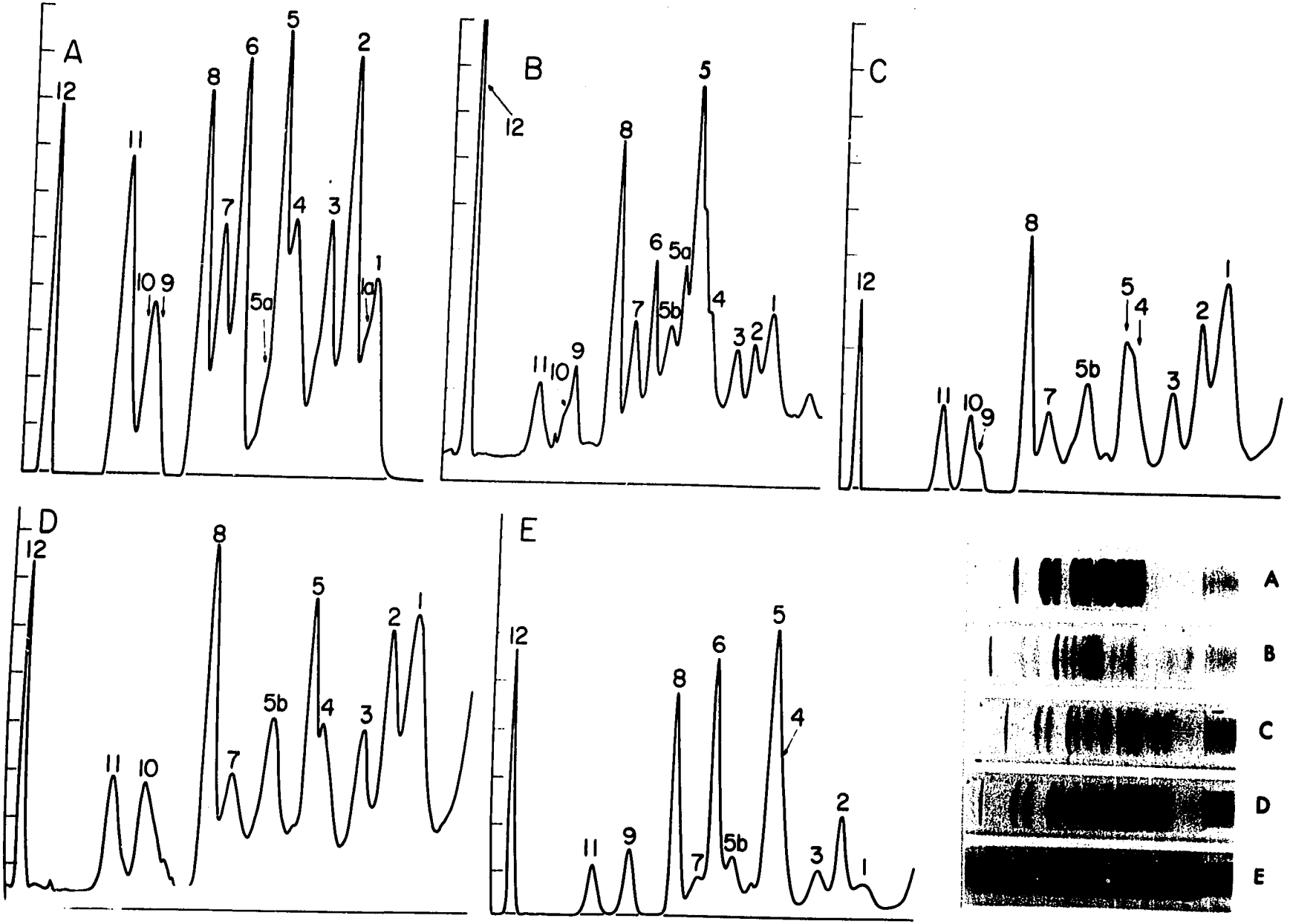
| Column | Buffer | $[K^+]$ M | $[Mg^{2+}]$ mM | $S_1^A$ | | $S_1^B$ | | $S_1^A + S_1^B = S_1$ | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | % Total* Protein | % Total* RNA | % Total* Protein | % Total* RNA | Protein | RNA |
| 1 | TMK | 0.05 | 0.3 | 65 | 4 | — | — | 65 | 4 |
| 2 | D | 3.4 | 100 | 0 | 0 | 64 | 3.4 | 64 | 3.4 |
| 3 | E | 3.4 | 10 | 17 | 0.8 | 41 | 2.8 | 58 | 3.6 |
| 4 | F | 3.4 | 1 | 38 | 1.6 | 24 | 1.7 | 62 | 3.3 |
| 5 | G | 3.4 | 0.3 | 41 | 2.1 | 22 | 1.5 | 63 | 3.6 |
| 6 | H | 0.05 | 100 | 38 | 0.3 | 21 | 3.4 | 59 | 3.7 |

maintaining 50S r-protein binding lies between 10 mM and 1 mM. In buffer G (3.4 M $K^+$ and 0.3 mM $Mg^{2+}$) 41% of the protein and over 50% of the 5S RNA was removed from the 50S subunits. This suggests that $Mg^{2+}$ is involved in binding 5S RNA to the 50S r-proteins and decreasing the $Mg^{2+}$ concentration may either weaken the 5S-r-protein binding or it may cause the dissociation of the r-proteins responsible for binding 5S to the 50S subunits. It has been suggested for E. coli ribosomes that all the proteins probably have specific binding sites on rRNA (26, 46, 51 and 55) and that $Mg^{2+}$ is somehow involved in this binding. The experiment described in Table VI suggests that $Mg^{2+}$ has a similar role in H. cutirubrum ribosomes.

Column 6 (buffer H) shows the effects of decreasing the $K^+$ concentration from 3.4 M to 0.05 M while the $Mg^{2+}$ concentration is kept constant at 100 mM. Suspending 50S ribosomes in buffer H removed 38% of the total protein but removed only 8% of the total 5S RNA. It is evident that a high $K^+$ concentration is required to maintain r-protein binding to 50S subunits, probably as Bayley suggested by neutralizing the negative charges on highly acidic proteins. However, it seems that the $K^+$ concentration does not play a significant part in normal 5S RNA binding to 50S subunits.

In the second part of the experiment shown in Table VI, the pellets $P_1^A$ obtained from the first fractionation in buffers E, F, G, and H were resuspended in TMK buffer. After a high speed centrifugation of these suspensions a supernatant $S_1^B$ and a pellet $P_1$ were obtained (Table VI). Protein and 5S RNA were recovered in all $S_1^B$ supernatants. This may represent the extraction of the remaining $S_1$ proteins from pellets $P_1^A$ since the sum of the per cent total protein in $S_1^A$ and $S_1^B$ is equivalent to the per cent total protein calculated for $S_1$. This assumption is further strengthened by the acrylamide gel profiles of $S_1^A$ and $S_1^B$ proteins (Fig 4 and 5).

The protein moiety in the supernatants $S_1^A$ and $S_1^B$ was analyzed on pH 8.7 urea-acrylamide gels (Fig 4 and 5). The distinct protein bands have been arbitrarily numbered from 1 to 12. Fig 4A represents the protein profile of the $S_1$ proteins and Fig 4B, C, D and E represent a comparison of the gel profiles of the $S_1^A$ supernatants formed in buffers E, F, G and H respectively. It is evident that nearly all the protein bands observed in $S_1$ are also present in the $S_1^A$ supernatants. However, some bands in $S_1^A$ are present in much smaller proportions than the corresponding bands in $S_1$. Although a successful subfractionation of


A
12
11
10
9
8
7
6
5a
5
4
3
2
1a
1
B
12
11
10
9
8
7
6
5a
5b
5
4
3
2
1
C
12
11
10
9
8
7
5b
5
4
3
2
1
D
12
11
10
8
7
5b
5
4
3
2
1
E
12
11
9
8
7
6
5b
5
4
3
2
1
A
B
C
D
E

F I G 4

Urea-acrylamide gels (pH 8.7) and their respective densitometer tracings of the proteins in the $S_1^A$ fractions obtained by treating 50S subunits with various salt concentrations. The centrifugation conditions are as described in Table VI. The scan rate was 2 cm/min and the chart speed was 2 inches/min. The full scale absorbance in all except B was 3 OD units. The full scale absorbance for B was 1.2 OD units.

