Characterisation of ribosomes from the thermoacidophilic archaeon *Sulfolobus acidocaldarius*

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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Dedicated to
MY PARENTS

CONTENTS

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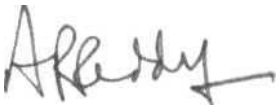
Certificate

This **is** to certify that the thesis entitled **Characterisation of ribosomes from the thermoacidophilic archaeon *Sulfolobus acidocaldarius*** is based on the results of the work done by **Mr Rampalli Srinivas** for the degree of **Doctor of Philosophy** under my supervision. This work has not been submitted for any degree or diploma of any University or Institution.



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DECLARATION

I hereby declare that the work presented in the present thesis entitled, **Characterisation of ribosomes from the thermoacidophilic archaeon** *Sulfolobus* ***acidocaldarius***, is entirely original and was carried out by me under the guidance of **Prof. T. Suryanarayana,** Department of Biochemistry, University of Hyderabad, Hyderabad, INDIA. I also declare that this has not been submitted before for the award of degree or diploma from any University or Institution.

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Rampalli Srinivas

ABBREVIATIONS

Da	daltons
DNase	Deoxyribonuclease
DEAE-	Diethylaminoethyl-
DTT	Dithiothreitol
EDTA	Ethylenedi amine tetra acetate
EF-	Elongation factor-
g	centrifugal field
hr	hours
IgG	Immunoglobulin G
KDa	kilodaltons
min	minutes
NP-40	Nonidet P-40
nm	nano meters
O. D	optical density
poly (U)	poly ribouridylic acid
RPM	revolutions per minute
RNP	ribonucleoprotein

r-proteins	ribosomal proteins
RNase	Ribonuclease
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyaciylamide gel electrophoresis
Tris	Tris (hydroxymethyl) amino methane
TEMED	N, N, N', N' - tetramethylethylene-diamine
UV	Ultra violet

CHEMICALS

Tris, Bis-tris, DNAse-I, Bovine serum albumin, NP-40, Novobiocin, Lithium Chloride, Spermine, Sucrose, Alumina, Agarose and Coomassie blue R-250 were purchased from Sigma chemical company, U.S.A. Acryiamidc, N-N-methylene bis-acrylamide and SDS were purchased from Serva Company, Germany.

DEAE-cellulose (DE-52) was from Whatman, England. Sephacryl S-200, Sepharose 4B and Sephadex G-50 were procured from Pharmacia Fine Chemicals, Sweden. Nitrocellulose paper was obtained from Schleicher and Schull, Germany. Anti rabbit IgG (goat) coupled peroxidase and 4-chloro-1-naphthol were from Pierce, U.S.A. Casaminoacids, Bactorryptone, Yeast extract, complete and incomplete Freund's adjuvant were purchased from Difco laboratories, U.S.A. DTT was obtained from Boehringer Mannheim, Germany. Triton X-100 was from Fluka laboratories, Switzerland.

TEMED, 2-mercaptoethanol and ammonium sulphate were obtained from Sisco Research laboratories, Bombay, India. Folin-Ciocalteau reagent was from Spectrochem Pvt. Ltd, India. All other chemicals used in this study were of analytical grade, available locally.

Chapter-1
INTRODUCTION

INTRODUCTION

Archaebacteria have been grouped as a third primary kingdom of cells in addition to eukaryotes and bacteria based on the ribosomal RNA sequence homologies and lipid composition (Woese & Fox, 1977) (fig. 1). Furthermore, they possess or lack typical biochemical markers of both prokaryotes and eukaryotes and have unique properties not found elsewhere (Woese *et al.,* 1978, 1983; 1990). None of these three groups appears to be more ancient than either of the other two from an evolutionary point of view.

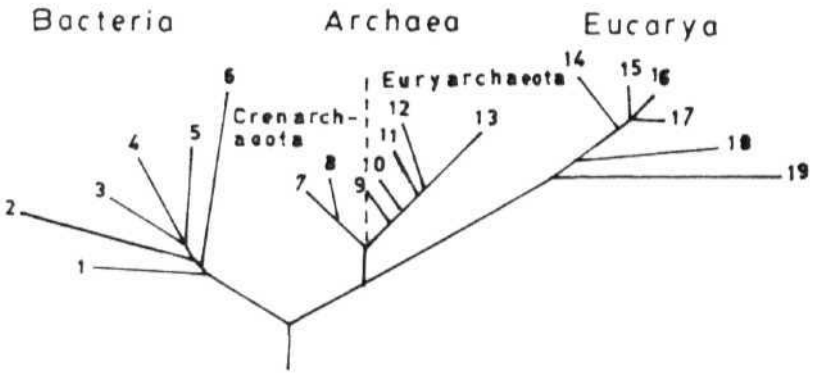
This classification in turn served to highlight rRNAs as excellent molecular chronometers (Fox *et al.,* 1980). Not only are rRNAs present in all organisms, but even their primary function within the translation has remained essentially unchanged.

From 1991, the term archaea was adopted to avoid the confusion that archaebacteria and bacteria are closely related (Garrett *et al.*. 1991). Archaea tend to be found in extreme habitats which normally prove fatal to the rest of the organisms such as high salinities, acidic pH and high temperatures. These conditions are probably similar to those that prevailed at the time when life first originated on earth (Ernst, 1983; Walker *et al.,* 1983). Archaea exhibit a diverse array of morphological forms comprising of both aerobic and anaerobic forms.

Diversity in the archaeal kingdom:

Archaea exhibit considerable phylogenetic diversity despite their restricted range of phenotypes. These unusual organisms can be conveniently divided according to their extreme environmental niches, into three broad phenotypes.

Fig. 1: Universal phylogenetic tree in rooted form, showing the three domains. Branching order and branch lengths are based upon rRNA sequence comparisons. The position of the root was determined by comparing sequence of pairs of paralogous genes that diverged from each other before the three primary lineages emerged from their common ancestral condition. The numbers on the branch tips correspond to the following groups of organisms. *Bacteria:* 1, the Thermotogales; 2, the flavobacteria and relatives; 3, the cyanobacteria; 4, the purple bacteria; 5, the Gram-positive bacteria; and 6, the green non-sulfur bacteria. *Archaea:* the kingdom *Crenarchaeota*: 7, the genus *Pyrodictium*; and 8, the genus *Thermoproteus*; and the kingdom *Euryarchaeota*: 9, the Thermococcales; 10, the Methanococcales; 11, the Methanobacteriales; 12, the Methanomicrobiales; and 13, the extreme halophiles. *Eukarya:* 14, the animals; 15, the ciliates; 16, the green plants; 17, the fungi; 18, the flagellates; and 19, the Microsporidia.
(The diagram was taken from Woese *et al.*, 1990).


Bacteria
Archaea
Eucarya
Crenarch-
aeota
Euryarchaeota
1
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3
4
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Figure.1

(a) Methanogens: Methanogens are a group of obligate anaerobes that reduce carbon dioxide to methanol. The biology of methane production involves a unique set of co-enzymes that appear to be confined only to these organisms.

(b) Halophiles: These are capable of growing in medium containing 2.5-5.2 M brine. Their purple membranes with their bacterio-rhodopsin have provided some of the most significant insights into the mechanisms of biological energy transductions.

(c) Sulphur dependent thermoacidophiles: These organisms include both aerobic and anaerobic species utilising inorganic forms of sulphur. Some of them are capable of aerobic chemolithoautotrophic metabolisms using H_2, CO_2 and elemental sulphur. In this group, many though not all, are thermoacidophiles (Stetter and Zillig, 1985; Yang *et al.*, 1985).

These organisms are infact closer to each other within the group rather than with any other organism from either of the two groups Some of their biochemical properties are unique, while others are shared with bacteria on one hand and with eukaryotes on the other (Fewson, 1986) (**Table-1**).

Within the archaeal kingdom there appears a clear and deep evolutionary division between the sulphur-dependent thermoacidophiles and the other two archaeal families, the methanogens and the halophiles (Pace *et a!.*, 1986; Woese and Olsen, 1986).

Biochemistry and molecular biology of archaea:

Archaea exhibit a great diversity in cell envelopes also (Kandler and Konig, 1985; Konig *et al.*, 1982). Neither muramic acid nor D-amino acids, which are typical components of bacterial cell walls, have been detected in any archaeon till today. Pseudomurein is a particularly well charactensed component of archaeal

Table- 1

Properties of **archaea**

Unique to archaea	**Similar to eukarya**	**Similar to bacteria**
Many 16S rRNA sequences	Initiator tRNA is methionine	Prokaryotic cellular organisation
Pseudomurein	RNA Polymerase subunit structure	70S ribosomes containing 16S, 23S and 5S rRNAs
Ether linked lipids with branched aliphatic chains	Elongation factor EF-2 sensitive to diphtheria toxin	Ribosomal RNA gene organisation
Certain structural features of 5S rRNA and tRNAs	Ribosomal "A" protein amino acid sequence	Occurence *in vivo* of restriction/modification and restriction endonucleases
Reverse gyrase	Introns in some tRNA and rRNA genes	
	CCA termini of tRNAs not coded in tRNA genes	
	DNA polymerase sensitive to aphidicolin	
	RNA polymerase insensitive to rifampicin and streptolydigin	
	Sensitivity to protein synthesis inhibitors, e.g anisomycin	
	Hybrid ribosomes with eukaryotic subunits	
	EF-1α containing 11 amino acid segment in thennophilic archaea	
	TATA-binding protein in the thennophilic archaeon *Pyrococcus woesei*	
	Presence of a molecular chaperone in *S. shibitae* related to the t-complex polypeptide-1	

envelopes, which is different from bacterial envelopes which contain murein (Kandler, 1982).

Archaea differ from bacterial and eukaryal cells in a variety of molecular respects. Unlike the straight chain fatty acids and fatly acid ester glycerol lipids that are characteristic of bacteria and eukarya, archaeal lipids are distinguished by having isoprenoid and hydro isoprenoid hydrocarbons and isopranyl glycerol ether lipids in their cell walls (Langworthy, 1985; De Rosa *et al.,* 1986). The archaeal lipids are distinct also in possessing a glycerol moiety having the 2,3, sn stereo configuration rather than the common 1,3 sn configuration The lipids can also serve as convenient chemical markers for the identification of archaea for establishing taxonomic relationships and to provide insights into the geochemical processes that could have predominated in the earliest episodes of the earth's history.

The presence of tetra-ethers in archaea allows the formation of membranes that in effect are lipid monolayers rather than bilayers. These monolayers are more heat stable than bilayers which might tend to melt apart (Langworthy *et al.,* 1982; Langworthy, 1985). Ether linkages in membranes are very resistant to acid or base hydrolysis, which presumably protect the organisms against hostile environments. Membrane fluidity at high temperatures encountered by some of the archaea, can apparently be adjusted by the presence of cyclopentanyl rings in terra-ethers (De Rosa *et al.,* 1980; 1986).

A ribosomal protein L12 from *Methanococcus* was overexpressed in *E. coli* and has been reported to be incorporated in *Halobacterium* 50S subunits yielding active ribosomes (Kopke *et al.,* 1990). Many molecular properties of sulphur-dependent thermoacidophiles are closer to eukaryotes than to other archaea and bacteria. For example, the sulphur-dependent archaeal 5S rRNA, like

eukaryotes, have triphosphorylated termini, in contrast to methanogens and halophiles which like the bacteria have 5' terminal monophosphate. The secondary structure of 5S RNA from *S. acidocaldarius* and *Thermoplasma* also resembles eukaryotes but differs more from other archaeal or bacterial patterns than does even eukaryotic 5S RNA (Fox *et al,* 1982). On the other hand, the t-RNA genes have been found in the spacers between the 16S and 23S rRNA genes of methanogens (Ostergard *et al,* 1987; Jarsch and Bock, 1983) and extreme halophiles (Leffers *et al,* 1987; Mankin and Kagramanova, 1986) but not in extremely thermophilic sulphur-dependent archaea (Leffers *et al,* 1987; Larson *et al,* 1986; Kjems *et al,* 1987). Significant amounts of long polyadenylated sequences are found in *S. acidocaldarius* mRNA that are similar to those in eukaryotic mRNAs (Ohba and Oshima, 1982).

Eukaryotic and sulphur-dependent archaeal rRNAs are highly modified (although the sites of modification differ), while the rRNAs of bacteria and the other archaea have much lower levels of modifications (Woese *et al,* 1984) The linkage of rRNA genes differs between sulphur-dependent organisms and methanogenic and halophilic archaea. As in bacteria, a close linkage of the 16S, 23S and 5S rRNA genes appears to be the rule in the latter two groups of archaea In the sulphur-dependent organisms, the 5S rRNA genes are unlinked to rRNA genes atleast in the case of *Thermoproteus* (Kjems *et al,* 1987), *Desulfurococcus* (Larsen *et al.*, 1986) and *Sulfolobus* (Reiter *et al.,* 1987).

Archaeal t-RNAs contain a great variety of modified bases including thiolated nucleosides, which are much more extensive in the sulphur-dependent organisms than in halophiles or methanogens (Gupta and Woese, 1980). All the archaeal RNA polymerases are insensitive to rifampicin (which inhibits initiation in bacteria), streptolydigin (which inhibits elongation in bacteria) and to a-

amanitin Silybin stimulates transcription mediated by RNA polymerase isolated from thermoacidophilic archaea and eukaryotic RNA polymerase I (Gropp *et al.*, 1986) RNA polymerases purified from a variety of archaea show features such as subunit composition, immunological relationships and antibiotic sensitivities like eukaryotic RNA polymerases (Schnable *et al.*, 1983). The division in the archaeal kingdom is further exemplified by the shape in ribosome and r-protein content between the two groups (Cammarano *et al.*, 1986), which led Lake to propose that the acidothermophilic bacteria can be grouped separately as another kingdom, the *eocytes* (Lake *et al.*, 1984).

Presence of introns in the genes encoding r-RNA and t-RNA has also been observed in archaea (Kaine *et al.*, 1983; Kjems and Garrett 1985, 1988, 1991). Examples include the small introns in t-RNA ft*") and t-RNA $^{(Ser)}$ of *Sulfolobus*. A 105 base pair intron in t-RNA $^{(Trp)}$ of *Halobacterium* and 622 base pair intron in the 23S r-RNA of *Desulforococcus* (Daniels *et al.*, 1985) were also detected. In all of these cases, the processing sites occur at positions analogous to the locations of processing sites in eukaryotes. In addition to the presence of introns, archaeal t-RNAs have many other eukaryotic characteristics. For example, the minor base 1-methyl adenine is common to eukaryotic and archaeal t-RNAs but not to bacterial t-RNAs (Gupta, 1985). Likewise, the initiator t-RNA is methionyl t-RNA and not N-formyl methionyl t-RNA (Bayley and Morton, 1978). As observed in the case of eukaryotic genes, most of the sequenced t-RNA genes in archaea lack the 3^1 CCA end (Wich *et al.*, 1984).

EF-2 is the only archaeal protein that is a substrate of fragment A of Diphtheria toxin and undergoes ADP-ribosylation It shares this property with the eukaryotic analogue, but not with the bacterial one (Pappenheimer *et al.*, 1983;

Kessel and Klink, 1980). The relative affinities of archaeal EF-1 for guanine nucleotides range near to the values for eukaryotic factors (Klink, 1985).

Recently it has been shown that the eukaryotic elongation factor EF-1a contained an 11-amino acid segment that was also found only in the thermoacidophilic archaea but not in bacteria (Rivera and Lake, 1992). A molecular chaperone from thermophilic archaeon *Sulfolobus shibitae* was shown to be related to the eukaryotic protein t-complex polypeptide-1 (Trent *et al.*, 1991). The transcription factor, TATA-binding protein has been reported to be a general transcription factor both in eukaryotes and thermophilic archaeon *Pyrococcus woesei* (Rowlands *et al.*, 1994). Pisani and Rossi, (1994) have characterised eukaryotic α-like Polymerase from *Sulfolobus solfataricus*. Sandman *et al.* (1990) have isolated and characterised a DNA binidng protein from the hyperthermophile *Methanothermus fenidus* that is most closely related to histones.

These findings have reinforced the close relation between archaea, particularly thermoacidophilic archaea and eukaryotes.

Bacterial Ribosomes:

Ribosomes are large ribonucleoprotein particles that are responsible for translation of the genetic code (Hardesty and Kramer, 1985; Chambliss *et al.*, 1980). As emphasised by Woese, their biological role links genotype with the phenotype and therefore the evolutionary origin of ribosomes are closely tied to the origin of life. A deeper understanding of the structure and function of ribosomes is therefore likely to lead not only to a description of the complex interplay of events in the protein synthesis, but also to have important influence on the theories of molecular evolution. As a complex RNP, the ribosome serves as a prototype for the study of an increasing number of newly discovered functional

RNPs such as spliceosomes (Brody and Abelson, 1985), signal recognition particles (Walter and Blobel, 1982), ribonuclease P (Guerner *et al*, 1983) and telomerase (Greider and Blackburn, 1987).

Extensive information on structural and functional aspects of both prokaryotic (*E. coli)* and eukaryotic (rat liver) ribosomes is now available based on the various experimental approaches (Monographs edited by Nomura *et al*, 1974; Hardesty and Kramer, 1986; Hill *et al.,* 1990; Nierhaus *et al.,* 1993).

Several experimental approaches such as chemical cross-linking (protein-protein; protein-RNA), affinity labelling with ribosome interacting molecules, chemical probing, chemical modification reagents, accessibility of r-proteins to proteolytic enzymes, genetic studies with mutant ribosomal proteins, electron microscopy, immunoelctron microscopy, neutron and small angle X-ray scattering and crystallography were made to understand the structure of the ribosome. The results of such experiments have been used to construct rather detailed models of the small (30S) subunit (Brimacombe *et al,* 1988; Stern *et al,* 1989; Capel *et al,* 1987, 1988; Schuler and Brimacombe, 1988) and preliminary models of the large (50S) subunit (Walleczek *et al,* 1988; 1989; Brimacombe *et al,* 1990). Some of the important results on ribosome, related to the present work are given in the following pages.

The *in vitro* reconstitution assays of 30S and 50S ribosomal subunits (Traub and Nomura, 1968; Nomura and Erdmann, 1970) have led to the discovery that the information required for the assembly of this complex organelles is entirely contained in the structures of the RNA and protein components. The *in vitro* assembly reaction essentially resembles the *in vivo* process The sequence of addition of purified r-proteins to 16S RNA, in reconstitution experiments led to the conclusion that S4, S7, S8, S13, S15, S17 and S20 of *E. coli* 30S subunits are

individually and specifically are able to bind to 16S RNA (Mizushima and Nomura, 1970; Held *et al.,* 1974). Moreover, the assembly of the 30S subunit from *E. coli* ribosomes occurs via two assembly domains, which are initiated by S4 and S7 (Nowotny and Nierhaus, 1988). In a similar fashion L1, L2, L3, L4, L6, L13, L16, L20, L23 and L24 of the *E. coli* 50S subunit have each been found to interact directly and specifically with 23S RNA (Stoffler *et al.,* 1971a & b; Harold and Nierhaus, 1987). Apart from these, L5, L18 and L25 of the *E. coli* 50S subunit, bind individually to 5S RNA (Gray *et al,* 1973).

The six ribosomal proteins S4, S7, S8, S15, S16 and S17 are necessary and sufficient for the 16S RNA to acquire a compactness similar to that within the 30S particle. The general conclusion is that the overall specific folding of the 16S RNA is governed and maintained by its own intramolecular interactions but the additional folding up or the stabilisation of the final compactness requires some ribosomal proteins (Serdyuk *et al.,* 1983). The early events in the assembly of *E. coli* 30S and 50S ribosomal subunits can be best visualised by scanning transmission electron microscopy and circular dichroism. These approaches made it possible to quantitatively analyse conformational changes induced in the rRNA by the binding of primary rRNA binding proteins This is evident from the electron micrographs and from the decrease in R_G values. These results provide direct evidence that 16S and 23S rRNA undergo significant structural re-organisations during the ribosomal subunit assembly process (Mandiyan *et al.,* 1989; Tumminia *et al.,* 1994).

A detailed pathway for *in vitro* assembly called the 30S assembly map was then elucidated using a stepwise reconstitution with purified r-proteins. The assembly map describes a complex set of inter-dependencies among r-proteins during assembly of 30S and 50S subunits. The proteins which interact with the

RNA (16S, 23S and 5S) specifically and independently are referred to as primary RNA binding proteins. Other proteins, which otherwise show no specific affinity for 16S, 23S and 5S RNA, are capable of assembly contingent in the presence of one or more primary RNA binding proteins. These are called secondary rRNA binding proteins. For example, the presence of primary rRNA binding protein S7 is both necessary and sufficient for the assembly of the secondary' rRNA binding proteins S9 and S19. Most of the structural alterations in rRNA are brought about by both primary and secondary binding proteins suggesting that the primary and secondary rRNA binding proteins make extensive interactions with rRNA. Assembly of the remaining proteins (tertiary binding proteins) requires the presence of one or more secondary binding proteins and sometimes other tertiary proteins such as S21 (Stern *et al.*, 1989; Stoffler-Meilicke and Stoffler, 1987)

One of the very early methods used to know the topographical arrangement of r-proteins is salt stripping. On incubation at a high salt concentration and a stepwise increase in ionic strength (Itoh *et al.*, 1968), groups of proteins sequentially split from the particles resulting in the formation of a series of protein deficient derivatives, i.e., a stepwise disassembly of ribosomal particles occurs. Stepwise disassembly of proteins from the *E. call* 30S subunits occur with increasing concentration of LiCl. Finally, at the highest LiCl concentration (3.5 M) the residual core particle contains just four ribosomal RNA binding proteins, S4, S7, S8 and S15 whose dissociation from RNA requires more drastic treatment e.g., a combination of high salt and urea Stripping of the *E. coli* 50S ribosomal subunit proceeds in a similar fashion (Serdyuk *et al.*, 1984).

Treatment of yeast 60S ribosomal subunits with 0.5 M LiCl was found to remove all but six of the ribosomal proteins. These core proteins were split off sequentially with increasing in LiCl concentration from 0.5 M to 1 M. The

susceptibility of ribosomal proteins to removal by LiCI corresponds quite well with their order of assembly as determined by Kruiswijk *et al.* (1978) and El-Baradi *et al.* (1984).

Primary and secondary rRNA binding proteins of the large subunit of the bovine mitochondrial ribosomes were studied using four different approaches. Binding of radio labelled RNA to western blotted proteins, disassembly of the intact ribosomal subunits with urea, binding of r-proteins to RNA in the presence of urea and binding of proteins extracted with LiCI to RNA (Piatyszek *et al.*, 1988). In contrast to results with *E. coli* ribosomes several mitoribosomal proteins remain core associated indicating a different structural organisation in these ribosomes (Scheiber and O'Brien, 1982).

The analysis of partially assembled and disassembled particles has allowed to dilineate a preliminary pattern of the topographical hierarchy of the *Sulfolobus* large subunit proteins (Altamura *et al.*, 1991). In a similar fashion, by varying the ionic concentrations, the primary rRNA binding proteins of *H. cutirubrum* were identified (Strom *et al.*, 1975).

The structural comparison of core particles with intact ribosomal subunits help to identify positions of several proteins on the ribosome. A detailed three-dimensional structural analysis of *E. coli* 50S subunits depleted of proteins L7/L12, along with its comparison to non-depleted subunits provided information that these proteins are responsible for the appearance of the elongate protuberance on the 50S subunit that has been termed the stalk. The stalk-like protuberances were found to be absent in the depleted subunit (Carazo *et al.*, 1988).

The large ribosomal subunit of the thermophilic fungus *Thermomyces lanuginosus* was treated with 2.96 M NH_4Cl to remove specific complements of the ribosomal proteins and the core particles thereby derived were visualised by

bright field transmission electron microscopy. By such studies a new characteristic projection was elucidated which showed a large depression or channel passing through the subunit. Such a channel has been perceived in the prokaryotic large ribosomal subunit under certain conditions and has been postulated to be the exit pathway for the nascent polypeptide chain (Harauz *et al,* 1994).

Incubation of 50S subunit of *E. call* with 4.2 M LiCl leads to 4.2c cores and the complementary split protein fractions sp 4.2, the latter containing quantitatively L24. Partial and total reconstitution experiments performed in the absence and presence of L24 demonstrates the crucial role of L24 in the early stages of assembly. However, this protein is dispensable for the subsequent steps of *in vitro* assembly and the *in vitro* translations (Spillman and Nierhaus, 1978). By using disassembly and *in vitro* reconstitution experiments the ribosomal components from *E. coli* 50S subunits involved in the reconstitution of peptidyl transferase activity were identified (Hampl *et al,* 1981).

The arrangement of r-proteins in relation to RNA were identified by an affinity chromatographic procedure which involves the covalent coupling of agarose to rRNA/tRNA (through an adipic acid dihydrazide spacer) to identify specific r-proteins which interact with rRNA. These bound proteins are later analysed by 2-D PAGE. Two ribosomal proteins L18 and L25 were identified to bind tightly to *E. coli* 5S RNA by this procedure (Burrell and Horowitz, 1977). 5S RNA, 5.8S RNA and t-RNA binding proteins from rat liver were identified by coupling 5S RNA, 5.8S RNA and t-RNA to sepharose (Metspalu *et al,* 1978). The proteins of the 40S subunit of rat liver ribosome did not bind to the 5S rRNA affinity column, nor did the proteins of either the large or small sub-particle of *E. coli* ribosomes (Ulbrich and Wool, 1978).

RNA-protein cross-linking has also been widely employed as a tool to elucidate rRNA-r-protein interactions (Budowsky and Abdurashidova, 1989; Brimacombe *et al,* 1988b). However, the most widely used method for inducing RNA-protein cross-linking is by direct irradiation with UV light. This has the advantage that it is a zero length cross-linking technique in contrast to the chemical methods (Maly *et al,* 1980; Stiege *et al.,* 1986; Brimacombe *et al.,* 1988), where the reagents involved are rather long. The precise analysis of intra-RNA cross-links generated *in situ* by UV irradiation is the most direct method for obtaining information about the topographical folding of rRNA in the ribosome, the interaction of rRNA with r-proteins, the t-RNA binding domain in 23S RNA, the arrangement of mRNA relative to t-RNA, the path of mRNA through the ribosome and several other functionally important sites (Stiege *et al,* 1983; 1986; Brimacombe and Stiege, 1985; Mitchell *et al,* 1990; Lim *et al,* 1992).

In another study, formaldehyde has been used to cross-link proteins to RNA within the 30S and 50S subunits (Moller *et al,* 1977). While formaldehyde is not an ideal reagent for this purpose as the cross-linking reaction is readily reversible, by using this, it was possible to localise partially a number of cross-linking points on the RNA.

In certain instances, r-proteins were blotted on to nitro-cellulose membranes and assayed for their binding affinity to 16S RNA. The same method was applied to determine protein-RNA interactions in spinach Chloroplast 30S ribosomal subunits, where, a set of only seven proteins was bound to Chloroplast rRNA in stringent conditions. They were identified to be S6, S10, S11. S14, S15, S17 and S22. These proteins also bind to *E. coli* 16S rRNA. The core particles obtained after treatment by LiCl of Chloroplast 30S ribosomal subunit contained three proteins (S6, S10 and S14) which are included in the set of seven binding proteins.

This set of proteins probably play a part in the early steps of the assembly of the Chloroplast 30S ribosomal subunit (Rozier and Mache, 1984).

Heterologous reconstitution:

E. coli r-proteins can associate with a variety of 5S, 16S and 23S RNA molecules. These results establish the existence of common functional regions in rRNA from prokaryotes and eukaryotes and such associations have been highly conserved (Thurlow and Zimmerman, 1978; Zimmerman *et al.*, 1980; Noller, 1984; 1991). Functionally active hybrid 30S ribosomes can be assembled *in vitro* from 16S RNA of one bacterial species *(B. stearothermophilus)* and ribosomal protein components of another distantly related species *(E. coli)* or of the reverse combination The activity of these particles was only 40-60% of that of subunits reconstituted from homologous components. These results suggested that there was substantial functional homology among the ribosomal constituents of both closely and distantly related bacteria and in particular between structures mediating interaction of RNA with protein. Since the 16S RNAs differ in nucleotide composition and base sequence and since the proteins displayed significant electrophoretic differences, it has been concluded that only particular regions of the 16S RNA and the ribosomal proteins required for the mutual interaction are conserved (Nomura *et al.*, 1968). Functionally equivalent counterparts for most of the proteins of *E. coli* 30S subunit including all those that bind directly to 16S RNA could be identified among the 30S proteins of *B. stearothermophilus* (Higo *et al.*, 1973).

Further experimental evidence for the evolutionary conservation of protein binding sites rRNA comes from the studies of Raue *et al.* (1985). Furthermore, it has been shown that ribosomal protein LU from *E. coli* and L15 from

S. cerevisiae bind to the same site on both yeast 26S and mouse 28S RNA (El-Baradi *et al.*, 1987a). The interaction of ribosomal protein L25 from yeast and L23 from *E. coli* with yeast 26S and mouse 28S RNA involves an evolutionarily conserved site (El-Baradi *et al.* 1985; 1987b).

The binding sites for most of the *E. coli* r-proteins on rRNA have been identified using a variety of cross-linking agents (Osswald *et al,* 1987; Greuer *et al,* 1987; Wower *et al,* 1981; Kyriatsoulis *et al,* 1986; Wiener and Brimacombe, 1987; Gregory *et al,* 1984; Guile *et al,* 1988; Ejebjerg *et al,* 1991). It is possible that similar analyses and comparison of binding sites for other ribosomal proteins will lead to a better understanding of the molecular processes that govern protein-RNA recognition in ribosomes.

Role of primary rRNA binding proteins:

Studies on the translational regulation of ribosomal protein biosynthesis indicate that certain ribosomal proteins that are known to bind directly to rRNA act as translational repressors by binding to polycistronic mRNAs usually encoding several r-proteins and in some cases other crucial proteins for transcriptional and translational processes (Nomura *et al,* 1982; 1984). The regulatory sites on these mRNA have been proposed to involve structures that mimic the rRNA binding sites for the regulatory r-proteins (Olins and Nomura, 1981; Gourse *et al,* 1981; Branlant *et al,* 1981). Most ribosomal protein operons contain one protein which behaves as a repressor. If protein synthesis outstrips rRNA synthesis the accumulating pool of free repressor will bind to the mRNA and represses translation of all the ribosomal proteins in that operon In rRNA abundance, however, the protein preferentially enters the assembly pathway leaving the mRNA free for translation. For example, it was found that synthesis of

L1 and LU, which are encoded within the same operon, is suppressed both *in vitro* and *in vivo* when the former protein is present in excess. In the absence of the LU promoter region, L1 fails to regulate its own production (Nomura *et al.*, 1980).

The binding site for *E. coli* protein L1 on the large subunit rRNA from *P. vulgaris* (Branlant *et al.*, 1981), *B. stearothermophilus* (Stanley *et al.*, 1978) and *D. discoideum* (Gourse *et al.*, 1981) have sequenced and compared to the corresponding region in *E. coli* 23S RNA. Similarities in both the primary and especially the secondary structure were revealed. This finding points to a conservation of the specific recognition and binding sites of protein L1.

Archaeal Ribosomes:

Archaeal ribosome is a 70S structure composed of two subunits with sedimentation values of SOS and 30S. Each 70S ribosome contains one molecule each of 5S, 16S and 23S rRNA (Visentin *et al.*, 1971). There is no evidence for a separate 5.8S rRNA molecule as is found in eukaryotic ribosomes.

One of the unusual features of ribosomal proteins from halobacteria is their high acidity, viz., more than 90% of the proteins have average isoelectric points of 3.9 (Strom *et al.*, 1975). Although, ribosomal proteins of methanogens are more acidic than the conventional bacteria, they are less acidic than the extreme halophiles. Jarell *et al.* (1984) have extended their experimentation to study the correlation between acidity of ribosomal proteins and internal salt concentrations. Among methanogenic organisms such as *hi. arboriphilus* possessing the highest internal K^+ concentration as high as 1.2 M were found to contain the highest number of acidic proteins and vice versa. *S. acidocaldarius* on the other hand

contains mainly basic proteins in its ribosome (Schmid and Bock, 1982) and has low internal salt concentration (Green *et al.*, 1983)

It is now evident from the studies of Cammarano *et al.* (1986), that there are two distinct classes of ribosomes within the archaeal kingdom. The ribosomes from extreme halophiles and most of the methanogens are similar in size in comparison to those of bacteria. Ribosomes from halophilic branch in general have bacteria-like protein content with few or no proteins greater than 30,000 daltons in mass. On the other hand, ribosomes from the sulphur dependent thermoacidophilic branch have a much higher eukaryote-like protein content with many proteins greater than 30,000 daltons in mass (Cammarano *et al.*, 1980). It is estimated that thermoacidophilic archaeal 30S subunits (52% by protein weight) are appreciably richer in protein than the corresponding bacterial particles (31% protein by weight) The greater protein content of the archaeal 30S subunits is accounted for by both large number and greater average molecular weight of the subunit proteins Thermoacidophile 30S subunits resemble more closely eukaryotic 40S subunits than do bacterial 30S subunits (Londei *et al.*, 1983). *C. acidophila* 30S subunits yielded 28 proteins whose combined mass is 0.6 X 10^6 daltons compared with 20 bacterial 30S proteins totalling 0.35 X 10^6 daltons mass, whereas eukaryotic 40S subunits contain about 0.74 X 10^6 daltons (Wool, 1980). It is concluded that the heavier than bacterial mass of *C. acidophila (S. solfatancus)* ribosomes resides primarily in their smaller subunits. C. *acidophila* ribosomes exhibit a typical eukaryotic 1:1 protein/RNA mass ratio of the small subunits (Londei *et al.*, 1983).

Since rRNA sizes are very nearly the same for all archaea, the approximate two-fold variation in protein content must cause some change in the size of the ribosomal subunits Electron micrographic studies of Lake *et al.* (1982), indicate

that the structure of the 30S ribosomal subunits from archaea show unique features and are significantly different from the 30S subunits of bacteria Small subunits from archaea, eocytes and eukaryotes contain a structure that resembles a duck bill, "the archaebacterial bill". The bill is not present in bacterial ribosomc. Eocytic and eukaryotic small subunits, in addition to containing the features of the archaeal subunits possess additional structures at the bottom of the subunit called the eukaryotic lobes. These lobes are absent in both bacteria and archaea and are present in reduced form in eocytes (Henderson *et al,* 1984). Lobes are present at the base of both eukaryotic and eocytic large subunits and a bulge near the L1 ridge is present in both eocytes and eukaryotes.

Within the three evolutionary lineages some structural feature of the ribosomal subunit is remarkably constant for e.g., as in the methanogens, the extreme halophiles and the thermoacidophiles all show the archaebacterial bill. This conservation of structure within each lineage implies that the ribosome structure has great evolutionary stability and it provides morphological data in support of the theory of Woese and Fox, (1977).

Two archaeal ribosomal domains have been studied in some detail and they are L7/L12 stalk (A protein domain) and 5S r-RNA protein domain. Casiano *et al* (1990) have isolated the L7/L12 stalk from the archaeon *Sulfolobus solfataricus.* The proteins in this complex are designated as *Sso* L12e- *Sso* L10e. The stoichiometry of the proteins in the complex was approximately four copies *of Sso* L12e to one copy of *Sso* LlOe similar to *E. cob* (L7/L12).L10 and eukaryotic $(P1)_2/(P2)_2$.PO, indicating that this element of quaternary structure is a major conserved structural feature of the ribosome (Kopke *et al,* 1989, Casiano and Traut, 1991; Casiano *et al,* 1990). A great deal of structural information is available about L1, L10 and L11 proteins from archaea (Ramirez *et al,* 1989a;

Shimmin *et al.,* 1989b). Both L1 and L11 bind directly to 23S r-RNA. The binding sites of these two proteins in the 23S r-RNA have been conserved in the three kingdoms (Beauclerk *et al,* 1985; El-Baradi *et al,* 1987a; Zimmerman *et al,* 1980).

The other domain which was studied more extensively in archaea is the 5S r-RNA protein domain from *H. cutirubrum* (Matheson, 1980b). There is a considerably greater homology between archaeal and eukaryotic 5S r-RNA binding proteins than between the equivalent bacterial and archaeal proteins.

The 50S subunit from *Halobacterium cutiruburm* when subjected to ionic environments depleted in either K^+ or Mg^{++} or both, specific classes of proteins are released from the subunit along with 5S r-RNA and by varying the ionic environments, the proteins which were least afTected were the early assembly proteins which interact with the r-RNA molecule (Strom *et al,* 1975).

Archaeal ribosomes lack binding sites for a number of bacterial 70S antibiotic inhibitors such as chloramphenicol and streptomycin, but possess sites for other 70S inhibitors. Similarly, they have binding sites for certain 80S inhibitors, such as anisomycin but lack sites for others such as cycloheximide (Elhardt and Bock, 1982; Cammarano *et al,* 1985; Sanz *et al.,* 1987).

Schmid and Bock, (1982) have found that the 30S and 50S subunits from *S. acidocaldarius* contains 27 and 34 electrophoretically distinguishable spots respectively. Three additional proteins on both the subunits (a, b, c') are present Using *in vitro* translational experiments, Londei *et al.* (1983), have shown that 1 M NH_4Cl washed ribosomal subunits are equally active as the parent crude particles. Accordingly, a purification protocol involving treatment of the crude subunits, 1M NH_4Cl was adapted as the best compromise to ensure removal of the most extrinsic proteins without releasing proteins required for synthetic activity.

The secondary structure regions in *C. acidophila* r-RNA (64% G-C) are substantially more than those in *E. coli* r-RNA (54-55% G-C) Whether this difference reflects structural adaptive changes to extremely hot niches or the archaeal ancestry of the former microbial species cannot be decided until other mesophilic and archaea are scrutinised (Cammarano *et al*, 1982a). Moreover, they have shown that the EF-Tu equivalent factor in the archaea {*Caldariella acidophila* (genus *Sulfolobus*)} does not cross react with antibodies against *E. coli* EF-Tu and the factor is totally insensitive to pulvomycin and kirromycin (antibiotics that inhibit bacterial EF-Tu). The pulvomycin and kirromycin reactions thus could provide new phyletic markers for archaeal ancestry (Cammarano *et al*, 1982b).

Cammarano *et al* (1983), compared secondary structural features of ribosomal RNA species within intact ribosomal subunits and efficiency of RNA-protein interactions in thermoacidophilic organisms and bacteria The increment in ribosome Tm values with increasing thennophily is greater than the increase in Tm for free r-RNA indicating that within the ribosome bihelical domains of thermophiles, r-RNA species are stabilised more efficiently than their mesophile counterparts by proteins or other components.

An *in vitro* polypeptide synthesising system was developed from thermoacidophilic archaeon *S. solfataricus*. The stimulation of phenylalanine incorporation by poly (U) was entirely dependent upon the addition of spermine (3 mM) and magnesium acetate (10 mM) concentration. The optimal temperature for phenylalanine incorporation was 75 °C (Friedman, 1985). According to requirements for polypeptide synthesis and degree of stability of the ribosomal subunit association, sulphur dependent thermophiles cluster into two groups Group I organisms *(D. mobilis, T. tenax, S. solfataricus)* harbour 70S monomers

composed of weakly associated subunits whose poly (phe) synthesising capacity is totally dependent on added spermine and drastically inhibited by monovalent cations. Group II organisms *(T. celar, T. acidophilum)* contain 70S particles composed of tightly associated subunits whose synthetic capacity is independent of spermine and totally dependent on monovalent cations. Spennine promotes poly (phe) synthesis on ribosomes of group I organisms by converting the peptidyl transferase centre into an active conformation while monovalent cations are inhibitory by preventing the interaction between the free ribosomal subunits (Londei *et al.,* 1986a). Interestingly, spermine (or thermine) is also essential for the successful reconstitution of functionally active 50S subunits of *S. acidocaldarius* ribosomes from the completely dissociated RNA and proteins The reconstituted archaeal subunits are essentially indistinguishable from the native ones by a number of structural and functional criteria. These results indicate that polyamines are essential structural components of *S. acidocaldarius* ribosomes and are involved in properly shaping and stabilising the quaternary packing of the 50S subunits (Londei *et al.*, 1986b).

Addition of homologous unfractionated t-RNA to the *in vitro* translational system from *S. acidocaldarius* enhances poly (U) directed phenylalanine incorporation at 65 °C more than two and a half folds. Supplementation with t-RNAs from *E. coli*, baker's yeast, wheat germ and rat liver was without effect Pre-incubation of ribosomes at 65 °C for 40 minutes in the absence of spermine impaired their ability to carry out poly (U) directed phenylalanine synthesis by 32% (Friedman, 1986).

Teixido *et al.* (1989) showed that the 50S subunits reconstituted in the absence of 5S rRNA are inactive in the protein synthesis and lack a few ribosomal proteins. Furthermore, it has been determined that in the course of the *in vitro*

assembly process, *S. acidocaldarius* 5S rRNA can be replaced by the corresponding RNA species of *E. coli*.

The early assembly proteins interact with the 23S rRNA in a temperature independent fashion forming a thermally stable core particle that can be subsequently converted into complete 50S subunit of *S. acidocaldarius* ribosomes. Most of the early assembly proteins of *S. acidocaldanus* ribosomes are able to bind co-operatively to heterologous 23S rRNA species from both archaea and bacteria thereby suggesting that the RNA-protein recognition sites are largely conserved within these two kingdoms. By contrast, no specific binding of the archaeal proteins to eukaryotic RNA could be demonstrated (Altamura *et al.*, 1991).

The relationship of ribosomal subunits from *S. aadocaldarius* to eukaryotic ribosomal subunits can best be exemplified by the capacity of archaeal and eukaryotic ribosomal subunits to form active hybrid ribosomes. Purified ribosomal subunits from *S. solfataricus* are able to recognise ribosomal subunits from the yeast *S. cerevisiae* forming hybrid monosomes that have been revealed by sucrose gradient analysis and are active in peptide bond formation. Both reciprocal combinations (archaeal 30S + eukaryotic 60S and archaeal 50S + eukaryotic 40S) are functionally active. No hybrid couples are formed between subunits of yeast and *E. coli* ribosomes (Altamura *et al.*, 1986).

Unlike eukaryotes, ribosomal proteins and subunits of DNA dependent RNA polymerase from archaea are not phosphorylated under *in vivo* or *in vitro* conditions tested (Skorko, 1984).

Homologous ribosomal proteins in different organisms:

Since ribosomes occur in all organisms ranging from bacteria to mammals, they are the ideal objects for evolutionaiy studies. Although these organisms have maintained a common function in protein biosynthesis. their structure has undergone drastic changes throughout the process of evolution This raises the question as to how different species of ribosomes can perform a common function despite their drastic structural differences and which parts of the individual ribosomal components are responsible for conservation of the basic function.

The immunological relatedness of ribosomal proteins from archaea with those from bacteria and eukaryotes has been analysed employing a variety of techniques like immunodiffusion, quantitative immunoprecipitation and immunoblotting (Schmid and Bock, 1981; Schmid *et al.,* 1984) The results from the semi-quantitative analysis may be summarised as follows (i) antisera directed against ribosomal proteins from methanogens *M. vanniellii* and *M. bryanti,* cross-reacted extensively with ribosomal proteins from halophiles There was no cross-reaction of the antibodies directed against *M. bryanti* ribosomes with ribosomal proteins from *S. cerivisiae*, *S. acidocaldarius* or *E. coli* under conditions in which proteins from the extreme halophile *H. halobium* were reacting.

