ISOLATION AND CHARACTERIZATION OF CERTAIN RIBOSOMAL DOMAINS; THE 5S RNA-PROTEIN DOMAIN FROM ESCHERICHIA COLI AND THE 'A' PROTEIN DOMAIN FROM WHEAT GERM.

by

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B.Sc., University of Salford, England, 1978

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
in the Department
of
Biochemistry and Microbiology

We accept this thesis as conforming
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ABSTRACT

Ribosomes are believed to be composed of many structurally and functionally important, protein-protein and RNA-protein domains. Two of these domains have been investigated. These are the 5S RNA-protein domain and the ribosomal 'A' protein domain.

(i) An attempt has been made to isolate the 5S RNA-protein domain from the large ribosomal subunit of the eubacterium Escherichia coli, as the first step in a project to isolate this domain from an archaebacterial source. E. coli 50S subunits were subjected to a low concentration of Mg^{2+} (2 mM), EDTA·Na_2(10 mM) and NH_4Cl (1 M), (A. Liljas, unpublished), as a possible method to remove the 5S RNA-protein complex from the ribosome. The suspension was centrifuged at 35,000 rpm for 15 hours in a Beckman Ti60 rotor and the supernatant obtained was passed through a 5-20% sucrose gradient. A fraction was obtained which contained several r-proteins and 5S RNA. In an attempt to purify the complex further, the fraction was passed through an S200 column. Although evidence suggests that a complex was obtained, attempts to identify the composition were not successful.

(ii) An attempt was also made to isolate the ribosomal

'A' protein domain, (equivalent to EL7/EL12-EL10 from E. coli), from the large subunit of wheat germ ribosomes, and to isolate and characterize its individual components. A putative complex has been found, of molecular weight 58,000, containing three r-proteins with molecular weights 15,000, 13,700 and 32,000 respectively. The N-terminal portions of each of these r-proteins has been sequenced. One protein (protein 8) was identified by its sequence as the ribosomal 'A' protein, while another (protein 7) has no homology with the N-terminal portion to protein 8. The third protein (protein 10) has been obtained and differs substantially from the other two proteins in molecular weight, but shows an identical N-terminal amino acid sequence to protein 7.

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LIST OF ABBREVIATIONS

A_{260}	absorbance at 260 nanometers
ATP	adenosine triphosphate
BHK	baby hamster kidney
-Me	-mercaptoethanol
CD	circular dichroism
DEAE	diethylaminoethylcellulose
DMAPN	3-dimethylaminopropionitrile
EF	elongation factor
EM	electron microscopy
fmet	formylmethionine
GDP	guanosine diphosphate
GTP	guanosine triphosphate
IEM	immune electron microscopy
IF	initiation factor
P_i	inorganic phosphate
mRNA	messenger RNA
NS	neutron scattering
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PMR	proton magnetic resonance
RF	release factor
RNAase	ribonuclease
r	ribosomal
SDS	sodium dodecyl sulphate
S	Svedberg unit

LIST OF ABBREVIATIONS CONTINUED

TEMED	N, N, N', N'-tetramethylethylenediamine
tRNA	transfer RNA
Tris	tris-(hydroxymethyl)-aminoethane

ACKNOWLEDGMENTS

I would like to thank Dr. A. T. Matheson for his guidance and support throughout this research and in preparation of this manuscript.

I express my sincere appreciation to Dr. W. Garland, Andrea Louie and Sandy Kielland for their technical assistance with amino acid sequencing and composition determinations. I would also like to thank Albert Labossiere for his kind assistance in photographic preparations and I would especially like to thank Dr. R. W. Olafson for his valuable discussions and advice in the amino acid sequencing work.

Appreciation is also extended to Jane Garland for her care and patience in typing this thesis.

Financial support was provided in part by a University of Victoria Fellowship and Scholarship.

I. INTRODUCTION

1. General Introduction

Ribosomes are ubiquitous supramolecular assemblies composed of RNA and protein and are associated with the translation of genetic information into protein structure. For this reason ribosomes are being studied at a molecular level with the aim that a topographical understanding of the ribosome will eventually lead to a total elucidation of the structure-function relationships associated with protein synthesis.

Ribosomes of eubacteria have a diameter of about 20 nm, a sedimentation coefficient of 70S and can dissociate into a large 50S and a small 30S subunit. These ribosomes contain about 65% RNA and 35% protein (1) (Fig. 1). On the other hand the cytoplasmic ribosomes of eukaryotes are somewhat larger, having a diameter of about 22 nm, a sedimentation coefficient of 73 - 80S and dissociate into a large 60S and a small 40S subunit. These ribosomes contain about 45% RNA and 55% protein (3) (Fig. 1).

In eukaryotic cells, ribosomes are also found in the cell nucleus and the cell organelles (mitochondria and chloroplasts). The ribosomes of mitochondria generally have smaller S values than cytoplasmic ribosomes (4). Mitochondrial protein synthesis is characteristic of prokaryotic protein synthesis and has led to the suggestion that mitochondria may have originated from parasitizing aerobic bacteria during eukaryotic evolution (5,6).

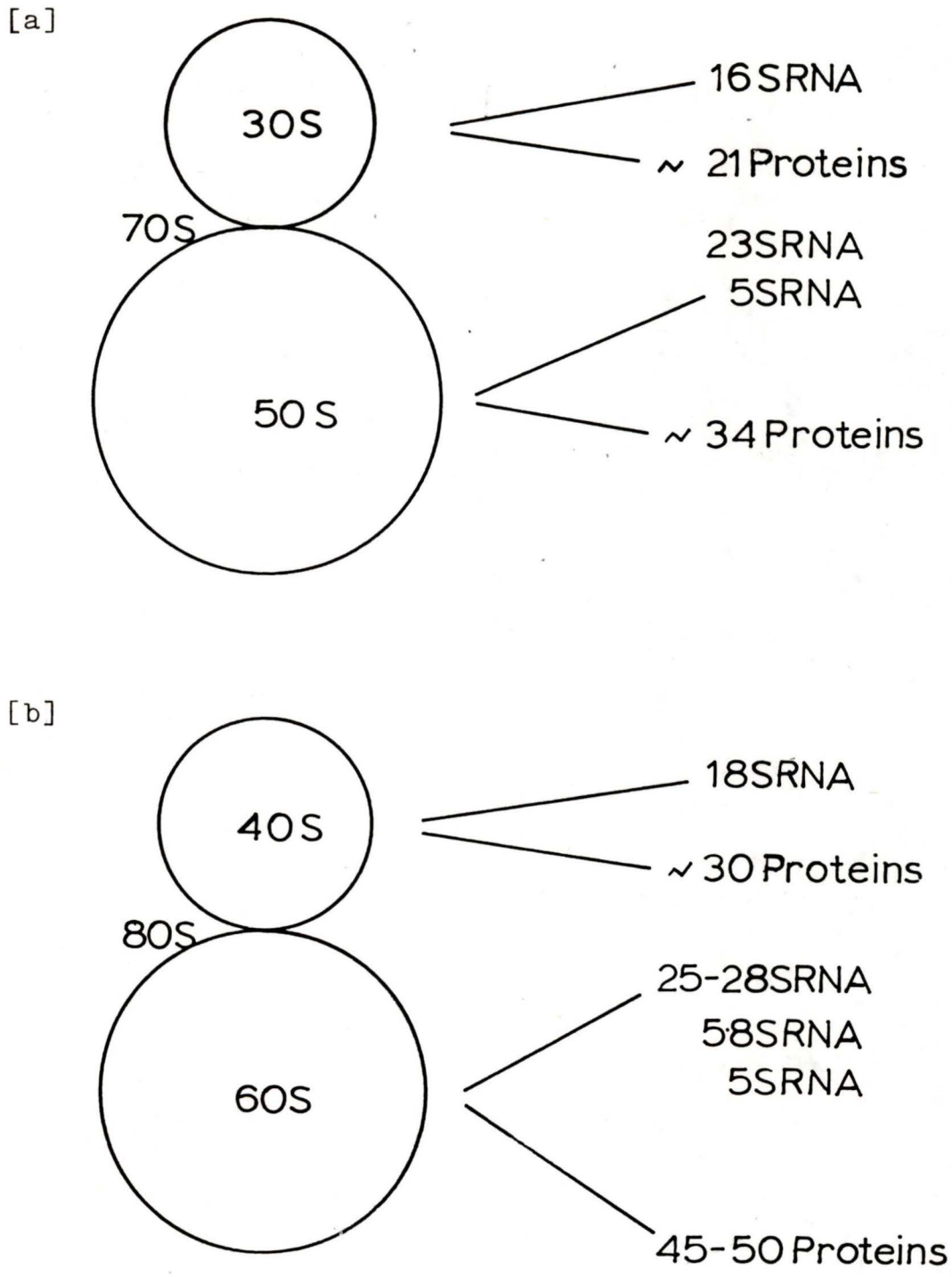


Figure 1. Subdivision of [a] eubacteria (2) and [b] eukaryotic (3) ribosomes into their molecular components.

Chloroplasts also have a protein synthesizing system that resembles that in mitochondria (4).

Comparative studies have been performed on the primary sequences of ribosomal proteins and ribosomal RNA and these studies have led to important new concepts in molecular evolution. From a comparison of the 16S (18S) rRNA sequences Woese and Fox (7) have postulated the revolutionary idea of three kingdoms or evolutionary lines of descent: the eubacteria or "true" bacteria, i.e., classical bacteria; the archaebacteria and the nuclear-cytoplasmic components of eukaryotes. This nomenclature will be used in this thesis and the evolutionary significance of this classification will be discussed in more detail later in the introduction.

A great deal of research has been done on the topography or 3D array of the RNA and protein components that make up the ribosome structure. One approach to this study has been the dissociation of the ribosomes into ribosomal domains (8, 9, 10). These domains are RNA-protein, RNA-RNA or protein-protein complexes that may be cooperatively related both structurally and functionally. Due to their ease of isolation, two domains have received wide attention, namely the 'A' protein domains and the 5S RNA-protein domains. These two domains form the basis of the research interest in this thesis.

2. Structure of the ribosome

A. Ribosomal proteins

(i) Isolation, purification and characterization

Ribosomal proteins are generally isolated using 67% acetic acid (11) or 2 M LiCl in 4 M urea (12). However it is believed by certain groups (13, 14) that if further physical studies are to be carried out, non-denaturing extractions must be employed using a high salt concentration sometimes in the presence of 50% ethanol (15). These authors believe the extraction of proteins using denaturing conditions alters the conformation of the proteins. However, since denatured r-proteins can be renatured, the isolation procedure may not be critical.

Extracted r-proteins may be separated by gel filtration and ion-exchange chromatography (16) and characterized using SDS-PAGE and 2D-PAGE (17, 18).

Characterization of r-proteins by the 2D-PAGE method of Kaltschmidt and Wittmann(17) has allowed a numbering system to be produced for the r-proteins of both the large and small subunits. Proteins of the small subunit are prefixed (S) and those of the large subunit prefixed (L). These proteins are then numbered according to their mobilities in the 2D system.

(ii) Primary structures of ribosomal proteins

The complete primary sequences of r-proteins are deduced by conventional methods (19).

Knowledge of the primary structure of r-proteins allows the predictions of possible secondary and tertiary conformations (20). Far more important in the case of this thesis is the use of primary structures for comparative studies which allows tentative evolutionary groupings amongst organisms (21).

While comparing homologous regions of r-proteins from various species of eubacteria it is of interest to note that different individual proteins from the ribosome of the same organism show no indication of strong homologies, with the notable exception of L7/L12 of Escherichia coli which differs only in an additional acetyl group at the N-terminal on L7 (22), and S20/L26 of E. coli which are the same protein (17).

Almost all the proteins from the 70S ribosomes of E. coli have been sequenced (19, 13, 23, 24) and many have been found to have modified amino acids. E. coli S5, S17 and S18 have acetylated N-terminals, while S11, L11, L16 and L33 have methylated N-terminals. Modified amino acids have also been identified in internal regions of the r-proteins, S12, L3, L7/L12 and L16 (13). At present no functional or structural significance has been attributed to the modified amino acids of these r-proteins.

Although the eukaryotic r-proteins have not been as extensively studied as eubacterial r-proteins, sequence data are available on many eukaryotic r-proteins including

Y44 and Y55 of Saccharomyces cerevisiae(25, 26) and S4, S6, S8, P2, L6, L7a, L18, L27, L30, L37, L37a and L39 of rat liver (3).

Many of the r-proteins of eukaryotes have phosphorylated amino acids which are thought to play some sort of translational regulatory role (27). For example, in BHK cells two major phosphorylated r-proteins have been identified; one is a 14K protein from the 60S subunit and the other a 31K protein, S6 from the small subunit. S6 is a multiphosphorylated protein and is thought to be the major phosphorylated species in several mammalian species (27). The 60S subunit phosphorylated protein has been observed as a closely migrating doublet comparable to the doublet isolated from Artemia salina and rat liver (28).

The amount of sequence data available on archaebacteria r-proteins is relatively small compared with the data available for eubacterial r-proteins. However, partial sequence data is available on a large number of the r-proteins from the extreme halophile Halobacterium cutirubrum, both for the 30S subunit (Yaguchi et al, unpublished) and the 50S subunit (Matheson et al, unpublished).

(iii) Secondary structures of ribosomal proteins

Secondary structures of r-proteins have generally been determined by 2 methods: [1] predictions based on primary amino acid sequences (19, 23, 24), and more importantly by [2] physical studies using CD (29).

[1] Secondary structures have been investigated by running predictive algorithms on computers and interpreting the results according to the methods of Burgess (30), Ponnuswamy and Scherga (31), Chou and Fasman (32), Nagano (33), Robson and Suzuki (34, 20). A particular secondary structure is assumed if three of four predictive methods indicate comparable results. These methods may predict helical regions, extended structures, turns and random coils. An example of a predictive method analysis of E. coli L23 indicated 35% helix, 24% turns or loops and at least 8% extended structure (35). Recently, secondary structures have been predicted for E. coli r-proteins S11 (23), L22 (19) and S10 (24).

[2] Secondary structure determination using CD may depend on the conditions by which the r-proteins are extracted (13). Proteins extracted using acetic acid show relatively low levels of secondary structure when compared to r-proteins isolated by salt extraction (14). It is also important to note that CD measurements are performed on r-proteins in solution and may not represent the secondary structures in the ribosome per se (13). Comparison of secondary structures of r-proteins determined by predictive methods and CD have been shown to be in close agreement for several r-proteins of E. coli: namely, S4, S8, L11, L7/L12 and L27. However, predictive methods have shown higher

α-helical content for r-proteins S20, S21 and L25 than that measured by CD (13).

The use of predictive methods should be regarded only as additional information confirming secondary structures deduced from physical methods and not as proof of secondary structure by itself.

(iv) Tertiary structures of r-proteins

Tertiary structures may be deduced by two methods: [1] predictions from secondary structures and [2] physical studies using PMR (29), calorimetry (29), intrinsic fluorescence (36) and crystallization (37, 38).

[1] Computer aided predictions of tertiary structure have been adopted in association with physical studies for the E. coli r-protein L7/L12 (39).

[2] Tertiary structures may be measured in solution using PMR. This method detects interactions of apolar residues and apolar side chains and provides information on specific interactions between amino acid side chains due to a particular tertiary folding of the protein molecule. This method has been used in studying the E. coli r-protein L7 (29).

Tertiary structure may also be deduced using calorimetry (29). As a protein is heat denatured, heat absorption peaks are observed that demonstrate conformational transitions within the protein molecule of an "order-disorder" type. This technique has also been used in studying E. coli

r-protein L7/L12.

Tertiary structures have further been studied using intrinsic fluorescence (36). An r-protein subjected to fluorescence radiates intrinsic fluorescence originating from tyrosinyl and tryptophanyl residues and gives information on the environment and localization of these emitting residues which are important in determining protein conformation. This technique has been used in the study of E. coli r-proteins S4, S7 and L7/L12 (36).

By far the most important physical technique for studying the tertiary structure of r-proteins is crystallization. Crystallization allows X-ray diffraction studies to be done and will ultimately lead to the complete tertiary structure of the entire r-protein. At present this technique has only been successful on r-protein fragments of E. coli L7/L12 (38).

B. Ribosomal RNA

(i) 5S RNA (5.8S RNA)

5S RNA can be isolated by phenolic extraction of either unbroken cells or isolated ribosomes. The 5S RNA is then separated from the other rRNA's and tRNA molecules by chromatography on Sephadex G-200 or by PAGE (40).

The first 5S RNA was sequenced from E. coli in 1967 (41) and was shown to contain 120 nucleotide residues and no minor bases. Since then over a hundred different

5S RNA molecules have been sequenced (42) allowing comparative studies to be performed and tentative evolutionary positions for various organisms to be suggested (43, 44).

Osterberg et al. (45) proposed a model for the overall shape of 5S RNA as an elongated "Y" with dimensions 40 x 80 x 125 Å. From chemical, enzymatic, physical and oligonucleotide binding studies 5S RNA is believed to have two stable conformations, an 'A' form as proposed by Fox and Woese (46) (Fig. 2) and a 'B' form distinct from the 'A' form. It is suggested that all the 'A' form helical regions are disrupted forming the 'B' form and a new helix is formed between residues 33-42 and 79-88 (29, 48, 49). The 'A' form is believed to be the conformation of 5S RNA within the ribosome (50). The significance of the 5S RNA 'B' form remains unclear.

Erdmann et al (42) have used IR spectroscopy to determine the base pairing in E. coli, Bacillus stearothermophilus and yeast 5 S RNA. They conclude from these studies and previous chemical, enzymatic and oligonucleotide binding studies that these small rRNA's are stabilized by a significant number of tertiary interactions. Erdmann et al (42) were able to simulate IR spectra from other proposed models and then compare them. The models found to be in best agreement with this data are shown in Figure 3 (45, 51-53).

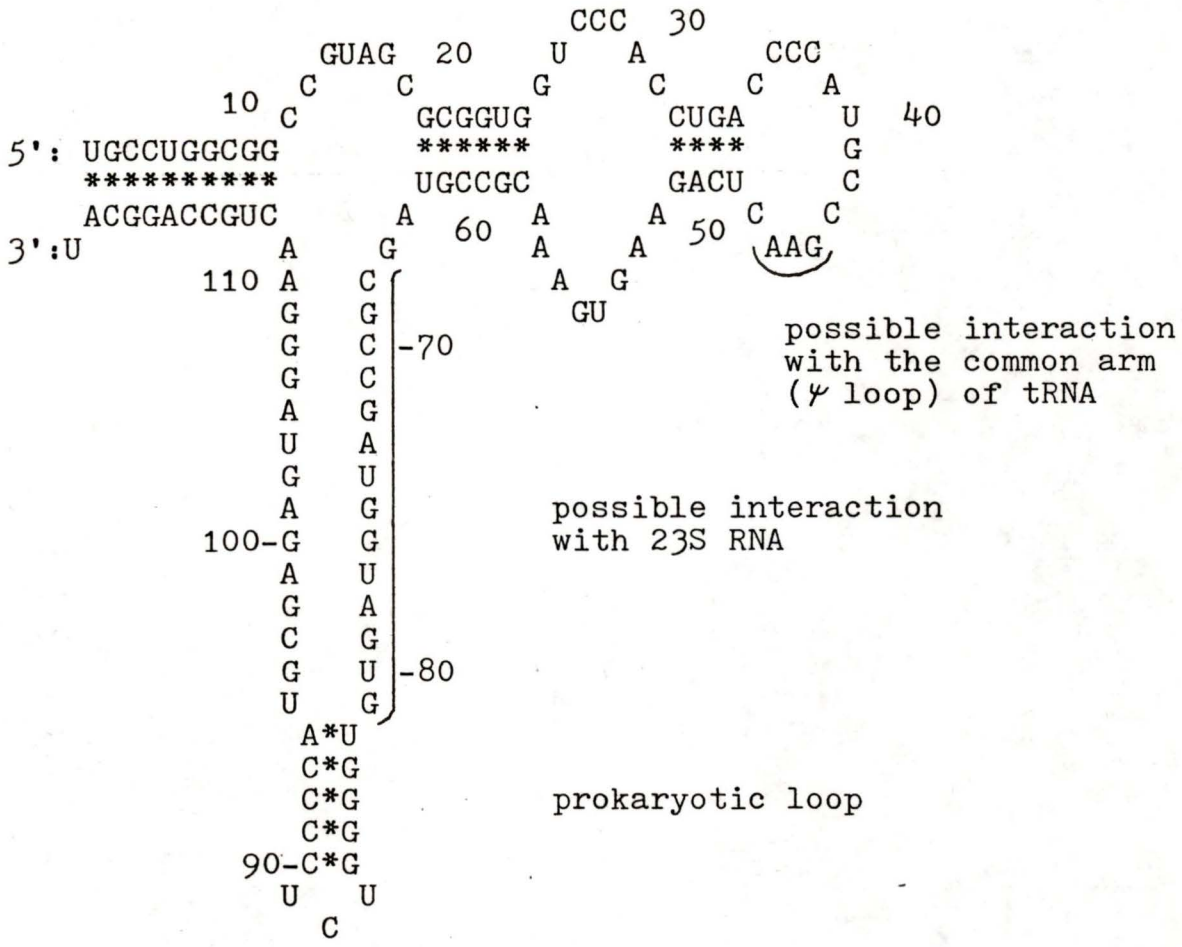
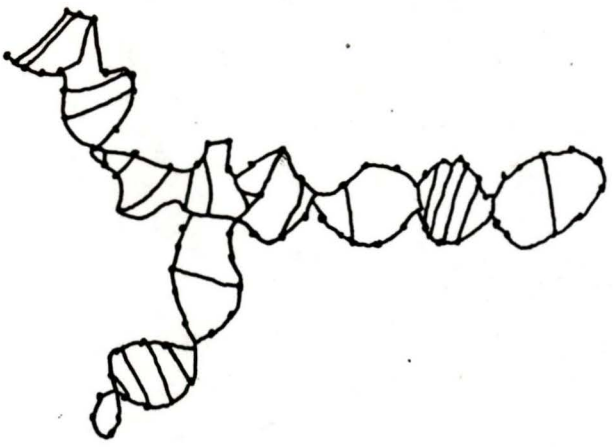


Figure 2. 'A' conformation for 5S RNA from E. coli as proposed by Fox and Woese (47). The diagram also shows possible sites of RNA-RNA interaction. See text for details.

Figure 3. 5S RNA Models in agreement with IR data from Erdmann et al (42).



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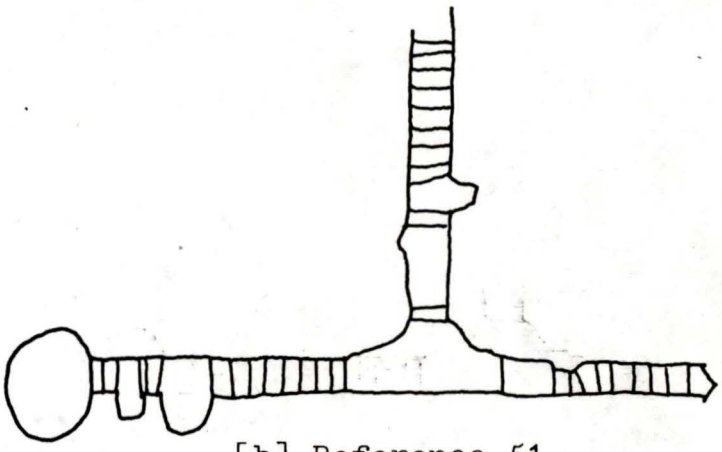

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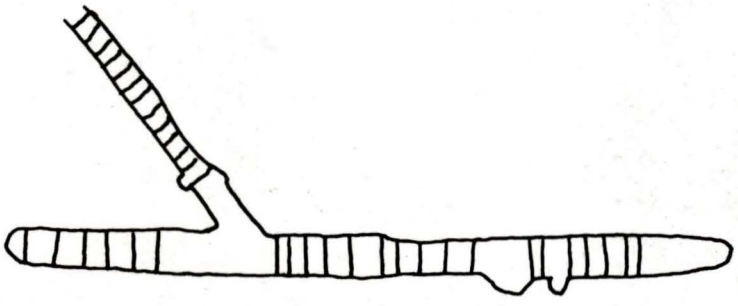

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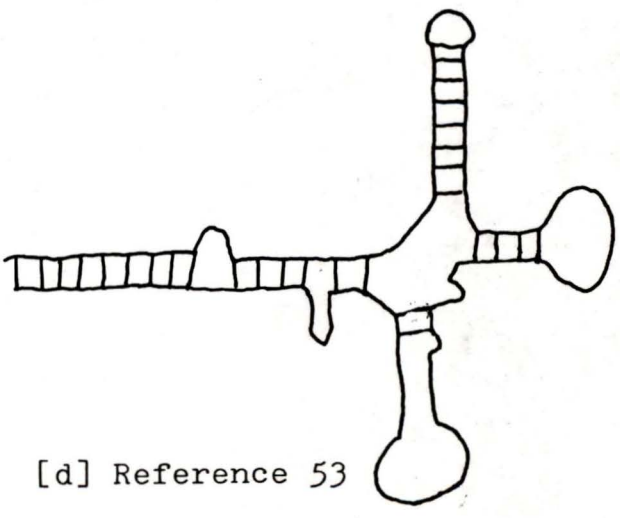


[d] Reference 53

Recent work on the determination of the secondary structure of 5S RNA of both prokaryotes and eukaryotes using slow tritium exchange (50), crosslinking (54), nuclease digestion and chemical modification (55) support the basic minimal secondary structure proposed by Fox and Woese (46).

The nucleotide sequence of 5.8S RNA from several species is now known (56, 57). The 5.8S RNA molecule contains approximately 158 nucleotides and estimations of secondary structure based on maximized base pairing and partial ribonuclease digestion indicate 5 base-paired regions, 3 forming a folding terminal and 2 forming secondary hairpin loops. The models are described as 'burp-gun' models.

5.8S rRNA has been released from 60S ribosomes with heat or formamide and it has been discovered that 5.8S RNA is hydrogen bonded to the high molecular weight component 26S RNA (58, 59). Although no function for 5.8S RNA is known, its binding to 26S RNA may be an integral part in ribosome structure and function. Furthermore, recent studies have indicated that 5.8S RNA is covalently linked in 23S RNA of eubacteria and this suggests a closer evolutionary likeness of eubacterial and eukaryotic ribosomes than had previously been thought (60).

(ii) 16S RNA (18S RNA)

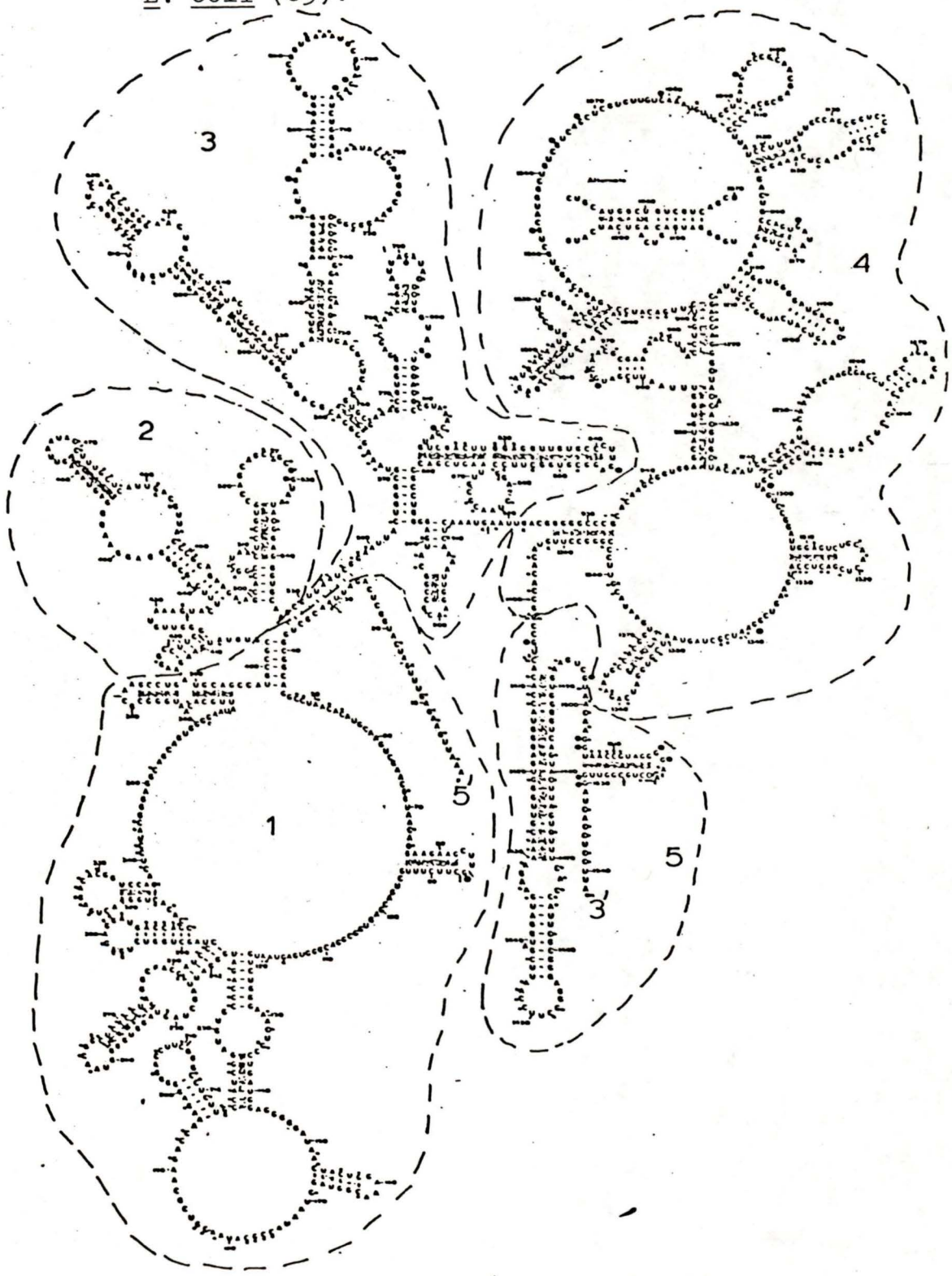
The complete nucleotide sequence of E. coli 16S RNA

has been deduced by two independent methods; [1] from the DNA sequence using the cloned rrnB gene (61) and [2] direct RNA sequencing methods (62). The results of these two techniques are essentially the same, testifying to the practical reliability of the methods used. The 16S RNA from E. coli was determined to contain 1541 nucleotide residues.

Recently a secondary structure model for 16S RNA from E. coli has been proposed (63). This model is based on accumulated information from computer-aided predictions, chemical and enzymatic data (61) [Fig. 4 (63)]. The 16S RNA chain may be divided into 5 structural domains [1-5] by virtue of several long range base pairing interactions. These domains are correlated with the regions of 16S RNA associated with protein binding studies and so it was proposed that protein binding may be related to the RNA structure itself (64). Furthermore, long range reversible interactions between positions 100 and 1060, 400 and 1050 suggest a 'switch' type of interaction in which the switch is closed in the 30S subunit and open in the 70S subunit. This mechanism would allow availability of a particular site only in the 70S ribosome. Such a site could be for aminoacyl tRNA binding (65). The model contains many various sized hairpin loops and 19 of these have been substantiated by comparative phylogenetic evidence (64).

16S RNA structures have also been studied using electron microscopy; however, the dataare conflicting (66,67).

Figure 4. Secondary structure model for 16S RNA from E. coli (63).



Sieber et al (66) studied the effects of changing ionic strength on the secondary conformation of 16S RNA under the electron microscope. Cations were found to affect the sedimentation coefficient of the 16S RNA molecule by affecting base stacking due to electrostatic repulsions of charged phosphate groups. This study showed the 16S RNA molecule to be a flat, round particle of diameter 18 ± 1.9 nm.

Spirin et al (67) suggests that the conditions used in the work of Siebar et al (66) may be too drastic a treatment for RNA. Spirin's group also indicate that the electron micrographs of Sieber et al. (67) show images comparable to their own, resembling a 'V' shaped contour similar to the 30S subunit.

It should be pointed out that the interpretation of electron micrographs is a highly speculative task for the untrained eye and one must exercise caution in accepting any one particular author's argument over the other without further evidence.

The 16S RNA molecule has also been used to investigate evolutionary patterns. Woese and Fox (7, 47) looked at homologous regions of both 16S RNA molecules and 18S RNA and proposed a three kingdom theory (to be discussed in greater detail in a later section of this thesis).

The 5'-terminal sequence of 18S RNAs have been deduced by Woese and Fox (7) and the primary sequence of 80% of 18S RNA from baker's yeast has been determined by analysis

of the nucleotide sequence of DNA genes for this rRNA (68).

(iii) 23S [25-28S] RNA

Long fragments of E. coli 23S RNA were sequenced in Ebel's laboratory in Strasbourg (69) and more recently the total primary sequence of 2904 nucleotides has been deduced using the 23S RNA gene of the rrnB operon (70).

A knowledge of the primary sequence of 23S RNA should ultimately lead to a proposal for the secondary conformation as has been done with 16S RNA. Proposed conformations to data are based on chemical and enzymatic studies (61).

23S RNA, like 16S RNA, has been studied with the E.M. and results suggest to the authors that the 23S RNA molecule in solution has similar dimensions (260 ± 20 Å) to that of the large subunit (230 ± 10 Å).

The large RNA's from eukaryotes are presently under investigation and the sequences for many of these are expected to be deduced in the near future.

C. Ribosomal topography

The individual functional steps in protein synthesis cannot be attributed to single ribosomal components, but rather complexes of several different components are involved (72). This realization has lent new urgency to the problem of elucidating the structure of the ribosome, which will in turn aid the understanding of ribosomal functions.

The topographical study of ribosomes has been advanced in recent years using several techniques, most notably,

electron microscopy (73, 74, 75), protein-protein and protein-RNA cross-linking (76, 77), RNA-RNA cross-linking (78) and neutron scattering (79).