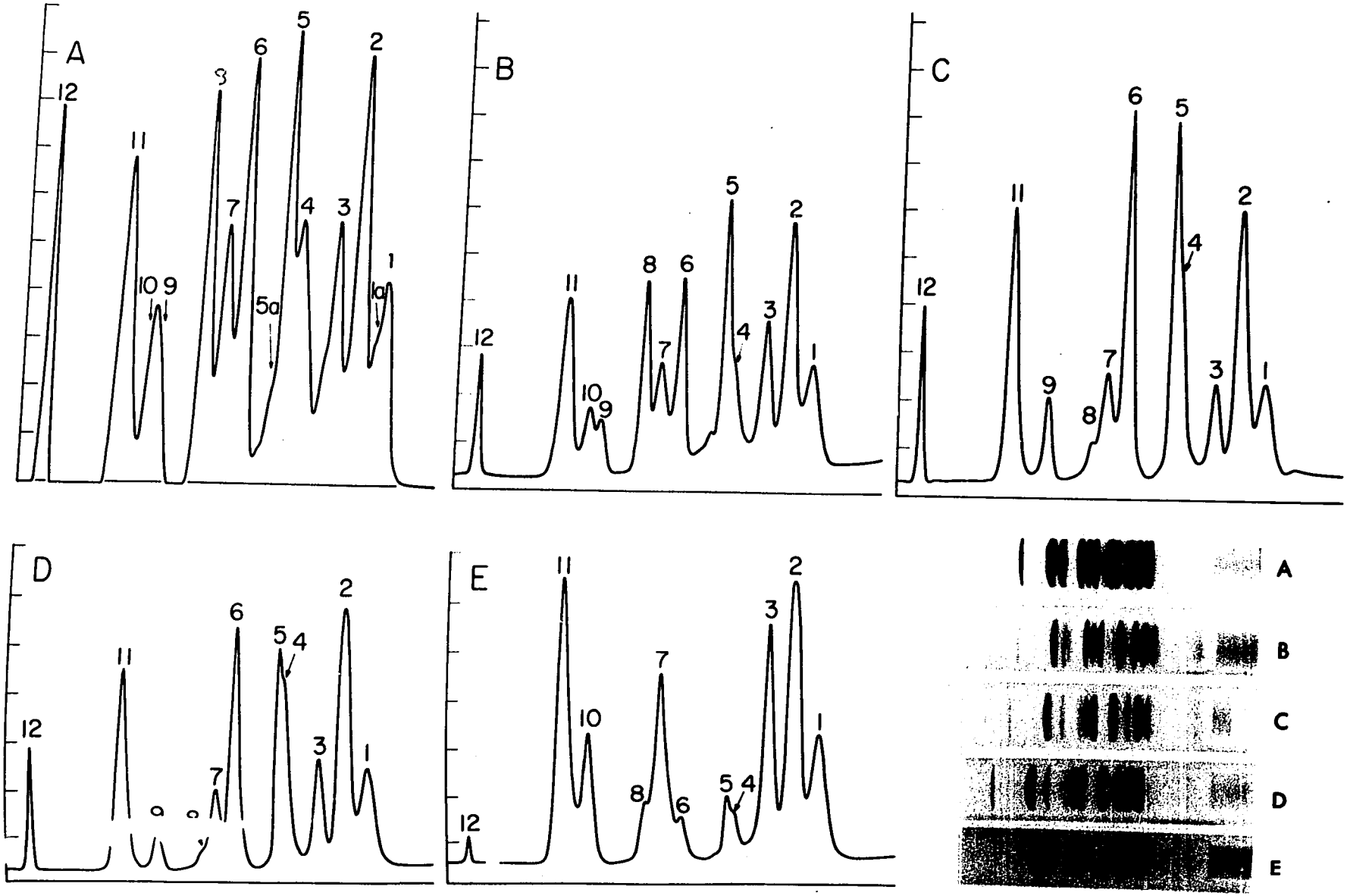
A. $S_1$ supernatant proteins of 50S subunits treated with TMK buffer.

B, C, D, and E represent $S_1^A$ supernatant proteins of 50S subunits treated with buffers E, F, G and H respectively.

$S_1$ proteins was not achieved there is evidence of selective removal or retention of certain proteins.

When the $Mg^{2+}$ concentration is lowered there is an apparent enrichment of protein bands 1, 5, 5b, 8, 10 and 12 in $S_1^A$ (Fig 4D). When $Mg^{2+}$ concentration is kept constant and $K^+$ concentration is decreased there is an enrichment of bands 5, 6, 8, 9 and 12 (Fig 4E). Therefore bands 1, 5b and 10 are removed specifically by a decrease in the $Mg^{2+}$ concentration. This apparent specific requirement for $Mg^{2+}$ to maintain binding to 50S subunits by proteins 1, 5b and 10 has an interesting implication in relation to the extraction of 5S RNA. It was observed in Table VI that a decrease in $K^+$ concentration caused the removal of ribosomal protein without the extraction of 5S RNA. But when $Mg^{2+}$ concentration was lowered both 5S RNA and protein were extracted. If the assumption that 5S RNA binding proteins are extracted with a decrease in $Mg^{2+}$ concentration is correct, then proteins 1, 5b and 10 may be involved in the binding of 5S RNA to 50S subunits.

Fig. 5 demonstrates that the pellets ($P_1^A$) obtained by treatment and centrifugation of 50S subunits in buffers E, F, G and H still contain most of the $S_1$ proteins which


A
12
11
10
9
3
7
6
5a
5
4
3
2
1a
1
B
12
11
10
9
8
7
6
5
4
3
2
1
C
12
11
9
8
7
6
5
4
3
2
1
D
12
11
a
a
7
6
5
4
3
2
1
E
12
11
10
8
7
6
5
4
3
2
1
A
B
C
D
E

# F I G 5

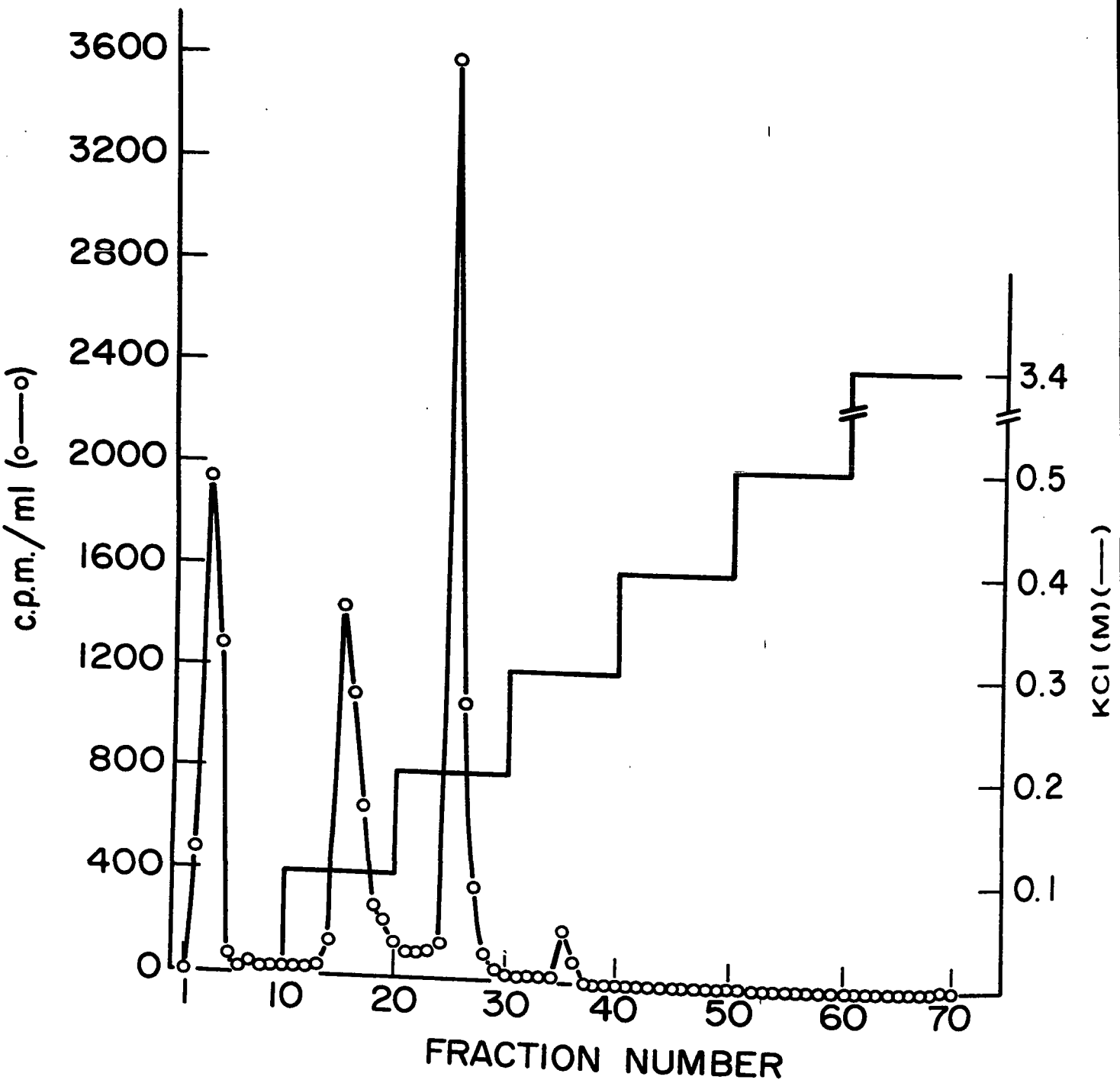
Urea-acrylamide gels (pH 8.7) and their respective densitometric tracings of the proteins in the $S_1^B$ supernatants (except Fig 5A) obtained by centrifuging the $P_1^A$ pellets in TMK buffer. Fig 5A is the same as Fig 4A. The centrifugation and scanning conditions were the same as in Fig 4. Fig 5B, C, D and E represent $S_1^B$ supernatant proteins extracted from the $P_1^A$ pellets and correspond respectively to Figs 4B, C, D and E.

can be extracted with a further treatment with TMK buffer. Although a sharply defined subfractionation of $S_1$ was not achieved a comparison of Fig 4E and 5E and 4D and 5D indicates the selective removal of a few of the proteins.

