PREPARATION OF RIBOSOMAL SUBUNITS BY GEL FILTRATION

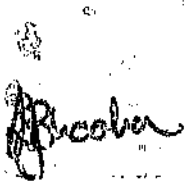
DEENA BHOOLIA

A Dissertation submitted in fulfilment
of the requirements for the degree of
Master of Science at the University
of the Witwatersrand

Johannesburg
January 1991

DECLARATION

I declare that this dissertation is entirely my own work and has not been presented for any degree at another University.



DEENA JETHALAL BHOOLIA

Johannesburg
January 1991

ABSTRACT

An attempt was made to separate ribosomal subunits by gel filtration on Trisacryl GF2000 and Sepharose 4B. Trisacryl GF2000, a synthetic gel, separated rat liver ribosomal subunits on a 135 cm column with a resolution of ~0,3, resulting in ~60 % impurity of each of the subunits. Rabbit reticulocyte and Escherichia coli ribosomal subunits were not resolved. Sepharose 4B, an agarose based gel, separated the subunits by adsorption chromatography rather than by gel filtration. At 4°C, the 40S subunits were eluted with a k_d ~0,20, but the 60S subunits adsorbed to the gel, and were eluted when the temperature of the column was increased to 25°C-35°C. This adsorption phenomenon seems to be a property of all agarose based gels studied here, including Sepharose 2B and Sepharose 6B, and is exclusive to mammalian ribosomal subunits. Analysis of the subunits by in vitro [^{14}C]polyphenylalanine synthesis showed no difference in the activities of ribosomal subunits prepared by gradient centrifugation or by Sepharose chromatography. Analysis of the subunits by acrylamide-agarose composite gels resulted in the resolution of subunits isolated from lower organisms in non-denaturing gel systems and subunits from mammalian tissue in denaturing gel systems only.

Gel filtration does offer a suitable method for the preparation of ribosomal subunits, but only if the adsorption properties of Sepharose gels are exploited.

This work is dedicated to

Ba, Archie, Deepak and Bhavna

and of course Anoop.

ACKNOWLEDGEMENTS

I should like to thank the following;

Professor K L Manchester, my supervisor, for valuable advice and discussions;

M Mouat for guidance; continued interest and encouragement;

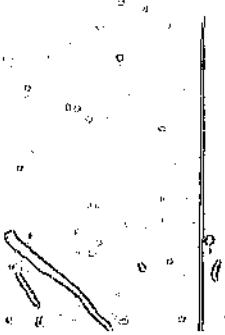
Members of the Department of Biochemistry, University of the Witwatersrand;

The CSIR and the University of the Witwatersrand for financial support.

TABLE OF CONTENTS

ABBREVIATIONS

ATP	Adenosine triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Ethylenediaminetetra-acetic acid
GTP	Guanosine triphosphate
HEPES	N-2-hydroxyethylpiperazine N'-2-ethanesulfonic acid
HETP	height equivalent of a theoretical plate
MSH	Mercaptoethanol
PEG	Polyethylene glycol 6000
poly(U)	poly-uridilic acid
RNA	Ribonucleic acid
SDS	Sodium Dodecyl Sulphate
TEMED	N,N,N',N'- Tetralmethylethylenediamine
Tris-HCl	Tris (Hydroxymethyl) aminomethan-hydrochloride



LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Ribosomes are the cellular organelles which partake in the biosynthesis of proteins. The main interest in the biochemistry of ribosomes is to know the structure and to define the exact function of each of the components in the particle. The present understanding of the structure-function relationship is still limited and this is mainly due to poor elucidation of the complete structure of the ribosome and its subunits. Without insight into the exact structure of the ribosome, its subunits and their components, the mechanism of protein biosynthesis will remain at a descriptive level.

The importance of analysing ribosomes and ribosomal subunits in biochemistry has necessitated the development of simple and efficient techniques for their preparation and characterization.

1.1 PHYSICAL CHARACTERISTICS OF PROKARYOTIC AND EUKARYOTIC RIBOSOMES AND RIBOSOMAL SUBUNITS

The ribosome of both prokaryotes and eukaryotes are characterized by their sedimentation coefficients which are directly correlated to their size and molecular weight. The ribosomes of prokaryotes are somewhat smaller than those found in the cytoplasm of eukaryotes. The former have a sedimentation coefficient of 70S (Svedburg units) and the latter a sedimentation coefficient of 80S. All types of ribosomes consist of two subunits of unequal size: those from 70S

ribosomes sediment at 30S and 50S and those from 80S ribosomes sediment at 40S and 60S. The sedimentation coefficient values are approximate values which depend on the origin of the ribosomes.

The total molecular weight of the 70S monosome from bacteria is ~2,8 x 10^6 daltons. The molecular weights of the large and small subunits of Escherichia coli ribosomes are ~1,8 x 10^6 and 0,7 x 10^6 daltons respectively. Chemical analysis of the ribosomes and ribosomal subunits of Escherichia coli show that the large subunit contains one molecule each of 23S ribosomal RNA (rRNA) and 5S rRNA and 34 ribosomal proteins and the small subunit contains one molecule of 16S rRNA and 21 ribosomal proteins. The sum of the molecular weights of the proteins and the rRNA's is equal to the molecular weights of the subunits.

Eukaryotic ribosomes are larger than prokaryotic ribosomes due to extra rRNA's and proteins. The large subunit of rat liver cytoplasmic ribosomes contains one molecule each of 28S rRNA, 5,8S rRNA and 5S rRNA and 45-50 ribosomal proteins, and the molecular weight of this 60S particle is ~2,8 x 10^6 daltons. The small subunit of rat liver ribosomes contains one molecule of 18S rRNA and 30 ribosomal proteins and has a total molecular weight of ~1,5 x 10^6 daltons.

1.2 MORPHOLOGY OF PROKARYOTIC AND EUKARYOTIC RIBOSOMES AND RIBOSOMAL SUBUNITS

There is now general agreement concerning the overall structure and

morphology of the Escherichia coli ribosome. The smaller subunit (30S) is divided into two unequal parts by an indentation and a region of accumulated negative stain (Lake, 1985). The parts are the head or upper third, and the base or lower two thirds. A region of the subunit, called the platform, extends from the base of the small subunit and forms a cleft between it and the head. The approximate dimensions of the small subunit is 23 nm x 11 nm (Wittman,1983). The large subunit of Escherichia coli ribosomes, like that of the small subunit, is an asymmetric ellipsoid with dimensions of 11,5 nm x 23 nm. It consists of a central protuberance, or head, and two protrusions inclined approximately 50 degrees to either side of the central protuberance. One of these, the "L7/L12 stalk," is at the right and contains the only multiple copy proteins present in the Escherichia coli ribosome. In a projection approximately orthogonal to this, the large subunit is characterized by a notch on the upper surface (Lake, 1985). In the monomeric ribosome of Escherichia coli the small subunit is positioned asymmetrically on the large subunit. This allows the platform of the small subunit to contact the large subunit, so that the partition between the head and the body of the small subunit is approximately aligned with the notch of the large subunit.

The morphology of eukaryotic ribosomes is still the subject of much speculation. Although definite progress has been made in the investigation of the ribosome structure by electron microscopy, unlike that of prokaryotic ribosomes, no definite model of eukaryotic ribosomes has been proposed. The general characteristics of the small ribosomal subunit (40S) of eukaryotic ribosomes is that it consists of an elongated portion which comprises up to two thirds of the

length of the particles along its axis. To one of the ends of this elongated portion, a "head" is attached. The length of the particle is about 25 nm whereas its width fluctuates between 8,5 nm and 14,5 nm (Kiselev et al, 1978). The 40S subunit shows a protuberance at the body directed to the stronger contrast line between the body and the head. It is assumed that this protuberance is involved in the contact between the 40S subunit and the 60S subunit inside the 80S ribosome (Bielka, 1982). The general view of the morphology of the large subunit of eukaryotic ribosomes shows a heart shaped structure with one blunt end and one pointed end. It has one concave side, in which a dense notch towards the blunted end can be observed. This notch is thought to be the flattened surface of the subunit. The pointed edge is eccentrically located pointing to the right of the subunit and the notch is on the left side. On the lower tip of the image, two lateral incisions can be seen which possibly represent a ring-like groove around the tip of the 60S subunit. The dimensions of the large subunits are ~24 nm x 20 nm (Beilka, 1982).

1.3 CHEMICAL CHARACTERISTICS OF RIBOSOMES OF PROKARYOTES

Proteins

The proteins of the small and the large subunits of *Escherichia coli* have been isolated by CM-cellulose chromatography and Sephadex gel filtration in the presence of urea. Proteins prepared in this degradative manner have been used mainly for immunological and amino acid sequencing studies. Efforts have been made to extract and purify the ribosomal proteins under native conditions so as to study

their secondary and tertiary structures. The complete amino acid sequences of 33 of the 55 proteins have so far been determined. The extent of homology of the proteins is no greater than would be expected on a random basis, except for the L7/L12 pair which have almost identical primary sequences and the S20/S26 pair which have identical primary sequences. Predictions of secondary and tertiary structures have been made once the primary structures were known.

Ribosomal RNA

In prokaryotic cells, ribosomes contain three ribosomal RNA molecules designated 5S, 16S, and 23S. These molecules contain about 120, 1540 and 2900 nucleotides respectively (Noller, 1984). Nucleotide sequences of the rRNA molecules have been deduced by DNA sequencing of the corresponding cloned rRNA genes.

General secondary structure models for the 16S and 23S rRNA's have been proposed (Noller, 1984). The 16S rRNA is subdivided into three major structural domains and one minor domain, by three sets of long range base paired interactions. Over 100 individual helices are distributed among six domains in the 23S rRNA molecule. As with the 16S rRNA, the six domains are defined by long range base-paired interactions. Studies on the binding of isolated proteins to rRNA indicate that the proteins are able to influence the secondary structure of the rRNA.

The Association of rRNA's and Ribosomal Proteins within the Ribosomal Subunits of Escherichia coli

Ribosomal protein topography, or the spatial arrangement of the proteins within the ribosomal subunits, is obviously important in the

correlation of structure and function. Using purified antibodies that bind to the specific proteins, the antigenic sites of all 21 proteins of the 30S ribosomal subunit have been located by immune electron microscopy.

Various proteins (S4, S7, S11, S12, S15, and S18) revealed multiple antibody binding sites at widely separated points on the 30S subunit surface (Brimacombe et al., 1978), thus indicating that these proteins have highly extended conformations. Other proteins showed slightly extended conformations (S2, S3, S5, S10, and S19) where their antigenic sites were separated by 5-8 nm. Proteins S1, S8, S13, S14, S20, and S21 showed single antigenic sites and S6, S9, S16, and S17 showed two, indicating that these proteins are probably globular. Since parts of the protein chain may be buried within the ribosomal subunit and inaccessible to the antibody, these findings may have to be viewed as just an estimate. Most of the antibody binding sites occur on the head of the subunit and, while there are several sites on the neck and the body, there is a notable absence of any site in the hollow between the head and the body.

Immune electron microscopy has also been applied to the localization of a specific region of the 16S rRNA (Politz and Glitz, 1977), where the 6-N,N dimethyl adenine residue near the 3' terminus of the 16S rRNA was located on the "platform" of the 30S subunit.

A three dimensional map of the locations of 19 of the 34 ribosomal proteins of the 50S subunit has been established. The proteins of the 50S subunit are more asymmetrically arranged in comparison to those of the 30S subunit, where immune electron microcopy revealed

that most of the antibody sites were clustered within the "crowned seat" region. Six proteins show elongated shapes when analysing their antibody attachment sites. The location of the L7/L12 protein was on the rod-like appendage (Lake, 1977).

The first direct measurements of neighbourhoods between ribosomal proteins and rRNA were made with the aid of bifunctional cross-linking reagents. A total of 34 pairs of cross-linked proteins from the 30S subunit have been clearly identified. If the protein cross-linking data are compared with the results of immune electron microscopy, good agreement is found only if the elongated nature of the proteins is taken into account. Immune electron microscopy and cross-linking data have been verified using neutron scattering and fluorescence techniques. In vitro reconstitution studies of the 30S subunit indicated that a few proteins - S4 (Nitta et al., 1986), S7, S9 (Chiaruttini et al., 1986), S8 (Gregory and Zimmerman, 1986), S15, S17, S20, and possibly S13, were able to interact specifically with the 16S rRNA molecule. The remaining proteins could be added to complexes of the RNA and one or more of the primary binding proteins. It should be noted, however, that protein binding sites, as stated earlier, are very much dependent on the RNA conformation as well as on the conformation of the proteins, and that proteins such as S7 (Brimacombe et al, 1978) are able to alter the conformation of the RNA, thus aiding the binding of other proteins. Similarly studies on the 50S subunit showed that ribosomal proteins attach to the 23S RNA from the 5' end towards the 3' end. Like the 30S subunit, certain proteins have a functional role in aiding attachment of other proteins to the rRNA, in particular L24 (Brimacombe et al., 1978) and L2 (Nag et al., 1986) which bind directly to the 23S rRNA.

1.4 THE CHEMICAL COMPOSITION OF EUKARYOTIC RIBOSOMES

Proteins

Ribosomal proteins are separated according to size by two-dimensional electrophoresis (Kaltschimdt and Wittman, 1970). The number of proteins varies between species and within a particular species. It also depends on the conditions used for electrophoresis and the preparation of the subunits. Generally rat liver ribosomes are made up of between 26-42 proteins in the small ribosomal subunit and between 34-46 proteins in the large ribosomal subunit (Bielka, 1982). Proteins are numbered according to their position on the two-dimensional electrophoretogram. The molecular weights of the ribosomal proteins of rat liver ribosomes range from 12 000-27 000 daltons for those found in the 60S subunit and 10 400-27 400 daltons for those found in the 40S subunit (Terao and Ogata, 1975).

The proteins of ribosomes can be prepared by CM-cellulose chromatography (Terao and Ogata, 1972) and gel filtration. Preparation of the individual ribosomal proteins enables the study of amino acid composition and analysis of the tryptic patterns of each ribosomal protein. The covalent structures of several proteins of rat liver ribosomes have been determined directly, including P2 (Lin et al., 1982), L37 (Lin et al., 1983), L39 (Lin et al., 1984) and S21 (Itoh et al., 1985). This task is being expedited by the application of recombinant DNA technology. Thus, the amino acid sequences of a number of rat ribosomal proteins have been deduced from the cDNA's, including S11 (Tanaka et al. 1985), S26 (Kuwano et al., 1985), S17 and L30 (Nakanishi et al., 1985), L35a (Tanaka et al., 1986), L19 (Chan et al., 1987), L31 (Tanaka et al., 1987) and S12 (Lin et al.,

1987).

Ribosomal RNA

Ribosomal RNA's constitute the backbone of the structure of the two ribosomal subunits. The large subunit contains three ribosomal RNA molecules, i.e. 28S rRNA, 5,8S rRNA and 5S rRNA, and the small ribosomal subunit contains one ribosomal RNA molecule, i.e. 18S rRNA.

The 28S rRNA has been sequenced using cloned rDNA fragments (Hadjiolov et al., 1984). It contains 4800 nucleotides and has an estimated molecular weight of 1,66 x 10^6 daltons. The secondary structure of the 28S rRNA consists of 7 domains. Each domain consist of numerous helices bound by long range base-pairing. The 18S rRNA contains 1 874 nucleotides and has an estimated molecular weight of 6,09 x 10^6 daltons (Chan et al., 1984). The secondary structure of the 18S rRNA is similar to the 16S rRNA of bacteria in that several domains are linked together by long range base-pairing, although there are major differences in sequence homologies and the helices formed (Chan et al., 1984).

The Association of Ribosomal Proteins and Ribosomal rRNA's within the Subunits of Eukaryotes

Protein-protein interactions are being determined by chemical cross-linking studies and by immune electron microscopy. Protein-RNA interactions, on the other hand are being determined by affinity chromatography, which is a process whereby mixtures of ribosomal proteins are passed through a Sepharose column containing the immobilized nucleic acid. Those proteins that bind to the immobilized nucleic acid are eluted and identified by gel

electrophoresis.

The 5S rRNA binding proteins revealed by this procedure are L19, L6, L18, L7, L8 and L35 (Ulbrich and Wool, 1978). Affinity chromatography (Metspalu et al., 1978) also shows that the proteins L5, L6, L7 and L18 bind to the 5.8S rRNA, all of which overlap with the proteins which bind to the 5S rRNA, showing close proximity of the 5S and 5,8S rRNA. It is thought that these proteins are located at or near the peptidyltransferase centre (Metspalu et al., 1978) and may be involved in the placement and/or displacement of rRNA molecules from their sites on the ribosome surface. The proteins of the 60S subunit, L8 and L6, and L19 have been shown to associate with the 5,8S rRNA and so has S13 and S9 of the 40S subunit proteins. Thus it is thought that the 5,8S rRNA participates in the formation of the ribosome by simultaneously binding small and large subunit proteins. Treatment of the large ribosomal subunit of rat liver with EDTA releases a 7S complex (Blobel, 1971) which consists of 5S rRNA and protein L5. This complex is absolutely essential in the biological function of the large subunit since it was shown to be active in ATP and GTP hydrolysis (Grummt et al., 1974). Therefore it is suggested that this complex is located at or near the ribosomal P-site of the 60S subunit.

Chemical cross-linking can be applied to estimate protein neighbourhoods within the ribosome and to identify proteins in the vicinity of the rRNA's. Chemical cross-linking using the cleavable bifunctional reagent, dimethyl 3,8-diaza-4,7dioxo-5,6dihydroxy-decan-bisimidate showed that proteins S2, S3 and S3a were in close proximity with each other and S15 was cross-linked to S15a and S5 to

S25. S3a and S15 were found to be cross-linked to eIF-2 (Westermann et al., 1979), thus confirming the neighbourhood of proteins S3 and S3a.

Immune electron microscopy provides information about the localization of antigenic sites on the surface of the particles with respect to the three dimensional arrangement of the particles. Immune electron microscopy showed S2 to be involved in the binding of eIF-2 (Noll et al., 1978), again confirming the close proximity of proteins S2, S3, and S3a. These proteins are located near the ribosomal P-site and the P-site is organized at least partially in the head region of the small ribosomal subunit (Noll et al., 1978). The topographical studies using immune electron microscopy demonstrate that some proteins (S5 and S17) are located in the head region of the small ribosomal subunit, whereas others (S7, S9 and S21) are found in the body part only. Proteins S2, S3, S3a and S6 have antigenic determinants in both regions of the small ribosomal subunit. By comparing data obtained by hydrodynamic methods (Behlke et al., 1979) and immune electron microscopy, the conclusion can be drawn that no major differences exist between the shape of ribosomal proteins found in isolation and those assembled in the ribosomal structure.

1.5 THE FUNCTION OF RIBOSOME AND RIBOSOMAL COMPONENTS

The ribosome mediated translation of mRNA into protein from aminoacyl-tRNA substrates can be divided into three phases: chain initiation, chain elongation and chain termination.

The first step in the initiation process in prokaryotes is the formation of the 30S preinitiation complex which involves the cooperative binding of three initiation factors to the 30S ribosomal subunit to form a 30S.IF-1.IF-2.IF-3 particle. All three factors bind contiguously near the 3' end of the 16S rRNA.

Following this, initiator fmet-tRNA$_f$ and mRNA specifically bind to the 30S complex to form the 30S initiation complex. The 30S subunit and the mRNA molecule bind at a site on the mRNA that includes the initiation codon, AUG or GUG. The 30S ribosomal subunit has the inherent property of specifically recognizing and selecting initiation sites on natural mRNA. The formation of the initiation complex is completed by the binding of GTP. GTP acts as a steric effector permitting stable association of IF-2 with ribosomes. Thus the 30S initiation complex contains bound fmet-tRNA$_f$ and mRNA. During binding of the 50S subunit, GTP is hydrolysed by the ribosome bound IF-2. Then IF-I, IF-2, GDP and Pi are released from the ribosomal complex. The 50S ribosomal proteins L7/L12 and protein L11 are involved in IF-2 dependent GTP hydrolysis. However they do not exhibit intrinsic GTPase activity.

Initiation of protein synthesis in eukaryotes is more complex since more factors are involved. Ribosomal subunits are produced as a consequence of the ribosomal cycle in protein synthesis. Ribosomal subunits then accumulate as a result of active dissociation of 80S ribosomes or because they are prevented from reassociating by other factors. Initiation factor eIF-3 binds covalently to the 18S rRNA (Nygard and Westermann, 1982) and various ribosomal proteins on the 40S subunits. eIF-3 binds at the ribosomal interphase (Moldave,

1985) and closely adjacent to the attachment sites for eIF-3, mRNA and the 60S ribosomal subunit. eIF-6 is another initiation factor which reacts with the 60S ribosomal subunit and thereby prevents the 60S subunit from reassociating with the 40S ribosomal subunit (Moldave, 1985).

The ternary complex, which carries initiator fmet-tRNA$_f$, is formed by the binding of eIF-2 to GTP in the formation of the eIF-2.GTP binary complex. This binary complex then binds initiator fmet-tRNA$_f$ to form the ternary complex, fmet-tRNA$_f$.eIF-2.GTP. The ternary complex then binds to the 40S.eIF-3 complex to form the 43S pre-initiation complex. Following the formation of the 43S pre-initiation complex, mRNA binds to the complex with the aid of various initiation factors and ATP. The ribosomal subunit recognizes a cap structure at the 5' end of the initiation codon on the mRNA and binds to this cap structure.

eIF-5 helps the binding of the 60S ribosomal subunit to the mRNA.43S preinitiation complex, in the formation of the 80S initiation complex. It also hydrolyses GTP to GDP and inorganic phosphate (Pi) after the binding of the 60S subunit. The eIF-2.GDP complex, eIF-3, and Pi are released.

Elongation, which is similar in both prokaryotes and eukaryotes, occurs after the formation of the initiation complex. The respective initiator tRNA is bound to a ribosomal site referred to as the P (donor) site. The initiation codon is located at the donor site. The next codon to be translated on the mRNA is in an open ribosomal position adjacent to the P-site, referred to as the A-site

(acceptor). A ternary complex consisting of the aminoacyl-tRNA, the appropriate elongation factor and GTP binds to the A-site. GTP is hydrolysed and the elongation factors along with GDP + Pi are released. When the two ribosomal binding sites are filled, that is the P-site with methionyl-tRNA and the A-site with newly bound aminoacyl-tRNA, a peptide bond is formed between the methionine and the incoming aminoacyl moiety. The formation of the peptide bond is catalysed by an enzyme, identified in prokaryotes as peptidyl transferase, which consists of one or more proteins of the 50S subunit. In order to free the A-site for the next aminoacyl-tRNA, a translocation step takes place. In this movement, the tRNA and the dipeptide attached is translocated from the A-site to the P-site. At the P-site the deacylated tRNA leaves the ribosome and the next codon of the mRNA enters the A-site. Translocation is aided by GTP and another elongation factor. During the reaction, GTP is hydrolysed and GDP + Pi and the elongation factor are released. The ribosome is then ready for another cycle of elongation.

Translation ends when a termination codon enters the ribosomal A-site. Three termination codons exist. They are UAA, UAG, and UGA. When the peptidyl-tRNA is at the ribosomal P-site and a termination codon is at the A-site, a protein factor, identified as RF in eukaryotes and RF1 and RF2 in prokaryotes, binds to the A-site in the presence of GTP in eukaryotes, and GTP and a third release factor in prokaryotes. The termination reaction involves the hydrolysis of the peptidyl-tRNA ester bond, the hydrolysis of GTP and the release of the completed peptide chain, the deacylated tRNA and the ribosome from the mRNA.

Ribosomes from all organisms are divided into two general functional regions. They are the translational domain, and the exit or secretory domain. These two domains are found on opposite ends of the ribosome. The translational domain includes the head and the platform of the small subunit and the L7/L12 stalk, the central protuberance and the L1 ridge of the large ribosomal subunit of Escherichia coli. The placement of the small subunit on the large subunit makes possible an operational distinction between 30S ribosomal proteins located on the exterior of the subunit and those at the interface between the two subunit . The proteins S4, S5, S8, and S12, located at the concave edge of the head of the subunit, are all involved in tRNA recognition and S3, S10, S14, and S19 are involved in tRNA binding. Recognition occurs on the exterior surface of the small ribosomal subunit of Escherichia coli (Lake, 1985). Proteins which occur at the platform (S6, S11, S15, and S18) and the 3' end of the 16S rRNA, which is also located at the platform of the small ribosomal subunit, have been linked to mRNA binding (Lake, 1985). IF-1, IF-2 and IF-3 bind across the cleft between S13, S19-II and S12 of the head of the small subunit and S11 on the platform, indicating that these ribosomal proteins are involved in the initiation of protein synthesis. Proteins L7 and L12 have unique properties. L12 is identical to L7 except that the amino terminus of L7 is acetylated. Both proteins are intimately involved in EF-Tu, EF-G and IF-2 dependent GTP hydrolysis. The L7/L12 stalk appears to be a universal ribosomal structure and therefore may be responsible for GTP hydrolysis in all functionally active ribosomes.

The morphological features of both ribosomal species are similar. Since most of the structural features present on the translational

domain of prokaryotes are also present in eukaryotes, it is suggested that the translational mechanisms might be the same in both cases.

1.6 THE PREPARATION AND ANALYSIS OF RIBOSOMAL SUBUNITS

The Dissociation of Ribosomal Subunits and Separation by Density Gradient Centrifugation

The dissociation of cytoplasmic ribosomes of both prokaryotes and eukaryotes into their respective ribosomal subunits is a physiological event occurring at the termination step of the biosynthesis of proteins. The subunits generated in this way are generally termed "native subunits".

The agents responsible for the association of the ribosomal subunits are the electrostatic binding forces of magnesium. Magnesium is known to stabilize the ribosomal particles of Escherichia coli, of yeasts, of plants and of animals. Furthermore the presence of magnesium is vital for maintaining the integrity of the ribosomal particles. Lowering the magnesium concentration causes the dissociation of the ribosomal subunits of Escherichia coli (Tissères et al., 1959). Complete dissociation is achieved at concentrations of ~0,25 mM magnesium. Restoration of the magnesium ion concentration to ~2 mM results in the reassociation of the ribosomal subunits which are active in polyphenylalanine synthesis.

Lowering the magnesium concentration does not dissociate mammalian ribosomes. It does, however, reduce the proportion of polysomes. Complete removal of the magnesium ions by chelating agents such as

EDTA results in the dissociation of eukaryote ribosomes. However, the application of EDTA is accompanied by the considerable unfolding of both the subunits and, during the subsequent separation procedure by ultracentrifugation, partial disassembly of protein and RNA components are unavoidable. The sedimentation values of such subunits obtained from rat liver are generally in the order of 50S and 30S for the large and small ribosomal subunits respectively (Martin et al., 1969; Lamfrom and Glowacki, 1962; Gould et al., 1966; Nolan and Arnstein, 1969), the lower sedimentation values being due to the partial disassembly of the protein and rRNA components. The changes that occur through EDTA treatment are irreversible, and subunits prepared in this way are unable to reassociate into active ribosomes.

The electrostatic binding forces of magnesium, responsible for subunit association, can be repressed by increasing the concentration of certain monovalent cations, thus dissociating the subunits. This dissociation is improved when the ribosomes are incubated at temperatures between 28°C and 37°C (Martin et al., 1969; Terao and Ogata, 1970). At these temperatures, the majority of the ribosomes dissociate into the 40S and 60S ribosomal subunits in 880 mM KCl while a small percentage sediments at 75S. The last fraction is a small fraction of the ribosomes bound to nascent protein which protects it from dissociating in high concentrations of KCl (Martin et al., 1971). Incubating the ribosomes in 1 M KCl at 37°C dissociates them into their subunits. However, density gradient analysis of the ribosomal subunits shows contamination of the 60S fraction with dimers of the 40S subunit (Terao and Ogata, 1970). One drawback in the use of high concentrations of KCl to dissociate

EDTA results in the dissociation of eukaryote ribosomes. However, the application of EDTA is accompanied by the considerable unfolding of both the subunits and, during the subsequent separation procedure by ultracentrifugation, partial disassembly of protein and RNA components are unavoidable. The sedimentation values of such subunits obtained from rat liver are generally in the order of 50S and 30S for the large and small ribosomal subunits respectively (Martin et al., 1969; Lamfrom and Glowacki, 1962; Gould et al., 1966; Nolan and Arnstein, 1969), the lower sedimentation values being due to the partial disassembly of the protein and rRNA components. The changes that occur through EDTA treatment are irreversible, and subunits prepared in this [illegible]e to reassociate into active ribosomes.

The electrostatic binding forces of magnesium, responsible for subunit association, can be repressed by increasing the concentration of certain monovalent cations, thus dissociating the subunits. This dissociation is improved when the ribosomes are incubated at temperatures between 28°C and 37°C (Martin et al., 1969; Terao and Ogata, 1970). At these temperatures, the majority of the ribosomes dissociate into the 40S and 60S ribosomal subunits in 880 mM KCl while a small percentage sediments at 75S. The last fraction is a small fraction of the ribosomes bound to nascent protein which protects it from dissociating in high concentrations of KCl (Martin et al., 1971). Incubating the ribosomes in 1 M KCl at 37°C dissociates them into their subunits. However, density gradient analysis of the ribosomal subunits shows contamination of the 60S fraction with dimers of the 40S subunit (Terao and Ogata, 1970). One drawback in the use of high concentrations of KCl to dissociate

mammalian ribosomes is that concentrations higher than 0,5 M tends to extract proteins from the ribosomal subunit. Clegg and Arnstein (1970) showed that up to 50% of the protein was dissociated from rat liver ribosomes when they were zonally centrifuged through a solution containing 1 M KCl. Concentrated salt washings appear to be acceptable for the 40S subunits of rat liver, since extra-ribosomal proteins are removed without the inactivation of the subunits (Reboud et al., 1972), whereas the 60S ribosomal subunit is inactivated by high salt washes, indicating that essential ribosomal proteins are lost.

Puromycin has been used to release nascent polypeptide chains from ribosomes in rat liver (Lawford, 1969; Blobel and Sabatini, 1971; von der Decken, 1973; Manchester, 1984), in rat muscle (von der Decken, 1973) and in rabbit reticuloctytes (Sundkivist et al., 1974). Puromycin is an antibiotic which inhibits protein synthesis by interacting with peptidyl-tRNA at the ribosomal peptidyltransferase centre, thus terminating the nascent peptide chain and releasing the ribosomal subunits. Individual subunits prepared from puromycin treated ribosomes were found to be inactive in synthesis of polyphenylalanine, but the 40S and 60S subunits together were active in polyphenylalanine synthesis in the presence of poly-U (Lawford, 1969). It has been shown that the extent of release of the nascent polypeptide is a function of the monovalent ion concentration and temperature. The release is enhanced when the KCl concentration is increased to 1 molar (Blobel and Sabatini, 1971). Incubating at 37°C results in the release of mRNA and the tRNA moiety of the peptidyl-tRNA , and this leads to complete dissociation of the polysomes into ribosomal subunits (Blobel and Sabatini, 1971). The

advantage of using puromycin as opposed to high salt for the preparation of ribosomal subunits is that a higher yield of active subunits is obtained with the former method.

The use of puromycin can be avoided if the ribosome population consists of particles mainly or completely free of nascent protein (Lawford, 1969; Blobel and Sabatini, 1971) or if precautions have been taken to terminate the nascent proteins by prior incubation of the polysomes with all the components necessary for in vitro protein synthesis. It is believed that the most active ribosomal subunits are prepared by in vitro peptide chain termination (Falvey and Staehelin, 1970). However, Sundkvist et al. (1974) has shown that there is no significant difference in the activities of the ribosomal subunits of rabbit reticulocytes prepared either way, that is by puromycin treatment or by incubating the polysomes with all the components necessary for in vitro protein synthesis.

In summary, the dissociation of eukaryotic ribosomes into reassociable subunits is more difficult than for prokaryotic ribosomes. Noninitiated 80S ribosomes dissociate more easily than polysomes carrying nascent proteins. Precautions must be taken so that the dissociation procedure does not damage the binding forces within the subunits to a greater extent than the forces interacting between the small subunit and the large subunit. Thus the dissociation behaviour of eukaryotic polysomes is different from that of prokaryotic polysomes. Because the interactions between the nonribosomal components promoting protein synthesis are basically the same in both prokaryotes and eukaryotes, the different responses observed with different dissociating agents may be attributable to

the more complex structure of the eukaryotic ribosome.

Separation of the Ribosomal Subunits by Gel Filtration

Column chromatographic methods for the purification of ribosomal subunits offer several advantages over traditional methods utilising sucrose gradient centrifugation. Purification of ribosomal subunits by column chromatography requires less work, is less expensive, takes a shorter time and can easily be applied to large quantities. Column buffers, in addition, avoid the use of sucrose solutions which are undesirable for subsequent manipulations in some cases.

Gel filtration is based on the principle of diffusion into and out of the stationary phase, where the diffusion is dependent on the bulk flow of the mobile phase and upon the Brownian motion of the solute molecules. The diffusion of the solute is characterized by the diffusion constant (K_d) which is defined as the fraction of the stationary phase which is available for diffusion of a given solute species.

Gel exclusion chromatography on agarose based resins has been widely used to separate high molecular weight macromolecules such as rRNA (Petrovic et al., 1971; 1973), total cellular RNA (Zeichner and Stern, 1977), polysomes (Darnbrough et al., 1973; Tai et al., 1973; Swiderski et al., 1979; Higgins and Mazurkiewicz, 1980), ribosomal subunits (Manchester, and Manchester, 1980) and proteins.