(i) Topography of the eubacterial small subunit

It has been suggested that the 30S subunit of E. coli ribosomes is composed of 'domains' (9) which may be observed by fragmenting the 30S subunit into two large ribonucleoproteins, one of which contains approximately 900 nucleotides in the 5' portion of the 16S RNA molecule and is associated with proteins S4, S5, S6, S8, S15, S16/17, S18 and S20, and a second fragment of approximately 500 nucleotides at the 3'-proximal portion of the 16S RNA which is associated with proteins S7, S9, S10, S14, and S19. An additional 150 nucleotide fragment at the 3'-terminal end was found to be unassociated with r-proteins.

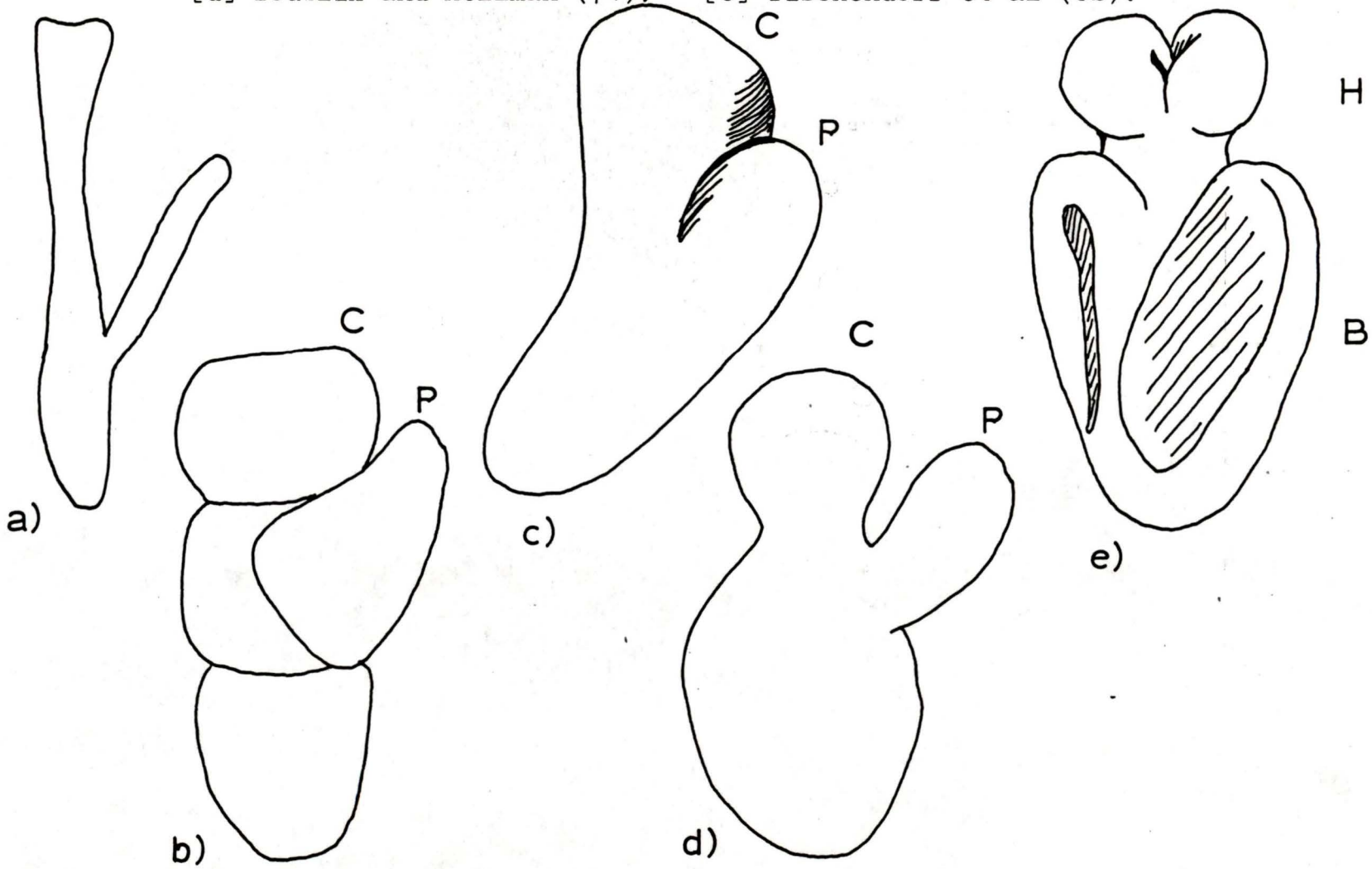
'Domains' have further been suggested by reconstitution of 30S subunits from its RNA and protein components (8, 80). These studies suggested three binding regions on the RNA molecule for r-proteins. The first group at the 5' end of 16S RNA contains proteins S4, S16, S17, and S20; the second group in the middle of the 16S RNA contains proteins S8 and S15, S6 and S18; and the third group at the 3'-proximal portion contains S7, S9, S13 and S19. The r-proteins underlined indicate the main proteins associated with the RNA, and these proteins appear to govern the interaction of the other proteins of that group.

Electron microscopic work of Spirin et al.(67) and Bogdanov et al (81) on the 16S RNA molecule has shown that the 16S RNA moleucle associated with only the four main proteins. S4, S8, S15, and S7, has morphological features of the 30S subunit. In fact, they also indicate that protein S4 alone with the 16S RNA molecule has a similar morphology to the 30S subunit.

The 30S subunit has been investigated by several physical methods and it is interesting, in the light of the present data, to compare and contrast the 30S models of various researchers with the model for 16S RNA alone (Figure 5). Many groups have studied the 30S subunit using electron microscopy (67, 73, 74, 75). Lake (73) suggests an asymmetric unit with a platform (p) and cleft (c) as the characteristic features. Boublik and Hellman (74) also suggest an asymmetric model with platform and cleft. However, Tischendorf et al (22) suggest a more symmetric model divided into a head (h) and body (b), with the head being segmented and the body comprising two unequally sized lobes. If these models are compared with that of Spirin et al (67) and his model for 16S RNA alone, it is obvious that the 'V' or 'Y' shaped conformation for the compacted 16S RNA molecule would suit all proposed models.

Although there exist major differences in the models, no greater discrepancies are more apparent than in the positioning of the individual ribosomal proteins. Neutron scattering (83) has indicated that RNA is packed

Figure 5. [a] Electron micrograph interpretation of 16S RNA (67). Electron micrograph interpretations of 30S subunit, [b] Spirin et al (67), [c] Lake (73), [d] Boublik and Hellman (74), [e] Tischendorf et al (82).



as RNA helices in a compact core which directs the formation of the RNA tertiary structure and binding of proteins on the periphery of the ribonucleoprotein molecule. If this is indeed the case, one would expect all or almost all the r-proteins to be identifiable by immune electron microscopy. Again, however, there is much conflict regarding the positions of r-proteins throughout the 30s subunit. A summary of the IEM data and neutron scattering studies for various models are shown in Figure 6.

Besides IEM, information on protein-protein neighbours has come from ribonuclease digests (8,9), chemical cross-links (78), fluorescent labelling (85), and neutron-scattering (79). Accumulation of this data has led Spirin et al (67) to propose a scheme of protein neighbours within the 30S subunit (Fig. 7). They suggest that the 30S subunit can be broken into three domains (1-3) as previously reported by Brimacombe et al (9), and these domains can then be interpreted in a composite 3 dimensional model (Fig. 6b).

Proteins are illustrated as numbered circles and it should be pointed out that this does not infer a globular nature for any of the proteins shown although this may be the case. In the comparison of r-protein positions for various authors shown in Figure 6, I have selected only several proteins that are most common in each author's work. Protein S6 is shown at the base of the 30S subunit in all cases studied. Proteins S12 and S8 are found at

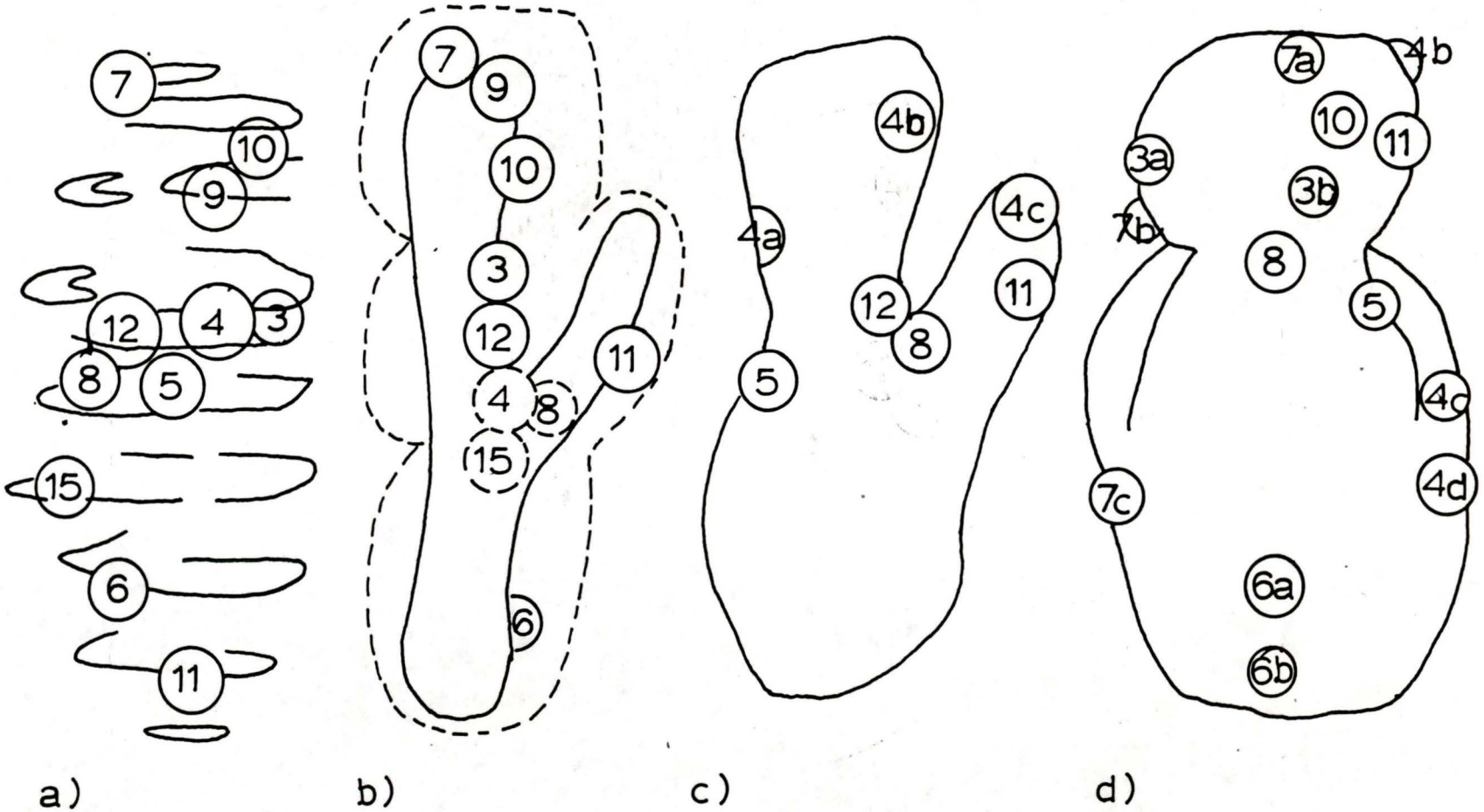
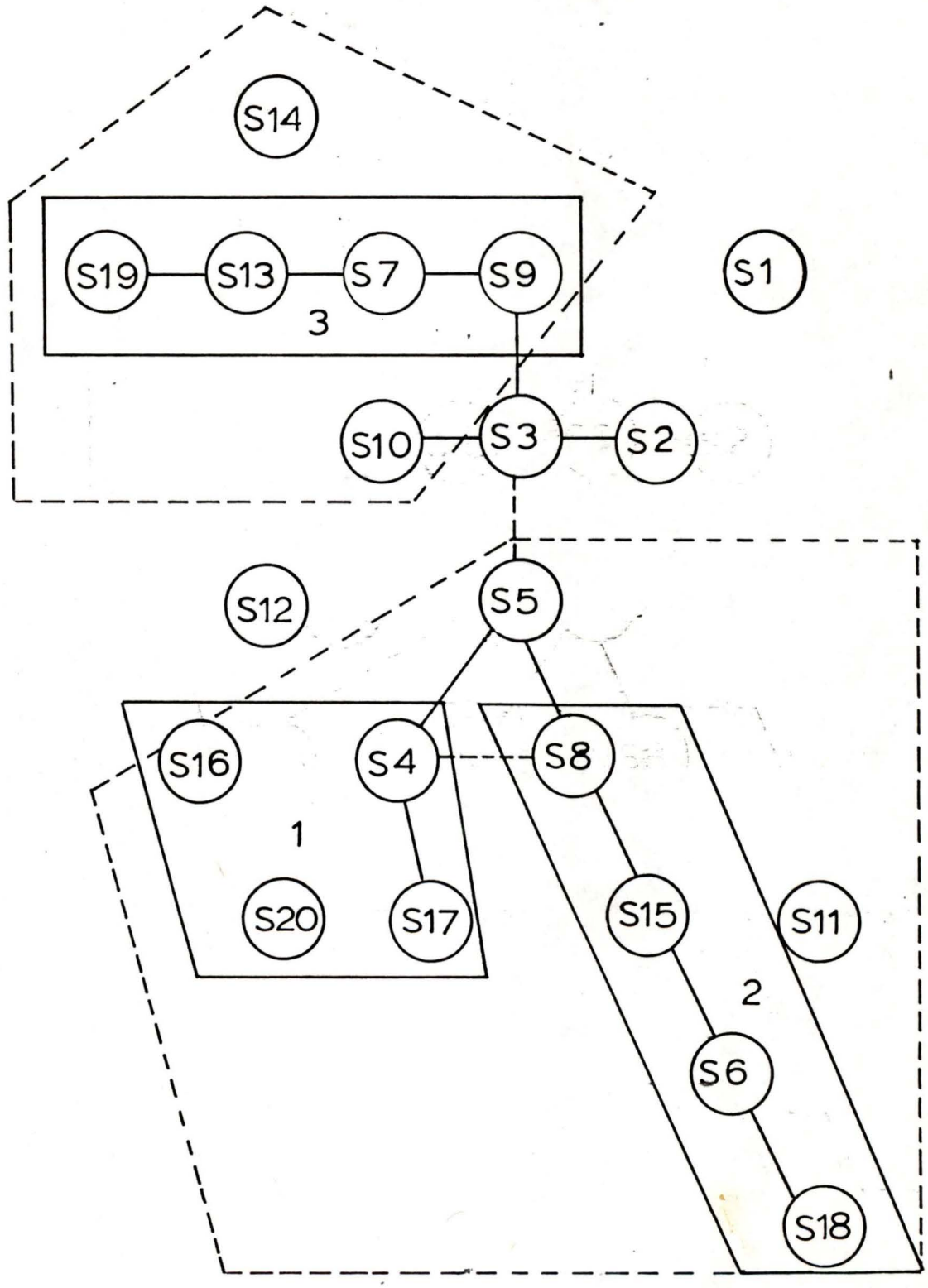


Figure 6. Position of r-proteins in the 30S subunit
[a] Neutron scattering model of Moore (83), [b] Spirin et al (67), a composite model of IEM, NS and ribonuclease data. The solid line indicates the RNA, and the dotted line the periphery of the r-proteins, [c] IEM model of Lake (84), and [d] IEM model of Tischendorf et al(82).

Figure 7. Ribonucleoprotein domains of the 30S subunit(67). Solid line shows neighbouring proteins on the RNA chain and dashed line shows RNase fragmentation.



the base of the cleft or in a central position in all models. Proteins S7 and S10 are at the apex of all models in which these proteins were studied. The remainder of the r-proteins are in varied positions and in the case of the IEM models of Lake (84) and Tischendorf et al (82) several proteins have more than one location, indicating a possible elongated nature. This could also suggest that the antibodies used may be contaminated. In the case of the model of Spirin et al (67), I also indicate the proposed position of the 16S RNA molecule within the 30S subunit.

(ii) The eukaryotic small subunit

Despite the larger size of the eukaryotic small subunit, it is strikingly similar in morphology to the prokaryotic 30S subunit (74,3). The 40S subunit is described as a 'curved and flattened prolate ellipsoid' with dimensions 230 x 140 Å (86) or 250 x 125 Å ± 10% (74). The 40S subunit may be divided into a 'head' (one-third) and 'body' (two-thirds) regions by a transverse portion seen under the electron microscope. Boublik and Hellman (74) describe three segments in the 40S subunit with a beak-like protrusion. A comparision of the 40S subunits from various electron microscopic studies are shown in Figure 8. One may visualize how the models would appear similar if the Boublik and Hellman (74) model was pointed beak first into the paper.

(iii) The eubacterial large subunit

Mild ribonuclease digestion of E. coli 50S subunits

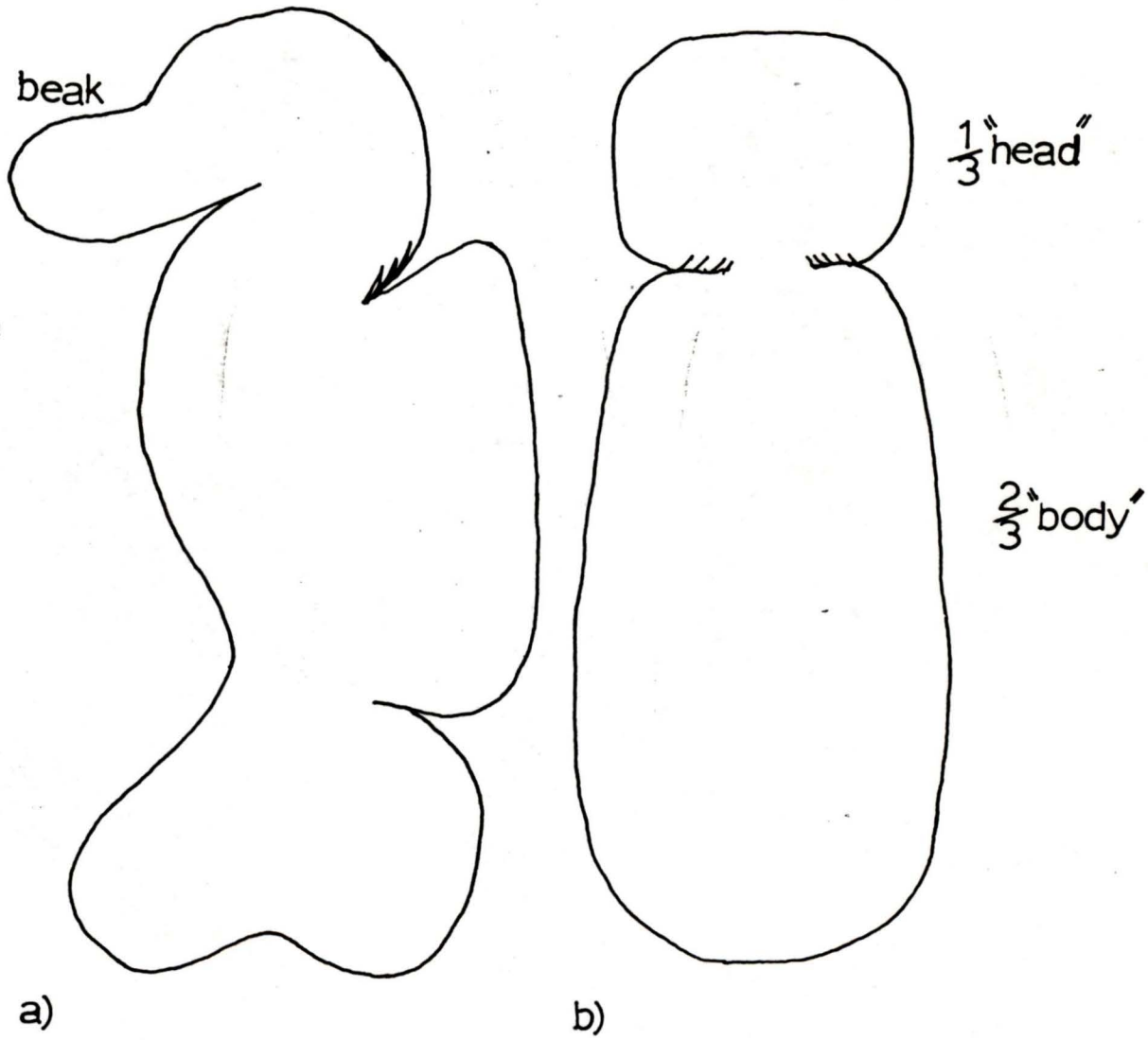


Figure 8. 40S subunit electron micrograph interpretations.
[a] Boublik and Hellman from A. Salina (74)
[b] Lake from Rat liver (86).

led Chen-Schmeisser and Garrett (87) to propose a scheme of three domains for this subunit. The RNase digestion products consisted of a 13S RNA-protein domain, an 11S RNA-protein domain and a central protein-RNA domain. It was further proposed that there is a stable 23S RNA-protein core within the 50S subunit consisting of proteins L3, L13, L17, L21, L22 and L24 (10).

Electron microscopic studies (71) on the morphology of 23S RNA within the 50S subunit indicate that the 23S RNA molecule can acquire a specific compact conformation morphologically similar to the 50S subunit. However, Sieber et al (66) suggest that the 23S RNA molecule described by Spirin et al (67) above is incorrect and the 23S RNA molecule is a flat structure, with a larger diameter than the 50S subunit, and that it undergoes conformational changes during ribosomal assembly. This agrees with the CD studies of Bogdanov et al (81) which also showed conformational changes during ribosomal assembly.

Several models have been proposed from electron microscopic studies for the 50S subunit. It is of interest to compare these with the 23S RNA molecule model from Spirin et al (67) (Fig. 9). The most notable difference between the 50S subunit molecules is the absence of the 'acidic-stalk' (L) in the model of Tischendorf et al (82). Furthermore, the model of Tischendorf et al (82) is more symmetric than the other models.

As with the small subunit, many techniques have been

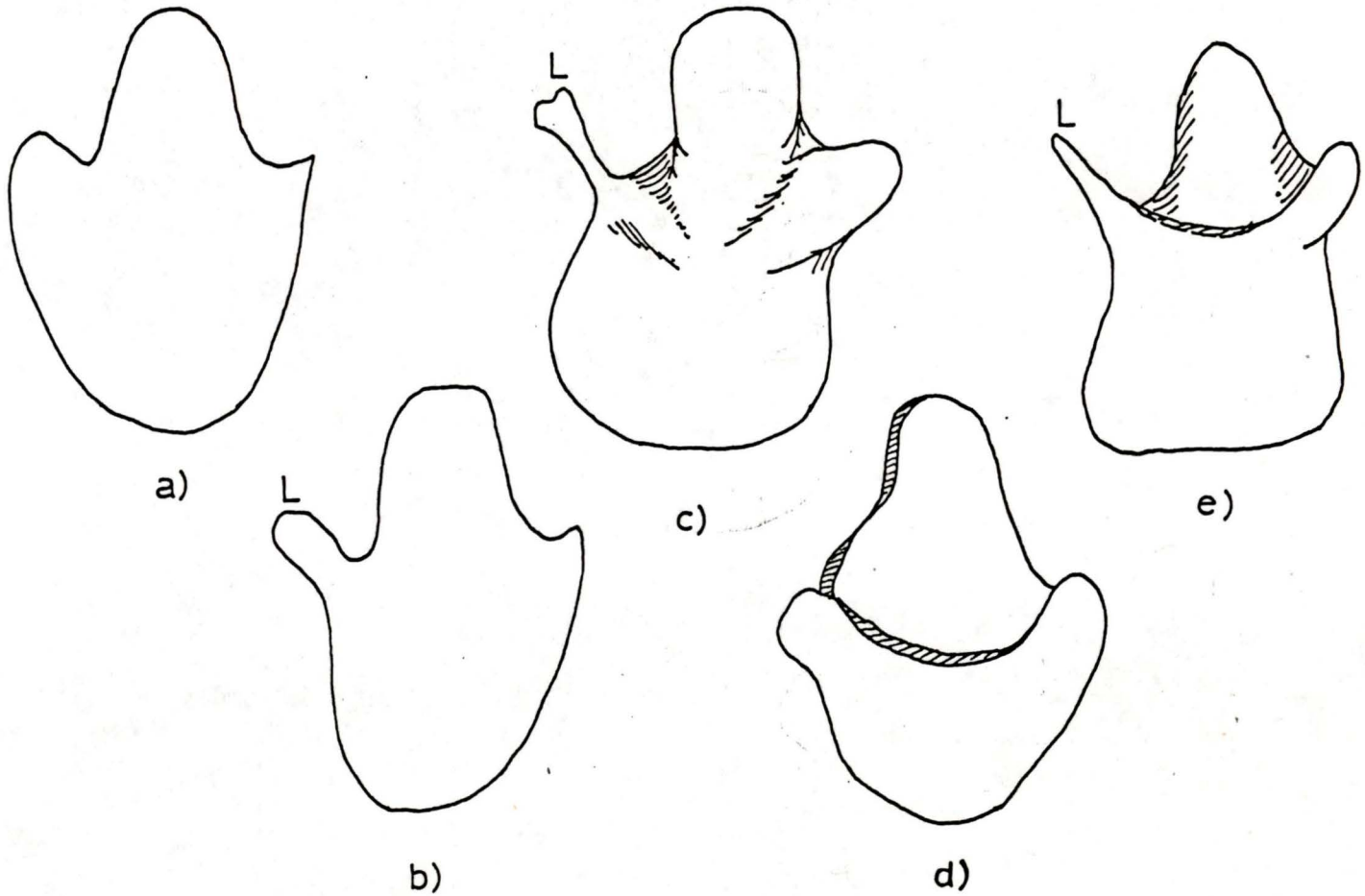


Figure 9.
[a] Electron micrograph interpretation of 23S RNA (71).
Electron micrograph interpretations of 50S subunits according to [b] Spirin et al (71), [c] Lake (73), [d] Tischendorf et al (82), and [e] Boublik and Hellman (74).

used in trying to identify the positions of the r-proteins in the large subunit, most notably IEM (73, 82) and protein-protein cross-linking (78). A comparison of the 50S r-protein positions deduced from the various techniques is shown in Figure 10.

It is obvious that a favourable comparison of r-proteins between the two IEM models is difficult. However, the association of L7/L12 and L10 shown in both Lake's (73) and Traut's (78) models are in agreement with studies on the isolation of these proteins as a complex (88). In contrast, the Tischendorf et al (82) model shows the L7/L12 r-protein to be associated as a "band" around the upper one-third of the 50S subunit. Obviously much more work must be done on determining the exact positions of the large subunit r-proteins.

An alternative method of studying the topography of the 50S subunit has been the isolation of complexes of RNA and protein and the investigation of the topography of these complexes individually. Examples of such complexes being investigated are the L7/L12-L10 complex from the eubacteria E. coli (89) and B. stearothermophilus (90), the HL3/4-L11 complex from the archaebacterium H. cutirubrum (91) and the 5S RNA-protein complexes from eubacteria (55), archae-bacteria (92) and eukaryotes (93). The structure of these complexes will be discussed in greater detail in a later section of this thesis.

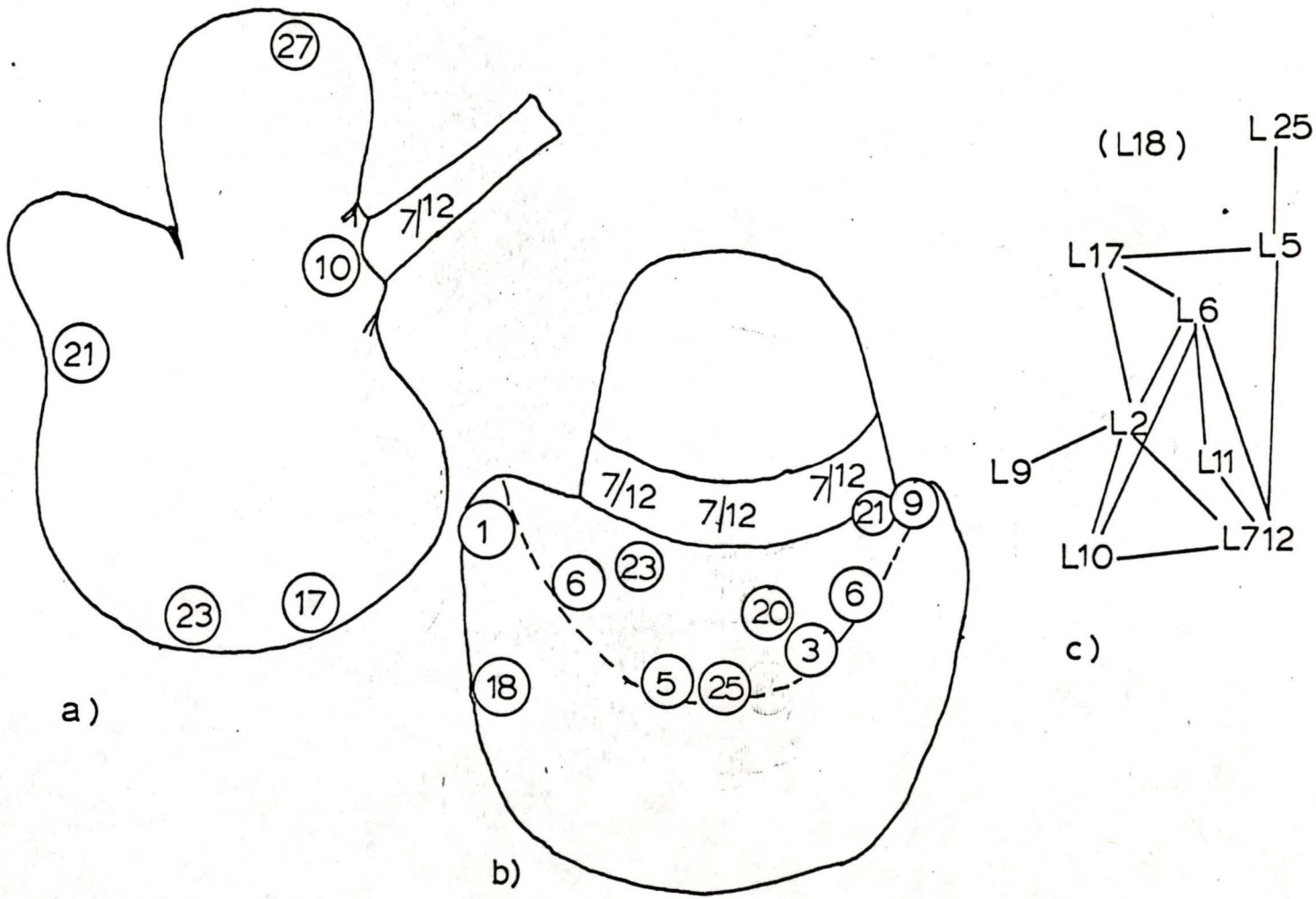


Figure 10. Protein locations on the 50S subunit as determined byIEM. [a] Lake's model (73), [b] Tischendorf et al model (82), [c] Protein associations as determined by crosslinking (78).

(iv) The eukaryotic large subunit

The large subunits of eukaryotes have been investigated using electron microscopy and a comparison of structures produced by various authors are shown in Figure 11. Lake (86) observes two types of electron microscope images. One is a round image of diameter 220 Å and the other is a "skiff" shaped asymmetric image. Lake (personal communication to ATM) has also observed a very small percentage of 60S ribosomes with "stalks". Boublik and Hellman (74) see the large subunit as a round image of diameter 260 Å ± 10% with a very apparent "stalk". The most notable difference between the two models is the knob-like protrusion of about 50 x 40 Å on the base of Boublik and Hellman's model (74)

(v) Topography of the eubacterial 70 S ribosome

Knowledge of the topography of the large and small subunits and electron microscopic studies of the 70S ribosome itself allow predictions of the orientation of the subunits in the ribosome. A comparison of the 70S models is shown in Figure 12. The models of Boublik and Hellman (74) and Stoffler et al (75) show the small subunit lying horizontally transverse to the large subunit with the groove and lobes of the 30S subunit orientated towards the seat of the 50S subunit. The Stöffler et al (75) model is more symmetric than the model of Boublik and Hellman (74). Lake's model (73) locates the small subunit 'platform' within an indentation of the large subunit.

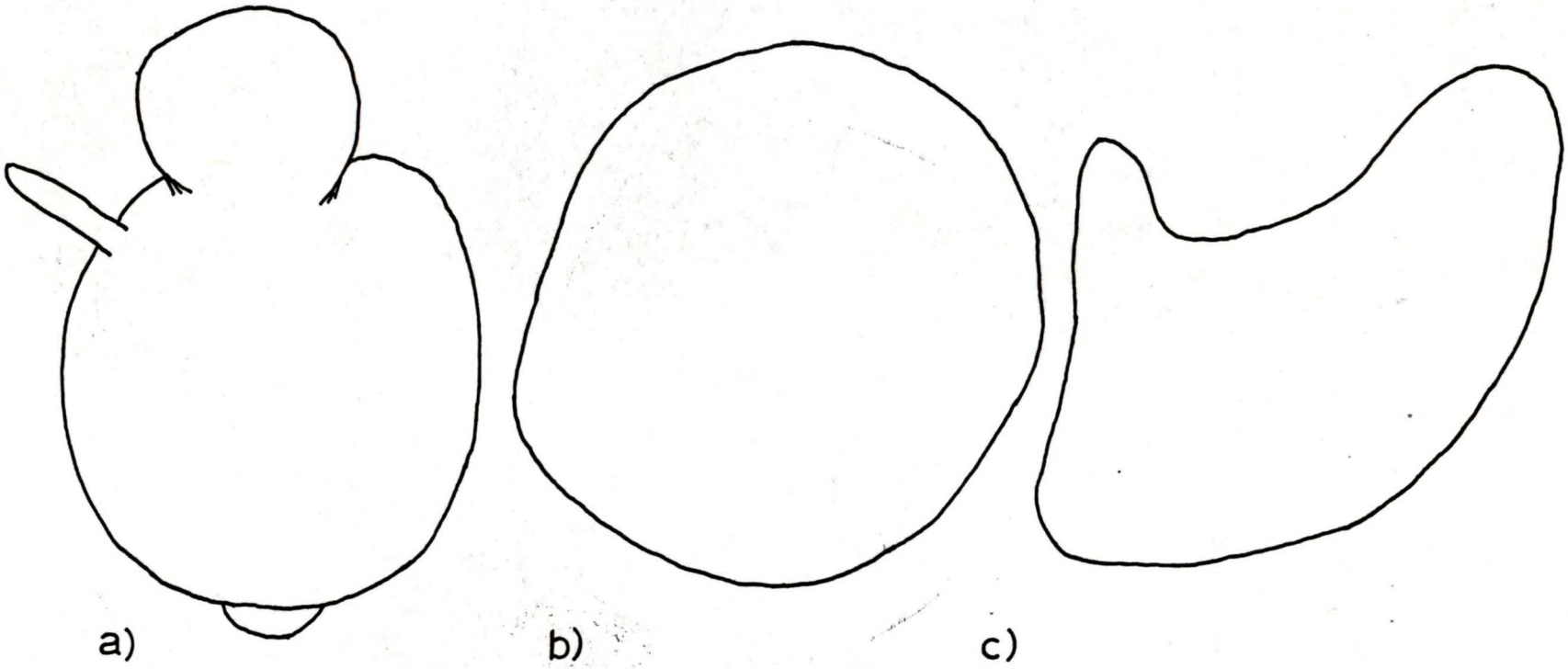


Figure 11. Electron micrograph interpretations of eukaryotic 60S subunits.
[a] Boublik and Hellman model of A. salina (74)
[b] Lake's "round" model of Rat liver (86)
[c] Lake's "skiff" shaped model of Rat liver (86).