4. Fractionation of 50S Subunit $S_1$ Proteins

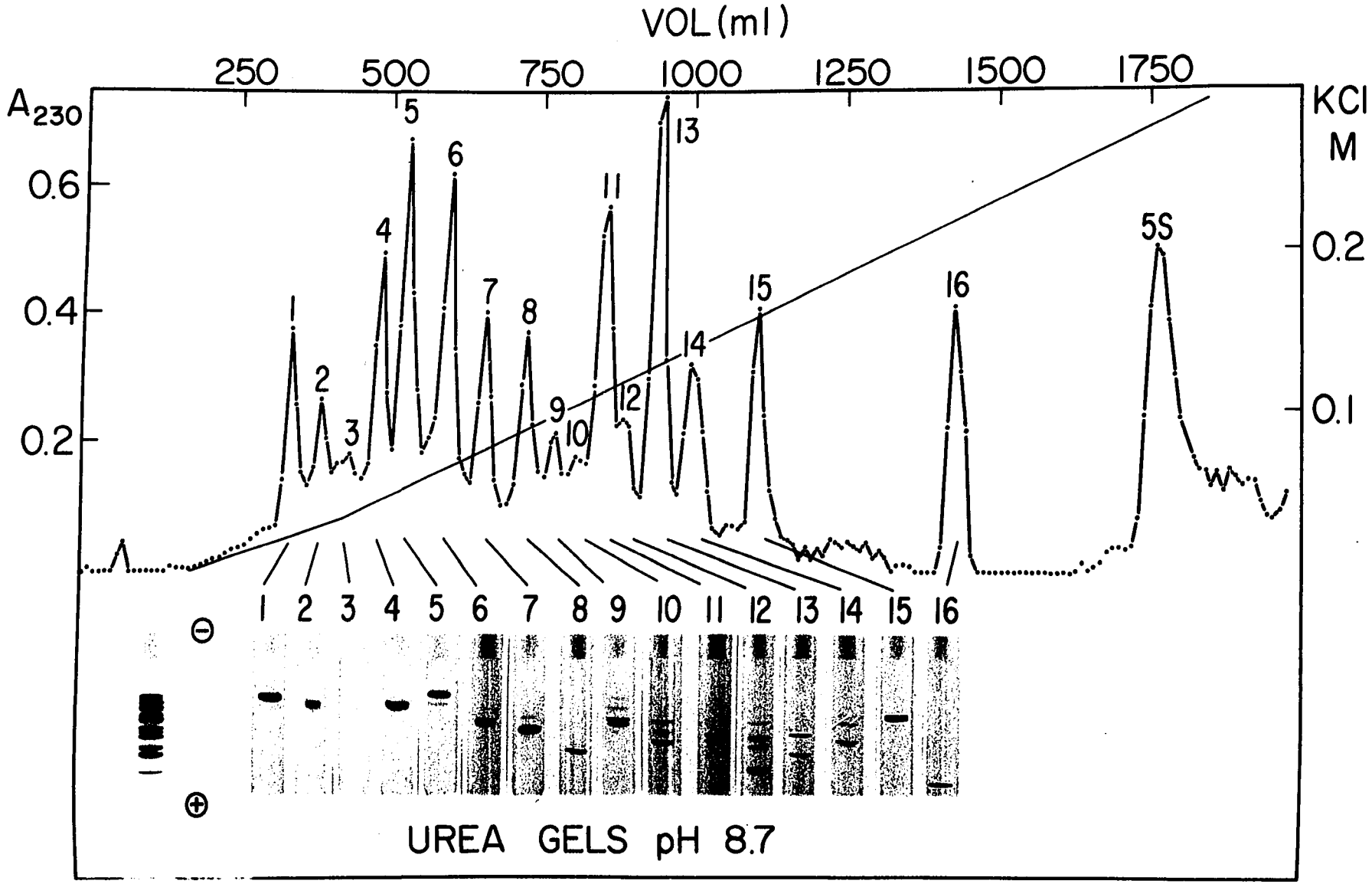
An H. cutirubrum 50S subunit $S_1$ sample labelled with $^{14}C$-lysine was fractionated by a step gradient of KCl on a column of DEAE cellulose to determine the conditions required for a large scale fractionation of individual $S_1$ proteins. Fig. 6 shows the results of the stepwise elution of the labelled $S_1$ proteins with increasing concentrations of KCl from 0 to 3.4 M. Some protein did not bind to the DEAE and was eluted with the starting buffer because the sample was in TMK buffer containing 50 mM KCl. Additional protein was eluted with 0.1, 0.2 and 0.3 M KCl resulting in the complete recovery of all the added $^{14}C$. On the basis of this experiment a linear gradient of 0 to 0.35 M KCl was used in a large scale fractionation of $S_1$ proteins.

Fig. 7 illustrates the results of a DEAE fractionation of $S_1$ proteins and 5S RNA. The sample applied was in Tris-urea buffer. The absorbance of each fraction was determined at 230 and 260 nm but only the former is recorded in Fig. 7. A number was arbitrarily assigned to each peak. The first $A_{230}$ peak was eluted with 0.02 M $K^+$. All the


3600
3200
2800
2400
2000
1600
1200
800
400
0
c.p.m./ml (o—o)
1
10
20
30
40
50
60
70
FRACTION NUMBER
3.4
0.5
0.4
0.3
0.2
0.1
KCl (M) (—)

F I G 6

Step-wise elution of H. cutirubrum $S_1$ proteins (labelled with $^{14}C$-lysine) on a DEAE cellulose column (0.5 x 15 cm). A sample containing a total of 70,000 cpm in a volume of 0.5 ml was applied to the top of the exchanger. The protein was eluted by the step-wise addition of 10 ml of increasing KCl concentration from 0 to 3.4 M. The eluant was in Tris-urea buffer. One ml fractions were collected and the amount of radioactivity in each fraction was determined by adding 0.2 ml aliquots to scintillation fluid.


VOL(ml)
250
500
750
1000
1250
1500
1750
A230
0.6
0.4
0.2
KCl
M
0.2
0.1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
5S
⊖
⊕
UREA GELS pH 8.7