Although gel filtration of proteins and polysomes (Darnborough et al., 1973; Higgins and Mazurkiewicz, 1980) is dependent exclusively on molecular weight, gel filtration of RNA on the same agarose based gels is dependent on the secondary structure of the RNA as the

presence of poly-adenylic acid (Petrovic et al., 1973; Zeichner and Stern, 1977). Similarly, gel filtration of ribosomal subunits is dependent on the secondary structure (Manchester and Manchester, 1980), and the elution behaviour of the ribosomal subunits is analogous to each subunit's respective rRNA's. No significant adsorption of either the 18S rRNA (Zeichner and Stern, 1977) or the 40S ribosomal subunit occurs (Manchester and Manchester, 1980). However, both the 28S rRNA and the 60S ribosomal subunits are adsorbed to the agarose gel at high ionic strength at 4°C. This also suggests that within the ribosomal subunit, the 28S rRNA is sufficiently exposed to interact with the gel. The adsorption is reversed when the temperature is increased to 35°C. The aforementioned properties of ribosomal subunits on agarose gels offer a distinct advantage in that it can be used to prepare ribosomal subunits without the need of a centrifuge.

Polysomes and ribosomes prepared from cell lysates by gel filtration on agarose gels appear to exhibit an amino acid incorporation activity higher than that obtained with ribosomes isolated as a pellet by centrifugation (Darnbrough et al., 1973; Higgins and Mazurkiewicz, 1980; Desai and Manchester, 1986). Furthermore Manchester and Manchester (1980) showed that ribosomal subunits prepared in this way exhibit an amino acid incorporation activity similar to subunits prepared by centrifugation on sucrose gradients.

Analysis of Ribosomal Particles by Gel Electrophoresis

Gel electrophoresis of ribosomal particles is possible with highly porous gels which are achieved with low concentrations of acrylamide. The gel electrophoretic separation of bacterial ribosomal

particles has been made possible by the development of composite gels, containing agarose and acrylamide (Peacock and Dingman, 1967). The addition of agarose to low percentage acrylamide gels forms a mechanically stable and yet very porous gel. The fine resolution provided by these gels permits the separation of large macromolecules which may differ only in conformation, as demonstrated by Ledoigt et al. (1975), Dhalberg (1974; 1979) and Szer and Leffler (1974).

As separation is based on the principle of molecular sieving, the electrophoretic behaviour of ribosomal particles is dependent on their size and their spatial configuration. The structure of both prokaryotic and eukaryotic ribosomal particles is influenced by their surrounding ionic conditions. Thus migration of the particles in acrylamide gels is inversely proportional to the cationic concentration of the buffer (Ledioqt et al., 1975).

Electrophoresis then provides a powerful analytical tool for studying the structure of polysomes (Dhalberg et al., 1969; Dhalberg, 1973), the structure of ribosomes (Talens et al., 1973) and the structure of ribosomal subunits (Talens et al., 1973; Szer and Leffler, 1974; Dhalberg, 1974; 1979).

Dhalberg et al. (1973) treated bacterial cells with streptomycin and showed that polysomes isolated from these cells migrate faster than polysomes isolated from normal cells in acrylamide-agarose composite gels. This phenomenon occurs because streptomycin causes stacking of the ribosomes on the mRNA, thus resulting in more compact polysomes. 70S Ribosomes and 50S and 30S ribosomal subunits of Escherichia coli can be separated on acrylamide gels. Electrophoretic studies carried

out by Talens and co-workers (1973), using the fine resolving power of polyacrylamide gel electrophoresis, have demonstrated the remarkable heterogeneity of Escherichia coli ribosomes and ribosomal subunits. The 70S ribosomes resolved into 4 subclasses and the 50S and 30S ribosomal subunits each resolved into 4 and 3 subclasses respectively. Two other groups of workers then elaborated on the finding by Talens et al. (1973) and showed, using the ribosomal subunits only, that the different subclasses are due to the loss of one or more ribosomal proteins. Thus, the slowest migrating subunit is the native subunit while the faster migrating forms are those without their constituent proteins (Szer and Leffler, 1974; Dhalberg, 1974; 1979). Furthermore, Dhalberg (1979) showed that by losing some of their proteins, such as the L7/L12 complex, the ribosomal subunits lose more than 95% of their ability to synthesize polyphenylalanine, even though the 4 subclasses of ribosomes together retains 40-100 % of their activity. Thus, using this technique, it is possible determine which class of ribosomal subunits is most active in a particular preparation.

Electrophoresis of ribosomal particles on acrylamide-agarose composite gels proved to be satisfactory with prokaryotes and lower eukaryotes only. Yeast ribosomes were the only eukaryotic species that gave good resolution on composite gels (Helser et al., 1981). Higher eukaryotes tend to give smeared patterns and it is thought that the smeared patterns are due to extra-ribosomal proteins which bind to the ribosomes. Removal of these proteins by high salt washes and treatment with non-ionic detergents may aid in the resolution of the ribosomes on acrylamide-agarose gels. However, this area of work still needs investigation. Rat liver ribosomes have been

electrophoresed on polyacrylamide gels (Ledoigt et al., 1975), so that adequate resolution of the ribosomes and the ribosomal subunits can be achieved on acrylamide disc gels.

1.7 THE AIMS OF THIS STUDY

The first part of the present investigation was prompted by the finding that gel filtration on Sepharose 4B was a suitable method for separation of ribosomal subunits by gel filtration (Manchester and Manchester, 1980). In the study carried out by Manchester and Manchester (1980), the separation of the subunits was not based on ideal gel filtration but rather on the adsorption properties of the gel by means of a two step preparation of the ribosomal subunits. A single step preparation would be less tedious to perform than a two step preparation.

Practical separations by gel exclusion chromatography are dominated by three considerations which are peculiar to these methods.

1. The course of the separation is determined by the nature of the stationary phase, the composition of eluent and the operating conditions. This makes the choice of the optimum stationary phase for a particular separation an important practical consideration.
2. The limitation $0 < K_d < 1$ restricts the effluent volume available for gel filtration separations by chromatography to a maximum value of V_i. The most important consequence of the second consideration is that large columns are necessary

for molecular sieve separations in order to provide elution volumes adequate for resolution.

3. Gel exclusion chromatography is carried out with eluents of constant composition and therefore no chemical and physical changes occur in the stationary phase.

At present the range of molecular sieve media available for chromatographic separation is vast. There are gel media made of natural fibres which are the cross linked dextrans, the polyacrylamides, the agarose gels, and then there are the synthetic media, which are rigid non-compressible sieve media which allow small and uniform particle sizes to be combined with relatively high flow rates and thus enabling a substantial increase in the column efficiency.

The molecular sieve media studied were agarose gels based on the work by Manchester and Manchester (1980) and this was expanded in the present case to include the study of gel filtration using a synthetic gel, that is Trisacryl GF2000 (Reactifs, IBF).

Trisacryl gels are synthesized by copolymerization of N-acryloyl-2-amino-2-hydroxymethyl-1,3 propandiol and a hydroxylated acrylic monomer. Trisacryl gels have a combination special advantages over existing gels. These include high separation efficiency due to the uniform particle size and high mechanical resistance which is due to the rigid non-compressible beads. Consequently they allow high linear flow rates. Column efficiency is not greatly altered by high flow rates. Since Trisacryl is a synthetic gel, it is not biodegradable. It is suitable for the separation of ribosomal subunits since it has a fractionation range of 150 000-15 million daltons.

Sepharose (Pharmacia) is a bead formed gel prepared from agarose that is stable under most conditions encountered in gel filtration. The mechanical strength and the porosity of Sepharose is dependent on the agarose concentration of the beads. The highest agarose concentration renders a gel with a relatively high mechanical strength. The extremely open gel structure of Sepharose extends the range of separations possible with gel filtration to include macromolecules with molecular weights as large as 40 million daltons. The disadvantage of Sepharose is that it displays some adsorption properties which may be due to the small number of sulphate and carboxyl groups present. However, the adsorption properties can be used as a basis for the purification of certain nucleic acids (Petrovic et al., 1973) and ribosomal subunits (Manchester and Manchester, 1980).

The second part of this study was prompted by the finding of Dahlberg et al.(1969) that polysomes from Escherichia coli can be separated on acrylamide-agarose composite gels by electrophoresis. The highly porous structure of the acrylamide-agarose gels allows the separation of large macromolecules whilst still maintaining a high mechanical strength. The physical properties of dilute acrylamide gels when supported by 0,5 % agarose are remarkable in view of the characteristics of each of the components separately. The 0,5 % agarose is just able to maintain itself without flaking and crumbling and the acrylamide possesses so little structure that it is almost fluid. Together they form a porous yet very rigid gel.

The fine resolution provided by these gels permits the separation of large macromolecules which may differ only in conformation or by the presence or absence of a single protein. The potential of this

technique offers great advantages in the study of ribosomes and ribosomal subunits of eukaryotes and can enhance the study on the structure of the ribosomal particles.

The separation of ribosomes and ribosomal subunits by electrophoresis in gels is different in several important ways from separation by ultracentrifugation. The properties of a ribosome which determines its mobility in an electric field is different from the properties which determine its sedimentation velocity in a gravitational field. In centrifugation, the large molecules move faster while in the electrophoretic analysis, the small molecules move faster. A much higher resolution is obtained by using electrophoresis instead of density gradient centrifugation. In addition, each fraction is very much less contaminated than in the case for centrifugation.

These differences open new possibilities in the analysis of ribosomes and ribosomal subunits of eukaryotes.

2.0 MATERIALS AND METHODS

2.1 MATERIALS

Trisacryl GF2000 was supplied by Reactifs, IBF, Sepharose 4B by Pharmacia, puromycin and [^{14}C]phenylalanine by Sigma, agarose (electrophoresis grade) by Biorad and Escherichia coli rRNA by Boehringer Mannheim. All other chemicals were of analytical grade and obtained from various sources.

2.2 ANIMAL CARE

Albino rats were obtained from the Central Animal Unit of the University. They were housed in cages and provided with a balanced cube diet and water. For the preparation of ribosomes the rats were starved overnight.

2.3 ESCHERICHIA COLI CELL CULTURE

Preparation of culture medium

Luria broth was prepared by dissolving 10 g tryptone (Difco), 5g yeast extract (Difco) and 5g NaCl in 800 ml distilled water. The pH of this solution was adjusted to pH 7,6 with NaOH. The broth was then put into 250 ml Erlenmeyer culturing flasks and sterilized by autoclaving for 30 min at 120°C. Filter sterilized glucose was added to each flask to a final concentration of 0,1 %.

Determination of the Growth Curve of Escherichia coli

A plate of Escherichia coli (K strain), grown on nutrient agar at 37°C for 48 hours, was donated by the Microbiology Department. 250 ml Luria broth was inoculated with a colony obtained from the nutrient agar plate and this was placed in a 37°C incubator to develop an overnight broth culture.

4 ml of the overnight broth culture was inoculated into 250 ml sterile Luria broth. A sample was taken at inoculation time (i.e. zero time) and the absorbance was read at 480 nm against a Luria broth blank. The inoculated broth was placed in a rotary shaker at 37°C. Samples were taken periodically and the absorbance was read as described above to obtain a growth curve (Figure 1). The growth curve was used to establish the time of harvesting of the Escherichia coli cells, i.e. the log phase, since it is during this phase that the bacterial cells are actively replicating and thus synthesizing proteins. During log phase, 40-50 % of the total mass of the Escherichia coli cells consist of ribosomes.

A Batch Culture of Escherichia coli K strain

4 ml of an overnight broth culture of Escherichia coli was inoculated into 250 ml flasks containing Luria broth. The inoculated flasks were then transferred to a rotary shaker and the bacteria cultured at 37°C with vigorous shaking. After 3 hours, the flasks were put into an acetone-ice bath to cool from 37°C to 0°C in ~20 seconds. The bacterial cells were harvested by centrifugation in a Sorvall SS34 rotor for 10 min. at 10 000 rpm. The pellets were resuspended in 10 ml sucrose buffer (10 mM Tris-HCl, pH 8, 25 % sucrose) and centrifuged again in an SS 34 rotor for 10 min. at

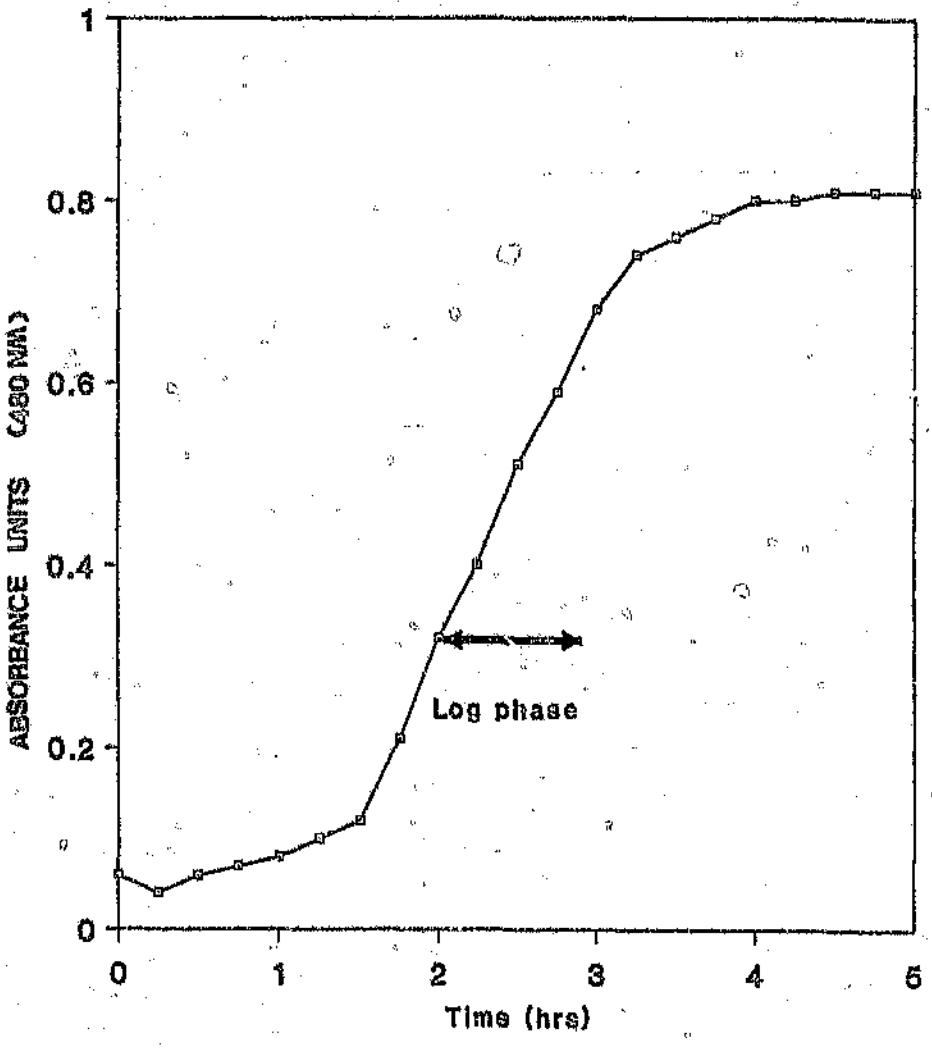


Figure 1. The growth curve of Escherichia coli. Escherichia coli was grown in Luria broth at 37°C with vigourous shaking. The growth curve was determined as described in section 2.3.

10 000 rpm. The cells were either stored as pellets at -70°C or the ribosomes isolated immediately after harvesting.

2.4 PREPARATION OF RIBOSOMES AND RIBOSOMAL SUBUNITS

The Preparation of Rat Liver Ribosomes and Ribosomal Subunits

Albino rats were starved overnight to reduce the glycogen content of the livers. The rats were killed by cervical dislocation. The livers were quickly removed and rinsed in ice-cold saline. The weight was measured and three volumes of 0,25 M sucrose in buffer A (100 mM KCl, 20 mM HEPES pH 7,8, 2 mM $MgCl_2$, 0,2 mM EDTA and 5 mM MSH) was added. The liver was homogenized in a Dounce homogenizer. The homogenate was centrifuged for 10 min. at 10 000 rpm in a Sorvall SS 34 rotor to pellet cells, cellular debris, nuclei, and mitochondria. To the post-mitochondrial supernatant, Triton X-100 was added to a final concentration of 1 % to release membrane bound ribosomes. The Triton X-100 treated supernatant was layered over 4 ml 1 M sucrose in buffer A in Ti 50 tubes. The discontinuous sucrose gradients were centrifuged for 1 hour at 4°C, and 50 000 rpm in an ultracentrifuge. The supernatant and sucrose layers were removed by suction with a water pump. The pellets were either stored at -20°C or dispersed in buffer B (500 mM KCl; 20 mM HEPES pH 7,8; 2 mM $MgCl_2$; 0,2 mM EDTA and 5 mM MSH). The purity of the ribosomal pellets were estimated spectrophotometrically by measuring the absorbance at 280 nm, 260 nm, 240 nm and 235 nm.

To prepare the ribosomal subunits, ribosomal pellets were dispersed in 1,5 ml buffer B per pellet. The A_{260} units were measured and

puromycin was added to a final concentration of 2 mM. The ribosomal suspension was then incubated at 37°C for 15 min. The cooled ribosomal suspension was layered over prepared 10-40 % linear sucrose gradients. The sucrose gradients were centrifuged at 20 000 rpm for 16 hours at 4°C in a SW 27 rotor. After centrifugation the gradients were fractionated and pumped through a 254 nm Uvicord. Fractions containing the ribosomal subunits were collected and pooled. The pooled fractions were centrifuged at 50 000 rpm in a Ti 50 rotor for 3 hours. The pellets were resuspended in 0,25 M sucrose in buffer A and recovery was measured in A_{260} units.

The Preparation of Rabbit Reticulocyte Ribosomal Subunits

Salt washed rabbit reticulocyte ribosomes were a kind gift from Mr M. Mouat of the Department of Biochemistry.

One pellet of reticulocyte ribosomes was resuspended in 10 ml buffer C (500 mM KCl; 3 mM $MgCl_2$; 20 mM Tris-HCl pH 7,6 and 5 mM MSH) and centrifuged in a Sorvall SS 34 rotor at 10 000 rpm for 5 min. to remove aggregated clumps of ribosomes. The supernatant was carefully removed and the A_{260} units measured.

To prepare ribosomal subunits, 2 mM puromycin and 2 mM GTP (final concentration) were added to 3 ml of ribosomal suspension which was 50 A_{260} units/ml. These solutions were incubated at 37°C for 30 minutes, cooled and layered over 10-40 % sucrose gradients which were made up in buffer C. The gradients were centrifuged in a SW 27 rotor at 20 000 rpm and 4°C overnight. After centrifugation the gradients were pumped through a 254 nm Uvicord. Fractions corresponding to the peaks recorded were collected and centrifuged at 50 000 rpm in a Ti 50 rotor at 4°C for 3 hours. The pellets were

stored at 20°C.

The Preparation of Escherichia coli Ribosomes and Ribosomal Subunits

Ribosomes of Escherichia coli were isolated by the method of Girbés et al. (1979). Approximately 7g bacterial cells (wet weight) were resuspended in sucrose buffer (10 mM Tris-HCl pH 8; 25 % sucrose) by slowly stirring with a glass rod. All operations were carried out on ice. To the suspension, freshly prepared solution was added, which consisted of 2 ml 20 mg/ml in 0,25 M Tris-HCl pH 8 and 2 ml 8 mM EDTA. The solution degraded the bacterial cell walls. After 10 min. of slow stirring, 10 ml of the freshly prepared lysis solution, which consisted of 6 ml 3% Triton X-100 in 6 mM Tris-HCl pH 7,8, 35 mM $Mg(CH_3COO)_2$; 90 mM NH_4Cl and 0,2 % sodium deoxycholate, was added. The solution was stirred for 5 min. during which time the viscosity increased tremendously, indicating the release of DNA and thus successful lysis of the bacterial cells. After 5 min. 0,75 mg DNase was added to the solution to degrade the DNA, consequently reducing the viscosity. After 15 min. the solution was centrifuged in an SS 34 at 10 000 rpm and 4°C for 10 min., to remove cell debris. The supernatant was carefully decanted and centrifuged again at 50 000 rpm in a Ti 50 rotor for 1,5 hours. The ribosomal pellets were either stored at -70°C or used to prepare ribosomal subunits.

Ribosomal subunits were prepared by resuspending a ribosomal pellet in 6 ml low magnesium buffer (10 mM Tris-HCl pH 7,6; 0,2 mM $MgCl_2$; 25 mM KCl and 5 mM MSH). The resuspension was dialyzed overnight with the same buffer. After measuring A_{260} units, 3 ml of ribosomal suspension (50 A_{260} units/ml) was loaded onto prepared

10-40 % sucrose gradients, which were made up in 10 mM Tris-HCl pH 7,6; 0,2 mM $MgCl_2$ and 25 mM KCl. The gradients were centrifuged in an SW 27 rotor at 19 500 rpm and 4°C for 16 hours. After centrifugation the gradients were fractionated. Fractions containing the ribosomal subunits were pooled and the ribosomal subunits recovered by centrifugation in a Ti 50 rotor at 50 000 rpm and 4°C for 3,5 hours. Pellets were resuspended in sucrose buffer (25 % sucrose; 10 mM Tris-HCl pH 7,6) and stored at -70°C.

2.5 THE PREPARATION OF RAT LIVER CYTOSOL

The cytosol from livers of fed rats was prepared by conventional differential centrifugation. Liver tissue was extracted from fed rats killed by cervical dislocation and rinsed in 0,9 % saline. 0,25M sucrose in buffer A was added (3 volumes) and the tissue homogenised using a Dounce homogeniser. The homogenate was centrifuged for 10 min at 10 000 rpm at 4°C in a Sorvall SS34 rotor. The supernatant was carefully removed and transferred to Ti 50 tubes and centrifuged at 50 000 rpm at 4°C for 1 hour to pellet the ribosomes. The supernatant was stored at -20°C.

2.6 THE PREPARATION OF *ESCHERICHIA COLI* CYTOSOL

Approximately 10g (wet weight) *Escherichia coli* cells were lysed by grinding with 3 parts alumina (Sigma) in a cold mortar until a thick flowing paste was obtained. DNase was added to a final concentration of 3 µg/ml to cleave the DNA thus reducing the viscosity of the

paste. After 10 min., 10 ml buffer (10 mM Tris-HCl pH 7,6, 50 mM KCl, 10 mM $Mg(CH_3COO)_2$ and 7 mM MSH) was added to suspend the paste into a free flowing slurry. The slurry was centrifuged at 16 000 rpm at 4°C for 10 min. to pellet the mitochondria, cells and cell debris. The supernatant was further clarified by repeating the centrifugation step. The supernatant was then centrifuged in a Ti 50 rotor at 50 000 rpm for 4,5 hours. The post ribosomal supernatant (S-100 extract) was stored at -70°C and the pellet was discarded.

2.7 PREPARATION OF LIVER GLYCOGEN

To prepare total glycogen from rat liver, the whole livers of fed rats were placed in liquid N_2 immediately after removal from the animal to minimize degradation (Geddes and Stratton, 1977). The tissue was ground in a pestle and mortar, then homogenized in 4 vol of 45% (w/v, final concentration) phenol for 4 min. at 25°C. The homogenate was stirred gently for 1 hour. The upper aqueous layer containing glycogen and RNA was removed. The RNA was digested with ribonuclease which had a concentration of 1 mg/10 ml. Glycogen was concentrated by dialyzing against 20 % polyethylene glycol in 0,25 M Tris-HCl pH 7,6 and 0,1 M NaCl.

2.8 SEPARATION OF THE DISSOCIATED RIBOSOMAL SUBUNITS BY SUCROSE DENSITY GRADIENT CENTRIFUGATION

Linear sucrose gradients were prepared by using a perspex gradient maker. 17 ml of each sucrose solution (i.e. the 10 % sucrose and 40

% sucrose solutions) was put into each of the chambers and mixed gradually while draining into 37 ml centrifuge tubes. The sucrose solutions were made up in the buffer specific for the isolation of each of the different species as indicated in section 2.4. The sucrose gradients were made up at 4°C, then loaded with 3 ml of the dissociated subunits and centrifuged at 4°C in an SW 27 rotor at 20 000 or 19 500 rpm for 16 hours. After centrifugation the gradients were fractionated using downward displacement and the eluate was pumped continuously through a 254 nm Uvicord. Fractions corresponding to the peaks recorded were collected and pooled. The ribosomal subunits were recovered by centrifugation at 50 000 rpm at 4°C for 3 to 3,5 hours.

2.9 GEL FILTRATION OF RIBOSOMAL PARTICLES OF TRISACRYL GF2000

Trisacryl GF2000 (Reactifs, IBF), supplied in its hydrated form, was mixed with an equal amount of buffer B, then de-aerated and left to attain equilibrium at the operating temperature. The slurry was packed into a Wright column (1,2 cm x 30 cm). The space below the bed support was filled with buffer before packing. This practice prevented air bubbles being trapped in the dead space under the net. The column was packed at a pressure of 30 ml/hr using a Pharmacia P-3 peristaltic pump to ensure that the maximum hydrodynamic pressure was not exceeded. The column was then equilibrated with three volumes of buffer B.

The separation of the solutes on a gel filtration column is dependent on the quality of the column packing. Thus, before the column was

used, the uniformity of the packing was checked by observing the progress of the coloured zone of a solution of blue dextran (0,2 %). It was discovered, however, that blue dextran was fractionated on the column and the peak emerged as a broad trailing peak. Thus, cytochrome C (Sigma) being a more homogeneous substance was used instead. With repeated packing of the Trisacryl GF2000 column, it was found that the column packed uniformly each time.

Since the separation of solutes is also dependent on the number of theoretical plates (N) of the column, the maximum number of theoretical plates was determined by running the 40S subunit of rat liver at various flow rates. The 40S subunit was resuspended in buffer B and 5 A_{260} units were applied to the column. N was calculated as described in section 3.4. Since the conformation of the solute determines its characteristics on the column and hence the estimation of N, it was useful to compare the maximum number of theoretical plates when running acetone at the optimum flow rate with that obtained when running the 40S subunit (see results section).

The solute behaviour on the column is determined by the characteristics of the column. Thus, the internal volume (V_i) and the void volume (V_o) were determined using tritiated water (^3H_2O) and glycogen respectively. Each sample was mixed with an appropriate volume of buffer B and 10 % glycerol to increase the density of the sample, then loaded onto the column with a micropipette allowing the sample to settle evenly on the top of the column bed.

The 40S and 60S subunit of rat liver and rabbit reticulocytes were used to calibrate the column. 5 A_{260} units of the sample were

applied to the column as stated above. The column was allowed to run with buffer B as the eluent, with continuous monitoring of the eluate at 254 nm (LKB Uvicord). Fractions of 1 ml were collected and the absorbance of each of the fractions were measured at 260 nm.

To determine the elution of the dissociated subunits of rat liver and rabbit reticulocytes, the ribosomes were resuspended in buffer B and treated with 1 mM puromycin and incubated at 37°C for 15 min. before being applied to the column as above. The column was allowed to run with buffer B as the eluent and the eluate was collected in 1 ml fractions. The absorbance of the fractions were measured at 260 nm.

All the above experiments were carried out at 4°C and 25°C. The column was packed at 4°C and to heat the column to 25°C, buffer B was warmed to 30°C, de-aerated and then passed through the column to stabilize the gel. The buffer treated in this way serves to heat the gel and also to absorb any air that might be released upon heating of the gel. The temperature of the column was maintained at 25°C by use of a water jacket through which water of a constant temperature passed.

Subsequent experiments were carried out on a 135 cm x 1,2 cm column. Trisacryl GF2000 was packed at a flow rate of 15 ml/hr to a bed height of 135 cm. The lower flow rate was used to accommodate for the increase in pressure in the longer column. In this way the maximum packing pressure was not exceeded.

The column was equilibrated with buffer B and the quality of the column packing was assessed by running cytochrome C through the

column. The consistency in the uniformity of the packing was again demonstrated in the larger column. Since the optimal flow rate for a particular gel is independent of the height of the column, it was not necessary to optimize the flow rate and 3 ml/hr was the flow rate used for subsequent chromatography operations. It was observed that on Trisacryl GF2000 the height equivalent of a theoretical plate (HETP) did not change significantly as the flow rate was increased.

Rat liver ribosomal subunits were chromatographed on the column at 4°C and at 20°C. The samples were applied to the column as before and eluted with buffer B at 3 ml/hr. A mixture of ribosomal subunits, obtained by incubating a sample of ribosomes in suspension in buffer B with 1 mM puromycin at 37°C for 15 min, was chromatographed on the column to determine whether the subunits would be separated on the column.

Rabbit reticulocyte ribosomal subunits were applied to the column and chromatographed with buffer B as the eluent at 20°C. A mixture of subunits obtained, by incubating ribosomes with puromycin at 37°C for 15 min. was applied to the column and eluted with buffer B at 3 ml/hr.

Escherichia coli ribosomal subunits were chromatographed on the column after reequilibration with a low magnesium buffer (25 mM Tris-HCl pH 7,6, 0,25 mM $MgCl_2$, and 25 mM KCl). The subunits were applied to the column with buffer and glycerol (10 % v/v). A mixture of ribosomal subunits was applied to the column and eluted with buffer. The eluate was monitored continuously at 254 nm and 2 ml fractions were collected. The absorbance of each of the fractions

3. RESULTS

3.1 SEPARATION OF RAT LIVER RIBOSOMAL SUBUNITS BY SUCROSE DENSITY GRADIENT CENTRIFUGATION

Ribosomes isolated from rat liver were dissociated with puromycin as described in section 2.4. The dissociation of the subunits was studied by subjecting the reaction mixtures to sucrose gradient centrifugation at 4°C.

Figure 2 shows the density gradient profile of rat liver ribosomal subunits. The subunits were well resolved, although the peaks do have some overlap. The relative heights and distance of separation of the 40S and 60S peaks were reproducible if the A_{260} units applied to the gradient did not exceed 50 A_{260}/ml. When the load was increased the peaks tended to broaden out and resolution decreased progressively.

To assess the yields of the ribosomal subunits the A_{260} units were measured and the concentration was calculated from an empirically derived coefficient (Peterman, 1964). The yields (Table 1) of the 2 subunits were good and they were comparable to the amount of ribosomes applied to the gradient after allowing for a certain percentage loss, since only fractions were collected as indicated in Figure 2.

The ultraviolet absorbance characteristics of the subunits are given in Table 1. The absorbance spectrum is characteristic of ribosomal

was measured at 260 nm. Fractions containing the ribosomal samples were analysed spectrophotometrically in the wavelength range 235 nm to 280 nm. The eukaryotic subunit fractions were concentrated by dialyzing against polyethylene glycol (20 % w/v) in buffer B with KCl concentration reduced to 100 mM and the Escherichia coli ribosomal fractions were concentrated by ultrafiltration using a millipore ultrafiltration unit. The ribosomal subunits were analysed by electrophoresis.

The column was heated as before by passing warmed de-aerated buffer through the column. A constant temperature was maintained at 20°C in a temperature controlled room.

2.10 GEL FILTRATION OF RIBOSOMAL PARTICLES ON SEPHAROSE 4B

Sepharose 4B (Pharmacia) was mixed with 1/3 of its volume of buffer B. The gel suspension was left in a 35°C water bath to attain equilibrium at the operating temperature. Before packing the gel into a 30 cm x 1.2 cm Wright column the gel suspension was de-aerated. The column was packed at a pressure of 48 ml/hr using a Pharmacia P-3 peristaltic pump, keeping the temperature at 35°C with the aid of a water jacket fitted onto the column. To equilibrate the column, buffer B was heated to 35°C, de-aerated, and passed through the column.

As with the Trisacryl GF2000 column, the uniformity of the column was checked using a solution of cytochrome C (2 mg/ml). Optimization of the Sepharose 4B column was carried out as before, the 40S subunit of rat liver was chromatographed at various flow rates, and N was

determined from the elution profiles obtained. 2,5 A_{260} units of 40S subunits in buffer B was applied to the column and eluted with buffer B. The column was characterized using ^3H_2O to determine the total column volume and glycogen to determine the void volume. The samples were mixed with buffer B and applied to the column. They were eluted with buffer B at the optimum flow rate at 35°C. The eluate from the glycogen run was monitored continuously with a 254 nm Uvicord. The 40S and 60S ribosomal subunits of rat liver, were applied to the column separately. About 5 A_{260} units of each subunit was applied to the column and eluted with buffer B at 35°C. Fractions (1,75 ml) were collected and recovery estimated. The mixture of ribosomal subunits was then applied to the column at 35°C to determine whether Sepharose 4B was a suitable gel for the separation of ribosomal subunits by pure gel filtration. The column was cooled to 4°C and equilibrated at this temperature with Buffer B (3 volumes). The 40S and 60S ribosomal subunits were applied to the column. The 40S ribosomal subunits eluted unretarded at 4°C but the 60S subunits adsorbed to the gel and the column was warmed to 35°C to elute the 60S ribosomal subunits. To determine the elution behaviour of a mixture of ribosomal subunits on the column, a sample of ribosomes was incubated with 1 mM puromycin at 37°C for 15 min. and applied to the column at 4°C. After elution of the puromycin peak, the column was warmed to 35°C to elute the 60S ribosomal subunits.

For all chromatography operations, the ribosomal subunits were collected and recovery estimated from the absorbance units at 260 nm. The ribosomal subunits were concentrated by dialysis against polyethylene glycol in buffer A.

The column was reequilibrated with a low magnesium buffer (25 mM Tris-HCl pH 7,6, 0,25 mM $MgCl_2$, 25 mM KCl) at 4°C. Ribosomal subunits of Escherichia coli were applied to the column. The eluate was monitored continuously and recovery of the fractions were assessed by measuring the absorbance at 260 nm.

The 60S subunits were applied to the column at various temperatures (4°C-25°C). The recovery was assessed from each run, and if the recovery was not good, the temperature of the system was increased to 30°C to elute any subunits that had adsorbed to the column. To determine whether the 60S ribosomal subunits were of similar size to the pores of the gel, the subunits were applied to Sepharose 6B (Pharmacia) and Sepharose 2B (Pharmacia). These gels were packed into a 15 cm x 1 cm column at 4°C and equilibrated with buffer B separately. The 60S subunits of rat liver were applied to the column and eluted with buffer B at 4°C. The subunits did not emerge from the column and therefore the column was warmed to 30°C to elute the subunits. To determine the elution volumes of the subunits on Sepharose 6B and Sepharose 2B the subunits were applied to the column and eluted at 30°C.