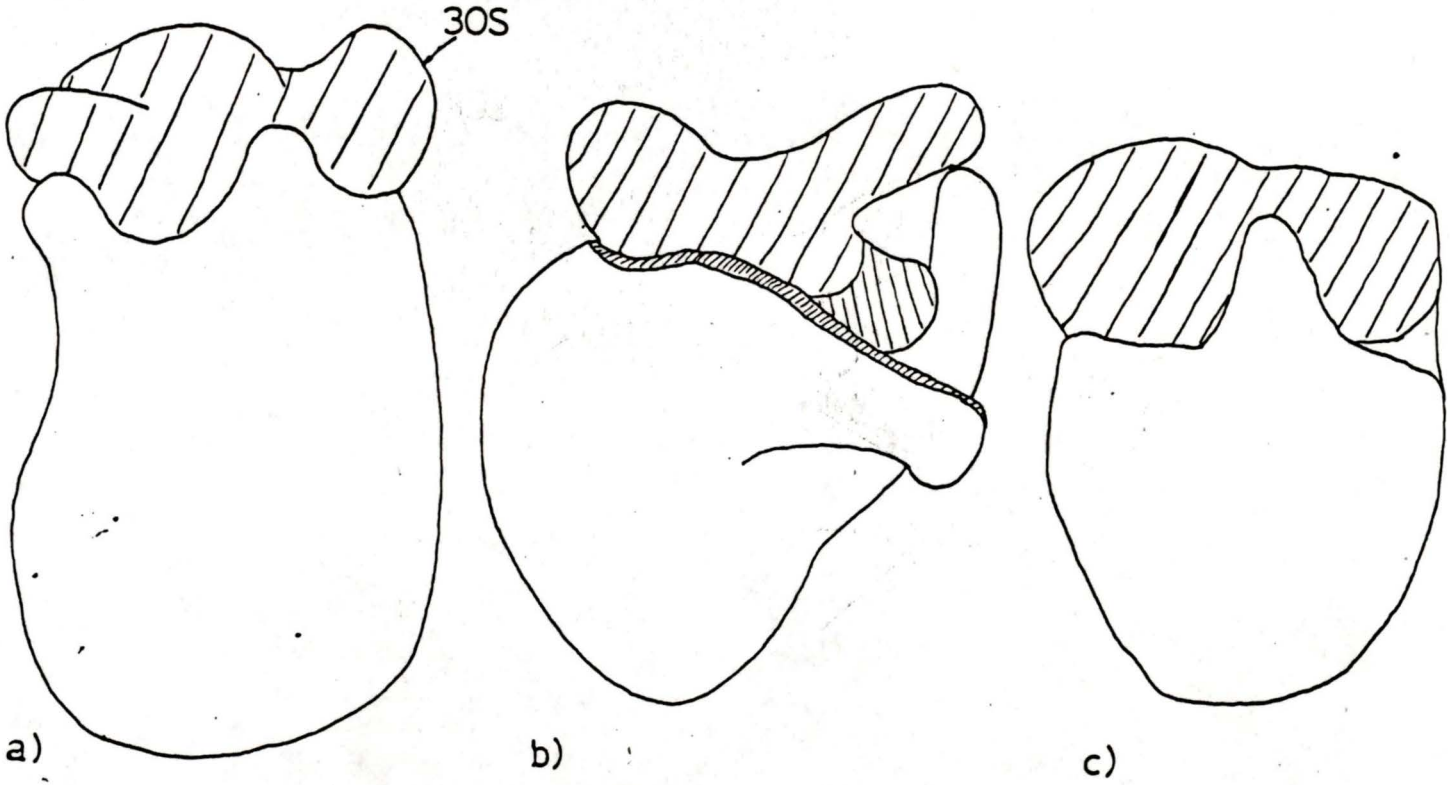


Figure 12. Electron micrograph interpretations of 70S ribosomes.
[a] Boublik and Hellman model (74)
[b] Lake model (73)
[c] Stoffler et al model (75)

This model is a rotation of 90° compared with the Boublik and Hellman model (74).

Cross-linking studies (78) have been useful in determining r-proteins associated with the subunit interface (Fig. 13). An incorporation of the cross-linking data and IEM studies are shown for the 70S model (Fig. 14). It is interesting to note the position of L7/L12 and L5, L25, part of the 5S RNA domain, near the ribosome interface. These domains will be discussed in more detail later in this thesis.

(vi) The eukaryotic 80S ribosome

A comparison of 80S ribosomes using electron microscope data from various groups is shown in Figure 15. Lake (86) observed two common images and concluded that the more rounded image was a lateral view of the first image ('skiff'-shaped). Boublik and Hellman (74) see the small subunit orientated lengthwise in the crown region of the large subunit. The models of the two authors are quite similar when viewed in the same projection.

At the present time, several groups are attempting to crystallize the ribosome particle to allow a 3-dimensional reconstruction of the ribosome. For example, naturally occurring crystalline arrays of eukaryotic ribosomes have been discovered in the oocytes of hibernating lizards (94). At present this technique has proven unrewarding, but there is hope that such ribosomes may allow complete elucidation of the 3D topography of the ribosome.

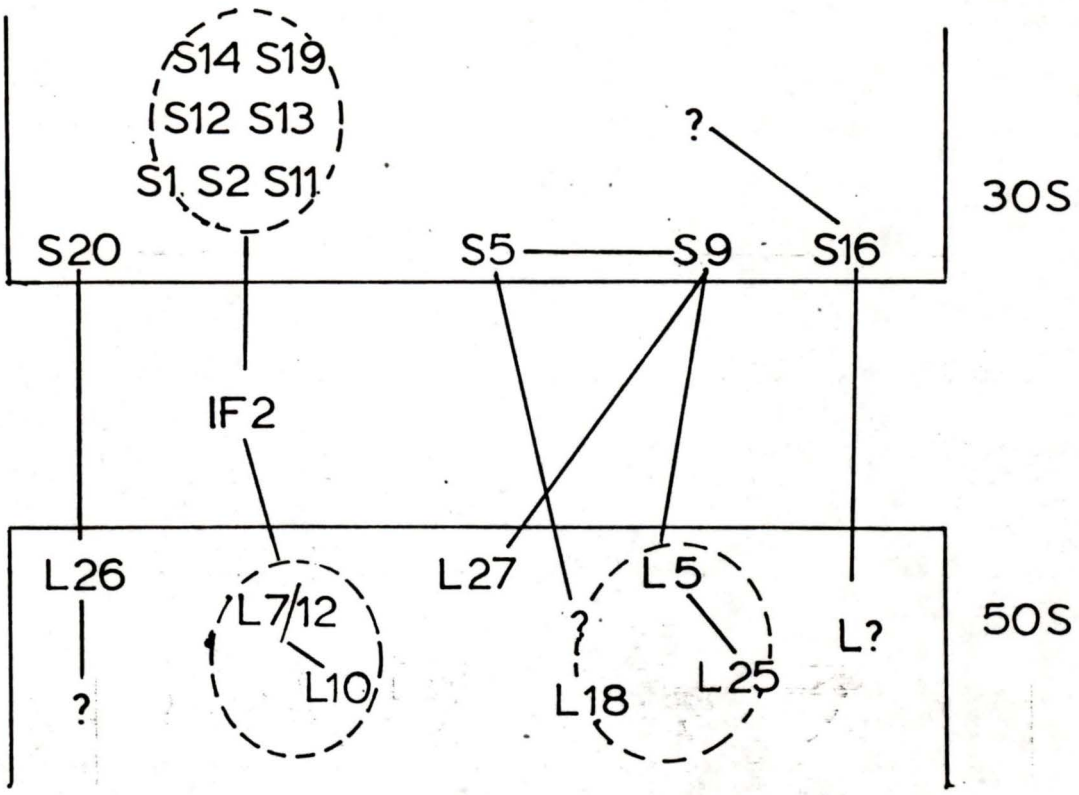


Figure 13. Cross-linked proteins at the 70S ribosome interface (78).

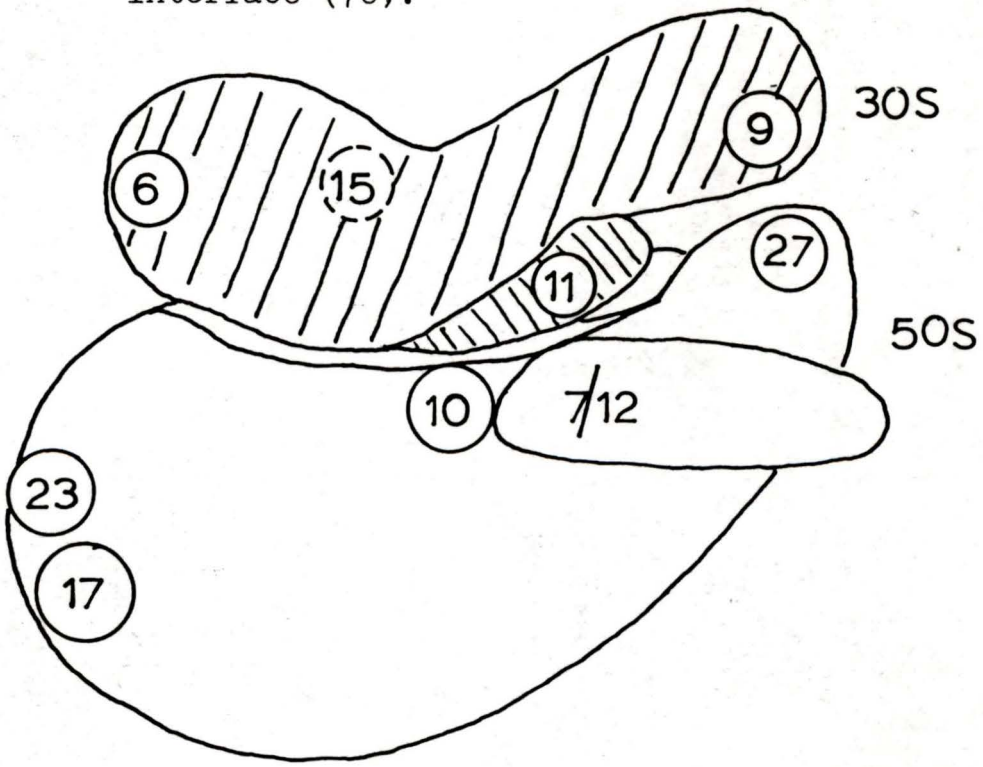


Figure 14. Lake's model (73) of 70S ribosome showing r-protein positions.

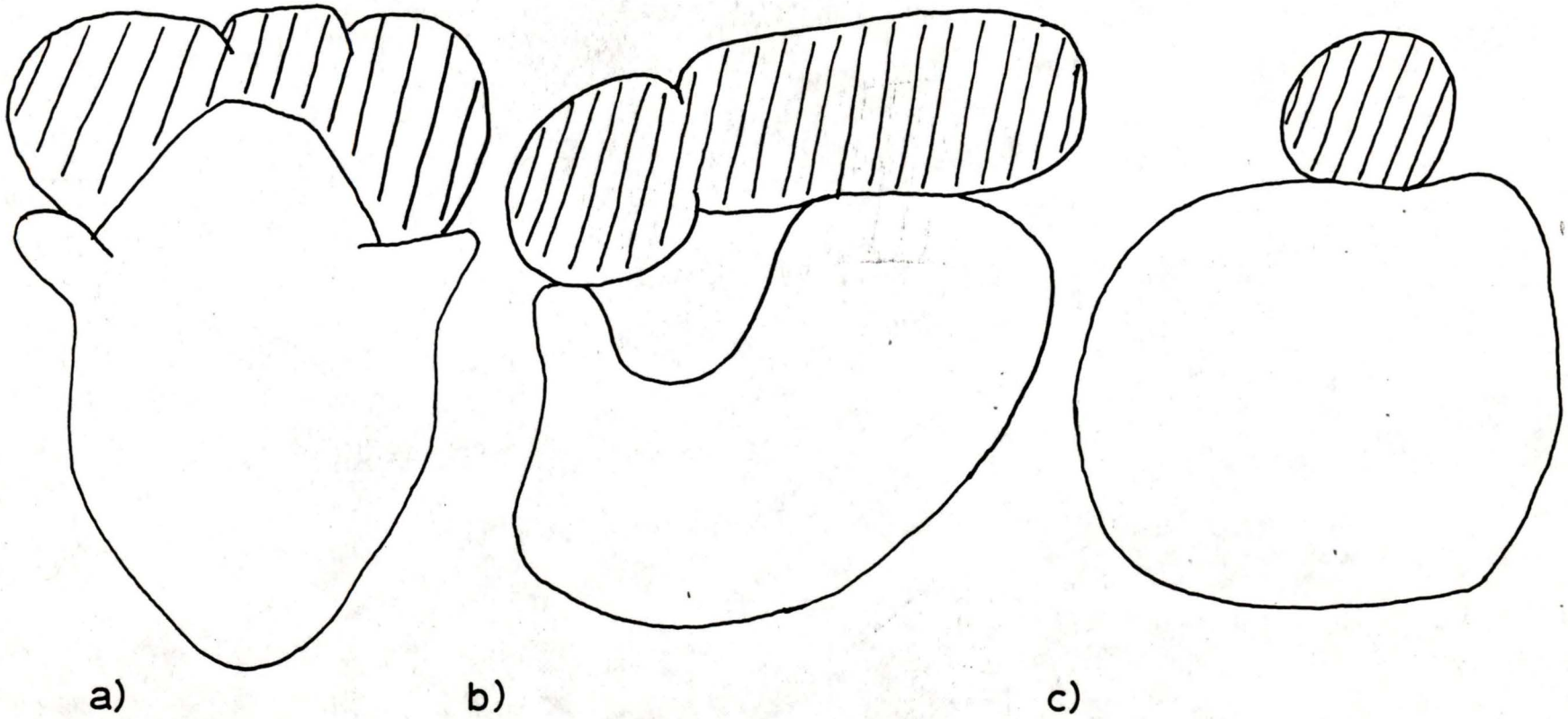


Figure 15. Electron micrograph interpretations of eukaryotic monosomes.
[a] Boublik and Hellman model from A. salina (74)
[b] Lake's "skiff" shaped model (86) [c] Lake's lateral view model (86)

3. A. The ribosomal 'A' protein domain

Ribosomal 'A' proteins are very acidic proteins found ubiquitously in large ribosomal subunits, and are equivalent to the ribosomal protein L7/L12 from E. coli (27). The term 'A' or acidic refers to the fact that they are normally the most acidic proteins in the ribosome and contain a large amount of glutamate and aspartate.

The complete amino-acid sequence of 'A' proteins from E. coli(22), the moderate halophile NRCC 11227 (Matheson, personal communication) and Bacillus subtilis (95) have been determined. In addition there is a large amount of N-terminal sequence data on 'A' proteins from other sources (96). The results of the sequence data will be discussed in a later section in relation to the evolution of the ribosome.

(i) The ribosomal 'A' proteins from eubacteria

The most extensively studied 'A' protein is L7/L12 from E. coli. L7 differs from L12 only in that it has an acetylated N-terminal serine (22). These proteins are 120 residues long (97) and exist as a dimer in solution (98).

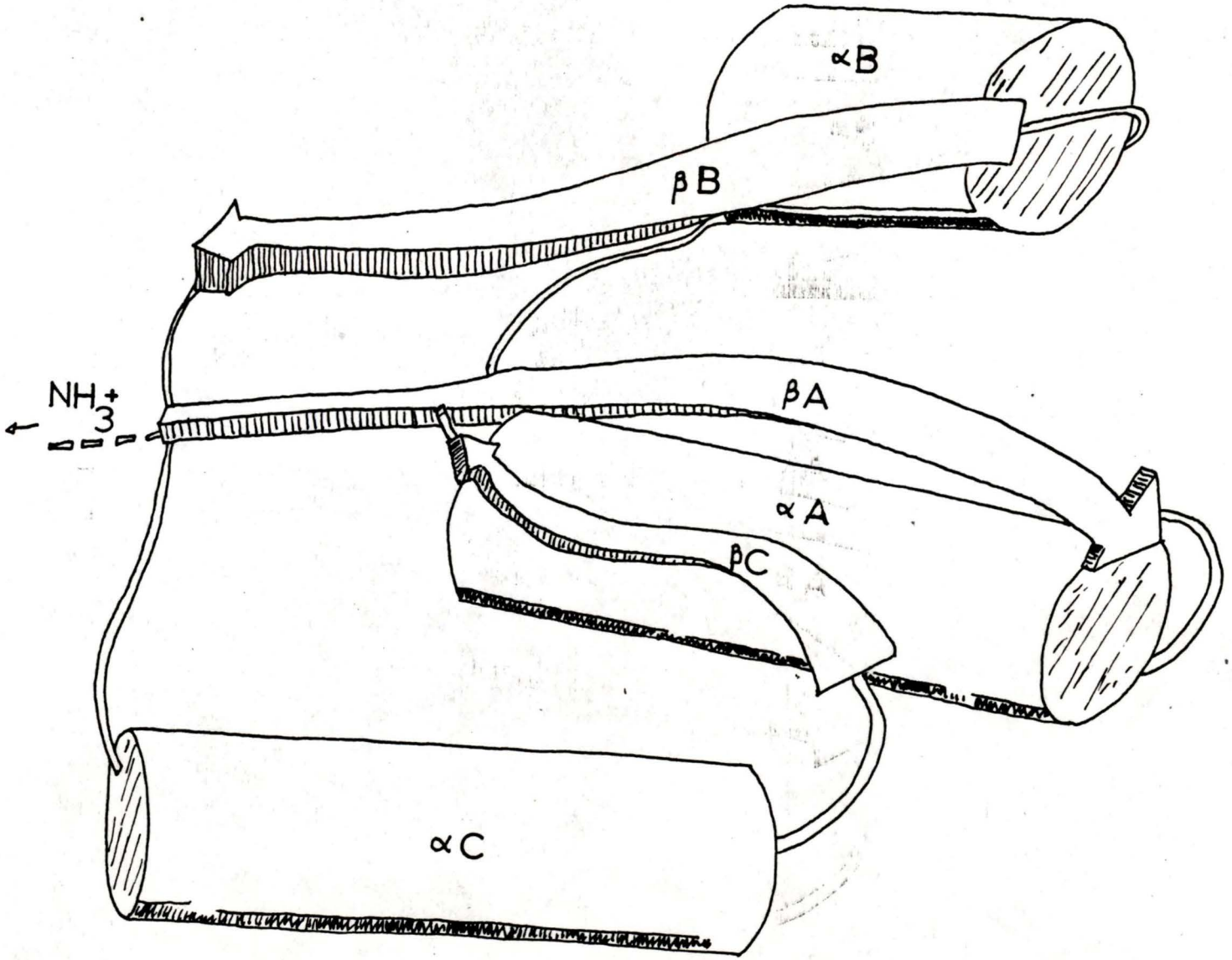
CD studies (99) on a mixture of intact proteins L7 and L12, and L7 alone (29) indicated 50-55% α-helicity. Similar studies using fragments of L7 suggested that the N-terminal fragment 1-73 has 50-55% helicity while the C-terminal fragment 72-120 has 25% helicity (29). Gudkov et al (29) determined that there was no β-structure in the L7 molecule. Calorimetric studies (29) indicated an ordered

array within the N-terminal fragment of L7 while PMR (29) data pointed to an importance of hydrophobic residues in maintaining the L7 protein as a dimer. The physical data was interpreted (29) in a model of the dimer as a 'dumbbell' in which two N-terminal helical regions interact with each other in an 'antiparallel' manner while the distal C-terminal parts are globular (39). Antiparallel implies that the N-terminal region of one molecule is near the C-terminal of the other. In total contradiction, Luer and Wong (100) suggest a 'parallel' model for the dimer which indicated the two N-termini are at one end, and C-termini at the other. The explanation for these contradictory results is not known at the present time. However, the results of both the preceding authors are consistent with L7/L12 as a stalk extending from the 50S subunit (84).

Two separate fragments 1-36 and 53-120 of the L7/L12 protein have been crystallized (38); however, a 3D structure has only been obtained for the 53-120 C-terminal fragment (Fig. 16). This structure is described as 'plum-shaped', of dimensions 20 x 20 x 35 Å, containing 38 residues (56%) in α-helices and 17 residues (25%) in β-strands. The presence of β-structure in L7/L12 is contradictory to the physical studies of Gudkov et al (29).

L7/L12 involvement in EFG and EFTu dependent GTPase activity has been demonstrated by removal of L7/L12 from 50S subunits (101). However, it was noticed that only the dimer of L7/L12 was required to restore almost

Figure 16. Tertiary structure of carboxy-terminal fragment (CTF) from E. coli r-protein L7/L12 (38). See text for details.



total EFG dependent GTPase activity (98). Cross-linking studies (78) have further indicated the involvement of L7/L12 with IF-2, RF1 and RF2 (Figure 13).

L7/L12 has been isolated from the 70S ribosome complexed to r-protein L10 in a ratio of 2:1 and 4:1 (89). Cross-linking experiments (78) have further established the association of L7/L12 with L10, and Dijk et al (102) have suggested that this complex is associated with 23S RNA through L10 and that the binding is stimulated by the r-protein L11.

An acidic protein BL13 has been isolated from B. stearothermophilus (90), and although its complete amino acid sequence is not known, its extensive homology with L7/L12 (21) has allowed Marquis et al (90) to suggest that the BL13 N-terminal also has high α-helical content (29).

BL13 and BL8 have been isolated from the ribosome as a 4:1 complex (90). It is suggested that the predominantly α-helical N-terminal one-third of BL13 interacts with BL8 on the ribosome similar to the interaction of L7/L12 with L10.

Reconstituted 50S subunits in which BL13 is replaced by the N-terminal fragment of BL13 have the same functional properties (translational) as the complete 50S subunit but polypeptide synthesis is reduced, as is EFG and GTP binding, indicating that the distal portion of BL13 is required in the EFG binding site (90).

(ii) Ribosomal 'A' proteins from archaebacteria

An acidic alanine-rich 50S subunit r-protein has been isolated from the extreme halophile H. cutirubrum (103). The secondary structure of this r-protein HL20 has been studied by CD and is estimated to have approximately 50% α-helical content (104).

An 'A' protein complex has also been isolated from this archaebacterium, comprising HL20 and HL11 (91). This complex is unusual in that it was isolated under conditions of high salt (3.4 M KCl) and was remarkably stable to denaturing conditions. This complex consists of the 'A' protein HL20 and HL11 in a 2:1 or possibly 4:1 ratio. (Liljas et al. unpublished. See ref. 64.) Experiments are in progress to determine whether the HL20-HL11 complex will bind 23S RNA as L7/L12 - L10 binds in the E. coli system.

(iii) Ribosomal 'A' proteins from 80S ribosomes

The NH_4Cl/Ethanol extraction (105) of eukaryotic 80S ribosomes, including rat liver (106), A. salina (106), S. cerevisiae(107, 108), BHK fibroblasts (27) and wheat germ (21), has yielded r-proteins comparable to L7/L12 of E. coli.

Two main acidic r-proteins eL12 and eL12' (molecular weight 13,000 and 13,500) and their phosphorylated derivatives eL12-P and eL12'-P have been isolated from the 60S subunit of A. salina (106), while in rat liver r-proteins P_1 and P_2 (molecular weight 15,200 and 16,000)

have been isolated from 60S subunits (106).

Similarly, two acidic proteins with a phosphorylated serine residue have been identified from the 60S subunit of BHK fibroblasts (27). The molecular weights of these two r-proteins are 13,500 and 14,500.

Sanchez-Madrid et al (108) have identified three acidic proteins in 60S subunits of S. cerevisiae; two of these, A_1 and A_2 (molecular weight 12,000), differ only in the degree of phosphorylation, while the third A_x (molecular weight 13,000) has been found in the cytosol as well as attached to the ribosome (107, 108). The acidic A_x protein binds the ribosome more tightly in its tri-phosphorylated form, and the authors concluded that the degree of phosphorylation of the r-proteins may be related to ribosome function. This may be visualized as a cycling 'on and off' of the acidic protein from the ribosome (27).

Early immunological data pointed to an equivalence of L7/L12 and several eukaryotic 'A' proteins (27), even though little sequence homology is evident between these proteins (21). It was earlier shown that L7/L12 could functionally replace 'A' proteins in several eukaryotic ribosomes (109). However, there have been no recent reports of interchange-ability of these 'A' proteins with L7/L12 and the immuno-logical studies appear successful only with unfractionated ribosomes or proteins in polyacrylamide, which may have led to artificial results (27). Furthermore, Sanchez-Madrid et al (108) report that there is a far less stringent

requirement for acidic proteins in S. cerevisiae for factor binding than in bacterial cells, although the r-proteins eL12 and eL12-P from A. salina have been shown to be involved in EF-1 dependent binding of amino acyl tRNA and EF-2 dependent GTP hydrolysis as in E. coli (106).

The only known similarities between L7/L12 and eukaryotic 'A' proteins are that they are extracted under the same conditions, are of similar molecular weight, are present in multiple copies and are most likely situated at the interface of the subunits (110, 111).

The research described in this thesis has attempted to determine whether an 'A' protein complex exists within the 60S subunit of wheat germ comparable to those isolated from E. coli, B. stearothermophilus and H. cutirubrum, and to ascertain a conformational and functional comparison with these organisms. Some indication of a conformational homology may be suggested from the E.M. studies of Boublik and Hellman (74) who visualize an 'acidic stalk' on A. salina 60S subunits. However, although Lake has observed these stalks (personal communication to ATM) he states that they are too few in number to be classed as significant. One may then consider the statement of Leader (27) concerning a cycling 'on and off' of the acidic proteins between the cytosol and ribosome; they may therefore only be observed under certain conditions, for instance during protein synthesis or just before this occurs, and this may account for the small number observed at any one

time.

B. 5S RNA-protein domains

5S RNA can be released from the large subunit of ribosomes as a 5S RNA-protein complex consisting of one molecule of 5S RNA and one to three proteins depending on the source of the ribosome (92, 87, 93, 112-118).

(i) Eubacterial 5S RNA-protein complexes

5S RNA-protein complexes have been obtained from B. stearothermophilus and E. coli by reconstitution of the RNA and protein (119), either using individual proteins or a total 50S protein mixture (113). Alternatively, native 5S RNA-proteins complexes have been isolated by mild treatment of 50S subunits with carrier-bound ribonuclease (87) or by a 1 M NH_4Cl/10mM EDTA extraction followed by isolation on a 5-20% sucrose density gradient or gel filtration (117).

B. stearothermophilus complexes contain 2 proteins, BL5 and BL22 while E. coli complexes have 3 proteins EL5 (equivalent to BL5), EL18 (equivalent to BL22) and EL25 (113). Recent cross-linking studies also suggest a fourth protein EL31' may also be associated with the E. coli 5S RNA-protein complex (120).

CD studies have shown that binding of r-proteins to 5S RNA changes the 5S RNA conformation and stability (121). It has been suggested that the binding of the r-protein increases stacking of specific single stranded bases in 5S RNA and aligns them in helical arrays resulting in a conformation that facilitates base pairing with 23S RNA

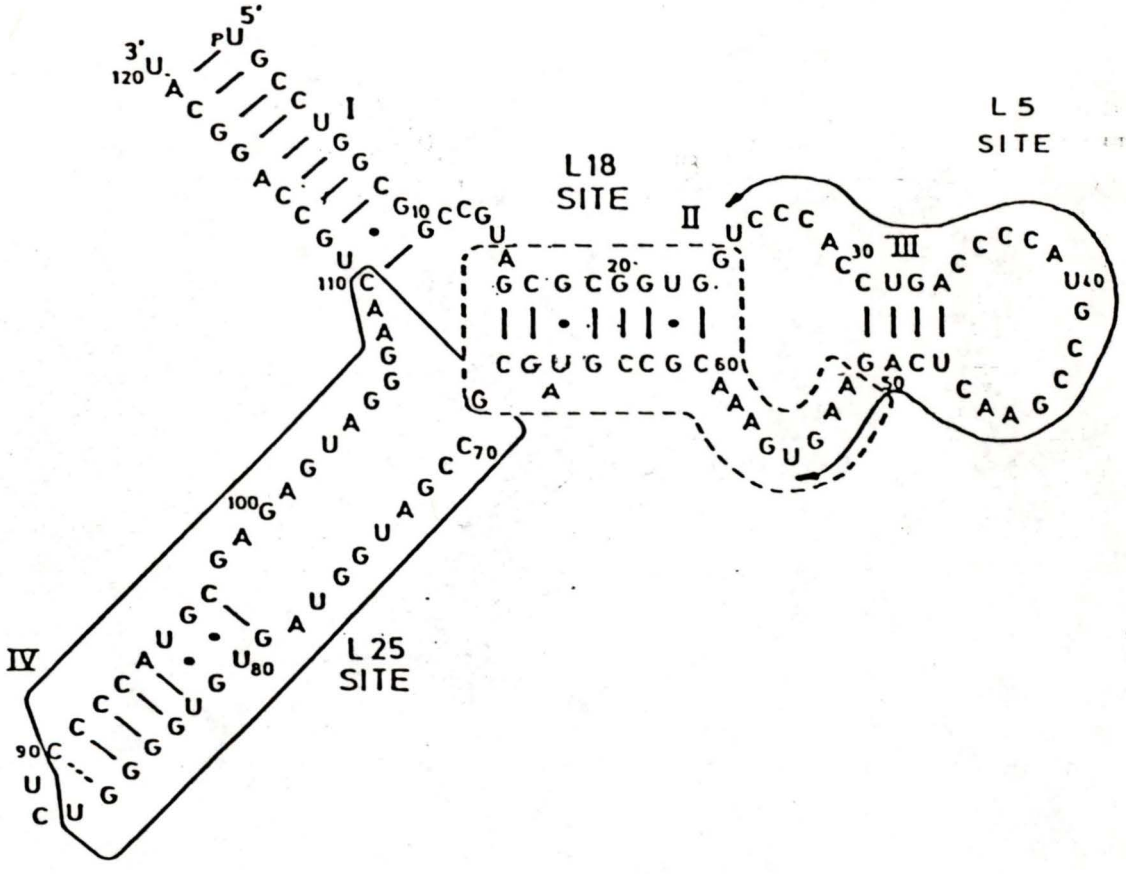
or tRNA or both (121).

5S RNA-complexed to a single protein has been studied in E. coli in order to determine the protein binding sites and conformational effects of the individual proteins on the 5S RNA molecule. For example, RNase A and T_2 digestion of the L25-5S RNA complex indicates the binding of L25 at nucleotides 1-11, 68-87 and 90-120 (122). However, it has also been observed that nucleotides 1-11 and 90-120 are equally resistant to ribonucleases in the presence and absence of r-protein L25 (123, 124). Douthwaite et al (123, 55) suggest that the binding site for L25 is most likely on nucleotides 69-110, but do not make any comment on the regions 1-11 and 90-120 mentioned above (Fig. 17). CD and fluorescence studies (125) indicate that binding of L25 causes a small conformational change on the 5S RNA which may stabilize the interaction of L18 binding.

Protein L18 is suggested to have an extended binding site on 5S RNA since it protects much of the 5S RNA from RNase digestion. The binding of L18 causes a major conformational change in RNA (125). A proposed L18 binding site is shown in Figure 17. Douthwaite et al (125) believe that L18 has a complex interaction with 5S RNA tertiary structure and its release on RNase A digestion may be due to a conformational change of the 5S RNA to an 'A' conformation.

The primary binding site for L5 is at residues 24-56 deduced by complexing ^{32}P-labelled 5S RNA with L5, L18 and

Figure 17. Binding sites for E. coli r-proteins L25, L18(122) and L5 (42) to 5S RNA. (See text for details.) Regions I-IV show base pairing regions.



L25, followed by digestion with ribonuclease (42) [Fig.17].

Both r-proteins L5 and L18 are required for the 5S RNA-protein complex to interact with 23S RNA and form an equimolar ribonucleoprotein complex (126), with the site of interaction on the 23S RNA molecule approximately 600 residues from the 3'-terminus (69). The significance of a complementary region at the 5'-terminus of 23S RNA and sequence 72-83 on 5S RNA remains unclear (126).