Constraints imposing conservativity could have been imposed on the primary rRNA binding proteins, because in case of primary rRNA binding proteins a co-evolution of the protein together with it's binding site at the RNA is necessary to maintain the binding of protein or RNA in different organisms

Ribosomal protein named L2 *(E. coli* nomenclature) is conserved in bacteria, eukaryotes and archaea Antibodies against *E. coli* L2 strongly cross reacted with one protein from *Methanococcus vanniellii* (ML7) and with one ribosomal protein from *S. cerevisiae* (YL6), but showed no cross-reaction with r-

proteins of rat liver or chicken liver. The antibodies against L2 either do not react or else reacted only weakly with ribosomal proteins from *S. solfataricus*, *T. tenax*, *D. mobilis* and *T. celar* (Schmid *et al,* 1984).

The *E. coli* homologues (Casiano *et al,* 1990) of L10 and L7/L12 are designated as *Sso* L10e and *Sso* L12e in *S. solfataricus*. *Sso* L10e is more tightly bound to the ribosome than its equivalent *E. coli* L10. The pentameric complex of L7/L12 and L10 of *E. coli* can be selectively extracted from the 50S subunits. *Sso* LlOe could not be extracted completely without removing many other proteins. This is also true for the eukaryotic anchor protein Po (Mitsui *et al.,* 1987; Uchiumi *et al,* 1987). *Sso* LlOe is considerably larger than L10 and resembles Po. The extra structural domains present in *Sso* LlOe and Po share significant similarities within their primary structures particularly in their C-terminal domain which contains a highly charged region that is also conserved in the C-terminal domain of the dimeric acidic proteins from both kingdoms (Ramirez *et al.,* 1989a; Tsurugi and Mitsui, 1989). This element is not present in *E. coli* L10 and L7/L12 and its function still remains unclear. These observations suggest that the *(Sso* $L12e)_4$.*Sso* LlOe complex is more closely related to the eukaryotic $(P1)_2/(P2)_2$.Po, complex than to the *E. coli* $(L7/L12)_4$.L10 pentamer.

The gene for a small basic ribosomal protein in *S. solfataricus* has been determined and the structure of the protein coded by this gene (L46e) has been confirmed by partial amino acid sequencing (Ramirez *et al.,* 1989b). The protein shows substantial sequence homology to the eukaryotic ribosomal proteins L39 in rat and L46 in yeast. There is no sequence homology to any of the bacterial ribosomal proteins suggesting that this protein is absent in the bacterial ribosome. All three proteins are small (50 aminoacids) and very basic. It should be noted that the two tryptophan residues are conserved in all the three proteins.

Regions of conserved sequence within the ribosomal RNAs and r-proteins are likely to contribute to the fonnation of structural domains that are especially important for the structure and function of the ribosome. A comparison of the primary structures of ribosomal proteins derived from different organisms is at present the only means of obtaining information about regions of conserved sequence and thus about the relatedness and evolution of these ribosomal components.

On the other hand, different organisms and their ribosomes have adapted to specific environmental conditions, such as high internal salt concentrations of halophilic cells or high temperatures suitable for the growth of thermophilic organisms. Therefore, ribosomes from different organisms will have special structural features In this context it is interesting to learn the changes in sequence that are correlated with particular adaptations.

Objectives and scope of the present investigation

Our current model of the ribosome structure and function is principally based on the *Escherichia coli* ribosomes. Ribosomes performing the same basic function in all living organisms, exist in three lineage specific morphological forms: bacterial, archaebacterial and eukaryotic. So far, ribosome diversity has been scarcely explored, yet considerably, more information on both eukaryotic and archaebacterial organelles is needed for understanding the evolution of the translational machinery.

In the present study, we analysed certain structural aspects of small and large ribosomal subunits of *S. acidocaldarius*, an acidothennophilic archaeon that thrives optimally at a temperature of 85-87 °C and a pH of 3.0. Studies on *S. acidocaldarius* ribosomes proves to be interesting because, they are derived from a species belonging to the most primitive archaeal group, the *crenarchaeota*, which are phylogenetically more closely related to the eukaryotes than to the bacteria (Woese *et al.*, 1990). Moreover, based on the ribosome morphology by electron microscopy it has been suggested that acidothermophilic bacteria can be grouped separately into another kingdom, the *eocytes* (Lake *et al*, 1984). Studies on the ribosome structure in thermophilic archaea may provide valuable information on the mechanisms involved in the stabilisation of nucleic acid-protein complexes at high temperatures.

S. acidocaldarius lacks a rigid cell wall and is in osmotic equilibrium with its surroundings. Inspite of low intracellular salt concentrations and high temperatures of growth, ribosomes from this organism are structurally stable and maintain translational efficiency and fidelity at very high temperature (85 °C). It is speculated that the differential stabilisation of rRNA secondary structure by

ribosomal proteins and other factors may contribute to the high degree of thennal tolerance of the ribosomes. Hence, we wanted to identify the proteins which bind strongly to rRNA and protect rRNA against thennal denaturation.

In the present study, we have developed a fractionation method which involves selective precipitation of ribosomal proteins with novobiocin. The same procedure was also used for the successful fractionation of ribosomal proteins from *E. coli*. Our results indicate that proteins with similar charge and molecular weight can be separated by this method. The procedure may have applications for the fractionation of other complex protein mixtures also.

RNA binding proteins from the large and small subunits of *S. acidocaldarius* ribosomes were identified using three different approaches. Sepharose 4B chromatography of *S. acidocaldarius* ribosomes, disassembly of 30S ribosomal subunits with different concentrations of LiCl and urea, disassembly of 30S ribosomal subunits with different concentrations of urea and different concentrations of LiCl separately.

Results from these three approaches allowed us to identify a set of 15-18 r-proteins in 50S subunit and a set of 6 r-proteins in 30S subunit which appear to be strong RNA binding proteins. The present work can be extended to understand the structural organisation of *S. acidocaldarius* ribosomes and the factors contributing to the thermal stability of the ribosomes.

Chapter-2
MATERIALS & METHODS

MATERIALS AND METHODS

Bacterial strains:

The organisms used in the present study viz., *Sulfolobus acidocaldarius* strain DSM 639 and *Escherichia coli* A19 were obtained respectively from Deutsche Sammulung Von Mikroorganismen, Gottingen, Germany, and from Dr. A. R. Subramanian, Max-Planck Institute for Molecular Genetics, Berlin, Germany.

Bacterial growth:

S. acidocaldarius was grown at 75 °C for 40-48 hours with vigorous aeration in a medium containing 0.1% yeast extract, 0.1% bactotryptone, 0.5% casaminoacids, 0.1% glucose, 0.02% sodium chloride, 0.13% ammonium sulphate, 0.03% potassium dihydrogen phosphate, 0.025% magnesium sulphate, 0.07% calcium chloride. The pH of the medium was adjusted to 3.0 with 1 M sulphuric acid (Kikuchi and Asai, 1984). The growth was followed by the measurement of absorbance at 650 nm after every 6 hours. The bacterial cells were harvested after neutralising the culture with 1 M Tris base (5 ml/ litre). The cell pellets were stored frozen at -80 °C.

E. coli was grown in Luria broth at 37 °C with vigorous aeration according to Minks *et al* (1978). The mid-logarithmic phase cultures were chilled and harvested by centrifugation and stored frozen at -80 °C.

Isolation of ribosomes and supernatant fraction (S-100):

This was carried out according to Minks *et al.* (1978). All operations were carried out at 0-4 °C. Cells were ground with double the weight of alumina until

soft and sticky and extracted with buffer (3 ml/g cells), containing 20 mM Tris-Cl (pH 7.6), 50 mM potassium chloride, 10 mM magnesium acetate and 7 mM 2-mercaptoethanol (TKM_{10} Me), in the presence of 2 μg/ml DNAse (RNAse free). The suspension was centrifuged for 10 minutes at 10,000 X g to sediment alumina and cell debris. The supernatant so obtained was again centrifuged at 30,000 X g for 30 minutes to obtain S-30 extract. The S-30 supernatant was centrifuged at around 1,00,000 X g for 4 hours 30 minutes to pellet ribosomes. The upper two-thirds of the supernatant (S-100) was collected, dialysed against $TKM_{10}Me$ buffer containing 10% glycerol for 6 hours and stored frozen in aliquots. The crude ribosomal pellet was suspended in $TKM_{10}Me$ buffer containing 1 M ammonium chloride and left at 4 °C for 15 hours. The ribosomes in 1 M ammonium chloride buffer were pelleted by centrifugation at 1,00,000 X g for 4 hours and the supernatant (ammonium chloride wash) was collected. The ribosomal pellet was suspended in $TKM_{10}Me$ buffer containing 15% glycerol and stored frozen at -80 °C.

Isolation of *S. acidocaldarius* ribosomes using gel filtration chromatography and ion-exchange chromatography:

In addition to the conventional method for the isolation of ribosomes using ultra-centrifugation, we have developed a method for the isolation of ribosomes from *S. acidocaldarius*, which involves gel-filtration and ion-exchange chromatography. The method which is described below is rapid in comparison to the ultra-centrifugation method and the ribosomes thus obtained are not contaminated with high molecular weight non-ribosomal proteins.

S. acidocaldarius cells (10 gms) were suspended in 25 ml of $TKM_{10}Me$ containing 0.5 M ammonium chloride buffer. The suspended cells were lysed

using 0.5% NP-40 and 0.1% Triton-X-100. The suspension was incubated on ice for 30 minutes. Pancreatic DNAse (100 μg) was added to the suspension and again incubated for 20 minutes. The suspension was centrifuged at 30,000 X g for 30 minutes. The supernatant (S-30) obtained was loaded on sephacryl S-200 column (bed volume 250 ml), which was previously equilibrated with $TKM_{10}Me$ containing 0.5 M ammonium chloride buffer. The column was eluted with the same buffer and fractions (5 ml each) were collected. The absorbance of diluted fractions was measured at 260 nm and 280 nm. The ribosome peak fractions in the void volume were analysed by slab gel electrophoresis and were pooled. The pooled fraction was dialysed against $TKM_{10}Me$ containing 50 mM ammonium chloride buffer and passed through a DEAE-cellulose column equilibrated with the same buffer. Non-specifically bound material was eluted using $TKM_{10}Me$ containing 100 mM ammonium chloride buffer. The DEAE-cellulose column was later eluted with $TKM_{10}Me$ containing 0.7 M ammonium chloride buffer. Absorbance of the column fractions was measured at 260 nm and 280 nm. The peak fractions of this elution were pooled, dialysed against $TKM_{10}Me$ and stored frozen at -80 °C.

Isolation of *S. acidocaldarius* ribosomes by ultra-centrifugation:

S. acidocaldarius cells (10 gms) were suspended in 25 ml of $TKM_{10}Me$ containing 0.5 M ammonium chloride buffer at 4 °C. NP-40 and Triton-X-100 were added to final concentrations of 0.5% and 0.1% respectively. The suspension was incubated at 4 °C for 30 minutes. Pancreatic DNAse (around 100 μg) was added and incubated for another 20 minutes. After complete lysis of cells, the suspension was centrifuged at 30,000 X g for 30 minutes. The S-30 fraction was centrifuged at 1,10,000 X g for 4 hours 30 minutes. The pelleted

ribosomes were washed and suspended in $TKM_{10}Me$ containing 1 M ammonium chloride buffer and left at 4 °C for 15 hours and clarified at 10,000 X g for 10 minutes to remove the aggregated matenal. The supernatant was centrifuged at 1,10,000 X g for 4 hours 30 minutes to pellet the salt washed ribosomes

Slab gel electrophoresis in the presence of SDS:

Protein samples were electrophoresed on 12.5% polyacrylamide slab gels containing SDS as described by Laemmli, (1970). The ratio of acrylamide to bis acrylamide was 30:0.8. Separating gel (12.5%) was in 0.375 M Tris-Cl (pH 8.8), 0.1% SDS. A stacking gel of 5% polyacrylamide in 0.125 M Tris-Cl (pH 6.8), 0.1% SDS was used. Protein samples were treated with 0.1% SDS and 1% 2-mercaptoethanol and heated at 65 °C for 15 minutes or boiled at 100 °C for 2 minutes. Electrophoresis was carried out at 120 Volts until the tracking dye reached the bottom of the gel. The electrode buffer was 0.05 M Tris, 0.38 M glycine, 0.1% SDS (pH 8.3). After electrophoresis the gels were washed in 7.5% acetic acid for 20 minutes and stained with 0.15% Coomassie brilliant blue R-250 in 50% methanol and 7.5% acetic acid for 2 hours. The gels were destained with a solution containing 5% methanol and 7.5% acetic acid.

Extraction of ribosomal proteins from ribosomes:

Ribosomal proteins (r-proteins) were extracted from *S. acidocaldarius* and *E. coli* ribosomes according to the method of Hardy *et al.* (1969). Two volumes of 100% acetic acid (glacial) containing 0.05% magnesium acetate were added to one volume of ribosomes (10 mg/ml) and left on ice for 45-60 minutes with constant stirring. After 1 hour, the suspension was centrifuged at 15,000 X g for 20 minutes. Supernatant was removed carefully and the pellet was re-extracted with

67% acetic acid containing 0.1 M magnesium acetate (0.5 volumes). Supernatant was collected after recentrifugation at 15,000 X g for 20 minutes to remove the precipitated RNA. Both the supernatants containing r-proteins were pooled and precipitated with 5 volumes of ice cold acetone. After 15 hours, the r-proteins were pelleted at 10,000 X g for 10 minutes in Sorvall SS-34 rotor. Ribosomal proteins were dissolved in 8 M urea and 7 mM 2-mercaptoethanol, dialysed against the same solution for 6 hours and stored frozen at -80 °C.

Estimation of protein:

The concentration of protein in different samples were determined according to LowTy *et al.* (1951) using bovine serum albumin as the standard.

Two dimensional gel electrophoresis:

Two dimensional gel electrophoresis (2-D electrophoresis) was performed according to the method of Geyl *et al.* (1981). Protein samples were in 6 M urea, 10 mM DTT and 10 mM bis tris-acetic acid pH 4.0. Basic fuchsin was used as a marker dye. Ribosomal proteins (100 µg in 100 µl) were placed on first dimensional (0.3 X 10 cm) 4% polyacrylamide gels in 0.0568 M bis tris-acetic acid (pH 5.0), 6 M urea, 6.5 mM EDTA and 0.1% bis acrylamide. The upper electrode buffer was 0.01 M bis tris-acetic acid (pH 4.0) and the lower electrode buffer was 0.18 M potassium acetate (pH 5.0). The first dimensional gel electrophoresis was performed at 110 Volts at 4 °C for 5-6 hours After the run the gels were removed and placed on the top of two dimensional slab gels (18.6% polyacrylamide and 0.48% methylene bis acrylamide, 1% of 5 N KOH and 6 M urea at pH 4.5).

Electrophoresis in the second dimension was performed at 15 °C and 110 Volts until the tracking dye (which had been used in the first dimension) was migrated to the bottom of gel. Electrode buffer in second dimension was glycine-acetic acid (0.28 M glycine at a pH of 4.5 adjusted with acetic acid). After the electrophoretic run, the gels were removed and stained in 0.15% Coomassie brilliant blue R-250 in 50% methanol and 7.5% acetic acid. The gels were then destained with a solution containing 5% methanol and 7.5% acetic acid.

Fractionation of r-proteins using novobiocin:

A fractionation method involving precipitation of r-proteins with novobiocin has been developed to fractionate ribosomal proteins of the archaeon, *Sulfolobus acidocaldarius* and bacterium, *Escherichia coli*. Ribosomal protein solutions at a concentration of 0.2 mg/ml were dialysed against 20 mM Tris-Cl (pH 8.0), 1 M ammonium chloride and 7 mM 2-mercaptoethanol. Novobiocin stock solution (10 mg/ml) in water was added to the dialysed ribosomal protein solution to a concentration of 200 µg/ml. The solution was mixed, incubated on ice for 30 minutes and centrifuged at 20,000 X g at 4 °C for 15 minutes. The supernatant was collected and the precipitated protein was dissolved in 8 M urea and 7 mM 2-mercaptoethanol. The concentration of novobiocin in the above supernatant was raised to 500 µg/ml and after incubation on ice for 30 minutes, it was again centrifuged to obtain proteins precipitated at this novobiocin concentration. The supernatant fraction was collected again and treated with 1000 µg/ml novobiocin. Both supernatant and precipitated protein fractions were obtained as above. The final supernatant fraction was treated with five volumes of acetone and the precipitated protein was dissolved in 8 M urea, 7 mM 2-mercaptoethanol. The precipitated protein at each novobiocin concentration was

estimated by Lowry's assay. Proteins in each novobiocin fraction were identified by two dimensional electrophoresis of Geyl *et al.* (1981). The numbering of the ribosomal proteins *S. acidocaldarius* was according to (Schmid and Bock, 1982) and those of *E.coli* according to Geyl *et al.* (1981).

Isolation of ribosomal core particles by hydrophobic chromatography:

Hydrophobic chromatography of *E. coli* ribosomes was developed by Kirillov *et al.* (1978) to dissociate *E. coli* ribosomes into subunits. Chromatography of *S. acidocaldahus* ribosomes yielded 50S subunit core particles and 30S subunits lacking some proteins. Ribosomes in low concentrations of magnesium acetate and high concentrations of ammonium sulphate are strongly adsorbed onto sepharose 4B. This method is based on the difference in the hydrophobic interaction of subunits with the agarose gel at very low concentration (or in the absence) of Mg^{++} ions.

All the experiments were performed in two stages at 4 ^{o}C. In the first stage *S. acidocaldarius* ribosomes were transferred by gel-filtration through a column of sephadex G-50 into a buffer containing 0.02 M Tris-Cl (pH 7.6) with desired concentrations of magnesium acetate and 0.6 M ammonium sulphate. Ammonium sulphate was added to a final concentration of 1.5 M to the pooled ribosome fractions eluting in the void volume of G-50 column.

Ribosomes were applied to a column of sepharose 4B (100 ml bed volume) equilibrated with the buffer containing 0.02 M Tris-Cl (pH 7.5), containing 1.5 M ammonium sulphate and desired concentration of magnesium acetate. The column was eluted with three volumes of reverse salt gradient of ammonium sulphate (1.5

M to 0.02 M) in the same buffer. Fractions were collected and analysed by measuring absorbance at 260 nm, after appropriate dilution.

Hydrophobic chromatography for the isolation of 50S core particles directly from S-30:

In this method, S-30 was prepared by lysing 15 gms of cells with Triton-X-100 and NP-40 as described previously. The S-30 obtained was dialysed against buffer containing 0.02 M Tris-Cl (pH 7.5), 1.5 M ammonium sulphate buffer for 72 hours. The precipitated material was removed by centrifugation at 15,000 X g for 15 minutes. The clear supernatant was loaded on sepharose 4B equilibrated with 0.02 M Tris-Cl (pH 7.5), 1.5 M ammonium sulphate buffer. Elution of the column was carried out by linear reverse salt gradient of 1.5-0.02 M ammonium sulphate in buffer containing 0.02 M Tris-Cl (pH 7.5). Absorbance of the fractions was measured at 260 nm after appropriate dilution and the fractions containing 50S core particle and 70S particle were pooled, dialysed against $TKM_{10}Me$ buffer and stored frozen at -80 °C.

Isolation of ribosomal subunits by density gradient centrifugation:

S. acidocaldarius and *E. coli* 70S ribosomes were dialysed against buffer containing high salt and low magnesium acetate (0.02 M Tris-Cl pH 7.6, 1 mM magnesium acetate, 0.5 M ammonium chloride). The dialysed ribosomal solution was clarified by low speed centiifugation, carefully layered on the top of 8-38% linear sucrose gradients and centrifuged for 2 hours 40 minutes at 40,000 RPM in VTi-50 Beckman rotor. Fractions were collected from the bottom of the gradient tube. The absorbance of the fractions was measured at 260 nm after appropriate dilutions. Fractions corresponding to 30S and 50S subunits were pooled The

subunits were immediately brought to 10 mM magnesium acetate by the addition of 1 M magnesium acetate and concentrated by DEAE-cellulose chromatography and were stored in aliquots at -80 ^{o}C.

Association and dissociation of ribosomal subunits and core particles:

The buffers used for association and dissociation were 20 mM Tris-Cl (pH 7.6), 50 mM ammonium chloride, 40 mM magnesium acetate, 3 mM spermine and 7 mM 2-mercaptoethanol (Buffer A). 20 mM Tris-Cl (pH 7.6), 1 mM magnesium acetate, 500 mM ammonium chloride, 7 mM 2-mercaptoethanol (Buffer B) respectively.

50S subunits (12 A_{260}) and 30S subunits (8 A_{260}) in 20 mM Tris-Cl (pH 7.6), 50 mM ammonium chloride, 10 mM magnesium acetate and 7 mM 2-mercaptoethanol were mixed and magnesium acetate and spermine were added to the concentrations of the buffer A. The mixture was incubated at 45 °C for 40 minutes and subsequently loaded onto a 8-38% linear sucrose gradient in buffer A. The sample was centrifuged at 40,000 rpm for 2 hours 20 minutes in a VTi-50 rotor. Fractions were collected from the bottom of the gradient tube. The absorbance of the fractions was measured at 260 nm after appropriate dilutions and the values plotted on a graph.

70S ribosomes (20 A_{260}) in 20 mM Tris-Cl (pH 7.6), 50 mM ammonium chloride, 10 mM magnesium acetate and 7 mM 2-mercaptoethanol were dialysed against buffer B (dissociating conditions), layered on a 8-38% linear sucrose gradient in buffer B and centrifuged at 40,000 rpm for 2 hours 20 minutes in a VTi-50 rotor. The fractions were collected from the bottom of the gradient tube and their absorbance analysed as before.

S. acidocaldarius 50S core particle (12 A_{260}) obtained by sepharose 4B chromatography of the 70S ribosomes (in the absence of Magnesium acetate) and (8 A_{260}) 30S subunits obtained by gradient centrifugation were mixed and incubated at 45 °C for 40 minutes in buffer A, by making appropriate additions. The sample was analysed as before on a 8-38% linear sucrose gradients.

Isolation of ribosomal RNA:

Total ribosomal RNA, 16 S RNA and 23 S RNA from *S. acidocaldanus* and *E. coli* were isolated using the following procedure. Equal volume of $TKM_{10}Me$ buffer saturated phenol was added to ribosomes or ribosomal subunits (10 mg/ml concentration) and vigorously shaken for 30 minutes at 4 °C The mixture was separated into two layers by centrifugation at 12,000 X g in a Sorvall RC5C centrifuge. The aqueous layer was carefully taken without disturbing the interface. The phenolization was repeated six times. All the 7 phenol phases were combined and extracted once with one volume of $TKM_{10}Me$ buffer. RNA present in aqueous phases was precipitated with 2 volumes of ethanol (pre-cooled at -20 °C) overnight. The precipitate was collected by centrifugation at 10,000 X g for 15 minutes in a SS-34 rotor in the Sorvall RC5C centrifuge. The pellet was dissolved in $TKM_{10}Me$ buffer, re-precipitated with 2 volumes of cold ethanol and dissolved in the appropriate buffer chosen for thermal analysis.

Thermal melting analysis:

Ribosomes, ribosomal subunits, core particles and rRNA (1 A_{260} each) in 1 mM Tris-Cl (pH 7.6) and 0.08 mM magnesium acetate was heated at a rate of 1 °C rise per minute and the increase in A_{260} was continuously recorded in Philips PU 8700 UV/Vis spectrophotometer equipped with a thermoprogrammer (model

PU 8764). Prior to heating, the contents were gently mixed and incubated for 5 minutes at the starting temperature. Low magnesium concentration was chosen for preventing aberrant Tm values.

Isolation of ribosomal subunit core particles:

The 30S ribosomal subunit of *S. acidocaldarius* is protein rich in comparison to *E. coli* subunit. Treatment of *S. acidocaldanus* with increasing concentrations of LiCl and urea produces a core particle which contains a well defined subset of ribosomal proteins.

LiCl and urea were added to a final concentration of 1 M LiCl/2 M urea or 2 M LiCl/4 M urea or 3 M LiCl/6 M urea or 4 M LiCl/8 M urea to 10 mg/ml of 30S subunit in 20 mM Tris-Cl (pH 7.6), 50 mM potassium chloride, 2 mM magnesium acetate and 7 mM 2-mercaptoethanol or 20 mM Tris-Cl (pH 7.6), 50 mM potassium chloride, 20 mM magnesium acetate and 7 mM 2-mercaptoethanol and incubated overnight at 4 °C with constant stirring The precipitated material was removed by centrifugation at 15,000 X g for 15 minutes. The core particles were separated from split proteins by centrifugation at 1,90,000 X g for 5 to 8 hours. The proteins present in the core particle were extracted with acetic acid and analysed by two dimensional gel electrophoresis.

Core particle of 30S subunits (10 mg/ml) was also isolated by treatment with only LiCl. Lithium chloride was added to a final concentration of 1 M LiCl, 2 M LiCl, 3 M LiCl, 4 M LiCl to 30S subunits obtained by ultra-centrifugation, and incubated overnight at 4 °C with constant stirring. The core particles were separated from split proteins by high speed centrifugation as previously described Core particles were also isolated by treating 30S subunits with different concentrations of urea (1 M, 2 M, and 4 M urea).

Antisera to subunits and isolation of antibodies:

Antibodies to 30S and 50S subunits of *S. acidocaldarius* and *E. coli* were raised in rabbits as described by Stoffler and Wittman (1971). Subunits (each 500 μg) was emulsified with Fruend's complete adjuvant and injected subcutaneously into rabbits at multiple sites. After four weeks, a booster injection of 200 μg protein in Fruend's incomplete adjuvant was given through subcutaneous route. Rabbit was bled after one week of booster injection through the pinna vein. Blood was allowed to clot at 30 °C for 2 hours and at 4 °C for 15 hours. Antiserum was collected after centrifugation of clotted blood. The antisera were heated at 55 °C for 30 minutes to inactivate the complement proteins and fractionated with ammonium sulphate. Serum (10 ml) was mixed with 6 ml of saturated ammonium sulphate in 0.02 M Tris-Cl (pH 8.0), 1 mM EDTA and stirred gently at 30 °C for 30 minutes. The precipitate was spun down at 4,000 X g for 45 minutes. IgG pellet was dissolved in small volumes of 20 mM Tns-Cl (pH 8.0) and 1 mM EDTA. IgG was re-extracted with one-third volume of saturated ammonium sulphate solution. IgG precipitate was collected by centrifugation at 4,000 X g for 45 minutes, dissolved in small volumes of 70 mM sodium phosphate buffer (pH 6.3) and dialysed against the same buffer.

The crude IgG fraction was further purified by ion-exchange chromatography on DEAE-cellulose. The ion-exchanger was packed into a column and equilibrated with phosphate buffer at room temperature. Dialysed ammonium sulphate fraction was applied on the column and eluted with the same buffer. Fractions of 1 ml volume were collected and absorbance at 280 nm was measured. IgG peak fractions were pooled and concentrated by adding equal volumes of saturated ammonium sulphate (precipitated at 50% saturation). The IgG precipitate was collected by centrifugation at 15,000 X g for 15 minutes, dissolved in

phosphate buffered saline and dialysed against the same buffer After dialysis, the concentrated antibody was distributed into aliquots and stored at -80 ^{o}C. Ouchterlony immunodiffusion was performed as per the standard protocol.

Immunoblotting of two dimensional gels:

This method was according to Towbin *et al.* (1979) with certain modifications. Ribosomal subunit proteins were extracted with acetic acid and subjected to two-dimensional gel electrophoresis of Geyl *et al* (1981). Proteins were transferred from two-dimensional gels to nitro-cellulose membrane using the electrode buffer (25 mM Tris, 192 mM glycine, 20% methanol), for a period of 8 hours at 7 Volts. After the transfer, the blots were removed from the blotting apparatus and air-dried Later the unbound sites were saturated by incubating the blots in blocking buffer containing 10 mM Tris-Cl (pH 8.1), 150 mM NaCl (T_{10} $NaCl_{150}$) and 3% BSA for 3 hours. The nitro-cellulose sheets were incubated with diluted antiserum (8:100) in blocking buffer for 4 hours and washed (6 X 5 minutes) with blocking buffer containing 0.05% NP-40. The blots were then incubated with peroxidase conjugated anti-rabbit IgG (1:1000) in blocking buffer for 2 hours. The membranes were again washed 6 times (6 X 5 minutes) with T_{10} $NaCl_{150}$ buffer. Finally, the blots were developed in the substrate solution The substrate solution was prepared freshly by adding 1 ml stock solution of 4-chloro-1-naphthol (3 mg/ml) in methanol and 10 µl of 30% H_2O_2 to 10 ml Tris-buffered saline ($T_{10}NaCl_{150}$) The reaction was terminated after 15 minutes by washing with distilled water and the membrane was air-dried.

Chapter-3
RESULTS & DISCUSSION

3.1: Isolation of ribosomes and fractionation of r-proteins

Apart from ultra-centrifugation, there are a few other methods to isolate ribosomes from bacteria. It was essential for our studies to ensure that the ribosomes contain little or no contamination by proteins of the supernatant fraction. We have employed three methods for the isolation of ribosomes from *S. acidocaldarius*. In the first method, ribosomes were isolated from alumina ground cells by ultra-centrifugation (Minks *et al.*, 1978). In the second method, ribosomes were isolated by gel filtration and ion exchange chromatography, after lysing the cells with low concentrations of Triton X-100 and NP-40. In the third method, ribosomes were isolated by ultra-centrifugation of cell lysates obtained by the use of Triton X-100 and NP- 40.

Isolation of ribosomes by different methods and analysis of r-proteins by 2-D gel electrophoresis:

The table summarises the isolation procedure of *S. acidocaldahus* ribosomes on a medium scale from 10-30 gms of cells using gel filtration and ion-exchange chromatography. The advantage of this technique is that the whole process is completed within a period of 24 hrs (**Table-2**).

Sephacryl S-200 is a very rigid gel filtration medium composed of cross linked allyl dextran which allows fast flow rates and excellent resolution even on large columns. Chromatography of S-30 extract on S-200 results in elution of the ribosomes as a sharp peak (**fig.** 2). The peak fractions obtained after S-200 chromatography were diluted to reduce the ammonium chloride concentration to 50 mM, and loaded on the DE-52 column pre-equilibrated with the same buffer as described in the materials and methods section. The non-specifically bound

Table 2

Purification of *S. acidocaldarius* ribosomes from 10 gms. of cells

Cell Lysis	1 hour
S-30 Supernatant	30 minutes
Sephacryl S-200 Chromatography	8-12 hours
DE-52 Chromatography	4 hours
Dialysis	**6 hours**

Experimental details in materials and methods section

Fig. 2: Sephacryl S-200 chromatography of *S. acidocaldarius* S-30.

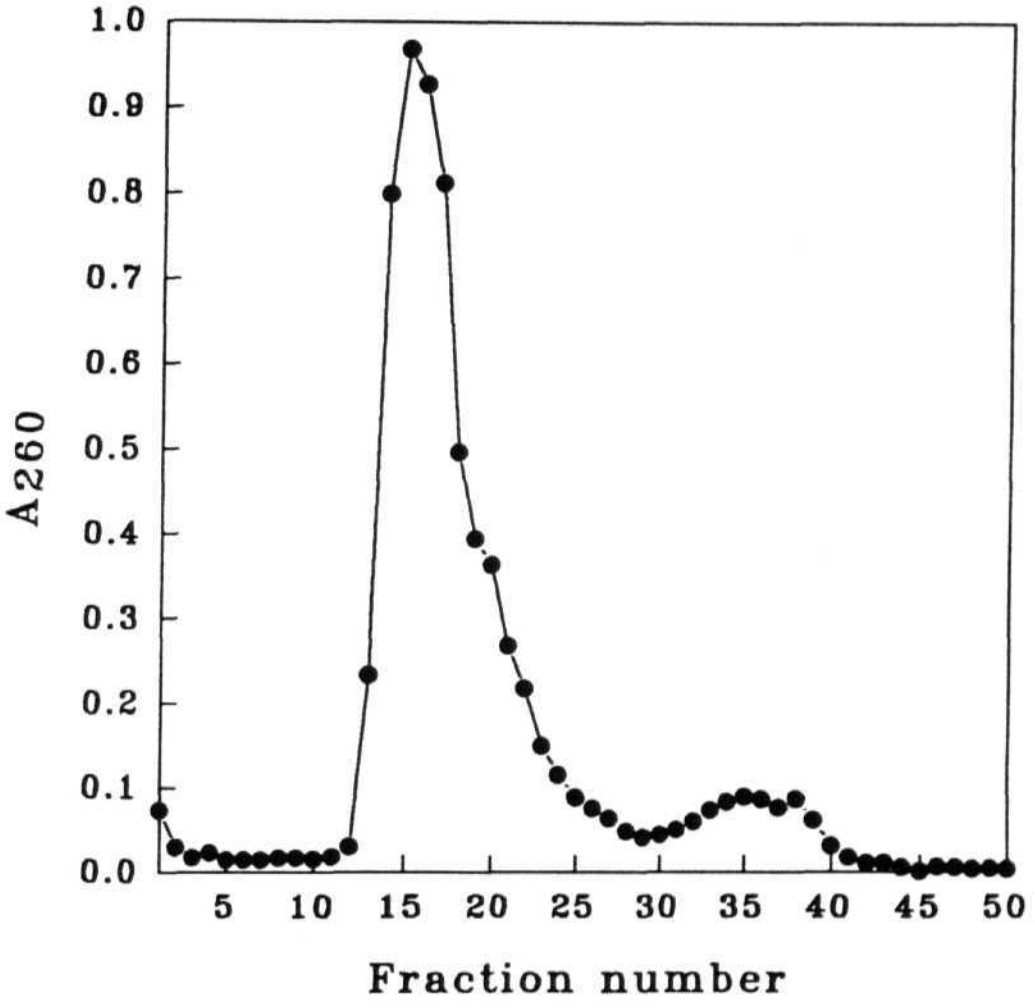

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
A260
5
10
15
20
25
30
35
40
45
50
Fraction number

Figure 2

material gets eluted at 0.1 M ammonium chloride while the pure ribosomes elute at 0.7 M ammonium chloride as depicted in fig. 3. The ribosomes were dialysed against $TKM_{10}Me_7$ containing 15% glycerol.

It was found that ribosomes isolated under gentle lysis conditions do not contain high molecular weight non ribosomal proteins. This technique is general and simple enough, so that it can be adopted for the isolation of ribosomes from other thermophilic organisms, provided, the concentration of Triton X-100 and NP-40 for the lysis of these organisms are optimised. The ribosomal protein pattern so obtained was almost similar to those published by Schmid and Bock, (1982). Along with ribosomes it is possible to isolate several small basic DNA binding proteins of 7 Kda, 8 Kda and 9 Kda which elute in the later fractions of the S-200 column. Grote *et al.* (1986) have also described a similar procedure for the isolation of ribosomes from *S. acidocaldarius* cell extracts (obtained by means of a french press), employing chromatography on Sephacryl column.

Ribosomes were also isolated by the conventional method viz., from the alumina ground cells as described earlier.

Cell extracts of *S. acidocaldarius* were obtained after lysis with non ionic detergents such as Triton X-100 and NP-40 and the ribosomes were isolated by ultra-centrifugation.

Ribosomes isolated by gel filtration and ion-exchange chromatography were compared with ribosomes isolated by ultra-centrifugation of cell lysates obtained by Triton X-100 and NP-40 and also with ribosomes isolated from alumina ground cells using ultra-centrifugation. Ribosomes isolated by the above three methods contain identical ribosomal protein patterns as analysed by two-dimensional gel electrophoresis (fig. 5). But in the case of ribosomes isolated from alumina ground cells and those from ultra-centrifugation of cell lysates, there is a

Fig. 3: DEAE-cellulose chromatography **of** *S. acidocaldarius* ribosomes.

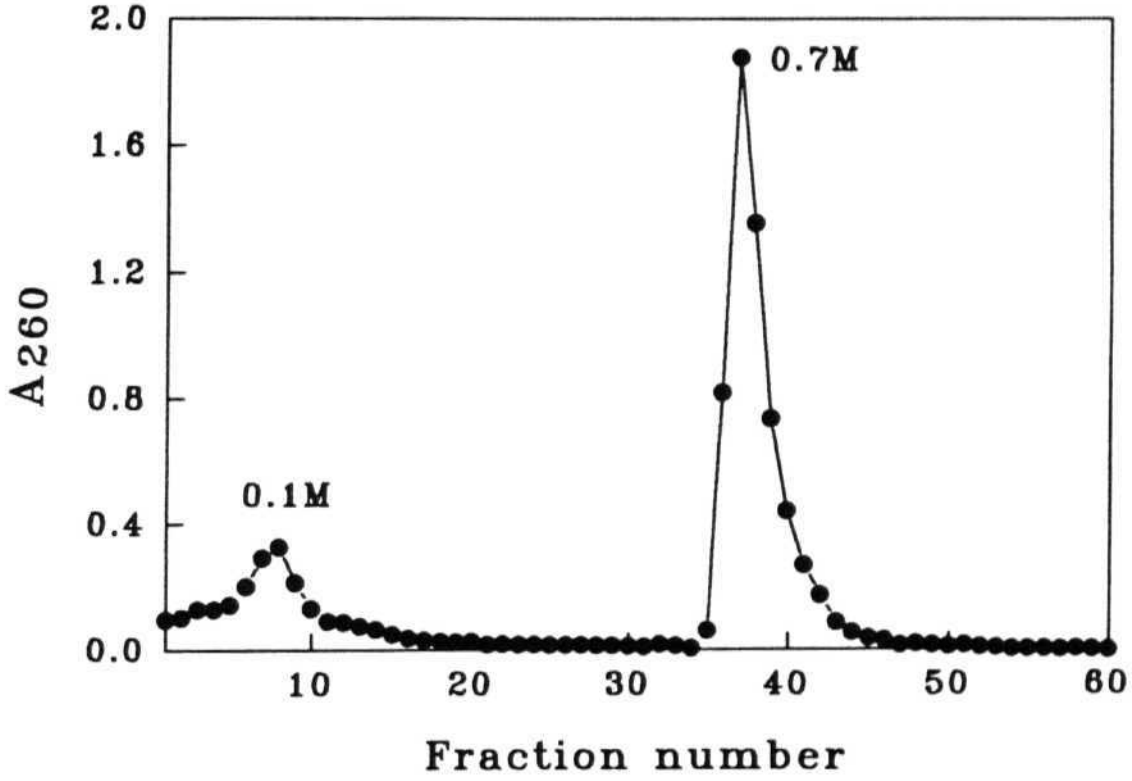
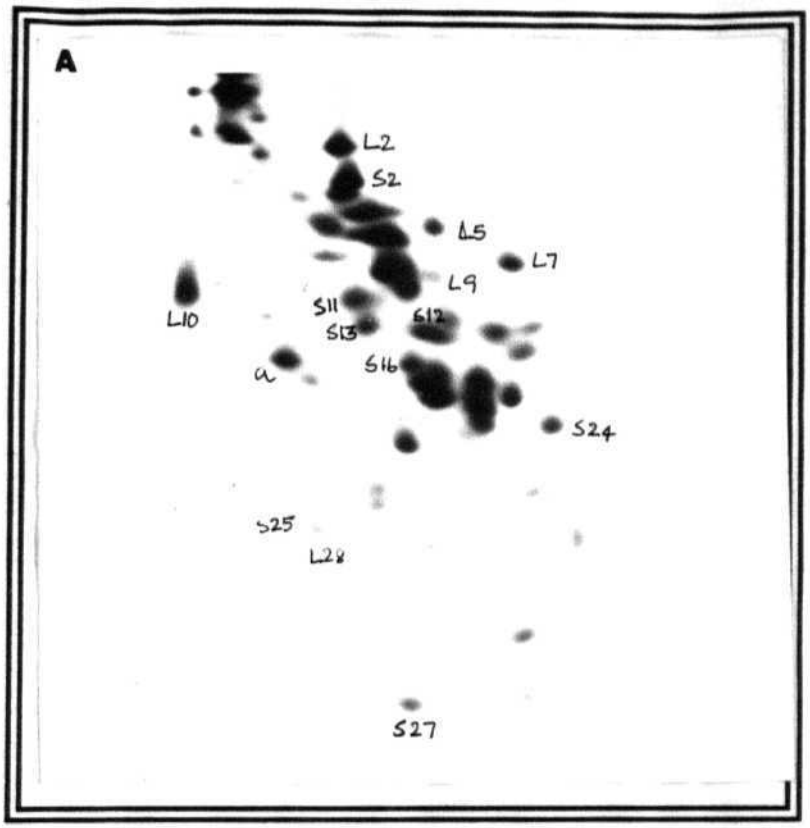
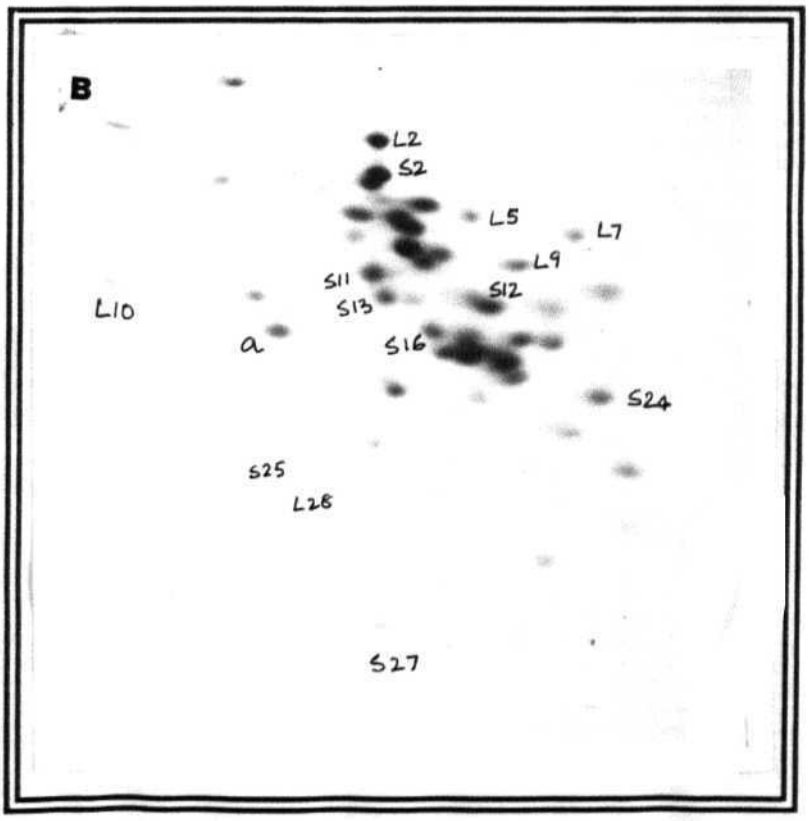
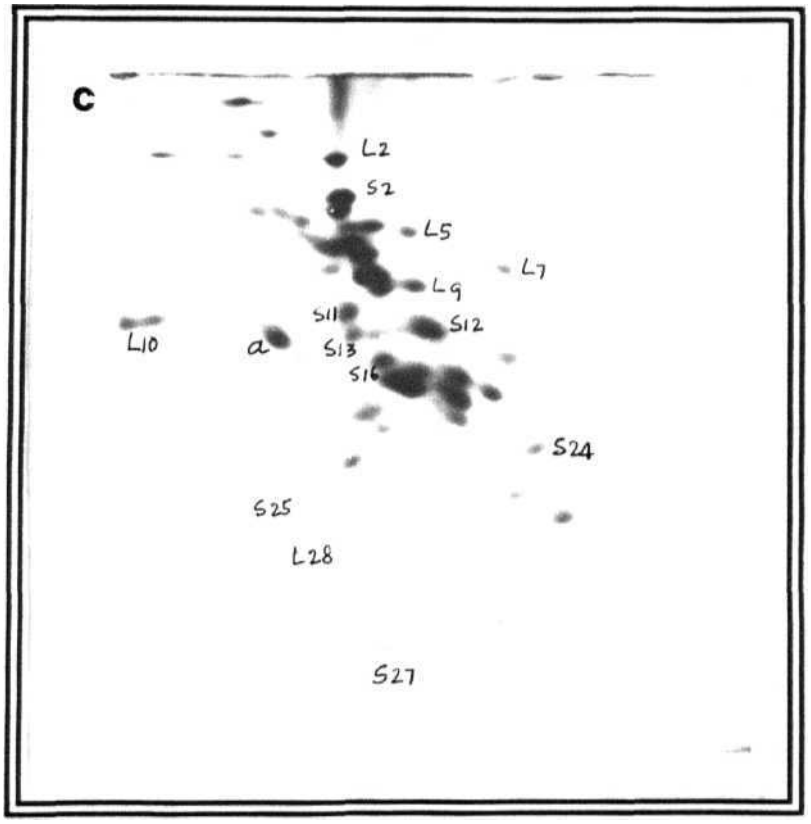


Figure 3

Fig. **5: Two-dimensional** gel electrophoresis **of** *S. acidocaldarius* r-proteins: (A) Ribosomes isolated from alumina ground cells, (B) Ribosomes isolated from cells lysed with Triton X-100 and NP-40, (C) Ribosomes isolated using ultra-centrifugation of cell lysates obtained by non ionic detergents. In each case, 150 μg of extracted r-proteins were used for electrophoresis.