2.11 ANALYSIS OF RIBOSOMES AND RIBOSOMAL SUBUNITS BY ACRYLAMIDE/AGAROSE COMPOSITE GEL ELECTROPHORESIS

Preparation of the Gel

Ribosomal particles were electrophoresed into vertical slab gels containing acrylamide in the various concentrations used (as described in legends to illustrations) and 0,5 % agarose, using a

vertical gel apparatus which had the following dimensions - 120 mm x 120 mm x 3 mm and the slots were 10 mm x 10 mm x 1 mm so that the slots were completely covered by gel. This practice facilitated resolution (Goodwin and Dahlberg, 1982).

The gels were made up by dissolving 0,33 g agarose (Biorad) in the appropriate volume of distilled water by heating for 1-2 min in a microwave oven. The dissolved agarose solution was then transferred to a 50°C water bath to cool the solution slowly to ~50°C. At 50°C, acrylamide, buffer (0,25 mM $MgCl_2$, 25 mM Tris-HCl pH8), and TEMED were added in their appropriate volumes. The temperature was then lowered to ~40°C by running tap water over the solution. Ammonium persulphate was added as a catalyst to polymerize the acrylamide. The gel solution was poured quickly into cooled clean glass plates to cause gelation of the agarose around the glass plates- this practice increased the stability of the gel. A cold slot former was then inserted quickly into the gel. The gel was left to set for 1 hour.

Preparation of the Sample

Ribosomal samples (in 0,2-0,4 A_{260} units) for electrophoresis were mixed with a volume of buffer (6 mM KCl, 25 mM Tris-HCl pH 7,6, 2 mM $MgCl_2$, 5 mM MSH, 10 % sucrose and 0,02 % bromophenol blue). If improved resolution was required an equal volume of agarose, 0,5 % in sample buffer, was added to the sample and this was gelled into place. The practice was not necessary for electrophoresis of Escherichia coli ribosomal subunits.

Sample Application and Running the Gels

The electrophoresis cell was placed vertically and buffer (0,25 mM $MgCl_2$, Tris-HCl pH8) was added to the top and bottom buffer reservoirs and the slot former was removed. The gel was pre-run for 1 hour at 60 V to remove the remaining unreacted persulphate and catalyst degradation products from the gel. The samples were then applied and electrophoresis carried out at the selected potential difference from the cathode to the anode. The time of electrophoresis depended on the voltage applied.

Staining of the Gels

After electrophoresis the gels were stained with a 0,1 % coomassie blue R 250 in 50 % methanol and 7,5 % acetic acid. The gels were left to stain for 4 - 16 hours and then destained by shaking first in 50 % methanol and 7,5 % acetic acid for 1-2 hours by replacing the destaining solution frequently and then changing to 10 % methanol and 7,5 % acetic acid. The gels were photographed and stored in 10 % methanol and 7,5 % acetic acid solution.

2.12 PROTEIN SYNTHESIS ASSAY

Activity of Ribosomal Subunits of Eukaryotes

The activity of the ribosomal subunits was assessed by their ability to incorporate [^{14}C]phenylalanine into protein, in the presence of poly(U). The concentrations of the solutions in the cell free system in a final volume of 250 ul were 1 mM ATP; 0,1 mM GTP; 4,5 mM phosphocreatine; 400 ul/ml poly(U); filtered cytosol; [^{14}C]phenylalanine and the various combinations of the subunits with the

magnesium concentration at 7 mM.

The energy charge mixture was made up of the following - 3 mg GTP, 30 mg ATP, 75 mg phosphocreatine and 2 ml buffer (500 mM KCl, 1 mM $MgCl_2$, 100 mM HEPES, and 0,1 mM EDTA pH 7,8). This mixture was made up to 10 ml. 50 µl 1 M $Mg(CH_3COO)_2$ was added with continuous stirring and the pH adjusted to pH 7 with 4 N KOH. The mixture was stored in 5 ml aliquots at -20°C and used as required. The poly(U) solution was made up by solubilizing 10 mg poly(U) in 4,9 ml buffer (100 mM KCl, 0,2 mM $MgCl_2$, 20 mM HEPES and 0,02 mM EDTA pH 7,8). To this solution 0,125 ml 1 M $MgCl_2$ was added. This mixture was stored at -20°C and used as required.

Prior to performing the protein synthesis assay, the cytosol from the livers of fed rats was filtered through a column of Sephadex G-25 (5 cm x 1 cm). The column was equilibrated and eluted with 0,25 M sucrose in buffer A. The gel filtered cytosol was used immediately.

The 40S subunits were made up to a concentration of ~0,2 mg/ml and the 60S subunits to ~0,5 mg/ml with 0,25 M sucrose in buffer A.

The assay mixture was made up by preparing a master cocktail containing 50 µl energy charge mixture, 50 µl poly(U) mixture and 50 µl filtered cytosol for each assay mixture and 10 µl [^{14}C]phenylalanine for every 6 assay tubes. All operations were carried out on ice. The various combinations of the subunits were added to each assay tube. 150 µl of the master cocktail was added to each assay tube. The assay solutions were mixed and incubated at 37°C for 5 min. The assay was stopped by cooling on ice. 100 µl samples of the

assay mixtures were absorbed onto filter paper discs which were air dried thoroughly and washed twice in 10 % trichloroacetic acid (TCA) solution and heated for 20 min. After another wash in cold TCA solution, the discs were washed with 95 % ethanol. Finally the discs were washed in ether and air dried. The filter paper discs were placed in 10 ml scintillation fluid (toluene containing 0,5 % 2,5-diphenyloxazole) and counted in a Beckman liquid scintillation counter.

Activity of Ribosomal Subunits of Prokaryotes

To assess the activity of the ribosomal subunits of Escherichia coli, the incorporation of [^{14}C]phenylalanine into protein in the presence of poly(U) was measured. The cell free reaction mixture in a total volume of 250 µl contained 1mM ATP, 0,4 mM GTP, 10 mM phosphoenolpyruvate; 10 µg/ml pyruvate kinase; 0,1 mg/ml poly(U); filtered S-100 extract (section); [^{14}C]phenylalanine; 80 mM NH_4Cl; 10 mM $Mg(CH_3COO)_2$; 50 mM Tris-HCl pH 7,8; 1 mM MSH and the various combinations of the subunits.

The energy charge mixture contained 5 mM ATP; 2 mM GTP; 50 mM phosphoenolpyruvate and buffer which was 400 mM NH_4Cl; 50 mM $Mg(CH_3COO)_2$; 250 mM Tris-HCl pH 7,8 and 6 mM MSH. The poly(U) mixture contained 0,5 mg/ml poly(U) in buffer which was 400 mM NH_4Cl, 50 mM $Mg(CH_3COO)_2$, 250 mM Tris-HCl pH 7,8 and 6 mM MSH. Stock solutions of the above mixtures was made up and stored at -20°C.

Just before performing the protein synthesis assay, the S-100 extract from Escherichia coli was filtered through a column of Sephadex G-25

to remove small molecular weight macromolecules. A master cocktail was prepared containing 50 µl energy charge, 50 µl poly(U) mixture, 50 µl freshly filtered S-100 extract and 2.5 ul pyruvate kinase (1 ug/ul) per assay tube and 10 µl [^{14}C]phenylalanine for every 6 assay tubes. The assay mixture was prepared by first placing the various combinations of the ribosomal subunits in each tube. 150 µl of the master cocktail was then added to each assay tube. The assay mixtures were mixed thoroughly by vortexing and then incubated at 37°C for 5 min. The assay was stopped by placing the tubes on ice.

100 µl samples of the cooled assay mixtures were absorbed onto filter paper discs which were air dried then washed and counted for radioactivity.

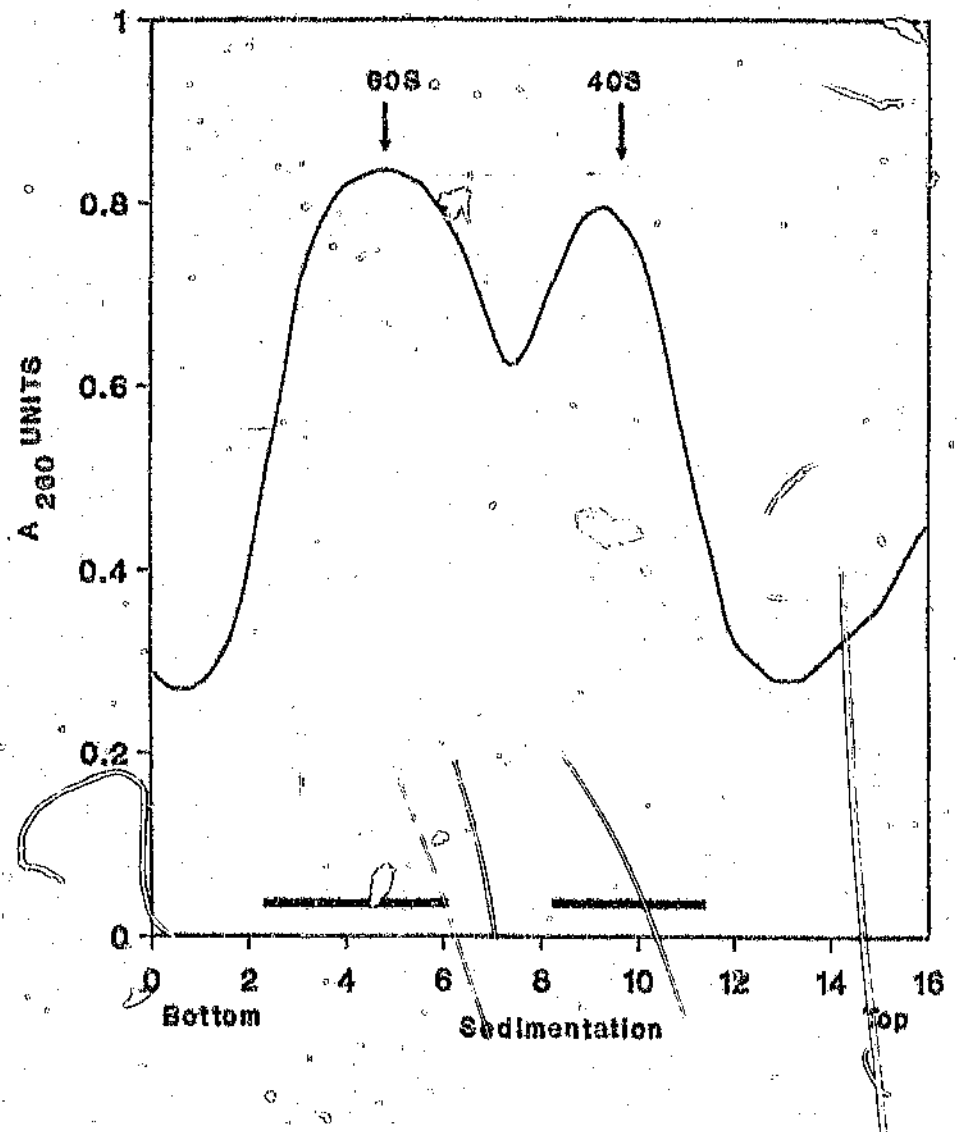


Figure 2. Sedimentation profile of rat liver ribosomal subunits.
Ribosomal subunits were separated by incubating with puromycin at 37°C and then fractionated on 10%-40% sucrose gradients in a SW27 rotor at 4°C and 20 000 rpm for 16 hrs. The bars indicated fractions collected.

Table 1 : Purification of ribosomal subunits by density gradient centrifugation

Ribosomal Species	Absorbance (nm)				Absorbance Ratios		Amount of Subunit (mg)
	280	260	240	235	260:235	260:280	
Rat Liver:							
60S	0,400	0,600	0,404	0,417	1,44	1,50	8,1
40S	0,286	0,470	0,310	0,319	1,47	1,64	3,2
Rabbit Reticulocyte:							
60S	0,187	0,321	0,191	0,188	1,71	1,72	4,3
40S	0,071	0,109	0,080	0,094	1,16	1,54	0,7
degraded 60S	0,126	0,232	0,137	0,145	1,60	1,84	1,6
Escherichia coli:							
50S	0,175	0,289	0,172	0,168	1,72	1,70	3,0
30S	0,091	0,158	0,098	0,109	1,45	1,74	1,6

Ribosomal subunits separated by density gradient centrifugation were recovered by centrifugation. The subunit suspensions were assessed spectrophotometrically. The amount of ribosomal subunits were calculated as in Petermann (1964).

particles in that a peak is reached at 260 nm and a trough at about 240nm.

Rat liver ribosomes are contaminated by ferritin when isolated by differential centrifugation. After gradient centrifugation it was noted that ferritin comigrates with the 60S ribosomal subunit.

3.2 THE SEPARATION OF RABBIT RETICULOCYTE RIBOSOMAL SUBUNITS BY DENSITY GRADIENT CENTRIFUGATION

Figure 3 shows the density gradient profiles of reticulocyte ribosomal subunits. Three peaks were observed. The first peak, which consisted of high molecular weight material, was the major peak. The second and third peaks were smaller, indicating a lower yield. Resolution of the three peaks was poor. Spectrophotometric analysis of the three peaks indicated the presence of ribosomal particles (Table 1).

The purity of the ribosomal material obtained from each peak was assessed by determination of the absorbance ratios, 260:235 and 260:280 (Table 1). The 260:235 ratios were all well above 1,45 and the 260:280 ratios were all close to 1,85 indicating a low degree of protein contamination. The high 260:280 ratio obtained for the material in the third peak indicates a loss of protein.

The nature and purity of the ribosomal components was assessed by analysis of their constituent RNA. Sodium dodecyl sulphate was employed to dissociate protein from ribosomal RNA and the samples

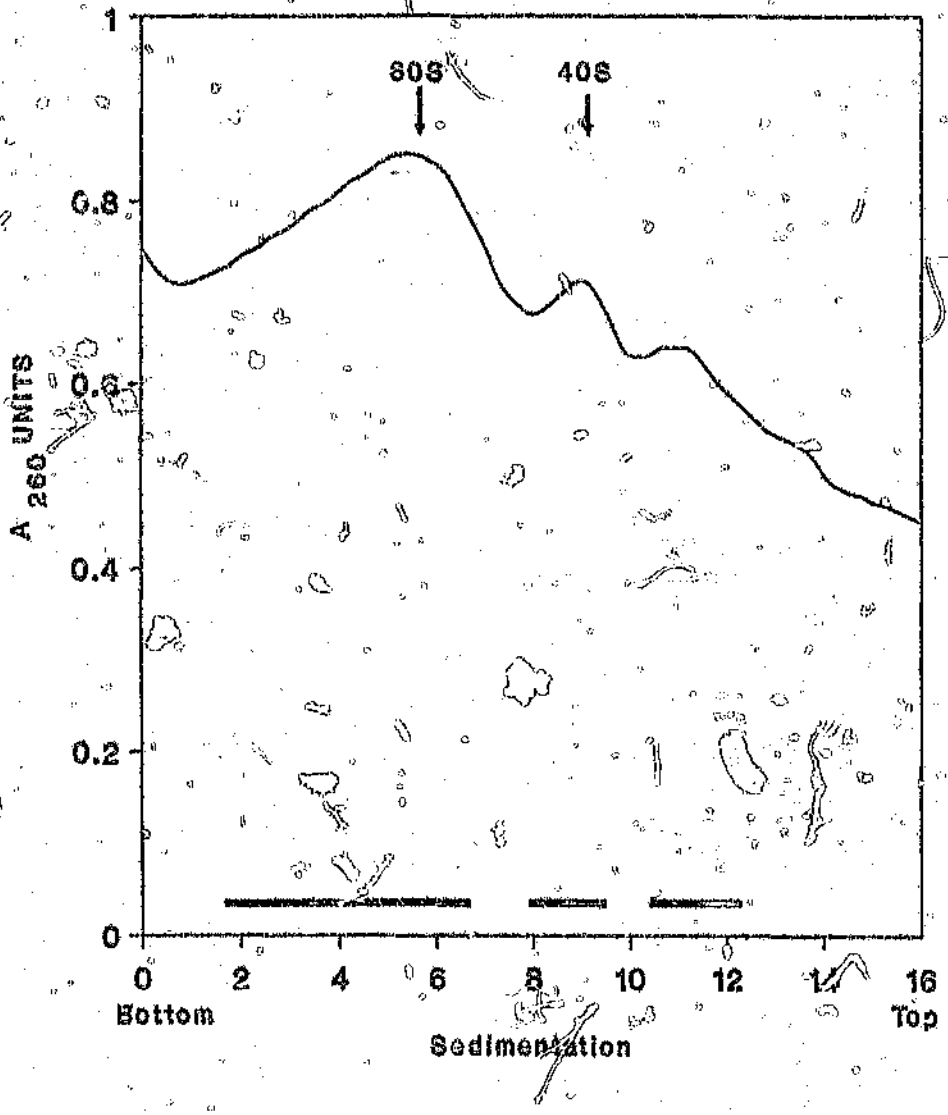


Figure 3. Density gradient profile of rabbit reticulocyte ribosomal subunits. Ribosomal subunits were obtained by incubating with puromycin and GTP at 37°C. The subunits were fractionated as in Fig. 2.

were analysed by acrylamide-agarose gel electrophoresis (not shown). The material within the first and third peaks contained predominantly 28S rRNA indicating that these peaks contained the 60S subunits of rabbit reticulocytes. The second peak contained predominantly 18S rRNA indicating the presence of 40S subunits. However the lower sedimentation rate and the high A_{260}:A_{280} ratio suggests that these particles were degradation products of the 60S subunits. The reason for this degradation is unknown. A possible reason is that the ribosomes were not freshly prepared but had been first treated, for other purposes, with high salt to remove initiation factors. It is known that high salt treatment of rat liver ribosomes results in removal of proteins (Clegg and Arnstein, 1970).

Generally the yield was not good. This was accounted for by the pellet obtained after gradient centrifugation. The pellet contained a large proportion of the ribosomal suspension applied to the gradient and it probably consisted of undissociated polysomes.

3.3 THE SEPARATION OF *ESCHERICHIA COLI* RIBOSOMAL SUBUNITS BY DENSITY GRADIENT CENTRIFUGATION

Escherichia coli ribosomes were dissociated into their component subunits by treating with a low magnesium buffer (0,2 mM Mg^{2+}). The subunits were separated by gradient centrifugation. The 50S and 30S peaks (Figure 4) were well resolved, but as for rat liver, resolution of the subunits depended on the amount of subunit suspension applied to the gradient. Resolution decreased significantly with loads higher than 80 A_{260}/ml. The 50S subunits were

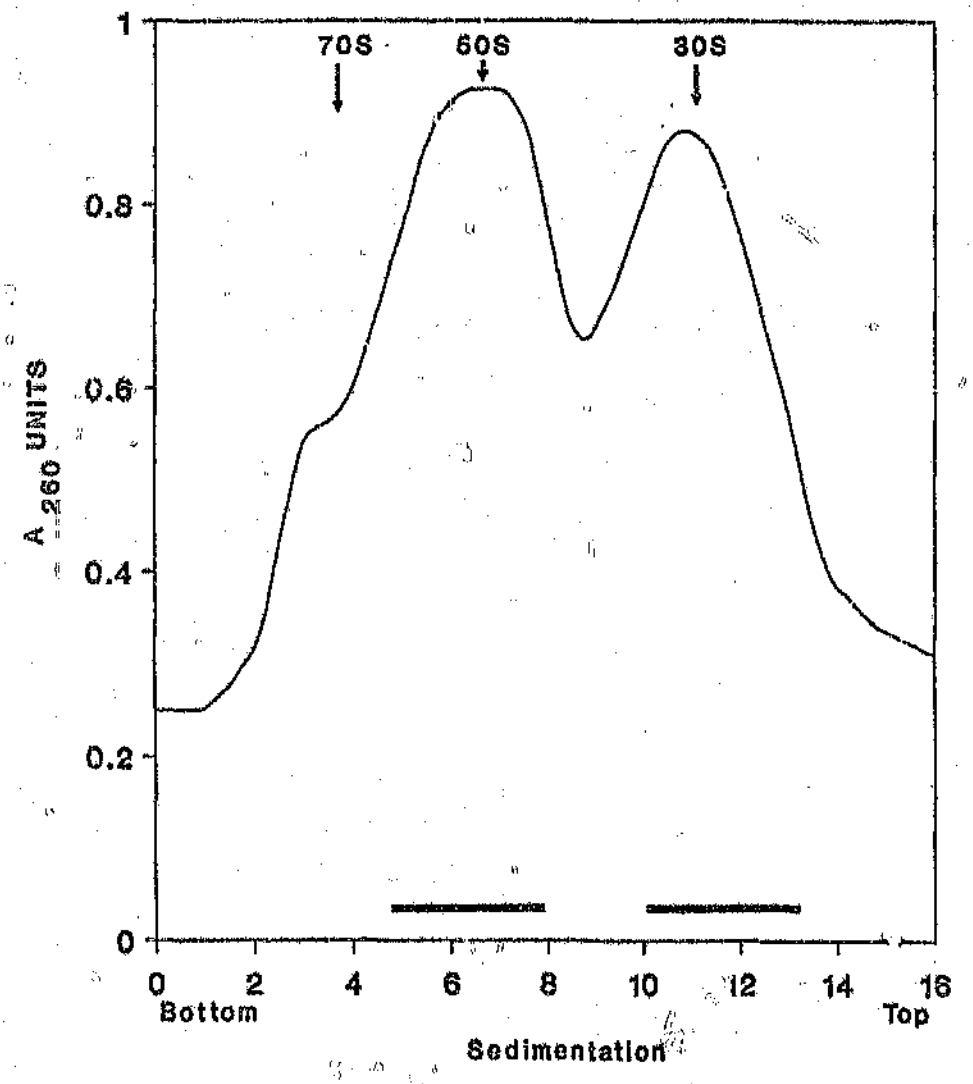


Figure 4. Density gradient profile of Escherichia coli ribosomal subunits.
Ribosomal subunits were obtained by resuspending the Escherichia coli ribosomes in a low magnesium buffer.
The subunits were fractionated as in Fig. 2.

preceded by faster migrating 70S ribosome "tight-couples" which appeared as a shoulder on the 50S subunit peak. The yields of the ribosomal subunits were assessed as for rat liver ribosomal subunits. The subunits showed good recovery (Table 1) and were comparable with the amount of ribosomes applied to the gradient.

The nature and purity of the ribosomal components were determined by analysis of the subunits by acrylamide-agarose gel electrophoresis (Figure 33) and by assessment of the absorbance ratios (Table 1). The absorbance ratios showed a low degree of impurities and were comparable with the published values, i.e. 260:280=1,85 and 260:235=1,45 (Petermann, 1964). Acrylamide-agarose gel electrophoresis (Figure 33) indicates no contamination of the 50S subunits with 30S subunits when the samples are collected within the regions shown in Figure 4.

3.4 GEL FILTRATION OF RIBOSOMAL PARTICLES ON TRISACRYL GF2000

Resolution of a gel filtration column is dependent on the number of theoretical plates (N) which is, amongst other things, determined by the flow rate of the eluent. Generally, the lower the flow rate, the greater N is, provided the flow rate is not so low as to increase longitudinal diffusion. N can be calculated from the elution profile:

$$N = 5,54 \frac{(V_e)^2}{(w_{1/2})^2}$$

where V_e is the elution volume of the solute taken at the top of the peak maximum, since gel filtration solutes have linear isotherms, thus giving symmetrical peaks, and $w_{1/2}$ is the width measured at an ordinate y/2, where y is the maximum ordinate. The height equivalent of a theoretical plate (HETP) can be determined from N:

$$HETP = \frac{L}{N}$$

where L is the length of the packed bed of column.

The maximum number of theoretical plates obtained for the 40S subunit of rat liver on a 30 cm column was about 185 (Table 2) and it was obtained when the flow rate was ~3 ml/h. During the investigations it was noted that N changes for different solute species and for the same solute under differing chromatographic conditions. N for acetone was found to be 560 (Table 3) at a flow rate of 3 ml/h on a 30 cm column. The optimum flow rate was not affected by the length of the column. It remained at 3 ml/h (Figure 5) when the length of column was increased to 135 cm. The resolving power of Trisacryl GF2000 was found to be very slightly dependent on the flow rate. This is demonstrated by the slow increase in the HETP as a function of flow rate on the 135 cm column (Figure 5).

To determine the elution characteristics of a certain solute the void volume (V_o) and internal column volume (V_i) are required since the elution of a solute is best characterized by a distribution coefficient(K_d).

$$K_d = \frac{V_e - V_o}{V_t - V_o - V_g} = \frac{V_e - V_o}{V_i}$$

TABLE 2 : Optimization of the flow rate of a Trisacryl GF2000 column.

FLOW RATE (ml/hr)	ELUTION VOLUME (ml)	$W_{1/2}$ (ml)	N	HETP (mm)
2,80	12,6	2,83	110	2,7
2,84	12,4	2,55	129	2,3
2,90	12,7	2,86	109	2,6
2,98	12,6	2,18	185	1,6
3,06	12,3	2,25	164	1,8
3,20	12,5	2,76	114	2,6

The 40S ribosomal subunits of rat liver were resuspended in buffer B. 5 A_{260} units were applied to the column in a total volume of 100 µl. The sample was eluted at 25°C. The eluate was monitored continuously at 254 nm. The V_e was taken at the peak maximum and N and HETP were calculated as described in section 3.4.

TABLE 3: Characteristics of Trisacryl GF2000 columns packed to bed heights of 30 cm & 135 cm.

SAMPLE	COLUMN LENGTH 30cm			135cm		
	Ve (ml)	N	HETP (cm)	Ve (ml)	N	HETP(cm)
Glycogen	9,3	-	-	45	-	-
Acetone	21,0	560	0,05	-		
^3H_2O	23,0	733	0,04	134	1795	0,08

The determination of V_o and $V_o + V_i$ was with glycogen (prepared as in section 2,5) and ^3H_2O respectively. The glycogen eluate was monitored continuously using a 254 nm Uvicord and the late of ^3H_2O was collected in fractions which were counted for [^3H] activity.

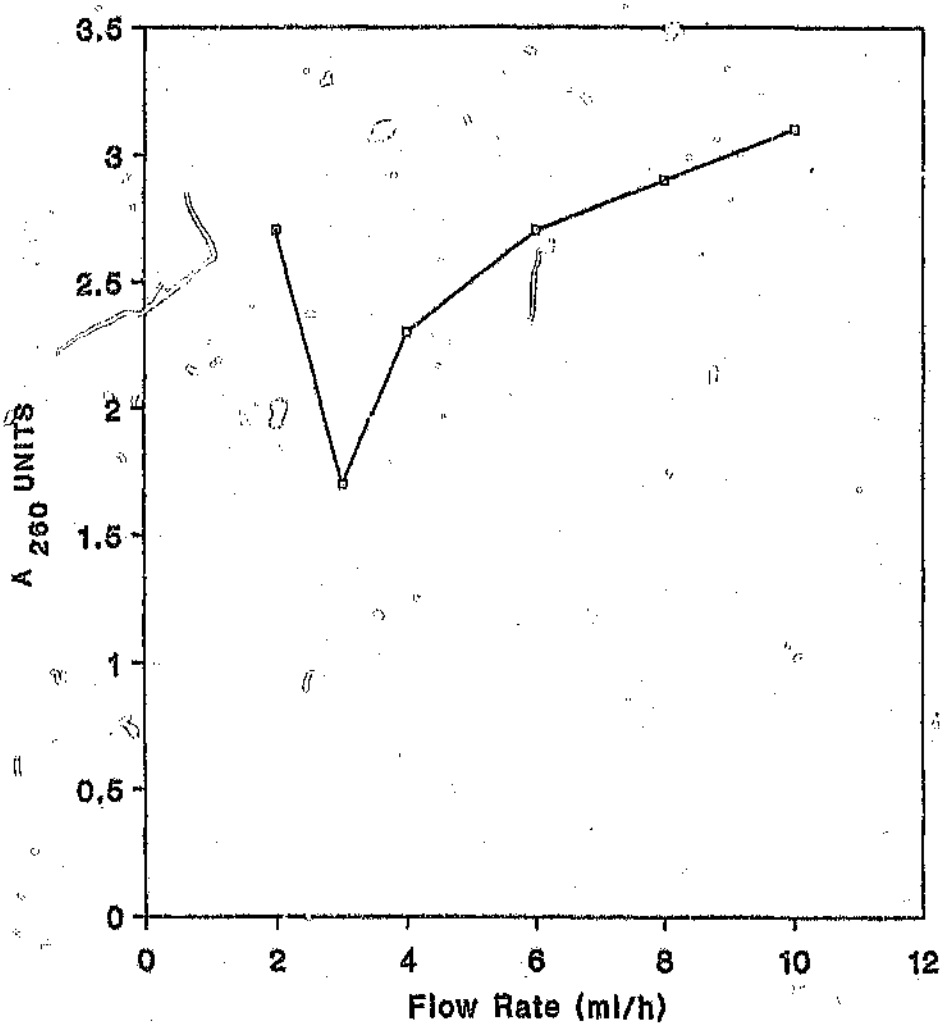


Figure 6. HETP variation as a function of flow rate. The 40S subunits of rat liver were used to determine the optimum flow rate of a 185 cm x 1.2 cm column of Trisacryl GF 2000. The HETP was calculated as shown in section 3.4.

where K_d represents the fraction of the stationary phase which is available for the diffusion of a given solute species, and

V_o is the void volume,

V_e is the elution volume,

V_t is the total column volume,

V_g is the volume of the matrix,

V_i is the internal volume.

V_o and (V_o+V_i) were determined using glycogen and ^3H_2O respectively (Table 3). Glycogen was used since the conventional V_o determinants such as high molecular weight DNA and dead bacteria were unsuitable for this particular gel matrix. The supposedly high molecular weight DNA is fractionated on the column and dead bacteria adsorbed to the gel.

Gel Filtration of Rat Liver Ribosomal Particles at 4°C and 25°C on 30 cm Trisacryl GF2000 Column

Preliminary experiments with Trisacryl GF2000 were carried out with a 30 cm column. The elution of the 40S and the 60S subunits of rat liver from this column is illustrated in Figure 6. The recorder trace shows symmetrical peaks. However the 60S subunit migrated with a slightly broader trailing zone than the 40S subunit. The broader peak of the 60S subunit is presumably due to the contamination of the 60S fraction with 40S subunits in the form of 55S dimers. It is known that 40S subunits exist in two forms, a monomer form (40S) and a dimer form (55S) (Wettenhall et al., 1973) and that the two forms exist in a dynamic equilibrium with one another (Manchester, 1984). 55S dimers sediment together with the 60S subunit in density gradients, hence the contamination of the 60S subunit (Manchester, 1984). The 40S fraction from density gradients elutes as a sharp

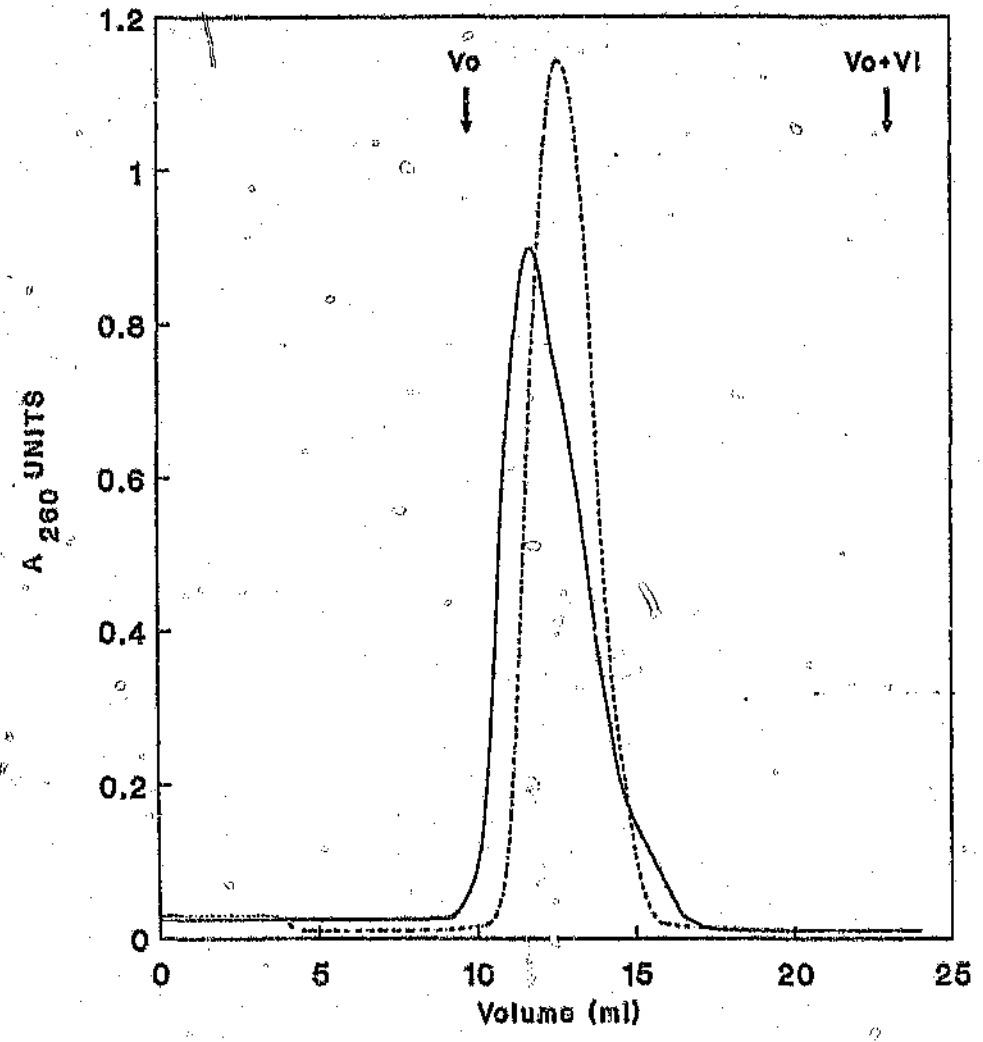


Figure 6. Gel filtration of rat liver ribosomal subunits on a 30 cm column of Trisacryl GF 2000 at 4°C.
10 A_{260} units of the 40S(---) and 60S(---) subunits were applied to the column and eluted with buffer B.

symmetrical peak. The peaks elute in a volume significantly greater than the void volume and less than the total column volume.
The ribosomes incubated with puromycin (Figure 7) eluted in a 11,6 ml which is the same elution volume as the 60S subunits (Table 4). However, the high HETP values indicate that the zone of the dissociated subunits is more diffuse than the zones of the individual subunits. The single peak obtained in Figure 7 shows that the 40S and 60S subunits were not resolved on the 30 cm Trisacryl GF 2000 column at 4°C.