Using IEM the 3'-end of 5S RNA has been located at the central protuberance of the 50S subunit (84). Almost all models for the 5S RNA, e.g., Figure 17, have residues 1-11 and 110-120 as part of a base paired region; therefore the 5' end of 5S RNA is also located at the central protuberance. If L25 is indeed bound to the 1-11 and 90-120 residues of 5S RNA (122), it also positions this protein at the central protuberance of the large subunit, which is in agreement with Lake (73). Furthermore, cross-linking data of Traut et al (78) indicates protein L5 is at the interface of the 50S and 30S subunits (Figure 13) and IEM data of Stoffler et al (75) also shows L5, L18 and L25 in a central position at the subunit interface.

Low angle neutron-scattering studies (42) have been used to determine <u>in situ</u> shapes of 5S RNA-protein complex components. The results suggest that L18 and L25 are globular, and 5S RNA is elongated. Furthermore, oligonucleotide binding, chemical modification, RNase digestion and IR spectroscopy suggest a high number of quaternary

interactions. Several models have been suggested in interpretation of this data (42, 45); however, since the secondary conformation of 5S RNA is still in dispute, it is difficult to favour one model over another.

Reconstitution of 50S ribosomes in the absence of 5S RNA has shown an inability of the 50S subunit to bind amino acyl tRNA (42). Inhibition of enzymatic and non-enzymatic binding of aminoacyl tRNA by a fragment TΨCG from tRNA, or an analogue of this fragment, indicated an interaction of 5S RNA 'C_{43}GAAC_{47}' and the tRNA loop IV sequence GTΨCG. However, there is evidence to dispute this interaction. If the sequence CC$_{43}$GAA is removed from the 5S RNA molecule and the remaining 5S RNA fragment rejoined with ligase and reconstituted into 50S subunits, active protein synthesizing 50S subunits are produced (127). The 5S RNA-protein complex has further been reported to have ATPase and GTPase activities (128). However, R. Villems (personal communication to ATM) was unable to detect ATPase or GTPase activity in E. coli 5S RNA-protein complexes. It is evident, therefore, from this data that there is considerable confusion as to the role of 5S RNA in the ribosome.

Because of the small size of 5S RNA and the 5S RNA-protein complex, any conformational change in the 5S RNA-protein complex might well result in little overall conformational change in the ribosome (55). Since the 5S RNA-protein complex is found at the subunit interface close to L16, a protein which is essential in peptidyl

transferase activity (75), to protein L11 that stimulates GTPase activity and to L7/L12-L10 which is involved in ribosomal factor binding, it would appear that the 5S RNA-protein complex may also have some important function, although at present this function remains to be determined.

(ii) The archaebacterial 5S RNA-protein complex

A 5S RNA-protein complex has been isolated from the archaebacterium H. cutirubrum (92). This complex is released by treating the 50S subunit, which requires 3.4 M. K^+ and 0.1 M Mg^{2+} for stability, with low levels of Mg^{2+} (0.3 mM). The complex was purified on agarose columns and was found to contain 5S RNA and 2 proteins HL13 and HL19, which from physical data (104) and sequence data (92), appear to be equivalent to E. coli r-proteins EL18 and EL5 respectively.

The secondary structure of the complex has been studied using ethidium-bromide probing, CD and limited nuclease digestion (128). The results indicate a decrease in secondary and tertiary confromation of 5S RNA when the r-proteins are bound. RNase digestion of the native 5S RNA-protein complex of H. cutirubrum indicates that the protection of the 3'-end of the RNA molecule is due to HL13 while HL19 confers no protection.

Attempts to find ATPase and GTPase activities of the H. cutirubrum 5S RNA-protein complex have been unsuccessful (92).

(iii)Eukaryotic 5S RNA-protein complexes

5S RNA-protein complexes have been isolated from several eukaryotic sources including rat liver (93, 114, 116, 129), rabbit reticulocytes (114) and yeast (130). These complexes generally have one protein bound to the 5S RNA. This protein, however, has a molecular weight equivalent to the sum of the molecular weights of the three E. coli 5S RNA binding proteins (131). 5S RNA-protein complexes from rat liver have been isolated by EDTA extraction and formamide extraction (93). EDTA extraction (114) and cross-linking with UV irradiation (132) indicate 5S RNA bound to protein L5. Similarly, one protein (YL3) has been isolated bound to yeast 5S RNA (131).

The rat liver 5S RNA-L5 complex has been shown to have aminoacyl tRNA and EF-2 dependent GTPase activity (133). 5S RNA-L5 may therefore be the centre of GTPase activity comparable to that suggested to occur in eubacterial 5S RNA-protein complexes (128, 134).

4. Function

Ribosomes associated with amino acyl tRNA's, soluble protein factors, GTP, mRNA, K^+, Mg^{2+}, NH_4^+ and other factors are responsible for the synthesis of polypeptides. The nature of the amino acids in the polypeptide chain is determined by the information contained within the mRNA (135). Protein synthesis has been extensively studied

and reviewed in several recent articles (135, 136, 137). The following will be a brief synopsis.

Protein synthesis can be conveniently broken down into three stages: (i) initiation, (ii) elongation and (iii) termination.

(ia) Initiation of protein synthesis in eubacteria

In protein synthesis an initiation complex is first formed between the ribosomal subunit, 30S, mRNA and fmet·$tRNA_f^{met}$ at the peptidyl transferase (P) site of the ribosome (138). The steps in the initiation process are shown below:

[1] $70S \xrightarrow{IF3} 50S + 30S\ IF3$

[2] $30S IF3 \xrightarrow[IF1]{mRNA} 30S mRNA$

[3] $30S\ mRNA \xrightarrow[GTP]{fmet\ tRNA_f^{met}} \xrightarrow[IF2]{} \xrightarrow{IF3} 30S mRNA : fmet\ tRNA_f^{met}$

(30S initiation complex)

[4] 30S initiation complex

$$\xrightarrow[IF1]{50S} \xrightarrow[IF2]{} \xrightarrow{GDP+P_i} 70S \text{ initiation complex}$$

70S ribosomes first dissociate into their 50S and 30S subunits, a process requiring IF3. The 30S subunit is then associated into an initiation complex by two steps: [1] IF3 dependent binding of mRNA to the 30S ribosome and [2] the IF2

binding of the fmet·tRNA to the mRNA-30S complex, a process requiring GTP (139). IF1 may also be required for binding mRNA to the 30S subunit.

Crosslinking data (78) has indicated possible locations of the initiation factors on the small subunit. IF3 has been linked with E. coli 30S subunit proteins S1, S11, S13, S19 and S21, while IF2 has been crosslinked to S1, S2, S11, S13, S14, S19 as well as L7/L12 of the 50S subunit (78). IF1 has been crosslinked to S1, S12, S13 and S19. One can infer from this data that all the initiation factors bind in approximately the same regions and that this is likely to be close to the interface of the small and large subunits.

A role for 16S RNA has been suggested in which the 3' end of 16S RNA is the binding site for the 5' end of mRNA (187). This mRNA:16SrRNA binding aligns the AUG codon for binding the anticodon in a 30S·fmet.tRNA complex (136,140).

Once the 30S subunit complex is formed, the 50S subunit re-associates with the 30S subunit accompanied by hydrolysis of GTP to GDP+P_i and release of IF1 and IF2. The fmet·tRNA is bound at the P site and interacts with the AUG codon on the mRNA. Beside the codon-anticodon interaction there is also some evidence to suggest that 5S RNA may interact with the aminoacyl tRNA and in binding to the P site (42). The 70S initiation complex is then ready for the elongation stage of protein synthesis.

(ib) Initiation of protein synthesis in eukaryotes

The steps in initiation of eukaryotic protein synthesis

are outlined below:

[1] $80S \xrightarrow{eIF3} 60S + 40S$

[2] $40S \xrightarrow{eIF3} 40SeIF3$

[3] $\text{met.tRNA} \xrightarrow[\text{GTP}]{\text{eIF2}} \text{met.tRNA}^{met}\text{:eIF2:GTP}$

[4] $40SeIF3 \xrightarrow[eIF4c]{\text{met.tRNA}^{met}\text{:eIF2:GTP}} 40SeIF3eIF4c\text{met.tRNA}^{met}\text{eIF2 GTP}$

[5] $40S\ eIF3eIF4c\ \text{met.tRNA}^{met}\ eIF2\ GTP \xrightarrow[\substack{eIF1\\eIF4a\\eIF4b}]{\text{mRNA, ATP}} 40S\text{ initiation complex}$

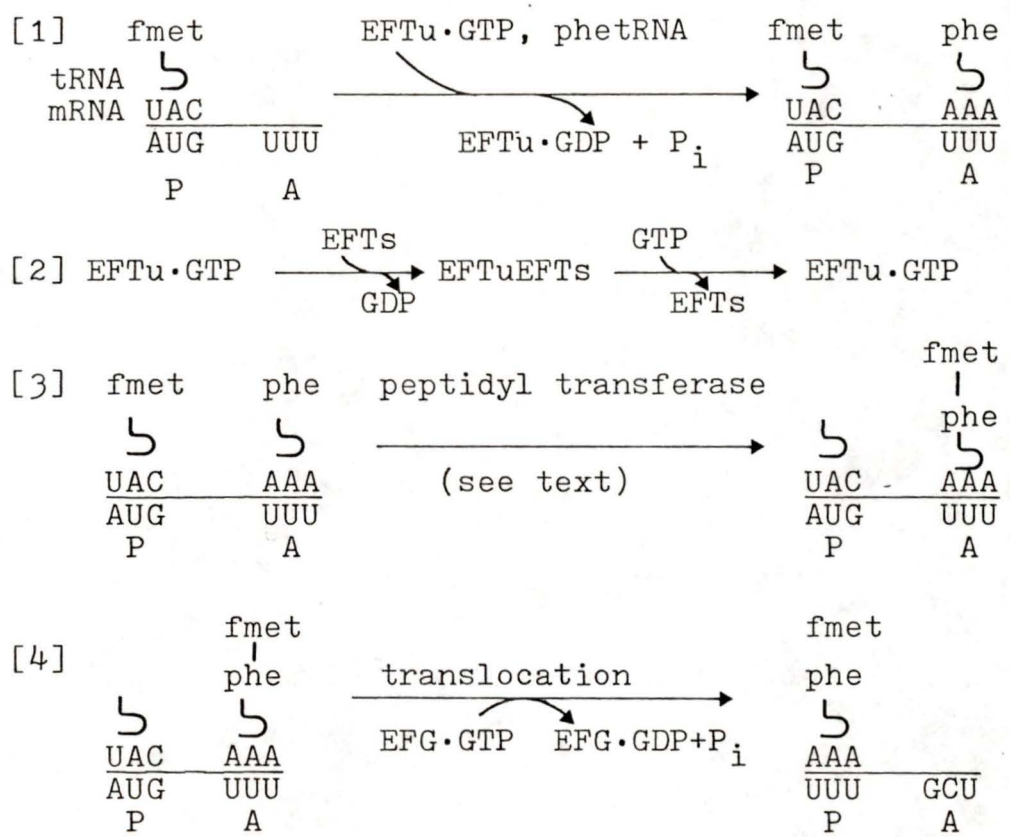
[6] $40S\text{ initiation complex} \xrightarrow[60S]{eIF5} \xrightarrow[GDP + P_i]{\text{initiation factor}} 80S\text{ initiation complex}$

The occurence of initiation factors in eukaryotes was first reported by Richard Sweet in 1968. Since that time many factors have found to be associated with the initiation process (139).

In eukaryotes, eIF3 complexes with the 40S subunit which in the presence of eIF4c associates with a met.tRNA_i^{met}· IF2·GTP complex. A 40S initiation complex is then formed on the binding of mRNA, a process requiring ATP, eIF1, eIF4a and eIF4b. In the presence of eIF5, the 60S and 40S subunits reassociate to form an 80S initiation complex (137). The importance of ATP in mRNA binding is unclear since no ATPase factors have been discovered.

(iia) Elongation in eubacteria

The specific binding of the initiation codon (AUG) on mRNA at the P site of the ribosome aligns the next triplet code at the aminoacyl (A) site (138) of the ribosome ready for addition of an amino acylated tRNA. The sequence of elongation events are summarized below (135, 136).



Binding of an incoming tRNA to EFTu and GTP forms an amino acyl tRNA.EFTu.GTP complex. EFTu.GTP is hydrolyzed to EFTu.GDP + P_i and released from the ribosome. EFTs interacts with EFTu.GDP to produce EFTu.EFTs followed by replacement of EFTs with GTP to regenerate the EFTu.GTP complex (140). The EFTu.GTP complex is then free to bind

another amino acyl tRNA.

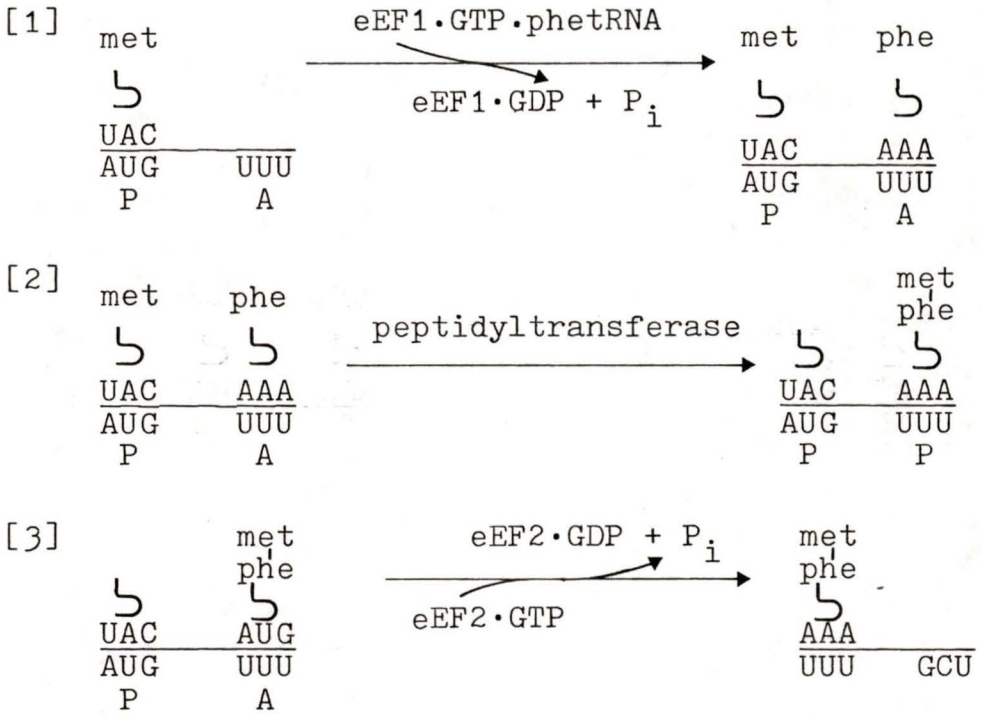
Once the A and P sites are occupied, a peptide bond is formed between the amino acids catalyzed by the ribosomal complex peptidyl transferase. Hampl et al (159) through reconstitution studies have shown that r-proteins L2, L3(4), L15, L16, L18, L20 and L24 associated with 23S RNA are essential for peptidyl transferase activity. They further showed that L20 and L24 are involved in assembly of this region, and proteins L13, L17, L21, L22, L27 and 5S RNA are involved in the reconstitution of activity. Furthermore, Stoffler et al (75) identified several r-proteins by IEM that they believed to be involved in the peptidyl transferase site, namely, L2, L11, L14, L16, L18,L23 and L27. Cross-linking data from Traut et al (78) identified L2-L11, L11-L14, L16-L23 and L16-L27 at the peptidyl transferase site. A comparison of results from the various researchers indicates r-proteins L2, L16, L18 and L27 are common in all models.

For peptide formation utilizing peptidyl transferase, the N-formyl methionyl residue at the P site is transferred and bonded to the NH_3^+ group of the amino acid residue at the 'A' site. This process is repeated during each elongation step. The transpeptidation reaction is followed by a translocation process: this process required EFG, a GTPase and GTP, and results in [1] release of deacylated tRNA in the P site, [2] translocation of the tRNA from the A site to the P site, and [3] movement of the mRNA so that

the next codon is positioned at the A site and the cycle repeats (135, 136).

(iib) Elongation in eukaryotes

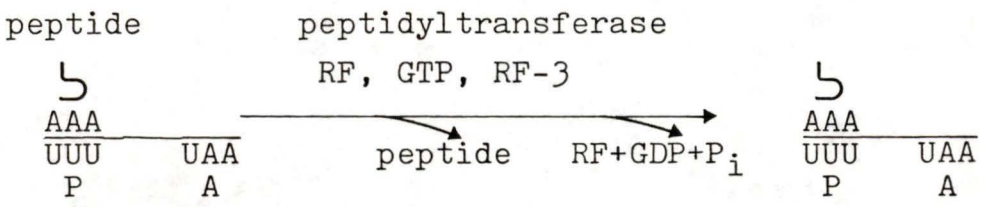
The elongation process in eukaryotes is outlined below:



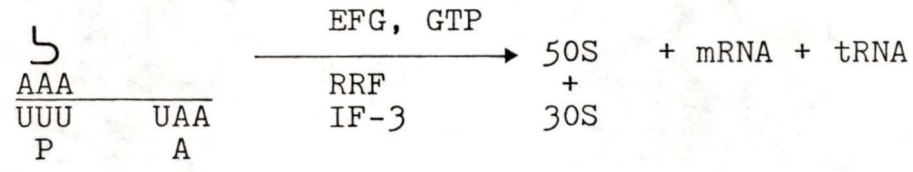
Elongation in eukaryotes is comparable to that in eubacteria with eEF1 equivalent to EFTu. eEF1 forms a complex with GTP which binds an amino acyl tRNA. This complex then binds to mRNA at the A site with hydrolysis of GTP and release of the eEF1·GDP complex. A peptide bond is formed as in eubacteria catalyzed by peptidyl transferase. The translocation process in eukaryotes requires the soluble factor eEF2, corresponding to EFG in eubacteria (137).

(iiia) Termination in eubacteria

[1]



[2]

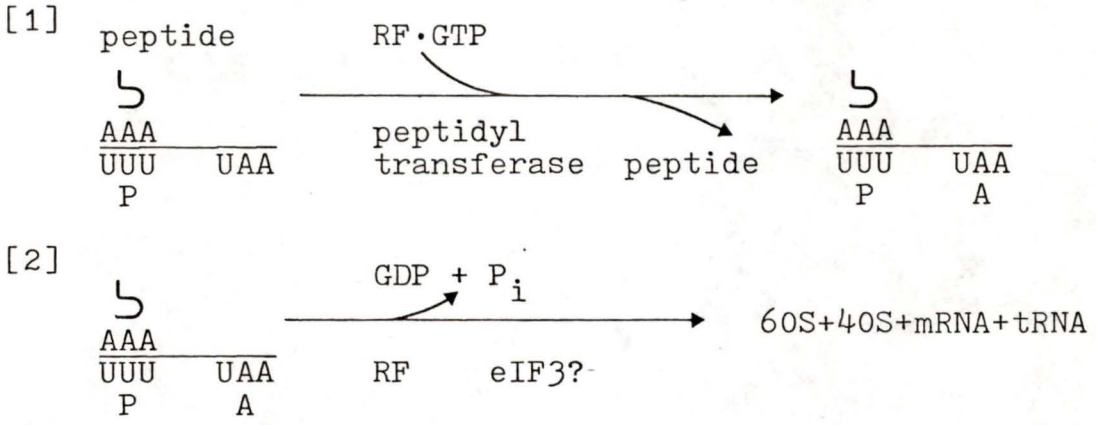


Termination is brought about by a termination codon, (UAA, UAG or UGA) on the mRNA at the A site. At this point the peptidyl tRNA is attached at the P site. In the presence of GTP and an RF, the completed polypeptide is released, leaving a deacylated tRNA at the P site. The GTP·RF complex is thought to induce release of the polypeptide by triggering the hydrolytic cleavage of its carboxyl terminal amino acid from the final amino acyl-tRNA bound to the ribosome. The particular RF used is determined by the codon. RF1 is specific for UAA or UAG and RF2 for UAA or UGA (140).

Peptidyl transferase is also believed to be involved in termination since peptidyl transferase inhibitors prevent peptide release. It is thought that the RF interaction with the terminator codon at the A site activates peptidyl transferase to release peptides. Once the peptide is released, the tRNA is then released and the ribsomes dissociates from the mRNA, a process involving EFG, IF-3, and the ribosome release factor RRF (135, 136).

(iiib) Termination in eukaryotes

Termination of protein synthesis in eukaryotes is outlined below:



Termination of protein synthesis requires a RF and GTP to bind the terminator region followed by peptidyl transferase hydrolysis of the peptidyl tRNA to release the peptide. GTP hydrolysis is followed by the release of RF, peptidyl transferase, tRNA and mRNA from the dissociated 80S ribosome (137). ATP and eIf3 may also be involved in the termination process (72).

(iv) Conclusion

The evidence so far presented indicates that the 'A' proteins may be associated with binding of soluble factors and GTP hydrolysis at several stages of the translation process and therefore play an important role in protein synthesis.

Data from both prokaryotic (89, 90) and archaebacterial (91) sources indicate the presence of an 'A' protein 'domain' in the large ribosomal subunit. This

domain is made up of four copies of the 'A' protein and one copy of another protein; e.g., 4 copies of L7/L12 and 1 copy of L10 in E. coli. However, the presence of such a domain in eukaryotes has not been demonstrated. It was decided therefore to search for this domain in a eukaryotic cell. Since considerable sequence data was already available for the 'A' protein from wheat germ, it was convenient to use this as the eukaryotic source.

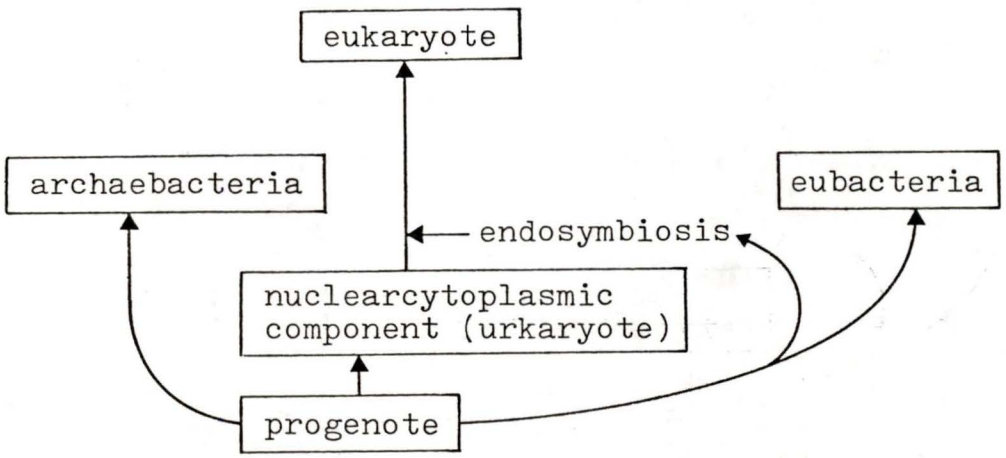
5. Ribosomal Evolution

Since translation is an essential function of all living organisms, it is expected that the cellular apparatus utilized in this process, the ribosome, has been conserved throughout evolution; and this is in fact the case. Although there are several physical differences between ribosomes of prokaryotes and eukaryotes (see earlier discussion), their function is equivalent. However, comparative studies on ribosomal RNA structures have led to revolutionary new ideas of cellular evolution being presented by Woese and his colleagues (7, 47).

Prior to 1977, the distinctions between organisms were made on the microscopic level and cell types were classed as characteristically prokaryotic or eukaryotic. This led most biologists to believe that living organisms arose from two lines of descent. This idea is seriously challenged today. It was the comparison of 16S and 18S RNA sequences that first led Woese and Fox (7, 47) to

propose a model of three kingdoms or evolutionary lines of descent. To date almost 200 species of bacteria and eukaryotes have been characterized by the classification of Woese and Fox (141). The three lines of descent include: [1] the eubacteria or true bacteria which includes the blue-green bacteria and chloroplasts, the gram-positive and the gram-negative bacteria (7); [2] the archaebacteria which to date includes the methanogens(142), extreme halophiles and thermoacidophiles (141) and [3] the nuclearcytoplasmic components in the eukaryotic group (5), which includes animal, plant, fungus and slime molds (7). Woese and Fox (47) suggest that there was a universal ancestor of the three kingdoms, the progenote, and that the three lineages diverged at a time when the cell's basic molecular processes were still being evolved and refined, with each lineage evolving its own independent solution to the translational mechanism. They further suggest that the nuclearcytoplasmic component, also referred to as a urkaryote (47), developed into the eukaryotic cell by an endosymbiotic pathway by engulfing certain eubacterial organisms, forming the eukaryotic cellular organelles (mitochondria and chloroplasts). There is much evidence available to support this concept (5, 6). Ribonuclease T_1 catalogues show homologies between plastid and blue-green algal 16S RNA nucleotide sequences but no homologies with cytoplasmic 18S RNA molecules in the same eukaryotic cell (5). Similar

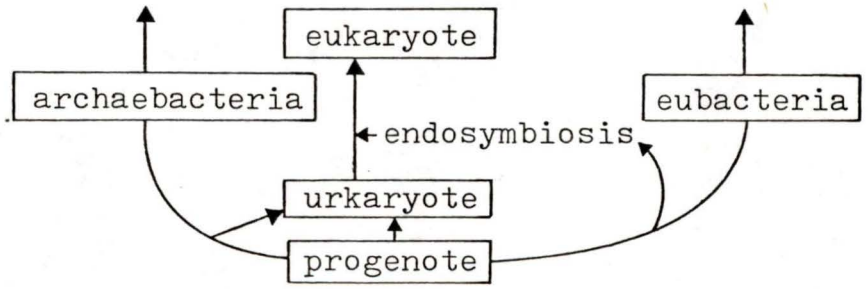
evidence has been presented by Edwards and Kossel (6) who have shown homologies between the 23S rDNA nucleotide sequence of Zea mays chloroplasts and E. coli 23S rDNA. A flow diagram representing these ideas of evolutionary origin is shown below. It should be pointed out that the origin of the eukaryotic nucleus is still far from clear.



As indicated earlier (7) the archaebacteria may be classified as a separate evolutionary line of descent. There is now much evidence in support of this idea. Although archaebacteria have several features resembling eubacteria, they also have several unique characteristics. Archaebacteria have many cell wall types, but none are the muramic acid-based peptidoglycan type. Cell membrane lipids of eubacteria and eukaryotes are composed of two straight-chain fatty acids bound at one end to a glycerol molecule through an ester linkage, whereas archaebacteria have a glycerol group linked to two branched hydrocarbon chains, with the connection between the glycerol and hydrocarbon chains being an ether link (143).

Furthermore, the modification patterns of 10 archaebacterial tRNA's have been analyzed and have been shown to be distinct from typical eubacteria and eukaryotes (144). Furthermore, Zillig and Stetter (145) have shown that the archaebacterial transcriptional machinery is as distinct as those of eubacteria and eukaryotes. For review see Zillig and Stetter (145).

In several features, the archaebacteria also resemble eukaryotes. Protein synthesis in the archaebacterium H. cutirubrum is initiated by methionine as in eukaryotes and not N-formyl methionine as in eubacteria, and the initiator $tRNA^{met}$ acceptor stem 5'-terminal base is base-paired as it is in eukaryotic initiators (146). A further evolutionary tool has been the ubiquitous ribosomal 'A' protein (147). Comparative sequence homologies of these r-proteins indicates a closer homology of archaebacterial 'A' proteins to eukaryotic 'A' proteins than to eubacterial 'A' proteins (21), and this has led to the suggestion that certain eukaryotic genes may be of archaebacterial origin (5, 141, 148). With this idea in mind one can redraw the earlier flow diagram with a link between archaebacteria and eukaryotes:



It must be made clear that although there is much evidence in favour of a three kingdom lineage, the interpretations of the data are speculative and the answer to our evolutionary origin will depend on further investigation within the cell itself.

II. MATERIALS AND METHODS

E. coli 5S RNA-protein Complex Extraction

The materials and methods described in this section were intended to allow isolation and identification of the 5S RNA-protein complex from E. coli and ultimately to use these procedures to isolate a 5S RNA-protein complex from an archaebacterium such as Methanobacterium thermoautotrophicum.

A. Growth of cells

Cells from a culture of E. coli K12 were transferred to 10 ml of trypticase soy broth (BBL Microbiology Systems) and grown to late-log phase (O.D. 0.55) at 37°C. A 10% inoculum of the E. coli cells was transferred to 200 ml of trypticase soy broth and again grown to late-log phase at 37°C. The total 200 ml of cells was then used to inoculate 10 litres of the trypticase soy broth in a 14 litre microferm fermentor (New Brunswick Scientific Co.). The cells were continuously aerated at 37°C and harvested in a Beckman continuous-flow rotor (JCF-2) at late-log phase. A typical growth curve is shown in Figure 18. Cells were washed in cold 10^{-2}M TMK buffer (Table 1).

B. Ribosome isolation

The washed cells were resuspended in 10^{-2}M TMK buffer (100g/100ml) and sonicated at 0°C in a sonifier cell disruptor (Bronson Sonic Power Co.) 20 x 30s with 30s cooling of the sonifier tip between each sonication. The sonicated cells were centrifuged at 10,000 rpm for

Figure 18. Growth curve (measured at 660 nm) for E. coli at 37°C in trypticase soy broth.

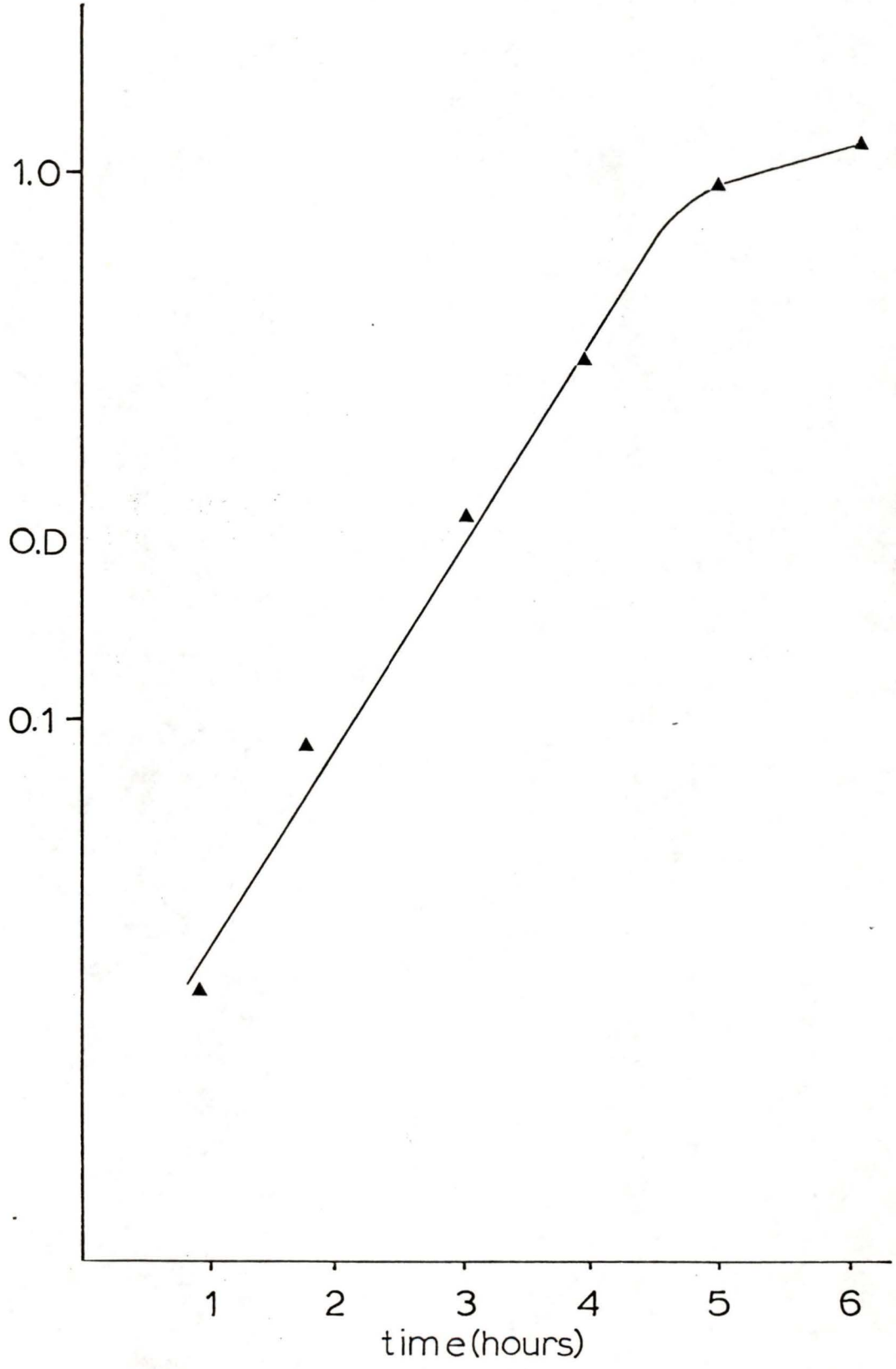


TABLE 1

Buffers used in washing, dissociating and extraction of ribosomes and ribosomal proteins.

	10^{-2}M TMK (pH 7.6)	10^{-4}M TMK (pH 7.6)	TSM (pH 8.0)
Tris	10mM	10mM	10mM
$MgCl_2$	10mM	0.1mM	10mM
KCl	50mM	50mM	--
Succinic Acid	--	--	0.3mM
β-Me	6mM	6mM	--

10 mins in a GSA Sorvall rotor. The 10K10 supernatant was centrifuged at 18,000 rpm for 20 mins in an SS34 Sorvall rotor, completing removal of all cell debris. The 18K20 supernatant was then centrifuged at 50,000 rpm in a Beckman Ti50·2 rotor for 2 hours and the pellet stored at -80°C.

C. Isolation of ribosomal subunits

70S ribosomes (50K/2 hour pellet) were resuspended in 10^{-4}M TMK buffer (Table 1), and dialyzed against 2 changes of 6 litres of the same buffer. The dialyzed ribosome suspension was made 2% with sucrose, loaded onto an 8-32% sucrose gradient in 10^{-4}M TMK and centrifuged at 29,000 rpm for 18 hours in a Beckman Ti15 rotor. Fractions (15ml) were removed from the rotor using a 60% sucrose cushion and the A_{260} values measured (Fig. 19). Subunit fractions were pooled and the 30S and 50S ribosomes isolated by centrifugation at 49,000 rpm for 36 hours in a Beckman Ti50·2 rotor. Identification of the 30S and 50S ribosomes was performed by total protein and RNA extractions (vide infra). The 50S and 30S ribosomal pellets were stored at -80°C.