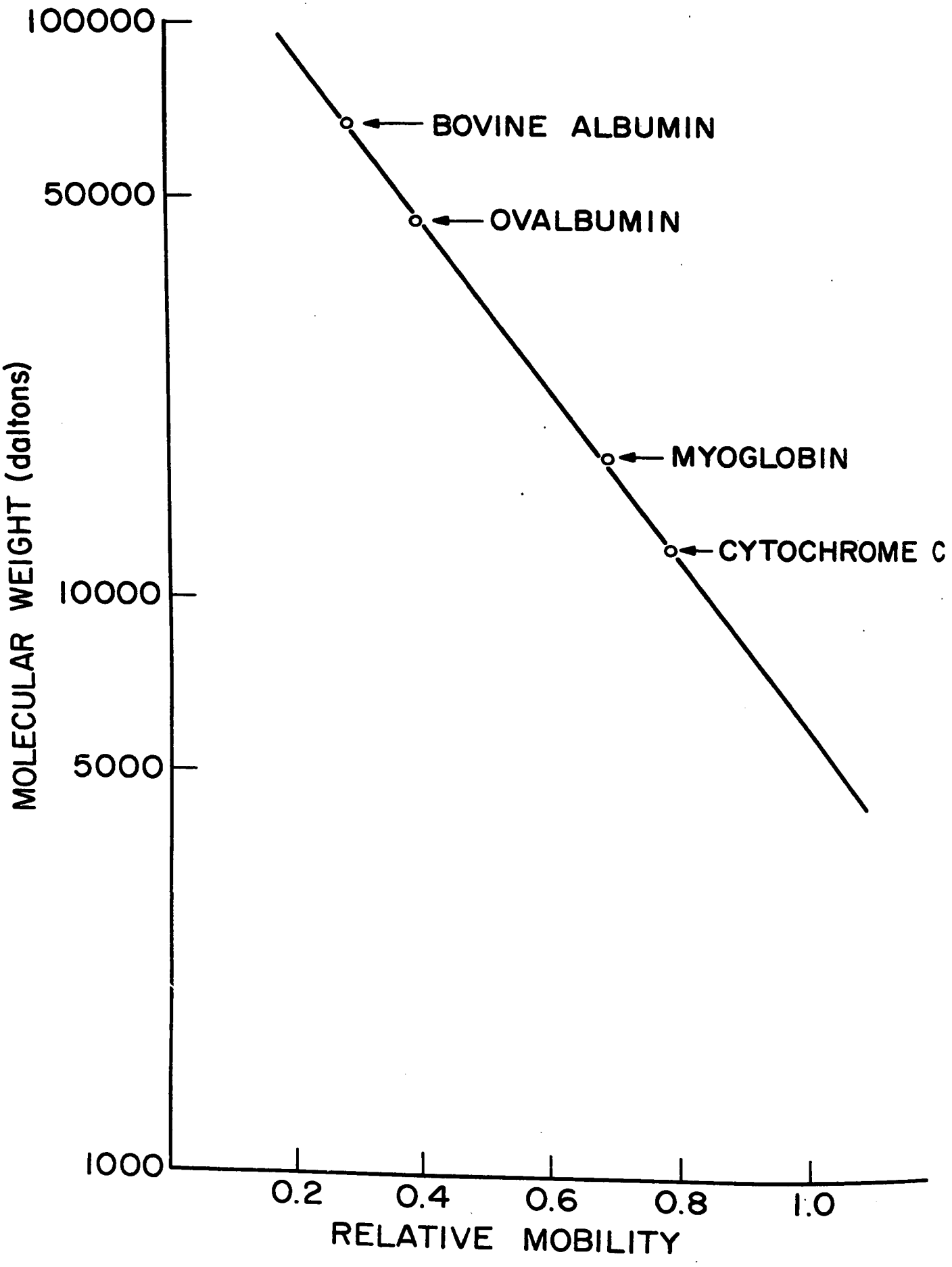
F I G 7

Chromatograph of H. cutirubrum $S_1$ proteins fractionated on a DEAE cellulose column (1.5 cm x 90 cm). A sample containing 200 $A_{230}$ units (about 100 mg of protein) was applied to the surface of the exchanger. The protein was fractionated with a 2 litre gradient of 0 to 0.35 M KCl in Tris-urea buffer at a flow rate of 20 ml per hour. Ten ml fractions were collected. Electrophoretic analysis of proteins on pH 8.7 gels was done on fractions containing the highest $A_{230}$ reading.

other peaks up to number 15 were eluted with 0.155 M $K^+$. The most acidic protein in the $S_1$ fraction, number 16, required a $K^+$ concentration of 0.215 M to elute it from the ion exchanger. The $A_{260}$ value for the last peak marked 5S was twice as high as the $A_{230}$ reading indicating the presence of nucleic acid. This material was later shown to be 5S RNA by acrylamide gel electrophoresis. Urea-acrylamide gels were used to determine the number of proteins present in the various fractions which had the highest $A_{230}$ values. The gel photographs in Fig. 7 illustrate that most of the proteins have been isolated to a high degree of purity. Therefore, the numbers assigned to the $A_{230}$ peaks also represent the number assigned to the proteins. Peaks 9, 10, 13 and 14 all contained two major protein bands and hence required further purification. Urea-acrylamide gels on samples from the 5S peak (without prior concentration of these fractions) indicated that this peak did not contain protein. About 19 distinct major protein bands and some minor indistinct bands are visualized on the basis of urea-acrylamide gels.

5. Molecular Weight Determinations of Isolated $S_1$ Proteins

SDS-acrylamide gels, prepared according to the method of Weber and Osborn (71), were used to determine


100000
50000
10000
5000
1000
MOLECULAR WEIGHT (daltons)
BOVINE ALBUMIN
OVALBUMIN
MYOGLOBIN
CYTOCHROME C
0.2
0.4
0.6
0.8
1.0
RELATIVE MOBILITY

F I G 8

Standard curve for molecular weight analysis of $S_1$ proteins on SDS-acrylamide gels according to the method of Weber and Osborn (71).

# T A B L E  V I

Table of molecular weights of isolated $S_1$ proteins determined by the method of Weber and Osborn (71). The values represent averages of duplicate runs.

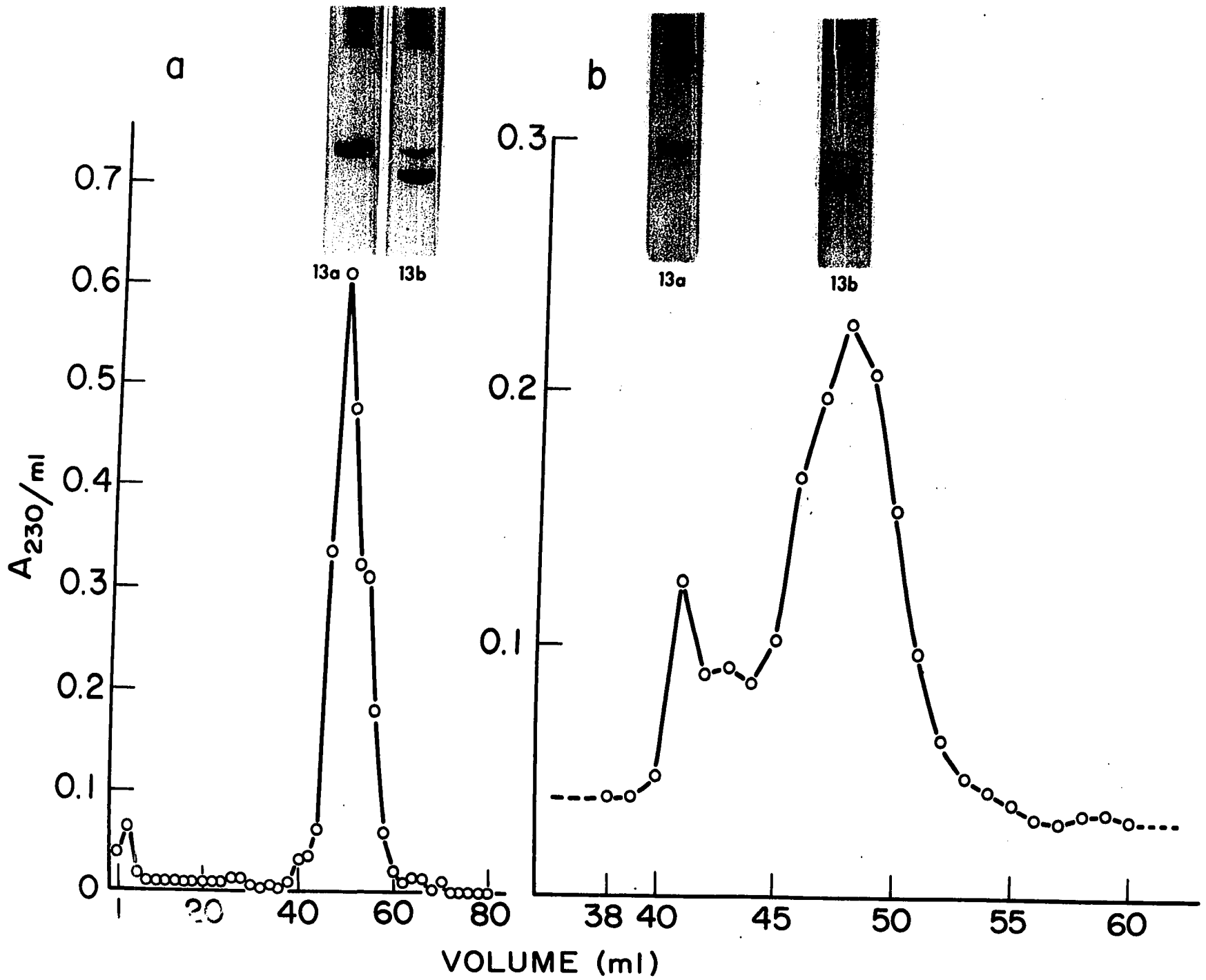
# T A B L E V I

| PROTEIN NUMBER | MOLECULAR WEIGHT (dalt) | PROTEIN NUMBER | MOLECULAR WEIGHT (dalt) |
|---|---|---|---|
| 1 | 13000 | 10a | 45000 |
| 2 | 9000 | 10b | 21000 |
| 4 | 19000 | 11 | 39000 |
| 5 | 39000 | 12 | 23000 |
| 6 | 22000 | 13a | 36000 |
| 7 | 11000 | 13b | 16000 |
| 8 | 9000 | 14 | 30000 |
| 9a | 41000 | 15 | 57000 |
| 9b | 28000 | 16 | 25000 |

the molecular weights (MW) and to confirm the purity of the isolated proteins. Fig. 8 represents the standard curve for MW determinations by the SDS-acrylamide gel electrophoresis method. Bovine serum albumin, ovalbumin, myoglobin and cytochrome C were used as standards. Table VI shows that the MW distribution of $S_1$ proteins is in the range of 9,000 to 57,000 with an average MW of 21,000. Although two major protein bands were visible for peak 14 on urea-acrylamide gels only one band was discerned on SDS-acrylamide gels. This suggested that protein 14 may either be in a monomer and dimer state in urea solutions and that the dimer is converted to a monomer after SDS treatment or that both proteins have the same molecular weight but different charges. The data in Fig. 10 makes the latter possibility unlikely.