To determine whether temperature would have an effect on the elution of the ribosomal subunits, the experiments with the 30 cm column were carried out at 25°C. The elution of the ribosomal subunits of rat liver is similar at 25°C and 4°C (Figure 8), except that longitudinal diffusion is decreased slightly at the higher temperature. Elution of the subunits is also slightly earlier than the elution at 4°C. The elution characteristics are unchanged. The 60S subunit chromatographic zone is broader than the zone of the 40S subunit. The difference in the HETP of the 40S subunit from 4°C to 25°C may be due to the 40S subunit aggregation at low temperatures, leading to the formation of dimers (Wool el al., 1979). Thus, on this column the 40S subunits appears as a broader zone resulting in a large HETP for that particular solute species.

Figure 7 shows the elution profile of a mixture of subunits at 25°C. The elution behaviour of the dissociated subunits at 25°C was similar to the elution of the dissociated subunits at 4°C. Both peaks illustrated in Figure 7 show a slight bit of trailing. At 25°C, less zonal diffusion is apparent, as indicated by the HETP

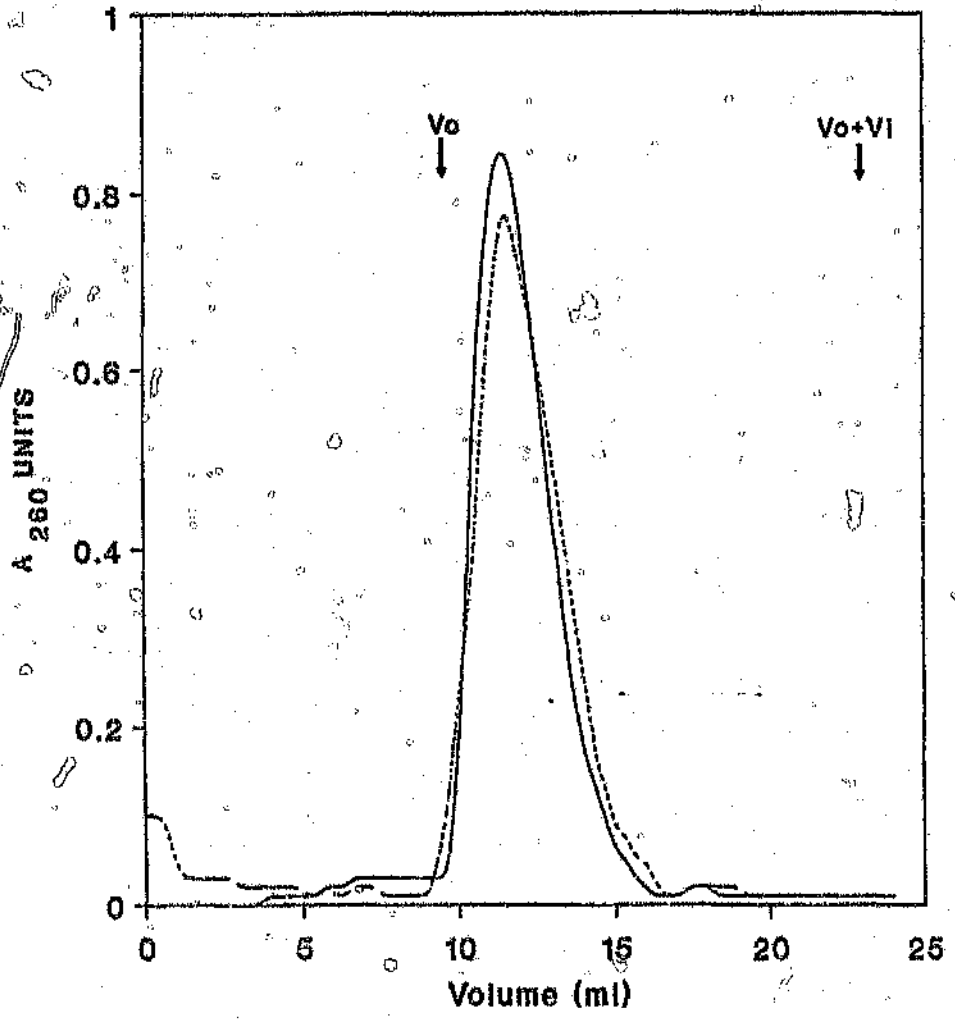


Figure 7. Gel filtration of a mixture of ribosomal subunits on a 90 cm Trisacryl GF 2000 column. A mixture of subunits obtained by incubating a sample of ribosomes with puromycin was applied to the column (10 A_{260} units) and eluted at 4°C(---) and 25°C(---).

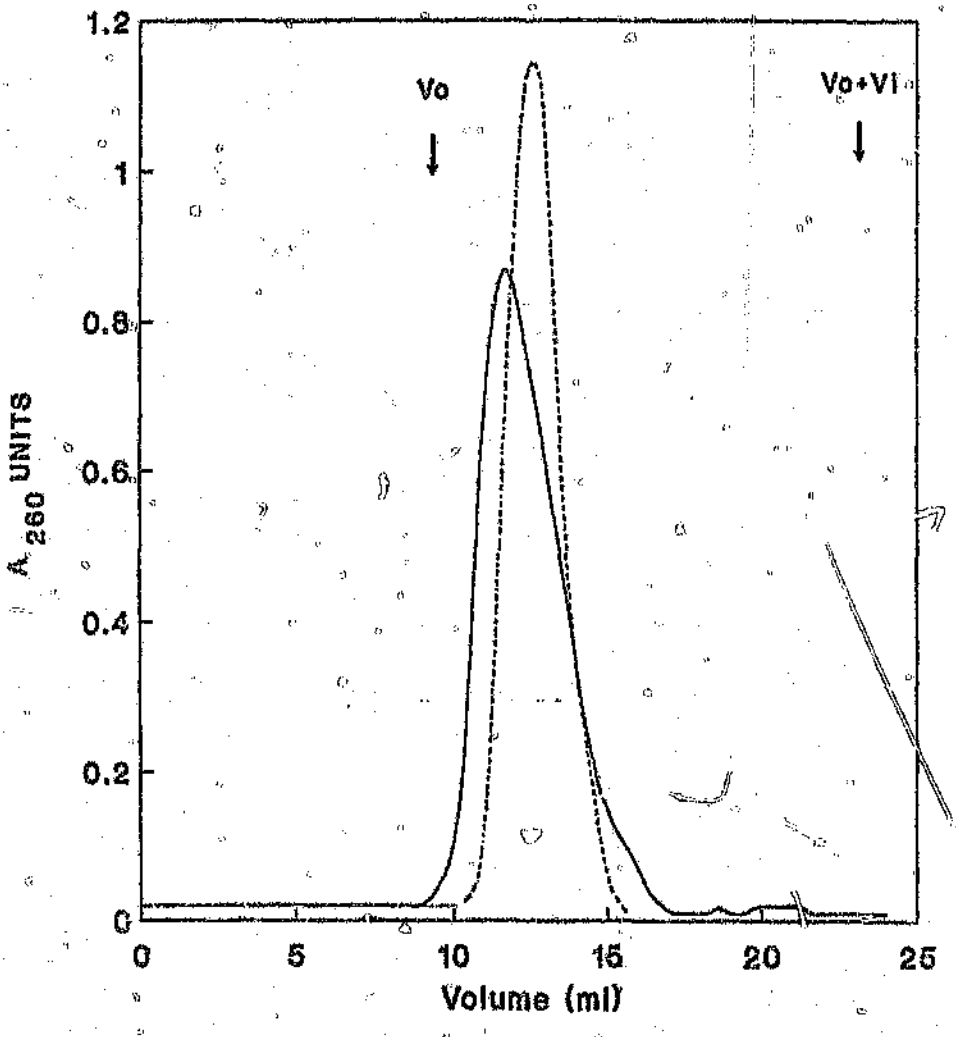


Figure 8. Gel filtration of rat liver ribosomal subunits on a 90 cm Trisacryl GF 2000 column at 25°C. The 60S(---) and the 40S(---) subunits were applied to the column at 25°C. .5 A_{260} units of each subunit was applied.

TABLE 4: The elution behaviour of rat liver ribosomal subunits on a 30 cm x 1,2 cm Trisacryl GF2000 column at 4°C and 25°C.

SOLUTES	V_e (ml)	K_d	N	HETP (mm)	Recovery (%)
4°C:					
40S	12,6	0,24	220	1,3	85
60S	11,6	0,17	83	3,6	73
dissociated subunits	11,6	0,17	78	3,8	64
25°C:					
40S	12,7	0,25	175	1,7	74
60S	11,7	0,18	81	3,6	65
dissociated subunits	11,5	0,16	100	3,0	75

The ribosomal particles were in suspension in buffer B. 5 A_{260} units of subunits in 100 µl were applied to the column and eluted at a flow rate of 3 ml/hr at 4°C and 25°C. The elute was monitored continuously and 1 ml fractions were collected. The recovery was measured by recording the A_{260} units of the fractions. Ribosomes were incubated with 1 mM puromycin at 37°C for 15 min prior to application.

K_d, N and HETP was determined as described in section 3.4 where V_o = 9,3 ml and V_i = 23,0 ml.

(Table 4). Although there is decreased zonal diffusion, the single peak in Figure 7 indicates that the 40S and 60S ribosomal subunits of rat liver were not resolved on the 30 cm column.

Gel Filtration of Rabbit Reticulocyte Ribosomal Particles at 4°C and 25°C on a 30 cm Trisacryl GF 2000 Column

To compare the elution behaviour between different species of eukaryotic ribosomal particles, rabbit reticulocyte ribosomal particles were chosen since they were readily available.

Figure 9 illustrates the elution of the 40S and 60S subunits of reticulocytes at 4°C. The 40S subunits elutes as a symmetrical peak with a narrow zone (HETP = 2,9 mm) at a volume of 11 ml (Table 5), while the 60S subunits peak shows slight trailing, eluting at a volume of 9,9 ml (Table 5). At 25°C (Figure 10) both subunits have similar elution characteristics, as at 4°C. However, the subunits elute slightly earlier at 25°C. The zone is narrower as judged by the HETP (Table 5) indicating a decrease in longitudinal diffusion. Both subunits elute closer to the V_0 than the rat liver subunits (Table 4) probably indicating a larger particle size. This observation could be due to the incomplete dissociation of the subunits, as observed with gradient centrifugation studies (Figure 3). Thus the 60S subunit fraction is contaminated with 80S particles and the 40S subunit fraction is contaminated with 60S particles.

Figure 11 shows the elution profiles of a mixture of ribosomal subunits at 4°C and at 25°C. Two peaks were obtained, eluting with K_d of approximately 0,0-0,02 and 0,14-0,16. The first peak elutes in the void volume. This peak could be a mixture of 40S, 60S

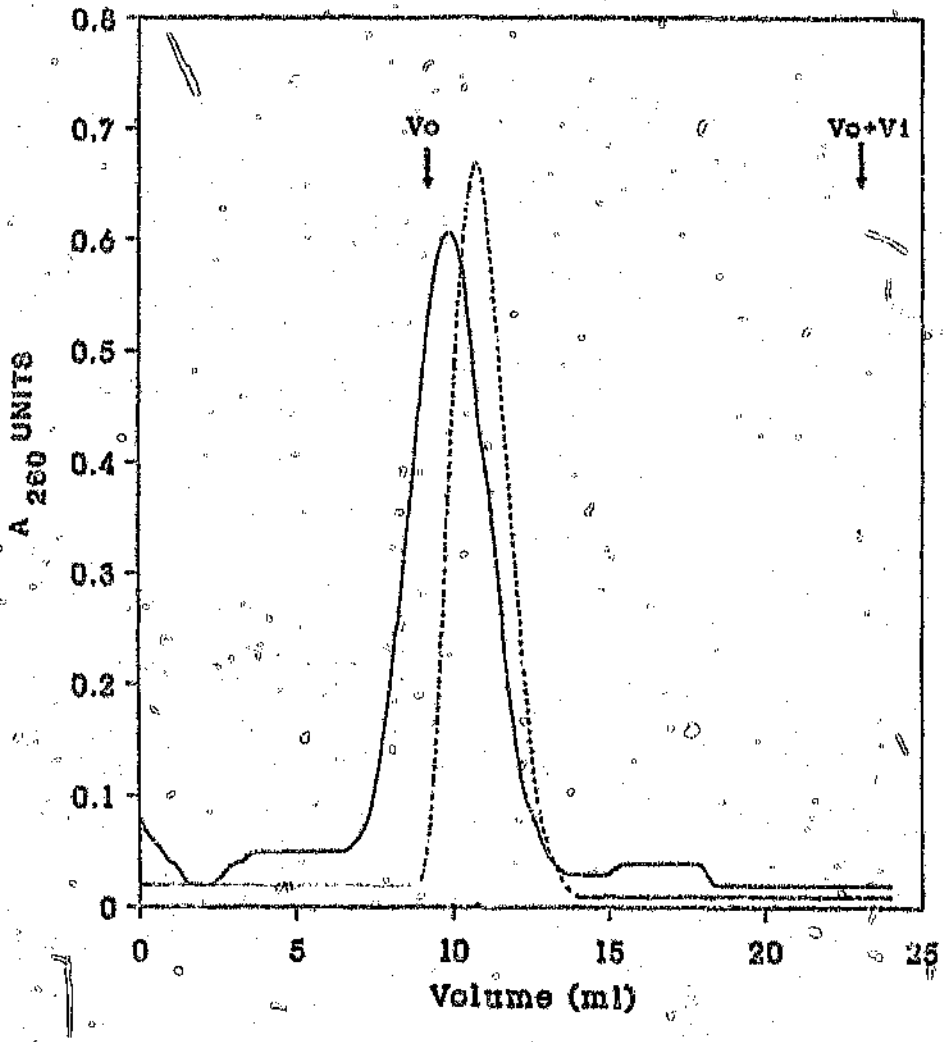


Figure 9. Gel filtration of rabbit reticulocyte ribosomal subunits on a 30 cm Trisacryl GF 2000 column at 4°C Ribosomal subunits isolated by density gradient centrifugation were applied to the column and eluted with buffer B at 4°C. 5 A_{260} units of the 60 S(---) and 40S(---) subunits were applied.

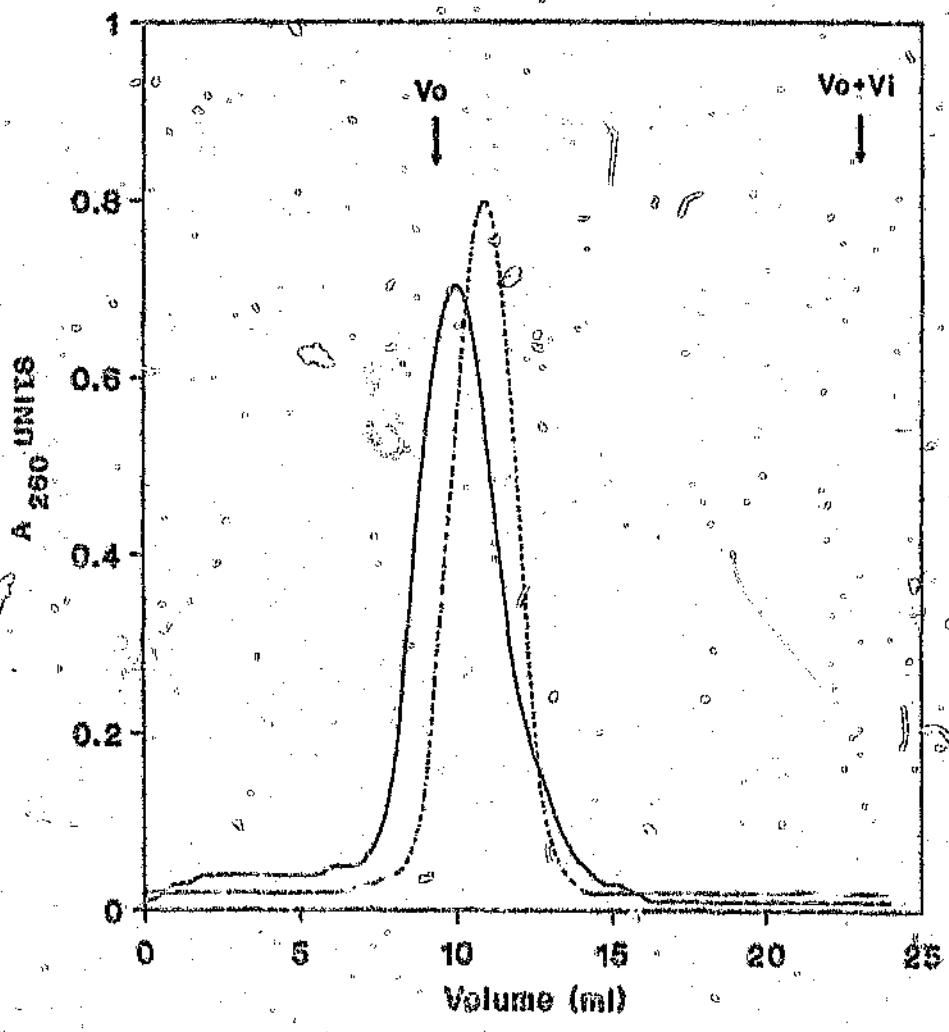


Figure 10. Gel filtration of rabbit reticulocyte ribosomal subunits on a 30 cm Trisacryl GF 2000 column at 25°C. 6 A_{260} units of the 40S(---) and 60S(---) ribosomal subunits were applied to the column and eluted with buffer B at 25°C.

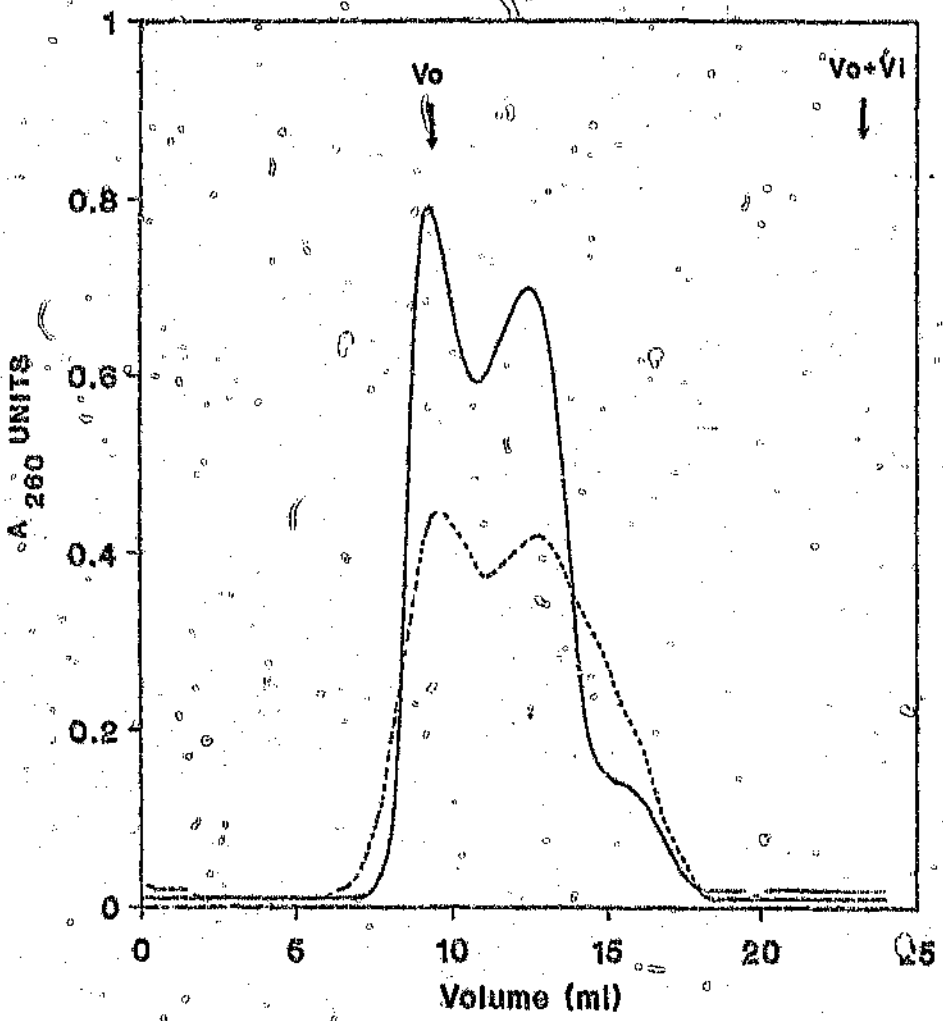


Figure 11. Gel filtration of a mixture of ribosomal subunits of rabbit reticulocytes.
A mixture of subunits was applied to the Trisacryl GF 2000 column and eluted with buffer B 4°C(----) and at 25°C(----).

TABLE 5 : Elution behaviour of rabbit reticulocyte ribosomal particles on a 30 cm Trisacryl GF2000 column.

SOLUTES		V_e (ml)	K_d	N	HETP (mm)	Recovery (%)
4°C :						
40S		10,7	0,10	131	2,2	88
60S		9,9	0,04	69	4,2	83
Dissociated ribosomes	(1)	9,6	0,02	-	-	-
	(2)	12,9	0,16	-	-	-
25°C :						
40S		10,9	0,12	174	1,7	81
60S		10,0	0,05	96	3,1	86
Dissociated ribosomes	(1)	9,3	0,0	-	-	-
	(2)	12,6	0,14	-	-	-

100 µl of a suspension of reticulocyte subunits ($5A_{260}$ units) were applied to the column and eluted with buffer B at 3 ml/hr and at 4°C and 25°C. Recovery was measured by recording absorbance at 260 nm. 10 A_{260} units of ribosomes were incubated with 2 mM puromycin and 2 mM GTP at 30°C for 15 min. prior to application. K_d, N and HETP were determined as in section 3.4.

V_0 is 9,3 ml and $V_0 + V_1$ is 23 ml.

and undissociated ribosomes. At 4°C the peak is broad and the descending side exhibits a slight shoulder. At 25°C, this peak is narrow and no shoulder is observed. The second peak elutes later than the 40S subunit and this peak could contain degraded subunits which are of lower molecular weight than the native 40S and 60S subunits. What was concluded, however, was that the native subunits did not separate on the column.

Gel Filtration of Rat Liver Ribosomal Subunits on Trisacryl GF2000 (135 cm Column) at 4°C and 20°C

It was apparent from the preliminary experiments with Trisacryl GF2000 that the ribosomal subunits would not be resolved on a 30 cm column and therefore a longer column was necessary.

Figure 12 illustrates the elution profiles of the 40S and 60S ribosomal subunits of rat liver at 4°C on a 135 cm column. The elution profile of the 40S subunit shows a symmetrical peak eluting with a K_d of 0,18. The 60S subunit eluted as a symmetrical peak as well but the descending side of the peak ended in a shoulder which occurred at a volume of ~62 ml. The material eluting in this shoulder had a slightly brownish colour. This brown solution has an absorbance maximum at 220 nm with a shoulder from ~240-~265 nm (Figure 13). This material was identified as ferritin. The ribosomal peaks elute at volumes which differ by 6,5 ml. Using equation 1 (in appendix) the resolution obtained on the 135 cm column is 0,21.

Figure 14 shows the elution profile of a mixture of ribosomal subunits at 4°C. The two peaks obtained indicate a separation of the subunits which elute with K_d of 0,17 and 0,11 for the 40S and

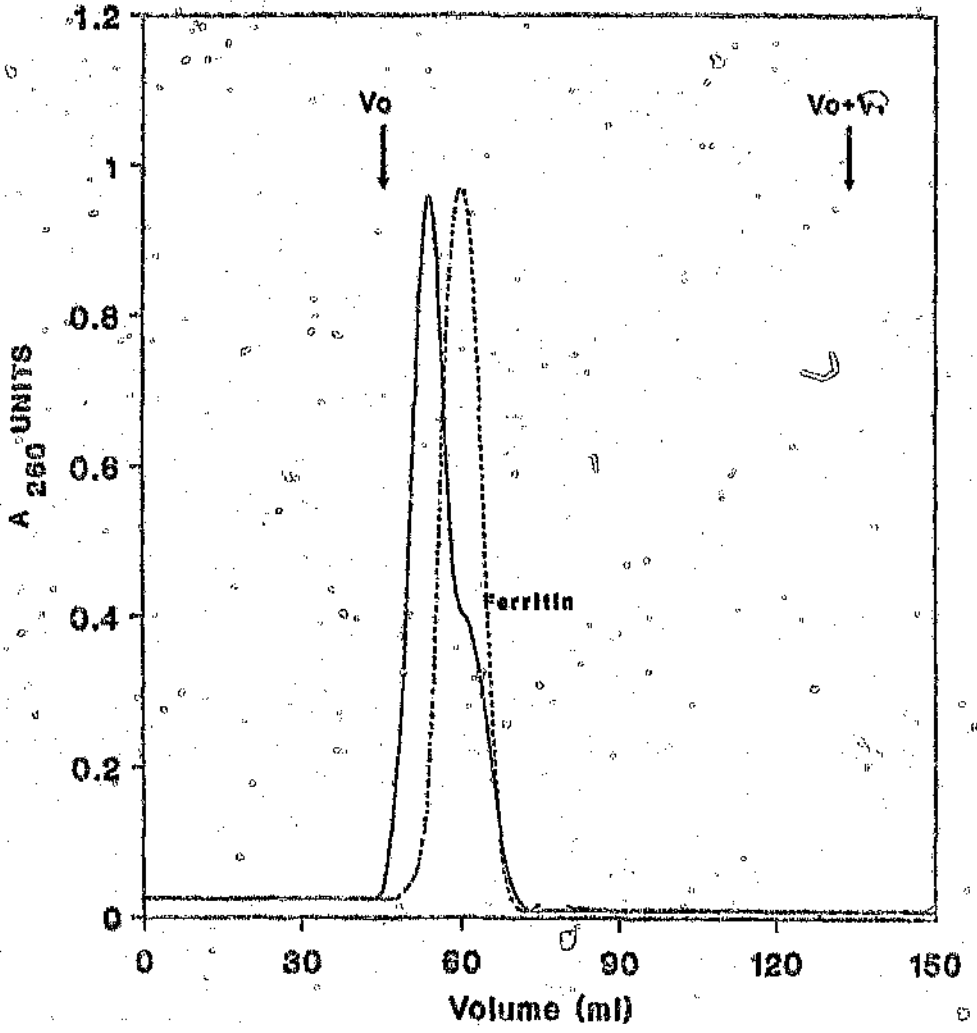


Figure 12. Gel filtration of rat liver ribosomal subunits on a 135 cm Trisacryl GF 2000 column at 4°C. 10 A_{260} units of the 40S(----) and the 60S(——) subunits were applied to the column and eluted with buffer B. Ferritin is the shoulder on the 60S subunits peak.

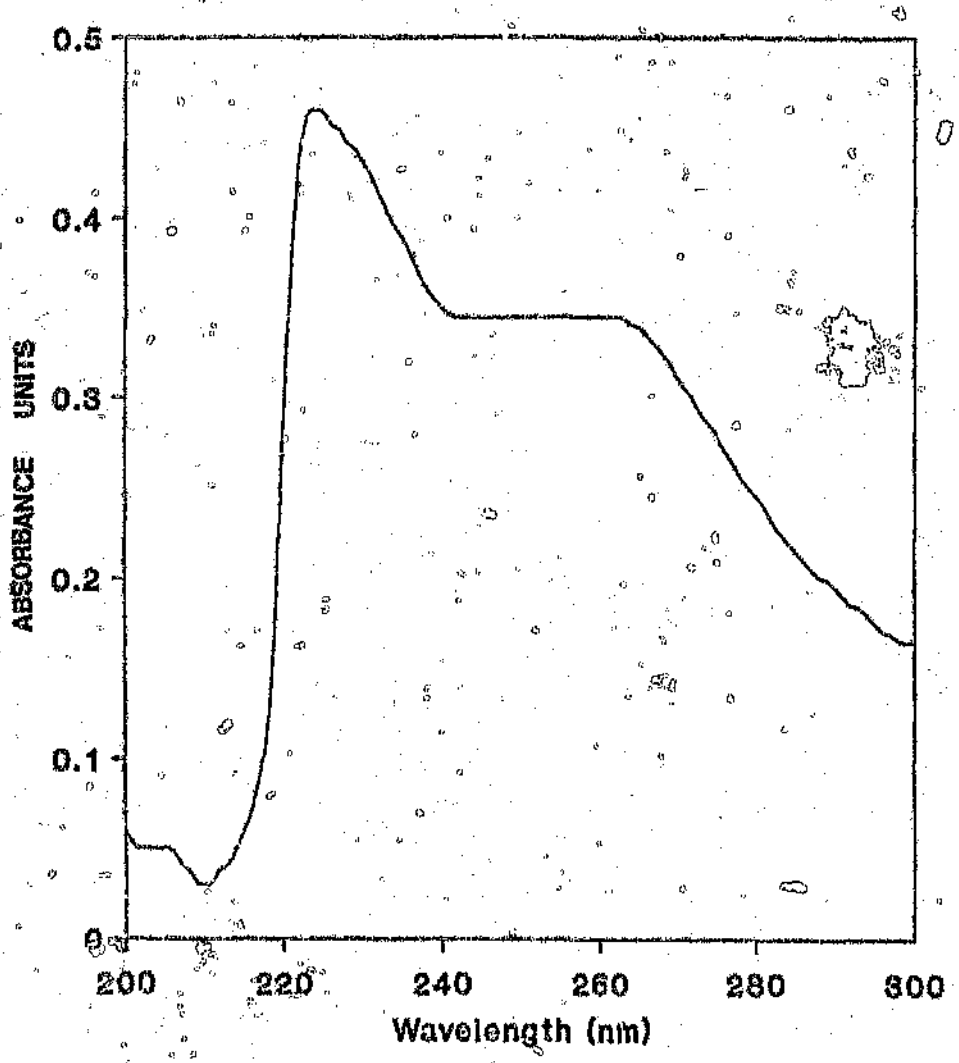


Figure 13. Ultraviolet absorption spectrum of the shoulder following the 60S ribosomal subunit peak in figure 12.
An absorption spectrum was taken of the brown fraction obtained when running the 60S subunits and the mixture of subunits on Trisacryl GF 2000.

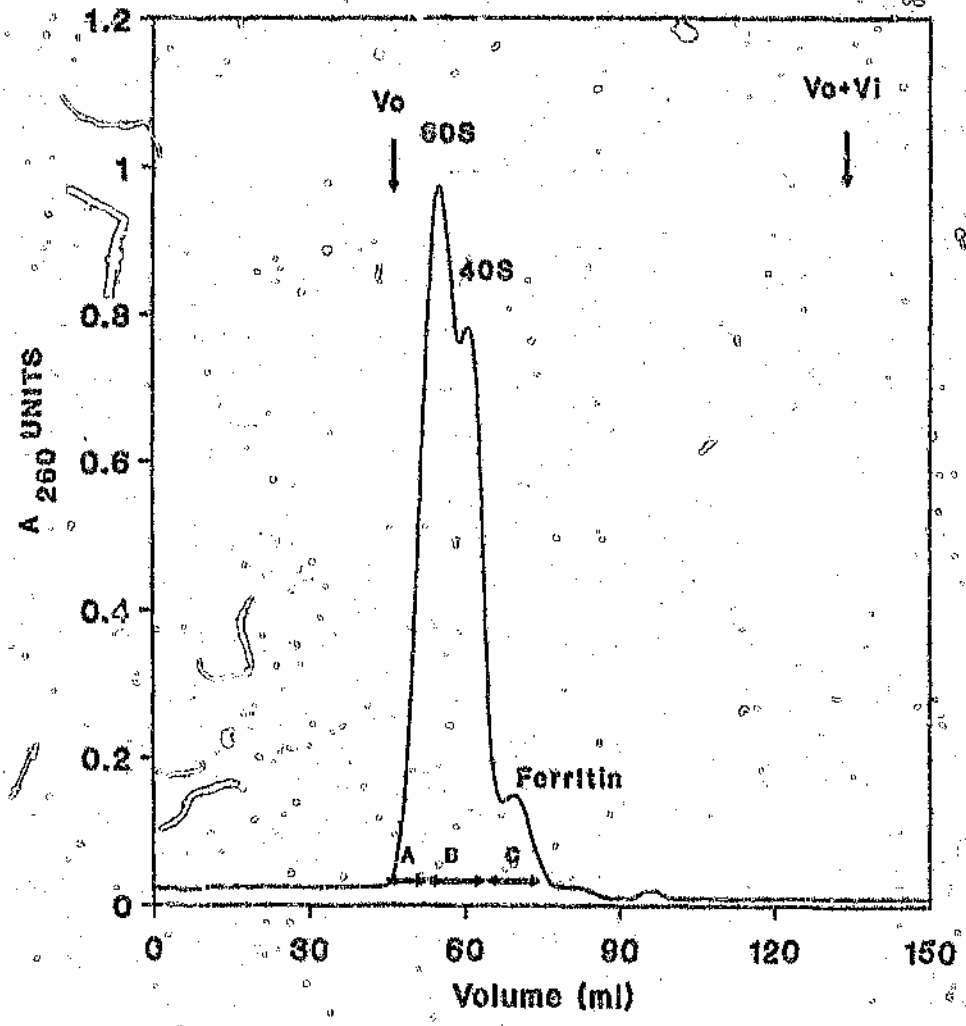


Figure 14. Gel filtration of a mixture of rat liver ribosomal subunits on a 135 cm Trisacryl GF 2000 column at 4°C. A mixture of subunits, (20 A_{260} units) were applied to the column and eluted with buffer B.