D. Protein isolation and purification

(1) Total protein extraction from 50S ribosomal subunits

Total protein was extracted from 50S subunits by the method of Hardy et al.(11). The 50S subunits were resuspended in TSM buffer (500 A_{260}/ml) [Table 1] at 0°C. In rapid succession, 0.1 volume of 1M $MgCl_2$ and 2 volumes

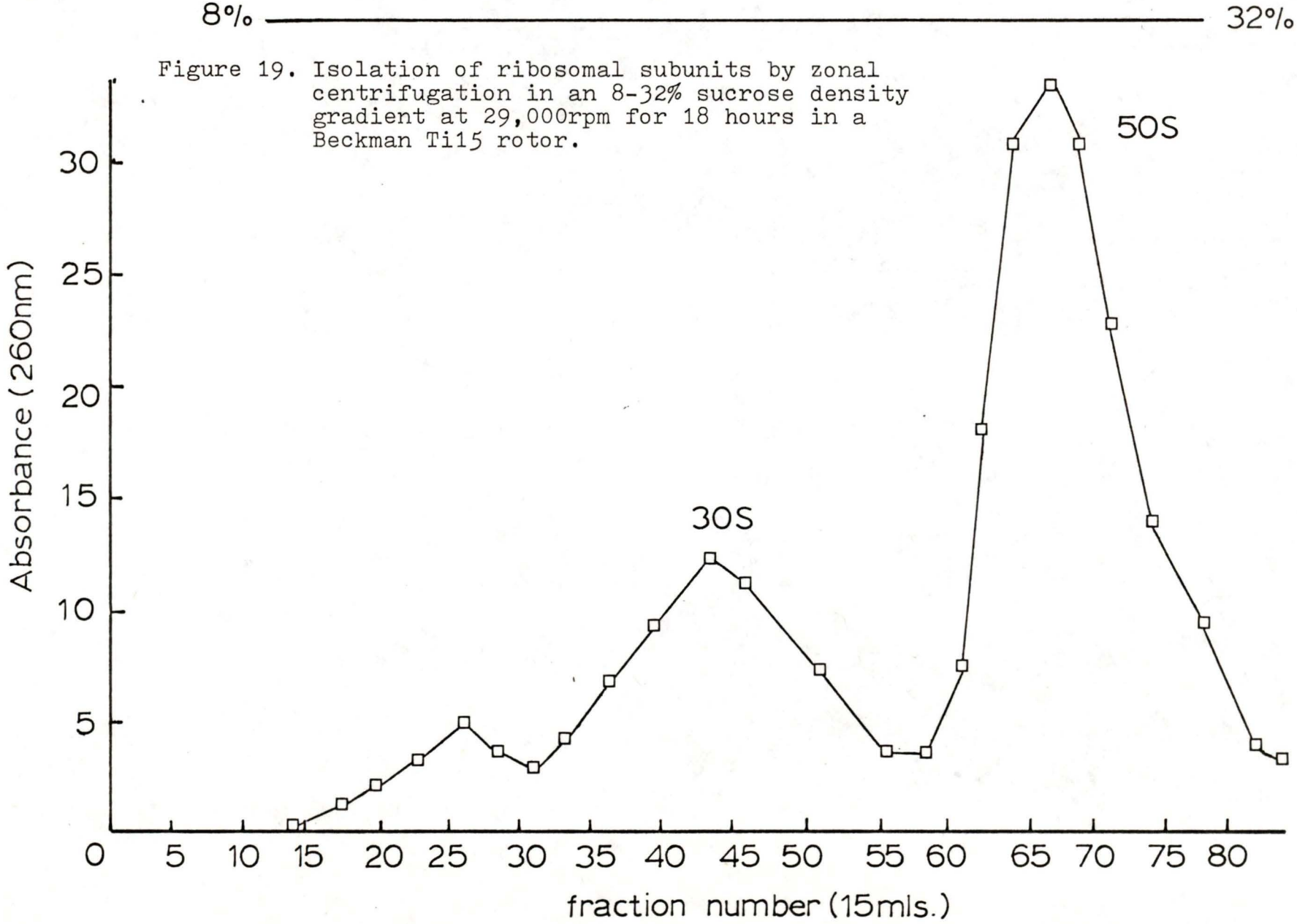


Figure 19. Isolation of ribosomal subunits by zonal centrifugation in an 8-32% sucrose density gradient at 29,000rpm for 18 hours in a Beckman Ti15 rotor.

of glacial acetic acid were added and stirred in an ice bath for 45 minutes. The suspension was centrifuged at 14,000 rpm for 10 minutes at 5°C in a Sorvall SS34 rotor. The 14K10 supernatant was dialyzed against 2 changes of 6 litres of deionized water, lyophilized, resuspended in water, redialyzed against water, lyophilized, and the protein stored at -80°C.

(ii) Polyacrylamide gel electrophoresis

(a) One-dimensional SDS gels

One-dimensional gels were run, with slight modification, according to the method of Neville (149). Solutions used in Neville SDS gels are shown in Table 2. Samples [1ug protein per protein band; protein was determined by the Lowry method (3)] were prepared for electrophoresis by mixing with an equal volume of solubilization reduction mix. Electrophoresis was carried out at 6mA/slab (120 x 140 x 1.5mm) through the stacking gel, as determined by the position of the bromophenol blue tracking dye, and 32mA/slab through the separating gel until the bromophenol blue was 1 cm from the bottom of the gel. Electrophoresis was towards the anode.

(b) Two-dimensional polyacrylamide gels

This technique was performed as described by Howard and Traut (18), adapted from the method of Kaltschmidt and Wittman (17). The solutions used in this method are shown in Table 3.

TABLE 2

Solutions for 14% Neville SDS gels (149)

1. 4 x Lower gel buffer (pH 9.18)

   0.1232N HCl
   0.698M Tris
   0.28M NaCl

2. 4 x Upper gel buffer (pH 6.1)

   0.1068M H_2SO_4
   0.2164M Tris

3. 10 x Reservoir buffer (pH 8.64)

   0.40M Boric Acid
   0.41M Tris
   1.0% w/v SDS

4. Stock acrylamide-stacking gel

   30% acrylamide: 2% bisacrylamide

5. Stock acrylamide-separating gel

   42% acrylamide: 0.36% bisacrylamide

6. Solubilization Reduction Mix

	1:1
SDS (w/v)	4%
β-mercaptoethanol(v/v)	10%
Upper gel buffer	2x
glycerol (v/v)	20%

TABLE 2 CONTINUED

7. Lower gel 14%

lower gel buffer (4x)	7.5ml
42% acrylamide:0.36% bisacrylamide	10.0ml
water	11.9ml
TEMED	42 µl
1% ammonium persulphate	0.6ml

8. Stacking gel 3%

Upper gel buffer (4x)	2.5ml
30% acrylamide:2% bisacrylamide	1.0ml
water	6.25ml
TEMED	20 µl
1% ammonium persulphate	0.24ml

TABLE 3

Solutions used for 2-D electrophoresis as described by Howard and Traut (18).

First dimension:

Separating gel, pH 8.7

6 M	urea
4 wt%	acrylamide
0.13 wt%	bisacrylamide
20 mM	Na_2EDTA
0.52 M	boric acid
0.4 M	Tris
0.45 ml/l	TEMED

polymerize with 5µl 10% ammonium persulphate per 1 ml gel solution

Running buffer, pH 8.2

10 mM Na_2EDTA

80 mM boric acid

60 mM Tris

TABLE 3 CONTINUED

Second dimension:

2D dialysis buffer, pH 5.2

8 M urea
40 mM acetic acid
10 mM KOH

2D separating gel solution, pH 4.5

6 M	urea
18.0 wt%	acrylamide
0.25 wt%	bisacrylamide
0.92 M	acetic acid
0.048 N	KOH
5.8 ml/l	TEMED

polymerize with 30 µl 10% ammonium persulphate per 1 ml gel solution

Running buffer, pH 4.0

0.18 M	glycine
6 mM	acetic acid

Samples were applied to 2 separate 1D tube gels, (60 x 5 mm), with one run from cathode to anode and the other from anode to cathode. Bromophenol blue and pyronin Y were used as tracking dyes respectively. Electrophoresis was carried out at 3 mA/tube for 30 minutes and then 6 mA/tube for 6 hours. The gels were removed from the tubes and dialyzed for 1 hour against dialysis buffer. (Alternatively, the gels could be stored at $5^{o}C$ prior to dialysis.)

While the 2D gel was polymerizing in the slab gel apparatus (120 x 140 x 1.5 mm), the 2 tube gels were placed with cathode and anode end facing each other (Fig. 20). Electrophoresis in the second dimension was towards the cathode at 30 V/slab for 30 minutes and then 80 V/slab until the pyronin Y tracking dye was within 1 cm of the bottom of the gel.

(c) Staining and destaining protein gels

Protein stains and destaining solutions are shown in Table 4 (152). Gels were generally left overnight in stain A, 4-5 hours in stain B, overnight in stain C and in stain D until the background had disappeared.

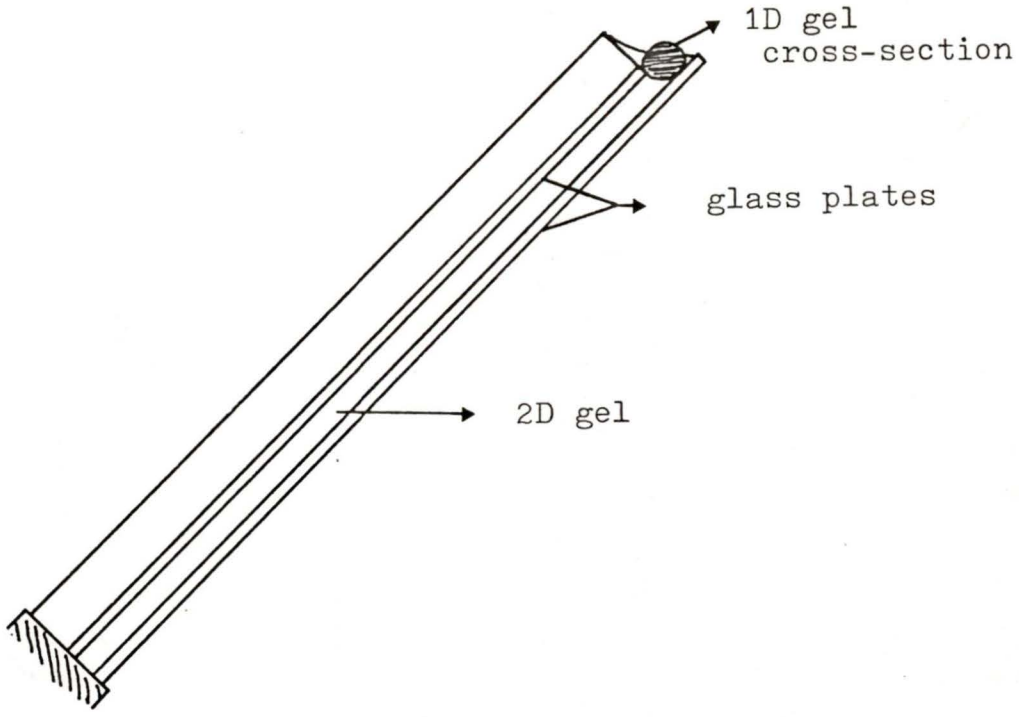
E. RNA isolation and identification

(i) Ribosomal RNA isolation

Ribosomal RNA was extracted from whole cells and 70S ribosomes by the method of Girard (151).

70S ribosomes (300 A_{260}/ml) were resuspended in 0.01 M EDTA-Na_2, 0.01 M Mg(acetate)$_2$, pH 5.1 (15 ml).

Figure 20. Apparatus for 2D gel run.



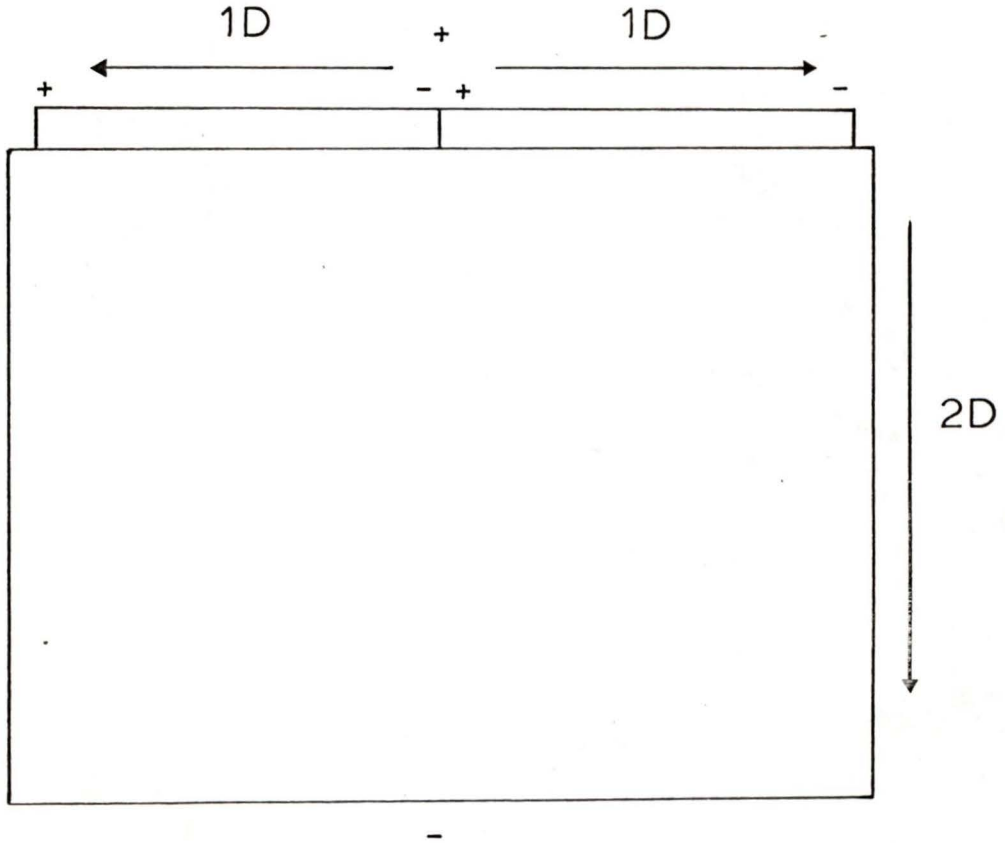


TABLE 4

(reference 152)

Protein Staining and Destaining Solutions

Stain (2 litres)	Isopropanol	Glacial Acetic Acid	Coomassie Blue %
A	25%	10%	0.03
B	10%	10%	0.003
C	--	10%	0.001
D	--	10%	--

SDS, 10% (0.7 ml) was added to the suspension in a 60 ml pyrex separating flask on ice. Phenol, (15 ml), was then added to the suspension and shaken vigourously in a 60°C water bath for 3 minutes. The flask was quickly placed in an ice bath to ensure prompt cooling. The phenolic and aqueous phases were separated by centrifugation at 10,000 rpm for 5 minutes in a Sorvall SS34 rotor. The phenolic layer was removed, while the white interphase was left with the aqueous layer. The above procedure was repeated with 10 ml and finally 7 ml of phenol. In the final step the aqueous layer was very carefully removed without the white interphase. The remaining phenol was removed by centrifugation at 10,000 rpm for 10 minutes in a Sorvall SS34 rotor. NaCl, 2 M, (0.1 volume) and 95% ethanol (2 volumes) were added to the aqueous phase and the suspension was left overnight at -20°C. The suspension was centrifuged at 30,000 rpm for 60 minutes in a Beckman Ti50·2 rotor and the RNA pellet stored at -80°C.

(ii) Isolation of 5S RNA

The RNA pellet from the phenol extract was redissolved in the extraction buffer (300 A_{260}/ml). This solution was loaded onto a Sephadex G-100 column, (95 x 1.6 cm), at a flow rate of 20 ml/hour and 5 ml fractions were collected. The A_{260} values of the fractions were read and an A_{260} profile plotted (Fig. 21). Fractions were pooled and the RNA precipitated with 2 volumes of

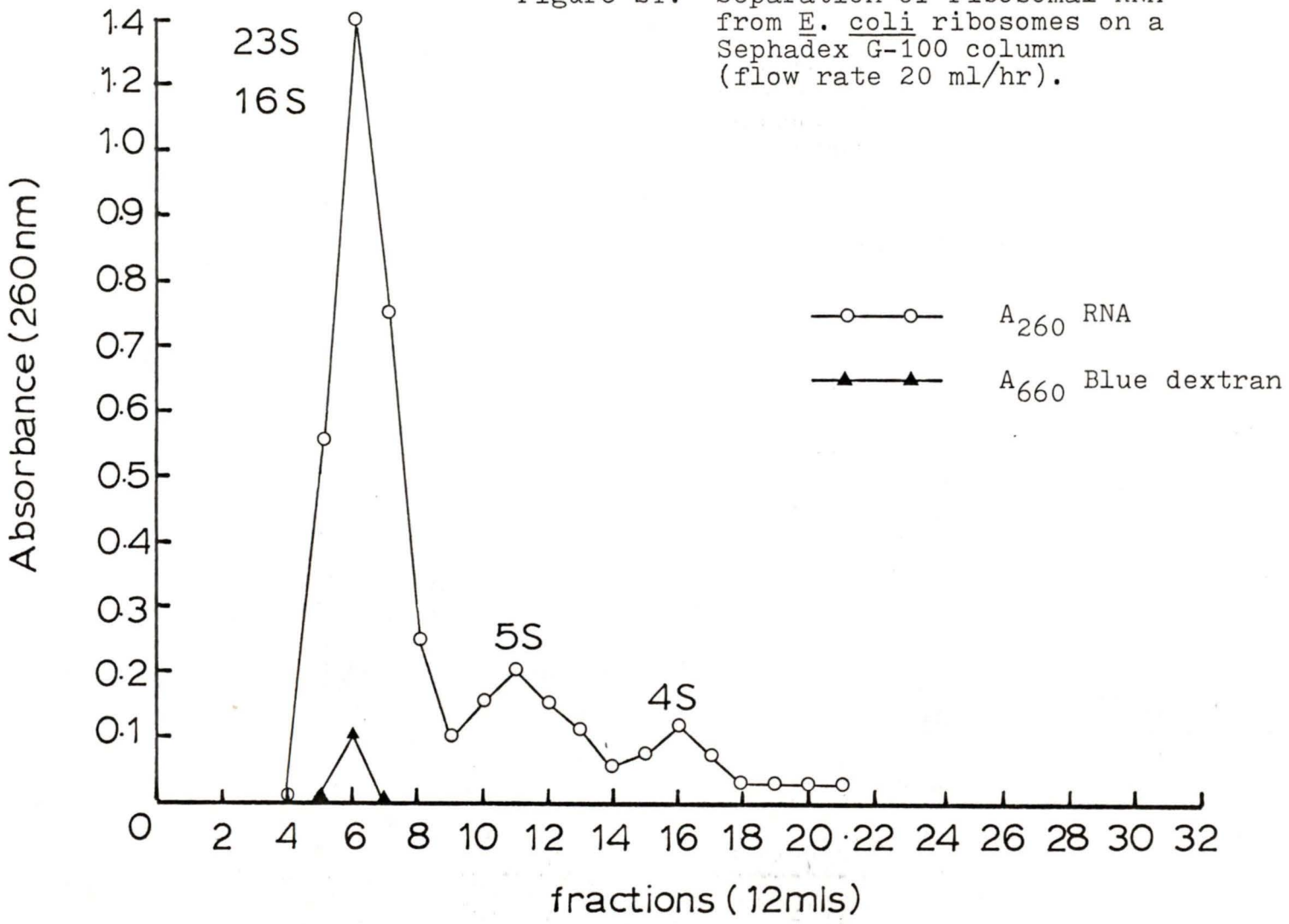


Figure 21. Separation of ribosomal RNA from E. coli ribosomes on a Sephadex G-100 column (flow rate 20 ml/hr).

95% ethanol at -20°C. The isolated RNA was run on both 7.5% (150) and 2% (153) polyacrylamide gels in order to identify both the small and large rRNA's respectively.

(iii) Polyacrylamide gel electrophoresis for RNA identification

(a) Polyacrylamide gels (7.5%)

Polyacrylamide gels (7.5%) were run according to the method of Loening (150). Solutions used in these gels are shown in Table 5. RNA samples (0.1 A_{260}'s) were equilibrated with the running buffer by addition of 0.2 volumes of 5x concentrated running buffer and 0.2% (w/v) SDS. The gels were run at 32 mA/slab towards the anode using bromophenol blue as the tracking dye.

(b) Polyacrylamide gels (2%)

Polyacrylamide gels (2%) were run according to the method of Peacock and Dingman (153). Solutions used in these gels are shown in Table 6.

Gels were prepared by refluxing agarose (0.8 g) in water(113 ml) at 110°C for 5 minutes. Buffer A (116 ml), DMAPN (10 ml) and stock acrylamide (16 ml), all at 45°C, were mixed with agarose and 1.6% ammonium persulphate (5 ml). A 10% polyacrylamide plug (0.5 cm) was also used to prevent the 2% gel from sliding out of the gel apparatus.

Samples (0.1 A_{260}) were run in 10^{-2} M TMK buffer with bromophenol blue as the tracking dye. Subcellular fractions were first treated with 0.1% SDS. Gels were pre-run for

TABLE 5

Solutions for 7.5% RNA gel electrophoresis (150)

Acrylamide stock solution:

12% w/v acrylamide
0.6% w/v bisacrylamide

Running buffer, pH 7.8

36 mM Tris
30 mM Na_2PO_4
1 mM EDTA

Gel composition

Acrylamide stock	6.25 ml
5 x concentrated running buffer	2.0 ml
H_2O	1.75 ml
TEMED	8 µl
10% ammonium persulphate	0.12 ml

TABLE 6

Solutions used in 2% RNA gel electrophoresis (153)

Buffer A

Tris	108 g
Na_2EDTA	9.3 g
Boric aicd	55 g

made to 1 L with dH_2O

20% stock acylamide

acrylamide	19 g
bisacrylamide	1 g

made to 100 ml with dH_2O

DMAPN

6.4% in dH_2O

10% acrylamide gels

10% stock acrylamide (15 ml)
10% AMP (0.1 ml)
TEMED (25 µl)

0.2% methylene blue RNA stain

0.2 M Na-acetate
0.2 M Acetic acid
0.2% methylene blue

45 minutes at 100 V and then at the same voltage for the sample run. RNA gels were stained for 30 minutes in 0.2% methylene blue solution and destained under running tap water.

F. Isolation and characterization of the 5S RNA-protein complex

Isolation of the 5S RNA-protein complex was attempted according to the methods of Anders Liljas (personal communication) and Chen-Schmeisser and Garrett (87). Solutions used in the extraction procedure of Liljas are shown in Table 7.

50S ribosomes were resuspended in buffer I (50mg/100ml) and stirred for 1 hour at 5°C. This suspension was dialyzed against two changes of six lites of the same buffer. It was then made 10 mM with EDTA-Na_2 and 1 M with NH_4Cl and stirred for 1 hour at 5°C. The suspension was centrifuged at 35,000 rpm for 15 hours in a Beckman Ti60 rotor. The "23S core pellet"[1] was stored at -80°C and the supernatant at 5°C.

The supernatant was made 2% with sucrose and loaded onto a 5-20% sucrose gradient in buffer II, and centrifuged at 29,000 rpm for 17 hours in a Beckman Ti50 zonal rotor.

1. The "23S core pellet" is the pellet remaining after the EDTA/NH_4Cl extraction of the 50S ribosomes. This core contains 23S RNA and any ribosomal proteins attached to 23S RNA as well as proteins that are not preferentially extracted under these conditions.

TABLE 7

Solutions Used for Isolation of the 5S RNA-protein complex

Buffer I

20 mM Tris, pH 7.6
2 mM $MgCl_2$
6 mM β-mercaptoethanol

Buffer II

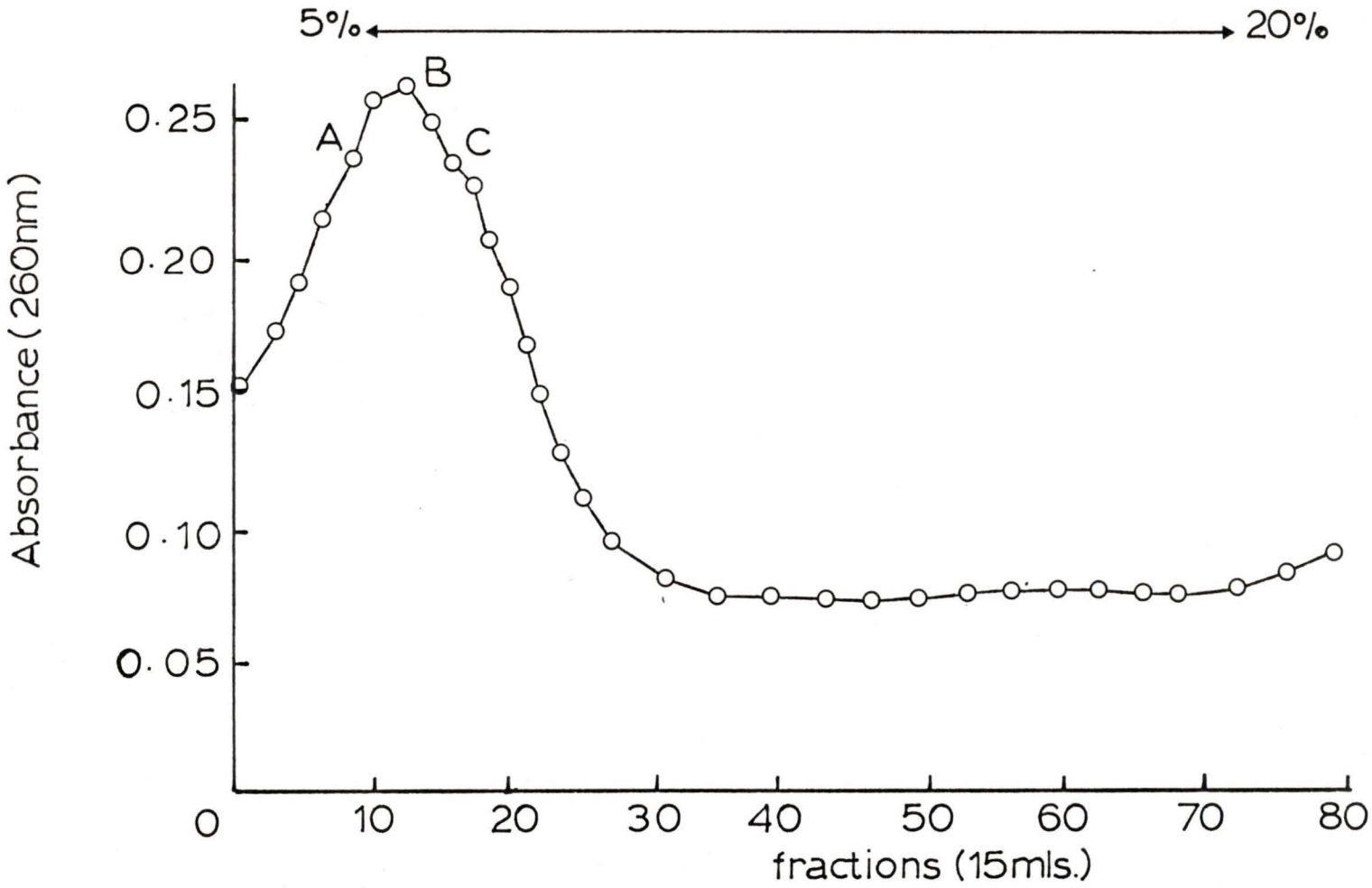
20 mM Tris, pH 7.6
10 mM $MgCl_2$
50 mM KCl
6 mM β-mercaptoethanol

Buffer III

50 mM Tris, pH 7.6
2 mM $Mg(Acetate)_2$
0.3 M KCl
1 mM β-mercaptoethanol

Fractions (15 ml) were removed using a 50% sucrose cushion and A_{260} values measured (Fig. 22). Fractions were run on 15%, 7.5% and 2% polyacrylamide gels. Fractions from the 5-20% sucrose gradient containing 5S RNA were precipitated with 95% ethanol at -20°C. The suspension was centrifuged at 14,000 rpm for 10 minutes in a Sorvall SS34 rotor and the pellet dissolved in buffer III before loading onto a Sephacryl(S200) column, (95 x 2.6 cm). Samples were run at 35 ml/hour and 4 ml fractions were collected and run on SDS polyacrylamide gels.

Figure 22. Isolation of 5S RNA-protein complex by zonal centrifugation in a 5-20% sucrose gradient. For details on fractions A, B and C, see Results and Discussion.



III. RESULTS AND DISCUSSION

The 5S RNA-protein complex from E. coli

Ribosomes are ribonucleoprotein complexes and as such are held together by many ionic, hydrophilic and hydrophobic interactions. Techniques adopted to isolate the 5S RNA-protein complex have utilized the breakdown of the ionic interactions by the chelating of Mg^{2+} ions or competition for Mg^{2+} binding sites with monovalent cations, either by decreasing the Mg^{2+} concentration or by raising the monovalent cation concentration (154).

At a Mg^{2+} concentration of 10^{-2}M the 70S ribosomes of E. coli remain intact, but if the Mg^{2+} concentration is lowered to 10^{-4}M, the 70S ribosomes dissociate into 50S and 30S subunits. The subunits may then be isolated by sedimentation in a sucrose gradient. Isolation of the 50S subunit provides a suitable starting point for extraction of the 5S RNA-protein complex.

A. Isolation and characterization of protein and RNA from E. coli ribosomes

RNA extracted from 70S ribosomes of E. coli and from whole cells of E. coli and H. cutirubrum was run on the 2% polyacrylamide gels of Peacock and Dingman (153). These polyacrylamide gels, stabilized with agarose, allow separation of the large rRNA molecules, 23S and 16S RNA, from the small RNA molecules 5S and 4S RNA. The rRNA's in Figure 23 may be identified when compared with the published gel profile for E. coli rRNA of Peacock and

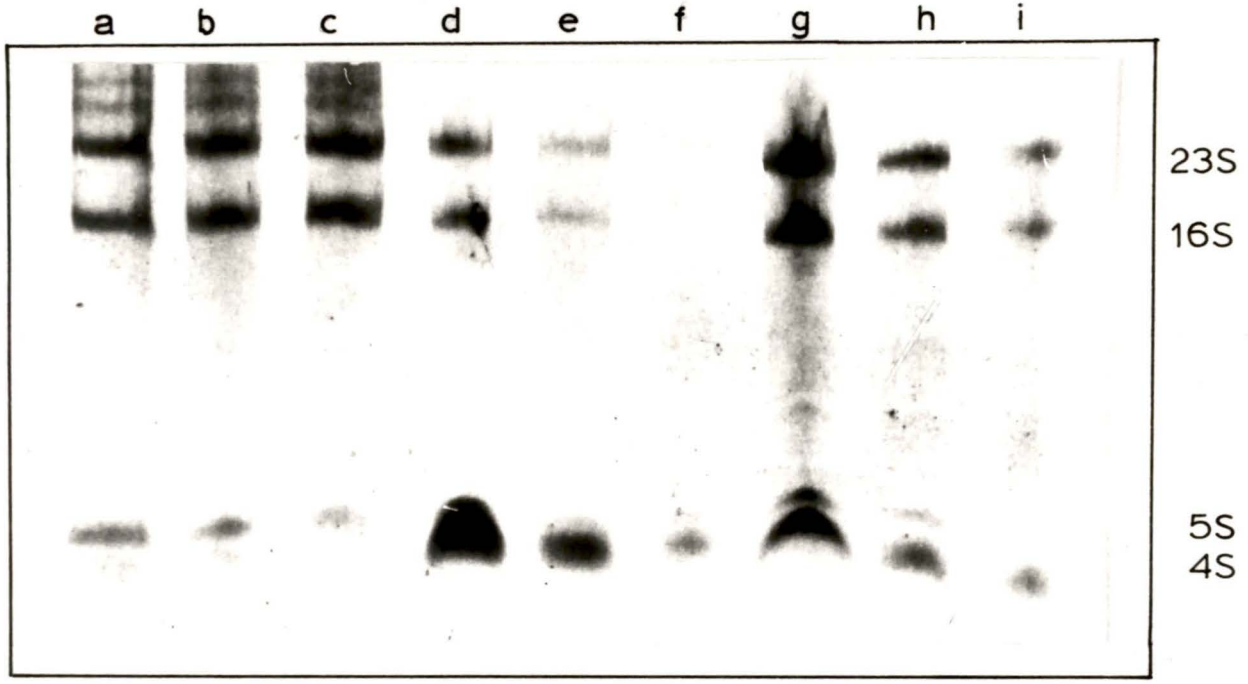


Figure 23. Separation of rRNA on 2% polyacrylamide gels.

Total RNA extracted from E. coli 70S ribosomes. (a) 1.0 A_{260}, (b)0.82 A_{260}, (c)0.68 A_{260}

Total RNA extracted from E. coli whole cells. (d)0.72 A_{260}, (e)0.36 A_{260}, (f)0.18 A_{260}

Total RNA extracted from H. cutirubrum whole cells. (g)0.87 A_{260},(h)0.48 A_{260},(i)0.24 A_{260}

Dingman (153). The proportions of 23S and 16S RNA appear to be in equimolar amounts, while the proportions of 5S RNA to 4S RNA vary depending on whether RNA is extracted from 70S ribosomes or whole cells. RNA extracted from the 70S ribosomes of E. coli show that the major proportion of RNA is ribosomal, as would be expected, with a small amount of 4S RNA contributed by tRNA associated with the ribosome at the time of the extraction. RNA extracted from whole cells of both E. coli and H. cutirubrum shows a higher proportion of 4S RNA when compared with RNA extracted from 70S ribosomes. This result is expected since whole cells contain cytosolic RNA as well as rRNA. RNA was also extracted from H. cutirubrum for comparative purposes as it was expected that an attempt would be made to isolate a 5S RNA-protein complex from an archaebacterium after isolating the 5S RNA-protein complex from E. coli.