A
L2
S2
L5
L7
L9
L10
S11
S12
S13
a
S16
S24
S25
L28
S27


B
L2
S2
L5
L7
L9
S11
S12
L10
S13
a
S16
S24
S25
L28
S27


c
L2
S2
L5
L7
L9
S11
S12
L10
a
S13
S16
S24
S25
L28
S27

clear contamination of high molecular weight non-ribosomal proteins which are not seen in case of ribosomes isolated by gel filtration and ion exchange chromatography as analysed by SDS-polyacrylamide gel electrophoresis (fig. 4).

2-D gel electrophoretic analysis of *S. acidocaldarius* ribosomal subunits:

Ribosomes isolated from Sephacryl S-200 and DE-52 were dialysed against low Mg^{++} buffer and dissociated into subunits by ultra-centrifugation in vertical rotors. Sucrose density gradient profiles of *E. coli* and *S. acidocaldarius* ribosomal subunits are shown in fig. 6A and 6B respectively. Ribosomal subunit proteins were extracted and analysed by two-dimensional gel electrophoresis (fig. 7A & 7B). The 30S subunit and 50S subunit of 5. *acidocaldarius* contain 27 and 34 electrophoretically distinguishable proteins respectively (fig. 7A). Three additional proteins are present in both 30S and 50S subunits. There was no detectable cross contamination of the subunits. It is interesting to note that there was no 30S protein with a molecular weight higher than 30,000 Da In the case of both 30S and 50S subunits there are three proteins called a, b, c which have migrated to identical positions. They may represent the ribosomal proteins present on both subunits like protein S20 and L26 of *E. coli* ribosomes (Wittman and Wirtman-Liebold, 1974; Stoffler, 1974). For comparison, 2-D gel patterns of *E. coli* 30S and 50S subunit proteins are given in fig. 7B.

The 2-D gel patterns of the subunits from *S. acidocaldarius* are very similar to those obtained by Schmid and Bock, (1982) indicating that the present isolation procedure yielded ribosomal subunits with the usual number of proteins.

Fig. 4:SDS-gel electrophoresis of ribosomes isolated by three different procedures:(A) Ribosomes isolated from alumina ground cells, (B) Ribosomes isolated from cells lysed with Triton X-100 and NP-40, (C) Ribosomes isolated using ultra-centrifugation of cell lysates obtained by non ionic detergents. In each case, 60 μg of extracted r-proteins were used for electrophoresis.

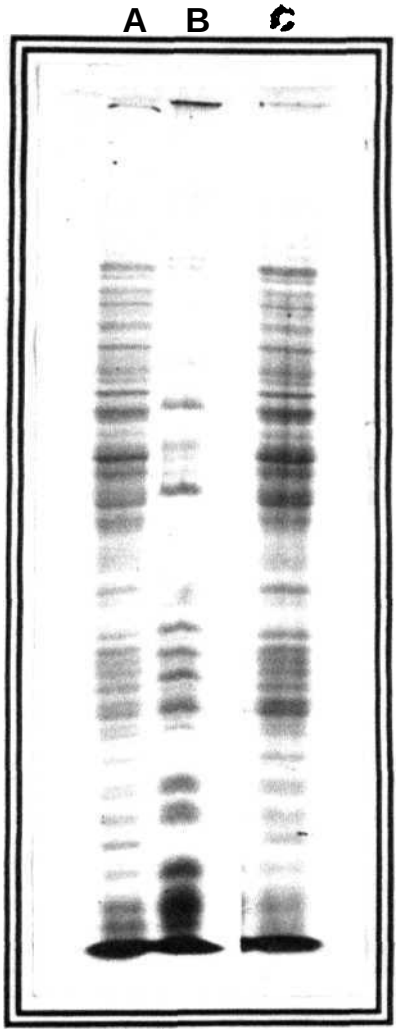


Figure-4

Fig. 6:Sucrose density gradient centrifugation profile of

(A) *E. coli* ribosomal subunits, (B) *S. acidocaldarius* ribosomal subunits.

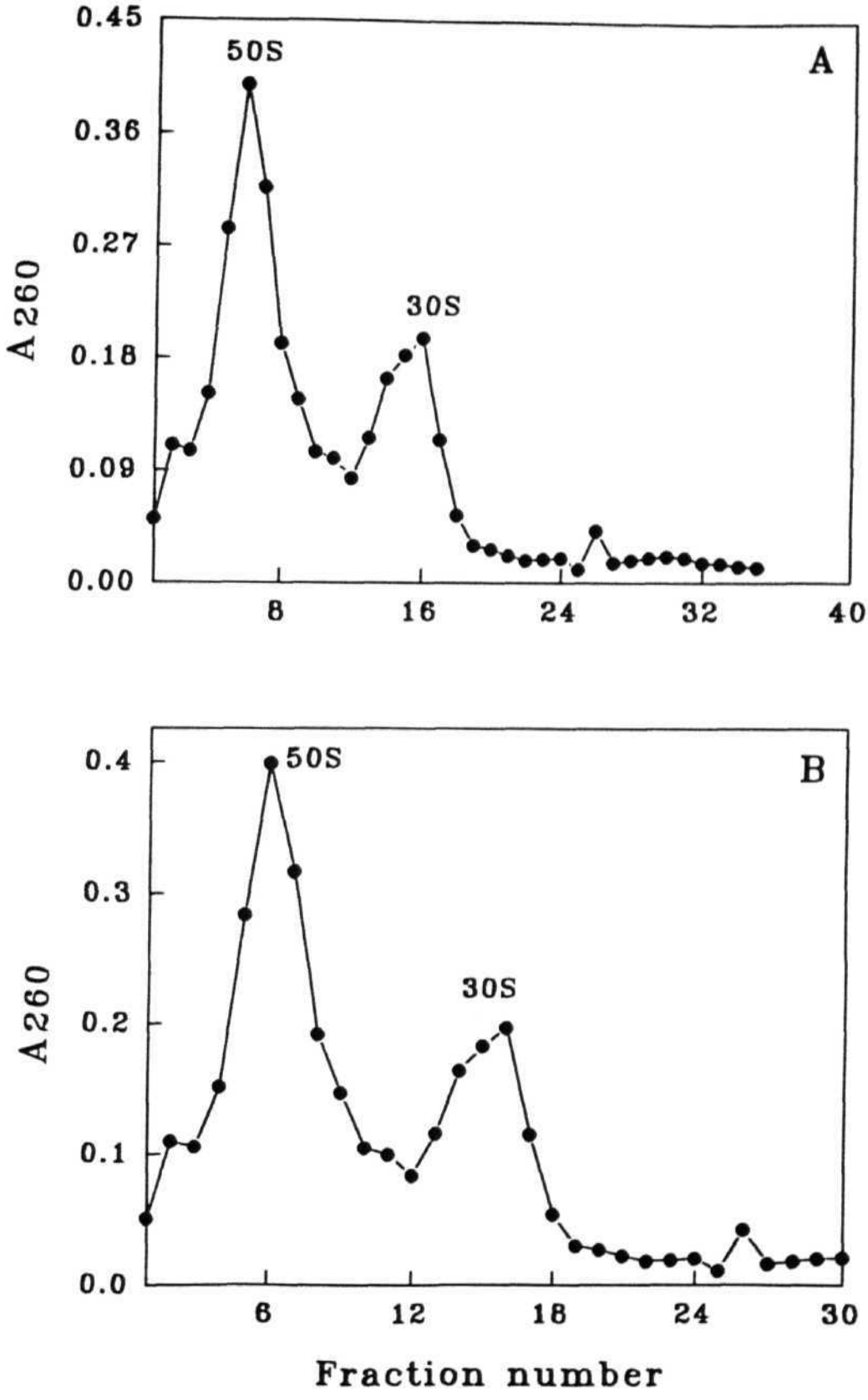
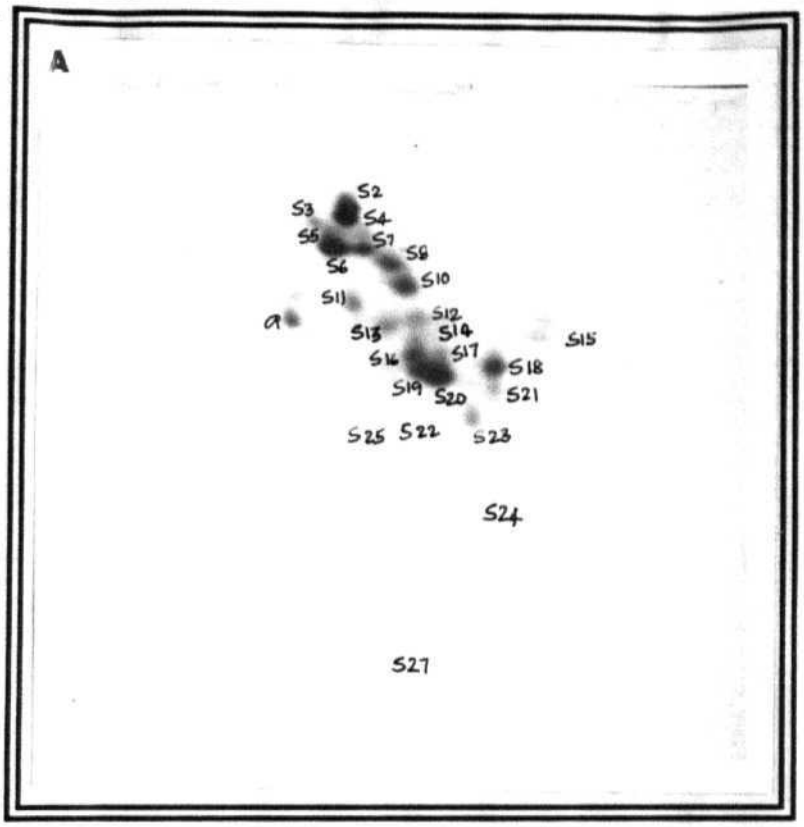

A
50S
30S
0.45
0.36
0.27
0.18
0.09
0.00
A 260
8
16
24
32
40
B
50S
30S
0.4
0.3
0.2
0.1
0.0
A 260
6
12
18
24
30
Fraction number

Figure 6

Fig. 7A:Two-dimensional gel electrophoretic pattern of *S. acidocaldarius.*
(1) 30S subunit proteins (100 μg), (2) 50S subunit proteins(150 μg).


A
S2
S3
S4
S5
S7
S8
S6
S10
S11
S12
a
S13
S14
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S16
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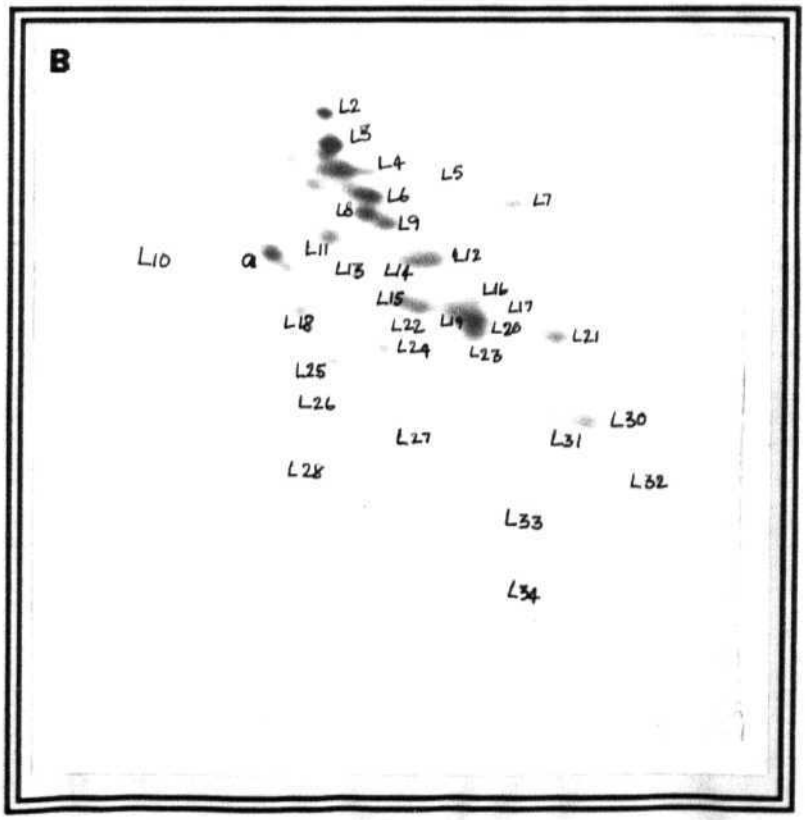
S27


B
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L16
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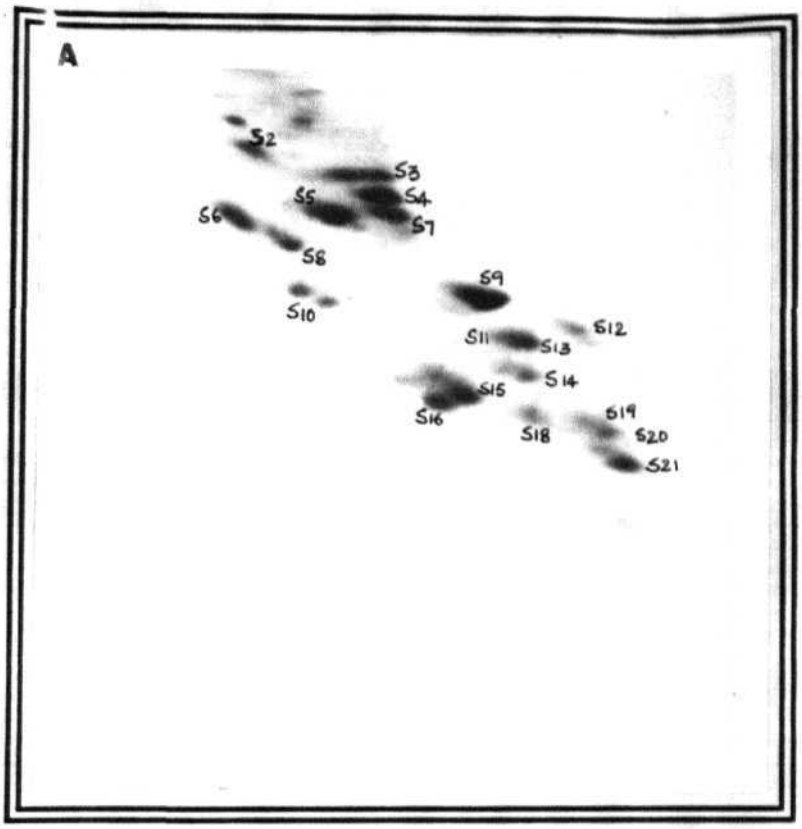
L31
L28
L32
L33
L34

Fig. **7B: Two-dimensional** gel electrophoretic pattern **of** *E. coll*

(1) 30S subunit proteins (100 μg), (2) 50S subunit proteins (150 μg).


A
S2
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S4
S5
S6
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S8
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S10
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S12
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S15
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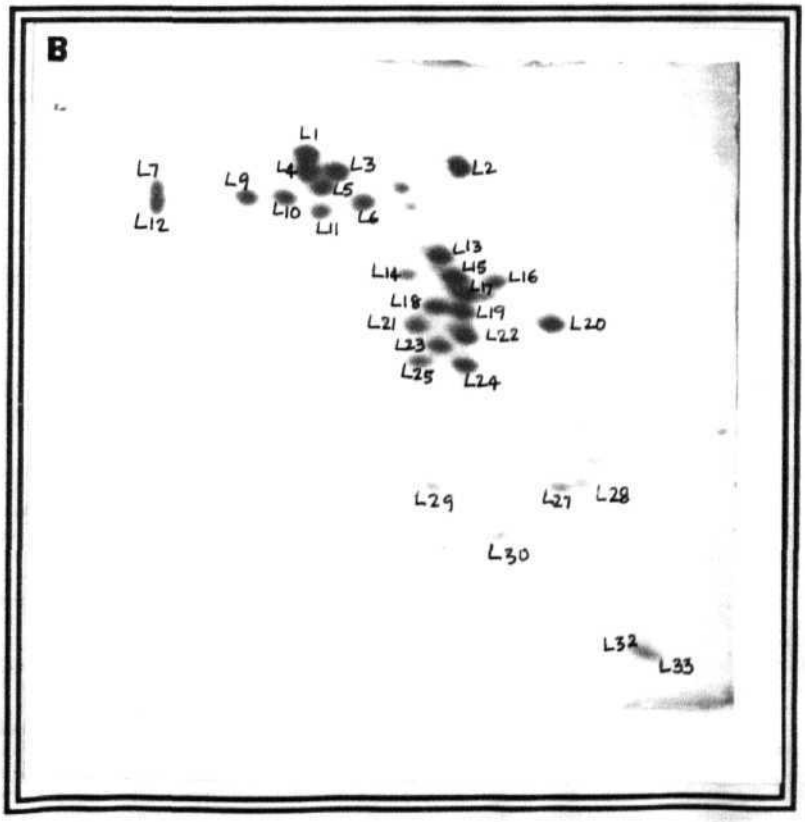
S18
S19
S20
S21


B
L1
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L3
L4
L5
L6
L7
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L30
L32
L33

Fractionation of ribosomal proteins using novobiocin:

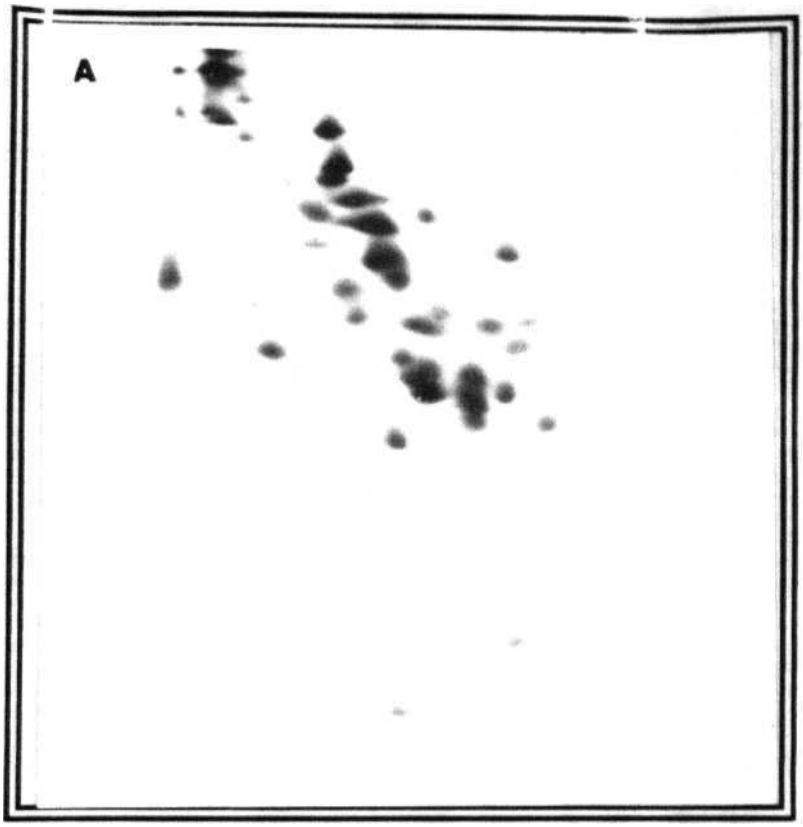
The ribosomal proteins from *S. acidocaldarius* and *E. coli* were fractionated by novobiocin as described in the materials and methods. Since higher concentrations of urea (> 4 M) and the presence of SDS (>0.1%) inhibits the precipitation of proteins by novobiocin, ribosomal protein solutions at a concentration of 0.2 mg/ml were dialyzed against 20 mM Tris-Cl (pH 8.0), 7 mM 2-mercaptoethanol buffer containing 1 M ammonium chloride. Low concentrations of ribosomal protein were chosen to ensure better solubility. It was also reported that precipitation by novobiocin was more efficient in higher ionic strength buffers (Brand and Toribara, 1976). Novobiocin (sigma) was dissolved in water so as to get a concentration of 10 mg/ml and stored at -20 °C in the dark (the antibiotic is susceptible to light induced cleavage that appears to interfere with its ability to precipitate histones). Precipitation of proteins by novobiocin was performed in 20 mM Tris-Cl (pH 8 0) and 1 M ammonium chloride at 4 °C as described in materials and methods chapter.

S. acidocaldarius ribosomal proteins were fractionated into four groups, corresponding to those precipitated with 200 μg/ml (fig. 8B), 500 μg/ml (fig. *SC),* 1000 μg/ml novobiocin **(fig.** 8D) and the proteins soluble at 1000 μg/ml novobiocin **(fig.** 8E). The proteins precipitated at each stage was dissolved in 8 M urea and 7 mM 2-mercaptoethanol and subjected to 2-dimensional gel electrophoresis. The same procedure was also successful for the fractionation of ribosomal proteins from *E. coli* (**fig. 9B-E).** In case of *E. coli,* only three groups of proteins were obtained, those corresponding to 500 μg/ml (fig. 9C), 1000 |ig/ml **(fig.** 9D) and those proteins soluble at 1000 μg/ml novobiocin (fig. 9E). Unlike *S. acidocaldarius,* in the case of *E. coli* no proteins were precipitated at 200 jag/ml novobiocin (fig. 9B). Proteins precipitated at each stage contained specific proteins

Fig. 8: Two-dimensional gel electrophoretic patterns of novobiocin fractions of *S. acidocaldarius* ribosomal proteins:

Fractionation of ribosomal proteins with novobiocin and electrophoresis were carried out as described in the text.

(A) Total ribosomal proteins 125 μg, (B) to (D) 200, 500, 1000 μg novobiocin precipitated fractions respectively and (E) 1000 |ig novobiocin supernatant fraction. 60 μg protein of each of the novobiocin fraction was electrophoresed.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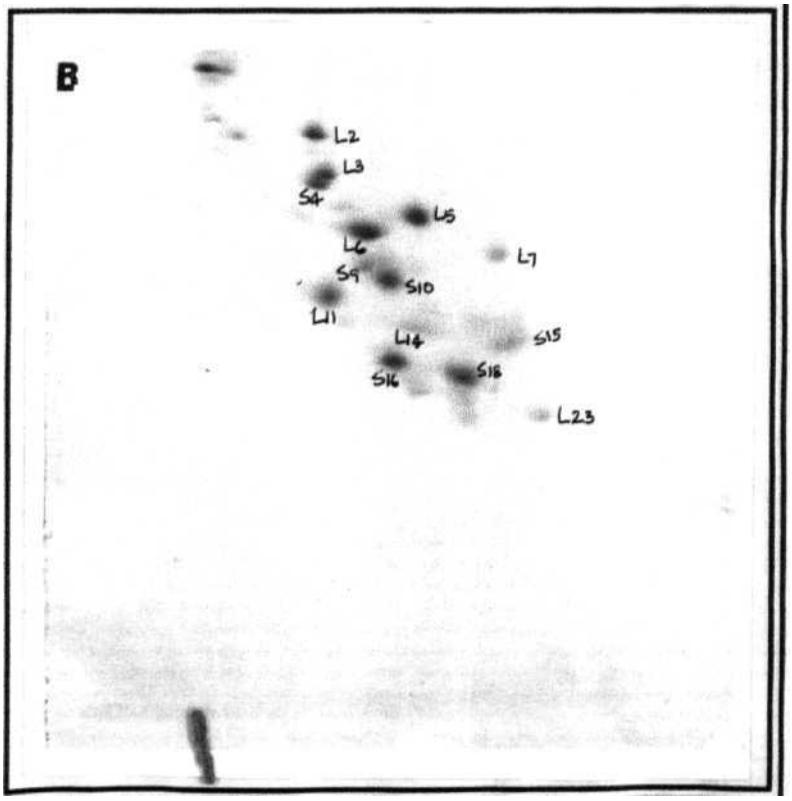
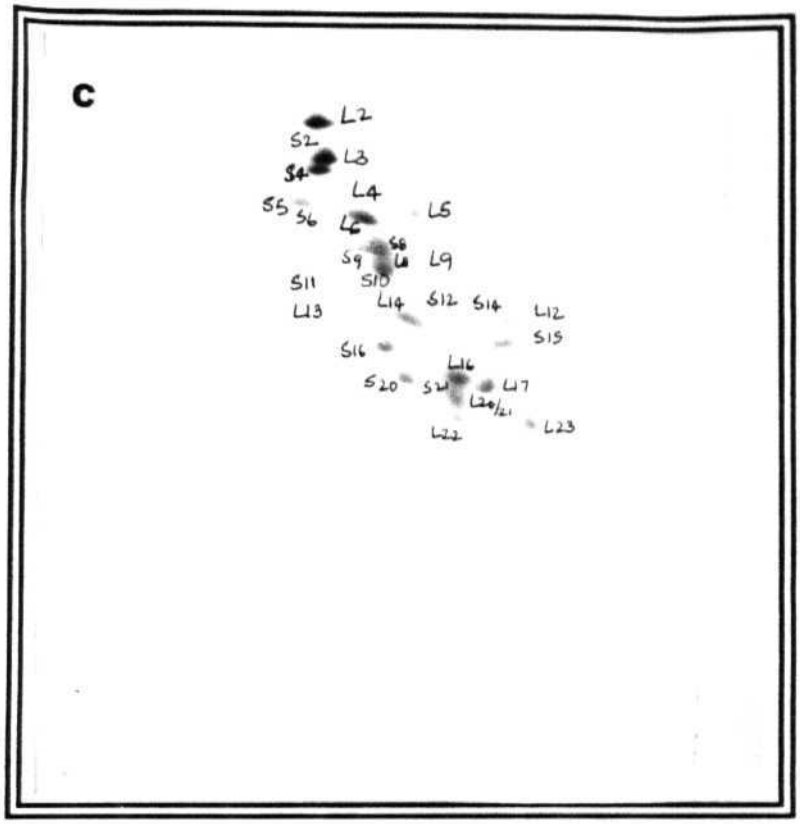
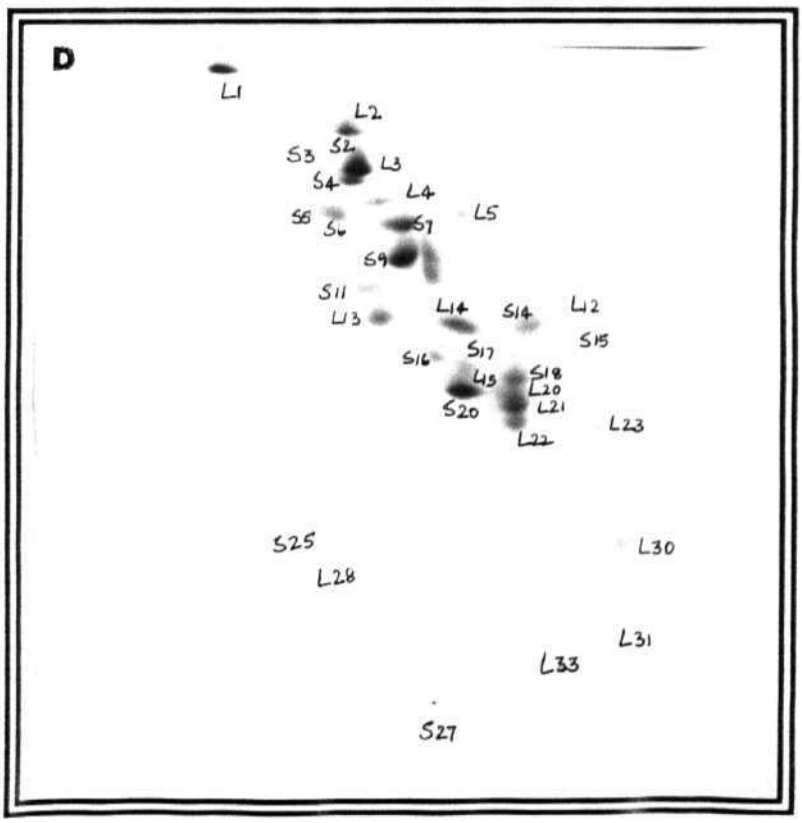

B
L2
L3
S4
L5
L6
L7
S9
S10
L11
L14
S15
S16
S18
L23


C
L2
S2
L3
S4
L4
S5
S6
L5
S9
L9
S11
S10
L13
L14
S12
S14
L12
S15
S16
L16
S20
L17
L20/21
L22
L23


D
L1
L2
S3
S2
L3
S4
L4
S8
S6
S7
L5
S9
S11
L13
L14
S14
L12
S15
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L23
S25
L30
L28
L31
L33
S27

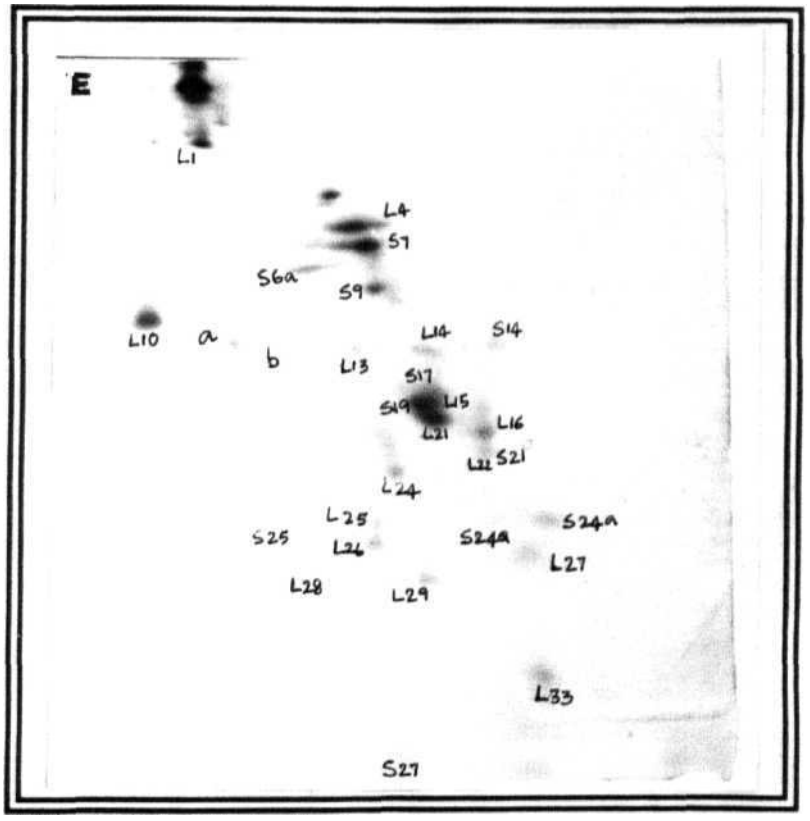
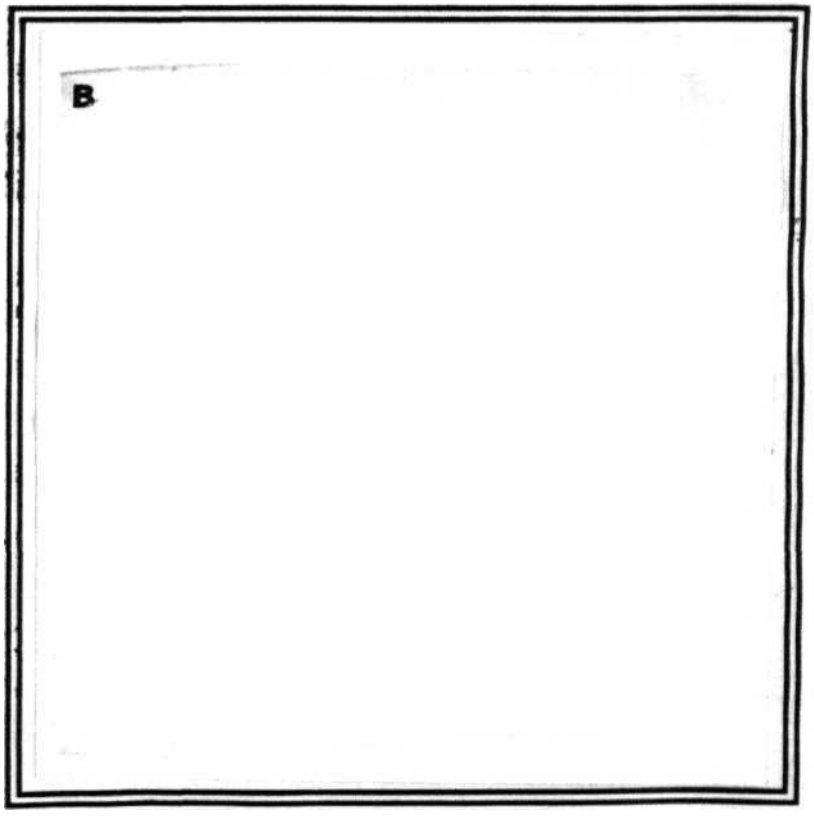
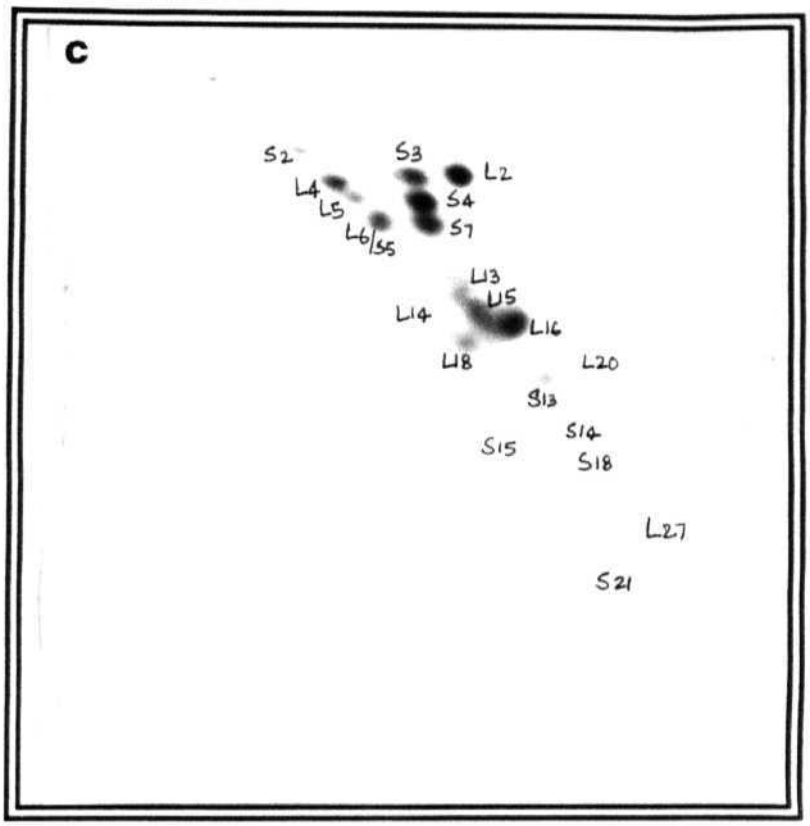
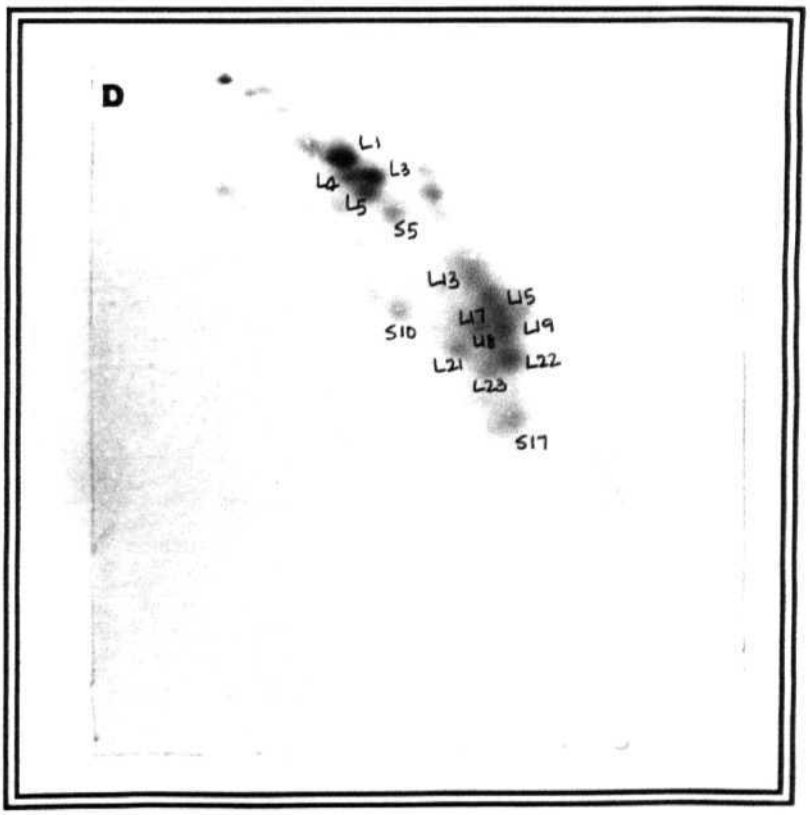

E
L1
L4
S7
S6a
S9
L10
a
b
L13
L14
S14
S17
S19
L5
L21
L16
L22
S21
L24
L25
S25
L26
S24a
S24a
L27
L28
L29
L33
S27

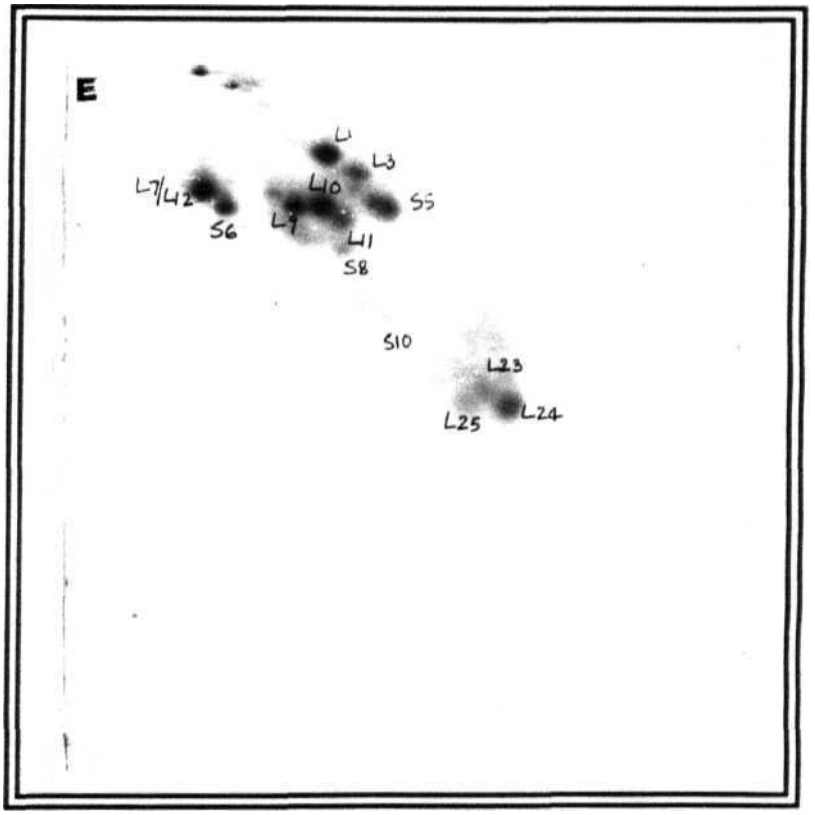
Fig. **9:Two-dimensional** gel **electrophoretic** patterns of novobiocin fractions of *E. coli* ribosomal proteins:
Fractionation of ribosomal proteins with novobiocin and electrophoresis were carried out as described in the text.
(A) Total ribosomal proteins 125 μg, (B) to (D) 200, 500, 1000 μg novobiocin precipitated fractions respectively and (E) 1000 (ig novobiocin supernatant fraction. 60 μg protein of each of the novobiocin fraction was electrophoresed.


A


B


C
S2
S3
L2
L4
L5
S4
S7
L6/S5
L13
L5
L14
L16
L18
L20
S13
S14
S15
S18
L27
S21


D
L1
L3
L4
L5
S5
L13
L5
L7
L9
S10
L8
L21
L22
L23
S17


E
L1
L3
L10
S5
L7/L12
S6
L9
L11
S8
S10
L23
L25
L24

which are present in large amounts. However, in each group there were small amounts of cross contaminating proteins belonging to other groups. This may be partly due to the contamination by small amounts of supernatant fluids at each step of the novobiocin fractionation and incomplete precipitation of some proteins at that novobiocin concentration. Different proteins precipitated at each novobiocin concentration were identified after comparing the two-dimensional gel patterns with those of 70S ribosomes and subunits of *S. acidocaldarius* and *E. coli*. The results are summarised in Tables **3** and **4.**

Discussion:

Ultra-centrifugation has been widely used to isolate ribosomes from a variety of organisms. In the literature, isolation of highly functionally active ribosomes without the use of ultra-centrifuge has been reported for *E. coli* ribosomes by Jelenc, (1980). We have modified this procedure for the isolation of ribosomes from *S. acidocaldarius*. We found that ribosomes isolated by ultra-centrifugation from alumina ground cells were contaminated with large amounts of high molecular weight non-ribosomal proteins as indicated by SDS-poly acrylamide gel electrophoresis. Like eukaryotic cells, *S. acidocaldarius*, can be lysed with non ionic detergents to prepare cell extracts. Earlier in our laboratory, *S. acidocaldarius* nucleoid was successfully isolated from detergent lysed cell extracts (Reddy and Suryanarayana, 1988; 1989). Ribosomes isolated by the procedure developed here, contain all the r-proteins as reported earlier for *S. acidocaldarius* ribosomes, isolated by a lengthy procedure involving ultra-centrifugation three times (Schmid and Bock, 1982).

Several techniques have been developed for the fractionation and purification of proteins. These include separation by precipitation with high salt

Table - 3

***Sulfolobus acidocaldarius* ribosomal proteins present in different novobiocin fractions**

Novobiocin fraction	Proteins present in large amounts	Proteins present in reduced amounts	Proteins present in trace amounts
200 µg/ml precipitate	L5, L6, L11, S10, S16, S18.	L2, L3, L7, L23, S4, S15.	L4, L17, L22, S9, S14, S20, S21.
500 µg/ml precipitate	L2, L3, L6, L8, L16, L17, S4, S8, S10.	L5, L12, L14, L20, L21, L22, S2, S9, S12, S14, S15, S16, S18, S20.	L9, L11, S5, S6, S9, S13.
1000 µg/ml precipitate	L5, L13, L14, L22, S2, S9, S14, S20, S21.	L2, L3, L16, L20, L23, L30, L31, S4, S10, S11, S16, S18, S27.	L4, L9, L12, L15, S3, S15, S17, S22.
1000 µg/ml supernatant	L1, L4, L6a, L10, L15, L21, S7, S17, S19.	L24, L25, L26, L27, L28, L29, L33, S2, S6a, S9, S14, S21, S24, S25, S26, S27, a .	L13, L14, L16, L22, S14, S18, S24, a, b, c.