60S ribosomal subunits respectively. The material obtained from the peaks was clear and spectrophotometric analysis confirmed the presence of ribosomal material. Ferritin appeared as a shoulder following the 40S subunit peak (V_e = 69,5 ml) indicating that this protein could be separated from ribosomes and the 60S subunits by gel filtration. It was difficult to estimate the exact A_{260} units recovered within each subunit peak because of the high degree of overlapping of the peaks. However, segments marked off on Figure 14 have ~44% in segment A, ~36% in segment B, and ~3% in segment C. It should be noted that both segments A and B are cross-contaminated by both subunits.

A change in the chromatographic conditions to a higher temperature resulted in a significant improvement in the resolution of the ribosomal subunits. This was apparent from the 2 to 3 fold increase in N (Table 6). The 40S subunits eluted in a symmetrical peak (Figure 15) which was narrower and sharper than at 4 °C. Similarly, the 60S subunit eluted in a narrower zone at 20°C than at 4°C. The 60S subunit peak was followed by a smaller well- resolved peak which was identified by its brown colour and ultra- violet absorption spectrum (Figure 13) as ferritin. The separation of ferritin from the 60S subunits illustrates how increasing the temperature results in a significant improvement in the resolution of the column. The recoveries of both the subunits was good and adsorption and retardation was not apparent, thus indicating that a change in temperature did not affect the surface properties of the gel.

Figure 16 illustrates the elution profile of a mixture of ribosomal subunits at 20°C. The subunits separated into two distinct peaks

TABLE 6 : The elution characteristics of rat liver ribosomal subunits on a 135 cm Trisacryl GF2000 column at 4°C and 20°C.

SOLUTES	V_e (ml)	K_d	N	HETP (mm)	Recovery/ Yield (%/A_{260})
4°C :					
40S	60,7	0,18	362	3,7	78
60S	54,2 61,7	0,10 0,19	242	5,5	88
Mixture of Ribosomal Subunits	1. 54,9 2. 60,4 3. 69,5	0,11 0,17 0,28	- - -	- - -	8,8 7,2 0,6
20°C					
40S	59,3	0,16	881	2,1	85
60S	54,6 62,2	0,11 0,19	389 -	4,0 -	76
Mixture of Ribosomal Subunits	1. 55,5 2. 60,0 3. 66,6	0,12 0,17 0,24	- - -	- - -	11,4 8,4 0,9

Rat liver ribosomal subunits were applied to the column and eluted with buffer B. K_d, N and HETP were calculated as described in section 3.4. 10 A_{260} units of the 40S and 60S subunits, and 20 A_{260} units of the mixture of subunits obtained by incubating a sample of ribosomes with puromycin, were applied successively.

V_o is 45 ml and $V_o + V_i$ is 134 ml.

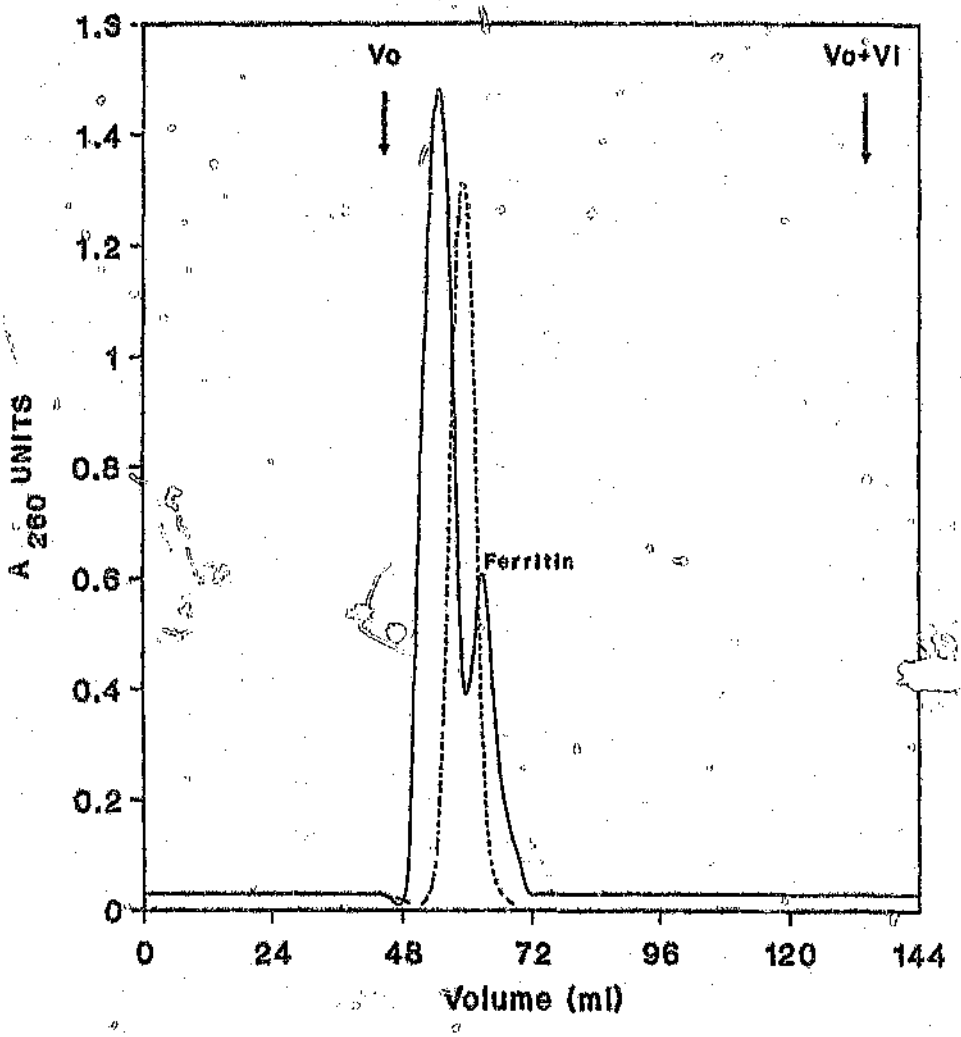


Figure 15. Gel filtration of rat liver ribosomal subunits on a 135 cm Trisacryl GF 2000 column at 20°C. 10 A_{260} units of the 40S(---) and 60S(---) subunits of were applied and eluted with buffer B.

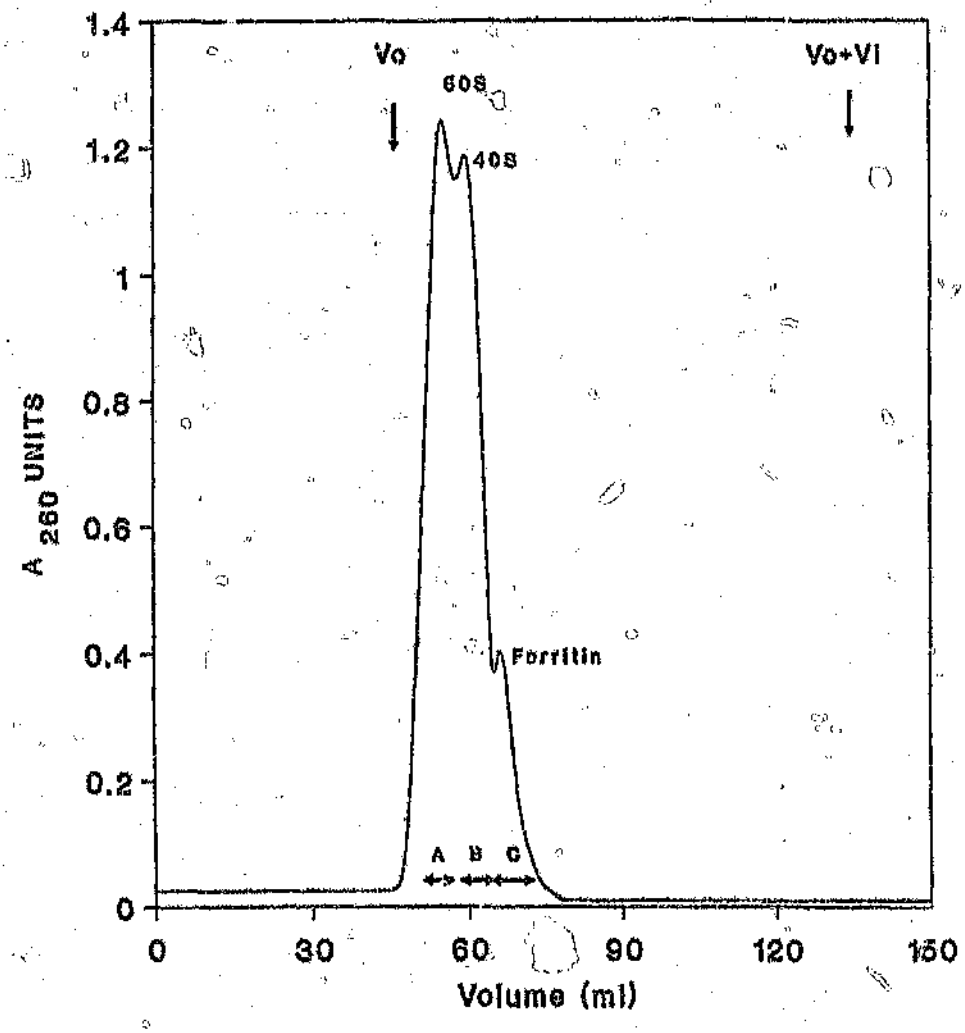


Figure 16. Gel filtration of a mixture of rat liver ribosomal subunits on a 135 cm Trisacryl GF 2000 column at 20°C.
20 A_{260} units of a mixture of subunits were applied to the column and eluted with buffer B.

representing the 60S subunit with a K_d of 0,12 and the 40S subunit with a K_d of 0,17. The material eluted from these peaks was clear and spectrophotometric analysis of the solutions showed ultraviolet absorption spectra which were characteristic of ribosomes. The ribosomal subunit peaks were followed by ferritin which appeared as a shoulder following the 40S peak, confirming the separation of ferritin from the subunits by gel filtration. The A_{260} units recovered within each peak were ~57% in segment A, 42% in segment B, and 4,8% in segment C as shown in Figure 16.

Puromycin eluted from the column at a volume of ~248 ml. It had a K_d of ~1,57 at both 4°C and 20°C, indicating that puromycin was retarded on the column. Previous work (Bhoolia, D.J., Honours project report) has shown that this retardation could be due to strong hydrophobic interactions. This was deduced when puromycin retained its adsorption properties when eluted with a high or low salt buffer, 8 M urea, or 50% dimethylformamide. These solvents excluded ionic, hydrogen bonding and weak hydrophobic interactions as being responsible for the adsorption of puromycin. Since puromycin is a dibasic compound with strong aromaticity, lowering the dielectric constant of the solvent, by increasing the concentration of ethanol, weakened the strong hydrophobic interactions which resulted in the elution of the compound within one column volume.

Ribosomal subunits obtained after gel filtration were concentrated either by ultrafiltration after reducing the KCl concentration to 100 mM or by dialysis against polyethylene glycol (PEG) in a buffer A. The concentrated subunits were assayed for activity by polyphenylalanine synthesis as shown in section 3.7.

Gel Filtration of Rabbit Reticulocyte Ribosomal Subunits on a 135 cm Trisacryl GF2000 column at 20°C

The previous experiment showed that the column efficiency improved somewhat at 20°C. Therefore the experiments to separate rabbit reticulocyte subunits by gel filtration were carried out at 20°C.

The elution profiles of the 60S and 40S subunit fractions are shown in Figure 17. The 60S subunit fraction eluted with a K_d of 0,11. The K_d value is similar to that obtained for the 60S subunit of rat liver. The material that was eluted from the peak was clear and it had an absorption spectrum which was characteristic of ribosomal particles. The A_{260} units recovered was good (Table 7). The 40S ribosomal subunit fraction eluted from Trisacryl GF2000 as a single peak having a K_d of 0,17. The K_d obtained was similar to that obtained for the 40S ribosomal subunits of rat liver. Spectrophotometric analysis showed a ultraviolet spectrum which was characteristic of ribosomal material. The material recovered from the 40S peak was ~87% of the amount loaded as judged by A_{260} units (Table 7).

Figure 18 shows the elution profile of a mixture of subunits. The figure shows two peaks which were not very well resolved. The first peak had a K_d of ~0,15. The second peak had a K_d of ~0,23. Spectrophotometric analysis of material obtained from both peaks were characteristic of ribosomal particles. The first peak eluted at a K_d which was in between the K_d's of the 40S and 60S subunits, and the second peak eluted much later with a K_d much larger than the K_d of the 40S subunits. This could suggest that the first peak contained a mixture of complete 40S and 60S subunits that were not

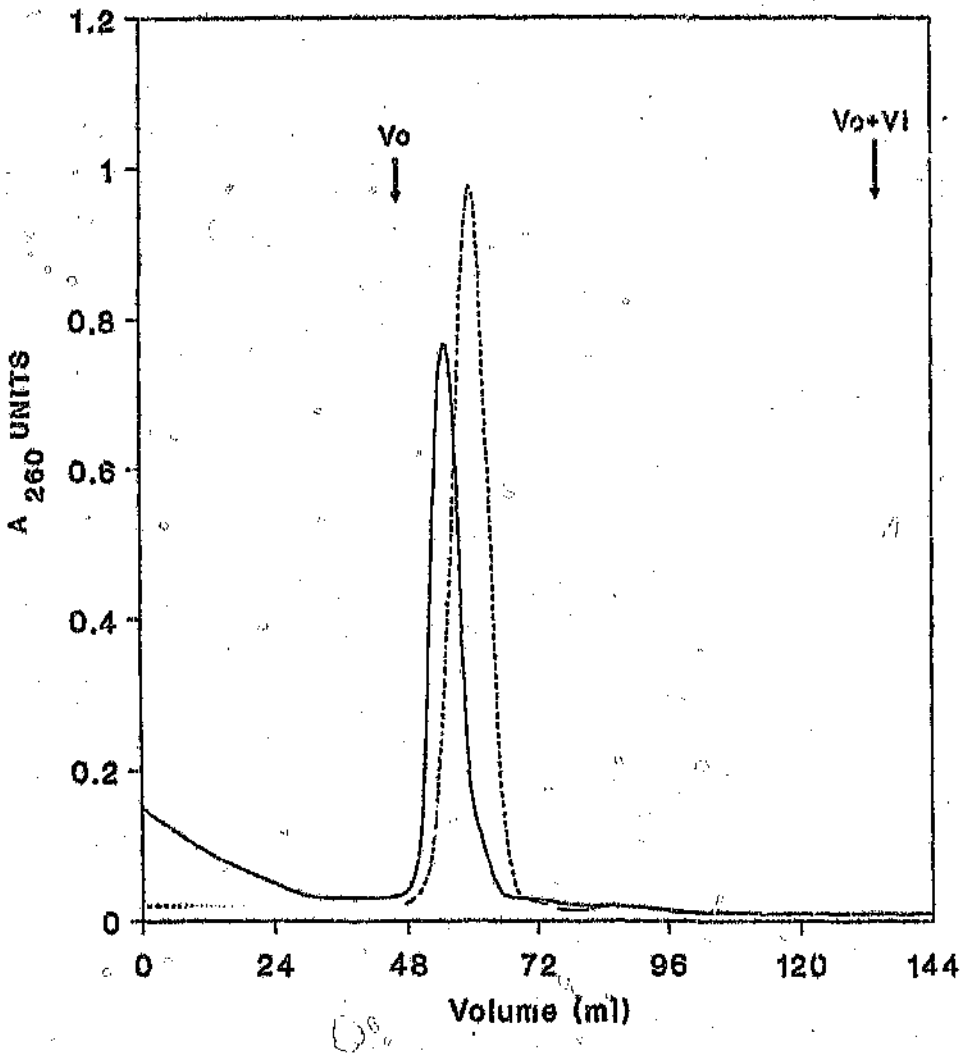


Figure 17. Gel filtration of rabbit reticulocyte ribosomal subunits on a 195 cm Trisacryl GF2000 column at 20°C.
The 40S(---) and 60S(---) subunits were applied to the column (10 A_{260} units) and eluted with buffer B.

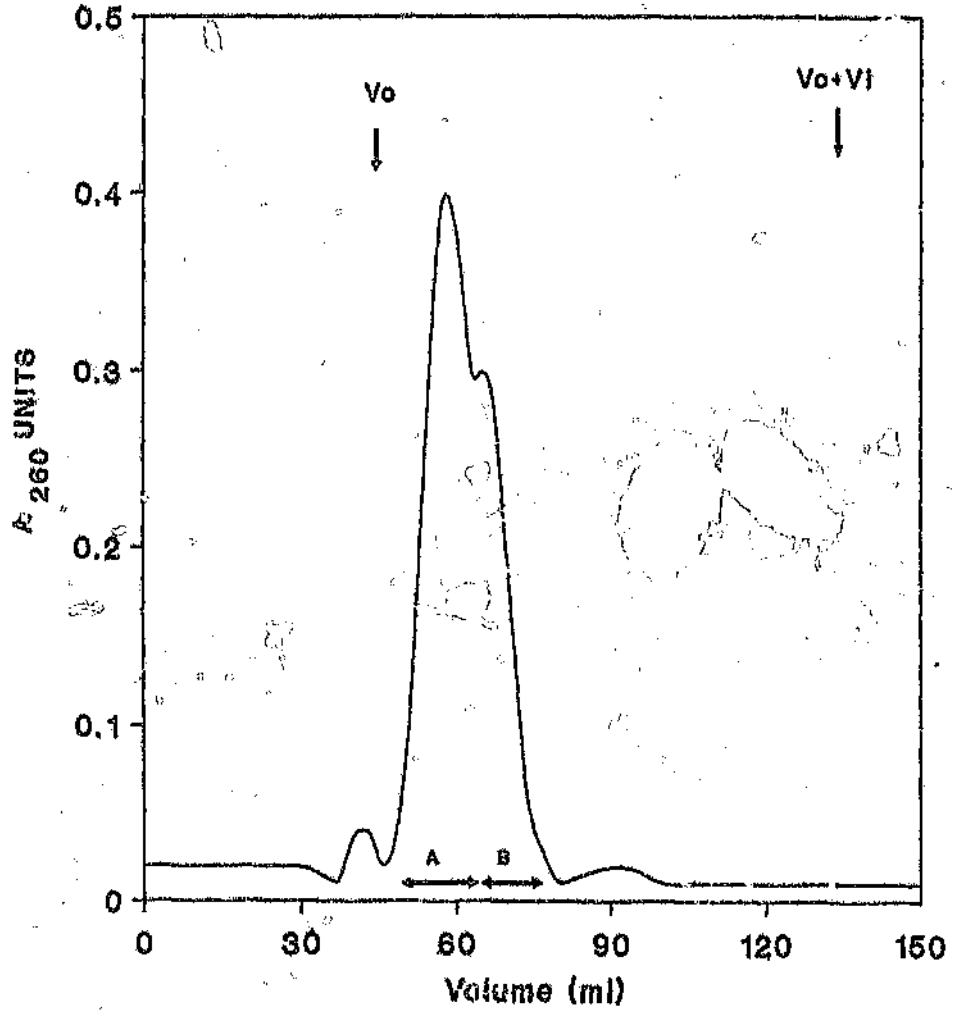


Figure 18. Gel filtration of a mixture of rabbit reticulocyte ribosomal subunits on a 135 cm column of Trisacryl GF 2000 at 20°C.
A mixture of subunits were applied to the column (10 A_{260} units) and eluted with buffer B.

TABLE 7 : The elution characteristics of rabbit reticulocyte ribosomal subunits on a 135 cm Trisacryl GF2000 column at 20°C.

SOLUTES	V_e (ml)	K_d	N	HETP (mm)	Recovery/ Yield (%/A_{260})
40S	60,0	0,17	327	4,0	87
60S	54,8	0,11	273	4,9	90
Mixture of subunits	58,1 65,1	0,15 0,23	- -	- -	7,2 2,8

10 A_{260} units of reticulocyte subunits and mixture of subunits was applied to the column and eluted with Buffer B. K_d, N and HETP were calculated as in section 3.4.

V_o is 45 ml and $V_o + V_i$ is 134 ml

resolved on the column and the second peak contained subunits that had been degraded due to high salt treatment during their preparation. The amount of material recovered in segment A in Figure 18 was 7,2 A_{260} units and 2,8 A_{260} units were recovered in segment B. Ribosomal fractions obtained after gel filtration were analysed for activity by polyphenylalanine synthesis, as shown in section 3.7.

Gel Filtration of Escherichia coli Ribosomal Subunits on Trisacryl GF2000 at 4°C

Escherichia coli ribosomal subunits were chromatographed on Trisacryl GF2000 to compare the elution characteristics of prokaryotes and eukaryotes. Figure 19 shows the elution profiles of the 30S and 50S subunits. The 30S subunit peak was preceded by a smaller peak which, upon spectrophotometric analysis, showed an absorbance spectrum characteristic of ribosomal particles. This peak contained 70S ribosomes. The 30S subunits eluted with a K_d of ~0,09 which was lower than the K_d of the 40S subunits of eukaryotic ribosomes. Similarly the 50S subunits eluted with a K_d ~0,06 which was also lower than the K_d of the 60S subunits of eukaryotic ribosomes. The elution behaviour of ribosomal subunits is unusual since prokaryotic subunits have a smaller molecular weight than eukaryotic subunits and therefore should elute later. This elution behaviour possibly indicates that the shape of eukaryotic ribosomal subunits is more elongated than prokaryotic subunits, thus causing a slight retardation of eukaryotic subunits.

Table 8 shows HETP values calculated (as in section 3.4) for the 30S and 50S subunits. The HETP values are much greater than those

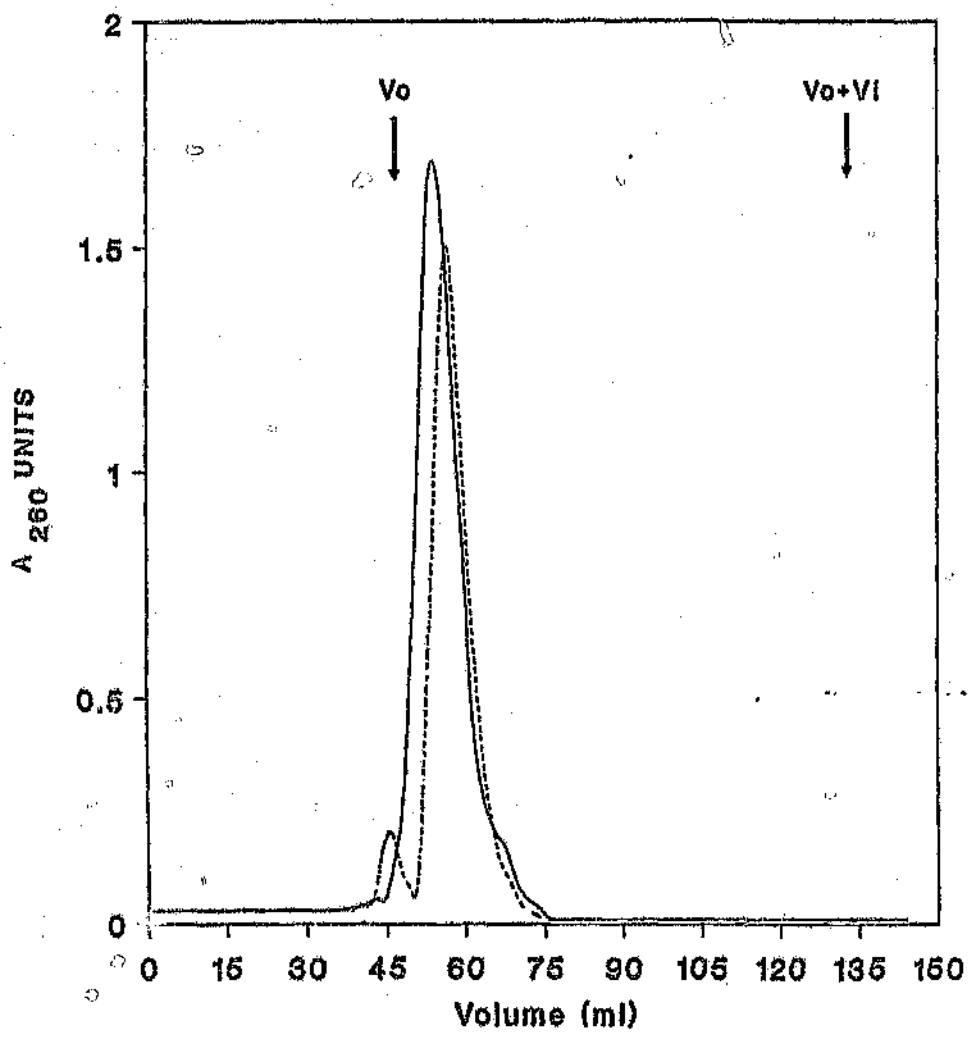


Figure 19. Gel filtration of Escherichia coli ribosomal subunits on a 135 cm column of Trisacryl GF 2000 at 4°C.
The 30S(---) and 50S(---) subunits were applied to the column. 20 A_{260} units of each subunit was applied and elution was with 25 mM Tris-HCl pH 7,6, 0,2 mM $MgCl_2$, 6 mM KCl.

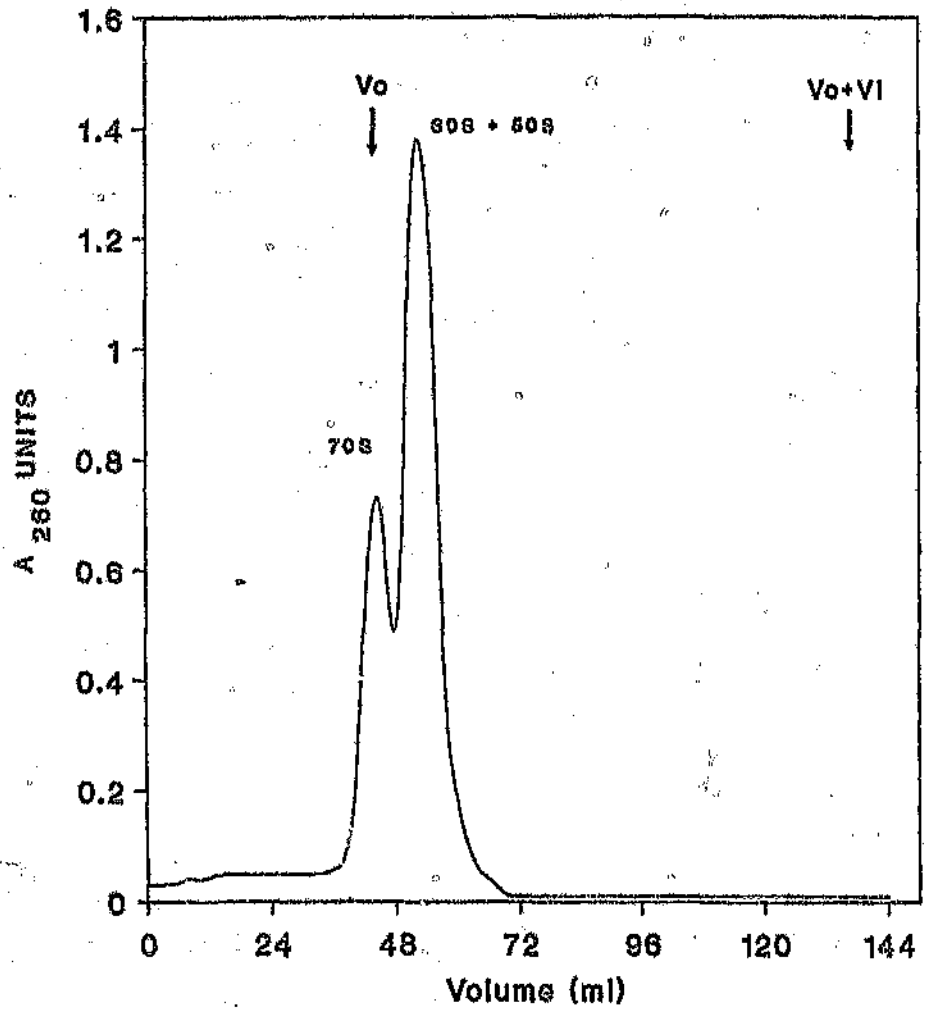


Figure 20. Gel filtration of a mixture of ribosomal subunits of Escherichia coli on a 135 cm column of Trisacryl GF 2000 at 4°C.
Ribosomes were dissociated in low magnesium buffer (as in Fig.19) and 20 A_{260} units of the mixture was applied to the column.

TABLE 8 : The elution characteristics of Escherichia coli ribosomal subunits on a Trisacryl GF2000 column at 4°C.

SOLUTES	V_e (ml)	K_d	N	HETP (mm)	Recovery/ Yield (%/A_{260})
30S	53,2	0,09	245	5,5	79
50S	50,4	0,06	243	5,6	82
Mixture of subunits	1. 45,0	0,00	311	4,3	6,7
	2. 52,5	0,08	294	4,6	11,8

Escherichia coli subunits in low magnesium buffer were applied to the column. 20 A_{260} units of each subunit and mixture of subunits was applied. K_d, N and HETP were calculated as in section 3.4.

V_o is 45 ml and $V_o + V_i$ is 134 ml.

obtained for rat liver subunits at 4°C (Table 6), indicating a greater extent of zone diffusion. Recovery of the subunits of Escherichia coli was good.

The small difference in elution volumes of the 30S and 50S subunits and the comparatively high degree of zone diffusion indicated that separation of the subunits would be unlikely. Figure 20 shows the elution profile of a mixture of ribosomal subunits of Escherichia coli, obtained by lowering the magnesium concentration to 0,2 mM. The profile shows 2 peaks, one eluting at the void volume which had an absorbance spectrum characteristic of ribosomal particles. It was deduced that this peak consisted of 70S tight couples. The second peak was the major peak which eluted with a K_d ~0,08 and it consisted of 30S and 50S subunits. Approximately 33% of the A_{260} units applied to the column were recovered in the void volume and 59% of the in the major peak. The single peak obtained upon elution of the ribosomal subunits indicates that separation of the subunits was not achieved. Subunits obtained after gel filtration were concentrated by ultrafiltration and analysed as shown in sections 3.6 and 3.7.

3.5 SEPARATION OF RAT LIVER RIBOSOMAL SUBUNITS BY SEPHAROSE 4B GEL FILTRATION

Rat liver ribosomal subunits were gel filtered on Sepharose 4B. At 4°C the 40S subunits eluted at a volume significantly greater than the void volume (V_0 ~9,0 ml determined using glycogen), but less than the total column volume, which for Sepharose 4B is approximately

equal to the internal volume + the void volume (~25,0 ml determined using ^3H_2O). The elution profile of the 60S subunits (Figure 21) appeared as an irregular profile. This indicated adsorption of the subunits to the gel. To elute the 60S subunits the column was warmed to 35°C. After warming, the 60S subunits appeared as a symmetrical peak which emerged in ~42 ml. All the A_{260} units of 60S subunits applied to the column were eluted after warming the column.

Figure 22 shows the elution profile at 4°C of a mixture of subunits. Suspensions of hepatic ribosomes and ribosomal subunits prior to separation always have a slightly opalescent appearance. The material responsible for this is of high molecular weight and elutes in the void volume. The absorbance spectrum of A_{220}-A_{360} shows a progressive decline with no clear peak at A_{260}. The second peak, is the 40S subunits. It is clear and shows a good ribosomal A_{280}-A_{235} profile. The yield of the 40S subunits was comparable with the quantitative conversion of the original ribosomes to subunits. The third peak was identified from the absorption spectrum as the puromycin peak which emerged at about the total column volume. After elution of puromycin, the temperature of the column was increased to 35°C to elute the 60S subunits. Almost all of the 60S subunits were recovered from the column.

The 40S and 60S subunits of rat liver were separated on Sepharose 4B because of the adsorption of the 60S subunits to the gel. To determine whether Sepharose 4B was a suitable gel for the separation of the 40S and 60S subunits by pure gel filtration, the experiments were repeated at 35°C since at this temperature, the 60S subunits

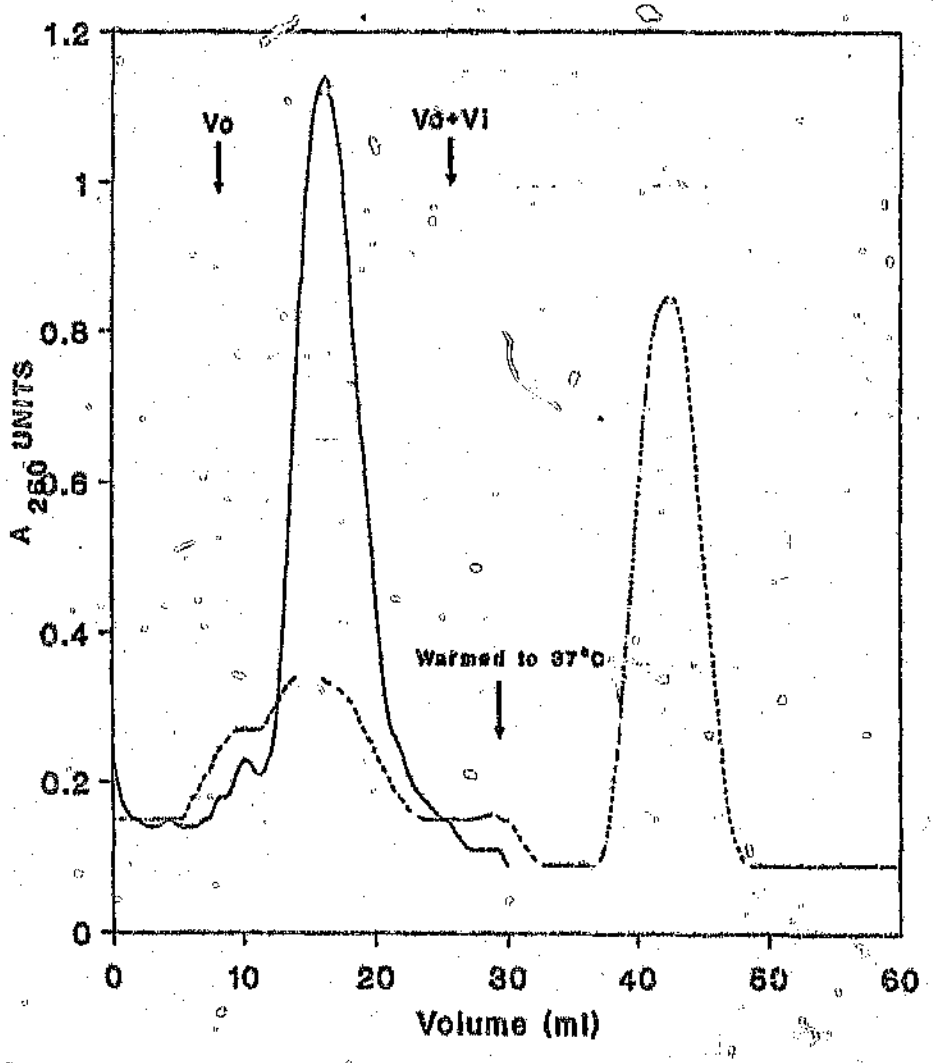


Figure 21. Gel filtration of rat liver ribosomal subunits on Sepharose 4B at 4°C.
Ribosomal subunits were applied to the column. Approximately 5 A_{260} units of the 40S(---) and the 60S(---) subunits were loaded. The 60S subunits were eluted after warming the gel.