Total RNA extracted from the 70S ribosomes of E. coli was passed through a Sephadex G100 column, a technique that effectively separated the large and small RNA's (Figure 21). Pooled fractions from the eluate of the G100 column and RNA from the 70S ribosomes of E. coli were run on the 7.5% polyacrylamide gel system of Loening (150) [Fig. 24]. Comparison of this gel and the published gel profile of Loening allows the 5S RNA to be identified and used as a marker for further investigation.

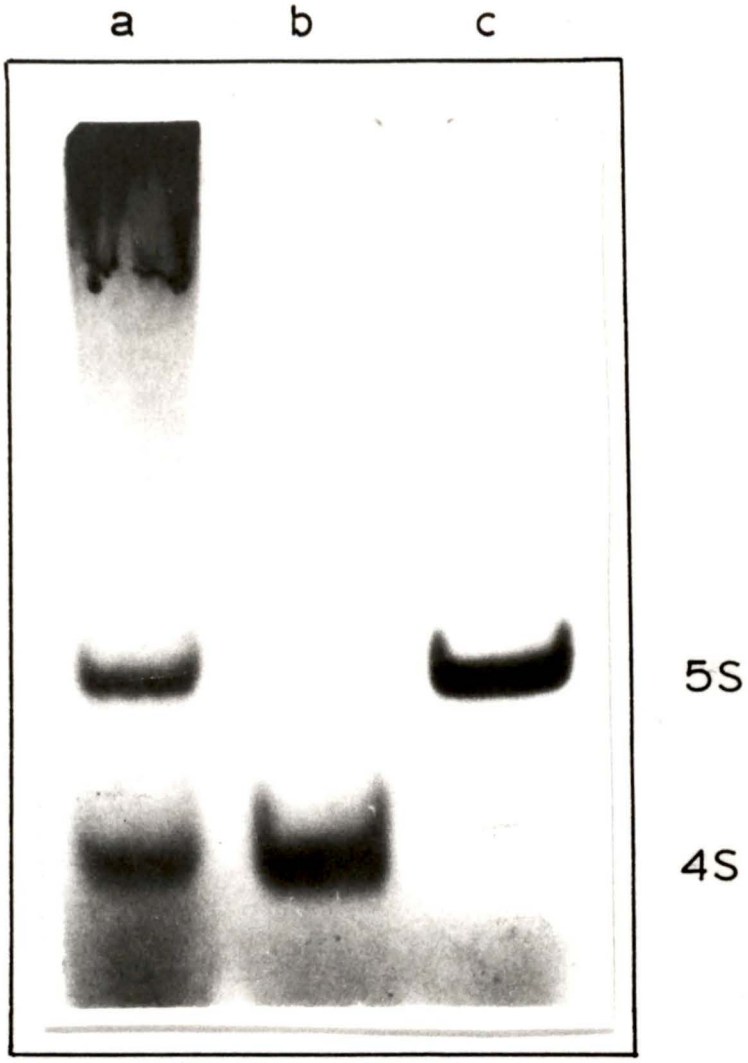


Figure 24. Separation of 5S and 4S RNA on 7.5% polyacrylamide gels.

(a) Total RNA extracted from E. coli 70S ribosomes.

(b) 4S RNA isolated from total RNA extracted from E.coli 70S ribosomes and run on a G100 column.

(c) 5S RNA isolated from total RNA extracted from E.coli 70S ribosomes and run on a G100 column.

Once rRNA markers had been established, it was also necessary to produce ribosomal-protein markers in order to determine r-proteins associated with 5S RNA in the 5S RNA-protein complex. A standard system for comparison of r-proteins uses the modification of the 2-dimensional polyacrylamide gel system of Kaltschmidt and Wittman (17) described by Howard and Traut (18). Since it had previously been determined (87) that E. coli 5S RNA is associated with r-proteins of the large subunit, a 2D gel of the total protein of the 50S subunit from E. coli was run (Fig. 25) as an aid in future identification of the 5S RNA-binding proteins.

B. Isolation of a 5S RNA-protein complex from E. coli

As previously mentioned, the ultimate aim of the first part of this thesis was to isolate the 5S RNA-protein complex from an archaebacterium using techniques primarily developed for the isolation of the E. coli 5S RNA-protein complex.

Two procedures were attempted in order to isolate the 5S RNA-protein complex from E. coli. The first method was that of Chen-Schmeisser and Garrett (87) in which 50S subunits were treated with carrier-bound RNAase A (Boehringer). However, due to poor solubility of the enzyme, an RNAase:23S RNA ratio of 1:3,000 (w/v) required for digestion could not be accurately determined and did not lead to satisfactory results. It was decided, therefore, that another method of isolation for the 5S RNA-protein

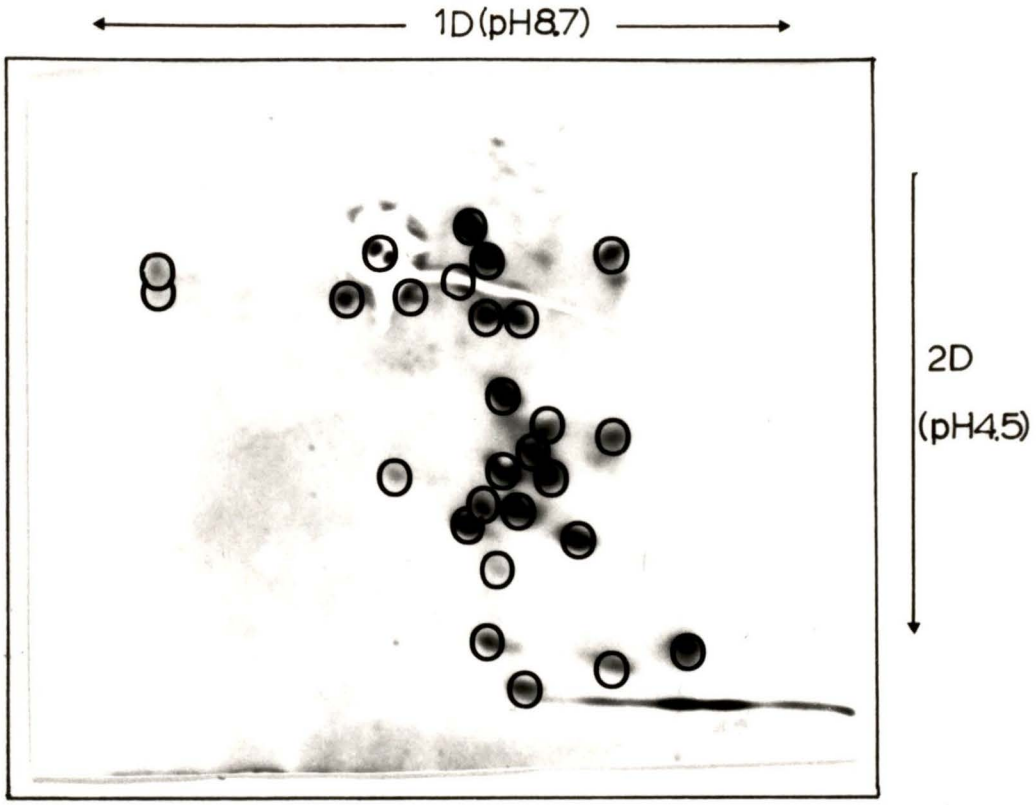


Figure 25. Total r-protein from E. coli 50S ribosomal subunits run on the 2D gel system of Howard and Traut (18).

complex would be adopted. The second technique attempted was that of Liljas et al (unpublished), in which the 5S RNA-protein complex was released from the 50S subunit by decreasing the Mg^{2+} concentration by chelating these cations with EDTA. This method effectively disrupts the ribosomal subunit by breaking ionic interactions between the ribosomal components.

From the work of Liljas et al.the majority of r-proteins and 23S RNA are pelleted down after EDTA treatment and a single ribonucleoprotein peak was observed at a concentration of 7% sucrose on a 5-20% sucrose gradient. They determined that this ribonucleoprotein peak contained the 5S RNA-L5~L18~L25 complex of approximate molecular weight 80,000. Previously, Chen-Schmeisser and Garrett (87) had separated 3 ribonucleoprotein fractions on a 5-20% sucrose gradient after digestion of the 50S subunit with RNAase A. They showed that a ribonuceloprotein peak at a concentration of 7% sucrose contained the 5S RNA—L5~L18~L25 complex, while two other peaks contained ribonucleoprotein fragments of 13S and 18S at sucrose concentrations of 17% and 19% respectively.

Adopting the method of Liljas et al, a single peak was observed at a sucrose concentration of 7% on a 5-20% sucrose gradient. Fractions from the peak were identified on 7.5% polyacrylamide gels and 15% SDS polyacrylamide gels (Figs. 26 and 27). Fraction C from the 5-20% sucrose gradient was shown to contain 5S RNA on 7.5% polyacrylamide

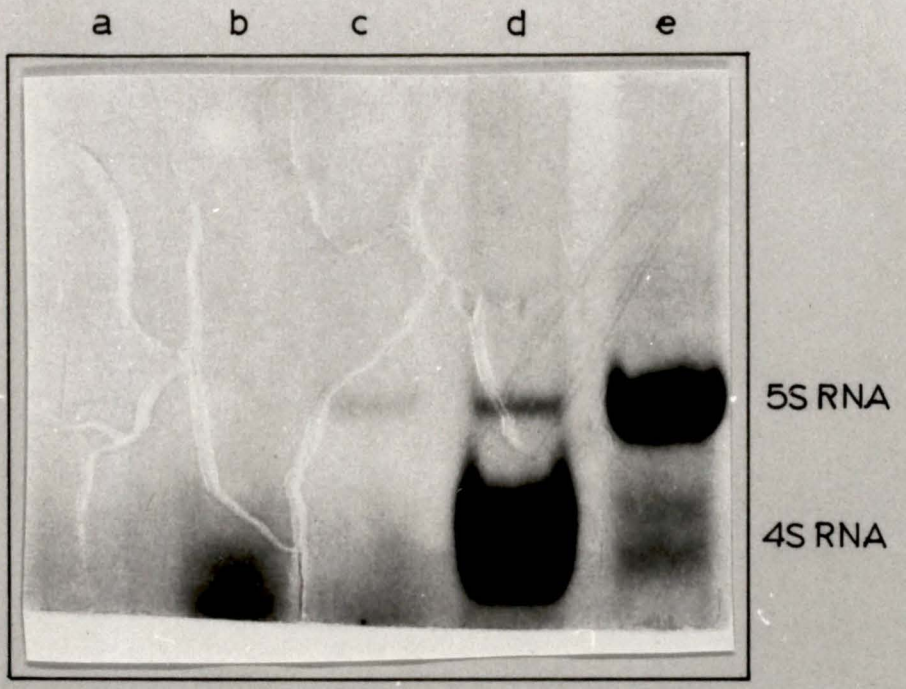


Figure 26. Fractionation of rRNA on 7.5% polyacrylamide gels.

(a) Fraction A from 5-20% sucrose gradient (Fig. 17)

(b) Fraction B from 5-20% sucrose gradient (Fig. 17)

(c) Fraction C from 5-20% sucrose gradient (Fig. 17)

(d) Marker 4S RNA

(e) Marker 5S RNA

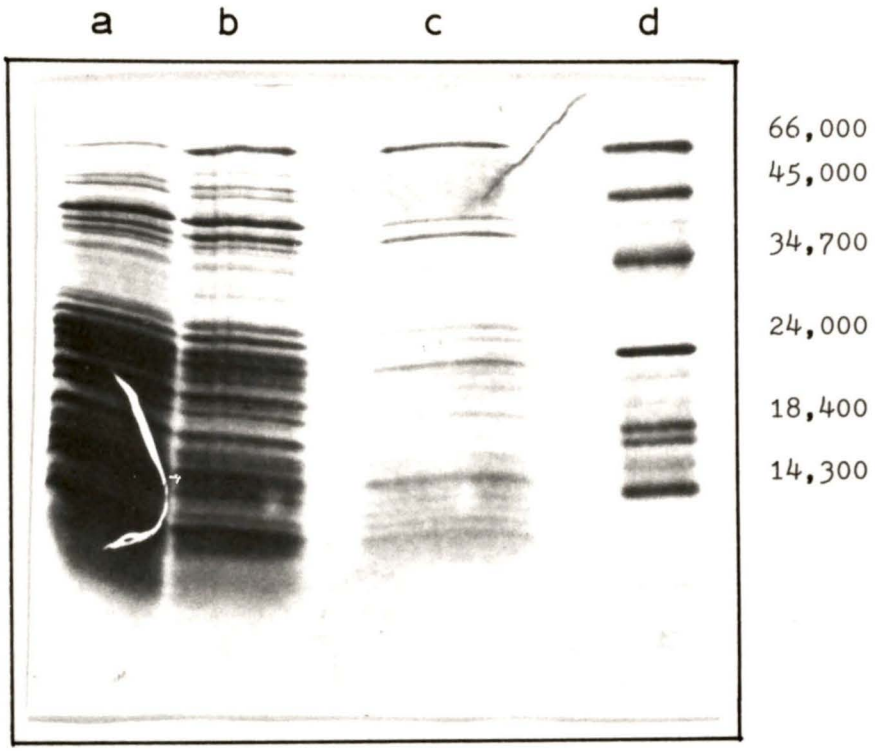


Figure 27. Separation of r-proteins on 15% SDS polyacrylamide gels.

(a) Fraction A isolated from 5-20% sucrose gradient (Fig.17)

(b) Fraction B isolated from 5-20% sucrose gradient (Fig.17)

(c) Fraction C isolated from 5-20% sucrose gradient (Fig.17)

(d) Molecular weight markers: bovine plasma albumin, 66,000; ovalbumin, 45,000; pepsin, 34,700; trypsinogen, 24,000; β-lactoglobulin, 18,400; lysozyme, 14,300.

gels. Since the E. coli 5S RNA-protein complex is known to contain only basic ribosomal proteins (87), fraction C was run on the basic side of the 2D gel system of Howard and Traut (18) [Fig. 28]. The ribosomal proteins in this fraction were identified by comparison to the 50S r-protein 2D gel pattern shown earlier (Fig. 25). The r-proteins identified were L1, L2, L3, L5, L11, L16, L18, L22, L24 and L25. It is interest to note that although Chen-Schmeisser and Garrett (87) showed L5, L18 and L25 as the major components in their 7% sucrose ribonucleoprotein fraction, they also had a major contamination of L1 and minor contaminations of L2, L6, L13, L14, L15, L22 and L24. Liljas et al did not show their 2D gel pattern for this fraction. One may conclude at this point that fraction C was likely to contain the 5S RNA—L5~L18~L25 complex but it was grossly contaminated.

In a further attempt to isolate and purify the 5S RNA-L5~L18~L25 complex, fraction C from the 5-20% sucrose gradient was passed through a Sephacryl S200 column. Fractions eluted from the column were too dilute to obtain accurate A_{230} (protein) and A_{260} RNA measurements. Each fraction from the column was concentrated by TCA precipitation and run on 15% SDS polyacrylamide gels (Fig. 29). The SDS gels indicated that partial fractionation of the proteins had occurred on the S200 column (see Fig. 27). Each of the fractions in the molecular weight range of 90,000 - 60,000, as estimated from partition coefficients

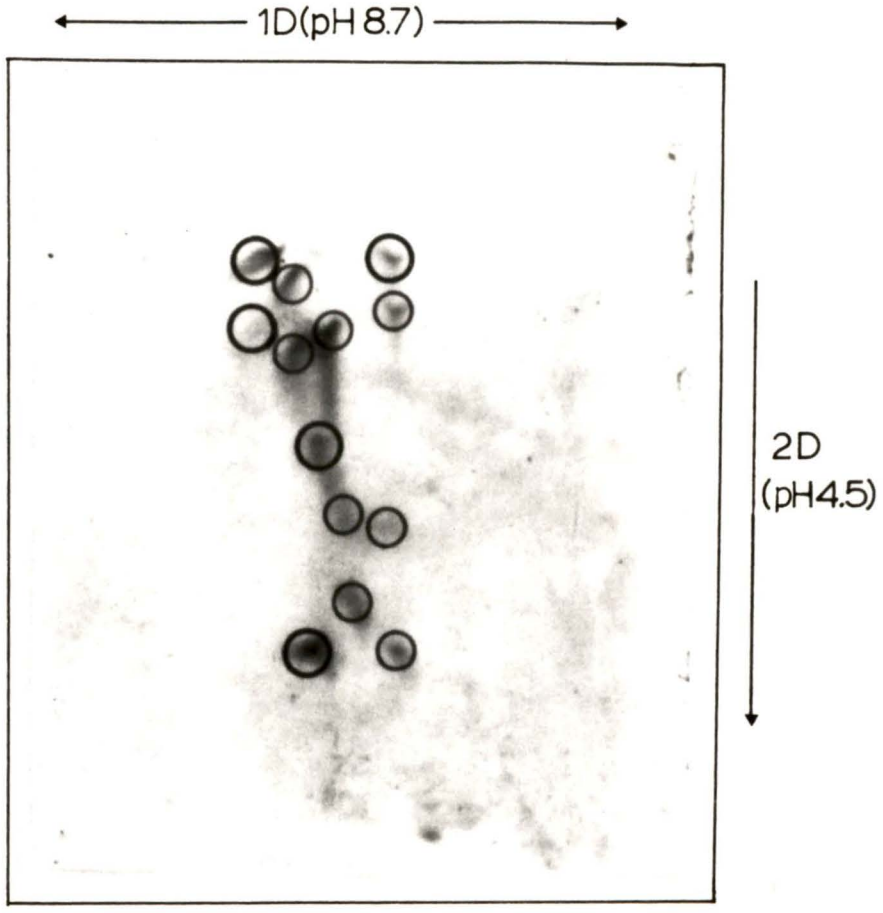


Figure 28. Two-dimensional polyacrylamide gel system of Howard and Traut (18) showing basic r-proteins of fraction C isolated from the 5-20% sucrose gradient (Fig. 17).

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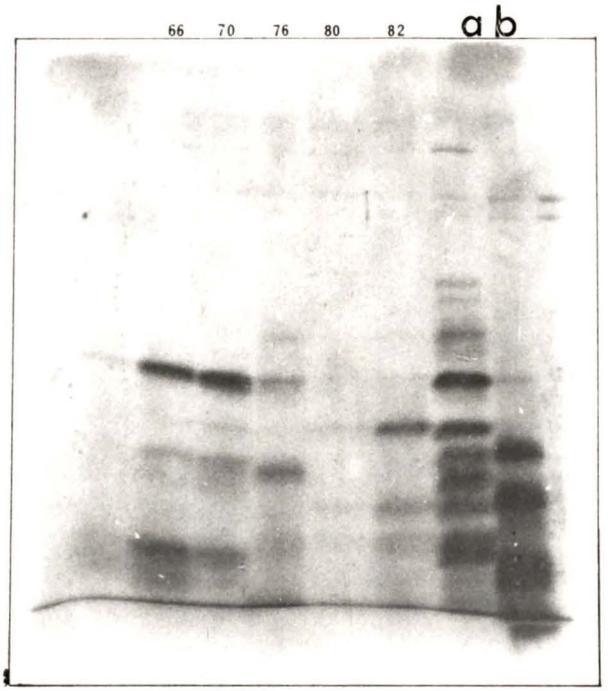


Figure 29. SDS-polyacrylamide gel of fractions eluted from an S200 column loaded with fraction C. (a) Fraction C.
(b) Molecular weight markers.

on the S200 column, contained 3 - 4 r-proteins.

In order to positively conclude that a 5S RNA L5 L18 L25 complex was present in any of the S200 column fractions, it would have been necessary to have shown 5S RNA in those fractions and the presence of the basic r-proteins L5, L18 and L25 as identified on 2D polyacrylamide gels in association with the 5S RNA.

Repeated attempts to isolate sufficient 5S RNA-protein complex were plagued with technical problems and since the amount of material available from the archaebacteria was limited, it was decided to terminate this problem in favour of the wheat germ ribosomal "A" protein project where an unlimited supply of material was available.

IV. ISOLATION AND CHARACTERIZATION OF RIBOSOMAL 'A' PROTEIN FROM WHEAT GERM RIBOSOMES

A. Introduction

The ribosomal 'A' proteins found in the large subunits of eubacterial, archaebacterial and eukaryotic ribosomes, show similar charge, size, amino acid composition as well as extensive N-terminal sequence homology (21). The conservation of these 'A' proteins within ribosomes of largely dissimilar organisms strongly suggests an integrally important requirement in structure or function within the ribosome.

In prokaryotes, there are multiple copies of a single 'A' protein within the large subunit that are involved in GTP dependent binding of certain translational factors and possibly in GTPase activity (90, 101). In the ribosome four molecules of this protein (EL7/L12 in E. coli) are associated with a less acidic r-protein of the large subunit (EL10 in E. coli) to form a 4:1 complex (89, 90, 91). Since sequence homologies have been found between the 'A' proteins in eubacteria, archaebacteria and eukaryotes (110, 111) and since a certain amount of functional interchange of this protein between different ribosomes is possible (109), it is of interest to determine whether eukaryotic ribosomes contain this protein in a specific domain similar to that found in eubacteria and archaebacteria.

To date no such comparable complex has been discovered in eukaryotes. The 'A' proteins in eukaryotes may be present in different forms such as those found in S. cerevisiae (108),

A. salina (109) and rat liver (106).

Previous studies on wheat germ ribosomes have shown that they also have several acidic r-proteins within the large ribosomal subunit (21, 154). The most acidic of these r-proteins was isolated, and from its unusual amino acid composition it appeared to have the properties of an 'A' protein. The first 41 residues of the N-terminal region of this acidic protein were then sequenced and shown to be highly homologous to 'A' proteins from several eubacteria, the archaebacterium H. cutirubrum and other eukaryotes. It was therefore of interest to isolate this acidic r-protein and the other acidic r-proteins present in the large subunit, characterize and partially sequence them to determine how many 'A' type r-proteins are present in eukaryotic ribosomes. These investigations would give an indication whether an 'A' protein domain exists comparable to that found in eubacteria and archaebacteria; and if not, they should provide a preliminary description of the 'A' protein domain in eukaryotic ribosomes.

B. Materials and Methods

1. Source of materials

Wheat germ embryos were a gift from Robin Hood Multifoods Ltd.

2. Ribosome isolation

80S ribosomes were extracted from wheat germ embryos by the method of Sikorski et al.(154). Wheat germ embryos (55 g) were suspended in 4:1 mixture of carbon tetrachloride/cyclo-

hexane (125 ml) and the cells homogenized in a blender (Galaxie Osterizer) 6 x 10s in buffer I (500 ml) [Table 8]. The suspension was centrifuged at 8,000 rpm for 10 minutes in a Sorvall GSA rotor. Tris-HCl (1 M) was added to the supernatant to bring it to pH 7.2. The solution was then centrifuged at 8,000 rpm for 10 minutes in a Sorvall GSA rotor. The 80S ribosomal pellet was isolated by centrifugation at 41,000 rpm for 2 hours in a Beckman Ti50.2 rotor. This pellet was resuspended in buffer I and the solution centrifuged at 41,000 rpm for 1 hour in a Beckman Ti50.2 rotor. The ribosomal pellet was stored at -80°C.

3. Isolation of ribosomal subunits

Ribosomal subunits were isolated by the method of Morrishey et al.(155). The 80S ribosomes (20 mg/ml) were resuspended in buffer II (Table 8), and centrifuged at 29,000 rpm for 17 hours at 5°C. in a Beckman Ti15 zonal rotor. Fractions (15 ml) were removed using a 60% sucrose cushion and A_{260} values were measured (Fig. 30). The subunit fractions were pooled and the ribosomal subunits isolated by centrifugation at 49,000 rpm for 36 hours in a Beckman Ti50.2 rotor. The isolated 40S and 60S ribosomal pellets were stored at -80°C. Ribosomal subunits isolated in a similar manner have been shown to be active in polyphenylalanine polymerization when recombined (154).

4. Protein isolation, purification and characterization

(i) Total r-protein extraction

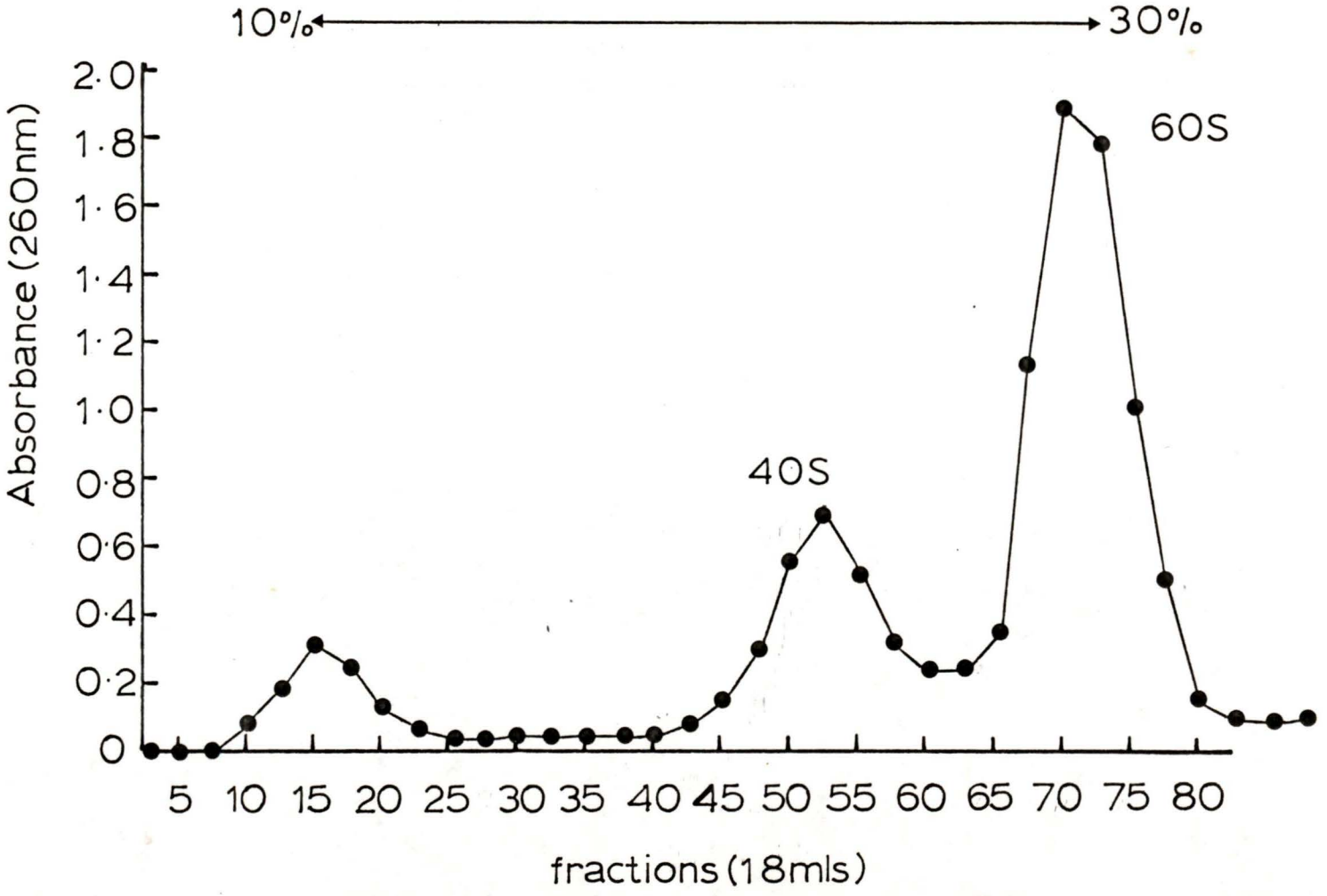
Total r-protein was extracted from both 80S and 60S

TABLE 8

Buffers used in the extraction of wheat-germ 80S ribosomes and subunits.

	Buffer		
	I(154)	II(155)	III(155)
Tris-acetate (pH 8.2)	50 mM	--	--
Tris-HCl (pH 7.6)	--	20 mM	20 mM
$Mg(Ac)_2$	5 mM	--	--
$MgCl_2$	--	3 mM	3 mM
KCl	50 mM	500 mM	300 mM
β-Me	5 mM	3 mM	3 mM

Figure 30. Isolation of ribosomal subunits by zonal centrifugation in a 10-30% sucrose density gradient at 29,000 rpm for 15 hours in a Beckman Ti15 rotor.



ribosomes by the method of Hardy et al (11) as described in Materials and Methods [Section II D(i)].

(ii) Polyacrylamide gel electrophoresis

(a) One-dimensional SDS gels

One-dimensional SDS gels were run as described in Materials and Methods [Section II D(ii)a].

(b) Urea gels, pH 8.7

Cox pH 8.7 urea gels were run according to the method of Cox (156). Solutions used in these gels are shown in Table 9. Samples were prepared in urea for electrophoresis using bromophenol blue as tracking dye. Electrophoresis was run at 32 mA/slab towards the anode.

(c) Two-dimensional polyacrylamide gels

Two-dimensional gels were run as described in Materials and Methods [Section II D(ii)b], with the difference that electrophoresis in the first dimension, towards the anode, was run at 6 mA/tube for 4 hours on 8% polyacrylamide gels.

(d) Staining and destaining polyacrylamide gels

As described in Materials and Methods [Section II D(ii)c].

(iii) Extraction of acidic r-proteins from ribosomes

Acidic r-proteins were isolated from wheat germ ribosomes according to the method of Hamel et al.(105). Ribosomes (200 A_{260}/ml) were resuspended in TMK buffer (50 mM Tris, pH 7.6, 2 mM $Mg(OAc)_2$, 0.3 M KCl, 1 mM β-mercaptoethanol). One third volume of 4 M NH_4Cl was added dropwise over ten minutes and stirred at 0°C. for 1 hour.

TABLE 9

Urea Gels

A. Solutions used in Cox 8.7 urea gels (156)

	A	B	C	D	U
Tris	12.1 g	3.0 g	--	--	--
HCl	1.37 ml	2.05 ml	--	--	--
TEMED	0.18 ml	0.5 ml	--	--	--
acrylamide	--	--	15.0 g	6.66 g	--
MBA	--	--	0.4 g	1.66 g	--
urea	48.0 g	48.0 g	48.0 g	48.0 g	48.0 g

Electrode buffer: 6.0 g Tris
2.88 g glycine
made to 2 litres with water

Persulphate solution 0.56%: 0.056 g ammonium persulphate
10.0 ml solution U

B. Gel preparation

Lower (separation) gel:

11.25 ml A
15.00 ml C
} degas, cool on ice

polymerize with 3.75 ml persulphate solution

Upper (stacking) gel:

1 ml B
1.5 ml D
1 ml U
} degas, cool on ice

polymerize with 0.5 ml persulphate solution

One volume of 95% ethanol (-20°C) was then added to the suspension over a 1 hour period, and the suspension stirred for 2 hours at 0°C. The suspension was centrifuged at 15,000 x g for 15 minutes at 5°C. The ribosomal core was stored at -80°C while the supernatant was stored at 5°C.

(iv) Purification of acidic r-proteins on DEAE-cellulose

DEAE-cellulose (DE52, Whatman) was equilibrated with column buffer (Table 10), and the column was packed at 30 ml/hr using a peristaltic pump. After dialyzing the sample against column buffer, it was loaded onto the column at 27 ml/hr using a peristaltic pump. Proteins were differentially eluted from the column using a continuous gradient of 0-0.3 M NaCl in column buffer. Fractions (10 ml) were collected and their conductivities and A_{230} values read (Fig. 31). Fractions collected from the DEAE-cellulose column were identified by running every second sample on Cox pH 8.7 urea gels, SDS polyacrylamide gels and 2D polyacrylamide gels.