Only the proteins whose spots are notably visible in the gel electropherograms are listed in the Table. Certain protein spots were faintly visualised and were exclusive to the group in which they are present. Faint spots may be due to weak staining by Coomassie blue, eg., low molecular weight basic proteins such as L32, L33, L34, S25, S26 and S27.

Table - 4

***E. coli* ribosomal proteins present in different novobiocin fractions**

Novobiocin fraction	Proteins present in large amounts	Proteins present in reduced amounts	Proteins present in trace amounts
200 µg/ml precipitate	---	---	L2, L4, L6, L16, S2, S3, S4, S5, S7.
500 µg/ml precipitate	L2, L4, L6, L14, L15, L16, L20, S3, S5, S7, S9, S13.	L5, L13, L14, L17, L20, L27, L30, S2, S14, S15, S18.	L27, L30, S21.
1000 µg/ml precipitate	L1, L3, L4, L5, L13, L15, L17, L19, L22.	L21, L23, S10, S16, S17.	L2, L6, L7/L12, L27, S2, S3, S4, S5, S6, S7, S16, S20.
1000 µg/ml supernatant	L1, L6, L7/L12, L9, L10, L11, L24, S5, S6.	L3, L13, L15, L19, L22, L23, L25, S8.	L13, L15, L17, L18, L19, L26, L29, L30, S19, S20.

Only the proteins whose spots are notably visible in the gel electropherograms are listed in the table. Certain protein spots were faintly visualised and were exclusive to the group in which they are present. Faint spots may due to weak staining by Coomassie blue, eg ., L29, L30, L32, S20, S21 etc.

concentrations (salting out), organic solvents, organic polymers and a variety of chromatographic procedures These methods have been widely used for the successful purification of a large number of proteins and enzymes. In the case of purification of ribosomal proteins extracted from ribosomes, no initial fractionation has been developed Total ribosomal proteins were processed by several chromatographic methods. Split proteins which are removed by salt washing of ribosomes were also used as starting material for purification. *S. acidocaidanus* ribosomal proteins could not be stripped in a step wise manner from ribosomes with increasing salt concentration. We have come across a report in the literature that novobiocin, a potent inhibitor of DNA gyfase, can precipitate purified histones in solutions (Cotten *et al.,* 1986). The precipitation of some purified proteins (histones, carbonic anhydrase, myoglobin, cytochrome-C and soyabean trypsin inhibitor) with novobiocin indicated that the effectiveness of precipitation was dependent on the arginine content as well as the arginine clusters in proteins. Those proteins with high arginine content were precipitated with low concentration of novobiocin (500 μg/ml), while those with the low arginine content, inspite of the basic nature of the proteins, either required high concentration of novobiocin for precipitation or did not precipitate at all (Cotten *et al.,* 1986). These observations on novobiocin precipitation of proteins prompted us to try novobiocin for differential precipitation of ribosomal proteins. As shown in the results section four groups of r-proteins were obtained by novobiocin precipitation. The relationship between the arginine content and clusters in *S. acidocaidanus* r-proteins and the precipitability by novobiocin could not be analysed as there is no information available on the amino acid composition/sequence of most of r-proteins from this organism. Therefore, *E. colt*

ribosomal proteins, whose amino acid composition and sequences are known were also fractionated by novobiocin.

In the case of *E. coli* ribosomes only three groups of proteins were obtained along with trace amounts of a few proteins precipitated at 200 fig/ml novobiocin. This may be due to the difference in the arginine content of the ribosomal proteins from the two organisms. *S. acidocaldarius* ribosomes apparently contain several ribosomal proteins with high arginine content such that they were precipitated using only 200 μg/ml novobiocin. It was reported (Qaw and Brewer, 1986) that there is a relationship between the arginine content of proteins and their thermal stability. Hence the precipitation of several ribosomal proteins of *S. acidocaldarius* by low concentrations of novobiocin (200-500 μg/ml) suggests that there may be a higher content of arginine in these proteins which in turn would imply an enhanced thermal stability of these proteins from this acidothermophilic organism.

Furthermore, a comparison of the fractionation patterns of the ribosomal proteins from the two organisms indicates that structurally homologous proteins identified between *S. acidocaldarius* and *E. coli* ribosomes are present in the same novobiocin fraction. Proteins denoted L1 and L10 in *S acidocaldarms* have been shown to be homologous to *E. coli* proteins L10 and L7/L12 These acidic proteins in both cases are present in the novobiocin 1000 jig/ml supernatant fraction. The two-dimensional gel electropherograms show that there is no relationship between the basicity of a protein and the concentration of novobiocin required for its precipitation. L24 which was not precipitated even by 1000 fig/ml novobiocin is more basic than the protein LU which was precipitated with 200 μg/ml concentration of the antibiotic. Furthermore, acidic proteins such as L10 of

S. acidocaldarius as well as one of the most basic proteins, L33, were present in the same group (1000 μg/ml novobiocin supernatant fraction).

Most of the proteins in the 500 fig/ml and the 1000 μg/ml novobiocin fractions of *E. coli* ribosomal proteins have a high arginine content (greater than 7 mole percent) except proteins S2 (5.4%) and Ll(4.1%). In the case of 1000 μg/ml soluble fraction, most of the proteins present had an arginine content lower than 7 mole percent except S6 (8.5%) and L23 (7.7%). These results therefore support the observation that precipitation by novobiocin is to a greater extent dependent on the arginine content of the proteins, the exceptions may be due to protein-protein interactions as well as the absence of arginine clusters in the proteins.

The individual groups of proteins fractionated by novobiocin may be used as starting material for the separation of individual proteins by ion exchange and gel filtration chromatography. We suggest that this method may also be useful for the fractionation of complex protein mixtures such as cell extracts.

3.2: Thermal stability of ribosomes and characterisation of 50S core particle

Adaptation of certain thermophilic organisms to natural hostile environments allows them to survive and grow even at temperatures close to (Brock *et al,* 1972; Brierly and Brierly, 1973; De Rosa *et al,* 1975), or higher than the boiling point of water (Morita, 1980). Thermally tolerant ribosomes that are capable of code translation with fidelity and efficiency are essential for bacterial growth and survival in extremely hot niches where, ribosomes from other organisms undergo denaturation. So the nucleic acid and protein interactions in these organisms may be stronger than in the conventional mesophilic organisms. In the present study, an attempt is made to understand the thermal stability of *S. acidocaldarius* ribosomes The rRNA and r-protein interactions were compared between *S. acidocaldanus* (which grows optimally at 87 °C and pH of 1-3) and mesophilic bacterium, *E. coli.* Thermal melting studies on rRNA and ribosomes from both the organisms were carried out Studies were also carried out on the ribosome core particles obtained by hydrophobic chromatography of ribosomes from *S. acidocaldarius* and *E. coli.*

Sepharose 4B chromatography of *S. acidocaldarius* and *E. coli* ribosomes:

S. acidocaldarius and *E. coli* ribosomes were also compared by hydrophobic chromatography using reverse salt gradient on sepharose 4B. Ribosomal particles containing defined set of r-proteins were isolated by different methods and their thermal stability was also studied The ribosomes are dissociated at low Mg^{++} concentrations and are brought to the verge of

precipitation at a high concentration of ammonium sulphate. Under these conditions they are strongly adsorbed onto sepharose 4B. The ribosomal subunits are eluted by a decreasing gradient of ammonium sulphate. The method is based on the difference in hydrophobic interaction of subunits with the agarose gel in the presence of very low concentrations of Mg^{++}.

Around 200 mg of 70S ribosomes in 10 ml buffer were applied to a column of sephadex G-50. Ammonium sulphate was added to a final concentration of 1.5 M to the ribosome fractions eluting from the column within the void volume. The ribosomes were applied to the column of sepharose 4B equilibrated with the buffer containing 0.02 M Tris-Cl (pH 7.5) containing 1.5 M $(NH_4)_2SO_4$. Elution was performed with three volumes of the same buffer but with a linear reverse salt gradient of $(NH_4)_2SO_4$(1.25 M-0.02 M) and 3 ml fractions were collected. The fractions were analysed by measuring absorbance at 260 nm.

The sepharose 4B chromatography was performed under three different buffer conditions viz., in the absence of Mg^{++}, in the presence of 1 mM Mg^{++}; and in the presence of 5 mM Mg^{++}.

When *S. acidocaldarius* 70S ribosomes were loaded and eluted on sepharose 4B in the absence of Mg^{++} using reverse salt gradient, ribosomes were fractionated into five groups as five different peaks (fig. 10) Each peak fractions were separately pooled and extracted with acetic acid for r-proteins and analysed by two-dimensional electrophoresis of Geyl *el al.* (1982), which revealed that each group contained predominantly a distinct set of proteins. The first peak contained stripped ribosomal proteins from both the subunits. These proteins were called as the split protein fraction-I. The second peak contained L7/L12 and some other ribosomal proteins from both the subunits. These proteins were called as split protein fraction-II. The third peak contained 30S particle containing some r-

Fig.10: Chromatography of *S. acidocaldarius* ribosomes on Sepharose 4B in **the** absence of Mg^{++} ions: Elution was carried out with 3 volumes of a linear reverse salt gradient {1.5 M- 0.02 M $(NH_4)_2SO_4$}.

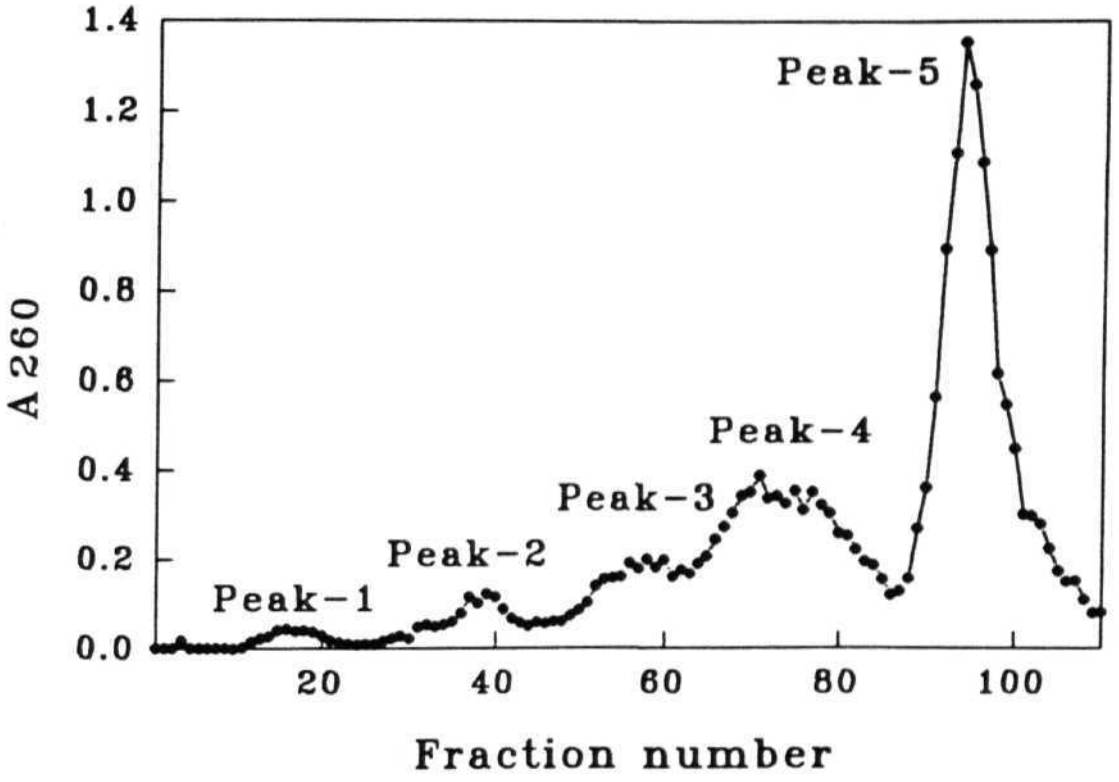

1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
A 260
Peak-1
Peak-2
Peak-3
Peak-4
Peak-5
20
40
60
80
100
Fraction number

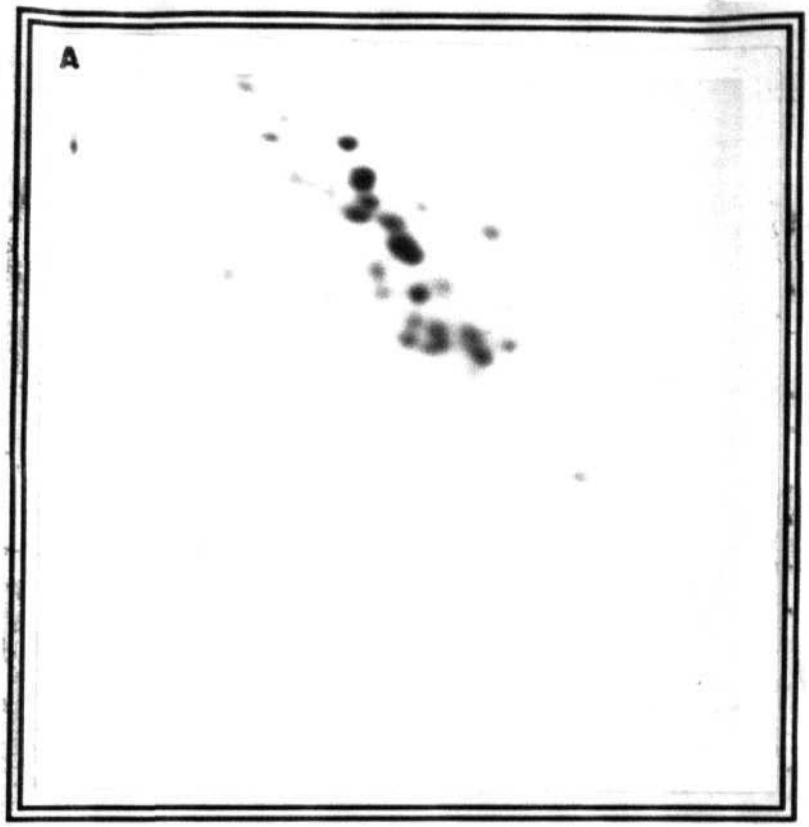
Figure 10

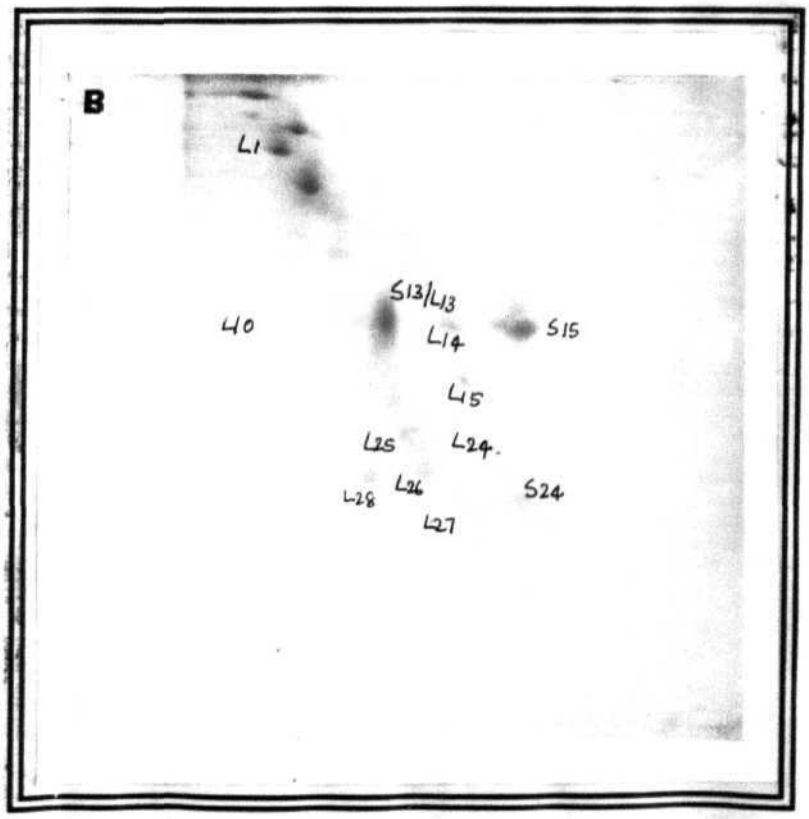
proteins in diminished amount. Peak 4 corresponds to the undissociated 70S particle which contains all the proteins from 30S and 50S subunits (albeit some proteins in reduced quantity). Peak 5 corresponds to SOS core particle and contains around 15-18 ribosomal proteins from the 50S subunit. The proteins present in each peak fraction were identified by comparing with the gel patterns of control 30S and 50S ribosomal subunit proteins (fig. 11 A; B; C; D; E; F) and the results are presented in **Table-5**.

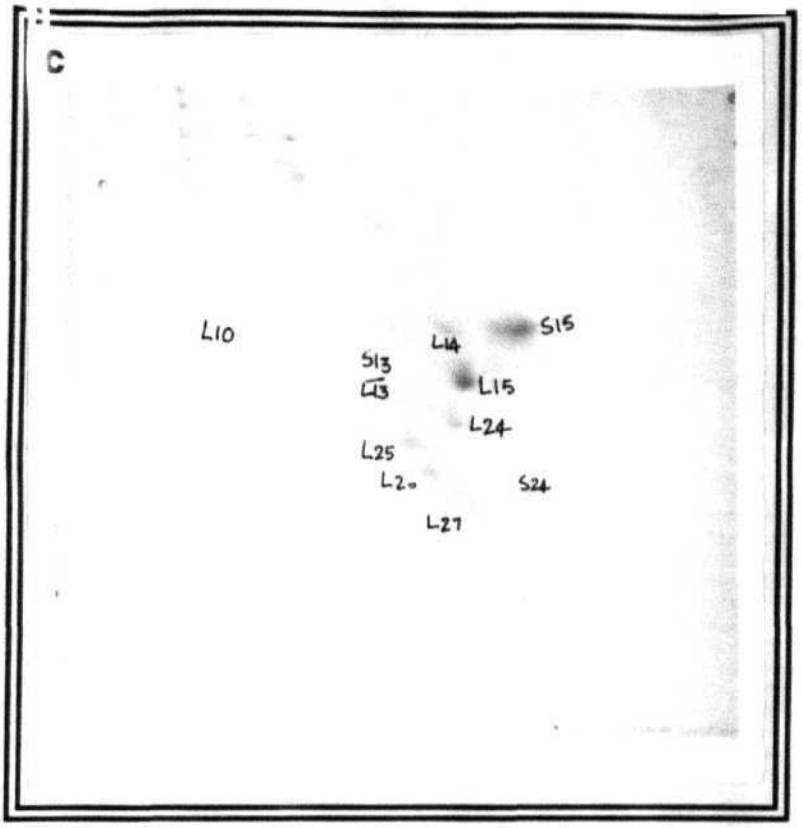
When *S. acidocaldarius* ribosomes were loaded and eluted from sepharose 4B in the presence of 1 mM Mg^{++} using reverse salt gradient, ribosomes were fractionated into three distinct peaks (fig. 12). In this case, the first peak represents 30S particle. The second peak represents the undissociated 70S particle containing all the ribosomal proteins from both the 30S and 50S subunits with some r-proteins in diminished amounts. The third peak contains 50S core particle proteins. The number of proteins in this group were from 15-18. The proteins present in each group were extracted with acetic acid. These extracted ribosomal proteins were subjected to two-dimensional gel electrophoresis and the proteins were identified as before (fig. 13A; B; C; D; Table- 6).

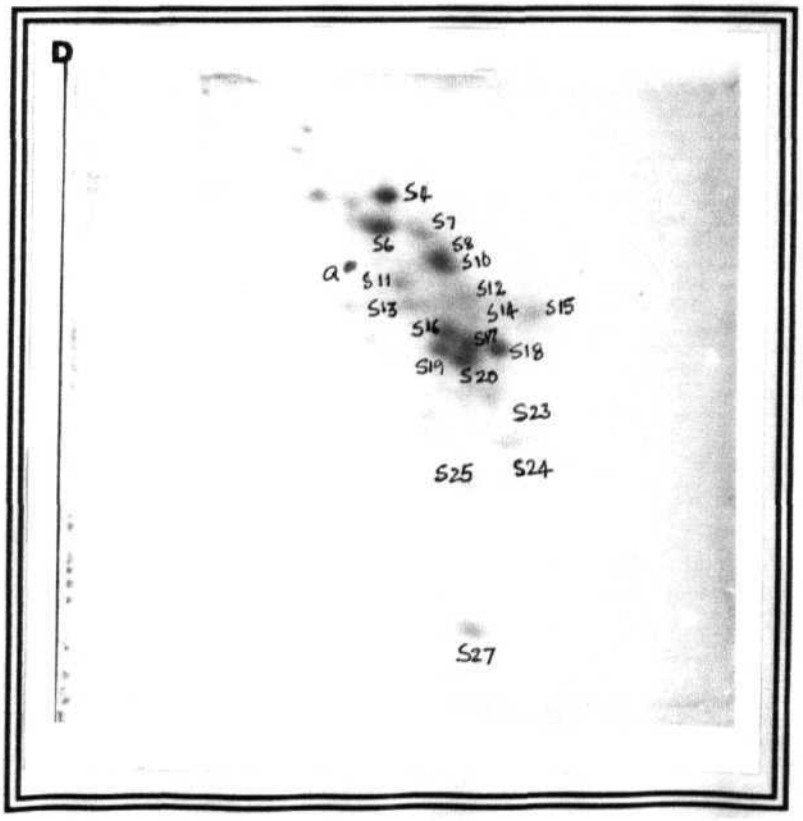
S. acidocaldarius ribosomes were chromatographed on sepharose 4B in the presence of 5 mM Mg^{++} using reverse salt gradient. The profile (fig. 14) was similar to that obtained when chromatography was performed in the presence of 1 mM Mg^{++}. Ribosomal proteins present in each peak were identified after two-dimensional gel electrophoresis (fig. 15A; B; C; D; Table-7). Although, the elution profiles (1 mM and 5 mM Mg^{++}) did not show any peaks corresponding to split proteins. The fractions eluting ahead of 30S peak contained some r-proteins (as in fig. 10).

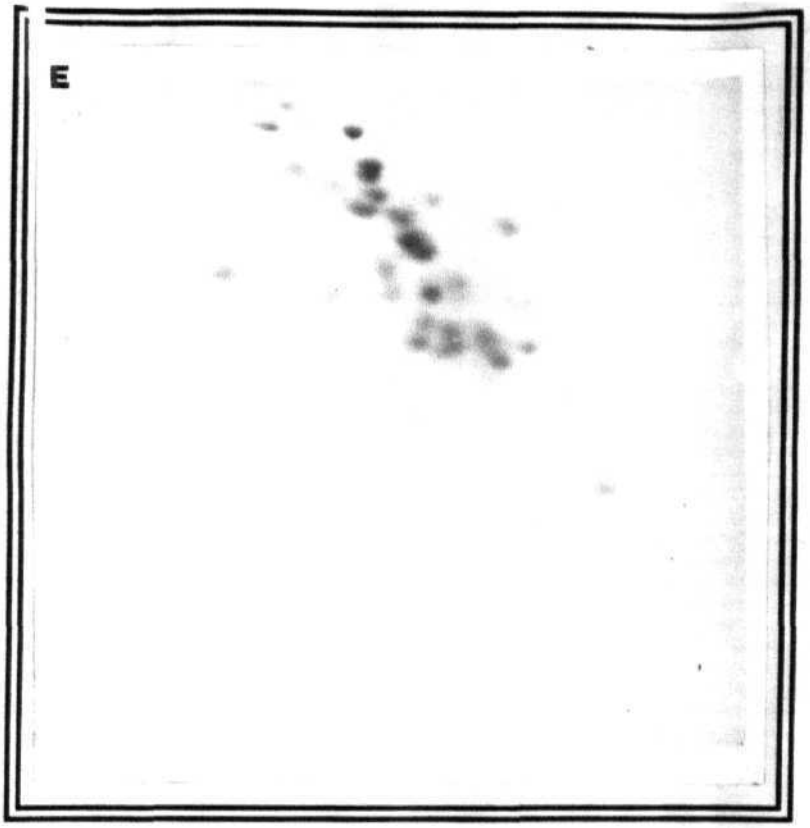
Fig. 11: Two-dimensional gel electrophoretic patterns of different peak fractions of sepharose 4B chromatography (no Mg^{++}): (A) Total ribosomal protein, (B) Peak-1 ribosomal proteins, (C) Peak-2 ribosomal proteins, (D) Peak-3 ribosomal proteins, (E) Peak-4 ribosomal proteins, (F) Peak-5 ribosomal proteins.


A


B
L1
S13/L13
L40
L14
S15
L15
L25
L24
L26
S24
L28
L27


C
L10
S15
L14
S13
L13
L15
L24
L25
L20
S24
L27


D
S4
S7
S6
S8
S10
a
S11
S12
S13
S14
S15
S16
S17
S18
S19
S20
S23
S25
S24
S27


E

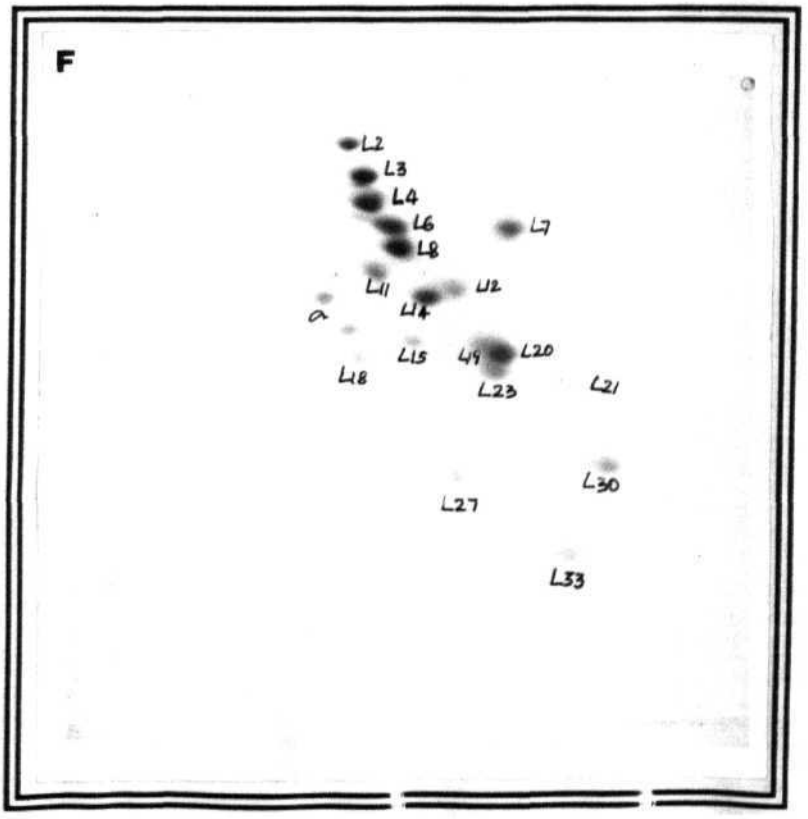

F
L2
L3
L4
L6
L7
L8
L11
U2
L14
a
L15
L9
L20
L18
L23
L21
L30
L27
L33

Table - 5

Sepharose 4B chromatography of *S. acidocaldarius* ribosomes in the absence of Mg^{++} ions.

Sepharose 4B Peaks	Proteins present
Peak-1 Split proteins	SI3. S15. S24. L1, LIO (faint). L13, L14 (faint). L15 (faint). L24 (faint). L25. L26, L27 L28.
Peak-2 Split proteins	S13, S15 (faint). S24. LIO (faint). L13, L14 (faint). L15, L24. L25. L26. L27
Peak-3 30S particle	S4. S6, S7, S8, SIO. S11, S12, SI3. S14, S15, S16, SI7. S18, SI9, S20. S23. S24. S25. S27. L10 (faint), a.
Pcak-4 70S particle	All 70S r-proteins are present
Peak-5 50S core particle	L2, L3, L4, L6, L7, L8, LU, L12, L14, L15, L18, L19, L20, L21, L23, L27, L30, L33, a, b.

Fig. 12: Chromatography of *S. acidocaldarius* ribosomes on Sepharose 4B in the presence **of 1 mM Mg^{++}** ions: Elution of ribosomes was affected by 3 volumes of a linear reverse salt gradient.

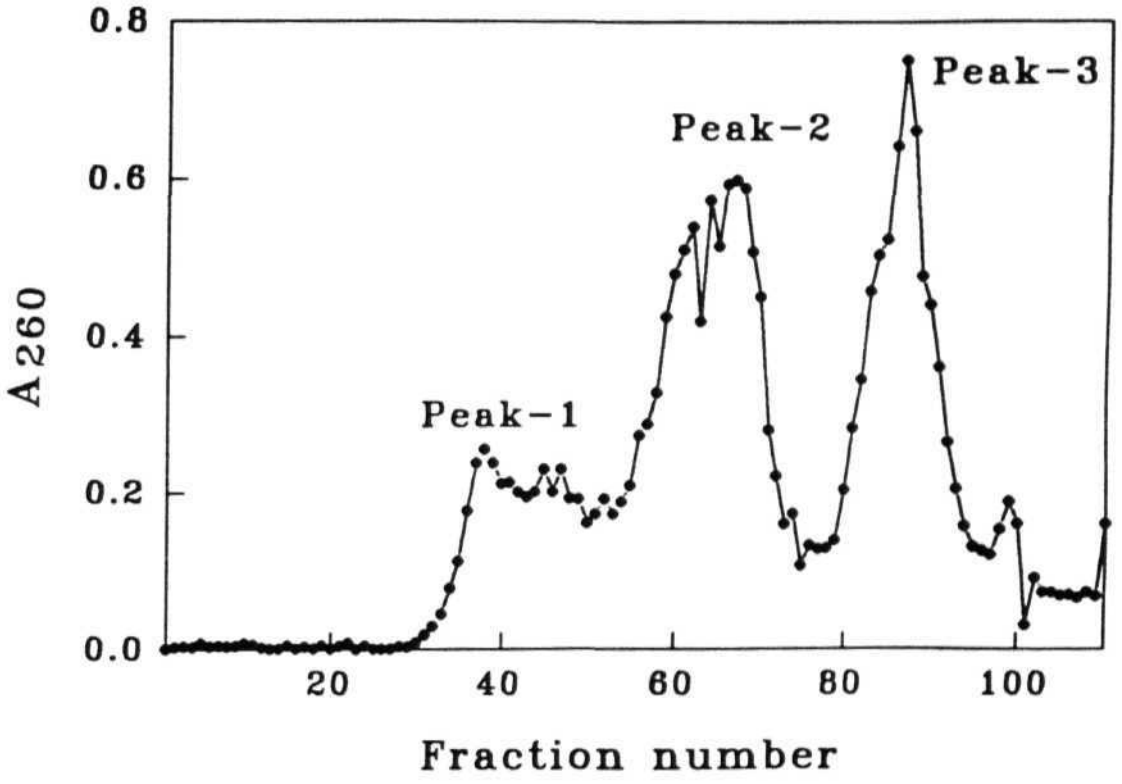
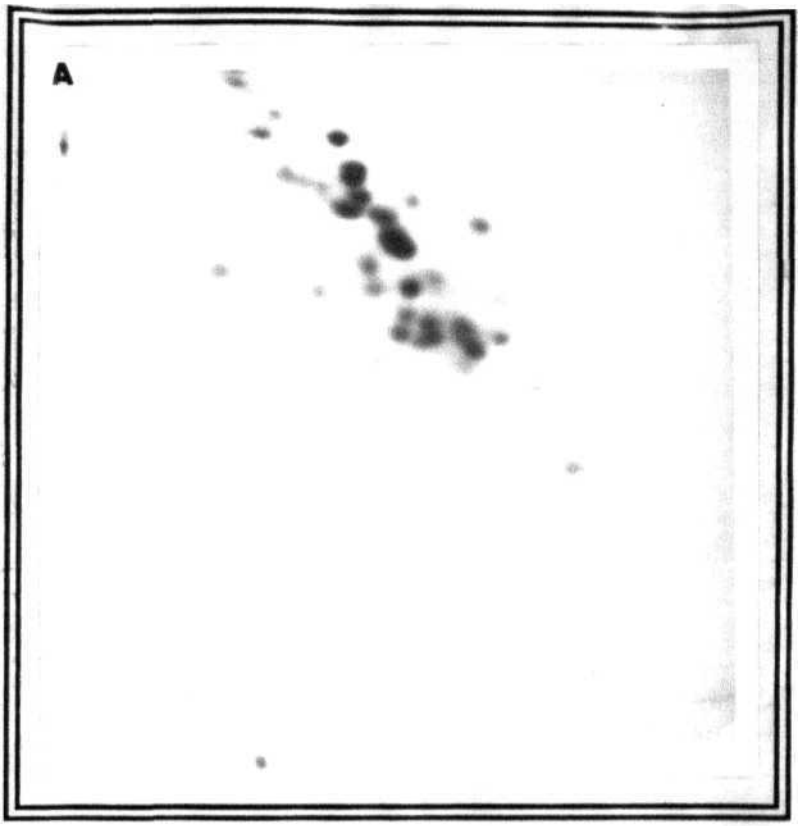
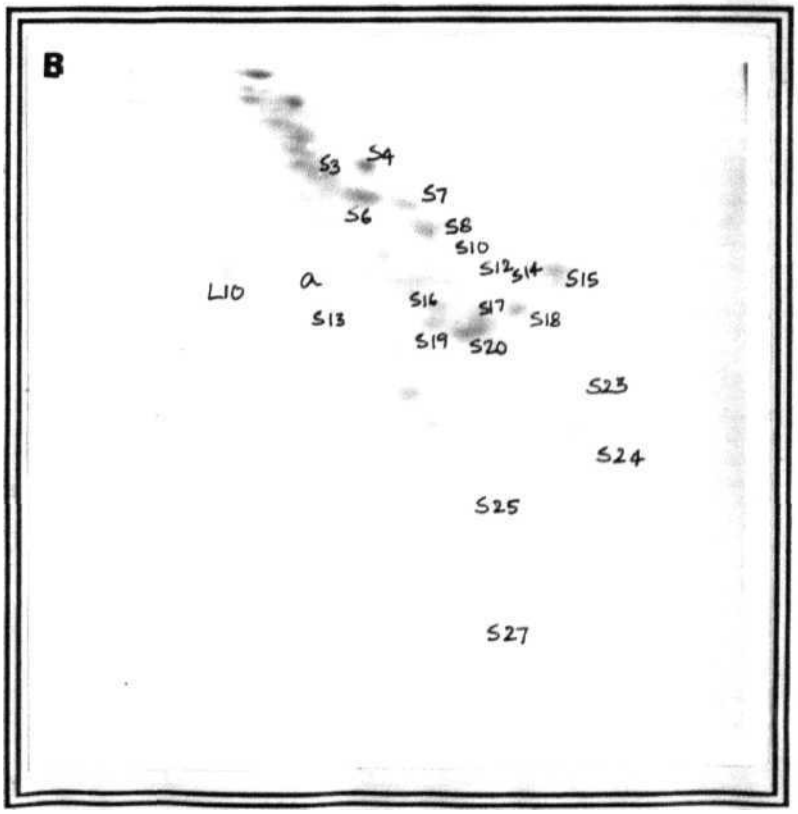

0.8
0.6
0.4
0.2
0.0
A260
Peak-1
Peak-2
Peak-3
20
40
60
80
100
Fraction number

Figure 12

Fig. 13: Two-dimensional gel electrophoretic patterns of different peak fractions of sepharose 4B chromatography (1 mM Mg^{++}): (A) Total ribosomal protein, (B) Peak-1 ribosomal proteins, (C) Peak-2 ribosomal proteins, (D) Peak-3 ribosomal proteins.


A


B
S3
S4
S7
S6
S8
S10
S12
S14
S15
L10
a
S16
S17
S18
S13
S19
S20
S23
S24
S25
S27


C

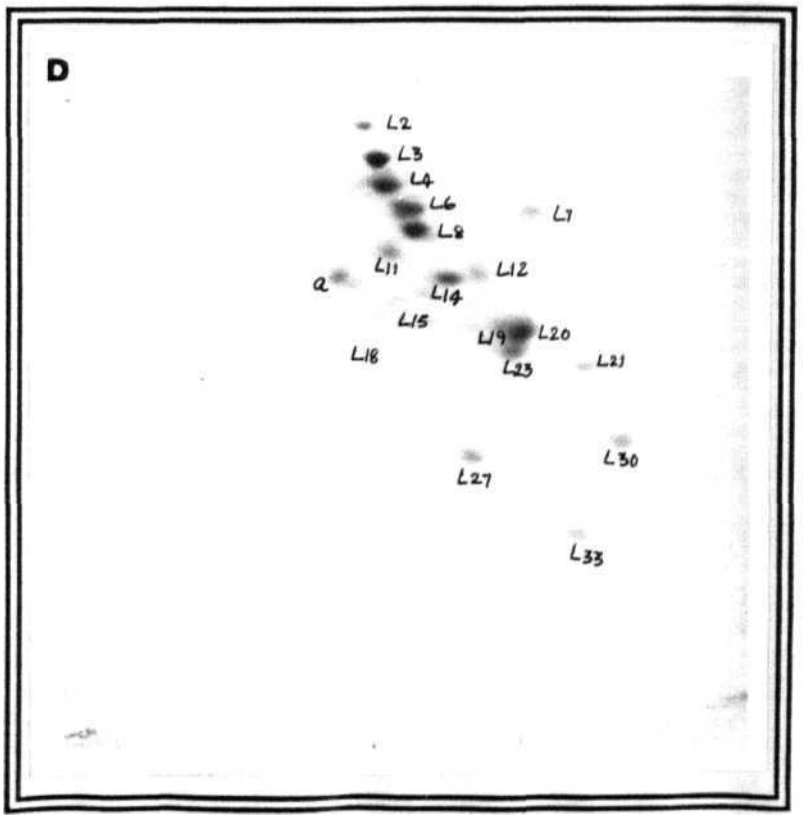

D
L2
L3
L4
L6
L7
L8
L11
L12
a
L14
L15
L19
L20
L18
L23
L21
L30
L27
L33

Table - 6

Sepharose 4B chromatography of *S. acidocaldarius* ribosomes in the presence of 1 mM Mg^{++} ions.

Sepharose 4B Peaks	Proteins present
Peak-1 30S panicle	S3. S4. S6. S7. S8. S10, S12, S13, S14, S15. S16. S17. SI8. S19, S20, S23, S24. S25. S27. L10 (faint), a.
Peak-2 70S Particle.	All 70S r-proteins are present
Peak-3 50S core particle	L2, L3. L4. L6. L7. L8. LU, L12, L14. L15. L18. L19, L20. L21. L23. L27, L30. L33. a.

Fig. 14: Chromatography of *S. acidocaldarius* ribosomes on Sepharose 4B in the presence of 5 mM Mg^{++} ions: Elution of ribosomes was affected by 3 volumes of a linear reverse salt gradient.