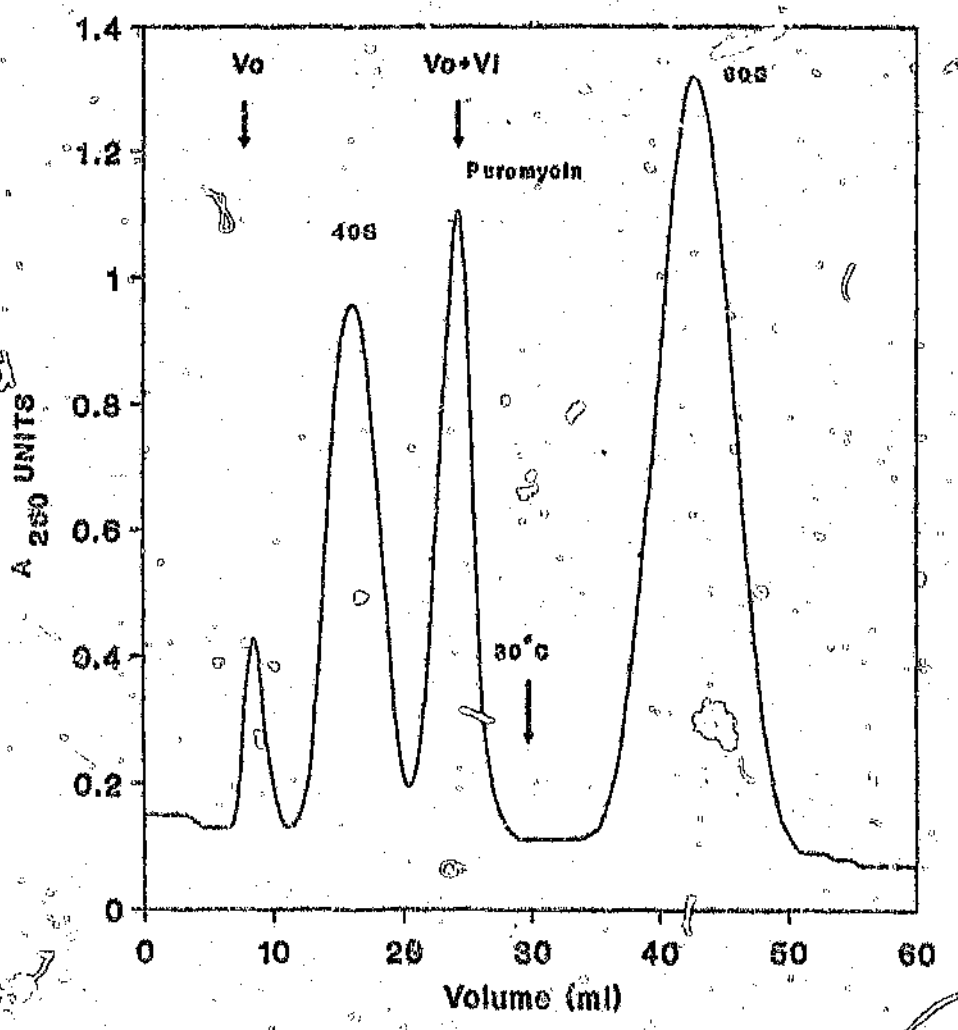


Figure 22. Gel filtration of a mixture of ribosomal subunits of rat liver on Sepharose 4B.
A mixture of subunits were applied to the column at 4°C. 20 A_{260} units were applied. After elution of the puromycin peak at 4°C, the column was warmed to 30°C to elute the 60S subunits.

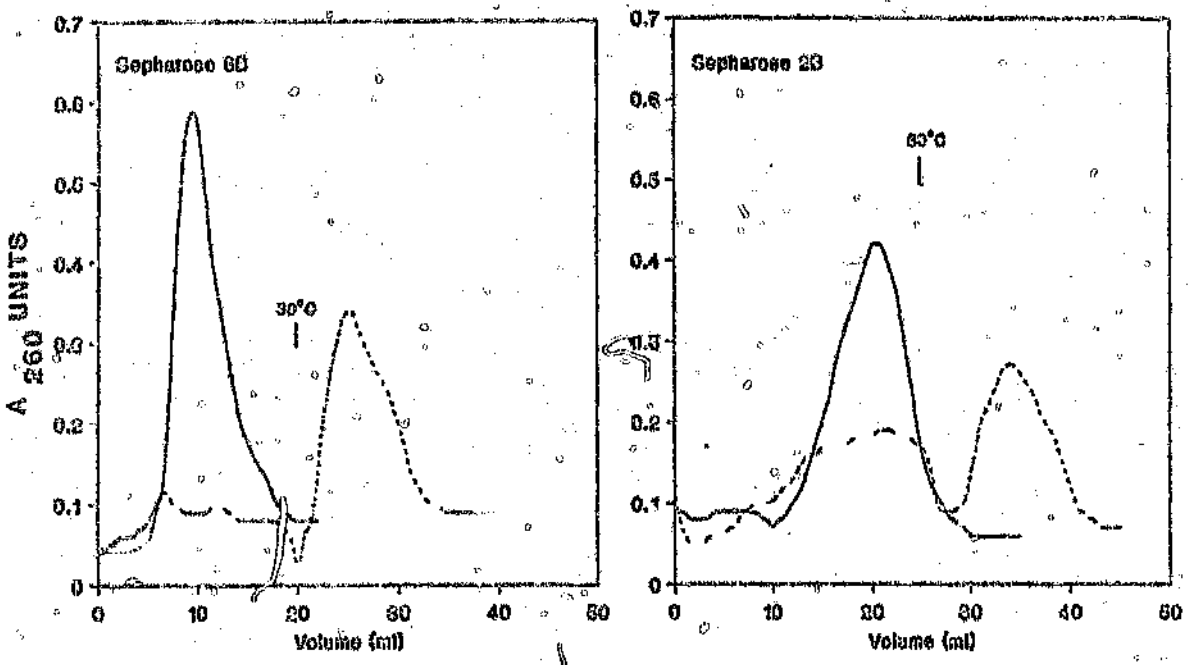


Figure 26. Gel filtration of the 60S ribosomal subunits of rat liver on Sepharose 6B and Sepharose 2B.
6 A_{260} units of the 60S subunits were applied at 4°C(- - -) and 30°C(---). Subunits that had adsorbed to the column at 4°C were eluted by heating the columns to 30°C.

TABLE 9 : The elution characteristics of rat liver ribosomal subunits on a 30 cm Sepharose 4B column at 4°C and at 35°C

SOLUTES		V_e (ml)	K_d	N	HETP (mm)	Recovery/ Yield %/A_{260}
4°C :						
40S		16,3	0,45	56	5,3	79
After warming 60S		42,3	2,08	-	-	95
Dissociated Ribosomes	1.	9,0	0	-	-	0,2
	2.	16,1	0,44	71	4,2	4,3
	3.	24,8	0,99	380	0,8	-
	4.	42,6	2,10	-	-	5,2
35°C						
40S		15,4	0,40	101	2,9	86
60S		14,6	0,35	51	5,8	92
Dissociated Ribosomes	1.	9,2	0,01	-	-	1,1
	2.	15,2	0,39	49	6,1	7,7
	3.	25,0	1,00	411	0,7	-

10 A_{260} units of rat liver subunits and a mixture of subunits were applied to the column at 4°C and at 35°C. At 4°C the 60S subunits adsorbed to the column and were eluted by heating the column to 35°C. K_d, N and HETP was calculated as in section 3.4.

V_o is 9 ml and $V_o + V_i$ is 25 ml

were eluted from the gel.

At 35°C, slightly different results were obtained. The 40S subunits eluted in a narrower zone than at 4°C, judging from the HETP (Table 9). The 60S subunits eluted in a broad chromatographic zone which emerged slightly earlier, but trailed more than the 40S subunit (Figure 23). Both the ribosomal subunits emerged at a volume greater than the void volume and less than the total column volume.

Gel filtration of a mixture of ribosomal subunits at 35°C resulted in the elution of three peaks (Figure 24). The first peak, emerging in the void volume, consisted of the heavy weight material found in liver ribosomal suspensions. The second peak contained a high percentage of the A_{260} units applied to the column having an ultraviolet absorbance profile characteristic of ribosomes. The peak was symmetrical but eluted in a broader chromatographic zone than both the 40S and 60S subunits (Table 9). The third peak eluted at the total column volume and was identified as puromycin. The amount of A_{260} units recovered was comparable with the amount applied to the column. This indicated that no material had adsorbed to the column. RNA analysis of the ribosomal peak at 35°C shows the presence of both the 18S and 28S rRNA (not shown), thus indicating that the ribosomal subunits were not separated by gel exclusion on Sepharose 4B.

The anomalous behaviour of the 60S subunits could be due to an intrinsic property of the 60S subunits. If the 60S subunits are of similar size to the pores of the gel, they would replace the water of solvation, and thus be retarded by the gel. This possibility was

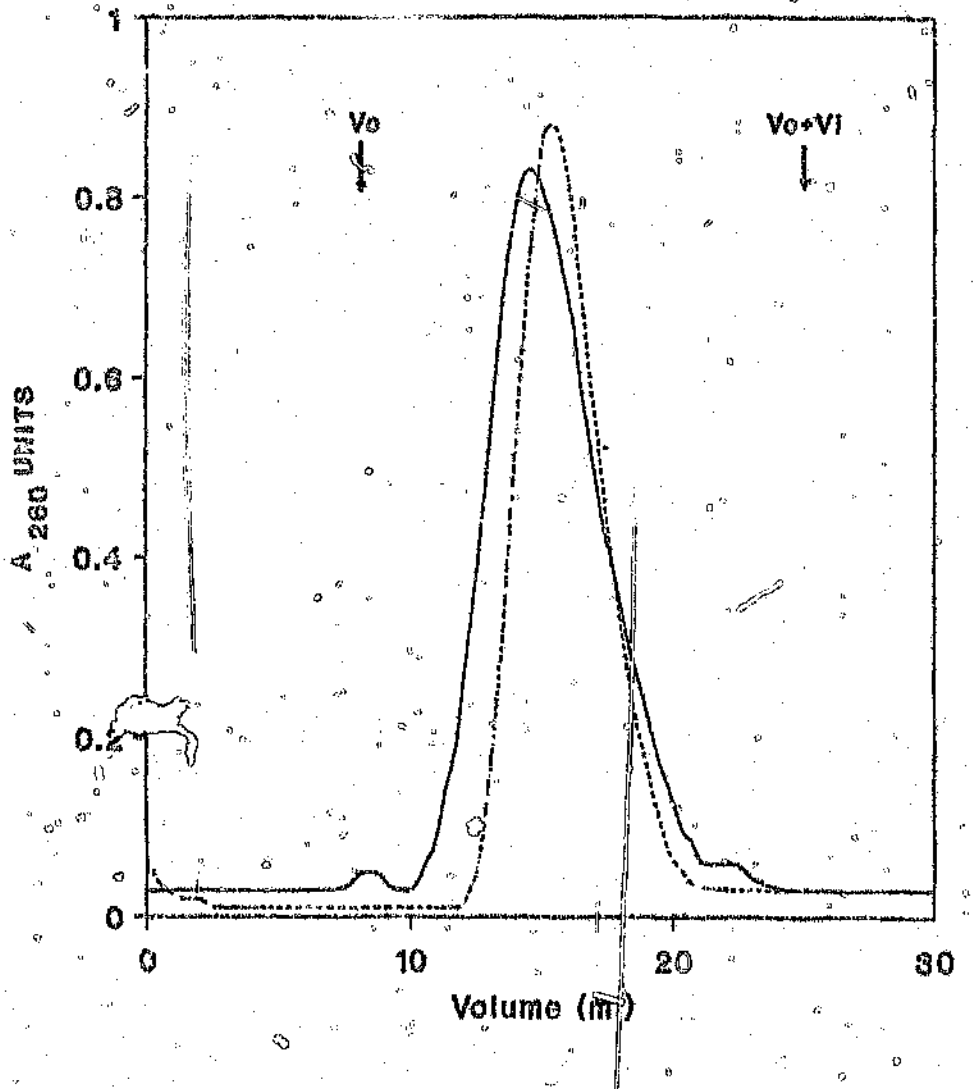


Figure 23. Gel filtration of ribosomal subunits of rat liver on Sepharose 4B at 35°C.
6 A_{260} units of the 40S(----) and 60S(---) subunits were applied and eluted at 35°C.

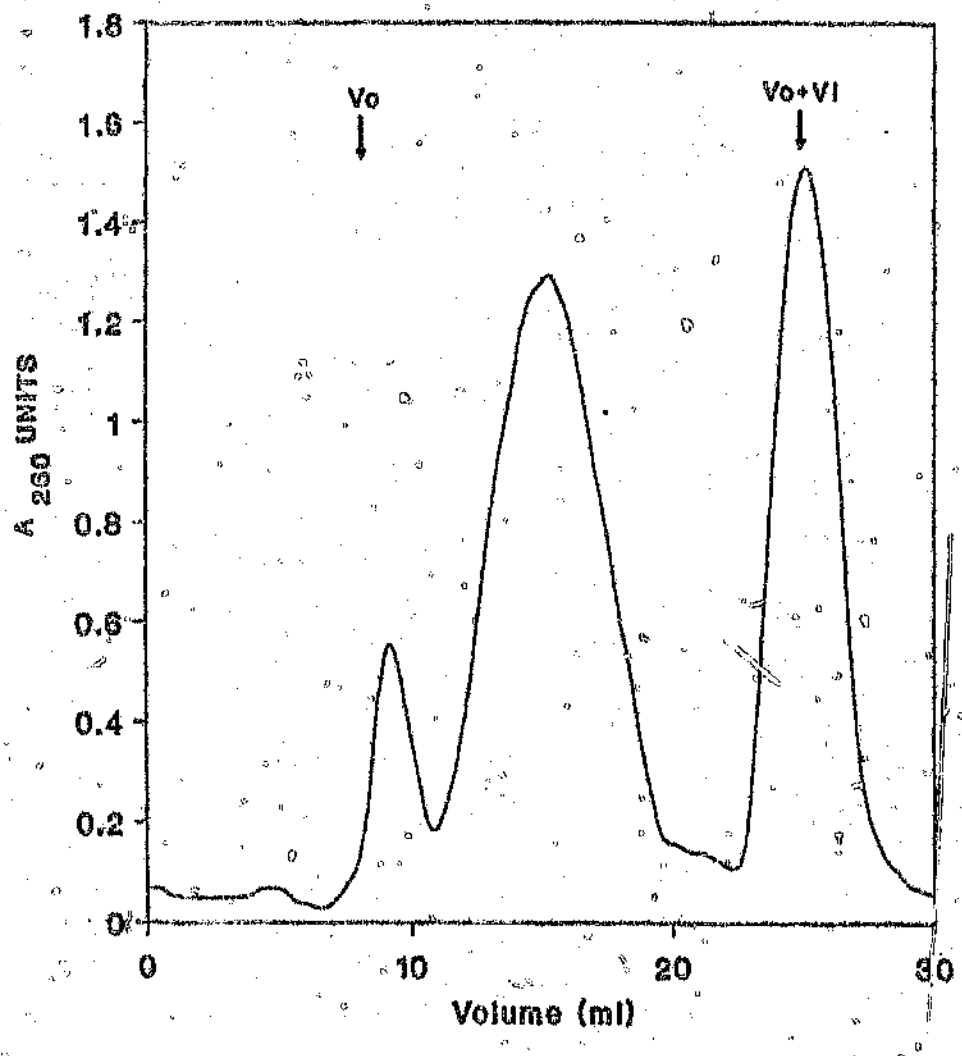


Figure 24. Gel filtration of a mixture of ribosomal subunits of rat liver on Sepharose 4B at 35°C. A mixture of ribosomal subunits (20 A counits) were applied to the column and eluted with buffer B.

resolved by applying the 60S subunits to a Sepharose column with a smaller pore size, such as Sepharose 6B, and to one with a larger pore size, such as Sepharose 2B. The 60S subunits were not eluted from either Sepharose 6B or Sepharose 2B at 4°C (Figure 25), but were eluted with a lower K_d from the Sepharose 6B than from the Sepharose 2B column when the temperature of the column was increased to 30°C. The 60S subunits eluted within a column volume when they were applied to both columns at 30°C (Figure 25). On Sepharose 6B, the peak emerged near the void volume and trailed slightly. On Sepharose 2B the peak showed a broad chromatographic zone. Thus the 60S subunit of rat liver adsorbed to agarose based resins at low temperatures.

The effect of temperature on the adsorption of the 60S subunits on Sepharose 4B was demonstrated by applying the 60S subunits to the column at various temperatures (Figure 26). It was observed that the 60S subunits adsorbed to the gel gradually as the temperature was decreased. Before being totally adsorbed to the gel, the subunits were retarded, as shown by the profile at 20°C. At 4°C-15°C, the subunits were completely adsorbed and were only eluted after warming the column to 30°C. At temperatures above 25°C elution of the subunits were unretarded.

The 50S subunits of Escherichia coli did not display the same behaviour as the 60S subunits of rat liver (Figure 27). Both the 50S and 30S subunits eluted in a volume in between the void volume and the total column volume. Almost all of the ribosomal subunits applied to the column were recovered based on the A_{260} measurements. The Escherichia coli subunits elute earlier than the

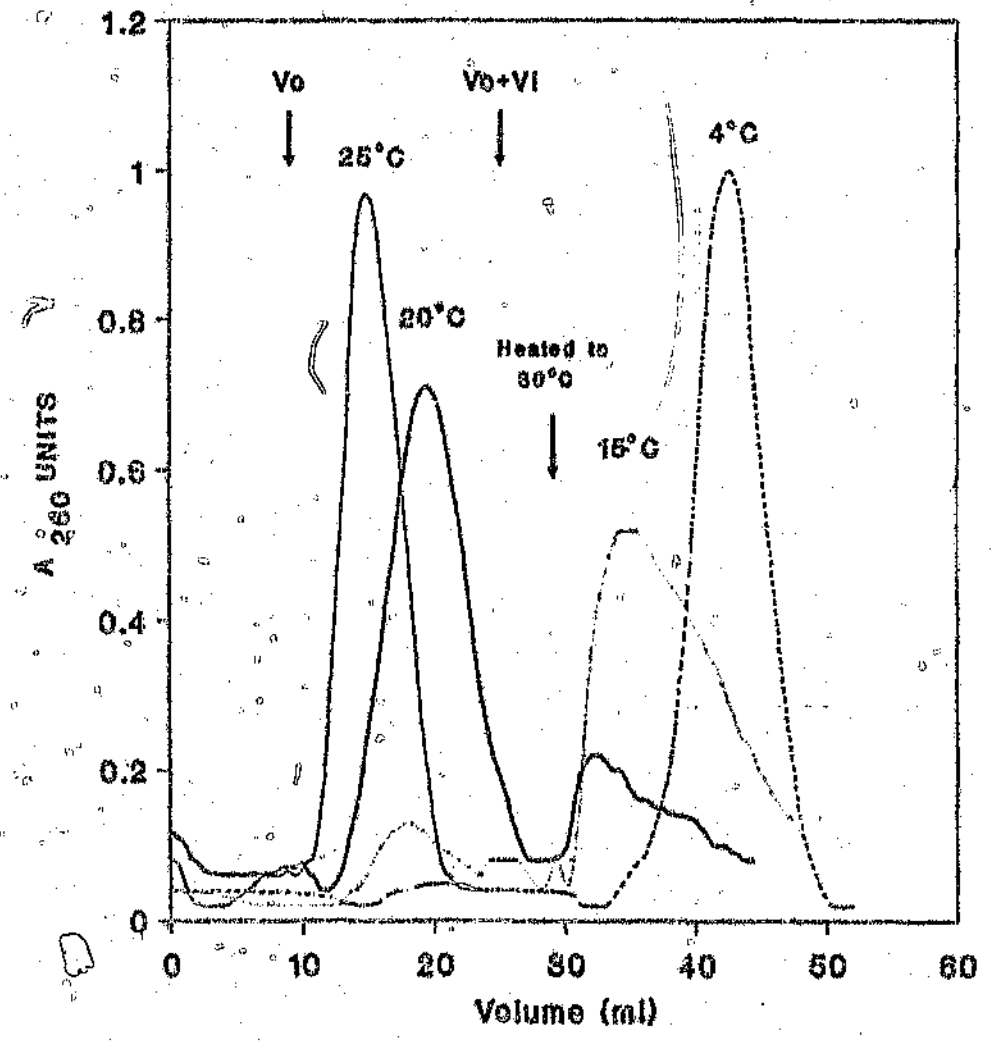


Figure 26. The effect of temperature on the adsorption behaviour of the 60S subunits of rat liver on Sepharose 4B.
5 A_{260} units of the 60S subunits were applied to the column at the temperatures indicated. The column was heated to 30°C to elute subunits that had adsorbed to the gel after each run, at the position indicated by the arrow.

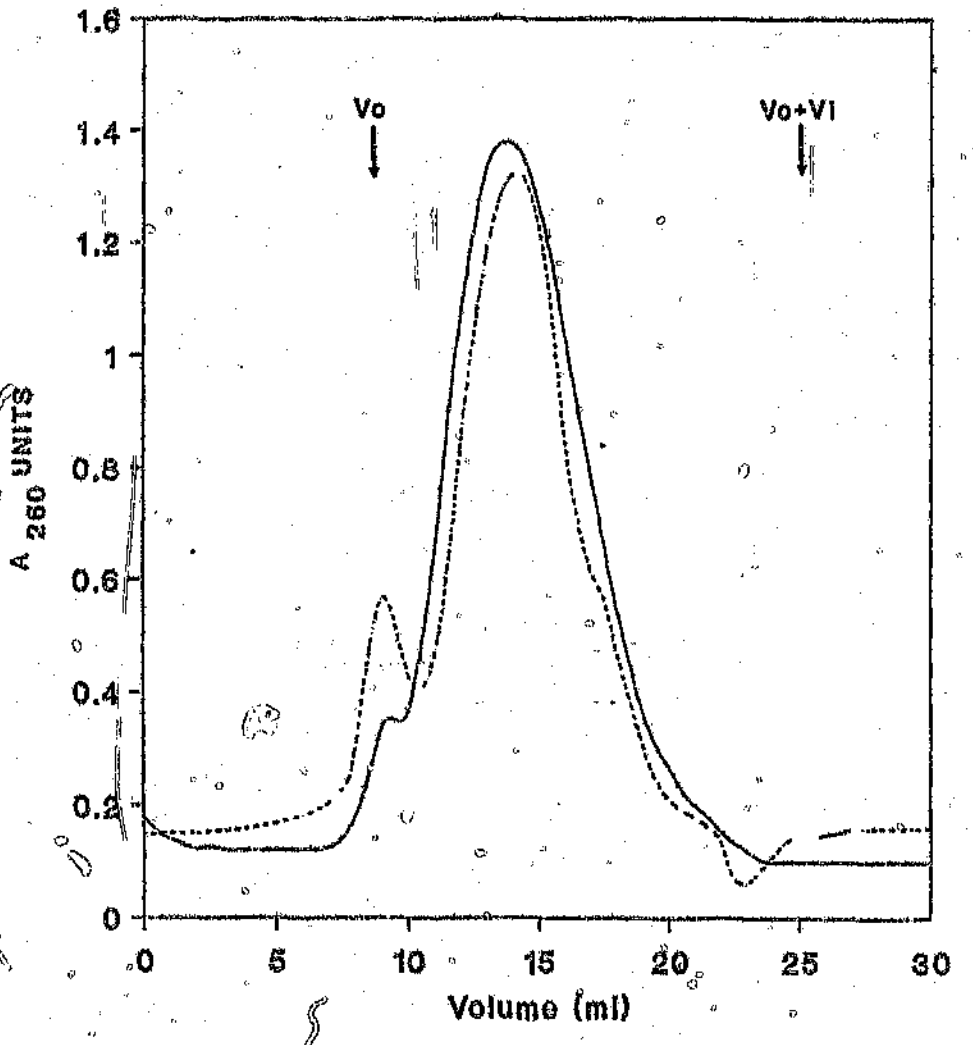


Figure 27. Gel filtration of Escherichia coli ribosomal subunits on Sepharose 4B at 4°C.
10 A_{260} units of the 30S(---) and 50S(---) of *Escherichia coli* were applied to the column and eluted with a low magnesium buffer (as in fig.19).

subunits of rat liver, as was the case with Trisacryl GF2000.

3.6 ANALYSIS OF RIBOSOMAL SUBUNITS BY ACRYLAMIDE-AGAROSE GEL ELECTROPHORESIS

Analysis of Escherichia coli ribosomal subunits

Composite gels containing both agarose and acrylamide made the gel electrophoretic separation of ribosomal subunits possible. Agarose provides mechanical stability to very weak acrylamide gels.

The gel apparatus used in this study was home made and did not have the dimensions of the slab gel apparatus described by Dahlberg et al. (1969). Therefore the conditions for electrophoresis had to be optimised in order to achieve adequate resolution. This was deduced after a preliminary electrophoresis experiment at a voltage of 200 V. After 4 hours of electrophoresis of Escherichia coli ribosomal subunits in 0,2 mM $MgCl_2$, 25 mM Tris-HCl pH 8 buffer, all that was observed was a smear across the lanes (not shown).

The optimization of electrophoretic conditions was carried out with ribosomal subunits of Escherichia coli isolated by gradient centrifugation, and electrophoresis was in a low magnesium buffer (0,2 mM $MgCl_2$, Tris-HCl pH 8) at various voltages and times (Figures 28a - 28e), where the times of electrophoresis depended on the voltage. The resolution was good between the range of voltages 60 V-120 V, and thereafter (150 V - 200 V) the resolution steadily decreased (Figures 28d - 28e). In Figure 28a - 28c, 5 bands are visible within each lane. The bands represented different forms of the 30S and 50S

Figure 28a. Electrophoresis of Escherichia coli ribosomal subunits in acrylamide-agarose gels at 60V
Ribosomal subunits were electrophoresed in 2,75% acrylamide-0,5% agarose gels in Tris-HCl (25 mM) and $MgCl_2$ (0,25 mM), pH 8 buffer for 6 hrs. at 60 V and 4°C. The gel was stained with Coomassie brilliant blue. Lanes 1-6 contained different concentrations of the 50S and 30S subunits.

Figure 28b. Electrophoresis of Escherichia coli ribosomal subunits in acrylamide-agarose gels at 90V
Ribosomal subunits were electrophoresed as in Fig. 28a except electrophoresis was at 90V for 6 hrs.

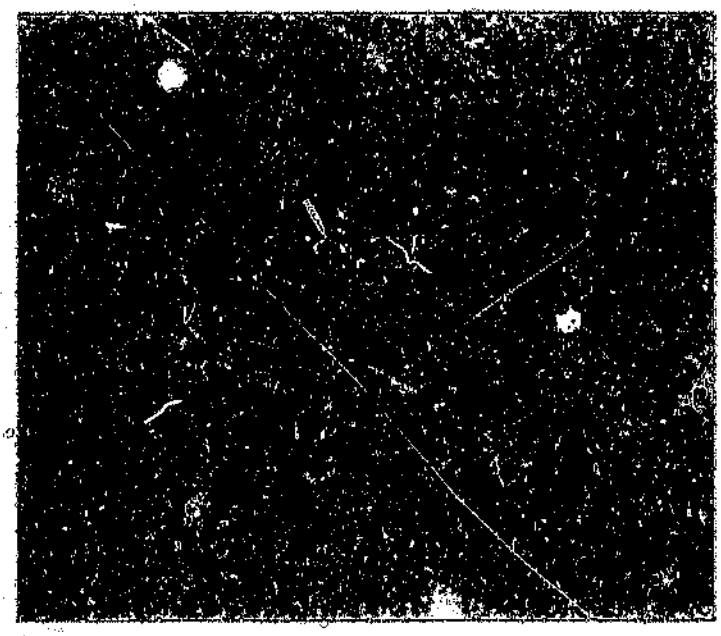


Figure 28c. Electrophoresis of Escherichia coli ribosomal subunits in acrylamide-agarose gels at 120V. Ribosomal subunits were electrophoresed as in Fig. 28a except that electrophoresis was at 120 V for 4 hrs.

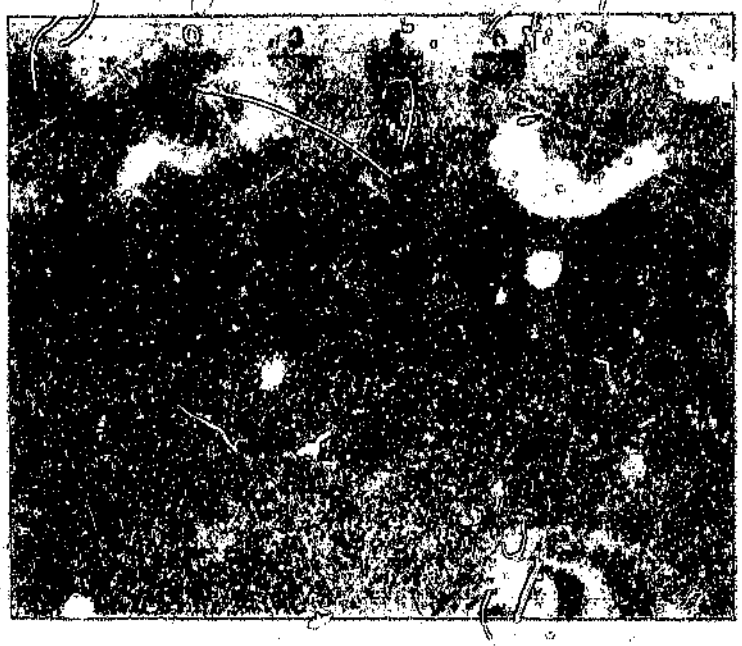


Figure 28d. Electrophoresis of Escherichia coli ribosomal subunits in acrylamide-agarose gels at 150V
Ribosomal subunits were electrophoresed as in Fig. 28a except that electrophoresis was at 150V for 3 hrs.

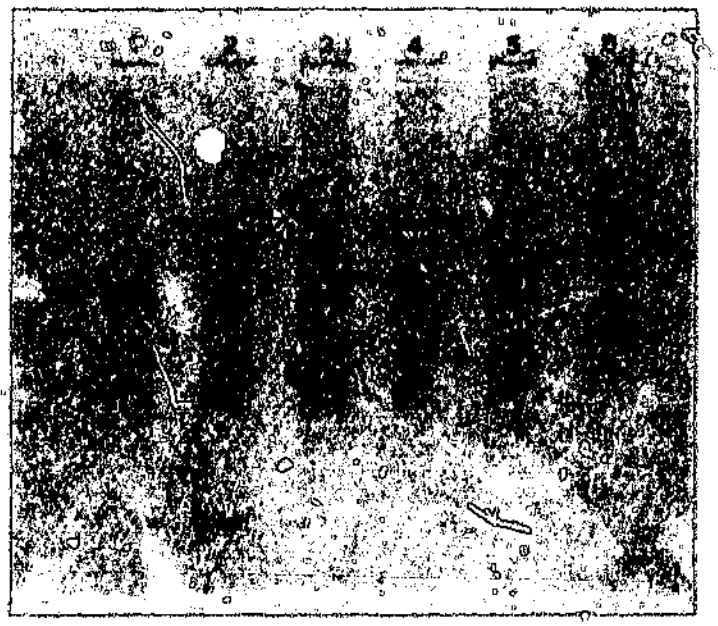


Figure 28e. Electrophoresis of Escherichia coli ribosomal subunits in acrylamide-agarose gels at 200V
Ribosomal subunits were electrophoresed as in Fig.28e except that electrophoresis was for 2 hrs at 200V.

subunits. The two fastest migrating bands were two different forms of the 30S subunits. The two forms arise from the loss of one of the ribosomal proteins and this results in one form being of lower molecular weight which migrates faster than the other (Dahlberg, 1974). Similarly the three slower migrating bands were three different forms of the 50S subunits (Talens et al., 1973). Again the three forms arise from the loss of one or more of the ribosomal proteins. In Figures 28d and 28e, the 5 bands were not as well resolved and the different forms of each subunit merged into a single diffuse band (Figure 28e). In Figures 28c - 28d, the different forms of the two subunits became more prominent when the amount of subunits applied to the gel was greater.

From the intensity of the bands it was observed that the slowest migrating form of the 50S subunits (S-50S) is the most prominent thus indicating that this form was in high concentration in the preparation. The 30S subunits were equally divided, judging from the staining intensity, and therefore in the ribosomal preparation only half the 30S subunits were native subunits.