6. Gel Filtration of Protein Samples 13 and 14 from the DEAE Fractionation.

Proteins 13a and 13b from peak 13 and proteins 14a and 14b from peak 14 were isolated by gel filtration on Sephadex G-100. Samples 13 and 14 from the DEAE fractionation were concentrated, applied to a Sephadex column and eluted with Tris-urea buffer. Fig. 9a illustrates the


a
b
13a
13b
13a
13b
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.3
0.2
0.1
$A_{230}$/ml
1
20
40
60
80
38
40
45
50
55
60
VOLUME (ml)

F I G 9

Gel filtration on a Sephadex G-100 column (1.5 cm x 90 cm) of proteins from the pooled fractions of peak 13 of the DEAE fractionation. The sample was eluted with Tris-urea buffer and the flow rate was 5 ml/hr.

a. Gel filtration of DEAE sample (peak) 13 collecting 2 ml fractions.

b. Fractions 26-29 from (a) were pooled, concentrated and reapplied to a Sephadex G-100 column. One ml fractions were collected.

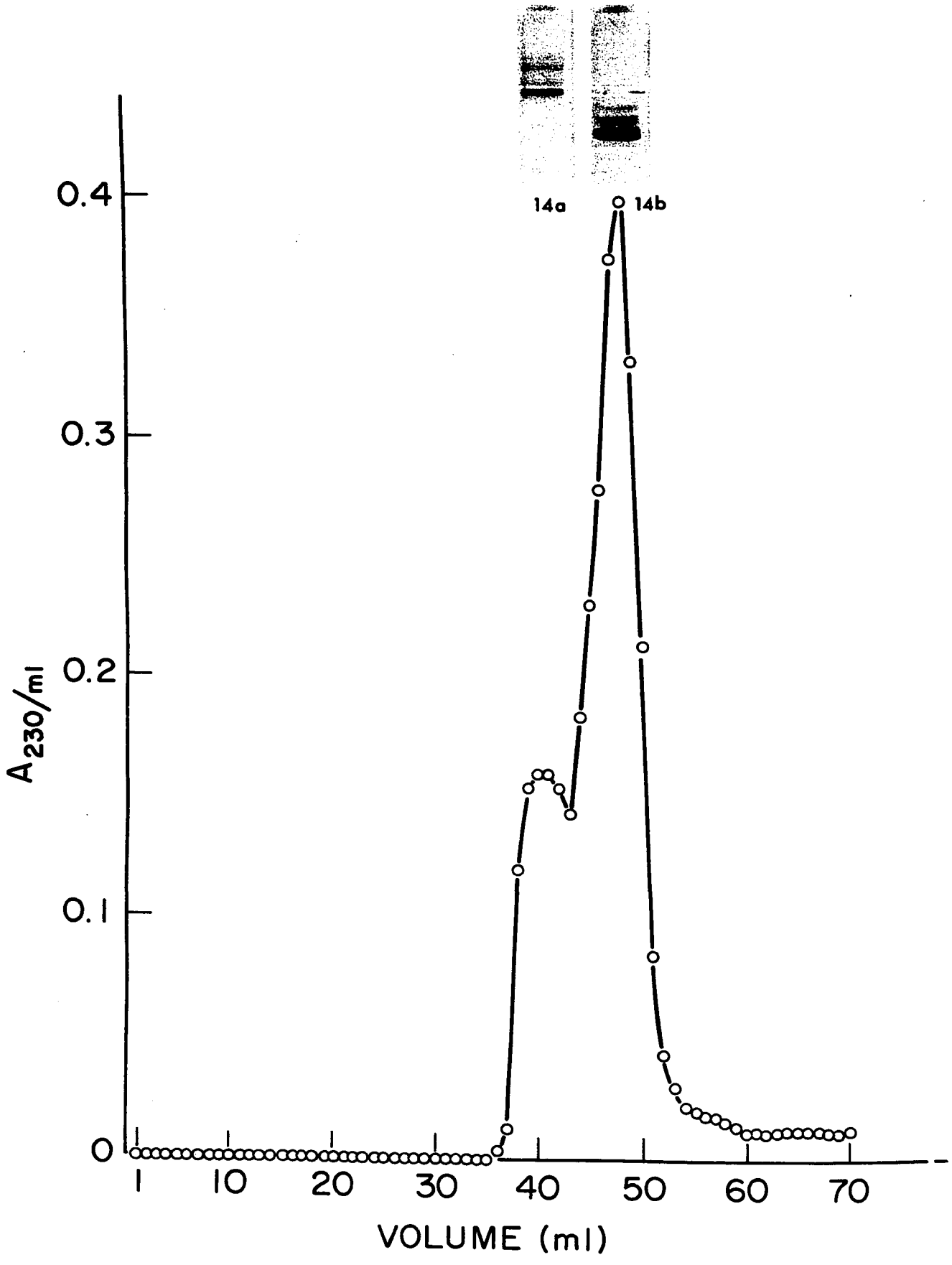
Fractions were analysed on pH 8.7 urea-acrylamide gels.

preliminary fractionation of sample 13 into protein 13a and 13b. Poor separation was probably the result of collecting fractions which were too large in volume. However, on the basis of acrylamide gel electrophoresis protein 13a was considered relatively pure. Fractions 26 to 29 from this gel filtration experiment were pooled, concentrated and fractionated once more on Sephadex G-100 (Fig. 9b). In this experiment one ml fractions were collected instead of 2 ml. Protein 13b was also purified in this manner with only slight contamination as evidenced on urea-acrylamide gels.

Sample 14 was similarily fractionated on Sephadex G-100 and proteins 14a and 14b were considered relatively pure on the basis of acrylamide gel electrophoresis (Fig. 10).

7. Amino Acid Analysis of Proteins Isolated from $S_1$

All the proteins that had been isolated to a high degree of purity were analysed for their amino acid content (Table VIII). The amino acid compositions were expressed as mole per cent. No attempt has been made to correct the data for the destructive hydrolysis of serine, methionine, cystine, threonine, and tyrosine or for the relative slow liberation of leucine, isoleucine, and valine from peptides.


14a
14b
0.4
0.3
0.2
0.1
0
$A_{230}$/ml
1
10
20
30
40
50
60
70
VOLUME (ml)

F I G 10

Gel filtration on a Sephadex G-100 column (as in Fig 9) of sample (peak) 14 from the DEAE fractionation of $S_1$ proteins. One ml fractions were collected with a flow rate of 5 ml per hour. Fractions were analysed on pH 8.7 urea-acrylamide gels.

The tryptophan and amide content of the proteins has not been measured. All the proteins analyzed exhibited a high proportion of the acidic amino acid residues aspartate and glutamate in comparison to E. coli 50S subunit proteins (17). Also in contrast to E. coli 50S subunit proteins H. cutirubrum 50S $S_1$ proteins exhibit a decrease in proportion of the basic amino acid residue lysine. The average ratio of the mole per cents of basic to acidic amino acid residues of the $S_1$ proteins is 0.4 whereas the similar ratio for all the E. coli 50S subunit proteins is 1.1.