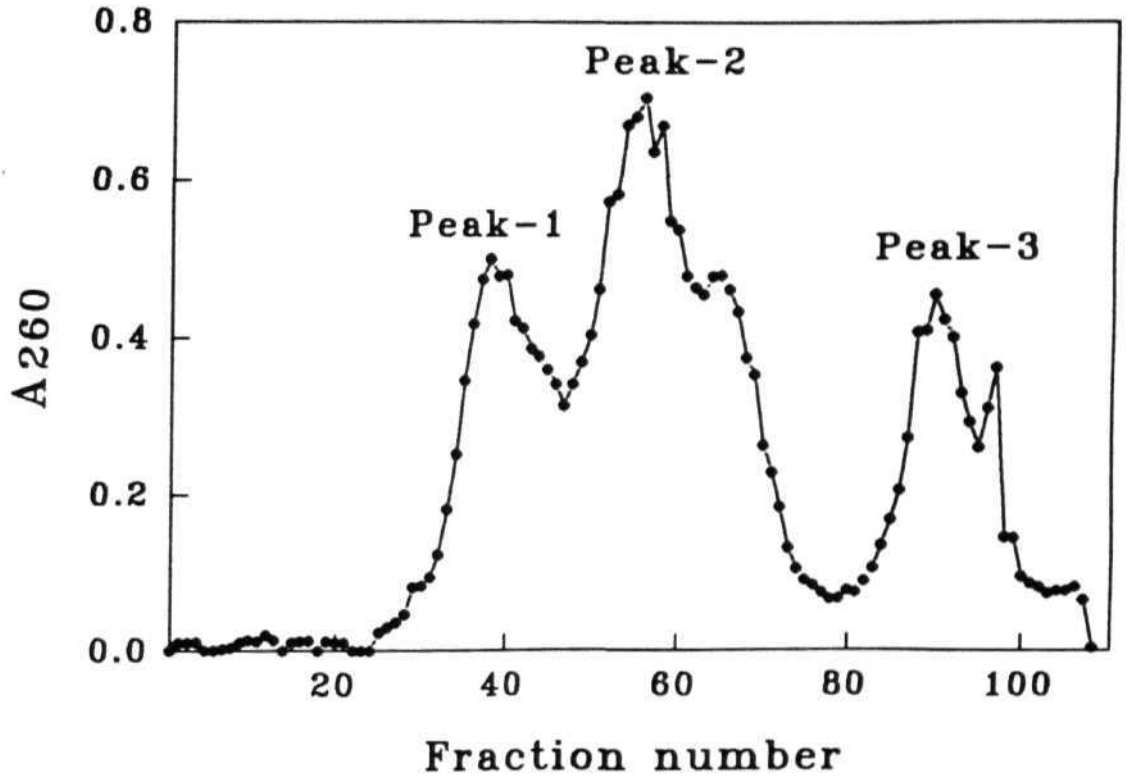
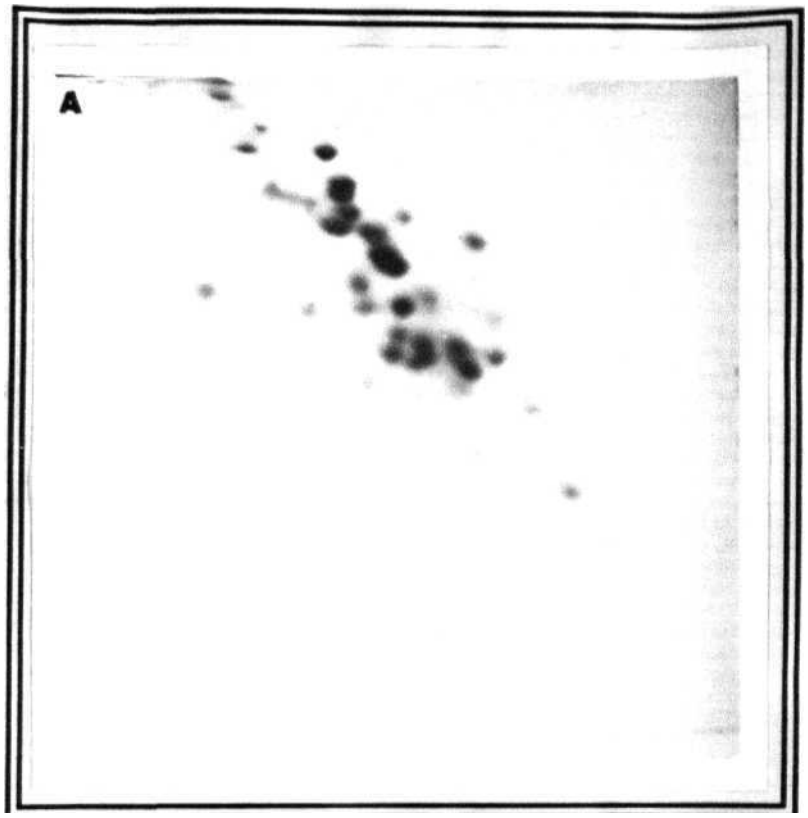
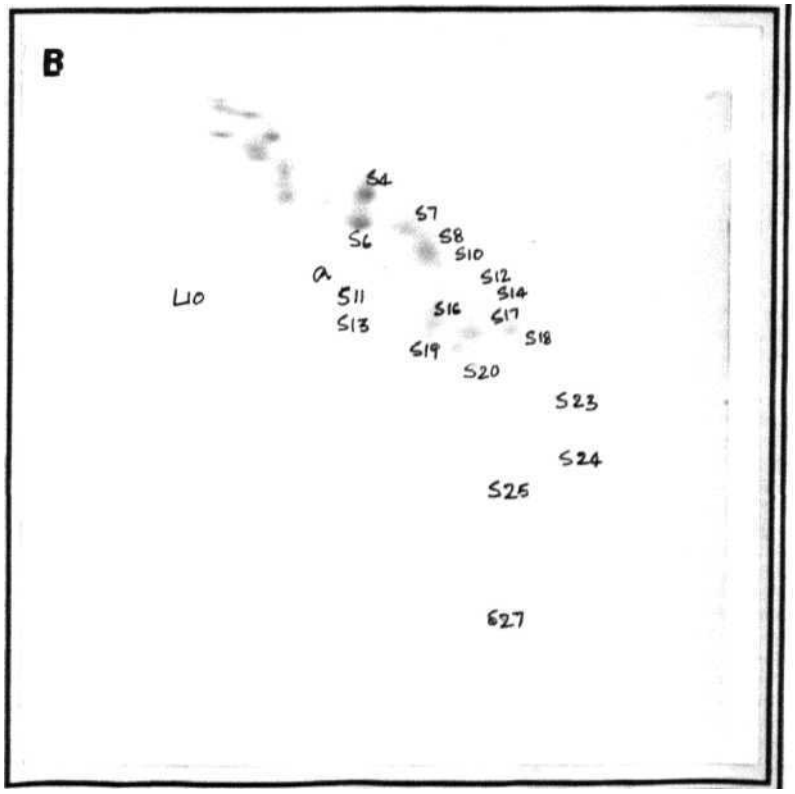
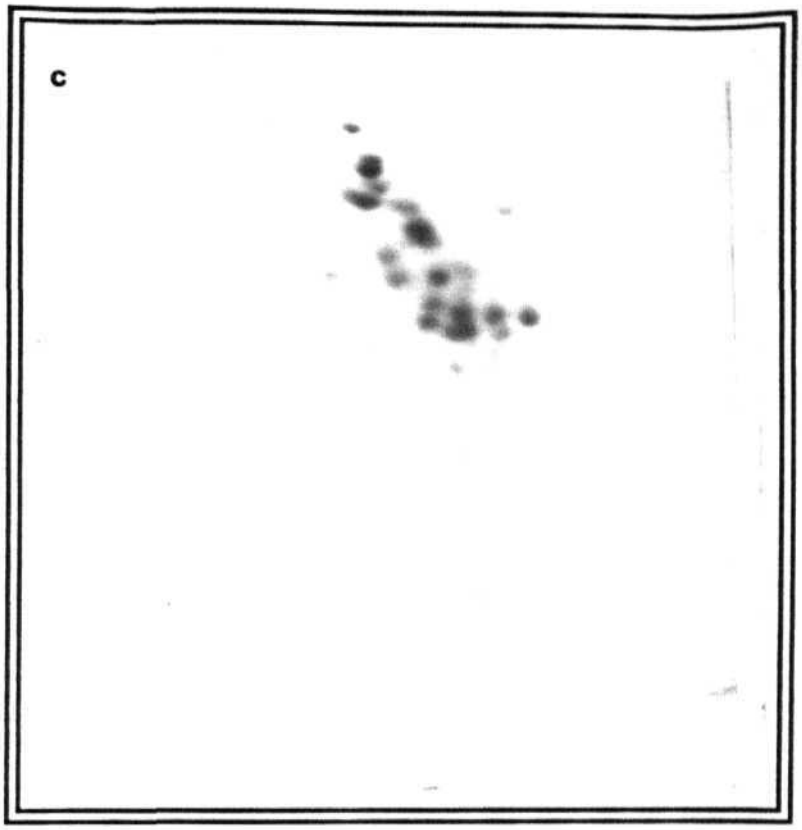

0.8
0.6
0.4
0.2
0.0
A260
Peak-1
Peak-2
Peak-3
20
40
60
80
100
Fraction number

Figure 14

Fig. 15: Two-dimensional gel electrophoretic patterns of different peak fractions of sepharose **4B chromatography (5 mM Mg^{++}):** (A) Total ribosomal protein, (B) Peak-1 ribosomal proteins, (C) Peak-2 ribosomal proteins, (D) Peak-3 ribosomal proteins.


A


B
S4
S7
S6
S8
S10
S12
S14
S11
S16
S17
S13
S18
S19
S20
S23
S24
S25
S27


c

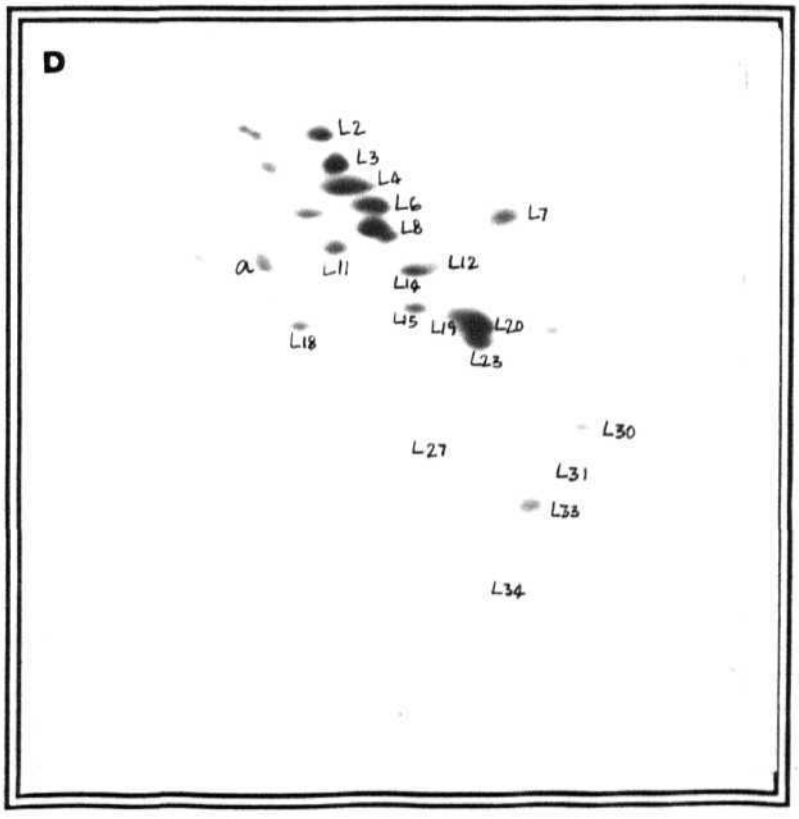

D
L2
L3
L4
L6
L8
L7
a
L11
L12
L14
L5
L19
L20
L18
L23
L30
L27
L31
L33
L34

Table - 7

Sepharose 4B chromatography of 5. *acidocaldarius* **ribosomes in** the presence of 5 mM **Mg^{++}** ions.

Sepharose 4B Peaks	Proteins present
Peak-1 30S particle	S4, S6, S7, S8, S10, S12, S13, S14, S15, S16, S17, S18, S19, S20, S23, S24, S25, S27, L10, a. b.
Peak-2 70S particle	All 70S r-proteins are present
Peak-3 50S core particle	L2, L3, L4. L6. L7, L8, LU, L12, L14. L15, L18, L19, L20, L23, L27, L30. L31,L33, L34, a.

We have performed sepharose 4B chromatography of *S. acidocaldarius* cell lysates. *S. aadocaldanus* S-30 was obtained by lysing the cells with Triton X-100 and NP-40 as described in the materials and methods section. The S-30 was dialysed against T_{20} 1.5 M $(NH_4)_2SO_4$ buffer for a period of 72 hours with four changes of buffer at 4 °C. After 72 hours, the precipitated material was removed by centrifugation of suspension at 15,000 RPM for 15 minutes. The clarified supernatant was loaded on to sepharose 4B and eluted with a reverse salt gradient. Four distinct peaks were obtained after elution (fig. 16), each peak was separately pooled and extracted with acetic acid for r-proteins and analysed by two-dimensional gel electrophoresis (fig. 17A; B; C). The first and second peaks contain S-100 proteins and stripped ribosomal proteins Whereas the third peak corresponds to undissociated 70S particles which contains all the ribosomal proteins from both the subunits. The fourth peak corresponds to the 50S core particle. The proteins present in both of these particles were identified (Table-8). The last peak obtained under all the conditions (of chromatography on sepharose 4B) is a 50S core particle which contains an identical set of proteins.

Sepharose 4B chromatography of *E. coli* 70S ribosomes in the absence of Mg^{++} ions:

For comparison, *E. coli* 70S ribosomes were loaded on sepharose 4B in the absence of Mg^{++} and eluted with a reverse salt gradient as described in the materials and methods section. As described previously by Kirrillov *et al.* (1978), *E. coli* ribosomes were fractionated into three distinct peaks, the first peak contained pure 30S subunits The second and third peaks contained two partially unfolded forms of 50S subunit showing a good separation on sepharose 4B (fig. 18). Two-dimensional gel electrophoresis of proteins of peak 1 shows that it

Fig. 16: Chromatography of *S. acidocaldarius* S-30 extract on Sepharose 4B in the absence of Mg^{++} ions: S-30 obtained from 20 grams of *S. acidocaldarius* cells was dialysed against buffer containing T_{20}, 1.5 M $(NH_4)_2SO_4$ for a period of 72 hours. The dialysed S-30 was chromatographed on sepharose 4B column as in fig. 1.

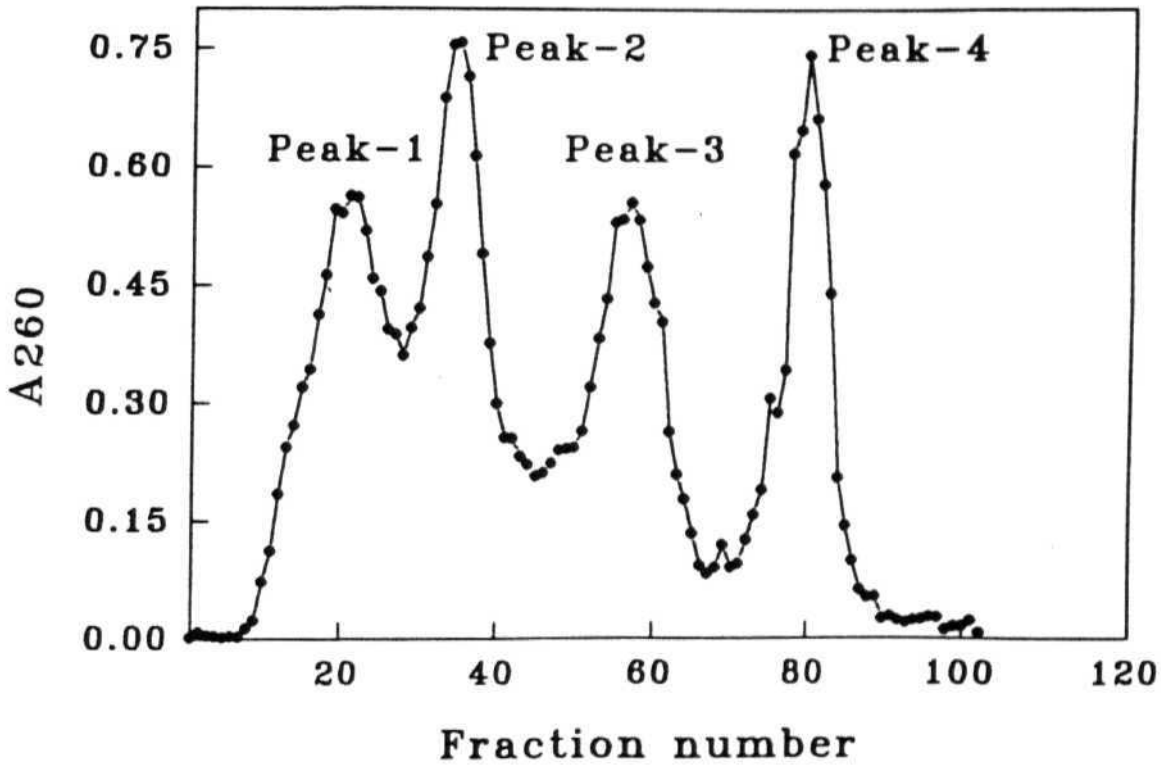
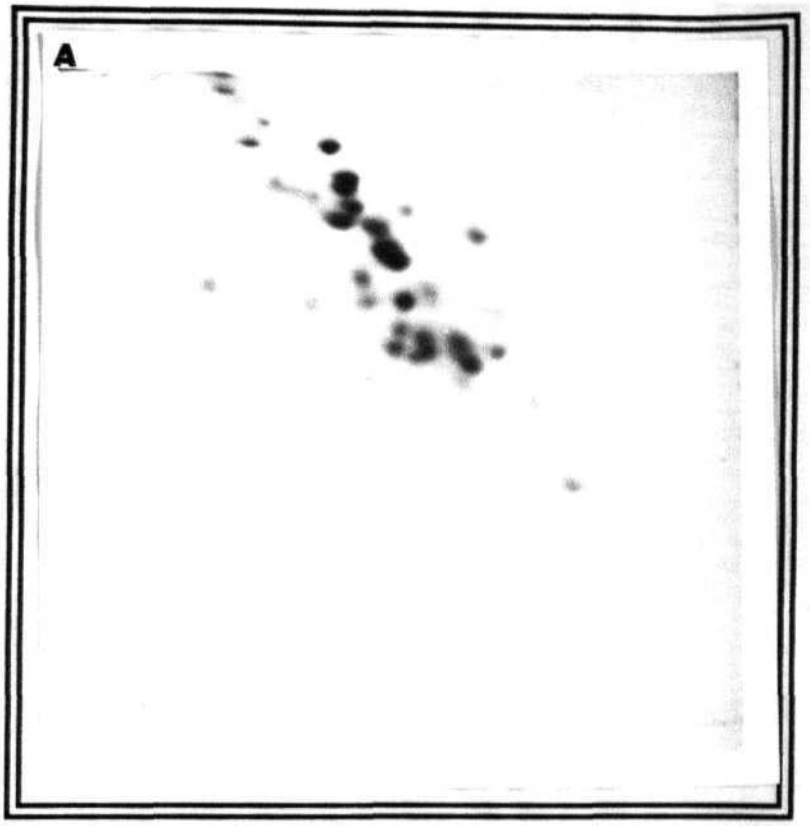
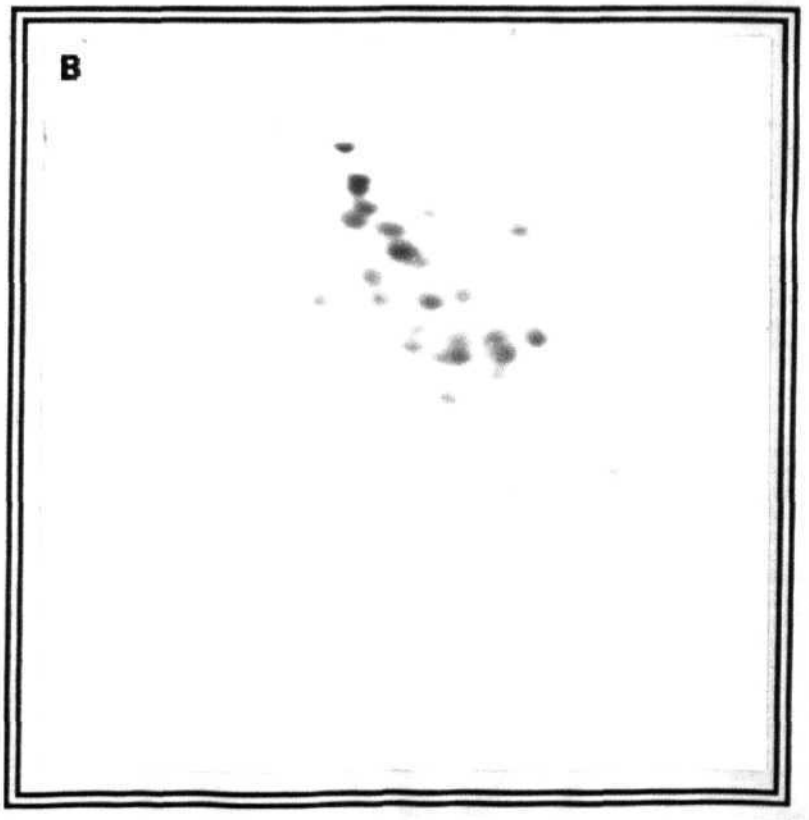

Peak-1
Peak-2
Peak-3
Peak-4
A260
0.75
0.60
0.45
0.30
0.15
0.00
20
40
60
80
100
120
Fraction number

Figure 16

Fig. 17: Two-dimensional gel electrophoretic patterns of different peak fractions of sepharose 4B chromatography (S-30):(A) Total ribosomal protein, (B) Peak-3 ribosomal proteins, (C) Peak-4 ribosomal proteins.


A


B

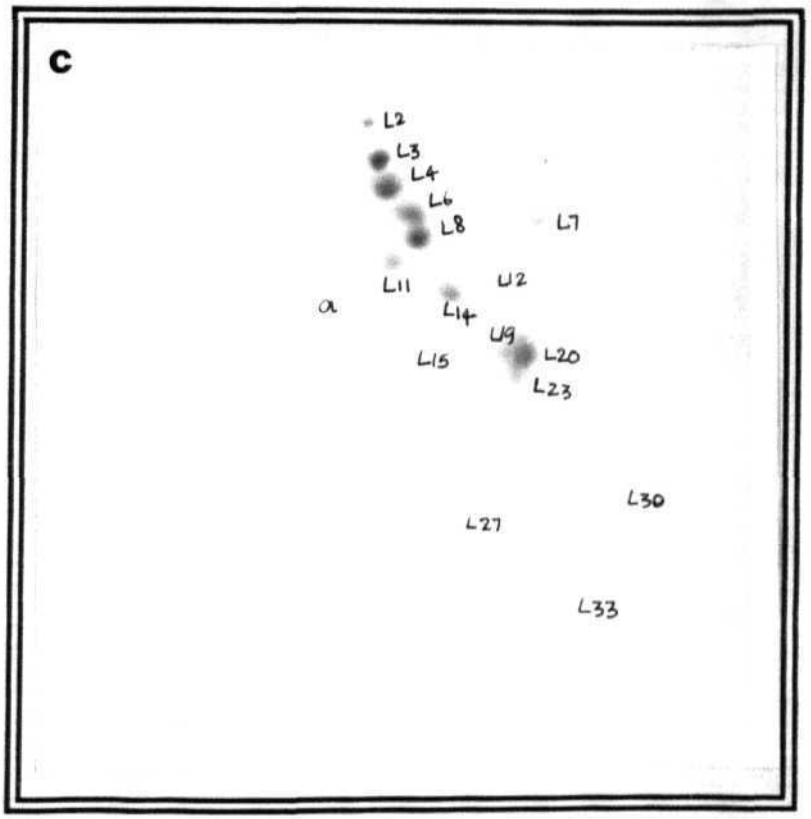

c
L2
L3
L4
L6
L8
L7
L11
L2
a
L14
L9
L15
L20
L23
L30
L27
L33

Table -8

Sepharose 4B chromatography of *V. acidocaldarius* S -30 in the absence of Mg^{++}.

Sepharose 4B Peaks	Proteins present
Peak-3 70S particle	All 70S r-proteins are present
Peak-4 50S core particle	L2. L3, L4. L6. L7. L8. L11. L12, LU. L15, L19, L20. L23, L27. L30. L33. a

Fig. 18: Chromatography of *E. coli* ribosomes on Sepharose 4B in the absence of Mg^{++} ions: Elution of ribosomes was affected by 3 volumes of a linear reverse salt gradient.

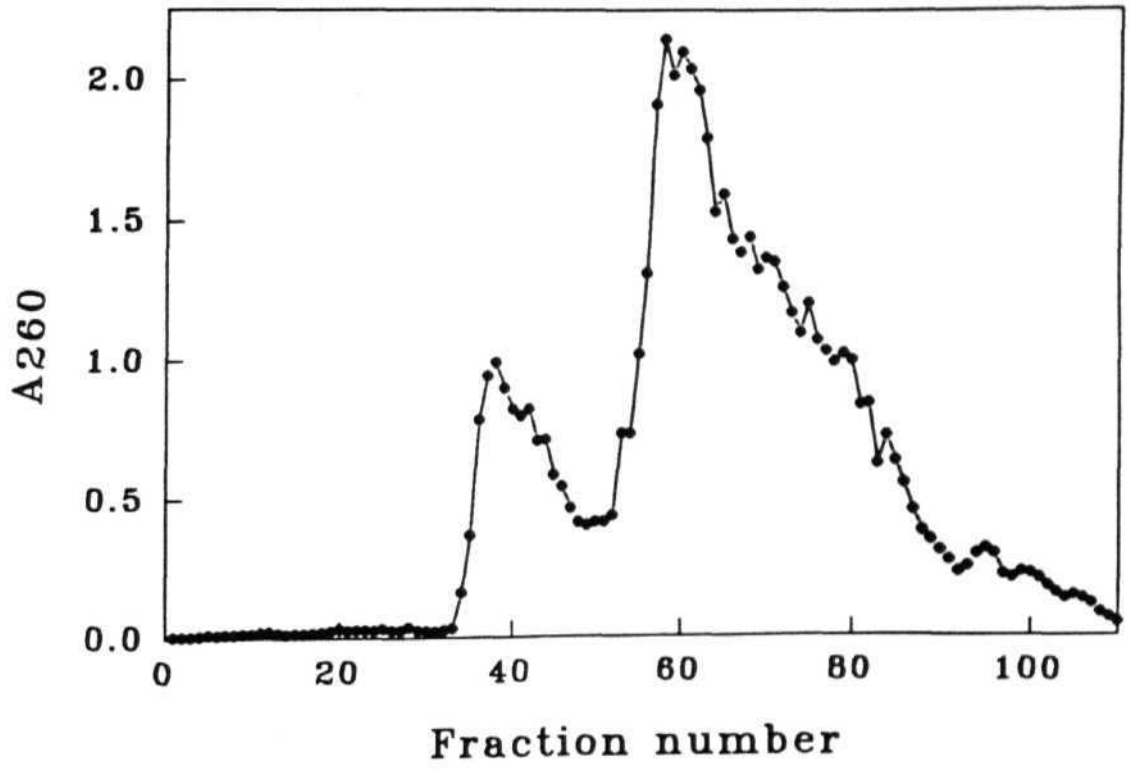


Figure 18

contains ribosomal proteins of 30S subunit whereas peak 2 and peak 3 contain some ribosomal proteins of *E. coli* 50S subunit as reported by Kirrillov *et al.* (1978).

Thermal melting analysis of ribosomes and rRNA from *S. acidocaldarius* and *E. coli*:

The thermal stability of *S. acidocaldarius* and *E. coli* ribosomes were monitored by following the increase in A_{260} with rise in temperature. The hyperchromicities vs temperature curves in fig (19A) show that the melting transitions *of S. acidocaldarius* ribosomes increase steeply after a threshold value. For instance, the melting range for *S. acidocaldarius* ribosomes was only 4 °C compared to 9 °C for the corresponding /:'. *coli* counterparts.

The tendency of ribosomes to aggregate on heating is avoided by keeping the Mg^{++} concentration low (0.08 mM). /:'. *coli* ribosomes appear to be stable upto 53-54 °C, after this temperature there was gradual increase in A_{260} The melting curve gave a Tm of 71 °C, *S. acidocaldarius* ribosomes do not show any increase in A_{260} until 73 °C and the melting curve gave a Tm of about 87 °C.

E. coli rRNA appeared to be stable upto 40 °C and displayed a Tm of 68 °C, whereas *S. acidocaldarius* rRNA which is found to be stable upto 50 °C, displayed a Tm of 80 °C (fig. 19B). In both cases, rRNA begins melting at temperatures much lower than the temperature at which ribosome denaturation is initiated, the melting transition being generally broader for rRNA than for ribosomes. These results indicate the large stabilisation of rRNA secondary structure by the binding of ribosomal proteins. However there are important quantitative differences between *E. coli* and *S. acidocaldarius* with respect to stability of rRNA in ribosomes

Fig. 19: Thermal melting profiles of ribosomes and rRNA from *S. acidocaldarius* and *E. coli*

(A) (o) *S. acidocaldarius* ribosomes.

(•) *E. coli* ribosomes.

(B) (o) *S. acidocaldarius* **rRNA.**

(•) *E. coli* rRNA.

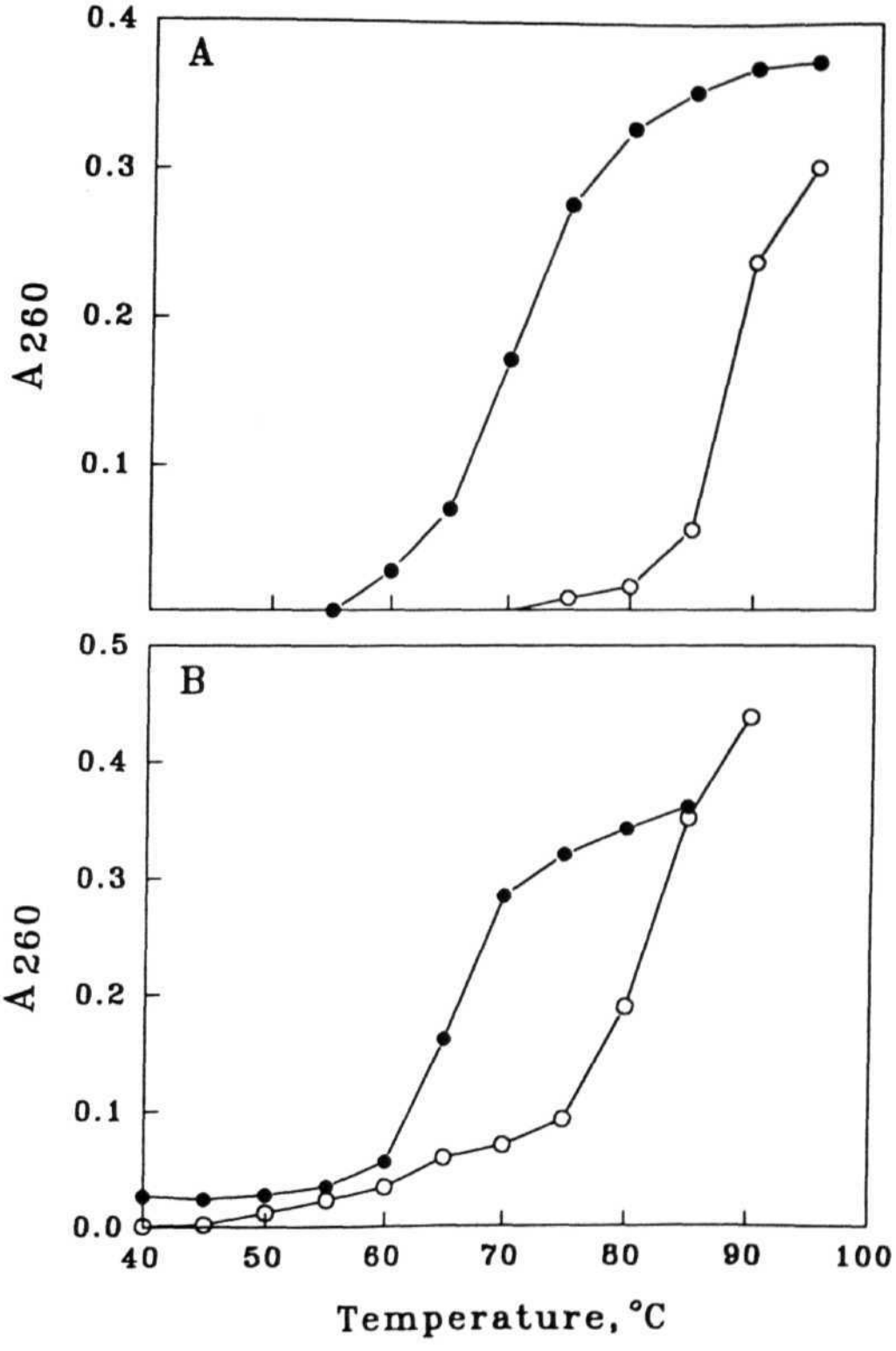

A
B
A 260
A 260
0.4
0.3
0.2
0.1
0.5
0.4
0.3
0.2
0.1
0.0
40
50
60
70
80
90
100
Temperature, °C

Figure 19

The significant result in fig (19A & B) is that the increment in ribosome stability with increasing thermophily is greater than the increment in stability for the isolated rRNA. This is evident by comparing the differences in δTm between ribosome and rRNA. The difference is small, only 3 °C for the *E. coli* rRNA and ribosomes, but is 8 °C for *S. acidocaldarius* rRNA and ribosomes. Accordingly, the ratio of ribosome Tm to that of rRNA Tm increases with increasing thermal tolerance of the organisms.

Thermal melting analysis of ribosomal subunits and ribosomal subunit rRNA from *S. acidocaldarius* and *E. coli:*

Intact salt washed ribosomal subunits were subjected to thermal melting analysis and compared to the free rRNA species melted in the same solvent (0.08 mM Mg^{++}). *S. acidocaldarius* 50S ribosomal subunit melts with a Tm of 87 °C (fig. 20A), which is equivalent to the Tm of *S. acidocaldarius* 70S ribosomes. In comparison to *S. aeidocaldarius* 50S subunits, 30S subunit melts with a Tm of 85 °C (fig. 20B) which is less by 2 °C. The free rRNA species from these subunits melt with a Tm of 80 °C (fig. 21 A) and 79 °C (fig. 21B) respectively.

E. coli large subunit rRNA melts with a Tm of 66 °C (fig. 21 A) and the small subunit rRNA melts with a Tm of 68 °C (fig. 21B) in comparison to ribosomal subunits which melt with a Tm of 72 °C (50S subunit fig. 20A) and 70 °C (30S subunit fig. 20B).

Thermal melting studies on the ribosomal particles obtained by the sepharose 4B chromatography were carried out The 30S particles, 70S particles and the 50S core particles obtained under different chromatographic conditions gave melting profiles with a Tm of 85.4 °C, 86.8 °C and 87 °C respectively (fig. 22, 23, 24, 25).

Fig. 20: Thermal melting profiles of ribosomal subunits from *S. acidocaldarius* **and** £. *coli*

(A) (o) *S. acidocaldarius* SOS ribosomal subunit.
(•) *E. coli* SOS ribosomal subunit.
(B) (o) *S. acidocaldarius* 30S ribosomal subunit.
(•) *E. coli* 30S ribosomal subunit.

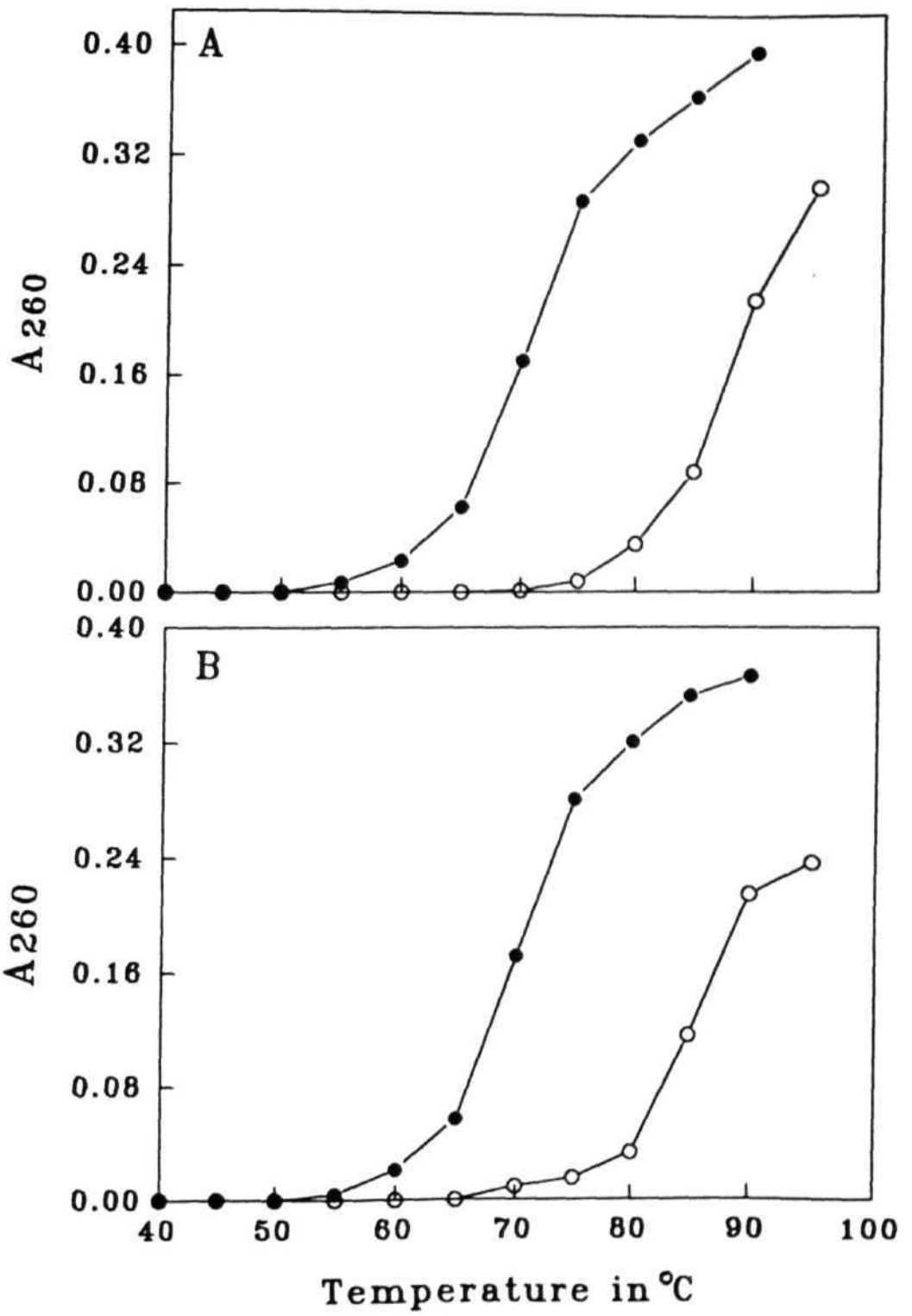

A
B
0.40
0.32
0.24
0.16
0.08
0.00
A260
40
50
60
70
80
90
100
Temperature in °C

Figure 20

Fig. 21: Thermal melting profiles of 23S and 16S rRNA from *S. acidocaldarius* and *E. coli*
(A) (o) *S. acidocaldarius* 23S rRNA, (•) *E. coli* 23S **rRNA.**
(B) (o) *S. acidocaldarius* **16S rRNA,** (•) *E. coli* **16S rRNA.**

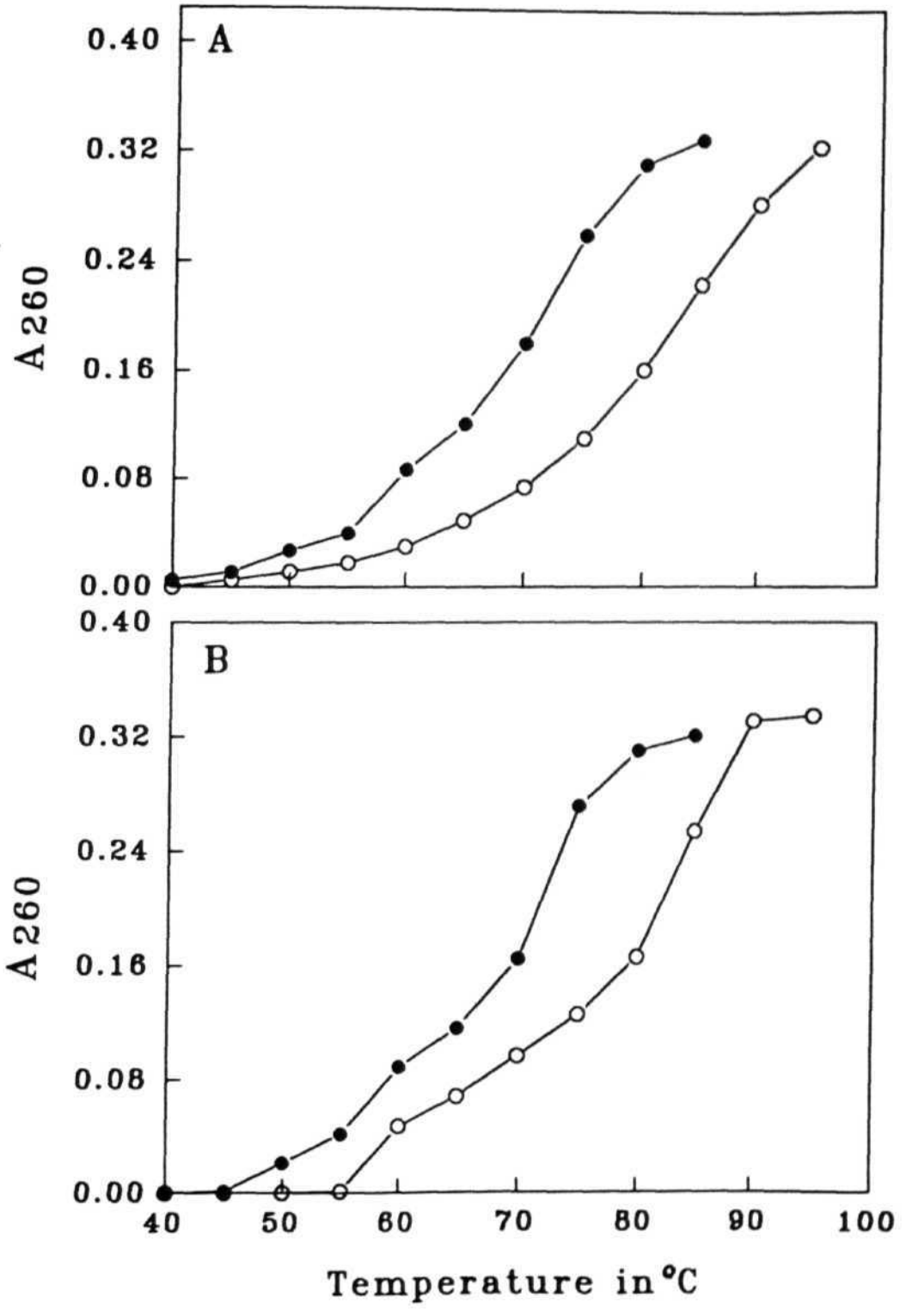

A
B
0.40
0.32
0.24
0.16
0.08
0.00
A 260
40
50
60
70
80
90
100
Temperature in °C

Figure 21

Fig. 22: Thermal melting profiles of ribosomal particles obtained by sepharose 4B chromatography of *S. acidocaldarius* **ribosomes (no Mg^{++}).**

(A) 30S particle (peak-3), (B) 70S particle (peak-4),
(C) 50S core particle (peak-5).

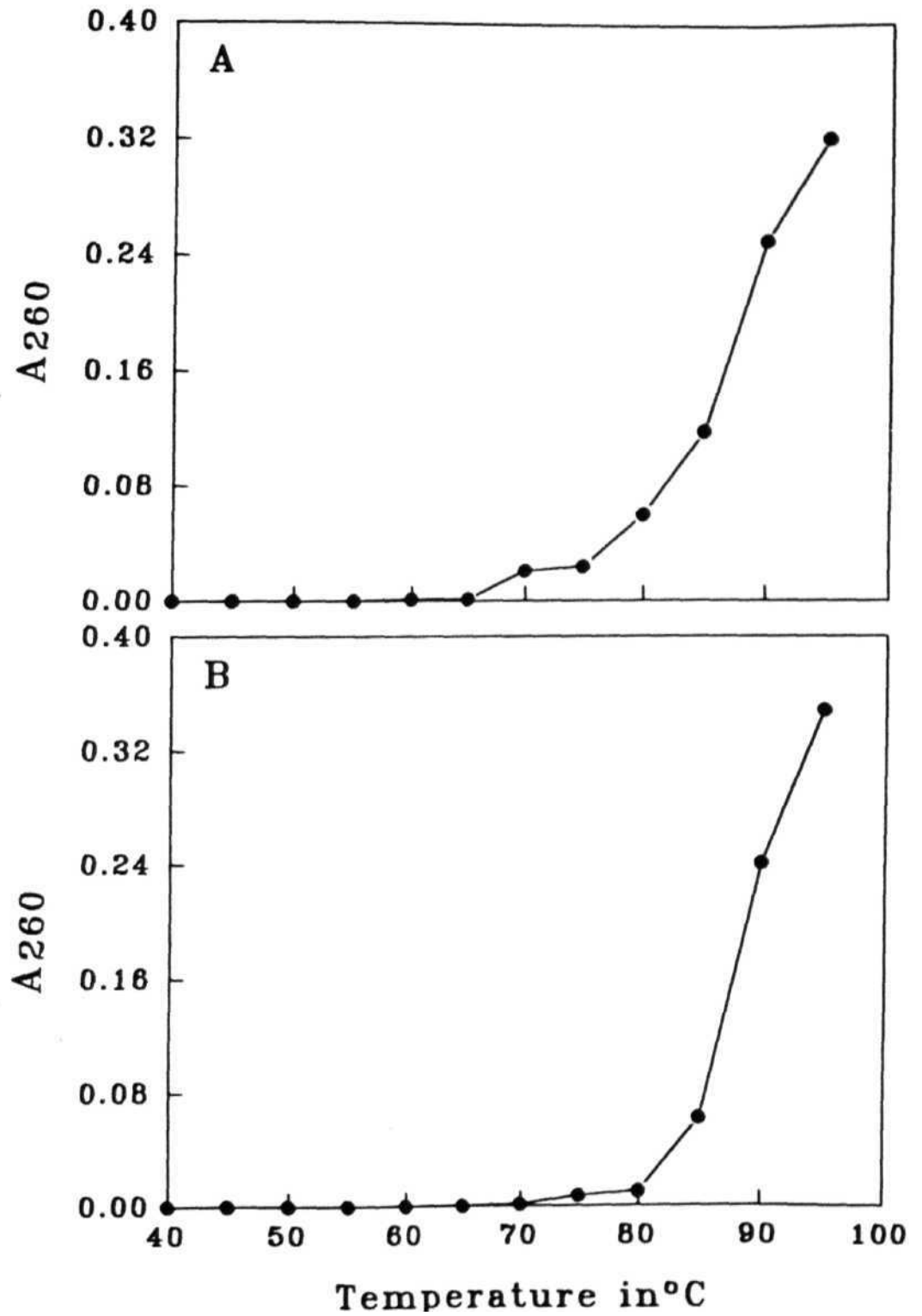

A
B
A260
A260
0.40
0.32
0.24
0.16
0.08
0.00
40
50
60
70
80
90
100
Temperature in°C

Figure 22

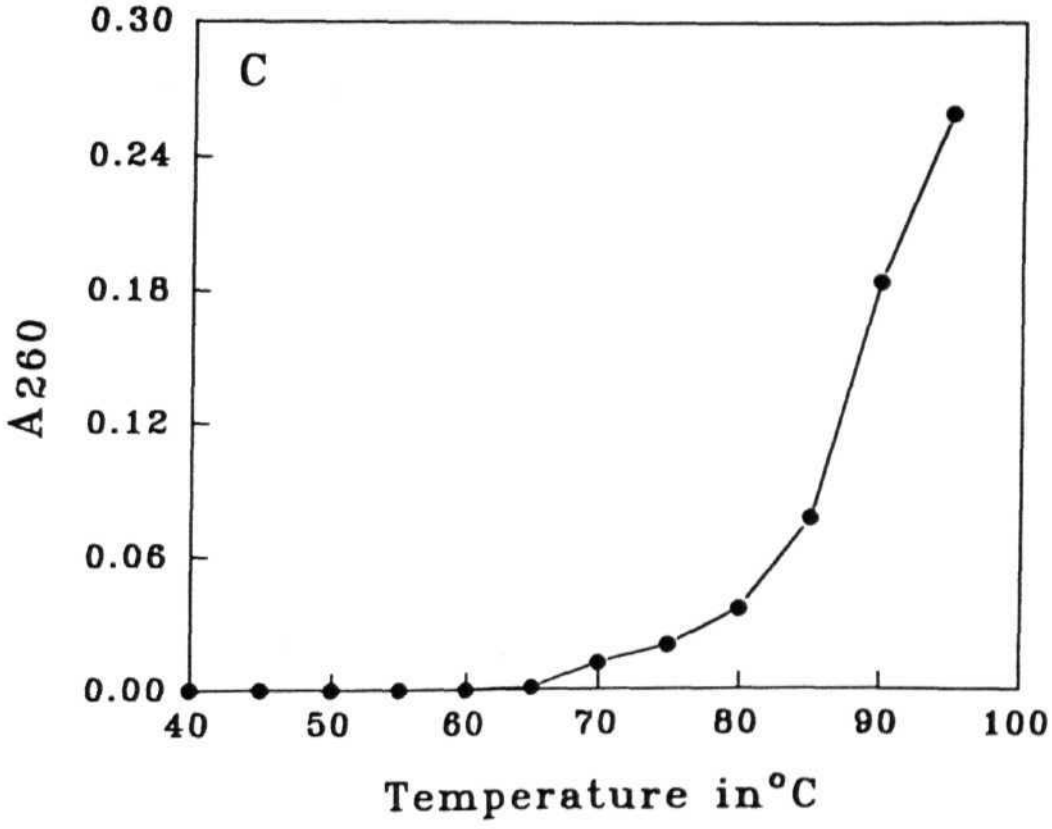

C
0.30
0.24
0.18
0.12
0.06
0.00
A260
40
50
60
70
80
90
100
Temperature in°C

Figure 22

Fig. 23: Thermal melting profiles of ribosomal particles obtained by sepharose 4B chromatography (1 mM Mg^{++}).
(A) 30S particle (peak-1), (B) 70S particle (peak-2),
(C) 50S core particle (peak-3).