The presence, in approximately equal proportions, of both ribosomal subunits in each preparation of ribosomal subunits indicates a certain amount of cross contamination which was a result of poorly resolved density gradient profiles due to an overloading of the gradients (in excess of 80 A_{260} units).

Analysis of ribosomal subunits of Escherichia coli eluted from Trisacryl GF2000

The ribosomal subunits of Escherichia coli gel filtered on Trisacryl

GF2000 were analysed by acrylamide-agarose gel electrophoresis (Figure 29). The 50S subunits migrated as two bands with the slower migrating band staining slightly more intensely than the faster migrating band. The fastest migrating band was absent in the gel filtered ribosomal subunits. The 30S subunits migrated as two bands, the F-30S form, being the faster migrating form, while the S-30S, the slower migrating form. The F-30S form was more prominent than the S-30S form. When the load was increased, the two bands of the 30S subunits were more diffuse and were poorly resolved.

When the pooled fractions obtained from a mixture of ribosomal subunits gel filtered on Trisacryl GF2000 were electrophoresed (Figure 29, lane 7 & 8), two bands were observed which corresponded to the S-50S band and the S-30S band indicating that the native forms of the subunits were present. The bands were more diffuse than the marker bands (30S and 50S subunits of Escherichia coli isolated by density gradient centrifugation), and if any other forms of the subunits were present it was not resolved on the gel. Since both subunit bands were observed, the results confirm that the ribosomal subunits on Trisacryl GF2000 were not resolved. The 30S subunit fraction was not contaminated by 50S subunits and similarly the 50S subunit fraction was not contaminated by 30S subunits which indicated good resolution on the density gradients. The 30S subunits migrated as two bands with the F-30S being the predominant band.

The analysis of rat liver ribosomal subunits on acrylamide-agarose gel electrophoresis

Ribosomes and ribosomal subunits of eukaryotes have not been distinctly separated on acrylamide-agarose composite gels. In an

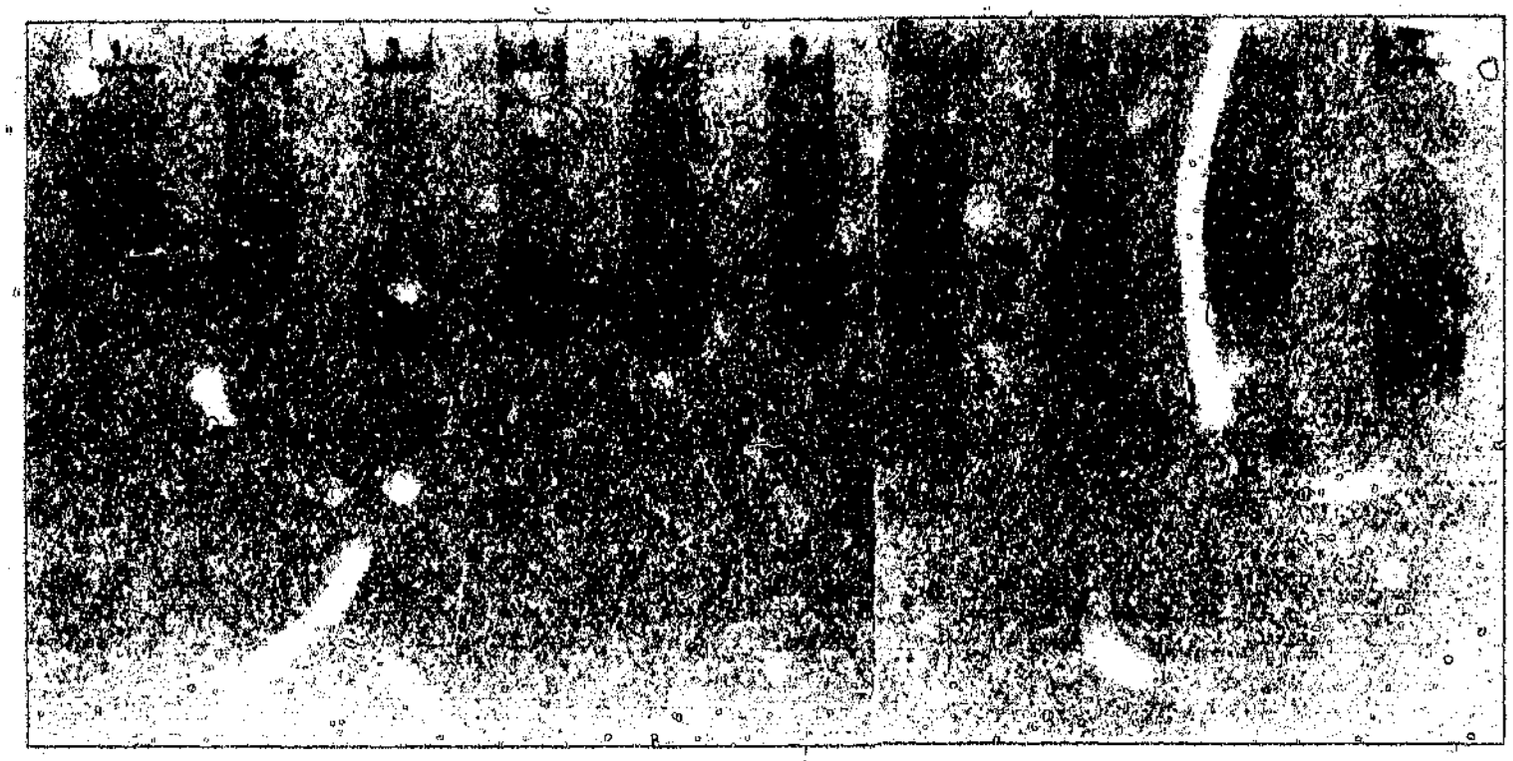


Figure 29. Acrylamide-agarose gel electrophoresis of Escherichia coli ribosomal subunits isolated by gel filtration and density gradient centrifugation.
The conditions of electrophoresis was as described in Fig.28c. Lanes 1-2 contained different concentrations of the 50S subunits, lanes 3-6, the 30S subunits, lanes 7-8 a mixture of subunits from gel filtration on Trisacryl GF2000. Lanes 9 and 10 contained the 50S and 30S subunits respectively isolated by density gradient centrifugation.

attempt to develop this technique as an analytical tool for eukaryotic ribosomes and ribosomal subunits, various buffering systems and electrophoretic conditions were studied. The first buffering system used in the study was the one used by Dahlberg (1974) (Figure 30). Ribosomal subunits of rat liver isolated by density gradient centrifugation were applied to the gel in sample buffer. Electrophoresis of the gel from the anode to the cathode at 120 V resulted in the migration of the 60S subunits as streaks to about 2 cm from the well interface. The leading edge was a defined zone. The top of each lane in which the 40S subunits were applied showed some material which migrated as streaks with an undefined leading boundary. These streaks may indicate contamination of the 40S subunit fraction by 60S subunits. As outlined earlier, the voltage gradient played an important role in the resolving power of the electrophoresis gel, and lower voltages produced better zones. Thus a lower voltage was applied to the above electrophoretic system and electrophoresis was carried out for a longer time (Figure 31). With the lower voltage, it was observed that the migration of the 60S subunits was retarded when compared to the run at 120 V. The 40S subunits showed an increased retardation along the margins of each zone. Decreasing the voltage did not increase the resolution of the electrophoretic system.

The electrophoretic system used by Helser et al. (1981) was used in this study to electrophorese rat liver ribosomal subunits (Figure 32). The migration was very poor. Both ribosomal subunits migrated as streaks rather than defined zones. The migration of the subunits was more retarded than with the previous buffer system (Figure 30). All of the above three systems use non-denaturing buffer systems. It

Figure 30. Acrylamide-agarose gel electrophoresis of rat liver ribosomal subunits.
Electrophoresis of the subunits was on a 2,25% acrylamide-0,5% agarose composite gel in 25 mM Tris-HCl, 0,25 mM $MgCl_2$ and 6 mM KCl buffer pH 8 at 120 V and 4°C for 6 hrs. Lanes 1-3 contained the 60S subunits and lanes 4-6 the 40S subunits.

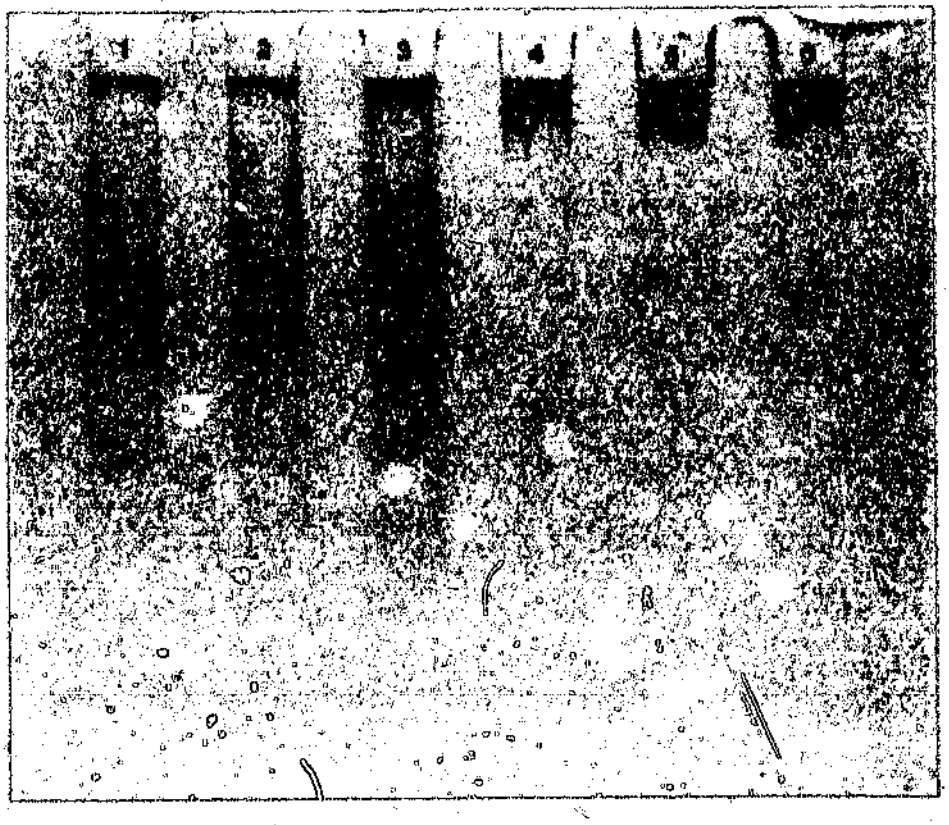


Figure 31. Acrylamide-agarose gel electrophoresis of rat liver ribosomal subunits.
Electrophoresis of the subunits was as in Fig30, except it was at 30V for 16 hrs.
Lanes 1-3 contained the 40S and lanes4-6 the 60S subunits.

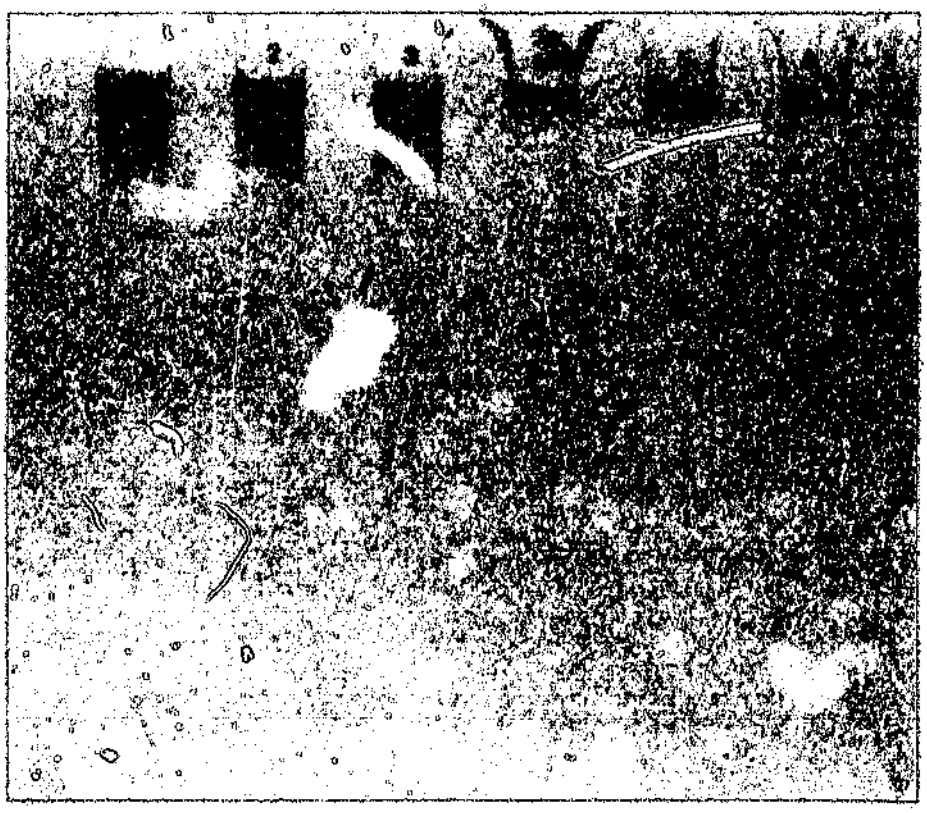


Figure 32. Acrylamide-agarose gel electrophoresis of rat liver ribosomal subunits.
Electrophoresis of the subunits was on a 2,25% acrylamide-0,5% agarose composite gel in 26 mM Tris-acetate, 10 mM magnesium acetate and 60 mM potassium acetate buffer pH 7.6 at 120 V and 4°C for 6 hrs. Lanes 1-3 contained various concentrations of the 40S subunits and lanes 4-6 the 60S subunits.

was evident from the above results that ribosomal subunits from higher eukaryotes were unable to migrate into non-denaturing electrophoretic systems.

The considerable buffering capacity of the Tris-Borate-EDTA buffer used by Peacock and Dingman (1968) permitted the separation of subunits of rat liver ribosomes (Figure 33 and 34). The 60S ribosomal subunits migrated as a single band whereas the 40S fractions from density gradients separated into two bands, one of which was the 60S subunit band, and another one, which migrated further along the lane and was more diffuse, was the 40S subunit band. At a lower voltage in the same buffer system the bands appear more diffuse (Figure 34). Figure 33 also shows the migration of Escherichia coli ribosomal subunits in the Tris-Borate-EDTA gel buffer system. The 50S subunits migrated as a single band whereas the 30S subunits resolved into three discrete bands, indicating that the 30S ribosomal subunits were excessively degraded. The migration of the ribosomal subunits is proportional to the molecular weight of the subunits and the ribosomal subunits of Escherichia coli migrate negative to the ribosomal subunits of rat liver.

3.7 ACTIVITY OF RIBOSOMAL SUBUNITS AND REASSOCIATED RIBOSOMES

Activity of rat liver ribosomal subunits

When separate ribosomal subunit fractions or reassociated ribosomes were assayed for their ability to synthesize proteins in a cell-free system, activities observed in the presence of template RNA show the 40S subunits alone had very little activity (Table 10) whereas the

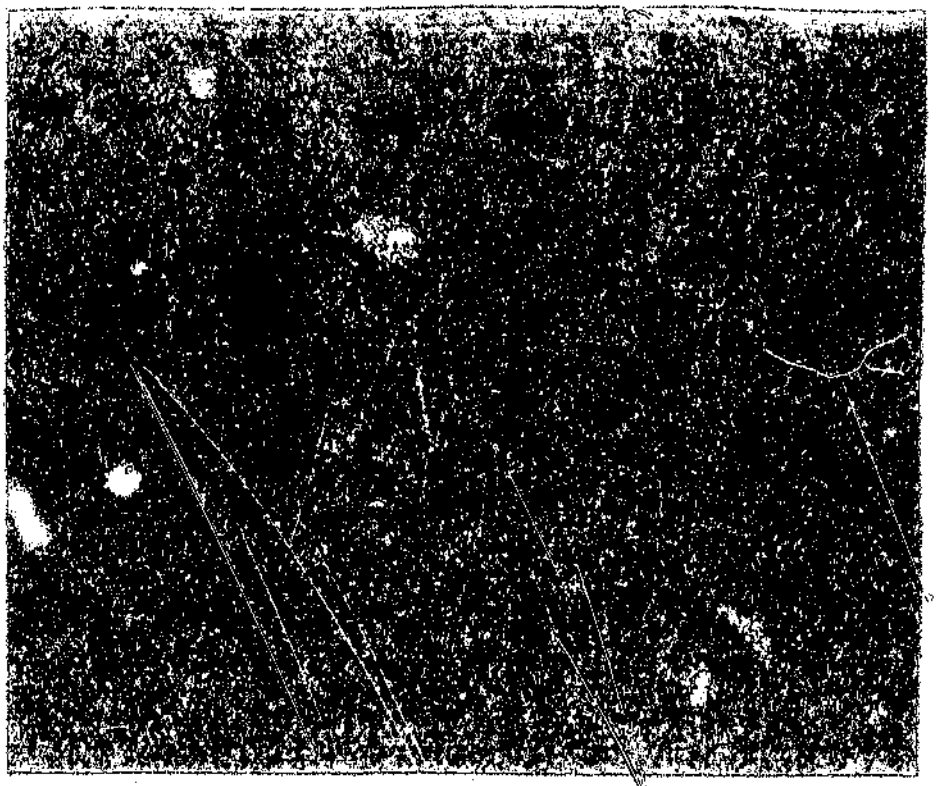


Figure 33. Acrylamide-agarose gel electrophoresis of rat liver and Escherichia coli ribosomal subunits. Electrophoresis of the subunits was on a 2,5% acrylamide-0,5% agarose composite gel in a Tris-borate-EDTA buffer pH 8,3 (Peacock and Dingman, 1968) at 120 V and 4°C for 8 hrs. The samples are the 40S subunits (lanes 1,2), and the 60S subunits of rat liver (lanes 3,4) and the 30S and 50S subunits of Escherichia coli (lanes 5,6).

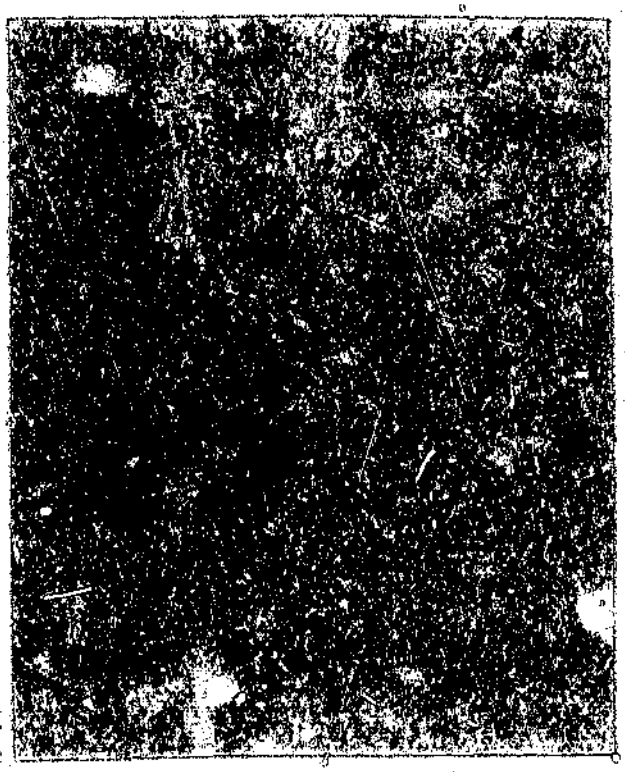


Figure 34. Acrylamide-agarose gel electrophoresis of rat liver ribosomal subunits.
Electrophoresis of the subunits was as in Fig 33 except it was for 16 hrs at 60 V.
The samples are the 40S subunits (lanes 1,2), the 60S subunits of rat liver (lanes 3,4).

TABLE 21 : Polyphenylalanine synthesis by ribosomal subunits of rat liver isolated by density gradient centrifugation

Ribosomal Species	cpm/tube
40S	1148
60S	2004
40S + 60S	7404
Blank	862
Control Ribosomes	7129

Poly U directed polyphenylalanine synthesis was as described in section 2.12. Each tube contained 10 µg 40S subunits and 25 µg 60S subunits as indicated.

60S subunits possess ~27% of the activity attainable with the combined 40S and 60S fractions, which accords with the presence of some 40S particles in the 60S fraction. The combined ribosomal subunits in the presence of template mRNA possess activity approximately equal to that of the control ribosomes. These results did not differ greatly when the method of preparation of the ribosomal subunits were varied. Ribosomal subunits prepared by gel filtration possess similar activities although the 60S subunits did appear to possess lower activity than 60S subunits prepared by gradient centrifugation. When pooled fractions from mixtures of subunits were assayed for activity, both the 60S and 40S subunits possessed ~20% of the activity of a combination of the 40S and 60S subunits (Table 11). The high degree of activity of the 40S and 60S subunits is in accordance with the low resolution obtained by gel filtration on Trisacryl GF2000 (Figure 14), thus the high degree of cross contamination of the subunits. This was apparent even when the subunits were separated at 20°C where both the 60S and 40S ribosomal subunits possessed a considerable amount of activity (Table 11). The polyphenylalanine synthesizing abilities of the ribosomal subunits were not affected by the temperature at which they were prepared. Ribosomal subunits prepared at 4°C or 20°C possess approximately equal activities to that of the control ribosomes (Table 11). When a complete separation of the ribosomal subunits was achieved, as with gel filtration using Sepharose 4B (Figure 22), the individual subunits of rat liver showed minimal activities, although the 60S subunits did possess ~13% of the activity of the combination of subunits (Table 12). This activity may be due to the 60S subunits having an intrinsic capability for polyphenylalanine synthesis (Manchester and Manchester, 1980). The combination of

TABLE II. Polyphenylalanine synthesis by ribosomal subunits of rat liver isolated by gel filtration on Ultrogel GF2000 at 4°C and 20°C

Ribosomal Species	cpm/tube
a) Subunits on column at 4°C	
40S	1171
60S	1812
40S + 60S	6561
Blank	201
b) Mixture of subunits separated at 4°C	
40S	1264
60S	1249
40S + 60S	6618
Blank	201
Control Ribosomes	6957
c) Subunits on column at 20°C	
40S	1299
60S	1460
40S + 60S	6480
Blank	505
d) Mixture of subunits separated on column at 20°C	
40S	1488
60S	1533
40S + 60S	7709
Blank	505
Control Ribosomes	7395

Ribosomal subunits were concentrated by dialysis against polyethylene glycol. Fractions from subunit peaks obtained from the mixture of subunits were pooled and concentrated. 10 µg 40S and 25 µg 60S subunits were in the assay mixture which was as described in section 2.12. Incubation was at 37°C for 5 minutes.

TABLE 12 Polyphenylalanine synthesis by ribosomal subunits of rat liver isolated by gel filtration on Sepharose 4B

Ribosomal species	cpm/tube
40S	960
60S	1136
40S + 60S	8293
Blank	868
Control Ribosomes	7560

The incubation mixture for the assay of protein synthesis was as described in section 2.12. The ribosomal subunits were concentrated by dialysis against polyethylene glycol. Each tube contained 10 µg 40S and 25 µg 60S subunits as indicated.

subunits possessed an even higher activity than the control ribosomes. The 60S subunits did not appear to lose any activity due to the heat treatment to elute them from the column.

The Activity of Rabbit Reticulocyte Ribosomal Subunits

The rabbit reticulocyte 40S and 60S subunits individually showed a considerable amount of activity (Table 13). The 40S subunits possessed ~40% and the 60S subunits possessed ~50% of the activity of the combination of ribosomal subunits. This high activity was attributed to the low resolution obtained on sucrose gradients. The degraded 60S subunits combined with 40S subunits showed an activity which was comparable with the 60S subunits, indicating that the degraded 60S subunits possessed activity comparable with the native 60S subunits.

The 40S and 60S subunits seemed to lose some activity after gel filtration. The 40S subunit possessed ~25% and the 60S subunit ~40% of the activity of the combination of subunits. The mixture of ribosomal subunits of rabbit reticulocyte gel filtered on ~~Trisacryl~~ GF2000 separated into 2 peaks (Figure 18). When the pooled fractions from each peak were assayed for activity the first peak showed activity which was comparable with the activity of the combination of subunits. The second peak possessed activity which was ~60% of the activity of the combination of subunits. A combination of the two peaks exhibited an activity comparable with the combination of subunits. The high activity of both peaks was in accordance with the poor resolution of the 60S and 40S subunits.

TABLE 13 Polyphenylalanine synthesis by ribosomal subunits of rabbit reticulocyte isolated by density gradient centrifugation and by gel filtration on Trisacryl GF2000

Ribosomal Species	cpm/tube
a) Subunits isolated by density gradient centrifugation	
40S	345
60S	393
40S + 60S	448
40S + degraded 60S	634
Blank	804
b) Subunits isolated by gel filtration	
40S	2065
60S	3369
40S + 60S	8959
Blank	804
c) Mixture of subunits separated by gel filtration	
Pooled fractions from peak 1 (50 µg)	7103
Pooled fractions from peak 2 (50 µg)	5251
Peak 1 (50 µg) + Peak 2 (50 µg)	8952

Ribosomal subunits were concentrated by centrifugation or by dialysis against polyethylene glycol as described in section 2.9. The incubation mixtures were as described in section 2.12 and each tube contained 10 µg 40S subunits and 25 µg 60S subunits as indicated.

The activity of Escherichia coli ribosomal subunits

The low cross contamination of subunits of Escherichia coli separated by density gradient centrifugation (Figure 4) resulted in low protein synthesizing activities of the individual subunits (Table 14). Both the 30S and 50S subunits exhibited ~10% of the activity of a combination of the subunits. Similarly the subunits after gel filtration exhibited ~10% of the activity of the combined subunits. The slightly higher activity of the mixture of subunits after gel filtration on Trisacryl GF 2000 exhibits the gentle nature of gel filtration as opposed to gradient centrifugation. The reassociation of Escherichia coli ribosomal subunits into active ribosomes depended on the magnesium concentration. The activity of the reassociated ribosomes was dependent on the addition of added template RNA, whereas if no poly U was added, activity was minimal (results not shown).

TABLE 14 Polyphenylalanine synthesis by ribosomal subunits of *Escherichia coli* isolated by density gradient centrifugation and by gel filtration on Trisacryl GF2000

Ribosomal Species	cpm/tube
a) Subunits isolated by density gradient centrifugation	
30S	503
50S	512
30S + 50S	4411
Blank	329
b) Subunits isolated by gel filtration	
30S	495
50S	510
30S + 50S	4952
Mixture of subunits on column (100ug)	5689
Blank	329

Ribosomal subunits were concentrated by centrifugation and by ultrafiltration. Each tube contained 20 ug 30S subunits and 50 ug 50S subunits as indicated. The incubation mixture was as described in section 2.12 and incubation was for 5 minutes at 37°C.

4. DISCUSSION

4.1 ISOLATION OF RIBOSOMAL SUBUNITS BY DENSITY GRADIENT CENTRIFUGATION

Before going on to discuss the various methods of isolation of ribosomal subunits, it is important to discuss briefly the optimum conditions for separation of the subunits. The complete dissociation of ribosomes into biologically active subunits is dependent on the release of the nascent peptide chain. For this to be achieved, a stringent set of conditions is required.

Ribosomal subunits aggregate readily, particularly at low temperatures. The aggregation is in part prevented by a high concentration of monovalent cations and low concentration of magnesium. The use of 500 mM KCl and 2 mM $MgCl_2$ is a compromise between seeking to maximise dissociation without producing conditions harsh enough for the subunits to unfold and denature or be stripped of their proteins.

Rat liver ribosomal subunits are often contaminated by non-ribosomal proteins (Manchester and Rosin, 1980) and this is illustrated by the low A_{260}/A_{280} ratios. An A_{260}/A_{280} ratio of less than 1,8 suggests that the ribosomal particles are contaminated by protein (Martin et al., 1971). Ferritin is a contaminant of the 60S subunits of rat liver. Ribosomes prepared from the post-mitochondrial supernatant by precipitation with magnesium are less contaminated with ferritin (Clemens and Pain, 1974).

High salt washes of ribosomes remove non-ribosomal proteins. In addition to this, some labile ribosomal proteins are also stripped

off by KCl concentrations up to 1 molar (Clegg and Arnstein, 1970). When reticulocyte ribosomes are treated with high salt to remove non-ribosomal proteins, some ribosomal proteins are also stripped off resulting in a greater rRNA/protein ratio.

Sundkivist and Howard (1974) prepared reticulocyte ribosomal subunits using puromycin and GTP and found that ribosomal subunits obtained from this method are structurally and functionally equivalent to ribosomal subunits prepared by the "runof " method. When we used the method described by Sundkivist and Howard (1974), large amounts of polyribosomes were pelleted upon density gradient centrifugation for reasons which are uncertain. However, it is known that ribosomes from different species and different tissues have different requirements for the optimal dissociation of ribosomal subunits (Martin et al., 1971).

Puromycin was not necessary for the dissociation of the ribosomal subunits of Escherichia coli since bacterial ribosomes dissociate easily when the associated magnesium is removed. A problem encountered when preparing Escherichia coli ribosomal subunits by density gradient centrifugation is that only small amounts of the ribosomes can be loaded to achieve good resolution. Subsequent recoveries of the subunits are small in comparison to recoveries of eukaryotic ribosomal subunits. To achieve a reasonable recovery, a zonal rotor would be required, since it can accommodate large quantities of sucrose solutions and ribosomes.

A feature of the sedimentation profiles obtained after gradient centrifugation of the subunits is the asymmetric shape of the peaks. It

has been explained in the general case as being due to inversion of solvent concentrations across the leading edge of the sample zone (Hinton and Dobrota, 1976). Manchester and Rosin (1980) found that using markedly convex or concave gradients or gradients up to 50% in sucrose did not change the shape of the profiles or improve the separation.

4.2 GEL FILTRATION OF RIBOSOMAL PARTICLES ON TRISACRYL GF2000

Gel filtration is based on the principle of diffusion into and out of the stationary phase. If ideal gel filtration behaviour is apparent then solutes should have a K_d of 0-1. Trisacryl GF2000 proved to be a suitable gel for the chromatography of ribosomal subunits since it displayed apparent ideal gel filtration behaviour. In general the column efficiency obtained in gel filtration chromatography are among the lowest found in any chromatographic system, exhibiting HETP values of 2 - 10 mm as opposed to affinity chromatography or ion exchange chromatography. The efficiency of the column is dependent on column geometry, column packing, particle size, flow rate, solvent properties, temperature and sample size (Morris and Morris, 1976).

Gel filtration chromatography of ribosomal subunits on a 30 cm Trisacryl GF2000 column revealed that a separation of the subunits could not be achieved since the resolution obtained was ~0,10.
For two solutes to be completely separated from each other the resolution of Rs = 1 is required. This corresponds to a cross contamination factor of 2,3% for two solutes present in approximately equal amounts.

To achieve ~97% separation a 20 m column would have been required in this study. A 20 m column is impractical for laboratory separations and therefore some resolution had to be compromised for a more practical length of column. Calculations indicated that with a 135 cm column, a resolution of ~0,47 would be achieved (equation 4, appendix). However, in practice, the resolution achieved for rat liver ribosomal subunits at 4°C was half that. The reasons for this could be due to the large zone dispersion which is expected with long columns. Zone dispersion is decreased remarkably by a change in one of the chromatographic conditions. Increasing the temperature to room temperature showed a substantial decrease in the zone dispersion. In the case of gel filtration, resistance to mass transfer effects and longitudinal diffusion are decreased by an increase in the temperature (Morris and Morris, 1976).

After optimizing the chromatographic conditions for rat liver ribosomal subunits, various species of subunits were chromatographed on the column. The separation achieved when chromatographing a mixture of ubunits of rat liver is not significant since the cross contamination factor is substantial.

An advantage of gel filtration chromatography, as opposed to density gradient centrifugation, is that ferritin, which is a contaminant of rat liver ribosomes, is separated completely from the 40S subunit at 20°C, whereas at 4°C ferritin appears as a shoulder on the 60S subunit peak. This is an illustration of the improved resolution obtained at 20°C as opposed to 4°C and with the 135 cm column as opposed to the 30 cm column.

An anomaly of the Trisacryl GF2000 gel is the substantial retardation of puromycin. Previous work has demonstrated that the adsorption of puromycin could be due to strong hydrophobic interactions. Puromycin is an antibiotic produced by Streptomyces albonigер. Puromycin is a dibasic compound with secondary and tertiary amide groups and it is heterocyclic. Aromatic adsorption is a common problem with gel filtration media and is often observed with the aromatic amino acids. These effects can usually be abolished by suitable choice of solvents. Since in this study the retardation of puromycin did not affect the chromatographic behaviour of the ribosomal subunits, we did not dwell further on this problem.

Reticulocyte ribosomal subunits chromatographed at 20°C showed elution characteristics similar to rat liver ribosomal subunits. The resolution obtained with reticulocyte ribosomal subunits on the 135 cm column was a third of that obtained with rat liver ribosomal subunits at 20°C and as a result the two subunits were not resolved on the column. In addition the poor recovery of the 40S subunits is of concern.

As stated previously high salt treatment of ribosomes results in the selective removal of ribosomal proteins (Clegg and Arnstein, 1970). The removal of proteins results in smaller, low molecular weight ribosomal subunits. These sediment slower than the complete subunits in sucrose density gradients. Similarly in gel filtration the elution of these subunits is retarded with respect to the complete ribosomal subunits. Clegg and Arnstein (1[illegible]) found with rat liver subunits that the 60S subunits were more susceptible to degradation than the 40S subunits. Our findings confirm this, and the degraded

ribosomal subunits appear to be 60S subunits.

When ribosomal subunits of Escherichia coli were chromatographed on Trisacryl GF2000 at 4°C in a low magnesium buffer the order of elution was 50S subunits and 30S subunits. However the difference in K_d was only 0,03. Both the subunits of Escherichia coli eluted much earlier than the 40S and 60S subunits of rat liver. Their K_d values show that the subunits did not enter the stationary phase freely. In density gradients, the subunits of Escherichia coli ribosomes sediment relative to their molecular weights in the same buffers as the mobile phase in column chromatography.