Bayley (64) made the interesting suggestion that a transformation of a non-halophile (E. coli type) ribosomal protein to a halophile (H. cutirubrum) ribosomal protein might come about by a misreading of the first letter of the lysine codons AAA and AAG. Reading guanine instead of adenine in both codons (therefore GAA and GAG) would result in the incorporation of glutamic acid instead of lysine in the protein. This might well result in a halophilic character of the protein. Reistad (77) suggested that mutation giving an error in the lysine codons may be instrumental in the evolution of an extremely halophilic cell from a non-halophilic cell, since the same high glutamic acid and low lysine content occur in different proteins as

# T A B L E V I I

Table of amino acid composition of isolated H. cutirubrum 50S $S_1$ proteins. Hydrolysis time was 20 hours at 110° and analysis was done by the accelerated method on a sequential single column Technicon analyser (66). The amino acid content is represented as moles per cent. Data has not been corrected for destructive hydrolysis of serine, methionine, cystine, threonine and tyrosine and for the relatively slow liberation of leucine, isoleucine and valine. The tryptophan and amide content of proteins has not been measured.

| AMINO ACID | PROTEIN NUMBER | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 4 | 5 | 6 | 7 | 8 | 11 |
| asp | 10.9 | 13.1 | 11.4 | 11.5 | 14.3 | 13.9 | 16.5 | 18.2 |
| thr | 6.0 | 6.9 | 5.2 | 6.7 | 7.4 | 5.3 | 6.2 | 7.1 |
| ser | 10.9 | 5.5 | 8.6 | 4.6 | 6.3 | 7.2 | 8.2 | 4.2 |
| glu | 12.1 | 15.4 | 15.9 | 14.3 | 16.3 | 14.1 | 15.7 | 11.7 |
| pro | 4.6 | 5.4 | 3.1 | 5.5 | 5.0 | 6.1 | 2.6 | 4.4 |
| gly | 8.7 | 9.1 | 10.1 | 9.6 | 9.3 | 7.4 | 5.5 | 6.8 |
| ala | 10.8 | 6.7 | 10.7 | 14.3 | 6.1 | 10.1 | 9.8 | 11.2 |
| val | 6.7 | 7.3 | 7.6 | 10.6 | 8.8 | 9.9 | 10.6 | 10.4 |
| cys/2 | + | 0.5 | + | 0 | 0 | + | 0 | 0 |
| met | 0.3 | 1.5 | 1.8 | 0 | 0 | 0.4 | 1.0 | 1.5 |
| ile | 3.4 | 4.4 | 3.9 | 0.9 | 4.2 | 3.4 | 5.9 | 2.8 |
| leu | 7.1 | 6.2 | 5.9 | 7.1 | 6.2 | 5.6 | 3.7 | 8.3 |
| tyr | 0.2 | 1.7 | 1.7 | 0 | 1.0 | 3.2 | 2.0 | 0.8 |
| phe | 1.5 | 4.1 | 2.4 | 1.7 | 4.2 | 3.4 | 2.3 | 2.0 |
| lys | 5.0 | 3.0 | 5.0 | 3.7 | 3.7 | 3.2 | 6.5 | 2.7 |
| his | 2.9 | 2.2 | 0.8 | 1.4 | 1.4 | 0.9 | 0.8 | 1.4 |
| arg | 8.7 | 7.2 | 6.3 | 8.0 | 5.9 | 6.1 | 2.8 | 6.6 |
| *basic / acidic | 0.7 | 0.4 | 0.5 | 0.5 | 0.4 | 0.4 | 0.3 | 0.4 |

*ratio of basic amino acid residues (lys, his and arg) to the acidic amino acid residues (asp and glu).

+represents trace amounts.

| AMINO ACID | PROTEIN NUMBER | | | | | | |
|---|---|---|---|---|---|---|---|
| | 12 | 13a | 13b | 14a | 14b | 15 | 16 |
| asp | 15.4 | 13.5 | 12.1 | 13.6 | 13.3 | 14.4 | 19.4 |
| thr | 7.6 | 7.6 | 4.7 | 6.1 | 6.5 | 6.7 | 1.9 |
| ser | 5.4 | 8.2 | 4.1 | 4.4 | 4.5 | 4.7 | 3.6 |
| glu | 19.3 | 14.6 | 19.0 | 16.8 | 16.4 | 17.1 | 21.1 |
| pro | 2.7 | 4.1 | 2.2 | 6.1 | 6.5 | 5.8 | 2.8 |
| gly | 7.6 | 10.4 | 7.2 | 11.0 | 11.0 | 10.5 | 5.7 |
| ala | 6.4 | 7.7 | 14.4 | 11.4 | 12.1 | 11.8 | 26.8 |
| val | 11.7 | 13.2 | 11.6 | 9.0 | 8.1 | 9.7 | 6.2 |
| cys/2 | 0 | 0 | 0 | 0.8 | 0.4 | 0 | 0 |
| met | 0.8 | 0.5 | 0.7 | + | + | 0 | 0.4 |
| ileu | 0.7 | 3.3 | 4.5 | 4.3 | 4.2 | 3.9 | 2.5 |
| leu | 6.8 | 4.2 | 7.2 | 7.0 | 7.0 | 5.6 | 5.6 |
| tyr | 1.0 | 1.0 | 1.5 | 1.3 | 1.6 | 1.8 | 1.4 |
| phe | 0.6 | 1.7 | 1.6 | 2.5 | 2.8 | 2.5 | 0.2 |
| lys | 2.7 | 3.7 | 6.5 | 3.2 | 3.1 | 3.6 | 0.9 |
| his | 1.5 | 2.8 | 0.3 | 0.8 | 0.6 | + | 0.7 |
| arg | 9.8 | 3.4 | 2.6 | 1.6 | 2.0 | 1.9 | 0.8 |
| basic / acidic | 0.4 | 0.4 | 0.3 | 0.2 | 0.2 | 0.2 | 0.1 |

well as different extreme halophilic organisms.

Protein 16 is a direct contradiction to the suggestion by Reistad (77) that high glutamic and aspartic content in proteins of extreme halophiles is consistent with the finding of low alanine content in the same proteins. It may be that protein 16 has some important functions which made it imperative to retain the high alanine content determined for this protein. Protein 16 is particularly interesting since the three amino acid residues, aspartate, glutamate and alanine constitute 66.5% of its total amino acid composition.

Möller and Widdowson (37) presented evidence for the occurrence of about 3 molecules of A-protein (the most acidic protein of E. coli ribosomes) per 70S ribosomes. The following comparison of E. coli A-protein with H. cutirubrum protein 16 suggests an interesting similarity as follows (also see Table VIII).

1. Both A-protein and protein 16 are the most acidic proteins of their respective ribosomes.
2. A-protein contains 24 mole per cent of alanine (17) and protein 16 contains 26.8 mole per cent of the same amino

# T A B L E VIII

Comparison of amino acid analyses of the most acidic proteins of E. coli and of H. cutirubrum ribosomes. Protein L7 of Kaltschmidt et al. (17) is similar to A-proteins of Möller and Widdowson (37). The amino acid content is represented as moles percent.

| Amino Acid | *E. coli* protein L7 (A-protein) | *H. cutirubrum* protein 16 |
|---|---|---|
| asp | 6.6 | 19.4 |
| thr | 3.0 | 1.9 |
| ser | 5.1 | 3.6 |
| glu | 14.9 | 21.1 |
| pro | 1.7 | 2.8 |
| gly | 6.9 | 5.7 |
| ala | 24.0 | 26.8 |
| val | 13.5 | 6.2 |
| met | 2.6 | 0.4 |
| ile | 3.4 | 2.5 |
| leu | 5.8 | 5.6 |
| tyr | 0.3 | 1.4 |
| phe | 1.5 | 0.2 |
| lys | 10.1 | 0.9 |
| his | 0.0 | 0.7 |
| arg | 0.8 | 0.8 |

acid. This indicates that both proteins probably have a high alpha helix content (78).

3. A-protein is one of the proteins easily removed from the surface of E. coli 50S subunits and is found in the split fraction (37). Similarly, protein 16 is also easily removed from the surface of H. cutirubrum 50S subunits and found in the split fraction.
4. Both A-protein and protein 16 have a molecular weight of about 25,000 (44).

As discussed in the introduction A-protein may have some significant importance in peptidyl transferase activity. It would be interesting to determine whether protein 16 may have a similar function as A-protein.

The major differences in H. cutirubrum $S_1$ proteins with the overall E. coli 50S ribosomal proteins are as follows. In $S_1$ proteins, lysine is relatively low, glutamic and aspartic acid are high and methionine is low and sometimes not present. The rest of the amino acid residues are very similar in their occurrence in H. cutirubrum $S_1$ proteins and E. coli r-proteins.

Protein 14a and 14b which were isolated by gel filtration are very similar in their amino acid composition

and probably are the respective dimer and monomer of the same protein.

It was mentioned earlier that on the basis of acrylamide gel electrophoresis the $S_1$ fraction proteins may be distinct from $P_1$ proteins. In our laboratory the $P_1$ proteins have also been purified (66) and their amino acid compositions are distinct from the amino acid compositions of the $S_1$ proteins. Therefore this evidence validates the original assumption.

8. 5S RNA

The 5S RNA isolated from the DEAE fractionation of $S_1$ was pooled, concentrated and dialysed against Tris-succinate buffer (0.01 Tris; 0.003M succinic acid; pH 8.0) to remove urea and salt. Then the RNA was precipitated with two volumes of absolute ethanol and left in the deep freeze overnight. The sample was centrifuged and resuspended in 0.5 ml of Tris-phosphate SDS buffer (72). The sample was applied to 3% SDS acrylamide gels (72) for RNA analysis and *E. coli* $Q_{13}$ ribosomal RNA and tRNA were used as markers. Fig. 11A illustrates the mobility of $Q_{13}$ rRNA and 4S RNA and Fig. 11B shows H. cutirubrum rRNA migrating at the 5S position and near the 4S position. The smaller peak could possibly be a breakdown of 5S, however, Aubert *et al.* (54) reported that when *E. coli* 5S RNA was exposed to urea solution and a high pH 5S RNA migrated as two distinct entities. They suggested that under these conditions two possible conformations of 5S rRNA were possible.