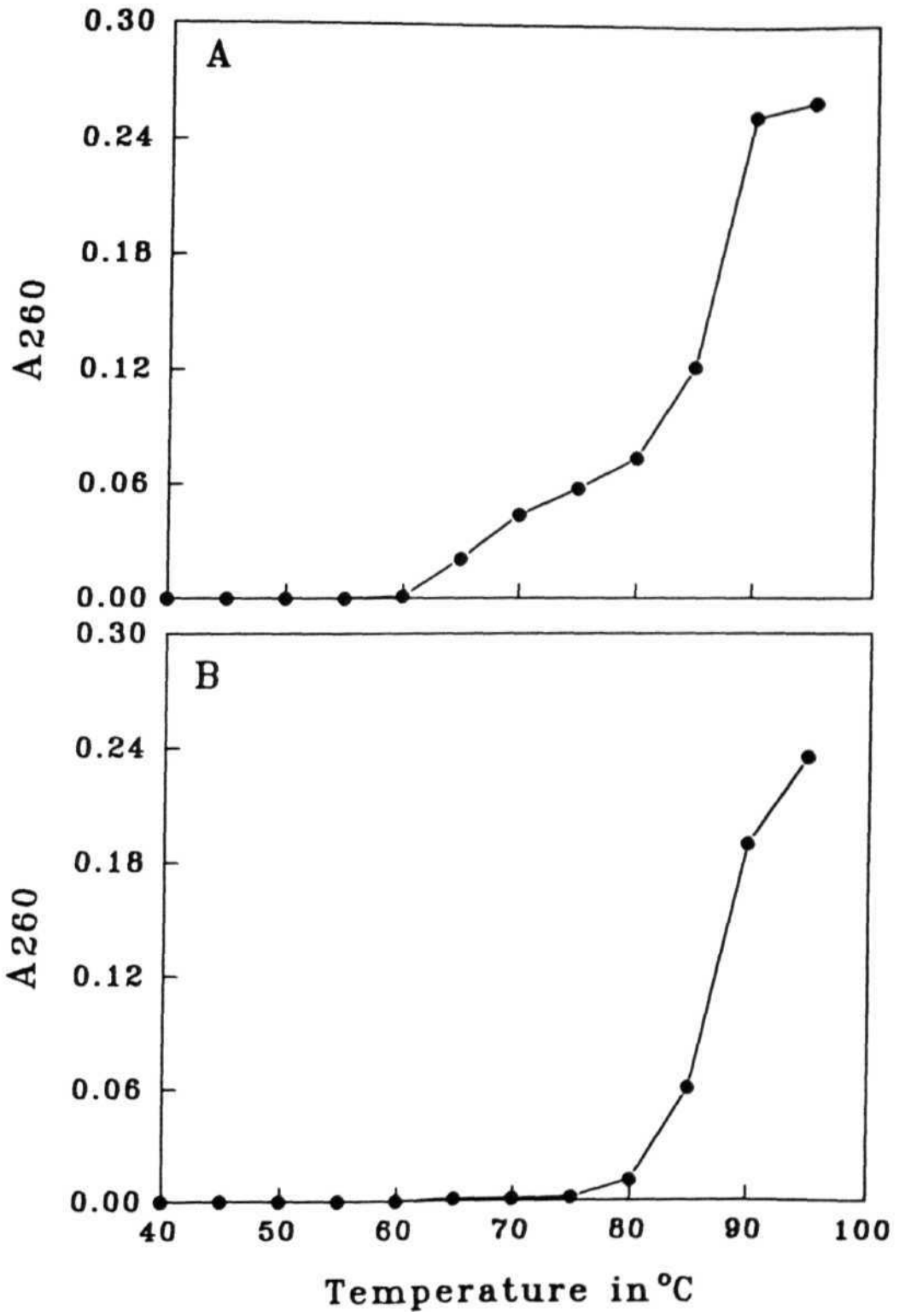

A
B
A260
A260
0.30
0.24
0.18
0.12
0.06
0.00
40
50
60
70
80
90
100
Temperature in °C

Figure 23

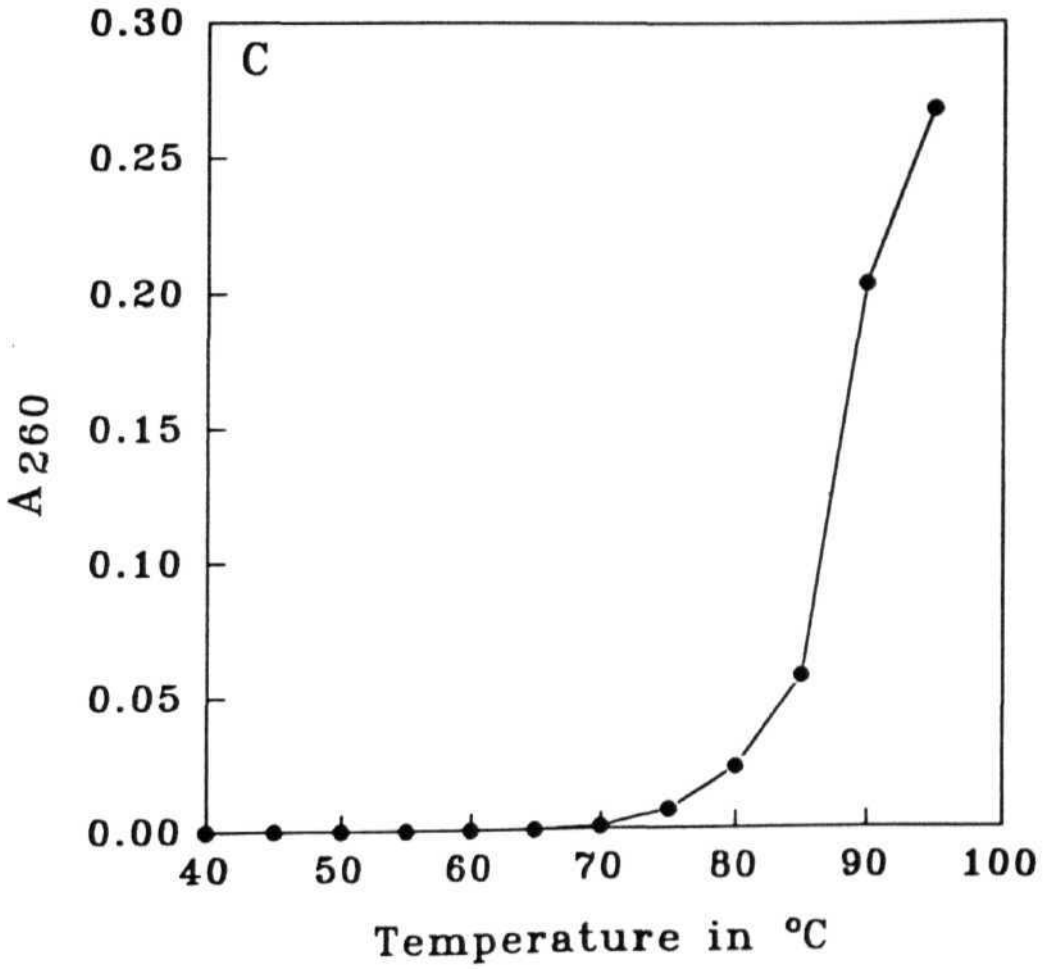

C
0.30
0.25
0.20
0.15
0.10
0.05
0.00
A 260
40
50
60
70
80
90
100
Temperature in °C

Figure 23

Fig. 24: Thermal melting profiles of ribosomal particles obtained by sepharose 4B chromatography (5 mM Mg^{++}).
(A) 30S particle (peak-1), (B) 70S particle (peak-2),
(C) 50S core particle (peak-3).

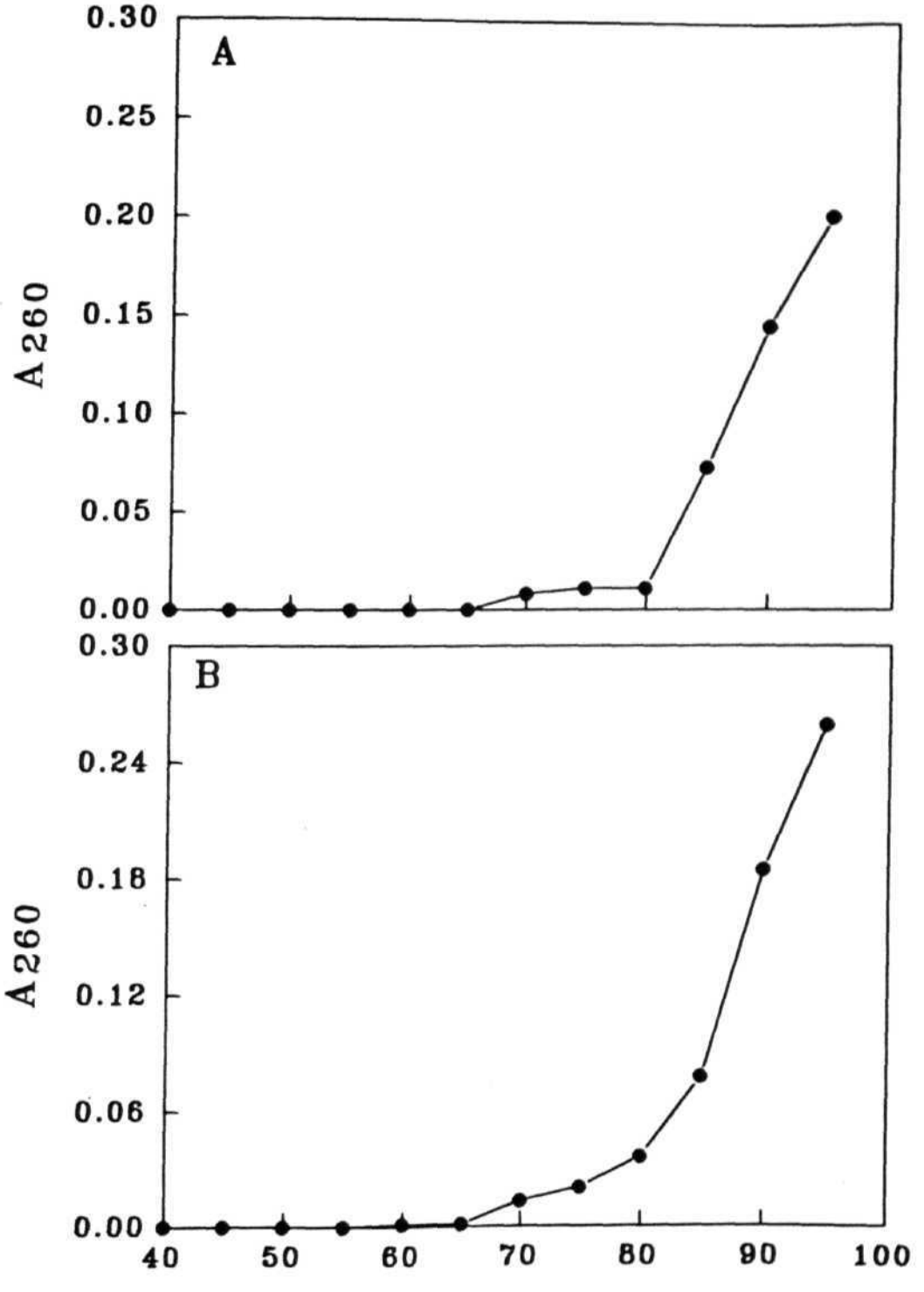

A
B
A260
A260
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.30
0.24
0.18
0.12
0.06
0.00
40
50
60
70
80
90
100
Temperature in°C

Figure 24

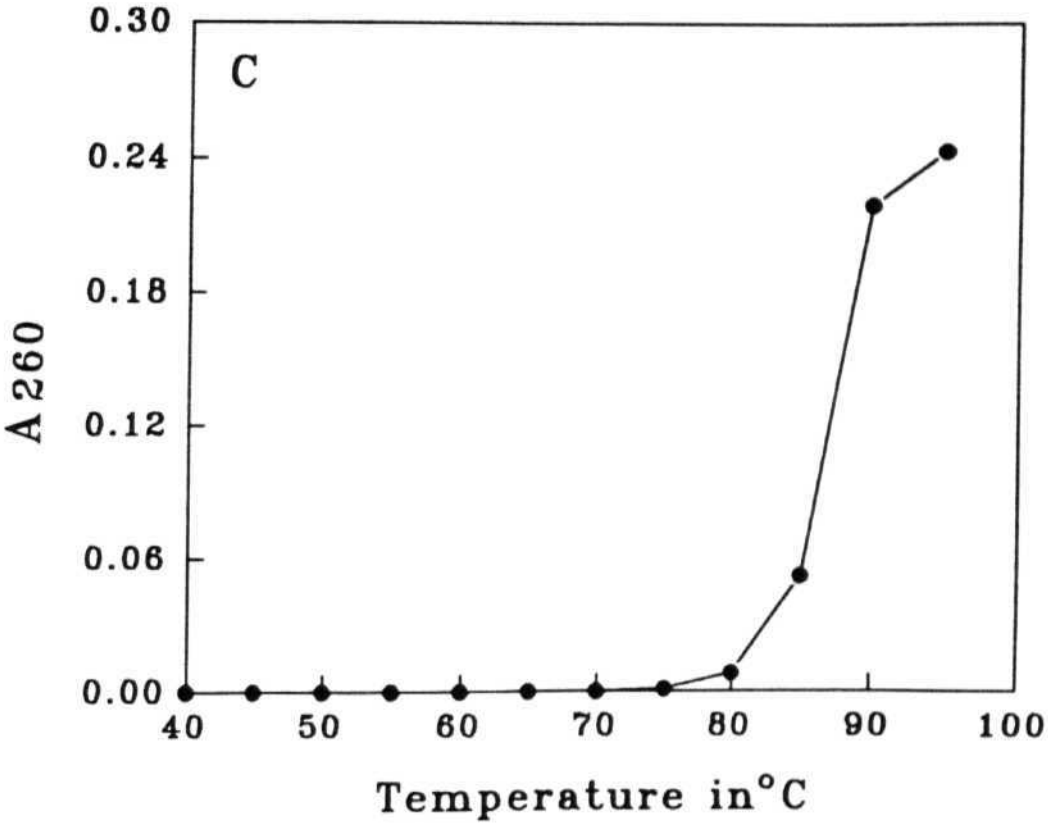

C
0.30
0.24
0.18
0.12
0.06
0.00
A 260
40
50
60
70
80
90
100
Temperature in°C

Figure 24

Fig. 25: Thermal melting profiles of ribosomal particles obtained by sepharose 4B chromatography (S-30).
(A) 70S particle (peak-3), (B) 50S core particle (peak-4).

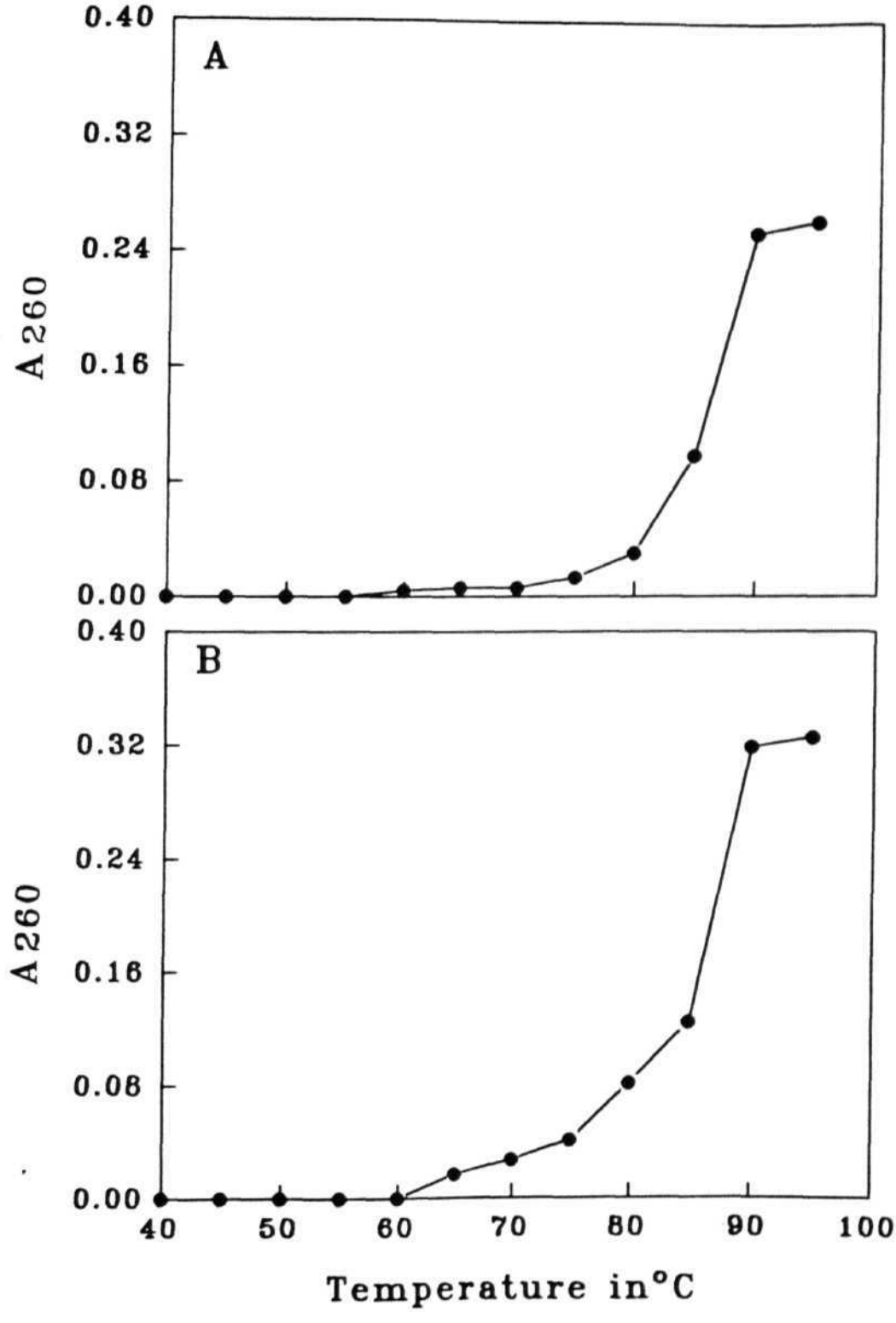

A
B
0.40
0.32
0.24
0.16
0.08
0.00
A 260
40
50
60
70
80
90
100
Temperature in°C

Figure 25

Association and dissociation of ribosomal subunits:

The ability of SOS core particles obtained by sepharose 4B chromatography to associate with 30S subunits to form 70S ribosomes was tested. As a control, *S. acidocaldarius* 50S and 30S subunits which were purified by gradient centrifugation were tested for reassociation in buffer containing 20 mM Tris-Cl(pH 7.6), 40 mM Mg^{++}, and 3 mM spermine. Control 50S and 30S subunits reassociated with the formation of 70S particle (fig. 26). When *S. acidocaldarius* 50S core particle was incubated with the 30S subunit under associating conditions it failed to reassociate to form 70S particle. The reason for this may be that the contact sites which are necessary for association are either absent or unavailable in the 50S core particle.

Discussion:

Results presented in this section show that the large and small ribosomal subunits of the extreme thermoacidophilic archaeon *S. acidocaldarius* are substantially more heat stable and exhibit a greater degree of co-operative interactions than the mesophilic organism, *E. coli*.

The remarkable heat resistance of *S. acidocaldarius* SOS and 30S ribosomal subunits in comparison to *E. coli* subunits can be accounted by the presence of increased G+C content of rRNA as well as efficient stabilisation of rRNA by ribosomal proteins (our results and Cammarano *et al.*. 1982; 1983). Tm of *S. acidocaldarius* free rRNA is only 12 °C higher than the Tm of *E. coli* rRNA, whereas the Tm *S. acidocaldarius* ribosomes is 19 °C higher than *E. coli* ribosomes. These results suggest that ribosomes are more heat stable than isolated rRNA indicating contribution of r-proteins for the thermal stability of ribosomes. Furthermore, the greater stability of the bihelical domains of rRNA within the

Fig. 26: *S. acidocaldarius* 30S ribosomal subunits were incubated with 50S subunits or 50S core particle for reassociation and analysed by sucrose density gradient centrifugation.
(o) 30S and 50S ribosomal subunits, (•)30S subunit and 50S core particles, (v) Control 30S and SOS subunits under dissociation conditions.

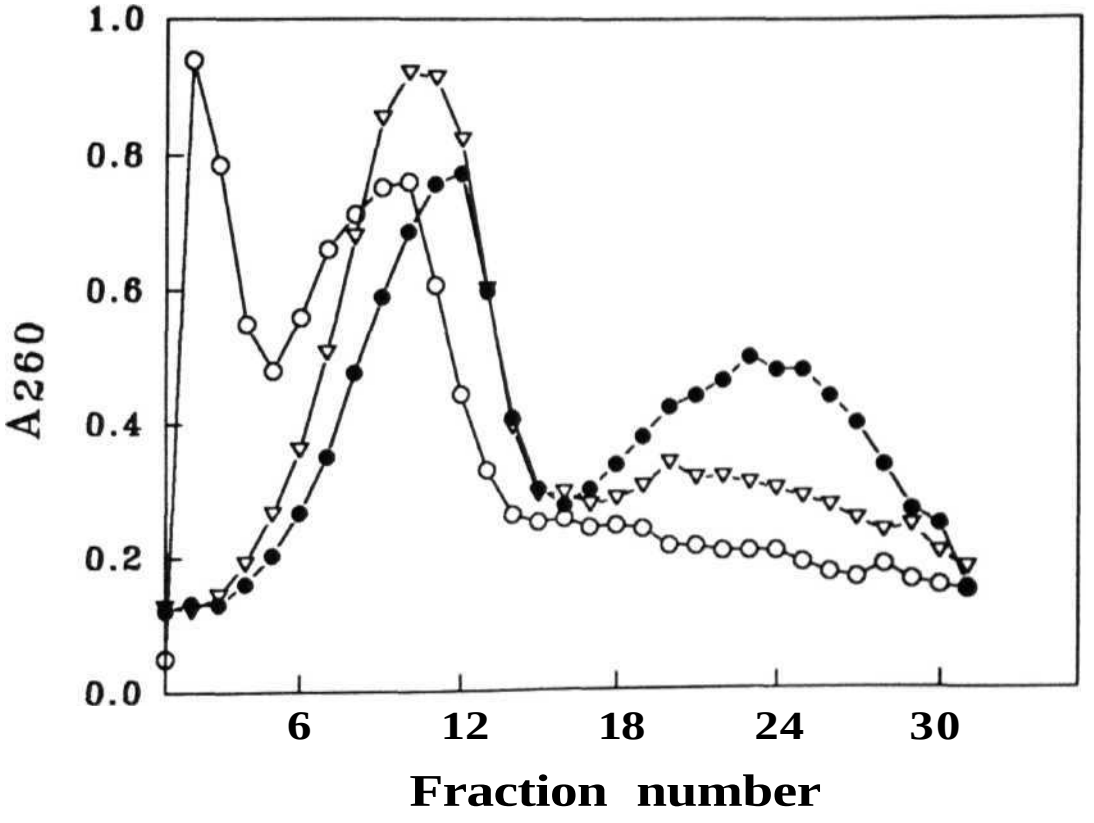


Figure 26

thermophilic ribosomes reflects more extensive interactions between functional groups of ribosomal proteins and rRNA domains. In ribosomes from *S. acidocaldanus,* bonds other than those maintained by Mg^{++} bridges are probably extensive to prevent the intra-particle packing from collapsing to an unfolded form under conditions that promote extensive unfolding, and presumably, loss of some ribosomal proteins from the mesophile ribosomes (Altamura *et al,* 1991).

The chemical composition of *C. acidophila* (genus *S. acidocaldanus)* ribosomes revealed that *C. acidophila* 30S subunits are about 280,000 Da heavier than bacterial 30S subunits, owing to both a larger number of proteins and a greater average molecular weight of the protein components In comparison to 30S subunits, *C. acidophila* 50S subunits are about 150,000 Da heavier than their *E. coli* counterparts. The increased protein to RNA ratio may possibly be related to the elevated thermal stability of *C. acidophila* ribosomes (Londei *et al.,* 1983). Our melting studies also revealed a conelation between the high temperature of growth of *S. acidocaldanus* and the high temperature (87 °C) required to start the melting of the thermophilic ribosomes.

In addition to ultra-centrifugation, the other method used for the isolation of *E. coli* ribosomal subunits is sepharose 4B chromatography using reverse salt gradients (Kirrillov *et al.,* 1978). Chromatography using reverse salt gradients was applied earlier for the separation of proteins (Sargent and Graham, 1964) and transfer RNA as well (Holmes *et al.,* 1975).

Unlike the case of *E. coli* 70S ribosome which gets completely dissociated into two subunits on sepharose 4B, a significant amount of 70S particle is present in the form of undissociated state in the case of *S. acidocaldanus* ribosomes. This could be due to the stabilisation of interaction between 30S and 50S subunits in

the presence of high salt concentration in the case of thermophilic ribosomes. The 30S particle and 50S core particle prepared by chromatography showed that they contain undegraded 16S and 23S rRNA molecules.

The *E. coli* 50S subunits were eluted from sepharose 4B in two different states, viz., (i) in a slightly unfolded 47S form and (ii) highly unfolded 33S form (Kirrillov *et al.,* 1978). Similar results (unfolding of SOS subunits into 46S and 28S forms) were observed earlier by Gavrilova *et al.,* (1966). In case of *S. acidocaldarius* there is only one form of 50S subunit core particle which elutes as the last peak. A significant amount of 50S core particle was obtained when the ribosomal sample was freshly prepared and the chromatography perfonned in the absence of Mg^{++}. A large amount of ribosomal proteins associated with the 50S subunit were stripped off and the 50S core particle that was tightly bound to the sepharose 4B through ionic interactions was eluted with a lower concentration of salt. Londei *et al.* (1986b), reported that *in vitro* reconstitution of 50S subunits require a two step procedure, viz , one at a temperature of 45 °C and the other at a temperature of 65 °C which bring about the conformational change in the core particle and allow the remaining proteins to be incorporated into 50S subunit. The protein composition of the core particle fomied in the first step of reconstitution is very similar to the protein composition of the 50S core particle obtained by us. The protein composition of 50S core particle was also similar to that of 50S core particle obtained by treatment with 2 M LiCl/4 M urea or 3 M LiCl/6 M urea or 4 M LiCl/8 M urea (Altamura *et al.,* 1991).

Unlike *E. coli* ribosomes, which undergo stepwise disassembly following treatments with increasing concentrations of LiCl (Nierhaus and Montejo, 1973), *S. acidocaldarius* large ribosomal subunits are relatively resistant to uncompacting treatments (Cammarano *et al.,* 1983). LiCI and urea treatment results in the

removal of some 12 external proteins, leaving behind a compact and thermally stable core particle. Attempts to remove additional proteins from the core resulted in the complete disaggregation of the particle, thus indicating that the component RNA and protein molecules are held together by very strong co-operative interactions. Hydrophobic bonds in protein/protein contact surfaces probably play an important role in conferring a high degree of stability to the thermophilic ribosome.

Preliminary studies of 50S core particles showed that they are inactive in reassociation with 30S subunits. Hence, the chromatographically isolated 50S core particles serve only as a source for the isolation of primary rRNA binding proteins. This method has no limitations with respect to the quantity of subunits to be isolated in one run. By increasing the volume of sepharose 4B column to several hundred milli-litres and increasing the content of amount of ribosomes loaded, we can prepare large quantities of ribosomal subunit core particles.

Ramakrishnan *et al.* (1986) reported that *E. coli* 30S subunits isolated by the method of Kirrillov *et al.* (1978) were active in protein synthesis *in vitro* and comigrated on sucrose gradients with subunits obtained by zonal sucrose gradient centrifugation. Their work shows that the subunits obtained by this technique were deficient in S3 and S14. These two proteins are removed from 30S subunits during chromatography and are eluted in a protein peak that elutes before 30S peak. Both S3 and S14 have been located on the periphery of the subunit by neutron triangulation technique (Moore *et al.*, 1986) These proteins are known to be on the periphery of the subunit and close to each other The assembly map of Mizushima and Nomura (1970) indicates that they are among the last proteins to bind to the 30S in the assembly of the subunit *in vitro.* In a similar fashion the two split fraction proteins of *S. acidocaldarius* which were obtained from

S. acidocaldarius on sepharose 4B chromatography, could be the last proteins to be assembled on both the subunits. *S. acidocaldarius* 30S subunit particle obtained under different conditions contained most of the ribosomal proteins indicating strong interaction of the 30S proteins with 16S rRNA under these conditions. Attempts were made to obtain 30S core particles by other methods (see chapter 3.3).

The stability of subunit particles was analysed by thermal melting experiments. The 30S particle and the SOS core particles which are obtained under different conditions when subjected to thermal melting analysis, melt with a Trn which is identical to that of 30S and 50S subunits In other words, the 50S core particle contains all the minimum i-proteins which are necessary for protecting the rRNA against thermal denaturation, and which may be directly interacting with the rRNA molecule. These proteins are the most likely candidates for early assembly proteins.

3.3: Characterisation of 30S core particle

Extensive work has been carried out on the protein-RNA interactions in *E. coli* ribosome (Held *et al,* 1974; Zimmerman *et al,* 1979, Wirrmann, 1982; Brimacombe *et al,* 1983; Wittmann, 1983). Concerning the 30S ribosomal subunit, it has been shown by Nomura's group and is generally accepted that seven ribosomal proteins (S4, S7, S8, S13, S15, S17, S20), independently and specifically bind to the 16S RNA (Held *et al,* 1974) Co-operative interactions between ribosomal proteins and 16S RNA have also been identified (Brimacombe *et al,* 1978). The results provide the basis for the assembly of the 30S subunits in *E. coli.*

30S subunit particle obtained by hydrophobic chromatography contained most of the 30S subunit proteins In order to know the primary 16S rRNA binding proteins, 30S core particle was isolated by salt/urea extraction of 30S subunits. In this chapter, we present the results obtained on treatment of *S. acidocaldarius* 30S subunits with different concentrations of LiCl and/or urea to isolate 30S core particles and identification of the primary 16S rRNA binding proteins.

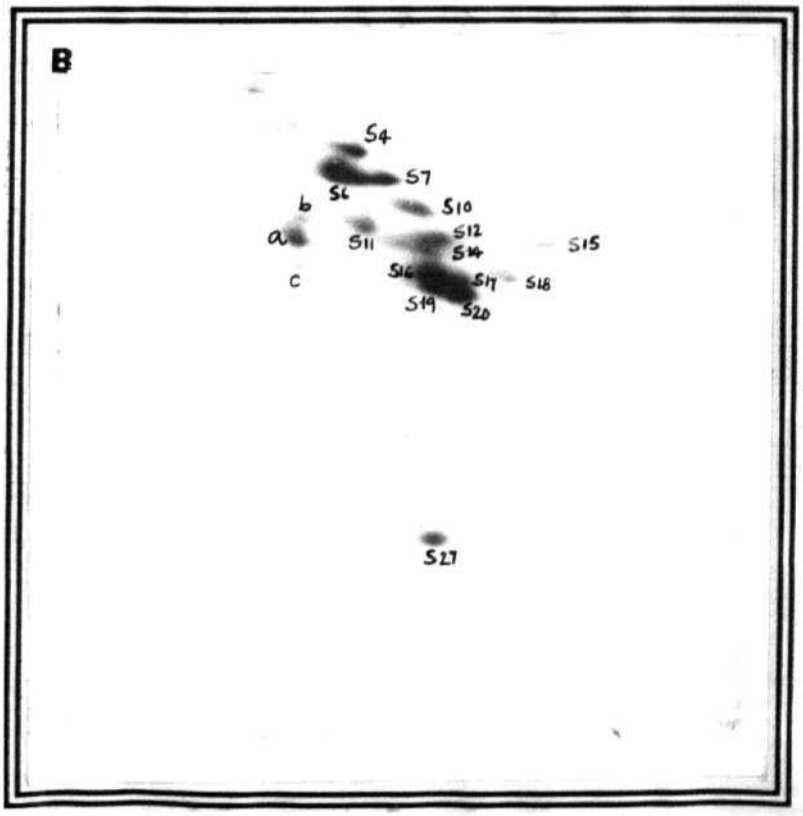
Isolation of 30S core particles:

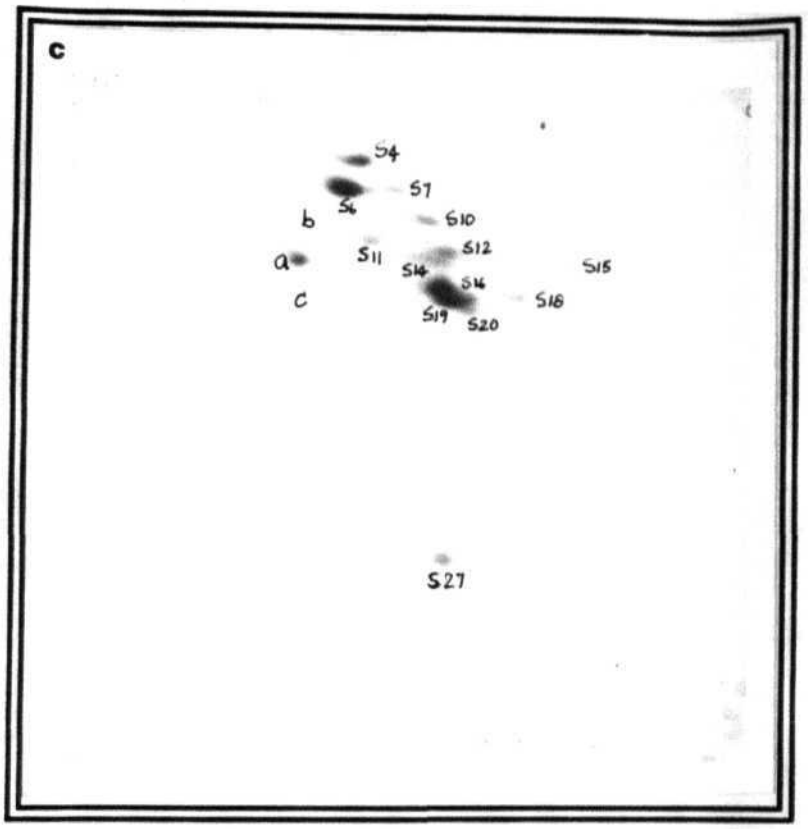
Treatment with LiCI:

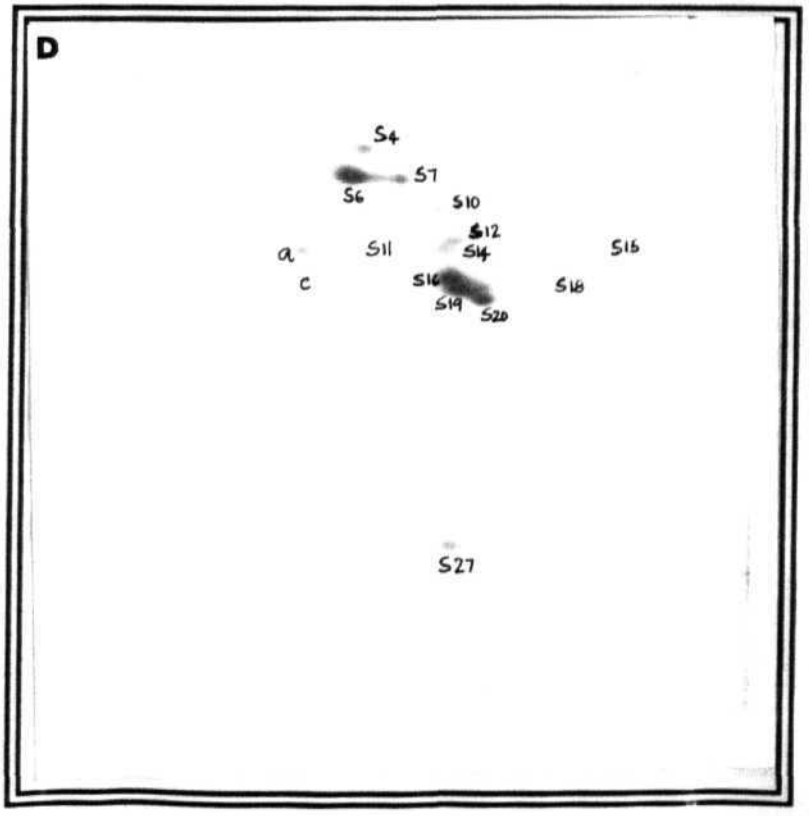
S. acidocaldarius 30S ribosomal subunits (obtained by density gradient) were treated with four different concentrations of LiCl (1 M, 2 M, 3 M, 4 M) as the only chaotropic agent in 20 mM Tris-Cl (pH 7.6), 2 mM Mg^{++}, 150 mM KCl, 7 mM 2-mercaptoethanol buffer Core particles were separated from extracted proteins by ultra-centrifugation. The proteins present in the core particles were extracted and analysed by two-dimensional gel electrophoresis (**fig. 27A, B, C,** D,

Fig. 27: Two-dimensional gel electrophoretic patterns of *S. acidocaldarius* 30S ribosomal core particle proteins obtained at different concentrations of LiCl:
(A) 30S ribosomal proteins, (B) 1 M LiCl core particle,
(C) 2 M LiCl core particle, (D) 3 M LiCl core particle,
(E) 4 M LiCl core particle, Sample loaded-125 μg.


A
S2
S3
S4
S5
S7
S8
S6
S10
S11
a
S12
S13
S14
S15
S16
S17
S18
S19
S20
S21
S25
S22
S23
S24
S27


B
S4
S7
S5
b
S10
a
S11
S12
S15
S14
S16
S17
S18
c
S19
S20
S27


C
S4
S7
S6
b
S10
S12
a
S11
S14
S15
S16
c
S18
S19
S20
S27


D
S4
S7
S6
S10
S12
a
S11
S14
S15
S16
c
S18
S19
S20
S27

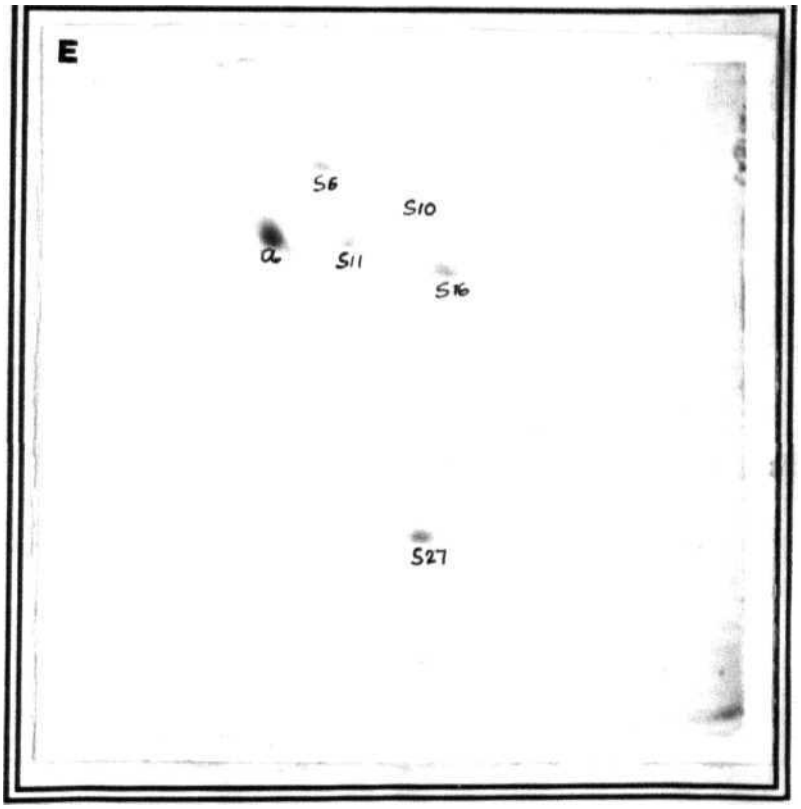

E
S6
S10
a
S11
S16
S27

Table - 9

.V. *acidocaldarius* 30S rihosomal proteins **present in** different LiCI core particles

LiCI Concentration	Proteins present
1 M LiCI 30S core particle	S4.S6.S7. S10. S11. S12. S14. SI5. SI6. S17. S18. SI9. S20. S27. a. b. c.
2 M LiCI 30S core particle	S4.S6.S7. S10. S11. SI2. S14. S15. SI6. S18. S19. S20. S27. a. b. c.
3 M LiCI 30S core particle	S4. S6. S7. SIO. S11. SI2. S14. S15. S16. S18. S19. S20. S27. a. c.
4 M LiCI 30S core particle	S6. S10. S11. S16. S27. a.