(v) Amino acid analysis

Samples of protein (0.08 - 0.25 mg) were hydrolyzed in 6N HCl in vacuo at 110°C for 23 hours. The HCl was evaporated in a desiccator containing NaOH and P_2O_5. The samples were dissolved in 500 µl of 0.2 N citrate buffer, pH 2.2, and filtered through a 0.45 µm millipore. The amino acid content was analyzed using a Beckman 1188L

TABLE 10

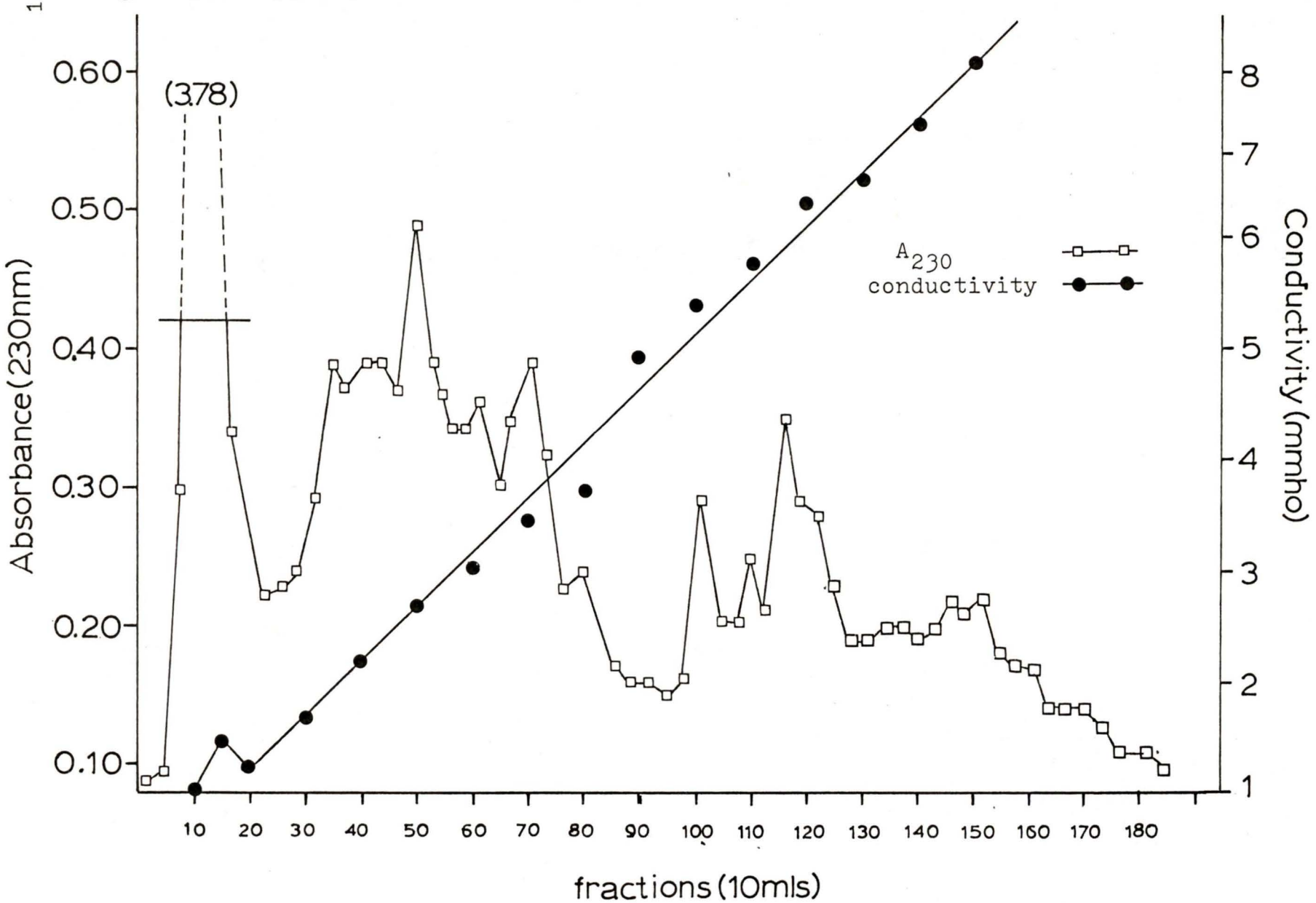
DEAE cellulose column running buffer, pH 8.0

*8 M urea	4.5 litres
Tris	7.26 g
dithiothreitol	0.093 g
40% methylamine	4.2 ml

made up to 6 litres with d H_2O

* 8 M urea prepared by cleaning with activated charcoal and passing through a dowex 50 column

Figure 31. Separation of acidic r-proteins on a DEAE-cellulose column.



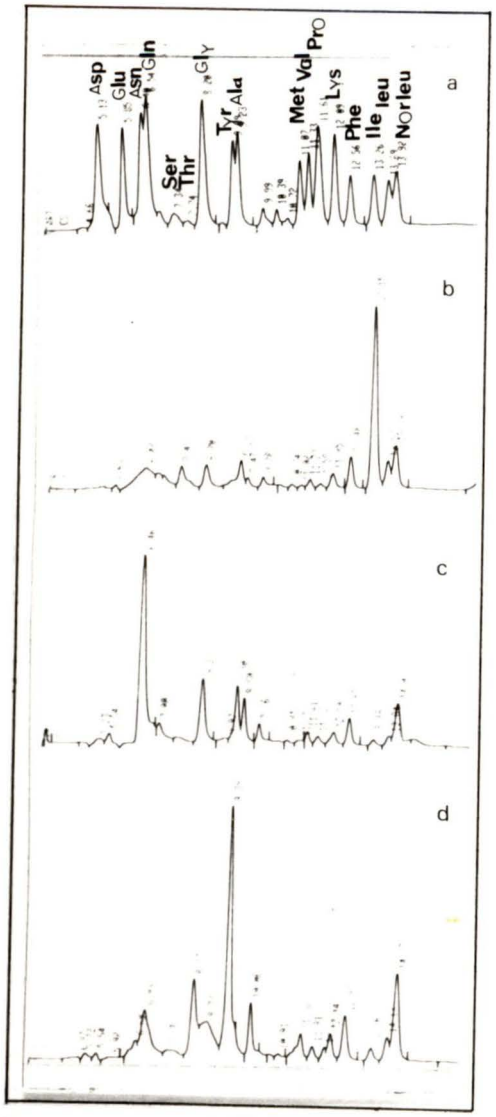
automatic amino acid analyzer. The mole percent of each amino acid was then calculated.

In order to determine the cysteine content in each sample, 0.1 mg of the protein was oxidized with performic acid (157), and then hydrolyzed as described above. The performic acid oxidation was achieved by the addition of methanolic formic acid [1 ml methanol:5 ml 99% formic acid] (0.32 ml) and performic acid reagent [incubation of 1 ml H_2O_2(30%) with 19 ml of 99% formic acid at 25°C for 2 hours] (0.53 ml), to the protein sample. The mixture was incubated in a test tube in a -7°C salt-ice bath for 2.5 hours. The reaction was stopped by addition of 25 ml of distilled water at 0°C. Samples were prepared for acid hydrolysis by lyophilization.

(vi) N-terminal sequence analysis of r-proteins

The amino acid sequence of the N-terminal region of the r-proteins (1-3 mg) was determined by automatic Edmann degredation using a Beckman model 890C sequenator with a 0.1 M quadrol program #121078. Automatic conversions of the thiazolinone derivatives were performed using a P6-autoconverter (Sequemat Inc.). The phenylthiohydantoin (PTH) derivatives were identified according to the method of Tarr (158) using a Beckman model 332 high pressure liquid chromatograph (HPLC). Typical PTH residues are shown in Fig. 32.

Figure 32. PTH amino acid profiles



(a) Standard PTH residues
(b) PTH isoleucine residue number 4 from 'A' protein 8, (Fig.43)
(c) PTH asparagine residue number 25 from 'A' protein 8 (Fig.43)
(d) PTH alanine residue number 32 from 'A' protein 8 (Fig.43)

Each PTH residue derivative has a characteristic retention time (compared to standard retention times) on the HPLC (High-pressure liquid chromatography). Since retention times vary from hour to hour, standard PTH residues were run after every five residues to allow accurate determination of amino acids.

(vii) 'A'-protein complex isolation on S200 columns

The acidic r-proteins extracted as in section 4 (i) were dialyzed against buffer III (Table 7, Section II), and concentrated to 5 ml in an Amicon diaflo ultrafiltration unit fitted with a PM 10 membrane. The acidic r-protein fraction (5 ml) was loaded onto an S200 column equilibrated with buffer III and run at 35 ml/hour. Fractions (4 ml) were collected and the A_{230} values read. Every second fraction from the S200 column was identified by running on SDS-polyacrylamide gels.

1. (i) Isolation of ribosomes and ribosomal subunits

The 80S ribosomes from wheat germ provided suitable starting material for extraction and isolation of acidic r-proteins. However, since the original intent of these experiments was to isolate the putative ribosomal 'A' protein domain (equivalent to EL7/L12 - EL10 in E.coli) from the 60S subunit, the 80S ribosomes were dissociated into their 40S and 60S subunits. In the presence of high concentrations (0.5 M) of monovalent cations, which effectively compete with Mg^{2+} binding sites holding the subunits in association, 80S ribosomes dissociate into their subunits (160).

(ii) Total r-protein from 80S ribosomes

For comparative purposes the total r-protein was extracted from 80S ribosomes and run on the 2D gel system of Howard and Traut (18) with the modifications described earlier in section B.4. The basic r-protein pattern shown on the 2D gel profile (Fig.33) agree favourably with those shown in the work of Sikorski et al (154). The acidic r-proteins from the 80S ribosomes shown on the left hand side of the 2D gel appear to be relatively poorly stained. Poor staining has been observed for many ribosomal acidic r-proteins (17).

(iii) Extraction of acidic r-proteins

Acidic r-proteins were selectively extracted from the 80S ribosomes and 60S subunits of wheat germ using the method of Hamel et al (105). The acidic r-protein fraction from 80S ribosomes [A80] and 60S subunits [A60] were run

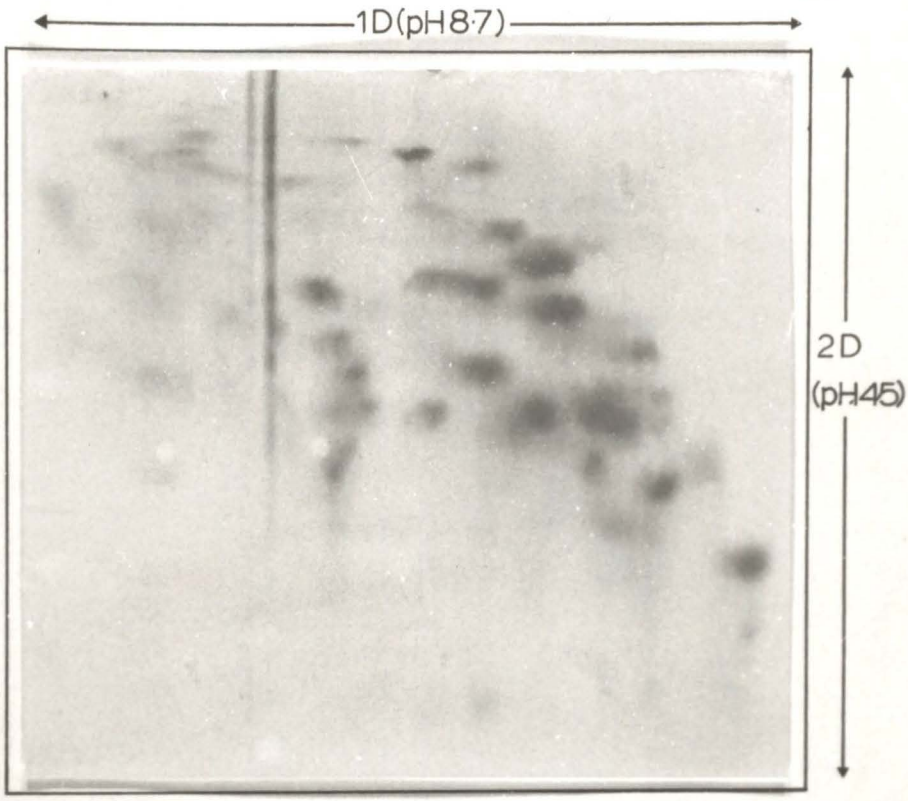


Figure 33. 2D gel profile of total r-protein extracted from 80S ribosomes of wheat germ.

on 15% SDS polyacrylamide gels (Fig. 34), Cox pH 8.7 urea gels (Fig. 35), and 2D polyacrylamide gels (Figs. 36 and 37).

(iv) Fractionation of acidic r-proteins

The SDS-polyacrylamide gel (Fig. 34) shows the A80 fraction to contain a large number of bands while the A60 fraction contains 3 major bands of molecular weights 13,700, 14,500, and 15,000, as well as some minor components. From the Cox pH 8.7 urea gels (Fig. 35), and the 2D gels (Figs. 36 and 37), it appears that there are approximately 11 acidic r-proteins in the A80 fraction, while there are 3 major proteins in the A60 fraction with several other acidic r-proteins. The resolution on the 2D gel of the A60 fraction is poor, but the individual proteins have been separated and are shown later in Fig. 42.

In order to further characterize these acidic r-proteins, it was necessary to isolate the individual proteins. The starting material used was 80S ribosomes since it is unclear whether dissociation into subunits causes loss of acidic r-proteins. [It has been previously reported to do so in other eukaryotic systems (106, 108), and there is some indication that this may occur in wheat germ.]

The A80 fraction was separated on a DEAE-cellulose column and the proteins were selectively eluted by a 0 - 0.3 M NaCl gradient. Cox pH 8.7 urea gels (Fig.38), SDS-polyacrylamide gels (Fig.39), and the A_{230} profile (Fig. 31, Section B) allowed selected fractions to be pooled. The pooled fractions were dialyzed against water

Figure 34. SDS-polyacrylamide gel (15%)
(a) Total r-protein extracted from wheat germ 80S ribosomes.
(b) Acidic r-protein fraction from the 80S ribosomes of wheat germ.
(c) Acidic r-protein fraction from the 60S subunits of wheat germ ribosomes.

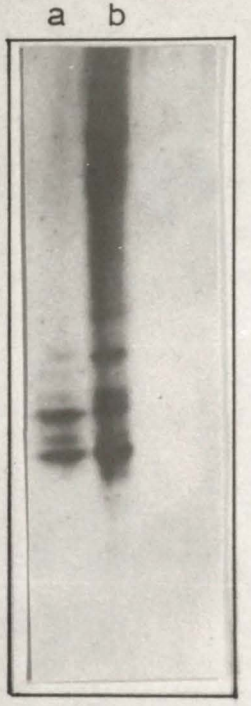


Figure 35. Cox pH 8.7 urea gels
(a) Acidic r-protein fraction from 60S ribosomal subunits of wheatgerm.
(b) Acidic r-protein fraction from 80Sribosomes of wheat germ.

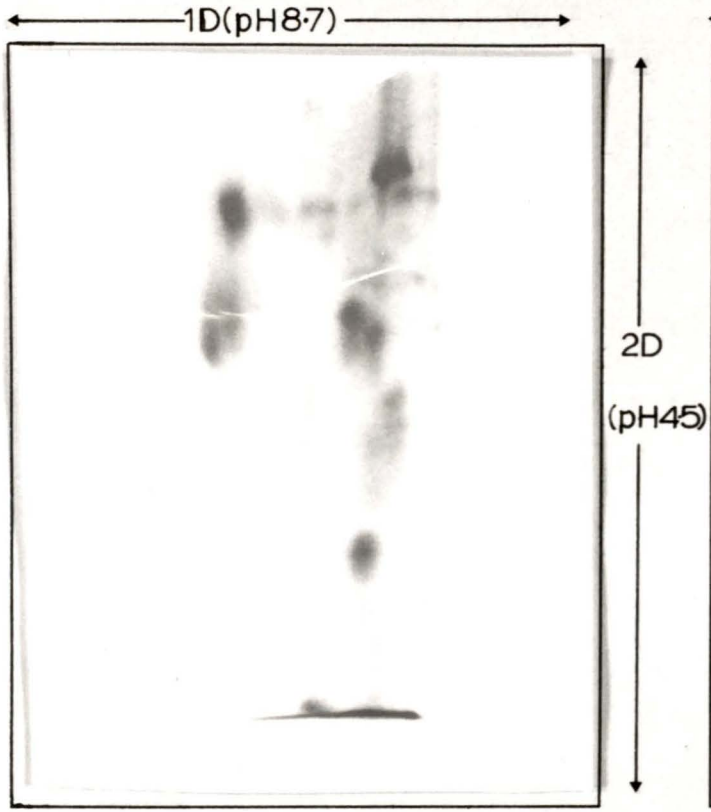


Figure 36.2D gel of the A80 fraction from wheat germ.

Figure 37. 2D gel of the A60 fraction from wheat germ.

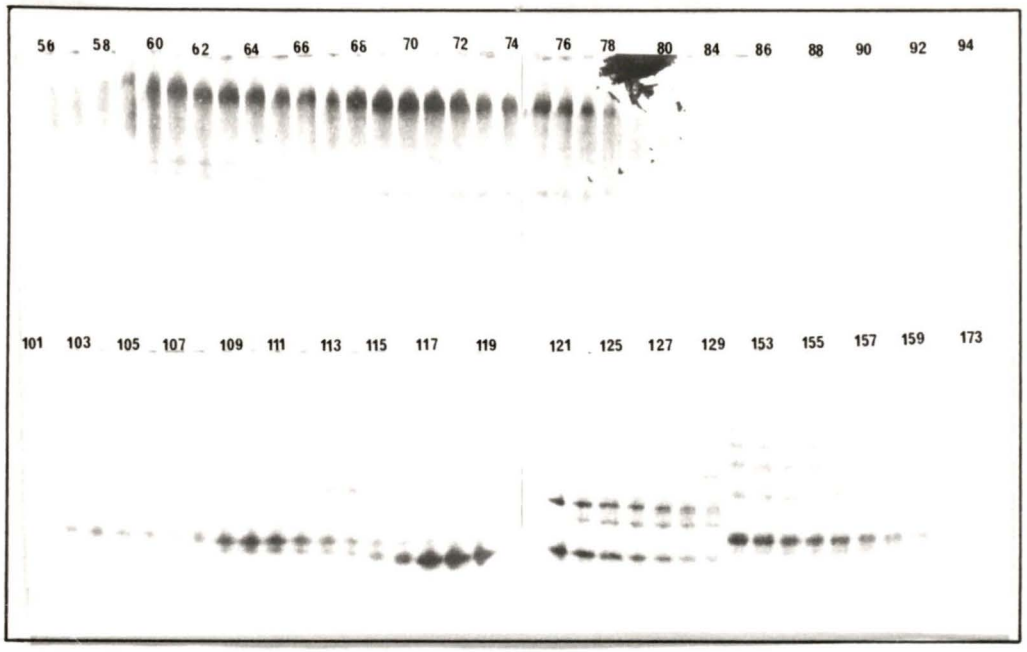


Figure 38. Fractions (10 ml) collected from a DEAE-cellulose column and run on Cox pH 8.7 urea gels.

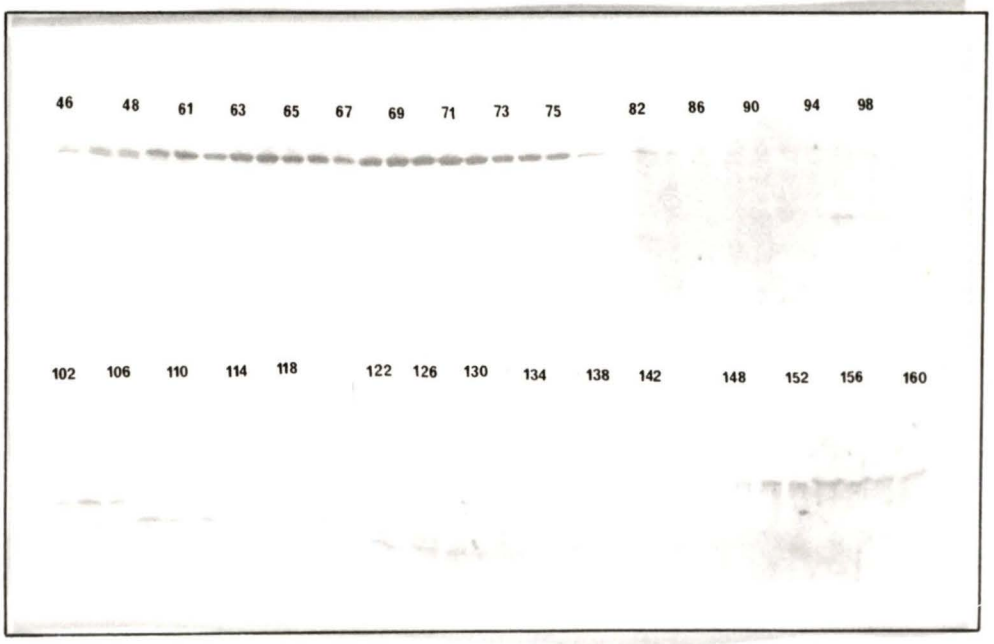


Figure 39. Fractions (10 ml) collected from a DEAE-cellulose column and run on 15% SDS polyacrylamide gels.

and lyophilized. The yields of the pooled fractions are shown in Table 11.

(v) Characterization of acidic r-proteins

The proteins were characterized by running the lyophilized samples listed in Table 11 on Cox pH 8.7 urea gels (Fig.40), SDS-polyacrylamide gels (Fig.41) and Howard and Traut 2D gels (Fig. 42).

A summary of estimated molecular weights for the individual proteins is shown in Table 11. The molecular weights were estimated by comparison of their mobilities on 15% SDS-polyacrylamide gels with known molecular weight standards (Fig.43). It is apparent from Figs.40-42 that 7 acidic r-proteins (fractions 2, 3, 4, 7, 8, 10 and 11) have been isolated in relatively pure form from the A80 fraction. The amino acid composition of these proteins were determined (Table 12). From the amino acid composition it is apparent that all the isolated proteins are acidic, as demonstrated by their low B/A residue mole % ratios. Other notable characteristics of these proteins are the high number of charged residues (32-41%) and the low number of aromatic residues (5.6% - 9%). Amongst the isolated proteins, sample 7 and 8 are the only ones, from their amino acid compositions, that are likely candidates as 'A' proteins; i.e., they have a low B/A residue mole % ratio, high alanine (17-18%) and low aromatic residues. The amino acid compositions of samples 7 and 8 are compared to eukaryotic 'A' proteins from rat liver, A. salina,

TABLE 11

List of Yields (mg) and Molecular Weights for Isolated Acidic r-proteins

Pooled fractions	Sample number	Lyophilized protein(mg)	Molecular weight SDS-PAGE
46-48	1	1.9	58,000; 57,000
60-62	2	2.5	58,000
63-66	3	2.5	58,000
67-78	4	6.8	58,000
83-92	5	2.3	--
99-106	6	2.6	14,500; 18,000; 20,000
107-113	7	3.6	15,000; 14,700
116-118	8	3.3	13,700
120-128	9	4.5	13,700; 14,500
152-160	10	3.5	32,000
173-176	11	0.6	48,000

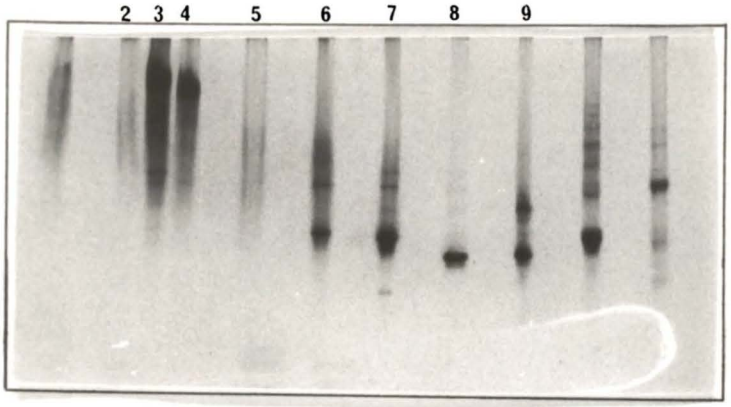


Figure 40. Cox pH 8.7 urea gel of pooled samples from DEAE cellulose column as indicated in Table 11.

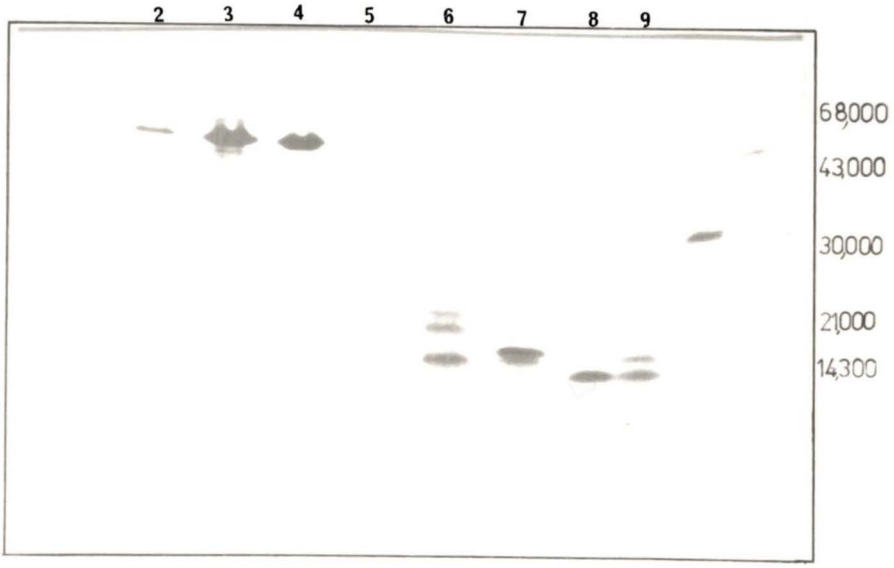


Figure 41. SDS-polyacrylamide gel of pooled samples from DEAE cellulose column as indiacted in Table 11.

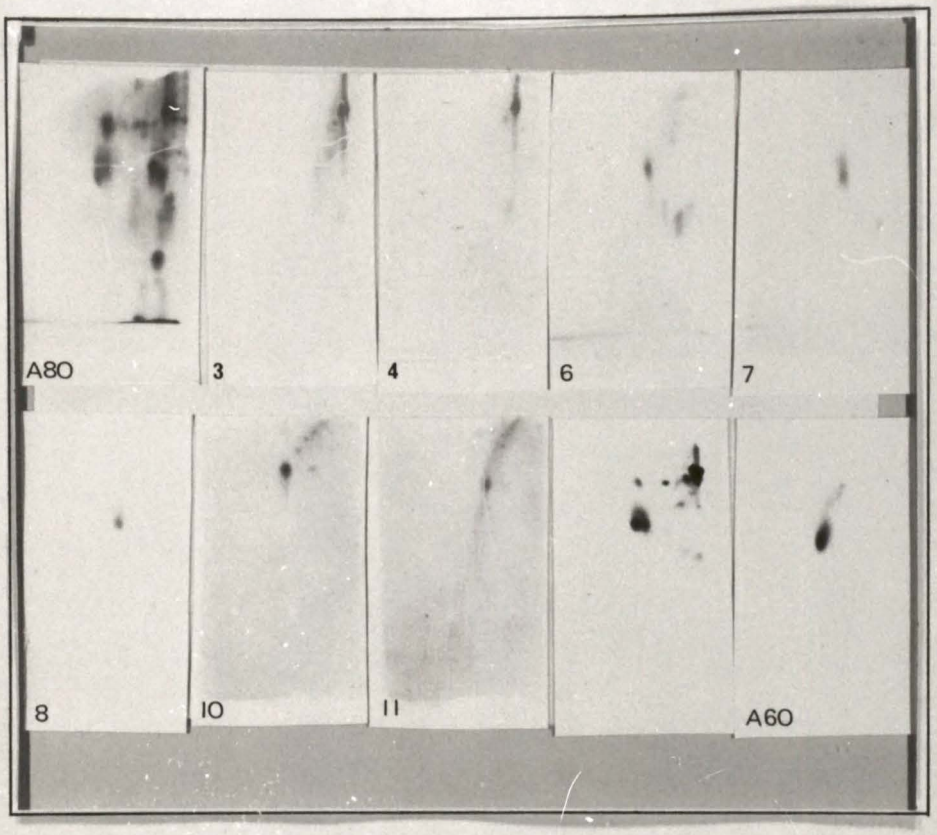


Figure 42. 2D Howard and Traut gels of individual samples from DEAE column, see Table 11. Also shown are the A80, A60 fractions and a combined sample gel (3, 4, 6, 7, 8, 10, 11).

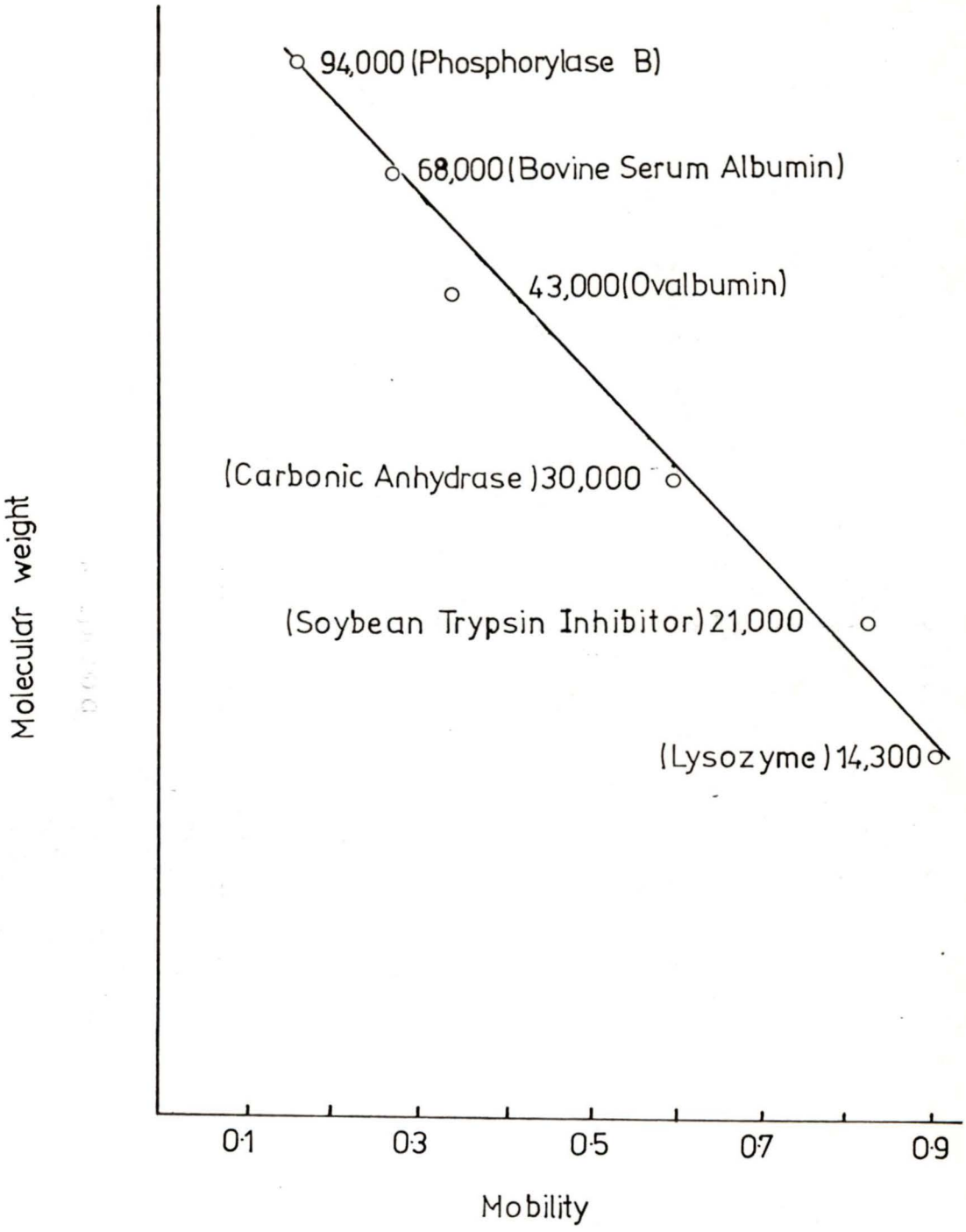


Figure 43. Standard curve of log molecular weights against electrophoretic mobility for standard proteins on an SDS polyacrylamide gel.

TABLE 12

Amino acid composition (mole %) of acidic r-proteins 2, 3, 4, 8, 10 and 11

Sample #	2	3	4	7	8	10	11
Aspartic acid	10.0	9.8	10.0	8.0	8.6	12.1	12.2
Threonine	4.8	4.1	4.1	2.6	1.2	4.4	4.7
Serine	5.6	5.6	4.6	8.2	8.6	7.7	8.4
Glutamic acid	15.1	15.3	15.9	14.4	14.0	15.4	14.8
Proline	5.6	5.6	6.1	4.1	3.4	4.7	4.2
Glycine	15.2	15.3	15.2	11.5	11.3	7.4	7.9
Alanine	10.4	11.4	11.0	17.0	18.1	12.6	10.4
Cystine	0	0	0	0	0	0	0
Valine	5.5	4.7	4.6	5.3	5.9	7.5	7.7
Methionine	0.9	1.1	1.5	1.3	1.5	1.0	1.1
Isoleucine	2.2	2.1	2.0	2.8	3.4	3.5	2.6
Leucine	5.1	5.4	5.0	5.4	7.6	6.1	5.0
Tyrosine	2.6	2.4	2.4	1.3	2.0	1.2	2.0
Phenylalanine	3.6	3.3	3.2	3.8	4.6	3.5	4.9
Histidine	0.7	0.4	0.6	3.9	0.2	0.9	1.6
Lysine	7.8	8.4	8.6	8.3	8.5	9.3	10.2
Arginine	4.9	5.0	5.3	2.1	1.2	2.8	2.4
A[a]	25.1	25.1	25.9	22.4	22.6	27.5	27.0
B[b]	13.4	13.8	14.8	14.3	9.9	13.0	14.2
B/A	0.53	0.55	0.56	0.64	0.44	0.47	0.53

a. A= acidic residues
b. B= basic residues

S. cerevisiae and to the previously reported value for the wheat germ 'A' protein (Table 13). It is apparent from Tables 12 and 13 that sample 8 closely resembles the previously reported value for the amino acid composition of the wheat germ 'A' protein, and both sample 7 and 8 are comparable to the other eukaryotic amino acid compositions.

(vi) Amino acid sequences of certain large subunit acidic r-proteins

The N-terminal portions of 3 acidic r-proteins from wheat germ (proteins 7, 8 and 10) were sequenced. The data for protein 8 identifies it as the 'A' protein previously sequenced by Visentin et al (Fig. 44). Certain discrepancies, which are underlined in Fig. 44, are apparent between the two sequences, and these are likely due to the different methods used in sequencing between the two laboratories. Heterogeneity at residue 3 had previously been reported by Visentin et al (21), but this heterogeneity was not found in our sample. The difference at residues 34-41 appears to be due to an extra alanine residue in the sequence of Visentin et al (21) which effectively puts the remaining residues out of phase by a single residue. The sequence also shows preliminary data for an additional 14 residues over and above those previously reported. Tentative amino acid residues are bracketed.