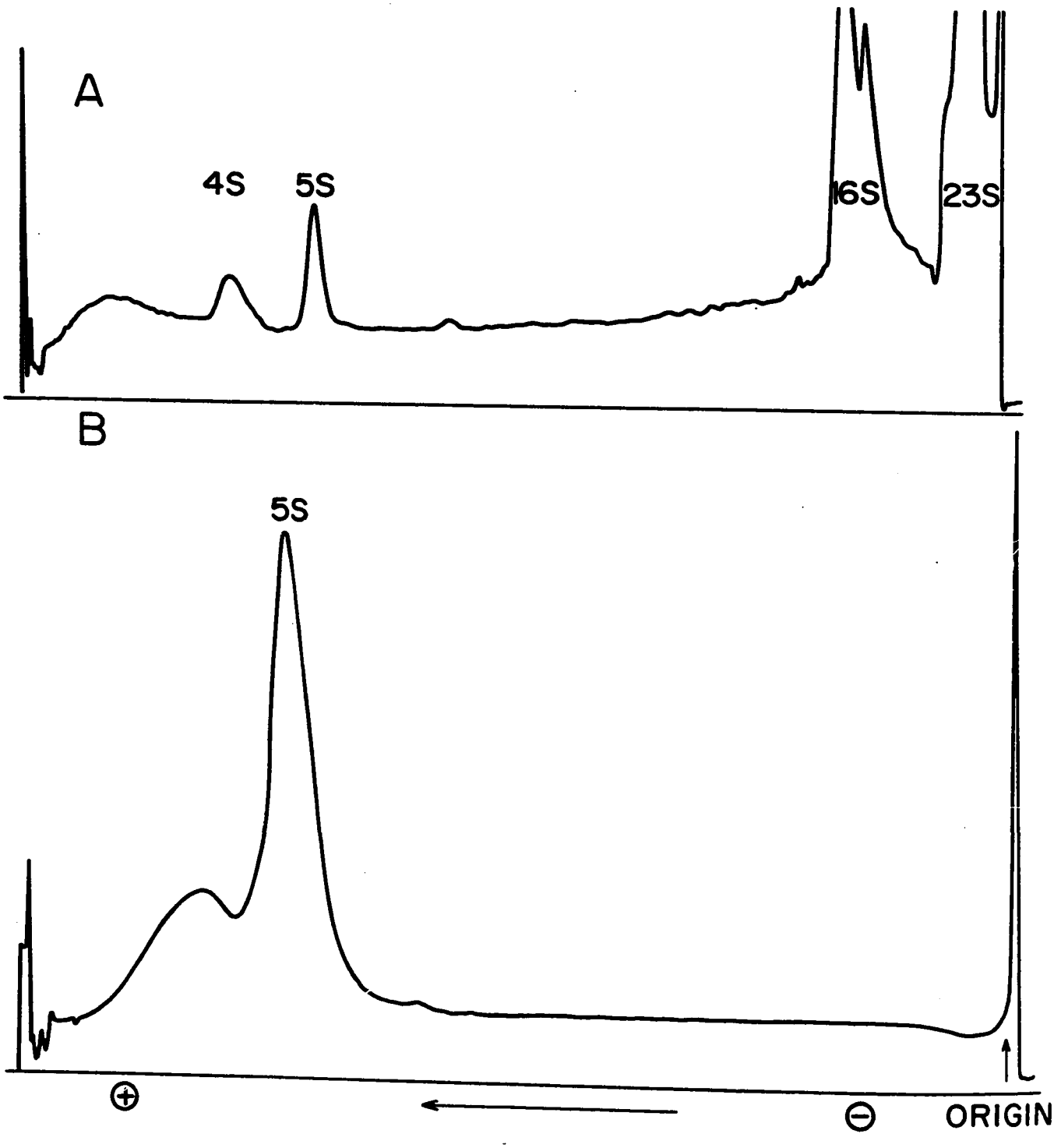
9. 5S rRNA-r-Protein Complex

To examine whether a 5S-protein complex still existed

after the DEAE fractionation of $S_1$ the SDS-acrylamide gel described in Fig. 11 was stained with coomassie blue for protein. Fig. 12A shows that a small peak appears not far from the origin of the gel after staining. This protein may have been bound to 5S RNA and released with SDS treatment, thus migrating as a separate entity from 5S RNA in the SDS-acrylamide gel shown in Fig. 12. There is a possibility that this protein band could be aggregate material which is eluted with the same K concentration as 5S RNA. This is unlikely, however, since the $S_1$ sample was in 6M urea with DTT at pH 8.0; conditions that minimize aggregation. It is also just as unlikely that aggregate material would migrate as a distinct band in SDS-acrylamide gels.

On page 49 it was mentioned that 5S RNA fractionated on the DEAE cellulose column (Fig 7) did not contain protein as determined by pH 8.7 urea-acrylamide gels. It is now apparent that the negative result was due to the fact that the sample applied to the gel was too dilute.

A CsCl equillibrium density gradient was formed to help confirm whether any specific 5S protein complex can be isolated from $S_1$. Fig. 13 illustrates the results of the CsCl density gradient centrifugation of the $S_1$ fraction. As expected the 5S RNA sedimented to the bottom of the tube giving an increased $A_{260}$ absorption in this region. There was also a slight increase in the $A_{260}$ at the top of the tube where all the $S_1$ proteins remained. The $A_{230}/A_{260}$ (not shown) was much higher at the top of the tube (indica-

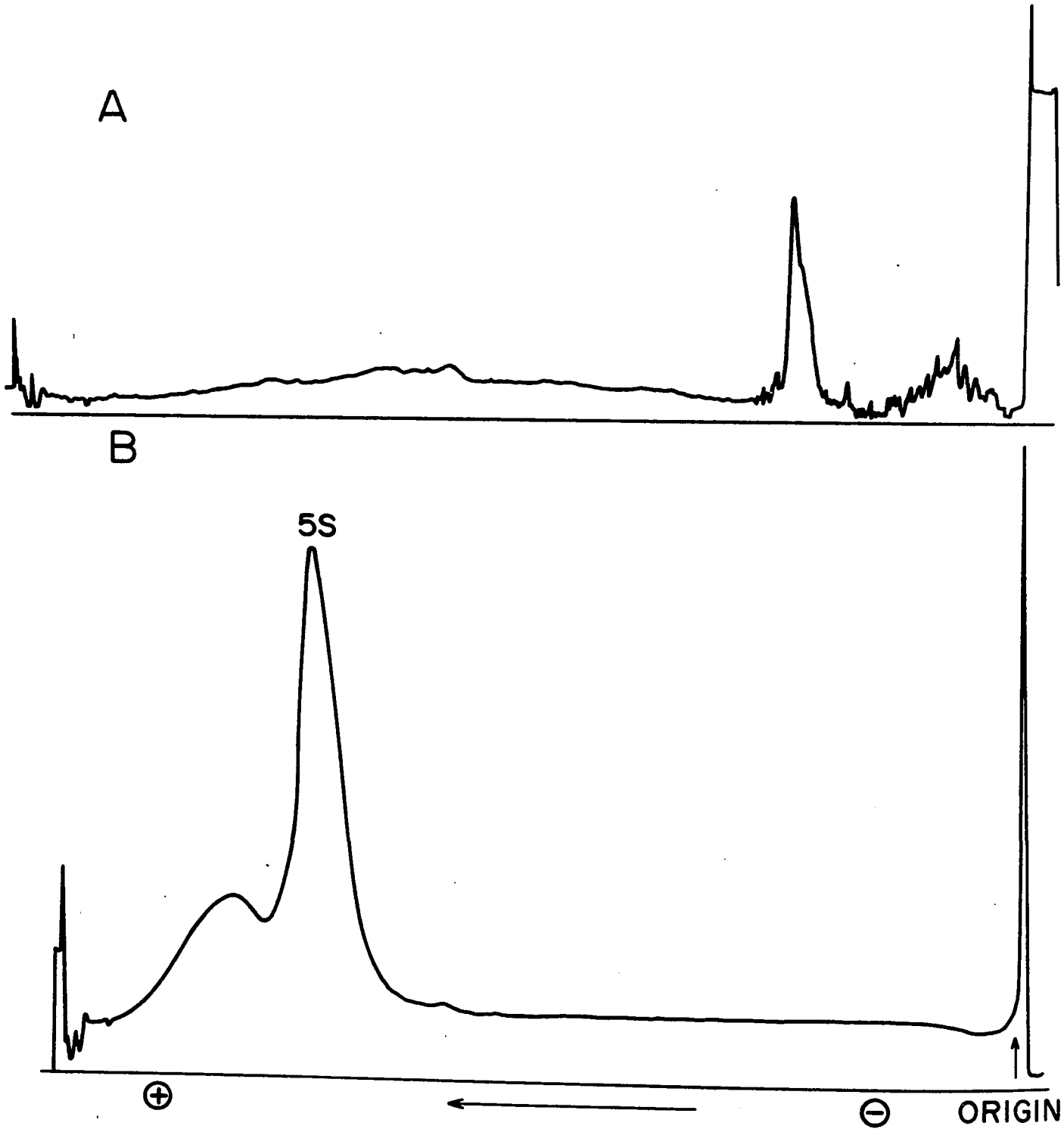

A
4S
5S
16S
23S
B
5S
⊕
⊖
ORIGIN

F I G 11

SDS-acrylamide electrophoretic analysis of rRNA (72) fractionated from $S_1$ on DEAE cellulose (see Fig 7). Three per cent acrylamide gels were used in the analysis. A sample of about 3 $A_{260}$ units was applied to each gel. A current of 3 mA/gel was applied. After completion of the electrophoresis run the gels were scanned in a Gilford spectrophotometer at 260 nm.