E). The proteins present in I M ; 2 M, 3 M and 4 M LiCl core particles were identified (Table-9) When the concentration of LiCl was increased from 2 M to 4 M, only six proteins were associated with the 16S rRNA and these could be binding to rRNA by very strong ionic or hydrophobic interactions.

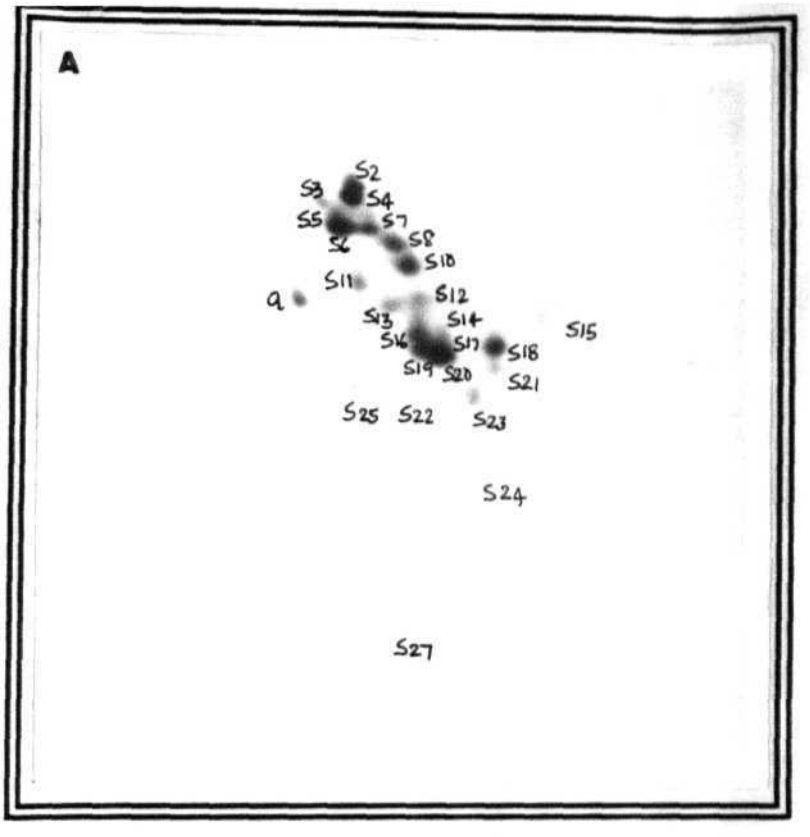
Treatment with urea:

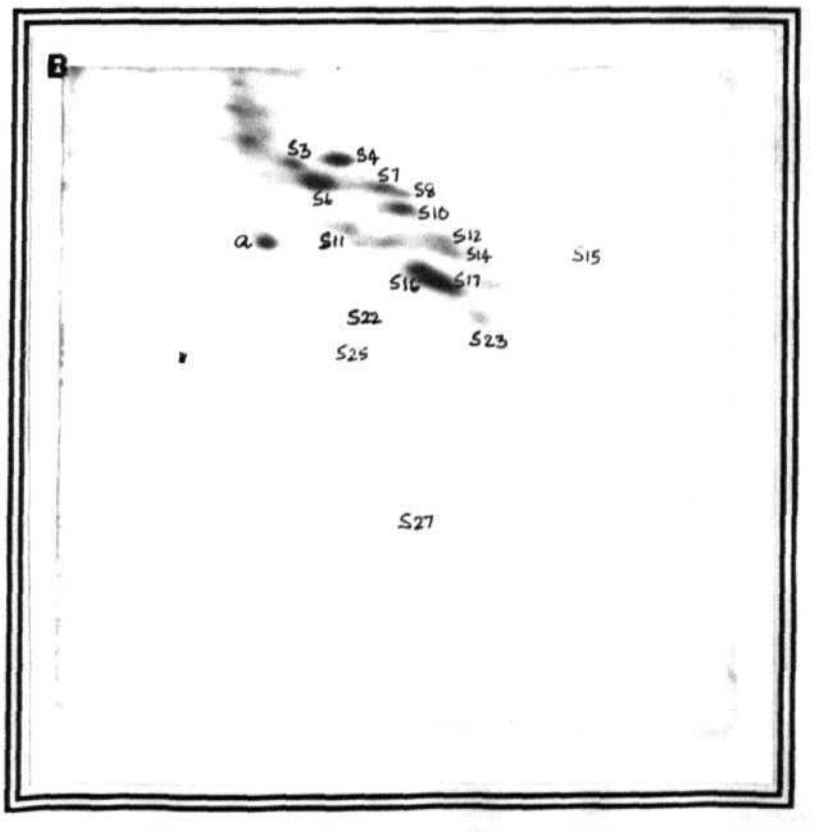
Urea was used in moderate ionic strength buffers (20 mM Tris-Cl pH 7.6, 2 mM Mg^{++}, 150 mM KCl, 7 mM 2-mercaptoethanol), as the only denaturant, to detect proteins that bind to RNA strongly. Core panicles were isolated from extracted proteins by ultra-centrifugation The proteins present in the core particles were extracted and analysed by two-dimensional gel electrophoresis (figs. 28A, B, C, D). The core particles obtained by 1 M, 2 M and 4 M urea contained similar set of proteins. The protein composition of these core padicles were similar to the 3 M LiCl core particles. The r-proteins present in urea core particles were identified (Table-10).

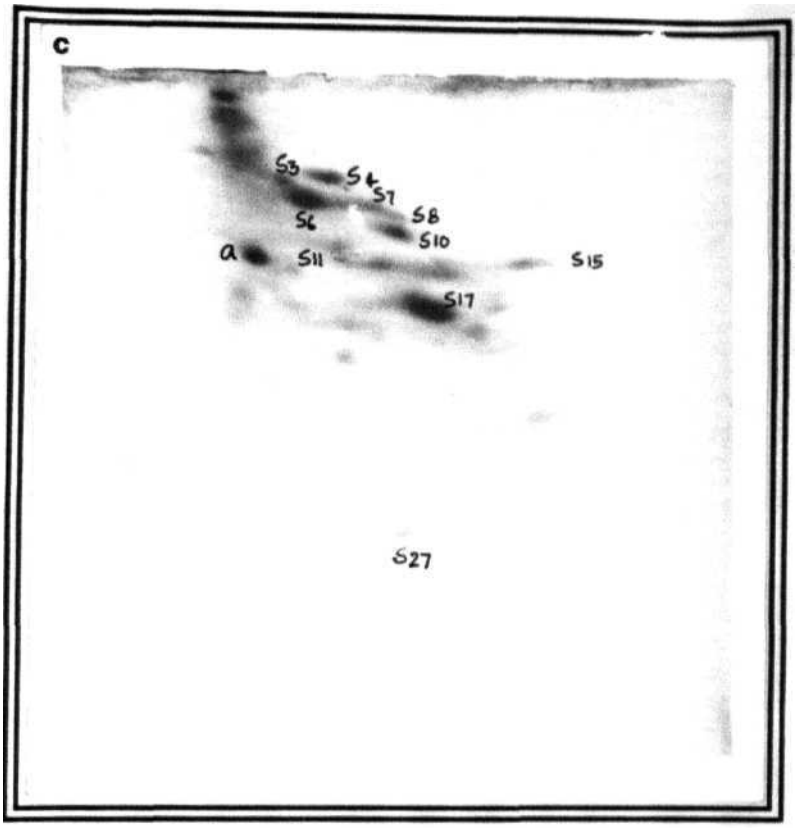
Treatment with LiCl and urea:

S. acidocaldarius ribosomal subunits were treated with four different concentrations of LiCl and urea to see the combined effect of chaotropic agent and a denaturant. The four concentrations used were 1 M LiCl/2 M urea, 2 M LiCl/4 M urea, 3 M LiCl/6 M urea and 4 M LiCl/8 M urea in 20 mM Tris-Cl (pH 7.6), 2 mM Mg^{++}, 150 mM KCl, 7 mM 2-mercaptoethanol or 20 mM Tris-Cl (pH 7.6), 20 mM Mg^{++}, 150 mM KCl, 7 mM 2-mercaptoethanol buffer Core particles were isolated by ulrra-centrifugation. The proteins present in the core particles were extracted and analysed by two-dimensional gel electrophoresis (figs. 29A, B).

Fig. 28: Two-dimensional gel electrophoretic patterns of *S. acidocaldarius* 30S ribosomal core particle proteins obtained at different concentrations of urea:
(A) 30S ribosomal proteins, (B) 1 M urea core particle,
(C) 2 M urea core particle, (D) 4 M urea core particle,
Sample loaded-125 μg.


A
S2
S3
S4
S5
S7
S8
S6
S10
S11
a
S12
S13
S14
S15
S16
S17
S18
S19
S20
S21
S25
S22
S23
S24
S27


B
S3
S4
S7
S8
S6
S10
a
S11
S12
S14
S15
S16
S17
S22
S23
S25
S27


C
S3
S4
S7
S6
S8
S10
a
S11
S15
S17
S27

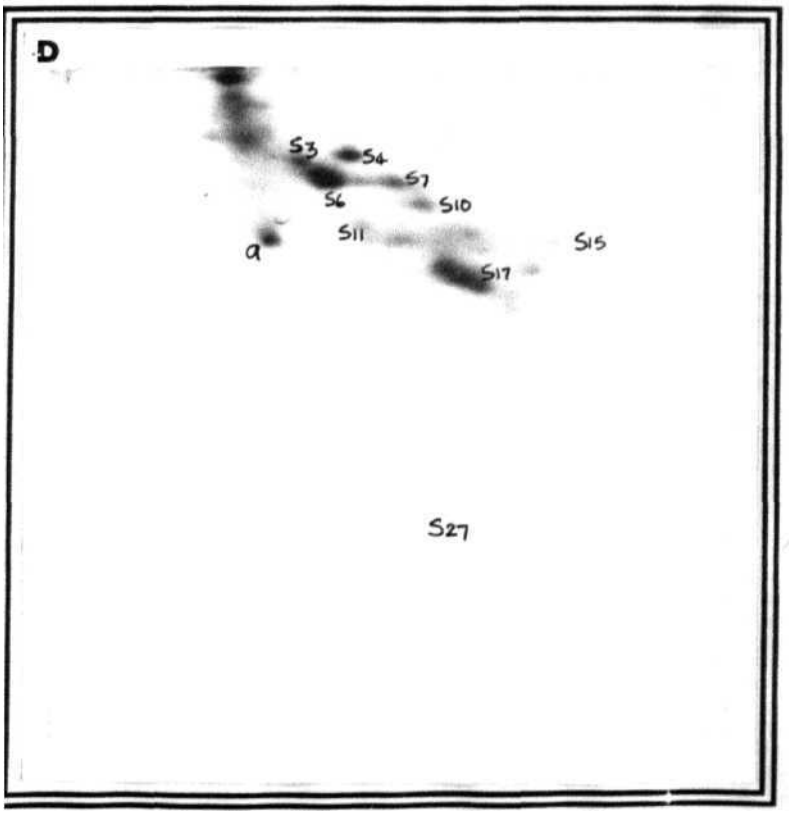
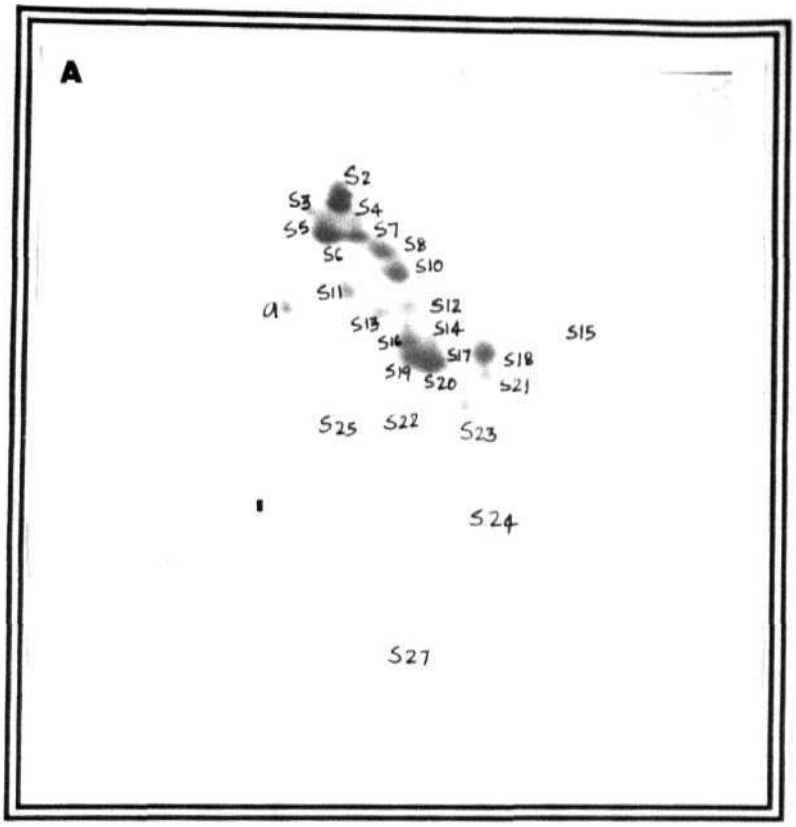

D
S3
S4
S7
S6
S10
S11
a
S15
S17
S27

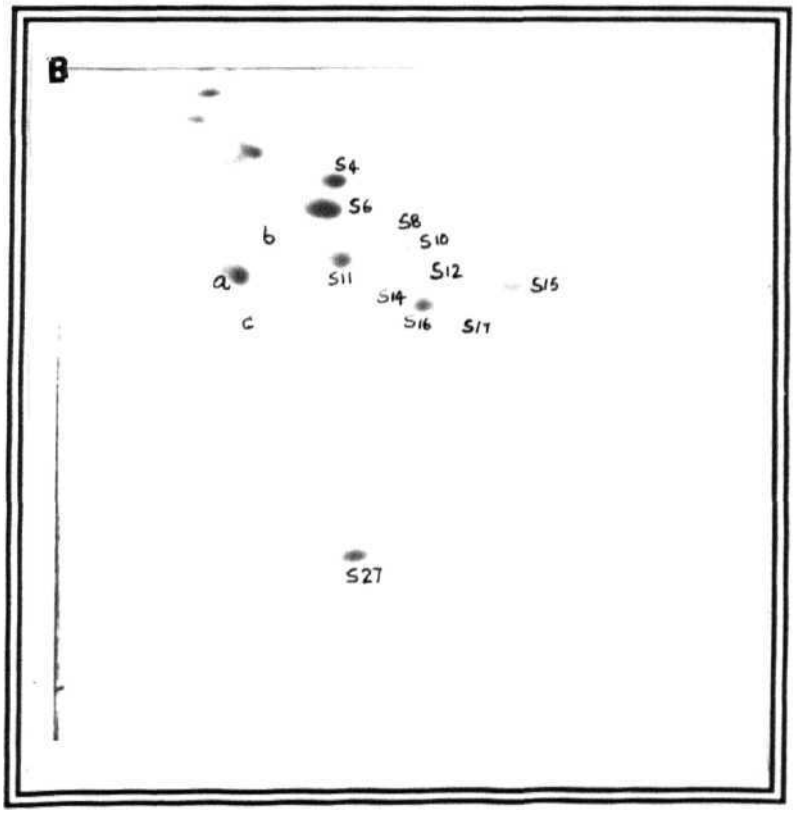
Table - 10

S. acidocaldarius 30S ribosomal proteins present in different Urea core particles

Urea Concentration	Proteins present
1 M Urea 30S core particle	**S3. S4.** S6. **S7. S8. S**10. S1**1.** S12. S14. S15. S16. S17. S22, S23. S25, S27. **a.**
2 **M Urea** 30S core **particle**	J S3. S4. S6. S7. S8. S10. S11. S15. S16. S17. S27. a J
4 M Urea 30S core particle	**S3. S4. S6. S7.** S10. **SI I.** S15. S16. **S17. S27.** a

Fig. 29: Two-dimensional gel electrophoretic patterns of *S. acidocaldarius* 30S ribosomal core particle proteins obtained at different concentrations of LiCl and urea:
(A) 30S ribosomal proteins, (B) 1 M LiCl/2 M urea core particle, Sample loaded-125 μg.


A
S2
S3
S4
S5
S7
S8
S6
S10
S11
a
S12
S13
S14
S15
S16
S17
S18
S19
S20
S21
S25
S22
S23
S24
S27


B
S4
S6
S8
b
S10
a
S11
S12
S15
S14
c
S16
S17
S27

Large number of the ribosomal proteins in (he 30S subunit were stripped off at a concentration of 1 M LiCl/2 M urea The proteins associated with the 16S rRNA in the 1 M LiCl/2 M urea core particles were identified (**Table-11**) The protein composition of the 1 M LiCl/2 M urea core particle was similar to 3 M LiCl core particles. At higher concentrations of LiCl/urea (2 M LiCl/4 M urea, 3 M LiCl/6 M urea) only protein-a was present in substantial amount, with trace amounts of S4, S6, S8 and S10. At 4 M LiCl/8 M urea concentration protein-a was also absent. Treatment with LiCl/urea at two different concentrations of Mg^{++} (2 mM and 20 mM) did not show any notable difference in the core particle protein composition.

Thermal melting studies were carried out on the core particles obtained by the three different treatments Core particles obtained by treatment with different concentrations of LiCl (**fig.** 30) or urea (**fig.** 31) melted with a Tm of about 84.5 °C. Melting analysis of the core particles obtained by 1 M LiCl/2 M urea gave a Tm of 85.7 °C (which is close to Tm of intact 30S subunit as in **fig.** 32).

Discussion:

Structural and functional studies on prokaryotic ribosomes have benefited enormously from the ability to separate ribosomal proteins and rRNA apart and to reassemble the dissociated ribosomal subunit components again into biologically active particles. One of the widely used techniques for controlled dissociation of ribosomal constituents involves treatment of ribosomal subunits with high concentrations of monovalent cations, notably LiCl. Such a treatment results in the removal of a portion of the ribosomal proteins, leaving discrete core particles with a well-defined subset of the ribosomal proteins (Homann and Nierhaus, 1971; Nierhaus and Montejo, 1973; Nierhaus and Dohme, 1974). Study of core particles

Table- 11

.V. *acidocaldarius* 30S ribosomal proteins present in different LiCl/Urea core particles

LiCl/Urea Concentration	Proteins present
1 M LiCl/2 M Urea 30S core particle	S4. S5. S6. S8, S10. S11, SI2. S14. S15. S16, S17. S27. a. b. c.

Fig. 30:Thermal melting profiles of LiCl 30S core particles from *S. acidocaldarius*:
(o) **1** M LiCl core particle,
(•) 2 M LiCl core particle,
(v) 4 M LiCl core particle.

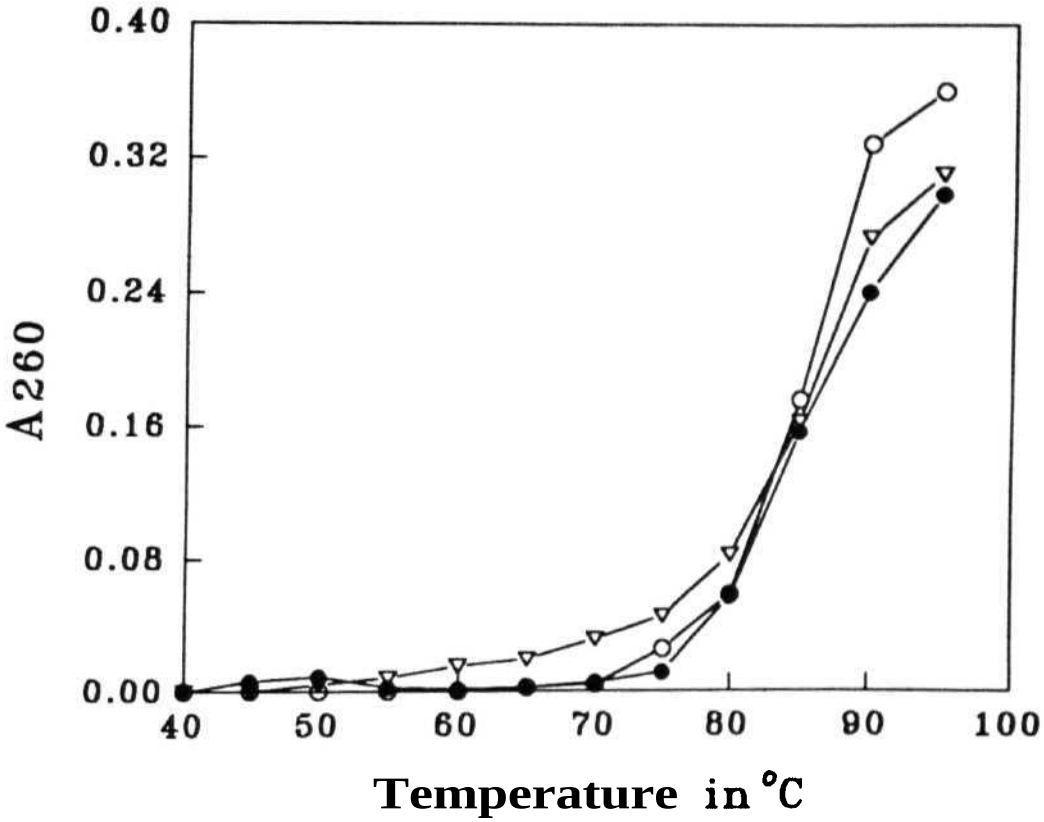

0.40
0.32
0.24
0.16
0.08
0.00
A260
40
50
60
70
80
90
100
Temperature in °C

Figure 30

Fig. 31:Thermal melting profiles of urea 30S core particles from *S. acidocaldarius*:
(o) **1** M urea core particle,
(•) 2 M urea core particle,
(v) 4 M urea core particle.

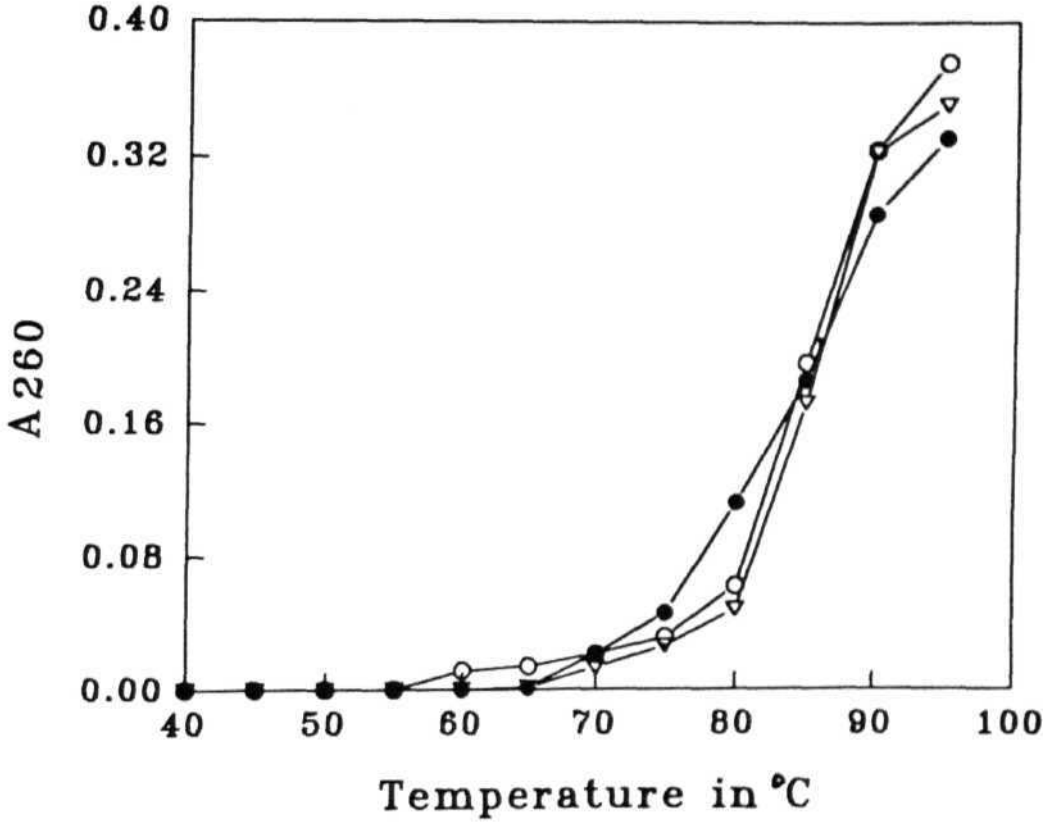

0.40
0.32
0.24
0.16
0.08
0.00
A 260
40
50
60
70
80
90
100
Temperature in °C

Figure 31

Fig. 32: Thermal melting profiles of LiCl/urea 30S core particles from *S. acidocaldarius*:

(o) 1 M LiCl/2 M urea core particles obtained in the presence of 2 mM Mg^{++}

(•) **1** M LiCl/2 M urea core particles obtained in the presence of 20 mM Mg^{++}

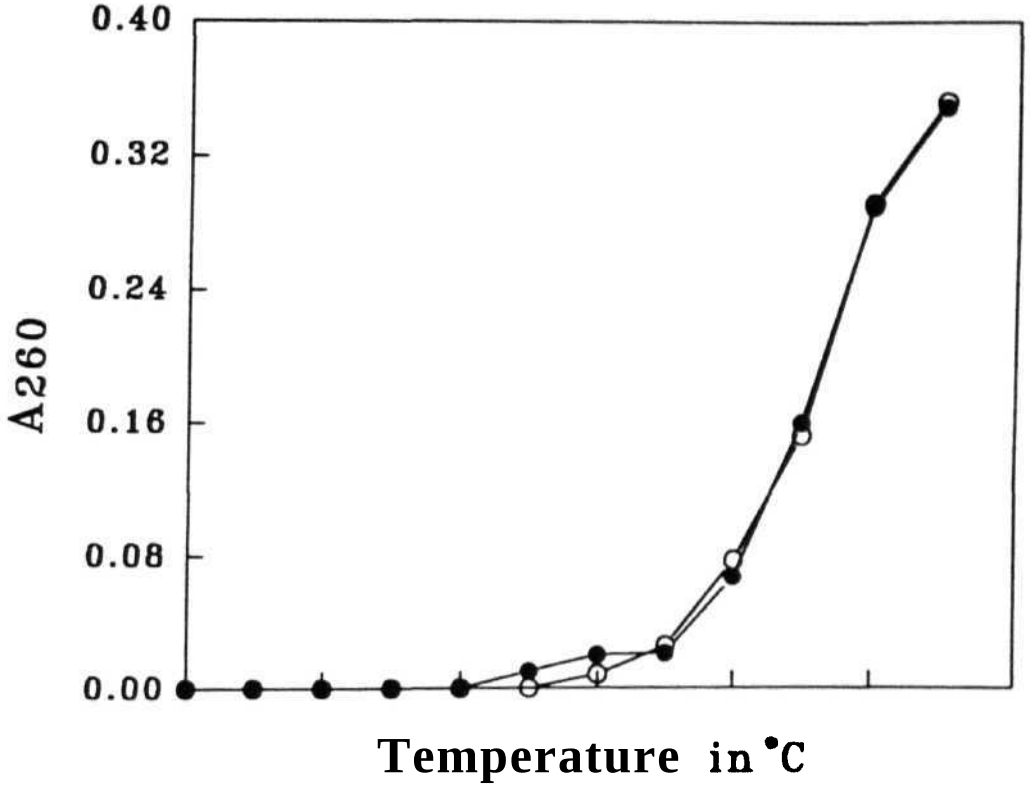


Figure 32

have been useful in establishing structure-function relationships for various prokaryotic ribosomal components (Nierhaus, 1980) and continue to be used for this purpose (Nowotny and Nierhaus, 1982; Miamets *et al.*, 1983). The identification of primary rRNA binding proteins in these core particles is particularly important because some of these proteins are surely expected to play a key role in providing the framework required for the correct assembly of ribosomes (Nowotny and Nierhaus, 1982; Bielka, 1979).

The dissection of *S. acidocaldarius* ribosome structure and function is still in its infancy compared to the extensive body of knowledge collected on prokaryotic ribosomes. *S. acidocaldanus* 30S ribosomal subunits are peculiar in that they are richer in protein content than the prokaryotic 30S ribosomal subunit (Londei *et al.*, 1983).

Here we report the identification *of S. acidocaldarius* small subunit proteins that are the primary 16S rRNA binding proteins and probably are the early assembly proteins The analysis of partially disassembled particles obtained by different conditions (LiCl, urea and LiCl/urea) has allowed us to delineate a preliminary pattern of the topographical hierarchy of .V. *acidocaldanus* 30S ribosomal subunit proteins into primary and secondary rRNA binding proteins

Proteins of the small subunit of .V *acidocaldanus* were extracted by washing subunits with a series of buffers containing increasing concentrations of LiCl as the only chaotropic agent and separately with increasing concentrations of urea as denaturant. The proteins were also extracted by increasing concentrations of LiCl and urea to see the combined effect of denaturant and chaotropic agent. Under all conditions *S. acidocaldanus* 30S ribosomal subunits are disassembled into specific groups of soluble proteins and a core particle containing proteins and rRNA.

S. acidocaldarius 30S ribosomal subunits when treated with increasing concentration of LiCl, majority of 30S proteins were removed between 2 M LiCl and 4 M LiCl concentrations In 4 M LiCl, rRNA is associated with 6 ribosomal proteins. The proteins which are stripped off from 30S subunit at 1 M and 2 M LiCl probably contain those proteins that had been predominantly held by ionic interactions. Proteins in the 4 M LiCl core particle may correspond to the primary 16S rRNA binding proteins. Extra proteins in 1 M LiCl/2 M urea; 4 M urea and 3 M LiCl core particles, other than the proteins in 4 M LiCl core particle, may represent those proteins that bind to the 16S rRNA subsequent to the binding of the primary rRNA binding proteins.

The degree of structural compactness of the core particles was analysed by thermal melting experiments. The 4 M LiCl core particle probably contains all the required r-proteins that directly interact with the rRNA molecule and protect it against thermal denaturation because the melting profiles of the core particles show Tm values close to that of the native 30S subunit

E. coli 30S subunits release S1, S2, S3, S14 and S21 at 1 M LiCl concentration. Incubation in 2 M LiCl leads to the splitting of the next portion of proteins S5, S9, S10, S12, S13 and S20. Between 3 to 3.5 M LiCl, S6, S18, S11, S19 and then S16 and S17 are released The residual 23S particles contain just four ribosomal RNA binding proteins S4, S7, S8, and S15 (Dijk and Littlechild, 1979; Littlechild and Malcolm, 1978). In contrast, yeast 40S subunits were found to be extremely sensitive to the salt as indicated by complete dissociation of the subunits at 0.5 M LiCl (El-Baradi *et al.*, 1984).

Unlike LiCl, urea treatment of 30S subunit upto 4 M concentration did not result in dissociation of majority of the r-proteins. Composition of the core

particles obtained by urea treatment suggest that 30S subunits are relatively resistant to urea treatment.

The proteins which are present in 4 M LiCl core panicles are identified to be primary rRNA binding proteins as they are associated with rRNA under highly stringent conditions. The resistance of these proteins to LiCl extraction compares well with that exhibited by the strong RNA binding proteins from other classes of ribosomes (Rozier and Mache, 1984; El-Baradi *et al.*, 1984; Nierhaus, 1980).

In *S. acidocaldarius* the 30S core particle is less stable than the 50S core particle. While the 30S core panicle is stable upto 1 M LiCl/2 M urea, the 50S core particle maintains its integrity even upto 4 M LiCl/8 M urea concentration. The results suggest that the *S. acidocaldarius* 50S subunit can withstand both thermal and chemical stresses better than 30S subunits.

Comparison of prokaryotic and eukaryotic core particles would help define both conserved and non-conserved features of ribosome structure. Furthermore, studies of ribosomal proteins and rRNA in thermophilic archaea may provide valuable information on the mechanisms involved in the stabilisation of nucleic acid and protein complexes at high temperatures and the degree of evolutionary conservation of ribosomal components in the primary kingdoms of the cell descent.

3.4: Homologous r-proteins between archaea and bacteria

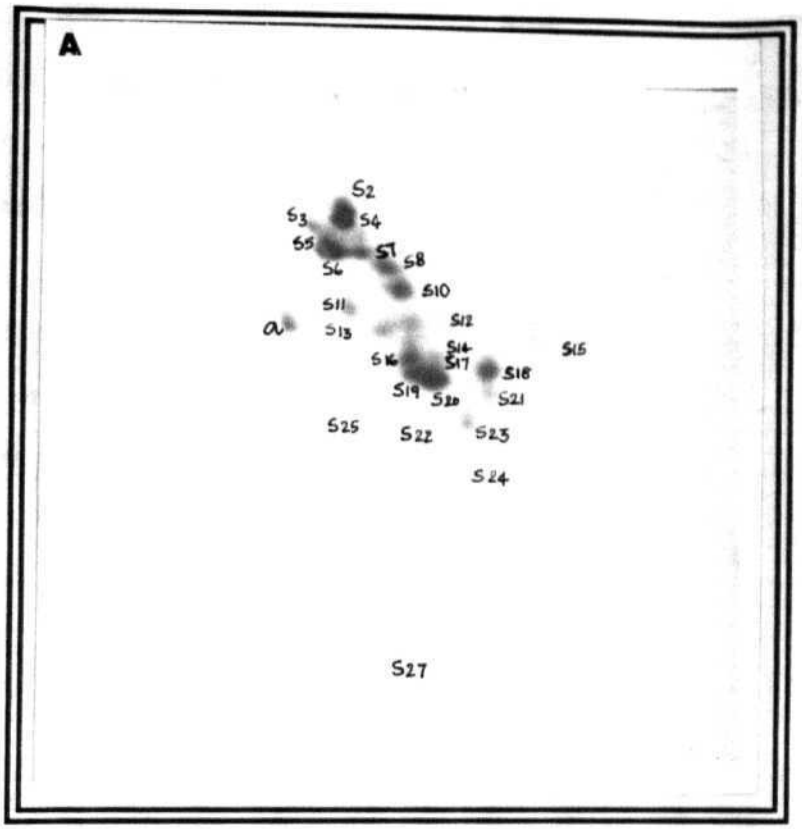
Cross-reactions between ribosomal proteins from archaea, bacteria and eukaryotes have been analysed by means of immunodiffusion, immunoprecipitation, and one- dimensional immuno electrophoresis (Schmid and Bock, 1981). It was found that the immunological relatedness was group specific and therefore closely paralleled the relationships delineated by the 16S rRNA oligonucleotide data (Balch *et al.,* 1979). The number of cross-reacting proteins was higher and the strength of their cross-reaction more intense when the organisms under comparison belonged to the same primary kingdom (Schmid and Bock, 1984). Only few cross-reacting proteins were detected when organisms from different kingdoms were compared. In the present work, immunologically homologous proteins between archaea *(S. acidocaldarius)* and bacteria *(E. coli)* were identified by two-dimensional gel electrophoresis of ribosomal proteins (from the two organisms) and immunoblotting

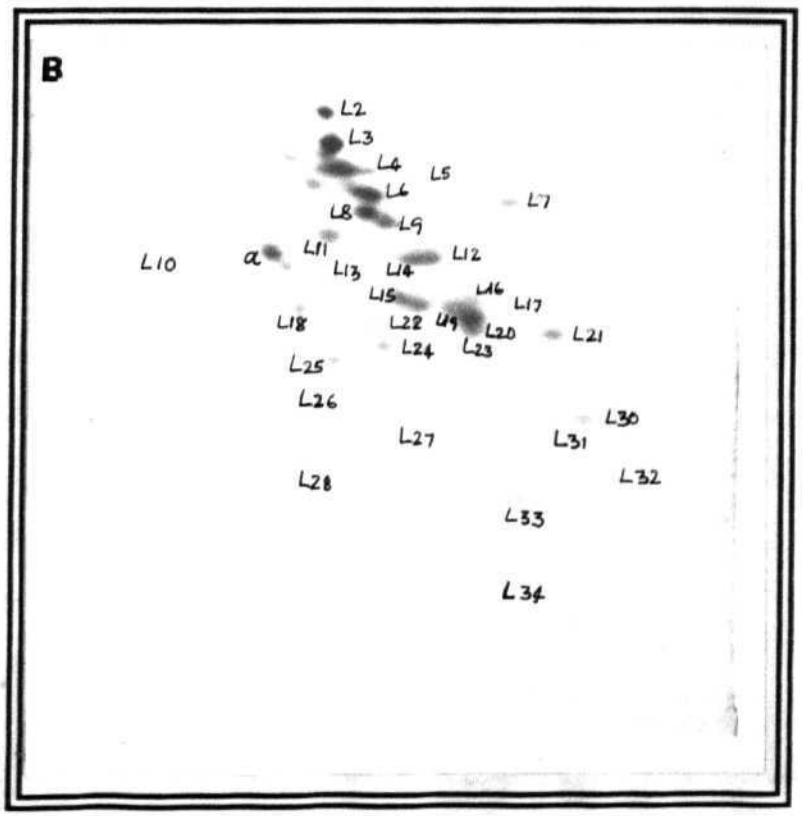
Immunological homology between archaeal and bacterial ribosomal proteins:

Ribosomal subunits were isolated from *S. acidocaldanus* and *E. coh* by using vertical gradient centrifugation. The purity of the ribosomal subunits was checked by two-dimensional gel electrophoresis. Antiserum was raised against 50S and 30S ribosomal subunits of *S. acidocaldanus* and *E. coli.* IgG was isolated from the antisera as described previously.

S. acidocaldanus 30S and 50S r-proteins were separated by two-dimensional gel electrophoresis (fig. 33) and transferred to nitrocellulose membranes. The membranes were immunoblotted with anti *E. coli* r-subunit

Fig. 33: Two-dimensional gel electrophoretic pattern of ribosomal proteins from *S. acidocaldarius*.
(A) 30S proteins (100 μg), (B) 50S proteins(150 μg).


A
S2
S3
S4
S5
S7
S8
S6
S10
S11
a
S13
S12
S14
S15
S16
S17
S18
S19
S20
S21
S25
S22
S23
S24
S27


B
L2
L3
L4
L5
L6
L7
L8
L9
L10
a
L11
L12
L13
L14
L16
L15
L17
L18
L22
L19
L20
L21
L24
L23
L25
L26
L30
L27
L31
L28
L32
L33
L34

antibodies. Control experiments were also performed to determine optimum concentration of antibody by electrophoresis of *E. coli* subunit proteins, which showed cross-reaction of all the r-proteins with homologous antibodies. The *S. acidocaldarius* 30S subunit proteins cross-reacting with anti *E. coli* 30S were found to be S5, S6 and S11 proteins (fig. 34). The *S. acidocaldarius* SOS proteins cross-reacting with anti *E. co/i* 50S were identified to be L8, LU and L16 (fig. 35). We have also performed immunoblotting experiments to identify the *E. coli* r-proteins (fig. 36) cross-reacting with anti *S. acidocaldarius* 50S and 30S antibodies. Proteins S5 and S18 (fig. 37) of *E. coli* 30S subunit strongly cross-reacted with anti *S. acidocaldahus* 30S and proteins L1, L3, L6 and L9 (fig. 38) of *E. coli* 50S subunit cross-reacted with anti *S. acidocaldarius* 50S.

Discussion:

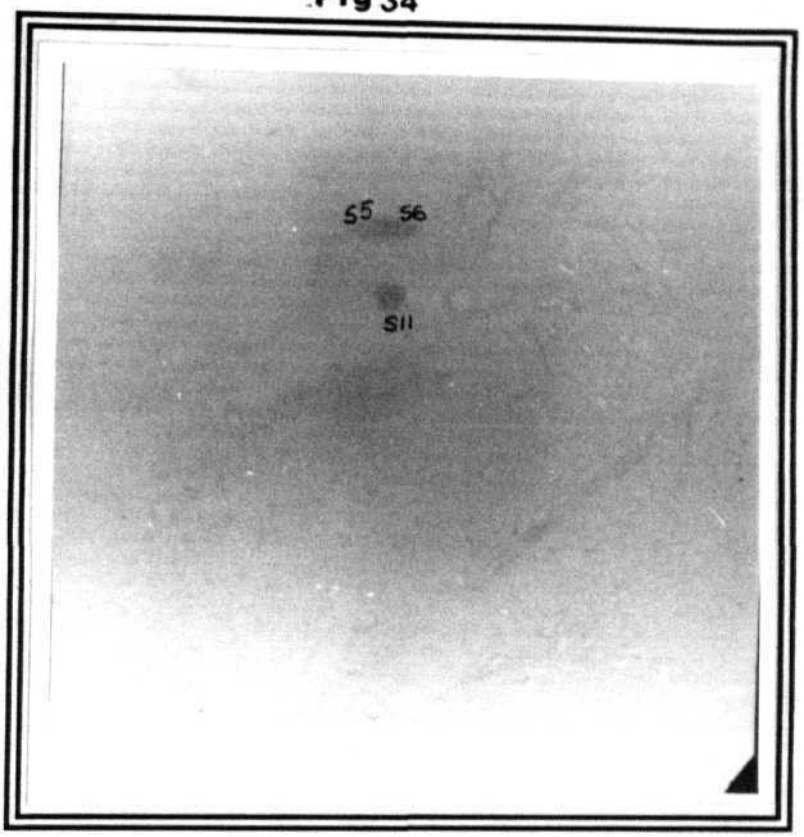
It is now evident from the studies by Cammarano *et al* (1986) that there are two distinct classes of ribosomes within the archaeal kingdom. The ribosomes from the extreme halophiles and most of the methanogens are similar in size and composition to those of the bacteria while those from the thermophilic archaea and several methanogens (Schmid and Bock, 1982) are larger in mass and contain significantly more proteins than do the bacterial ribosomes. Antisera raised in rabbits against r-proteins of *Methanobacterium bryantii* cross-reacted with the ribosomes within the methanogens and with a member of the extreme halophiles. With the methods and the anti-total protein sera employed, there was no detectable cross-reaction with ribosomal proteins or ribosomes from *Sulfolobus* species, eubacteria or yeast (Schmid and Bock, 1984)

Out of all archaeal organisms, *S. acidocaldanus* is least characterised in terms of immunological homology to bacteria and eukaryotes. The

Fig. 34: Immunoblot of *S. acidocaldarius* 30S **r-proteins with anti** *E. coli* 30S.

Fig. 35: Immunoblot **of** *S. acidocaldarius* 50S r-proteins with anti £. *coli* 50S.