The N-terminal portion of protein 7, which from its amino acid composition indicated it might also be an 'A'

TABLE 13

Amino acid composition of proteins 7 and 8 compared with other eukaryotic ribosomal 'A' protein amino acid compositions

Sample #	7	8	SC[a]	AS[b]	WG[c]	RL[d]
Aspartic acid	8.0	8.6	9.3	7.4	8.1	10.5
Threonine	2.6	1.2	2.1	2.9	2.0	1.8
Serine	8.2	8.6	7.9	6.4	9.0	5.9
Glutamic acid	14.4	14.0	16.6	17.3	12.6	11.6
Proline	4.1	3.4	2.2	4.0	2.1	7.7
Glycine	11.5	11.3	12.5	14.0	10.4	10.5
Alanine	17.0	18.1	20.3	18.5	20.3	20.4
Cystine	0	0	0	0	0	0
Valine	5.3	5.9	5.1	2.4	5.0	6.8
Methionine	1.3	1.5	1.5	3.7	2.1	0.7
Isoleucine	2.8	3.4	3.8	3.8	4.8	4.8
Leucine	5.4	7.6	8.9	8.1	8.8	7.1
Tyrosine	1.3	2.0	1.4	1.7	2.0	0.5
Phenylalanine	3.8	4.6	2.5	1.4	3.5	3.3
Histidine	3.9	0.2	0.2	0	0.2	1.0
Lysine	8.3	8.5	6.2	8.0	8.0	6.5
Arginine	2.1	1.2	0.7	0.7	1.4	0.0
A[e]	22.4	22.6	25.9	24.7	20.7	22.1
B[f]	14.3	9.9	6.7	8.7	9.4	6.5
B/A	0.64	0.44	0.26	0.35	0.45	0.29

a. SC= *S. cerevisiae* 'A' protein
b. AS= *A. salina* 'A' protein
c. WG= Wheat germ 'A' protein
d. RL= rat liver 'A' protein
e. A= acidic residues
f. B= basic residues

Figure 44. N-terminal amino acid sequence of sample 8 compared to 'A' protein from wheat germ identified by Visentin et al (21). X=unidentified residues. Underlined residues indicate differences in the sequence between the two laboratories.

	1				5					10
Protein 8	Met	Lys	Leu	Ile	Ala	Ala	Tyr	Leu	Leu	Ala
Visentin et al	Met	Lys	Phe Leu	Ile	Ala	Ala	Tyr	Leu	Leu	Ala
					15					20
	Tyr	Leu	Gly	Gly	Asn	Ser	Ser	Pro	Ser	Ala
	Tyr	Leu	Gly	Gly	Asn	Ser	Ser	Pro	Ser	Ala
					25					30
	Ala	Asp	Val	Lys	Asn	Ile	Leu	Asp	Ser	Val
	Ala	Asp	Val	Lys	Asp	Ile	Leu	Asn	Ala	Val
					35					40
	Gly	Ala	Gln	Asp	Glu	X	Lys	Leu	Glu	Phe
	Gly	Ala	Glu	Ala	Asn	Glu	Glu	Lys	Leu	Glu
					45					50
	Leu	(Gly)	Ala	Glu	Leu	(Lys)	(Gln)	X	(Asp)	Ile
	Phe	Leu								
					55					
	(Thr)	(Glu)	(Val)	Ile	Ala					

protein has been sequenced (Fig.45). No sequence homology is evident between the N-terminal amino acid sequence portion of the major component in sample 7 and the 'A' protein in sample 8. The HPLC amino acid profiles for protein 7 indicated the presence of a second minor protein which from its N-terminal sequence (Fig. 46) was tentatively identified as being similar but not identical to the ribosomal 'A' protein present in sample 8.

The third protein that was partly sequenced was protein 10, as it was believed that this may be part of an 'A' protein complex containing the major proteins in sample 7 and 8 (see next section). A preliminary N-terminal amino acid sequence of protein 10 is shown in Fig. 47. The N-terminal amino acid sequence of protein 10 was found to be identical to that of the major protein present in sample 7. These results were surprising since protein 10 has a molecular weight of 32,000 while protein 7 has a molecular weight of 16,000. Furthermore, from the data obtained on Cox pH 8.7 urea gels, proteins 10 and 7 appear to have equivalent acidities (Fig.40). One possible explanation is that protein 10 is a gene fusion product with the gene for protein 7 and the gene of another acidic r-protein which as yet has not been identified. Such gene fusion products have previously been identified in the ribosomal 5S RNA-protein domains of eukaryotes (131), (see introduction).

Figure 45. N-terminal amino acid sequence of wheat germ r-protein present in sample 7.

1				5					10
Gly	Val	Phe	Thr	Phe	Val	Tyr	Leu	Asp	Ala
				15					20
Gly	Ala	Glu	Pro	Ser	Ala	Lys	Asn	Gln	X
				25					30
X	Glu	Leu	(Glu)	Ala	Ile	Ala	Phe	Thr	Pro
	32								
Tyr	Asp								

Figure 46. Tentative N-terminal amino acid sequence of the minor contaminant in sample 7. A comparison of this sequence and that shown in Fig. 44 indicates that the minor contaminant is similar but not identical to the ribosomal 'A' protein sample 8. The underlined residues indicate apparent differences in the two proteins. X = unidentified residues.

1				5					10
X	Lys	Leu	Ile	Ala	<u>Asp</u>	<u>Gly</u>	(Leu)	X	Ser
				15					20
Tyr	Leu	<u>Phe</u>	Gly	Asn	<u>Pro</u>	X	Pro	X	<u>Lys</u>
									30
X	Asp	Val	<u>Thr</u>	(Asp)	<u>Ser</u>	Leu	X	X	Val

31
Gly

Figure 47. N-terminal amino acid sequence of wheat germ acidic r-protein sample 10.
X = unidentified residues

1				5					10
Gly	Val	Phe	Thr	Phe	Val	Tyr	Leu	Asp	Ala
				15					20
Gly	Ala	Glu	Pro	Ser	Ala	X	Asn	Gln	Ala
				25					30
Gly	Glu	Leu	Glu	(Ala)	X	X	Ala	X	Pro
				35					40
Tyr	Asp	Leu	Pro	Ile	Glu	Leu	X	Phe	Gln

X (Ser) X (Gly)

2. Isolation of an 'A' protein complex domain

Under non-dissociating conditions it is possible to isolate protein-protein complexes or protein-RNA complexes from ribosomes by fractionation of the extract on gel filtration columns such as S200. Using this procedure the A60 fraction was passed through an S200 column in an attempt to isolate an 'A' protein complex (equivalent to EL7/L12-E10 in E. coli). Fractions (4 ml) collected from the S200 column were run on 15% SDS-polyacrylamide gels (Fig. 48). Using molecular weights determined from protein coefficients for the column, it was apparent that several specific proteins were present in a fraction that eluted in the molecular weight range of 52-58,000. Since the individual proteins in these fractions have molecular weights much lower than 52-58,000, it would suggest that these proteins are present as a complex. The proteins in this apparent complex have tentatively been identified as proteins 7, 8 and 10. If each of these proteins were present in one copy then the complex would have a molecular weight approximately 58,000 when fractionated on an S200 column. Within the limits of error for determining molecular weight ranges using partition coefficients and SDS-polyacrylamide gels, it is likely that samples 7, 8 and 10 do in fact form an 'A' protein complex. This possibility is currently under investigation.

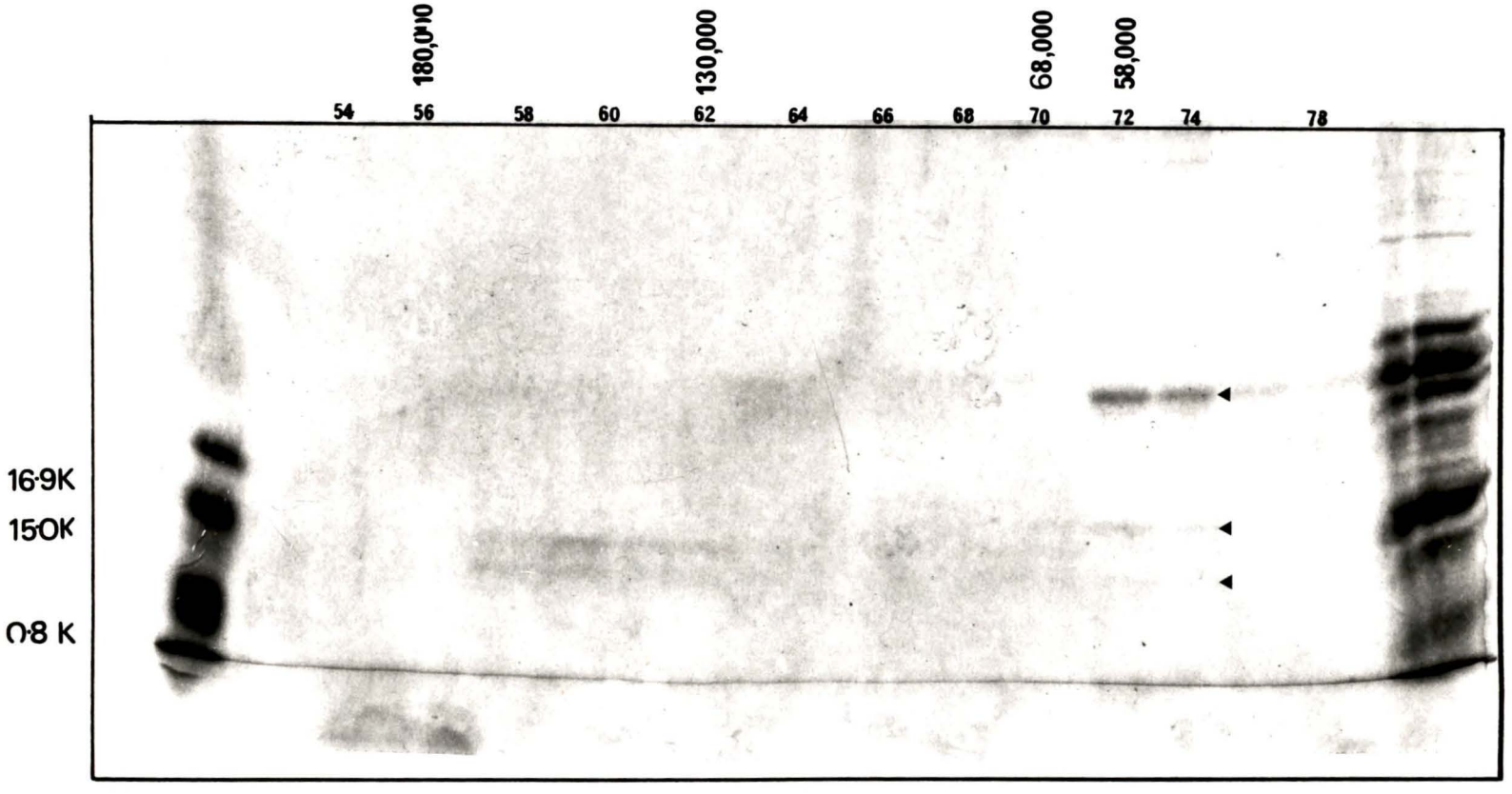


Figure 48. SDS polyacrylamide gel of fractions from the A60 fraction run on S200 column at 35 ml/hr.

V. CONCLUSIONS

Evidence presented for the differences in size and number of r-proteins and RNA within ribosomes of prokaryotes compared with eukaryotes suggests that there are likely to be morphological differences in these ribosomes. Electron microscopic studies of ribosomes from prokaryotes and eukaryotes definitely support this suggestion. One large characteristic difference in morphology between the prokaryotic and eukaryotic large subunits is the 'acidic stalk' present in prokaryotes. Isolation of this 'acidic stalk' from prokaryotes has shown it to be a complex containing 4 molecules of the 'A' protein (EL7/L12 in E. coli, molecular weight 12,206), and one molecule of a larger acidic r-protein, (EL10 in E. coli, molecular weight 17,736).

Since there are conflicting reports concerning the presence of an 'acidic stalk' in eukaryotes, (Lake, personal communication to Dr. A. T. Matheson), it was of interest to determine the 'A' protein environment in eukaryotes. Using wheat germ as my source of ribsomes, I have obtained evidence for a complex containing 3 acidic r-proteins, one of which from amino acid sequence data has been identified as the 'A' protein, (molecular weight 13,700). The second protein is slightly less acidic, (molecular weight 15,000), and its N-terminal amino acid sequence shows no homology with the 'A' protein. The third r-protein,(molecular weight

32,000), has an identical N-terminal amino acid sequence to the second protein but is much larger in size. One may speculate, therefore, that this 32,000 molecular weight protein may be a gene fusion product of the 15,000 molecular weight protein and another as yet undetermined r-protein.

When one compares these results with those obtained from prokaryotes, it is evident that substantial changes have occurred between these two kingdoms, and the evidence suggests that the eukaryotic 'A' protein domain may be more complex. The putative eukaryotic 'A' protein domain contains three different proteins while the prokaryotic 'A' protein domain contains two proteins, although the 'A' protein is present as a tetramer in prokaryotes. At this time, it is not possible to predict whether more than one copy of a protein is present within the eukaryotic domain. Since it appears that the two domains are significantly different, this might account for the fact that an 'acidic stalk' has not been observed in eukaryotes.

Since the evidence for the wheat germ 'A' protein complex is preliminary, further investigations must be carried out to duplicate and expand these results. It would be interesting to see if this complex can be re-constituted from its components and further, see if it can be bound to 28S RNA in a similar manner to that found in E. coli for the binding of the 4:1 (EL7/12:EL10) protein

domain to 23S RNA.

A further development of this work would be to ascertain a function for this eukaryotic 'A' protein domain.

It is of interest to note that comparative studies on the ribosomal 'A' protein indicate sequence homology between the archaebacterial and eukaryotic 'A' protein, but little sequence homology with eubacterial 'A' protein. However, when the above results for the wheat germ 'A' protein domain are compared with the results from archaebacteria and eubacteria, it is apparent that the eukaryotic 'A' protein domain is substantially different from that of archaebacteria and eubacteria, where the 'A' protein domains are very similar. The evolutionary significance of these observations remains to be determined.

LITERATURE CITED

1. Brimacombe, R., Stoffler, G., Wittman, H.G. 1978. Am. Rev. Biochem. 47: 217-49.

2. Wittman, H.G. 1976. Eur. J. Biochem. 61: 1-13.

3. Wool, I.G. 1980. in Ribosomes (G. Chamblis, G.R. Craven, J. Davies, U. Davis, L. Kahan and M. Nomura, eds.) pp 797-824. Cold Spring Harbor Lab. N.Y.

4. Chua, N.-H., Luck, D.J.L. 1974. in Risobomes (Nomura, M., Tissieres, A. and Lengyel, P., eds.) pp 519-539. Cold Spring Harbor Lab. N.Y.

5. Doolittle, W.F. 1980. TIBS 5: 146-149.

6. Edwards, K., Kossel, H. 1981. Nucl. Acids Res. 9: 2853.

7. Woese, C.R., Fox, G.E. 1977. Proc. Nat'l. Acad. Sci. USA 74: 5088-5090.

8. Zimmerman, R.A. 1974. in Ribosomes (Nomura, M. Tissieres, A. and Lengyel, P., eds.) pp225-269. Cold Spring Harbor Lab. N.Y.

9. Brimacombe, R., Nierhaus, K.M., Garrett, R.A., Wittman, H.G. 1976. Progr. Nucl. Acid. Res. And Mol. Biol. (Cohn, W.E. ed) 18: 1-44.

10. Kuhlbrandt, W., Garrett, R.A. 1978. FEBS Lett. 94: 207-211.

11. Hardy, S. J.S., Kurland, C.G., Voynow, P. Mora, G. 1969. Biochemistry 8: 2897-2905.

12. Sherton, C.C., Wool, I.G. 1974. Mol. Gen. Genet. 135: 97-112.

13. Wittman, H.G., Littlechild, J.A., Wittman-Liebold, B. 1980. in Ribosomes (G. Chamblis, G.R. Craven, J. Davies, U. Davis, L. Kahan and M. Nomura, eds.) pp 53-88. Cold Spring Harbor Lab. N.Y.

14. Lemieux, G., G. Lefevre, J.-F., Daune, M. 1974. Eur. J. Biochem. 49: 185-194.

15. Morrison, C.A., Bradbury, E.M., Littlechild, J., Duk, J. 1977. FEBS Lett. 83: 348-352.

16. Zimmerman, R.A., Stöffler, G. 1976. Biochemistry 15: 2007-2017.

17. Kaltschmidt, E., Wittman, H.G. 1970. Anal. Biochem. 36: 401-412.

18. Howard, G.A., Traut, R.R. 1974. Methods in Enzym. 30: 526-539.

19. Wittman-Liebold, B., Greur, B. 1980. FEBS Lett. 121:105.

20. Wittman-Liebold, B., Robinson, S.M.L., Dzionara, M. 1977. FEBS Lett. 77: 301-307.

21. Visentin, L.P., Yaguchi, M., Matheson, A.T. 1979. Can. J. Biochem. 57: 719-726.

22. Terhorst, C., Moller, W. Laursen, R., Wittman-Liebold, B. 1972. FEBS Lett. 28: 324-328.

23. Kamp, R., Wittman-Liebold, B. 1980. FEBS Lett. 121:117

24. Yaguchi, M., Roy, C., Wittman, H.G. 1980. FEBS Lett. 121: 113.

25. Itoh, T., Wittman-Liebold, B. 1978. FEBS Lett.96: 399-402.

26. Matheson, A.T., Moller, W., Amons, R., Yaguchi, M. 1980. in Ribosomes (G. Chamblis, G.R. Craven, J. Davies, U. Davis, L. Kahan and M. Nomura, eds.) pp 297-332. Cold Spring Harbor Lab. N.Y.

27. Leader, D.P. 1980. Molecular aspects of cellular regulation. 1: 203-233.

28. Amons, R., Moller, W. 1978. Eur. J. Biochem. 91: 553-556.

29. Gudkov, A.T., Khechinashvili, N.N., Bushuev, V.N. 1978. Eur. J. Biochem. 90: 313-318.

31. Burgess, A.W., Ponnuswamy, P.K., Scheraga, H.A. 1974. Isr. J. Chem. 12: 239-86.

32. Chou, P.Y., Fasman, G.D. 1978. Ann. Rev. Biochem. 47: 251-276.

33. Nagano, K. 1974. J. Mol. Biol. 84: 337-72.

34. Robson, B., Suzuki, E. 1976. J. Mol. Biol. 107:327-56.

35. Wittman-Liebold, B., Greuer, B. 1979. FEBS Lett. 108: 69-74.

36. Gerard, D., Lemieux, G., Laustriat, G. 1975. Photochemistry and Photobiology. 22: 89-95.

37. Appell, K., Djik, T., Epp, O. 1979. FEBS Lett. 103: 66-70.

38. Leijonmarck, M., Eriksson, S., Liljas, A. Nature 286: 824-826.

39. Gudkov, A.T., Behlke, J., Vtiurin, N.N., Lim, V.I. 1977. FEBS lett. 82: 125-129.

40. Monier, R., Feunteun, J. 1964. Methods in Enzym. 20: 494-502.

41. Brownlee, G.G., Sanger, F., Barrell, B.G. 1967. Nature 215: 735-736.

42. Erdmann, V.A., Appel, B., Digweed, M., Kluwe, D., Lorenz, S., Luck, A., Schreiber, A., Schuster, L. 1980. Genetics and evolution of RNA polymerase, tRNA and ribosomes. pp 553-568.

43. Hori, H., Osawa, S. 1979. Proc. Nat'l Acad. Sci. 76: 381-385.

44. Hori, H., Osawa, S. 1980. Genetics and evolution of RNA polymerase, tRNA and ribosomes. (S. Osawa, H. Ozeki, H. Uchida, and T. Yura, eds.) pp 539-552.

45. Österberg, R., Sjoberg, B., Garrett, R.A. 1976. Eur. J. Biochem. 68: 431-487.

46. Fox, G.E., Woese, C.R. 1975. Nature 256: 505-507.

47. Woese, C.R., Fox, G.E. 1977. Proc. Nat'l Acad. Sci. 74: 5088-5090.

48. Weidner, H., Yuan, R., Crothers, D.M. 1977. Nature 266: 193-194.

49. Kemkhadze, K. Sh., Odinstov, U.B., Semenkov, Yu. P., Kirillov, S.V. 1981. FEBS Lett. 125: 10

50. Faber, N.M., Cantor, C.R. 1981. J. Mol. Biol. 146: 223-239.

51. Madison, J.T. 1968. Ann Rev. Biochem. 37: 131-148.

52. DeBuy, B., Weissman, S.M. 1971. J. Biol. Chem. 246: 447-461.

53. Luoma, G.A., Marshall, A.G. 1978. Proc. Nat'l Acad. Sci. 75: 4901-4905.

54. Thompson, J.F., Wegnez, M.R., Hearst, J.E. 1981. J. Molec. Biol. 147: 417-436.

55. Garrett, R.A., Douthwaite, S., Noller, H.F. 1981. TIBS 5: 137-139.

56. Nazar, R.N., Sitz, T.O., Busch, H. 1975. J. Biol. Chem. 250: 8591-8597.

57. Rubin, G.M. 1974. Eur. J. Biochem. 41: 197-202.

58. Nazar, R.N. 1978. J. Biol. Chem. 253: 4505-4507.

59. Nazar, R.N., Sitz, T.O. 1980. FEBS Lett. 115: 71-76.

60. Jacq, B. 1981. Nuc. Acid. Res. 9: 2913.

61. Brosius, J., Palmer, M.L., Kennedy, P.J., Noller, H.F. 1978. Proc. Nat'l. Acad. Sci. 75: 4801-4805.

62. Ehresmann, C., Stiegler, P., Carbon, P., Ebel, J.P. 1977. FEBS Lett. 84: 337-341.

63. Noller, H.F., Woese, C.R. 1981. Science 212: 403-412.

64. Noller, H.F. 1980. Ribosomes (G. Chamblis, G.R. Craven, J. Davies, U. Davis, L. Kahan and M. Nomura, eds.) Cold Spring Harbor Lab. N.Y.

65. Herr, W., Chapmen, N.M., Noller, H.F. 1979. J. Mol. Biol. 130: 433-449.

66. Sieber, G., Tesche, B., Nierhaus, K.H. 1980. Eur. J. Biochem. 106: 515-523.

67. Spirin, A.S., Serdyuk, I. N., Shpungin, J.L., Vasiliev, V.D. 1979. Proc. Nat'l Acad. Sci. 76: 4867-4871.

68. Rubstov, P.M., Musakhanov, M.M., Batchikova, N.V., Shryabin, K.G. 1979. Doklady Akad. Nauk. SSR. 248:760-762.

69. Branlant, C., Krol, A., Sriwidada, J., Brimacombe, R. 1976. Eur. J. Biochem. 70: 483-492.

70. Brosius, J. Dull, T., Noller, H. 1979. Proc. Nat'l Acad. Sci. 77: 201-204.

71. Vasiliev, V.D., Zalite, O.M. 1980. FEBS lett 121: 101-104.

72. Caskey, C.T., Tomkins,R., Scolink,E., Caryk, T., Nirenberg, M. 1968. Science 162: 135.

73. Lake, J.A. 1976. J. Mol. Biol. 105: 131-159.

74. Boublik, M., Hellman, W. 1978. Proc. Nat'l. Acad. Sci. 2829-2833.

75. Stoffler, G., Bald, R., Kastner, B., Luhrmann, R., Stoffler-Meilicke, M., Tischendorf, G., Tesche, B. 1980. in Ribosomes (G. Chambliss,G.R.Craven, J. Davies, U. Davis, L. Kahan and M. Nomura, eds.) Cold Spring Harbor Lab. N.Y. pp 171-206.

76. Kenny, J.W., Sommer, A., Traut, R.R. 1975. J. Biol. Chem. 250: 9434-9436.

77. Baumert, H.G., Skold, S.E., Kurland, C.G. 1978. Eur. J. Biochem. 89: 353-359.

78. Traut, R.R., Lambert, J.M., Boileau, G., Kenny, J.W. 1980. in Ribosomes (G. Chambliss,G.R. Craven, J. Davies, U. Davis, L. Kahan and M. Nomura, eds.) pp 89-110.

79. Langer, J.A., Engelman, D.M., Moore, P.B. 1978. J. Mol. Biol. 119: 463-485.

80. Nierhaus, K.H. 1980. in Ribosomes (G. Chambliss, G.R. Craven, J. Davies, U. Davis, L. Kahan and M. Nomura, eds) pp 267-296. Cold Spring Harbor Lab. N.Y.

81. Bogdanov, A.A., Kopylov, A.M., Shatsky, I.N. 1980. Subcellular Biochemistry I.

82. Tischendorf, G.W., Zeichhardt, H., Stoffler, G. 1975. Proc. Nat'l. Acad. Sci. 71: 4688-4692.

83. Moore, P.B. 1980. in Ribosomes (G. Chambliss, G.R. Craven, J. Davies, U. Davis, L. Kahan and M. Nomura, eds.) pp111-134. Cold Spring Harbor Lab. N.Y.

84. Lake, J.A. 1980. Ibid. pp 207-236.

85. Huang, K., Fairclough, R.H., Cantor, C.R. 1975. J. Mol. Biol. 97: 443-70.

86. Lake, J.A., Nonomura, Y., Sabatini, D.D. 1974a. in Ribosomes (G. Chambliss, G.R. Craven, J. Davies, U. Davis, L. Kahan, and M. Nomura, eds.) Cold Spring Harbor Lab. N.Y.

87. Chen-Schmeisser, U., Garrett, R.A. 1976. Eur. J. Biochem. 69: 401-410.

88. Peterson, I., Hardy, S.J.S., Liljas, A, 1976. FEBS lett. 64: 135-138.

89. Peterson, I., Liljas, A. 1979. FEBS Lett. 98: 139-144.

90. Marquis, D.M., Fahnestock, S.R. 1980. J. Mol. Biol. 142: 161-179.

91. Matheson, A.T., Yaguchi, M. 1980-81. International Cell Biology. 103-110.

92. Smith, A., Matheson, A.T., Yaguchi, M., Willick, G.E., Nazar, R.N. 1978. Eur. J. Biochem. 89: 501-509.

93. Peterman, M.L. 1974. Methods in Enzymol. 30: 346-349.

94. Unwin, P.N.T. 1977. Nature 269: 118-122.

95. Itoh,T. Wittman-Liebold, B. 1978. FEBS Lett. 96: 392-394.

96. Yaguchi, M., Matheson, A.T., Visentin, L.P., Zucker, M. 1980. Genetics and evolution of RNA polymerase, tRNA and ribosomes. pp 585-600.

97. Terhorst, C., Moller, W., Laursen, R., Wittman-Liebold, B. 1973. Eur. J. Biochem. 34: 138-152.

98. Koteliansky, V.E., Domogatsky, S.P., Gudkov, A.T. 1978. Eur. J. Biochem. 90: 319-323.

99. Boublik, M., Brot, N., Weissbach, H. 1973. Biopolymers 12: 2083-2092.

100. Luer, C.A., Wong, K.P. 1979. Biochemistry 18: 2019-2027.

101. Schrier. PhD dissertation. 1977. See Reference 63.

102. Dijk, J., Littlechild, J., Garrett, R.A. 1977. FEBS lett. 77: 295-300.

103. Oda, G., Strom, A.R., Visentin, L.P., Yaguchi, M. 1974. FEBS lett. 43: 127-130.

104. Willick, G.E., Williams, R.E., Matheson, A.T. 1978. FEBS lett. 85: 279-282.

105. Hamel, E., Koka, M., Nakamoto, T. 1972. J. Biol. Chem. 247: 805-814.

106. Van Agthoven, A.V., Krieh, J., Amons, R., Moller, W. 1978. Eur. J. Biochem. 91: 553-565.

107. Zinker, S., Warner, J.R. 1976. J. Biol. Chem. 251: 1799-1807.

108. Sanchez-Madrid, F., Conde, P., Vazquez, D., Bellesta, J.P.G. 1979. Biochem. Biophys. Res. Comm. 87: 281-291.

109. Moller, W., Slobin,L.I.,Amons,R.,Richter, D. 1978. Proc. Nat'l. Acad. Sci. 72: 4744-4748.

110. Horak, I., Schiffman, D. 1977. Eur. J. Biochem. 79: 375-380.

111. Lastick, S.M., McConkey, E.H. 1976. J. Biol. Chem. 251: 2867-2875.

112. Burrell, H.R., Horowitz, J. 1977. Eur. J. Biochem. 75: 533-544.

113. Horne, J.R., Erdmann, V.A. 1972. Molec. Gen. Genet. 119: 337-344.

114. Blobel, G. 1971. Proc. Nat'l Acad. Sci. 68: 1881-1885.

115. Ulbrich, N., Wool, I.G. 1978. J. Biol. Chem. 253: 9049-9052.

116. Metspalu, A., Saarma, M., Villems, R., Ustav, M., Lind, A. 1978 Eur. J. Biochem. 91: 73-81.

117. Liljas, A., Erikson, S., Skold, S.E. personal communication.

118. Isoda, N., Taraka, T., Ishikawa, K. 1981. J. Biochem. Tokyo. 90: 551-554.

119. Gray, P.N., Garrett, R.A., Stoffler, G., Monier, R. 1972. Eur. J. Biochem. 28: 412-421.

120. Fanning, T.G., Traut, R.R. 1981. Nuc. Acids. Res. 9: 993-1003.

121. Fox, J.W., Wong, K.-P. 1978. J. Biol. Chem. 253: 18-20.

122. Gray, P.N., Bellemare, G., Monier, R., Garrett, R.A., Stoffler, G. 1973. J. Mol. Biol. 77: 133-152.

123. Douthwaite, S., Garrett, R.A., Wagner, R., Feunteun, J. 1979. Nuc. Acid. Res. 6: 2453-2470.

124. Zimmeramn, R.A. 1980. Ribosomes (G. Chambliss, G.R. Craven, J. Davies, U. Davis, L. Kahan and M.Nomura,eds) pp 135-170. Cold Spring Harbor Lab. N.Y.

125. Bear, D.G., Scheich, T., Noller, H.F., Garrett, R.A. 1977. Nuc. Acid. Res. 4: 2511-2526.

126. Spierer, P., Wang, C.C., Marsh, T.L., Zimmerman, R.A. 1979. Nuc. Acid. Res. 6:1669-1682.

127. Pace, N., Pace, B., Matthews, B. 1980. See reference 55.

128. Nazar, R.N., Willick, G.E., Matheson, A.T. 1979. J. Biol. Chem. 254: 1506-1512.

129. Terao, K., Uchiumi, T., Ogata, K. 1980. Biochim. Biophys. Acta 609: 306-312.

130. Nazar, R.N., Yaguchi, M., Willick, G.E., Rollin, C.F., Roy, C. 1979. Eur. J. Biochem. 102: 573-582.

131. Willick, G.E., Nazar, R.N., Van, N.T. 1980. Biochemistry 19: 2738-2742.

132. Terao, K., Takaahashi, Y., Ogato, K. 1975. Biochim. Biophys. Acta 402: 230-237.

133. Ogata, K., Terao, K., Uchiumi, T. 1980. J. Biochem. 87: 517-524.

134. Gaunt-Klopfer, M., Erdmann, V.A. 1975. Biochim. Biophys. Acta 390: 226-230.

135. Lengyel, P. 1974. In Ribosomes (M. Nomura, A. Tissieres and P. Lengyel, eds.) pp 13-52. Cold Spring Harbor Lab. N.Y.

136. Kurland, C.G. 1977. Ann. Rev. Biochem. 46: 173-200.

137. Weissbach, H. 1980. In Ribosomes (G. Chambliss, G.R. Craven, J. Davies, K. Davis, L. Kahan and M. Nomura, eds.) pp 377-412. Cold Spring Harbor Lab. N.Y.

138. Boublik, M. 1980. Roche Institute of Mol. Biology Annual Report. pp 18-24.

139. Ochoa, S. 1980. Ann. Rev. Bioch. 49: 1-31.

140. Armstrong, F.B., Bennett, T.P. 1979. Biochemistry. Oxford University Press. New York.

141. Woese, C.R. 1981. Scientific American

142. Fox, G.E., Magrum, L.J., Balch, W.E., Wolfe, R.S., Woese, C.R. 1977. Proc. Nat'l. Acad. Sci. 74: 4537-4541.

143. Langworthy, T. 1977. Biochim. Biophys. Acta. 487: 37-50.

144. Woese, C.R., Gupta, R. 1980. Current Microbiology 4:

145. Zillig, W., Stetter, K.O. 1980. Genetics and evolution of RNA polymerase, tRNA and ribosomes. (S. Osawa, H. Ozeki, H. Uchida and T. Yura, eds) pp 525-538.

146. Bayley, S.T., Morton, R.A. 1978. Critical reviews in microbioloby. 6: 151-205.

147. Matheson, A.T., Nazar, R.N., Willick, G.E., Yaguchi, M. 1980. Genetics and evolution of RNA polymerase, tRNA and ribosomes. (S. Osawa, H. Ozeki, H. Uchida and T. Yura, eds.) pp 625-638.

148. Matheson, A.T., Yaguchi, M., Balch, W.E., Wolfe, R.S. 1980. Biochim. Biophys. Acta. 625: 162-169.

149. Neville, D.M. 1971. J. Biol. Chem. 246: 6328-6334.

150. Loening, U.E. 1967. Biochem. J. 102: 251-257.

151. Girard, M. 1967. Methods in Enz. 12a: 581.

152. Fairbanks, G., Steck, T.L., and Wallach, D.F.H. 1971. Biochemistry 10: 2606.

153. Peacock, A.C., and Dingman, C.W. 1967. Biochemistry 6: 1818.

154. Sikorski, M.M., Przybl, D., Legocki, A.B., Kudlicki, W., Gasior, E., Azlac, J., Borkowski, T. 1979. Plant Science Letters. 15: 387-397.

155. Morrishey, J., Hardesty, B. 1974. Methods in Enz. 29: 728-729.

156. Cox, PhD. Thesis. CA. 1964

157. Hirs, C. H. W. 1967. Methods in Enz.XI: 197-203.

158. Tarr, G.E. 1981. Annal. Biochem. 111: 27.

159. Hampl, H., Schulze, H., and Nierhaus, K.H., unpublished. See Reference 80.

160. Sperrazza, J.M., Moore, M.N., Spremulli, L.L. 1981. Biochemistry. 20: 5073-5079.

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ISOLATION AND CHARACTERIZATION OF CERTAIN RIBOSOMAL DOMAINS; THE 5S RNA-PROTEIN DOMAIN FROM ESCHERICHIA COLI AND THE 'A' PROTEIN DOMAIN FROM WHEAT GERM.

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April, 1982