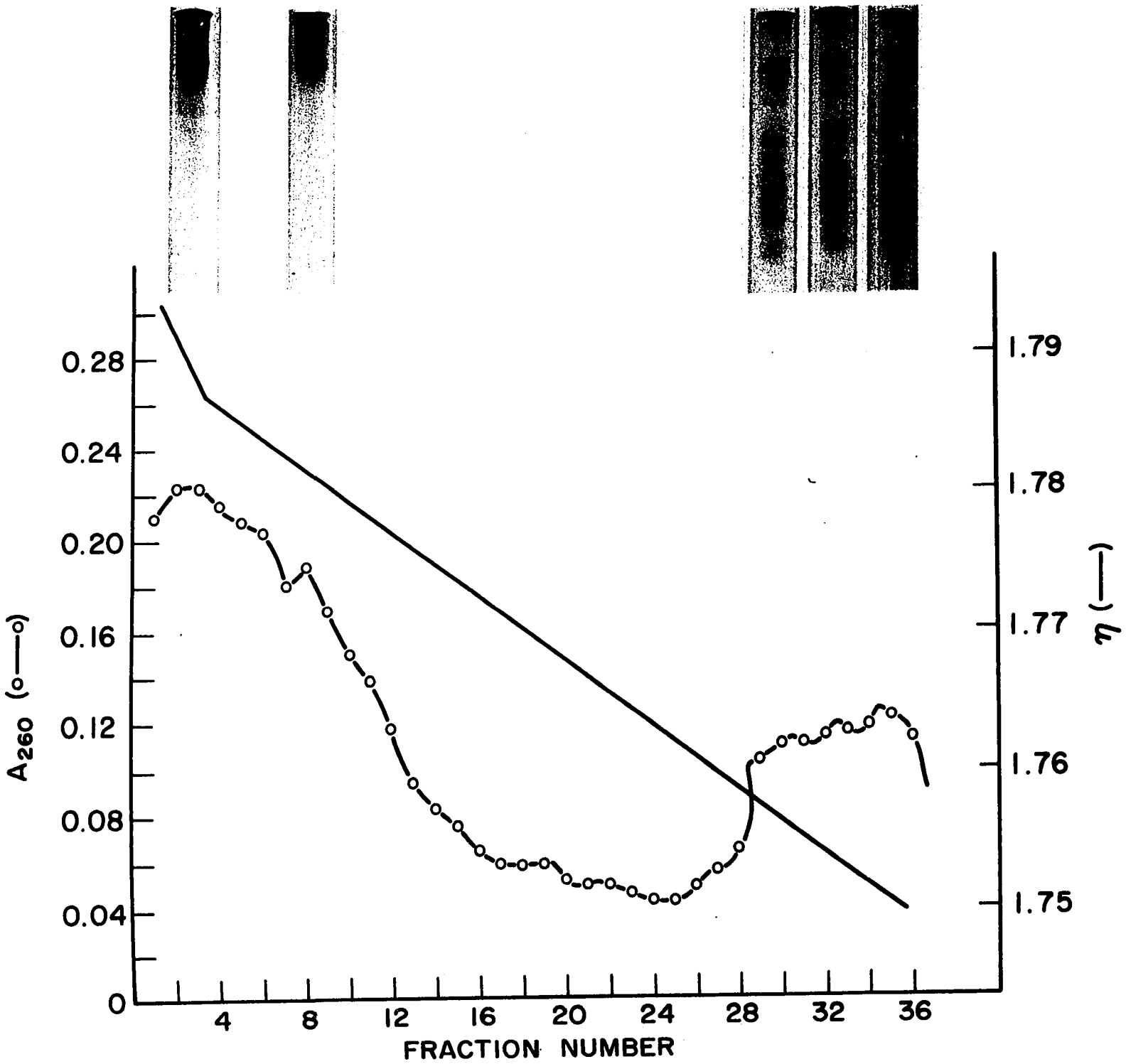
A. E. coli $Q_{13}$ rRNA and 4S RNA were used as standards (66).

B. H. cutirubrum 5S rRNA isolated by fractionation of $S_1$ on DEAE cellulose.


A
B
5S
⊕
⊖
ORIGIN

F I G 12

Determination of protein in SDS acrylamide gel shown in Fig 11B. Fig 12B is the same as Fig 11b. Fig 12A is the same gel as above stained with coomassie blue for protein and scanned at 570 nm.


0.28
0.24
0.20
0.16
0.12
0.08
0.04
0
$A_{260}$ (o—o)
1.79
1.78
1.77
1.76
1.75
η
4
8
12
16
20
24
28
32
36
FRACTION NUMBER

F I G 13

Centrifugation of $S_1$ sample on CsCl equillibrium density gradient. A sample of about 4.5 $A_{260}$ units was mixed with a CsCl solution. Centrifugation was at 35,000 rpm at 20° for 40 hours. Ten drop fractions were collected. SDS-acrylamide electrophoresis (86) was done on fractions 2, 8, 30, 32, and 34 for an analysis of the protein.

ting the presence of protein) than at the bottom of the tube. SDS-acrylamide gels were used to determine whether any proteins had migrated down to the bottom of the tube. SDS-acrylamide gels in Fig. 13 show that perhaps one or two proteins are found in fraction 2. Gels on fractions from the top of the tube showed about 10 bands (which were unfortunately not very distinct). The only plausible explanation for the presence of protein at the bottom of the tube is that they sedimented to this position in a complex with 5S RNA. Peterman (79) recently pointed out that there may be one or two proteins tightly bound to 5S RNA of E. coli 50S ribosomal subunits and that these proteins could only be liberated upon exposure of the complex to high urea concentrations. Much more work must be done on both the E. coli and H. cutirubrum systems before the concept of a specific 5S-protein interaction can be accepted. A starting point for a more thorough analysis of 5S-protein interaction in H. cutirubrum ribosomes could be the $S_1$ subfractionation scheme described in this thesis (Fig. 4), since by this method 5S RNA and protein can be removed from 50S subunits with some degree of selectivity.

# C H A P T E R IV

## Conclusions and Summary

1. When 50S ribosomal subunits of H. cutirubrum are exposed to low ionic strength buffer a group of 18 proteins and 5S RNA are extracted and found in the supernatant fraction ($S_1$) after centrifugation while the remainder of the proteins and 23S RNA are found in the pellet ($P_1$).

2. The binding of $S_1$ proteins to 50S subunits is influenced by changes in $Mg^{2+}$ and $K^+$ concentrations whereas the binding of 5S RNA to 50S subunits is dependent on $Mg^{2+}$ concentration. Certain $S_1$ proteins seem to be specifically removed by a decrease in the $Mg^{2+}$ concentration while the removal of other $S_1$ proteins seems to be affected solely by a decrease in $K^+$ concentration. Some $S_1$ proteins are removed by a decrease in concentration of either $K^+$ or $Mg^{2+}$.

3. Eighteen proteins and 5S RNA have been isolated from the $S_1$ fraction by ion exchange column chromatography and gel filtration. These 18 proteins are distinct entities on the basis of MW determinations and amino acid compositions. Also Visentin *et al.* (66) have

purified the $P_1$ proteins and on the basis of MW and amino acid compositions these proteins ($P_1$) are distinct from the $S_1$ proteins.

4. Evidence is presented which suggests that some $S_1$ proteins may form the binding site for 5S RNA. Furthermore, one or two proteins of the 50S subunit are tightly bound to 5S RNA and this complex is dissociated by SDS treatment.

5. The MW range of $S_1$ proteins is 9,000 to 57,000 daltons.

6. The ratio of the basic amino acid residues to the acidic amino acid residues is in the range of 0.1 to 0.7 indicating that $S_1$ proteins are all acidic. In contrast E. coli 50S subunits contain only about 4-5 acidic proteins (39).

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