Fig 34



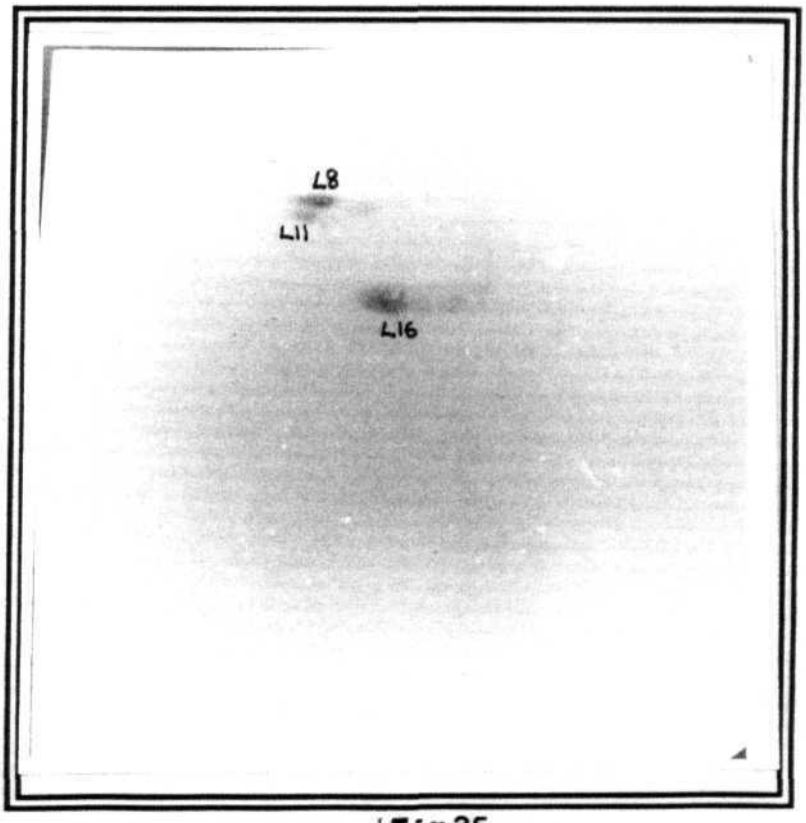
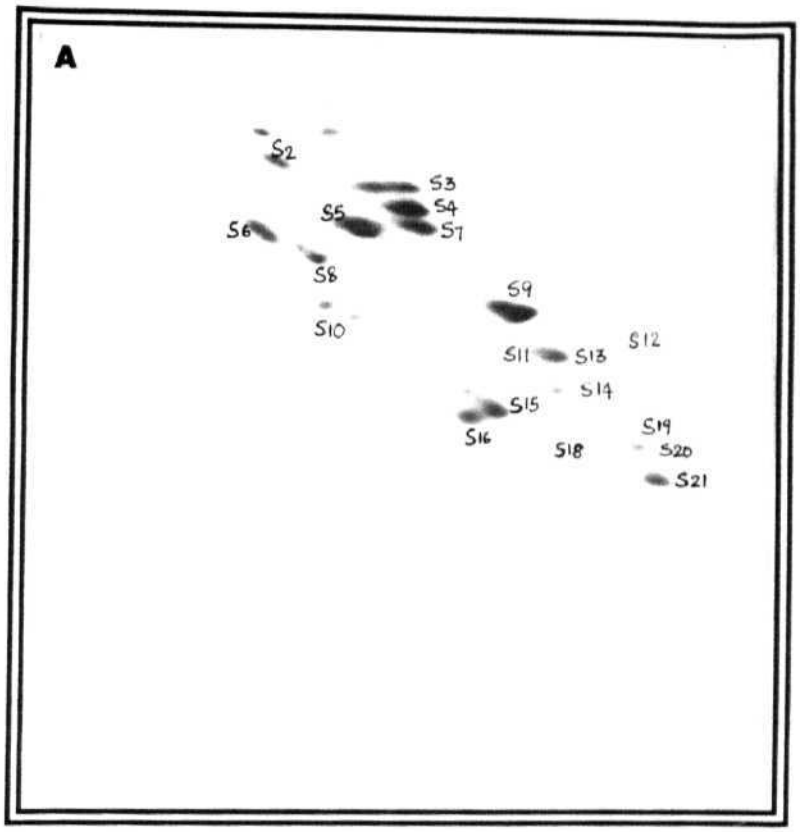


Fig 35

Fig. 36:Two-dimensional gel electrophoretic pattern of ribosomal proteins from *E. coli.*
(A) 30S proteins (100 μg), (B) 50S proteins (150 μg).


A
S2
S3
S4
S5
S6
S7
S8
S9
S10
S11
S13
S12
S14
S15
S16
S18
S19
S20
S21

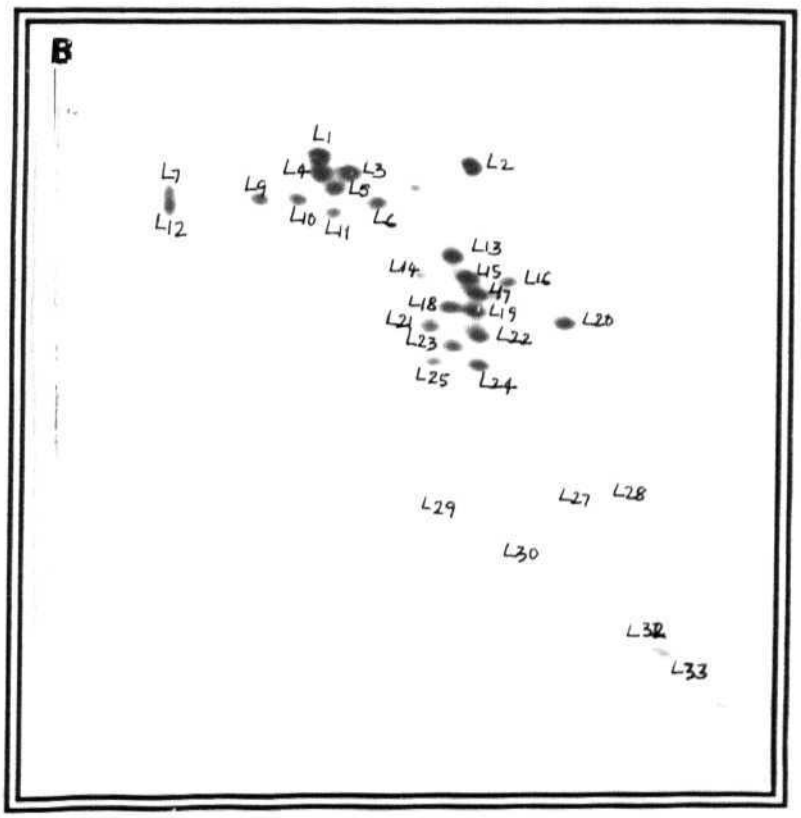

B
L1
L2
L3
L4
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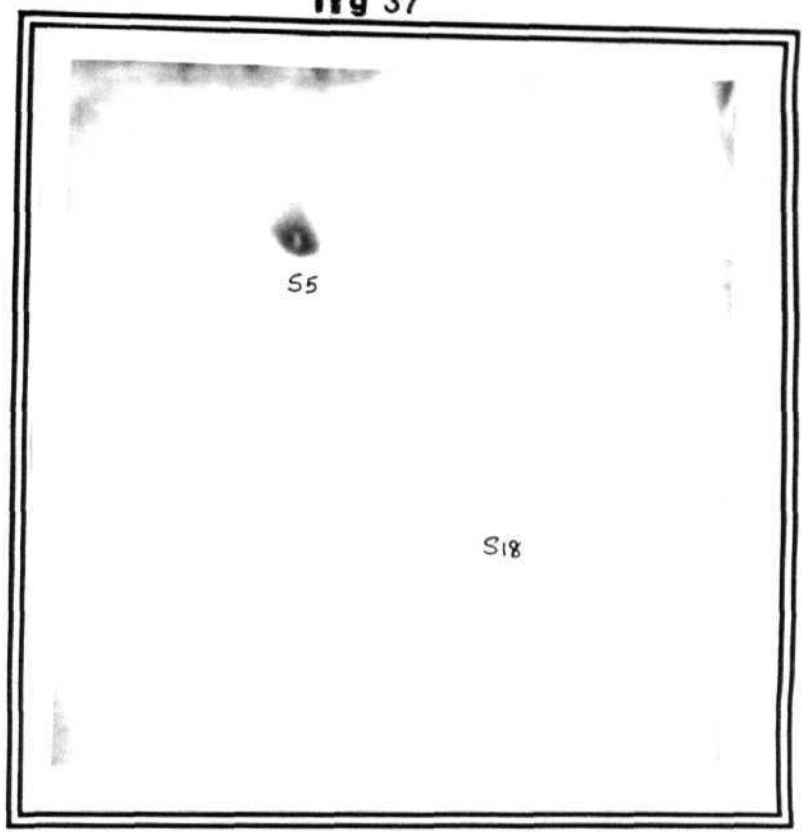
Fig. 37 : Immunoblot of *E. coli* 30S r-proteins with anti *S. acidocaldarius* 30S.

Fig. 38 : Immunoblot of *E. coli* SOS r-proteins with anti *S. acidocaldarius* 50S.

Fig 37



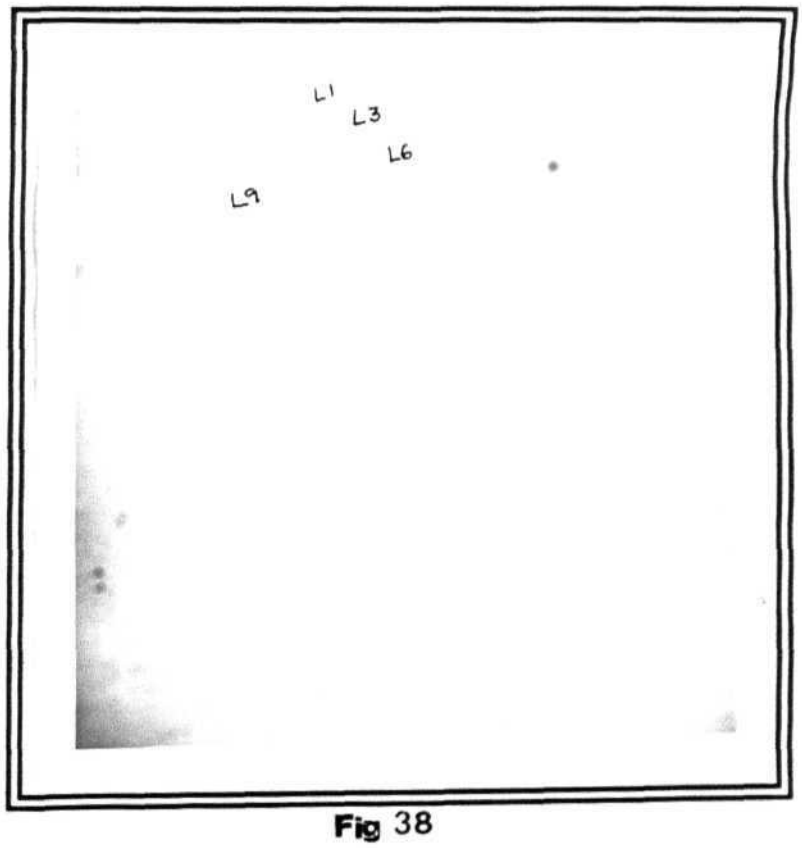


Fig 38

S. acidocaldarius proteins S5, S6 and S11 of small subunit and L8, L1land L16 of large subunit were found to be immunologically homologous to *E, coli* r-proteins. Interestingly these proteins were present in 30S core particle obtained by 1 M LiCl and 2 M urea and the 50S core particle obtained by sepharose-4B chromatogrphy. In *S. acidocaldanus,* except S5, all the proteins which are cross-reacting are identified to be primary rRNA binding proteins

We could not detect cross-reaction of *S. acidocaldanus* L7/L12 with *E. coli* 50S antiserum and vice-versa, eventhough *E. colt* L7/L12 and *S. acidocalarius* L7/L12 share several properties similar to each other such as low molecular weight; relative acidity; selective release from ribosome by high salt/ethanol and dimeric structure (Casiano *el al..* 1990). A probable explanation is that in this particular case either the proteins are not immunologically homologous or the immunological epitopes derived from the tertiary and quartemary structures of L7/L12 are not available for cross-reaction on the nitro-cellulose membrane.

We are unable to detect cross-reaction of *S. acidocaldarius* L2 with total 50S antiserum of *E. coli* and vice versa It has been reported that the antiserum raised against *E. coli* L2 protein did not react or else reacted only very weakly with ribosomal proteins from *S. solfataricus, 7 tenax, D mobilis* and 7. *celer.* When ribosomal proteins of cytoplasmic ribosomes from eukaryotes were tested, the antibodies reacted with one ribosomal protein each from *S. cerevisiae* and *P. anserina* but showed no reaction with r-proteins of the rat liver or chicken liver (Schmid *el al.,* 1984).

One of the 30S protein of *S. acidocaldanus* and *E. colt* which showed cross-reaction with *E. coli* and *V. acidocaldanus* 30S antiserum is protein S5 and is conserved in all life forms (Wittmann-Liebold and Greuer, 1978, Kimura, 1984, Scholzen and Arndt, 1991, All-Robyn *et al.,* 1990). S5 sequences are now known

from 13 prokaryotic, eukaryotic and archaeal organisms, although somewhat larger in later group of organisms. The structurally important residues that are crucial to its stuctural integrity are highly conserved, which reflects the invariance of the S5 structure. The molecule contains two distinct a/p domains that have structural similarities to several other proteins that are components of ribonucleoprotein complexes (Ramakrishnan and White, 1992). The *B. stearothermophilus* protein S5, is 55% homolgous to its *E. coli* counterpart (Wittmann-Liebold and Greuer, 1978; Kimura, 1984) and its function is almost certainly identical in the two organisms.

The *E. coli* large subunit proteins which are cross-reacting with *S. acidocaldarius* 50S antiserum are L1, L3, L6 and L9. The L6 molecule contain many structurally important residues that are highly conserved and which therefore show that its overall fold is invariant between life forms (Kimura *et al,* 1981; Cerettii *et al,* 1983; Suzuki *et al,* 1990; Scholzen and Arndt, 1991; Golden *et al,* 1993). Immune electron microscopy localizes L6 to the same site in the *B. stearothermophilus* and *E. coli* (Hackl and Stoffler-Meilicke, 1988). A related and probably homologous eukaryotic protein labelled L9 has been identified in rat liver ribosomes (Suzuki *et al,* 1990) The other *E. coli* ribosomal protein whose amino acid sequence is highly conserved in five organisms (three bacterial and two Chloroplast) is L9. L9 has an important role in ribosome function, since it is present in all viable strains of *E. coli* (Dabbs *et al,* 1986) and anti L9 antibodies reduce protein synthesis by some 75% (Nag *et al,* 1991). Its general role may be best demonstrated by the 50S assembly map (Herold and Nierhaus, 1987) which show that L9 and L1 are the only proteins that interact directly with the 3' end of 23S r-RNA without the cooperation of other large subunit ribosomal proteins. L9

may be the essential scaffold molecule that maintains correct folding of this region of 23S r-RNA (Hoffman *et al.*, 1993).

At present we do not know the correspondence between the proteins showing cross-reaction with heterologous antisera in *S. acidocaldarius* and *E. coli*. Further work is needed to determine the counterparts in *S. acidocaldarius* and *E. coli* among the cross-reactive proteins. Our studies also support the earlier observation that archaea contain far fewer proteins that are homologous to bacterial r-proteins.

Chapter-4
SUMMARY

SUMMARY

Chapter-1:

This chapter gives a brief overview of literature pertaining to archaeal molecular biology and biochemistry. A detailed account on archaeal translational apparatus, the ribosomal structure, organisation and assembly is presented. Salient features of bacterial ribosomes (*E. coli*) have also been described. The role of primary r-RNA binding proteins and the significance of homologous ribosomal proteins from different organisms has been documented. Objectives and scope of the present investigation have also been mentioned in this chapter.

Chapter-2:

In this chapter, the methodologies described arc growth of organisms, isolation of ribosomes, ribosomal subunits, ribosomal proteins, fractionation of r-proteins by novobiocin, chromatographic procedure for the isolation of 50S core particles, thermal melting analysis of ribosomes, isolation of subunit core particles by salt extractions, electrophoresis in the presence of SDS, two-dimensional gel electrophoretic technique and immunoblotting procedure etc.

Chapter-3.1:

In this chapter, the ribosomes isolated by different methods from *S. acidocaldarius* were analysed for total r-proteins by SDS-poly acrylamide gel electrophoresis and two-dimensional gel electrophoresis. It was found that the ribosomes isolated under gentle lysis conditions do not contain high molecular weight non ribosomal proteins. A fractionation method involving precipitation of proteins with novobiocin has been developed to separate ribosomal proteins of

S. acidocaldarius.Four groups of proteins were obtained corresponding to those precipitated with 0.2, 0.5, 1.0 mg/ml novobiocin and proteins soluble at 1.0 mg/ml novobiocin. The same procedure was also successful for the fractionation of ribosomal proteins from *E. coli*. Two-dimensional gel electrophoresis showed that proteins present in each group were distinct with only a minor contamination of proteins from other groups. Our results indicate that proteins with similar charge and molecular weight can be separated by this method. This procedure may have applications for the fractionation of other complex protein mixtures.

Chapter-3.2:

In the present study an attempt is made to understand the thermal stability of *S. acidocaldarius* ribosomes. *S. acidocaldanus* ribosomes are more heat stable than the *E. coli* ribosomes because of higher GC content in r-RNA and stronger r-RNA and r-protein interactions. Sepharose 4B chromatography of *S. acidocaldarius* cell lysates and ribosomes yielded 50S core particle and split proteins. The proteins which are associated with r-RNA in 50S core particle strongly protect the r-RNA against thermal denaturation and are possibly the early assembly proteins. These proteins were identified as L2, L3, L4, L6, L7, L8, L11, L12, L14, L15, L18, L19, L20, L21, L23, L27, L30, L33, a, and b. The split proteins which were identified (S13, S15, S24, L1, L10, L13, L14, L15, L24, L25, L26, L27, and L28) could be the late assembly proteins. The chromatographically isolated 50S core particle could serve as a best source for the purification of primary r-RNA binding proteins. This method has no limitations with respect to the quantity of core particle isolated in one run.

Chapter-3.3:

S. acidocaldarius 30S particle obtained by hydrophobic chromatography contained most of the 30S subunit proteins. Core particles were isolated by treatment of 30S subunit with different concentrations of salt and urea The proteins which are present in 4 M LiCl core particles were identified as S6, S10, S11, S16, S27 and a, which are primary r-RNA binding proteins as they are associated with r-RNA under highly stringent conditions The primary r-RNA binding proteins protect 16S r-RNA strongly against thermal denaturation The other set of proteins which are associated with 30S subunit after primary r-RNA binding proteins were identified as S3, S4, S7, S8, S12, S14, S15, S17, S18, S19, S20, b and c. These proteins were called secondary r-RNA binding proteins

Chapter-3.4:

Ribosomes from *S. acidocaldarius* and *E. coli* were analysed for the presence of homologous proteins using immunoblotting technique. Antibodies raised against subunits (30S and 50S) of *E. coli* and *S. acidocaldarius* were used in the present study. *S. acidocaldarius* r-proteins S5, S6 and S11 and L8, LU and L16 cross-reacted with anti *E. coli* subunit antibodies. These proteins were members of the primary r-RNA binding proteins *E. coli* r-proteins S5 and S18 and L1, L3, L6 and L9 cross-reacted with anti *S. acidocaldarius* subunit antibodies.

Chapter-5
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REFERENCES

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Pages 221-229

NOVEL FRACTIONATION OF PROTEINS USING NOVOBIOCIN AS A PRECIPITATING AGENT: APPLICATION TO RIBOSOMAL PROTEINS FROM THF ARCHAEBACTERIUM *SULFOLOBUS ACIDOCALDARIUS* AND *ESCHERICHIA COLI*

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Summary: A fractionation method involving precipitation of proteins with novobiocin has been developed to separate ribosomal proteins of the archaebacterium *Sulfolobus acidocaldarius*. Four groups of proteins were obtained corresponding to those precipitated with '0.2, 0.5 and 1.0 mg/ml novobiocin and the proteins soluble at 1.0 mg/ml novobiocin. The same procedure was also successful for the fractionation of ribosomal proteins from *Escherichia coli*. Two dimensional gel electrophoresis showed that proteins present in each group were distinct with only a minor contamination of proteins from other groups. Our results indicate that proteins with similar charge and molecular weight can be separated by this method. This procedure may have applications for the fractionation of other complex protein mixtures.

Introduction:

Several techniques have been developed for the fractionation and purification of proteins. These include separation by precipitation with high salt concentrations (salting out), organic solvents, organic polymers, and a variety of chromatographic techniques. These methods have been widely used for the successful purification of a large number of enzymes and other proteins. In the course of our study of ribosomal proteins from the acidothermophilic archaebacteriurr. *Sulfolobus acidocaldarius*, we found that the ribosomal proteins could not be stripped from the ribosomes by high salt treatment to isolate split proteins [Ref. 1 and our unpublished results]. It has been reported that novobiocin can precipitate purified histories from

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Fractionation of ribosomal proteins with novobiocin:

Since high concentrations (> 4 M) of urea inhibit the precipitation of proteins by novobiocin, ribosomal protein solutions at a concentration of 0.2 mg/ml were dialysed against 20 mM Tris-HCl pH 8.0 1.0M ammonium chloride and 7 mM 2-mercaptoethanol. Low concentration of ribosomal proteins was chosen to ensure the best solubility. Novobiocin stock solution (10 mg/ml) in water was added to the dialysed ribosomal protein solution to a concentration of 0.2 mg/ml. The solution was mixed, stored on ice for 30 min. and centrifuged at 20,000 g at 4 C for 15 min. The supernatant fluid was collected. The precipitated protein was dissolved in 8 M urea and 7 mM 2-mercaptoethanol. The concentration of novobiocin in the above supernatant was raised to 500 ug/ml and after storage on ice for 30 min it was again centrifuged to obtain the proteins precipitated at this novobiocin concentration. The supernatant fraction was collected again and treated with 1000 ug/ml novobiocin. Both supernatant and precipitated protein fractions were obtained as above. The final supernatant fraction was treated with 5 volumes of acetone and the precipitated protein was dissolved in urea/2-mercaptoethanol. The precipitated protein at each novobiocin concentration was dissolved in urea, 2-mercaptoethanol.

Two dimensional gel electrophoresis:

This was carried out essentially as described in [8]. The numbering of the ribosomal proteins of S. *acidocaldarius* was according to [9]. and those of E. *coli* according to [8].

Results and Discussion:

Fig.1 shows the results obtained after the fractionation of *S. acidocaldarius* ribosomal proteins with novobiocin. The four groups of proteins obtained gave distinct two dimensional patterns indicating that each group contained specific proteins which were present in larger amounts. However, in each fraction there were small amounts of cross-contaminating proteins belonging to other fractions. This may be partly due to contamination by small amounts of supernatant fluids at each step of the novobiocin fractionation procedure and an incomplete precipitation by some proteins at that novobiocin concentration. The different proteins precipitated at each step of novobiocin concentration were identified after comparison with two dimensional gel patterns of 30S and 50S proteins (Table 1). There was some overlap in the positions of certain ribosomal proteins. This may also be the reason for an apparent similarity in the visualized spot positions of a few proteins in the different

Fig.1 Two dimensional gel electrophoretic patterns of novobiocin fractions of S. *acidocaldarius* ribosomal proteins: Fractionation of ribosomal proteins with novobiocin and electrophoresis were carried out as described in the Text.
(A) Total Ribosomal Proteins 125 µg,
(B) to (D) 200, 500, and 1000 *uq* novobiocin precipitated fraction respectively, and (E) 1000 µg novobiocin supernatant fraction.
60 µg protein of each of the novobiocin fractions was electrophoresed.

Table I

S. acidocaldarius ribosomal Proteins present in different novobiocin fractions

Novobiocin fraction	Proteins Present in large amounts	Present in reduced amounts	Present in trace amounts
200 µg/ml Precipitate	L5, L6, L11, S10 S16, S18	L2, L3, L7, L23, S4,S15	L4, L17, L22, S9 S14, S20, S21
500 µg/ml Precipitate	L2, L3, L6, L8, L16 L17, S4, S8, S10	L5, L12, L14, L20, L21, L22, S2, S9, S12, S14, S15, S16, S18, S20,	L9, L11, S5, S6 S9, S13
1000 µg/ml precipitate	L5, L11, L14, L22 L22, S2, S9, S14 S20, S21	L2, L3, L16, L20, L23, L30, L31, S4, S10, S11, S16, S18, S27	L4, L9, L12, L15 S3, S15, S17, S22
1000 µg/ml Supernatant	L1, L4, L6a, L10 L15, L21, S7, S17 S19,	L24, L25, L26, L27, L28, L29, L31, S2, S6a, S9, S14, S21, S24, S25, S26, S27, a	L13, L14, L16, L22, S14, S18 S24a, b, c

Only the proteins whose spots are notably visible in the gel electrophorograms are listed in the Table. Certain protein spots were faintly visualized and were exclusive to the group in which they are present. Faint spots may be due to weak staining by Coomasie blue, e.g., low molecular weight basic proteins such as L32, L33, L34, S25, S26, S27.

Fig.2 Two dimensional gel electrophoretic patterns of novobiocin fractions of E. *coli* ribosomal proteins:
Fractionation of ribosomal proteins with novobiocin and electrophoresis were carried out as described in the Text.
(A) Total Ribosomal Proteins 125 µg,
(B) to (D) 200, 500, and 1000 µg novobiocin precipitated fraction respectively, and (F) 1000 *uq* novobiocin supernatant fraction.
60 *uq* protein of each of the novobiocin fractions was electrophoresed.

fractionated groups of proteins. It was snown by [2] that the precipitability of a protein by novobiocin is related to its arginine content. There is no information available on the amino acid compositior of most of the ribosomal proteins from S. *acidocaldarius*. Therefore, E. *coli* ribosomal proteins whose amino acid compositions [19] and sequences are known, were fractionated by novobiocin (Fig.2,. In the case of E. *coli* ribosomes only 3 groups of proteins were obtained. Proteins in each group were also identified (Table 2). Only trace amounts of a few proteins precipitated at 200 ug/ml novobiocin. This may be due to

Table II

E. coli ribosomal proteins present in different novobiocin fractions

Novobiocin fraction	Proteins Present in Large amounts	Present in reduced amounts	Present in trace amounts
200 µg/ml precipitate			L2, L4, L6, L16, S2,S3, S5, S4, S7
500 µg/ml precipitate	L2, L4, L6, L14, L15, L16, L20, S3, S5, S7, S9, S13	L5, L13, L14, L17 L20, L27, L30, S2 S14, S15, S18,	L27, L30, S21
1000 µg /ml precipitate	L1, L3, L4, L5, L13, L15, L17, L19, L22	L21, L23, S10, S16 S17,	L2, L6, L7/L12, L27, S2, S3, S4, S5, S6, S7, S16, S20
1000 µg/ml supernatant	L1, L6, L7/L12, L9, L10, L11, L24, S5, S6	L3, L13, L15, L19 L22, L23, L25, S8	L13, L15, L17, L18, L19, L26, L29, L30, S19, S20

Only the proteins whose spots are notably visible in the gel electropherograms are listed in the Table. Certain protein spots were faintly visualized and were exclusive to the group in which they are present. Faint spots may be due to weak staining by Coomasie blue, e.g., L29, L30, L32, S20, S21 etc.

difference in the arginine content of the ribosomal proteins from the two organisms. S. *acidocaldarius* ribosomes apparently contain several ribosomal proteins with high arginine content such that they were precipitated using only 200 ug/ml novobiocin. . It was reported [11] that there is a relationship between the arginine content of proteins and their thermal stability. Hence the precipitation of several ribosomal proteins of S. *acidocaldarius* by low concentrations of novobiocin (200-500 ug/ml) suggests thatthere may be a higher content of arginine in these proteins, which in turn would imply an enhanced thermal stability of these proteins from this acido-thermophilic organism.

Furthermore, a comparison of the fractionation patterns of the ribosomal proteins from the two organisms indicates that structurally homologous proteins identified between *S. acidocaldarius* and *E. coli* ribosomes are present in the same novobiocin fraction. Proteins denoted by L1 and L10 in *S. acidocaldarius* have been shown to be homologous to E. *coli* proteins L10 and L7/L12 [12]. These acidic proteins in both cases are present in the novobiocin 1000 ug/ml supernatant fraction. The two dimensional gel electropherograms show that there is no relationship between the basicity of a protein and the concentration of novobiocin required for its precipitation. In Fig 1, L24 which was not precipitated by 1000 ug/ml novobiocin is more basic than protein LU which was precipitated with 200 ug/ml of the antibiotic. Furthermore, acidic proteins such as L10 of S. *acidocaldarius* as well as one of the most basic proteins, L33 were present in the same group (1000 ug/ml novobiocin supernatant fraction).

Most of the proteins in the 500 ug/ml and the 1000 ug/ml novobiocin fractions of E. *coli* ribosomal proteins have a high arginine content (greater than 7 mole per cent) except proteins S2 (5.4%), S5 (5.0%) and L1 (4.1%). In the case of 1000 ug/ml soluble fraction, most of the proteins present had an arginine content lower than 7 mole per cent except S6 (8.5%) and L23 (7.7%). These results therefore support the observationof [2]

that precipitation by novobiocin is dependent on the arginine content of the proteins. The exceptions may be due to protein-protein interactions as well as the absence of arginine clusters in the proteins.

The individual groups of proteins fractionated by novobiocin may be used as starting material for the separation of individual ribosomal proteins by ion exchange chromatography and gel filtration. We suggest that this method may be useful for the initial fractionation of complex protein mixtures such as cell extracts.

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Effect of ethanol on hepatic ribosomal proteins and mRNA

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Abstract

Levels of RNA, mRNA and separation of ribosomal proteins from control and ethanol treated rat liver, showed no change in total RNA content, but poly(A$^+$)mRNA was reduced significantly in ethanolic rats Ribosomal proteins S2, S3a, S3b, S4, L3, L4, L4a, L10a and L15 were found substantially reduced in experimental rat livers This study suggests decrease in poly(A$^+$) mRNA coupled with loss of ribosomal proteins must be responsible for decreased protein synthesis in chronic alcoholism. (Mol Cell Biochem **115**: 14.V147. 1992)

Key words: ethanol, ribosomes, protein synthesis

Introduction

Chronic ethanol consumption results in altered protein metabolism [1]. The effect of ethanol on protein synthesis is ambiguous, as unchanged [2J. increased [3] and reduced [4] in corporation of labelled amino acids into proteins has been reported. These differences are due to varying doses of ethanol used. Following long term ethanol consumption, inhibition of RNA and protein synthesis [5] and changes in the reassociation properties of 40S and 60S ribosomal sub units have been reported [6]. Recent studies from our laboratory have documented protein defects and loss of many membrane associated proteins from liver [7] and brain [8] of chronic alcoholic rats. Ethanol induced changes in ribosome reassociation properties and altered protein metabolism require further study and this would help in understanding the mechanism of altered protein synthesis. Since ribosomal integrity and mRNA are prerequisite for efficient protein synthesis, we investigated changes in ribosomal proteins and poly(A$^+$(mRNA of chronic alcoholic rat liver.

Materials and methods

Chronic ethanol treatment of experimental animals

Wistar male and female rats of 30 ± 2 days age, weighing approximately SO ± 10 gms were used in this study. The animals were housed individually with access for food and water ad lib atleast for 7 days prior to experimentation The environmental condition in the animal house was adjusted for constant temperature and also a day-night cycle of 12 hr with light from 7 A.M to 7 P.M. The food given to animals was standard laboratory rat feed. Control animals were given tap water while experimental animals received ethanol as 5% (v/v) pre-

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pared from absolute ethanol as their only drinking fluid throughout the experimental period which was up to 10 weeks. Results of fluid consumption indicates that 7.5 gms of ethanol/Kg body weight day was consumed by each experimental rat. The protocol adapted to study the effect of chronic ethanol treatment is essentially followed from published procedures [9J. Another set of control animals were also maintained on tap water containing isocaloric sucrose substituted for ethanol. Following 10 weeks of ethanol treatment, no significant differences were observed in the protein profiles of control and sucrose-fed rats Therefore in subsequent studies comparison was made between control (tap water-fed) and ethanol liquid fed rats only. Changes in the body weight, food and fluid intake were monitored, every day between 10A.M. to 11 A.M. Blood alcohol concentrations (BAC) were measured with Alcohol dehydrogenase, as suggested in Sigma Chemical Company procedure No. 332-UV. The animals were sacrificed at the end of experimental period and livers were excised for isolation of poly(A⁺)mRNA. ribosomal proteins and also for the incorporation of ^{3}H-Leucine into total hepatic cellular proteins.

^{3}H-Leucine incorporation into the total proteins of liver

Protein synthesis was determined by injecting ^{3}H-Leucine intra peritoneally into both normal and experimental animals in a volume of 0.1 ml saline/100 gm body weight and contained 33.3 μCi of ^{3}H-Leucine The liver was homogenized in 10% Trichloroacetic acid, filtered through Whatman filter paper. The filter paper was washed twice with 5% TCA. Ethanol and Ethanol : Ether (3 : 1), finally the filter paper was dried and the radioactivity was measured in a liquid scintillation counter using Bray's mixture

Isolation of cellular RNA and poly(A⁺)RNA

Total cellular RNA from liver was isolated by homogenizing the tissue in guanidinium isothiocyanate and extracting the RNA with phenol as described [10]. The poly(A⁺)mRNA was isolated by using messenger affinity paper (mAP) following manufacturers instructions (Amersham). One square centimeter of mAP was soaked with 5 ml of buffer A (0.5 M NaCl. 0.01 M Tris-HCl (pH 6.8), 0.001 M EDTA) and total RNA was spotted. The paper was washed twice with 5 ml each of 0.5 M NaCl, dipped in 75% ethanol and dried The poly(A⁺)mRNA on the paper was recovered by placing in distilled water in a sterile microfuge tube for 10 min at 70° C. The RNA was estimated by measuring absorbance at 260 nm.

Isolation of ribosomes and extraction of ribosomal proteins

All the steps in ribosomal isolation were carried out under chilled conditions using absolute steriware, unless otherwise specified, to minimize protease and nuclease activities. The ribosomes were isolated [11] by homogenizing the tissue in 0.25 M sucrose made in medium A (50mM Tris-HCl pH 7 8, 12 5 mM MgCI2 and 80 mM KCl) The homogenate was centrifuged at 13,000 g for 15 min and the supernatant was further subjected to centrifugation at 78,000 g for 2 hr. The crude ribosomal pellet was suspended in medium A and then treated with NP-40 and Triton X 100 and centrifuged at 13,000 g for 15 min The supernatant was diluted with 0.15 volumes of medium A and layered on 5 ml of 0.5 M sucrose cushion (prepared in medium A), and centrifuged at 105,000g lor 2.30 hrs to obtain pure ribosomes [11]. The ribosomal proteins were extracted as described |12J, precipitated with 5 volumes of acetone and dissolved in 6 M urea and 10 mM dithiothreitol.

Two dimensional electrophoresis (IDE)

Ribosomal proteins (100 μg) from control and ethanolic rat livers were subjected to two dimensional electrophoresis as described earlier [13] using 4% acid-urea gels in the first dimension and 18% slab gels in the second dimension The proteins in the gel slabs were stained with Coomassie blue and were identified [14]

Results and discussion

In the present study ethanol was administered to rats as sole drinking fluid in the form of 5% ethanolic water and the experiments were carried out over a period of 10 weeks. The experimental group of rats did not show any overt behavioral or pathological symptoms excepting that their sleeping time was increased and the animals were flaccid. The righting reflex indicated they

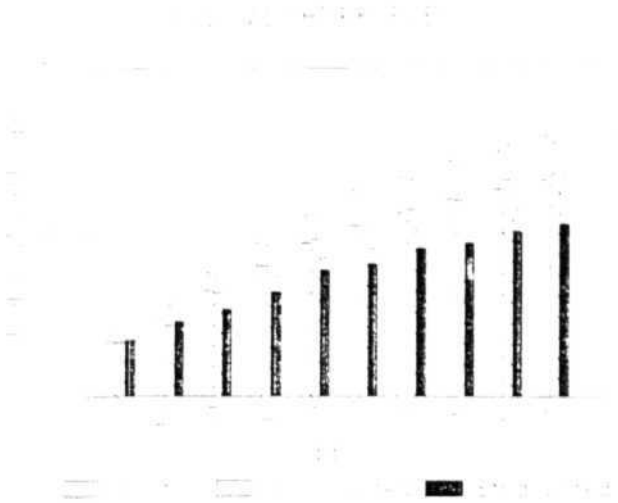

Fig 1 Changes in body weight in Control. Sucrose-fed and chronic ethanol-fed rats Each bar is represented as mean i SD of 10 samples

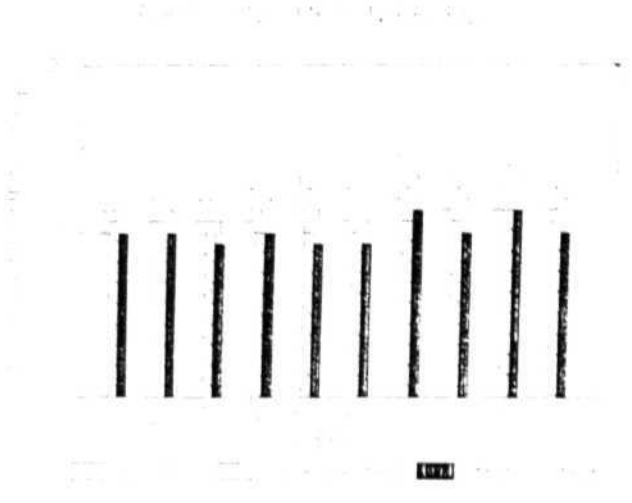

Fig 2 Changes in food intake pattern in Control. Sucrose-fed and chronic ethanol-fed rats Each bar is represented as mean ± S.D of 10 samples

were intoxicated. The alcohol dose consumed by animals did not cause any mortality and throughout the experimental period. the animals were healthy but inebriant.

A 9% decrease in body weight was observed in the experimental group of rats when compared to water or sucrose fed rats (Fig. 1). The decrease in body weight observed in ethanol treated group of rats agrees with earlier studies (15). There was no significant variation in the food consumption in the experimental group of animals when compared with the controls (Fig. 2). These results were in agreement with the values reported [16]. The average consumption of ethanol was 7.5 g/ Kg body weight/day. From the fluid consumption pattern (Fig. 3), as well as from ethanol levels in blood. blood alcohol concentration (BAC) was noticed to be 0.15%. suggesting that the animals were under intoxication range. The fluid intake pattern suggested that the absolute amount of ethanol consumed was relatively consistent. Data from food consumption in the experimental group of animals is not altered significantly (Fig. 2). suggesting that alcohol fed and pair fed control animals enjoy nutritional adequacy and further suggest that the results are due to specific effects of alcohol but not due to starvation or vitamin deficiency like in the case of under fed animals. Changes in body weight which remained more or less same in all groups indicated the adequacy of our attempt to maintain equal nutritional status.

Chronic ethanol treatment had no influence on the total RNA content (Table 1). but the poly(A')mRNA content has decreased by 50% in ethanol treated rats (Table 2). The recovery of the poly(A')mRNA by messenger affinity paper was found to be equal to that of the mRNA isolated by oligo dT-cellulose chromatography [16]. Analysis of ribosomal proteins by two dimensional electrophoresis (Fig 4). showed that certain ribosomal proteins are present in substantially reduced amount or are missing from ethanol treated rat liver ribosomes. Based on the 2DE gel pattern and Madjar

Fig 3 Changes in fluid intake pattern in Control. Sucrose-fed and chronic ethanol-fed rats Each bar is represented as mean t S.D of 10 samples

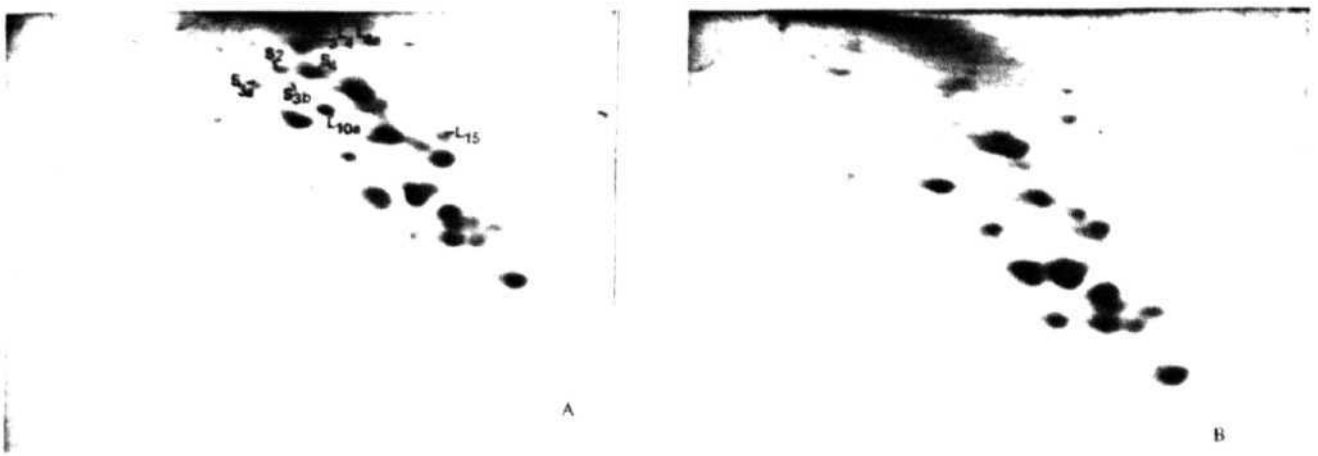

Fig. 4. Ribosomal proteins separated on two dimensional electrophoresis as described in methods from the liver of control (A) and chronic ethanol treated (B) rats. The proteins are identified according to Madjar's nomenclature [14]. Ribosomal proteins missing in alcohol treated rat liver are shown with dotted circles in panel B, while they are marked in panel A.

nomenclature proteins S2, S3a, S3b, S4, L3, L4, L4a, L10a, and L15 are found in reduced amounts and or missing in ribosomes of ethanol treated rats. There are no changes in poly(A⁺)mRNA and ribosomal proteins of liver from control and pair-fed rats (data not shown).

The majority of mRNA's before being translated have to be polyadenylated to form poly(A⁺)mRNA, without it, mRNA can not be translated by the ribosomes. Since the poly(A⁺)mRNA content is decreasing, it appears that either polyadenylation process itself is inhibited, or the poly(A')mRNA is being degraded soon after its formation. Nevertheless, the decrease in poly(A") (mRNA can certainly be one of the underlying factors affecting protein synthesis. ^{3}H-Leucine incorporation experiments showed that protein synthesis is indeed affected in liver due to chronic ethanol treatment (Table 3). Intact ribosomes are as important as mRNA for efficient translation. The ribosomal proteins in association with rRNA form binding sites for different factors of translation. Any qualitative variation in the ribosomal proteins could have a remarkable influence on the translation process. The ribosomal proteins S2, S3a, S3b, S4, L3, L4, L4a, L10a and L15 which are found to be missing or present in very low amounts in the ethanolic rat liver ribosomes have all been shown to be involved in initiation and/or elongation steps of protein synthesis. Protein S3a has been implicated in the binding of initiation factor eIf2 and mRNA binding. Proteins S2, S3a, S4, S5, are present near the initiation factor eIf3 binding site. The large subunit proteins form part of the ribosomal decoding site (see review 18). It is not known at present, whether the synthesis of these proteins is affected or their association with rRNA is affected in liver during long term ethanol consumption. The results presented here suggests that changes in ribosomal protein composition and decreased poly(A⁺) mRNA may contribute to the molecular mechanism of alcohol altered protein synthesis in the liver.

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Table 1. Total cellular RNA in control and ethanol treated rat liver

Treatment	A260/280	Total RNA (mg/gm tissue)
Control	1.72 ± 0.092	2.03 ± 0.22
Ethanol treated	1.88 ± 0.043	2.39 ± 0.62

Values are mean ± S.D. of 5 samples in each group.

Table 2. Poly(A⁺)mRNA levels in control and ethanol treated rat liver

Treatment	Total RNA loaded	mRNA recovered
Control	100 μg	10.2 ± 3.5 μg
Ethanol treated	100 μg	5.0 ± 1.9 μg

Values are mean ± S.D. of 5 samples in each group.

Table 3 Incorporation of ^{3}H-leucine into proteins of liver from control and chronic ethanol treated rats

Treatment	CPM/mg protein
Control	1301 *1* 201
Experimental	955 i 118
Difference	- 27%.

Mean *t* S.D of 5 wimples in each group $p < 0.05$

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