In gel filtration chromatography, two factors affect the elution properties of solute molecules. These are the molecular weight of the solutes and the dimensions of the molecules. The molecular weights of the 30S and 50S ribosomal subunits of Escherichia coli are $0,7 \times 10^6$ daltons and $1,8 \times 10^6$ daltons respectively,and are substantially smaller than the molecular weights of the 40S ($M_r = 1,5 \times 10^6$) and 60S ($M_r = 2,79 \times 10^6$) subunits of rat liver. X-ray scattering analysis of the ribosomal subunits of Escherichia coli estimate the dimensions of the 30S subunits as 5,5 nm x 22 nm x 22 nm and the 50S subunits at 11,5 nm x 23 nm x 23 nm (Wittmann, 1983) and electron microscopic analysis of the ribosomal subunits of rat liver estimate the dimensions of the 40S subunits as 25 nm x 13 nm and the 60S subunits as ~24 nm x 20 nm (Bielka, 1982). Therefore, on the basis of molecular weights and molecular dimensions, the ribosomal subunits of Escherichia coli should elute retarded with respect to the ribosomal subunits of rat liver. The reasons for the bacterial subunits actually eluting earlier than the

eukaryotic subunits are uncertain.

The resolution achieved with Escherichia coli ribosomal subunits was ~0,11 and therefore the ribosomal subunits of Escherichia coli did not separate out. However the 70S tight couples in ribosomal suspensions were eluted in the void volume. The exclusion of 70S tight couples, with the dimensions of ~23 nm in diameter and M_r >2,8 x10^6. is perplexing since the large subunits of rat liver with larger dimensions and greater molecular weights are not excluded by the stationery phase.

4.3 THE ISOLATION OF RIBOSOMAL SUBUNITS BY SEPHAROSE 4B GEL FILTRATION CHROMATOGRAPHY

Separations of macromolecules and other solutes by gel filtration is determined almost exclusively by the nature of the stationary phase and only secondarily by the composition of the eluent and by the operational conditions. In this study we have observed that separation is dependent on both the nature of the stationary phase as well as the operating conditions.

Gel filtration of ribosomal subunits on Sepharose 4B results in the adsorption of the 60S ribosomal subunit of rat liver at 4°C but the adsorption is reversible with an increase in the temperature, as observed by Manchester and Manchester (1980). The adsorption of the 60S ribosomal subunit was similar to that of its component 28S rRNA (Petrovic et al., 1971; 1973; Zeichner and Stern, 1977) and to that observed with ribosomes on Sepharose 4B (Hradec and Kriz, 1978).

Experiments at various temperatures revealed that the extent of the adsorption progressively diminished as the temperature was increased. At 25°C all of the 60S subunits eluted within a single bed volume. This observation supports the idea that either a surface property of the gel was responsible for the adsorption, or that the 60S subunits had dimensions which were similar to the size of the pores within the gel matrix.

The latter hypothesis was examined using agarose gels with larger pore sizes (Sepharose 2B) and smaller pore sizes (Sepharose 6B). Ribosomal subunits of similar size to the pores in Sepharose 4B would be excluded from Sepharose 6B, and would elute within one column volume from Sepharose 2B, being able the enter the gel phase freely. However the adsorbtion property was apparent with all Sepharose gels as observed by other workers (Hradec and Kriz, 1978; Manchester and Manchester, 1980).

The similarity in behaviour of the 28S ribosomal RNA (Petrovic et al., 1971; 1973; Zeichner and Stern, 1978), the ribosomal subunits (Manchester and Manchester, 1980) and the ribosomal monomer (Hradec and Kriz, 1978; Higgins and Mazurkiewicz, 1980) suggests that these macromolecules are adsorbed to agarose based resins by a physical interaction due to the 28S rRNA and it also suggests that within the large ribosomal subunits and the ribosomal monomers, the 28S rRNA is sufficiently exposed to interact with the gel. This fact is verified by the partial tryptic digestion of ribosomal proteins (Bielka, 1982) and ribonuclease treatment of ribosomal subunits (Cox, 1969). The structural integrity of the small ribosomal subunits of rat liver is largely destroyed by tryptic digestion, whereas the sedimentation

coefficient of the large subunit is nearly identical to the native subunits (Bielka, 1982), and ribonuclease treatment of the large ribosomal subunits results in the degradation of a considerable amount of ribosomal RNA (Cox, 1969).

The nature of the physical interaction between the 28S rRNA and agarose gels is uncertain. The rRNA is totally excluded from the gel with low ionic strength buffers or by raising the temperature (Petrovic et al., 1973), whereas the ribosomal subunits elute as an irregular profile from the void volume and extend well past the total column volume when the ionic strength of the buffer is reduced and is only excluded from the gel when the temperature is increased (Manchester and Manchester, 1980). These observations suggest that ionic interactions are not responsible for the adsorption of the 60S subunits to agarose based gels. Hydrophobic interactions can be excluded since they are strengthened by a rise in temperature (Nemethy and Scheraga, 1962). Hydrogen bonds, however, play an important role in maintaining the stability of ribosomes (Peterman et al., 1972) since liver ribosomes are highly hydrated. The disruptive effects of the higher temperatures on the 60S ribosomal subunit/28S rRNA - Sepharose adsorption phenomenon parallels the effect of high temperatures on water, where hydrogen bonds are broken by an increase in temperature. Thus, the adsorption of 60S subunits may be due to hydrogen bonding.

Since the adsorption of the 60S subunit of rat liver is attributed to the position and the properties of the 28S rRNA, this observation suggests that the 28S rRNA differs from the 23S rRNA of Escherichia coli subunits. The 28S rRNA of rat liver ribosomes (Hadjiolov et

al., 1984) has several regions of high sequence homology with the 23S rRNA of Escherichia coli ribosomes (Noller, 1984). The homologous regions are those that have been conserved through the evolution of the ribosomes. The excess of nucleotides in the 28S rRNA is accounted for mainly by the presence of eight distinct G + C-rich segments of different length inserted within the regions of high sequence homology (Hadjiolov et al., 1984). The 28S rRNA is also highly G + C enriched where a high proportion of the AU base pairs in the conserved regions of the RNA molecule are substituted by G-C base pairs (Hadjiolov et al., 1984). Thus, a high proportion of the entire 28S rRNA molecule is composed of G-C base pairs or G + C-rich segments. Although the biological role of the G-C enhancement remains to be elucidated, the findings in this study suggests that it may be the G-C enhancement which could be responsible for the interaction of the 28S rRNA, 60S ribosomal subunits and 80S ribosomes with agarose based resins.

The aim of this study was to isolate ribosomal subunits by a method that would be simpler than density gradient centrifugation. This was achieved by exploiting the properties of Sepharose column (Manchester and Manchester, 1980). The advantages over gradient centrifugation is that it avoids the use of sucrose solutions and subjecting the ribosomal subunits to high centrifugal pressures. Another, and possibly more important advantage, is that it avoids repeated preparation of separation material since chromatography columns, once packed properly, can be used repeatedly. However the method described above does have two disadvantages. Firstly, it does not employ ideal gel filtration principles to effect a separation, and secondly, it is a two step procedure instead of a one step gel

filtration process. Altering the conditions of the chromatographic system does render ideal gel filtration behaviour, and ribosomal subunits of rat liver eluted within the included volume. The HETP calculated for the 60S and 40S subunits are 5,8 mm and 2,9 mm respectively. These values are 200-500 times greater than the HETP values normally found in gel filtration chromatography and are also larger than HETP values obtained with Trisacryl.

The resolution achieved on the Sepharose 4B column was calculated to be 0,06. For two solutes to be adequately separated the resolution should equal approximately one. The cross contamination factor then is 1% and t = 2,3 (defined in appendix). Thus, the number of theoretical plates required would be approximately 7000. This would require a column length of ~50 m which is unrealistic.

4.4 ANALYSIS OF RIBOSOMAL SUBUNITS BY ACRYLAMIDE-AGAROSE GEL ELECTROPHORESIS

The term electrophoresis is used to describe the migration of a charged particle under the influence of an electric field. The rate of migration of charged macromolecules depends on the size and the shape of the molecules, the charge carried, the applied current and the resistance of the medium. Acrylamide-agarose gel electrophoresis has become a useful tool for the analysis of macromolecules. This sensitive technique has revealed attributes of macromolecules not previously detectable by analytical density gradient centrifugation.

Previous reports on electrophoretic properties of ribosomes in free

solution have been summarized by Peterman (1964). It was clear from their work that ribosomes had a substantial negative charge. Hjerten et al. (1965) reported that ribosomal subunits could be electrophoretically separated in porous polyacrylamide gels.

In this study it was observed that the resolving power of the gel is directly affected by the field strength of the electric field created. The field strength is expressed as the potential gradient in volt per centimeters across the migration channel. Therefore a higher voltage increases the potential gradient across the migration channel if the length of the channel remains unaltered. A higher potential gradient results in an increase in the current which subsequently increases the heat generated. An increase in the temperature of an electrophoretic system causes an increase in the rates of diffusion of the charged particles. When we used the voltage used by Dahlberg et al. (1969), the ribosomal subunits migrated as diffuse bands because the potential gradient in our system was greater than the potential gradient in the system used by Dahlberg et al. (1969).

Tokimatsu et al. (1981) found that reducing the magnesium concentration of the gel buffer to 0,2 mM resulted in the dissociation of Escherichia coli ribosomal subunits with considerably more structure than if they were dissociated with EDTA (Bowman et al., 1971). In low magnesium buffers, three forms of 50S subunits are observed (Tokimatsu et al.,1981). The three bands represent the native subunits which are the slowest migrating (S-50S), subunits lacking four copies of ribosomal proteins L7/L12 which migrate slightly faster (M-50S), and subunits lacking proteins L7/L12 and additional

proteins which migrate the fastest (F-50S).

Isolation and characterization of the two forms of the 30S subunits observed reveal that the primary difference resides in the presence or the absence of the ribosomal protein S1 (Dahlberg, 1974; 1979). The presence of mRNA in ribosomal samples consistently yields F-30S subunits. The reason for this is that in the presence of mRNA in ribosomal preparations an mRNA-30S complex is formed (Dahlberg, 1974). This complex requires the presence of S1 (Van Duin and Kurland, 1970). The complex is dissociated when exposed to EDTA or to low magnesium ions during electrophoresis. S1 has a strong affinity for mRNA (Van Duin and Kurland, 1970) and thus dissociates from the 30S subunit and remains associated preferentially with the mRNA. The presence of mRNA in ribosomal preparations is dependent on the initial state of the bacterial cells before harvesting. Cells cooled rapidly are arrested in a state of protein synthesis and thereby ensuring that the majority of the ribosomes are associated with mRNA. In slowly cooled cells, the majority of the ribosomes are in 70S monomer form or in subunit form and therefore don't have associated mRNA. In these cells the S-30S subunit form would predominate (Dhalberg, 1974).

The high degree of resolution obtained by the electrophoretic separation of subunits makes this a good technique for analysing subunits prepared by gradient centrifugation and by gel filtration. The simple advantage over RNA analysis by SDS-polyacrylamide gel electrophoresis is that this technique requires minimal handling of the sample.

The success achieved with the resolution of bacterial ribosomes and ribosomal subunits has not been achieved with eukaryotic ribosomes and ribosomal subunits, except for yeast ribosomes (Helser et al., 1981). Electrophoretic separation of yeast ribosomes revealed that trisomes were resolved on a 2,5 % acrylamide-0,5 % agarose composite gel. Equal resolution of higher eukaryotes was not achieved owing to the high degree of non-ribosomal proteins attached to the native ribosomal subunits. These proteins result in a considerable heterogeneity of the size of ribosomal subunit which migrate on acrylamide-agarose gels as streaks rather than discrete bands. A large proportion of non-ribosomal proteins are removed from ribosomes by treatment of the ribosomes with non-ionic detergents and high salts. The method of isolating ribosomes and subunits in this study employs both treatments. However, electrophoresis of these ribosomal subunits did not afford the separation of the subunits into discrete zones.

The ribosomal subunits do migrate as discrete zones in denaturing buffering systems such as that used by Peacock and Dingman (1968). For some reason, unknown as yet, rat liver ribosomal subunits only migrate into acrylamide-agarose gels as discrete zones in denaturing buffers whereas they are unable to migrate or migrate very slowly in non-denaturing buffers. There are a number of parameters which differ in the two systems and any one of these parameters could be the reason for the ribosomal behaviour displayed.

Other workers (Lediogt et al, 1979) have found that the ribosomal subunits of Tetrahymena pyriformis, Euglena gracilis, Chlorella vulgaris and rat liver migrate as discrete zones on polyacrylamide

disc gels with acetate ion buffers. However, the ionic strength of these buffers were higher than those used in this study. Higher ionic strength buffers give slower rates of migration, but in practice produce sharper zones of separation than low ionic strength buffers.

4.5 THE ACTIVITY OF RIBOSOMAL SUBUNITS AND REASSOCIATED RIBOSOMES IN POLYPHENYLALANINE SYNTHESIS

The aim of this study was to develop and optimize a new method for the preparation of ribosomal subunits, the majority of which function in polypeptide synthesis if recombined and supplied with artificial messenger RNA (mRNA). The latter factor is a crucial component of the protein synthesizing system since the reconstituted ribosomal subunits are not functional in protein synthesis in the absence of added messenger RNA. This factor also demonstrates the effectiveness of the dissociation reaction. Conditions for the dissociation of the subunits are important for the complete release of nascent peptide chains, tRNA and mRNA (Blobel and Sabatini, 1971), since mRNA and tRNA associated with rat liver ribosomes are not released by treatment with puromycin alone, they are only released by treating with puromycin and incubating at 37°C.

Greater activities are achieved with Sephadex filtered cytosol than with unfiltered cytosol because of the presence of unlabelled amino acids in the unfiltered cytosol.

A polyphenylalanine assay of ribosomal particles reveals two things.

The first and most important is related to the activity of the ribosomal particles, and the second is the extent of cross contamination.

When assaying ribosomal fractions isolated by gel filtration of Trisacryl GF2000, an activity comparable to subunits isolated by gradient centrifugation is observed. This activity was only observed in fractions obtained from running individual subunits on the column. When assaying fractions of a mixture of ribosomal subunits from the Trisacryl GF2000 column, both the 40S and 60S fractions have substantial activities, illustrating the extent of cross contamination.

Successful separation of the ribosomal subunits is achieved by a two step gel filtration procedure on Sepharose 4B. The 40S subunit fraction displays negligible activity while the 60S subunit possesses ~13% of the activity of the combined subunits. On Sepharose 4B there is no reason to believe that the 60S subunits are contaminated with 40S subunits. However, it has been suggested that the 60S subunit has an intrinsic ability to synthesize proteins (Manchester and Manchester, 1980; Manchester and Rosin, 1980).

Rabbit reticulocyte subunits assayed in the same system as rat liver subunits did not show any substantial difference in activity as the rat liver subunits. However, the 40S and 60S subunits isolated by gradient centrifugation possess substantial activity indicating a high degree of cross contamination. The cross contamination of the 60S subunit is due to the ribosomal subunits not dissociating under the conditions described. This led to poor resolution and a low

yield of 40S subunits. The third peak isolated by gradient centrifugation did possess substantial activity. The third peak on density gradient profiles (Figure 3) consisted of ribosomal particles from which some of the proteins had been removed by salt treatment. The activity of these particles suggest that the proteins that were removed are not essential in initiating protein synthesis. Ribosomal fractions of rabbit reticulocytes isolated by gel filtration also have activities similar to fractions obtained by gradient centrifugation showing that the activities of the subunits are not affected by the method of preparation.

Gel filtered ribosomal subunits of Escherichia coli also possess an activity which is comparable to subunits isolated by gradient centrifugation.

4.6 GENERAL DISCUSSION AND CONCLUSIONS

The 40S and 60S subunits of eukaryotic ribosomes, with M_r of 1,5 x 10^6 daltons and 3,0 x 10^6 daltons, are normally separated from each other by centrifugation on sucrose gradients and are isolated by fractionation of the gradients. In an attempt to overcome some of the limitations of preparing ribosomal subunits by density gradient centrifugation, this study was undertaken to provide a rapid method for the preparation of the subunits.

The synthetic matrix, Trisacryl GF2000, that was studied as a medium for separating the subunits by gel exclusion chromatography, was described as having a separation range of 1,2 x 10^5 - 1,5 x 10^7

daltons. Theoretically, two solutes of molecular weight $3,0 \times 10^6$ daltons and $1,5 \times 10^6$ daltons would elute with a K_d of 0,33 and 0,48 respectively on this gel. A baseline separation of the two solutes should then be possible if the number of theoretical plates is not less than 800 for the peak of the 60S subunit and 400 for the 40S subunit. The maximum resolving power of this matrix was well in excess of 600-800 on the 30 cm and the 135 cm column. Under the experimental conditions used, the ribosomal subunits eluted much earlier than the calculated K_d, suggesting that the calculated molecular weight is much higher than the actual molecular weights, but ferritin with a molecular weight of 5×10^5 daltons elutes with a K_d of 1,8, approximating its molecular weight to be $5,8 \times 10^6$. Thus, we could not conclude that the ribosomal subunits exhibit anomalous apparent molecular weight under the conditions described.

Escherichia coli subunits elute much earlier than the subunits of rat liver. In gel filtration chromatography molecular weight and size affect the elution properties of solutes. A likely possibility for this behaviour on the Trisacryl GF2000 column is that the relationship of K_d to log M_r is not linear. This could be due to the high resistance to mass transfer exhibited by gel filtration columns in general and in particular the Trisacryl GF2000 column.

On Sepharose 4B, the 60S subunits show adsorption to the gel. Separation of the subunits is achieved using this adsorption property. The explanation for the binding of the 60S subunits to the gel is not entirely known. The binding is not critically dependent on the pore size, and the 28S rRNA also behaves in an anomalous manner on Sepharose gels. The large subunits of Escherichia coli

ribosomes do not behave similarly to the large subunits of mammalian ribosomes. Possibly, the extra segments in the 28S rRNA as opposed to the 23S rRNA may be interacting with the gel. Evidence has shown that the interaction is not due to ionic (Manchester and Manchester, 1980) or hydrophobic interactions. It is speculated that hydrogen bonds which are important in maintaining the stability of ribosomes and are broken by an increase in temperature may be responsible.

A comparison of the Trisacryl GF2000 and Sepharose 4B gels, indicates that the former did not exhibit the adsorption phenomenon with the 60S subunit observable with the Sepharose gels, but conversely interacted in some way with puromycin so as to give retardation. It is not known why the subunits elute as they do on Trisacryl GF2000 and Sepharose 4B. It is possible that the protein to RNA ratio, the molecular weight, and other physical factors such as column size, geometry, and temperature affect the elution position.

Acrylamide-agarose gels have opened up new avenues for the analysis of ribosomal particles without excessive pretreatment. Ribosomal subunits could be electrophoretically separated in porous polyacrylamide gels (Hjerten et al., 1965). However, the weak gels made it difficult to handle. The addition of agarose to the gels does not change the electrophoretic properties of the gel but it does provide mechanical stability.

We have found that mammalian ribosomal subunits do not migrate under conditions applicable to Escherichia coli (Dahlberg, et al., 1969) and lower eukaryotic ribosomes (Helser et al., 1981). Instead mammalian subunits migrated as discrete bands in denaturing

electrophoretic systems. One possibility for this is that in denaturing gel systems, the subunits are unfolded extensively and the loss of the tertiary structure allows easy penetration and manoeuvering in the pores of the gel, thereby allowing separation of the subunits of different sizes.

In conclusion, it can be said that ribosomal subunits of eukaryotes can be separated by gel filtration on agarose gels in two steps. In the preparation of ribosomal subunits this method is superior to the conventionally used gradient centrifugation in that chromatography is more gentle on the subunits than centrifugation. Chromatography avoids the use of sucrose solutions, it is less time consuming, and once properly packed, the column can be used repeatedly if well maintained. The synthetic gel may provide better resolution than Sepharose 4B but the behaviour of the ribosomal subunits on the gel does not make it a suitable gel for their preparation.

As an analytical technique of ribosomal subunits, acrylamide-agarose gels could only be used in denaturing gel systems for the separation of mammalian ribosomal subunits whereas lower eukaryotes and Escherichia coli ribosomal subunits could be separated in non-denaturing gel systems.

APPENDIX

The resolution of two adjacent chromatographic zones may be defined as

$$Rs = \frac{V_e}{4\sigma_v} \qquad (1)$$

where V_e represents the difference in the elution volumes taken at the zone maxima and σ_v represents the corresponding standard deviations of the peaks expressed in volume.

To obtain optimal separation (99%) of the two zones on the column, the resolution (Rs) should be approximately equal to 1. The cross contamination factor then is 1% and t = 2.3 where t is defined by the following equation:

$$y = y \exp\left(\frac{t^2}{2}\right) = \frac{1}{\sigma\sqrt{2\pi}} \cdot \exp\left[-\frac{(x - x)^2}{2\sigma^2}\right] \qquad (2)$$

where x & y are the abscissa and ordinate respectively. x is the mean value of the variable x and y is the corresponding maximum ordinate. Using the value of t from equation (2), the approximate number of theoretical plates could be calculated for 2 subunits whose elution volumes are known for any extent of cross contamination according to the following equation:

$$\sqrt{N} = \frac{t_r \cdot (Ve_1 + Ve_2)}{Ve_2 - Ve_1} \qquad (3)$$

The bed height (L_1) required to give Rs = 1 can be calculated from

$$L_1 = \frac{L}{Rs^2}$$

where Rs is the resolution obtained with a column length L.

REFERENCES

Behlke, J., Theise, H., Noll, F., and Bielka, H. (1979) FEBS Letters 106, 223-226.

Bielka, H. (1982) The Eukaryotic Ribosome, Springer-Verlag, Berlin, Heidelberg, New York.

Blobel, G. (1971) Proc. Natl. Acad. Sci. USA 68, 1881-1885.

Blobel, G. and Sabatini, D. (1971) Proc. Natl. Acad. Sci. USA. 68, 390-394.

Bowman, C.M., Dahlberg, J.E., Ikemura, T., Konisky, J. and Nomura, M. (1971) Proc. Natl. Acad. Sci. [illegible] 4-960.

Brimacombe, R., Stöffler, G. and Wittmann, H.G. (1978) Ann. Rev. Biochem. 47, 21-219.

Chan, Y.-L., Gutell, R., Noller, H.F. and Wool, I.G. (1984) J. Biol. Chem. 259, 224-230.

Chan, Y.-L., Lin, A., McNally, J., Peleg, D., Meyuhas, O. and Wool, I.G. (1987) J. Biol. Chem. 262, 1111-1115.

Chiaruttini, C., Milet, M., Hayes, D.H. and Expert-Bezancon, A. (1986) Eur. J. Biochem. 160, 363-370.

Clegg, J.C.S. and Arnstein, H.R.V. (1970) Eur. J. Biochem. 13, 149-157.

Clemens, M.J. and Pain, V.M. (1974) Biochim. Biophys. Acta 361, 345-357.

Cox, R.A. (1969) Biochem. J. 114, 753-767.

Dahlberg, A.E. (1974) J. Biol. Chem. 249, 7673-7678.

Dahlberg, A.E. (1979) Methods in Enzymol. LIX, 397-401.

Dahlberg, A.E., Dingman, C.W. and Peacock, A.C. (1969) J. Mol. Biol. 41, 139-147.

Dahlberg, A.E., Lund, E. and Kjeldgaard, N.O. (1973) J. Mol. Biol. 78, 627-636.

Darnbrough, C., Legon, S., Hunt, T. and Jackson, R.J. (1973) J. Mol. Biol. 76, 379-403.

Desai, N., and Manchester, K.L. (1985) Inter. J. Biochem. 17, 1053-1060.

Dingman, C.W. and Peacock, A.C. (1968) Biochemistry 7, 668-674.

Falvey, A.K. and Staehelin, T. (1970) J. Mol. Biol. 53, 1-19.

Geddes, R. and Stratton, G.C. (1977) Biochem. J. 163, 193-200.

Girbés, T., Cabrer, B. and Modolell, J. (1979) Methods in Enzymol. LIX, 353-361.

Goodwin, G.H. and Dhalberg, A.E. (1982) Electrophoresis of Nucleoproteins in Gel Electrophoresis of Nucleic Acids : A Practical Approach IRL Procs. (ed. Pickwood, D and Hames, B.D.) IRL Press, Oxford, Washington.

Gould, H.J., Arnstein, H.R.V. and Cox, R.A. (1966) J. Mol. Biol. 15, 600-618.

Gregory, R.J., and Zimmermann, R.A. (1986) Nucl. Acid Reser. 14, 5761-5776.

Grumunt, F., Grumunt, I. and Erdmann, V. (1974) Eur. J. Biochem. 43, 343-348.

Hadjiolov, A.A., Georgiev, O.I., Nosikov, V.V. and Yavachev, L.P. (1984) Nucl. Acid Reser. 12, 3677-3693.

Haga, J.Y., Hamilton, M.G. and Petermann, M.L. (1970) J. Cell Biol. 47, 211-214.

Hamilton, M.G., Pavlovec, A. and Peterman, M.L. (1971) Biochemistry 10, 3424-3427.

Heiser, T.L., Baan, R.A. and Dhalberg, A.E. (1981) Molec. and Cellular Biol. 1, 51-57.

Higgins, J.A. and Mazurkiewicz, J.E. (1980) Prep. Biochem. 10, 317-330.

Hinton, R. and Dobrate, M. (1976) Density Gradient Centrifugation North Holland, Amsterdam.

Hjertén, S., Jerstedt, S. and Tiselius, A. (1965) Anal. Biochem. 11, 211-218.

Hradec, J., and Kriz, O. (1978) Biochem. J. 173, 349-352.

Itoh, T., Otaka, E. and Matsui, K.A. (1985) Biochemistry 24, 7418-7423.

Kaltschmidt, E. and Wittman, H.G. (1970) Proc. Natl. Acad. Sci. USA 67, 1276-1282.

Kanai, K., Castles, J.J., Wool, I.G., Stirewalt, W.S. and Kanai, A. (1969) FEBS Letters 5, 68-72.

Kiselev, N.A., Stel'masachuk, V.K.A., Lutson, H. and Noll, F. (1978) J. Mol. Biol. 126, 109-115.

Krystosek, A., Cawthon, M.L. and Kabat, D. (1975) J. Biol, Chem. 250, 6077-6085.

Kurland, C.G. (1977) Ann. Rev. Biochem. 46, 173-200.

Kuwano, Y., Nakanishi, O., Nabeshima, Y., Tanaka, T. and Ogata, K. (1985) J. Biochem. 97, 983-992.

Lake, J.A. (1977) Proc. Natl. Acad. Sci. USA 74, 1903-1907.

Lake, J.A. (1985) Ann. Rev. Biochem. 54, 507-530.

Lamfrom, H. and Glowacki, E.R. (1962) J. Mol. Biol. 5, 97-108.

Lawford, G.R. (1969) Biochem. Biophys. Res. Comm. 37, 143-149.

Ledoigt, G., Curgy, J.J., Stevens, B.J. and André, J. (1975) Biochim. Biophys. Acta 407, 222-239.

Ledoigt, G., Stevens, B.J., Curgy, J.J. and André, J. (1979) Exp. Cell Res. 119, 221-229.

Lin, A., Chan, Y.-L., Jones, R. and Wool, I.R. (1987) J. Biol. Chem. 262, 14343-14351.

Lin, A., McNally, J. and Wool, I.G. (1983) J. Biol. Chem. 258, 10664-10671.

Lin, A., McNally J. and Wool, I.G. (1984) J. Biol. Chem. 259, 487-490.

Lin, A., Wittmann-Liebold, B., McNally, J. and Wool, I.G. (1982) J. Biol. Chem. 257, 9189-9197.

Lill, R., Robertson, J.M. and Wintermeyer, W. (1986) Biochemistry 25, 3245-3255.

Lutsch, G., Noll, F., Theise, H., Enzmann, G. and Bielka, H. (1979) Molec. Gen. Genet. 176, 281-291.

Manchester, K.L. (1984) Biochim. Biophys. Acta 781, 279-285.

Manchester, K.L. and Alford, T. (1979) Biochim. Biophys. Acta 563, 155-162.

Manchester, K.L. and Manchester, J.E. (1980) FEBS Letters 110, 177-180.

Manchester, K.L. and Rosin, I. (1980) S.Afr. J. Science 76, 126-128.

Martin, T.E., Rolleston, F.S., Low, R.B. and Wool, I.G. (1969) J. Mol. Biol. 43, 135-149.

Martin, T.E., Wool, I.G. and Castles, J.J. (1971) Methods in Enzymol. 20, 417-429.

Metspalu, A., SaarMa, M., Villems, R., Ustav, M. and Lind, A. (1978) Eur. J. Biochem. 91, 73-81.

Moldave, K. (1985) Ann. Rev. Biochem. 54, 1109-1149.

Morris, C.J. and Morris, P. (1976) Separation Methods in Biochemistry, Pitman Publishers.

Nag, B., Tewari, D.S., Etchison, J.R., Sommer, A. and Traut, R.R. (1986) J. Biol. Chem. 216, 13892-13897.

Nakanishi, O., Oyanagi, M., Kuwano, Y., Tanaka, T., Nakayama, T., Mitsui, H., Nabeshima, Y. and Ogata, K. (1985) Gene (Amst) 35, 289-296.

Nemethy, G. and Scheraga, H.A. (1962) J. Phys. Chem. 66, 1773-1780.

Nitta, N., Kuge, O., Yui, S., Negishi, K. and Hayatsu, H. (1986) Eur. J. Biochem. 157, 427-432.

Nolan, R.D. and Arnstein, H.R.V. (1969) Eur. J. Biochem. 9, 445-450.

Noll, F., Bommer, U.-A., Lutsch, G. and Bielka, H. (1978) FEBS Letters 87, 129-131.

Noller, H.F. (1984) Ann. Rev. Biochem. 53, 119-162.

Nygard, O. and Westermann, P. (1982) Biochim. Biophys. Acta 697, 263-269.

Peacock, A.C. and Dingman, C.W. (1967) Biochemistry 6, 1818-1824.

Peterman, M.I. (1964) The Physical and Chemical Properties of Ribosomes, Elsevier Publishing Co. Amsterdam, London, New York.

Petermann, M.L. and Pavlovec, A. (1971) Biochemistry 10, 2770-2774.

Petermann, M.L., Pavlovec, A. and Hamilton, M.G. (1972) Biochemistry 11, 3925-3932.

Petrovic, S., Novakovic, M. and Petrovic, J. (1971) Biochim. Biophys. Acta 254, 493-495.

Petrovic, S.L., Petrovic, J.S. and Novakovic, M.B. (1973) Biochim. Biophys. Acta 308, 317-323.

Politz, S.M. and Glitz, D.G. (1977) Proc. Natl. Acad. Sci. USA 74, 1468-1472.

Povey, N. and Holm. R.A. (1970) J. Chromat. 46, 33-39.

Raymond, S. (1962) Clin. Chim. Acta 8, 455-467.

Reboud, A.-M., Buisson, M. and Reboud, J.-P. (1972) Eur. J. Biochem. 26, 354-359.

Sundkivist, I.C. and Howard, G.A. (1974) FEBS Letters 41, 287-291.

Swiderski, R.E., Johnson, S.A., Larkins, B.A. and Graham, D.E. (1979) Nucl. Acid Reser. 6, 3685-3701.

Szer, W. and Leffler, S. (1974) Proc. Natl. Acad. Sci. USA 71, 3611-3615.

Talens, A., van Diggelen, O.P., Brongers, M., Popa, L.M. and Bosch, L. (1973) Eur. J. Biochem. 37, 121-133.

Tai, P.C., Wallace, B.J., Herzog, E.L. and Davis, B.D. (1973) Biochemistry 12, 609-615.

Tanaka, T., Kuwano, Y., Ishikawa, K. and Ogata, K. (1985) J. Biol. Chem. 260, 6329-6333.

Tanaka, T., Kuwano, Y., Kuzumaki, T., Ishikawa, K. and Ogata, K. (1987) Eur. J. Biochem. 162, 45-48.

Tanaka, T., Wakasugi, K., Kuwano, Y., Ishikawa, K. and Ogata, K. (1986) Eur. J. Biochem. 154, 523-527.

Terao, K. and Ogata, K. (1970) Biochim. Biophys. Res. Comm. 38, 80-85.

Terao, K. and Ogata, K. (1972) Biochim. Biophys. Acta 285, 473-482.

Terao, K. and Ogata, K., (1975) Biochim. Biophys. Acta 102, 214-229.

Terao, K., Takahashi, Y. and Ogata, K. (1975) Biochem. Biophys. Acta 402, 230-377.

Tissières, A., Watson, J.D., Schlessinger, H. and Hollingworth, B.R. (1959) J. Mol. Biol. 1, 221-223.

Tokimatsu, H., Strycharz, W.A. and Dahlberg, A.E. (1981) J. Mol. Biol. 152, 397-412.

Ulbrich, N. and Wool, I.G. (1978) J. Biol. Chem. 253, 9049-9052.

Van Duin, J. and Kurland, C.G. (1970) Mol. Gen. Genet. 109, 169-176.

von der Decken, A. (1973) Eur. J. Biochem. 33, 475-480.

Westermann, P., Hewmann, W., Bommer., U.A., Bielka, H., Nygard, O. and Hultin, T. (1979) FEBS Letters 97, 101-104.

Wettenhall, R.E., Wool, I.G. and Sherton, C.C. (1973) Biochemistry 12, 2403-2411.

Wittman, H.G. (1976) Eur. J. Biochem. 61, 1-13.

Wittman, H.G. (1983) Ann. Rev. Biochem. 52, 35-65.

Wool, I.R. (1979) Ann. Rev. Biochem. 48, 719-754.

Zeichner, M. and Stern, R. (1977) Biochemistry 16, 1378-1382.