THE UNIVERSITY OF MANITOBA

In Vitro Formation of Subparticles of the *Escherichia coli* 50S Ribosomal Subunit

BY

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TO PA

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ABSTRACT

The sequential binding of ribosomal proteins to RNA species of the E. coli 50S ribosomal subunit to form ribonucleoprotein particles of increasing sizes was a highly temperature dependent process. During sequential incubation from $0^{o} \longrightarrow 37^{o} \longrightarrow 55^{o}$, 23S RNA - associated particles increased in S-value from 28S $\longrightarrow$ 32S $\longrightarrow$ 43-48S (when analyzed in 1.0mM Mg^{++}), respectively, accompanied by simultaneous binding of more 50S ribosomal proteins. Reconstituted particles sedimenting at 28, 32 and 43-48S in 1.0mM Mg^{++} increased in S-value to 32, 37 and 48S, respectively, in 10mM Mg^{++}.

Formation of a single 48S peak in the sedimentation profile was achieved only in the presence of excess 50S ribosomal proteins (50S protein/23S RNA ratio of 3.0). Previous incubation at 37^{o} was absolutely required when 48S particles were formed at 55^{o}. However, addition of 10mM spermidine to the reconstitution mixture prior to incubation not only increased the rate of 48S particle formation but also abolished this requirement so that 48S particles could be formed on direct incubation at 55^{o}. The 50S ribosomal proteins bound in each type of reconstituted particles were

quantitated after bidimensional electrophoresis by means of dual-label technique.

Not all 48S particles formed at 55° could associate with 30S ribosomal subunits to yield 70S complexes. However, all 48S particles formed at 50° in the presence of excess 50S ribosomal proteins and 10mM spermidine were able to associate with 30S ribosomal subunits in a specific and stoichiometric manner. Also, the latter 48S particles exhibited an association activity almost equal to that of 50S ribosomal subunits. The presence of 5mM spermidine was required for the stabilization of the 70S complexes against dissociation.

Both 37S (or 32S) and 48S reconstituted particles were partially active in IF-dependent coupled and uncoupled GTP hydrolysis, and EF-dependent uncoupled GTP hydrolysis. However, both were inactive in fMet-tRNA binding, peptide bond formation and poly(U)-directed polypeptide synthesis. Inclusion of various ribosomal and nonribosomal factors in reconstitution did not yield fully functional 50S subunits.

Although 37S reconstituted particles formed at 37° could not associate with 30S ribosomal subunits, incubation of these particles at 50° in the presence of exogenous 50S ribosomal proteins converted them to 45S particles which could do so. The proteins that became bound under these conditions were L2, L11, L15, L18 and L25, and conferred upon the particles the ability to associate with 30S ribosomal subunits.

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ABBREVIATIONS

A_{260}	absorbance at 260 nm
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
c.p.m.	counts per minute
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediamine tetraacetic acid
EF	elongation factor
fMet-tRNA	formylmethionyl-transfer RNA
50SP	50S proteins
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
IF	initiation factor
MRE	Microbiological Research Establishment
mRNA	messenger RNA
PEP	phosphoenolpyruvate
poly U	polyuridylic acid
p.s.i.	pounds per square inch
RNA	ribonucleic acid
RNase	ribonuclease
r.p.m.	revolutions per minute
rRNA	ribosomal RNA
S	Svedberg unit

Abbreviations Continued

SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
Tris	tris (hydroxymethyl) aminomethane
tRNA	transfer RNA

I. INTRODUCTION

Nomura and Traub (1968) first reconstituted functional E. coli 30S ribosomal subunits from 16S RNA and purified 30S ribosomal proteins. They have shown that in vitro subunit assembly depends exclusively upon ribosomal molecular components. Their reconstitution system has proven to be a very powerful tool in the study of structure and function of the 30S ribosomal subunit (Nomura, 1973). While data about the small ribosomal subunit have rapidly accumulated, attempts at reconstituting functional E. coli 50S ribosomal subunits have met difficulties. Nomura and Erdmann (1970) were able to reconstitute Bacillus stearothermophilus 50S ribosomal subunits that were active in polypeptide synthesis. Presumably, the heat stability of the 50S ribosomal molecular components from the thermophilic organism was responsible, in part, for this success. There have been 2 reports of successful reconstitution of functionally active E. coli 50S subunit. The first, by Maruta, Tsuchiya and Mizuno (1971), has not been verified by other workers, while the second, by Nierhaus and Dohme (1974), if proven consistent, should be invaluable for the functional analysis of molecular components of the E. coli 50S

ribosomal subunit.

A number of workers investigated RNA-protein interactions in E. coli 50S subunit and found that eight 50S proteins formed stable and specific complexes with RNA from 50S subunit (Stoffler, Daya, Rak and Garrett, 1971). Other workers reported that four 50S proteins could be involved in the quaternary complex of 23S RNA and 5S RNA (Gray and Monier, 1971; Gray, Garrett, Stoffler and Monier, 1972). Still others tried to elucidate protein-protein interactions using iodination (Craven and Gupta, 1970), fluorescent dyes (Huang and Cantor, 1972), bifunctional cross-linking reagents (Kurland, Green, Schaup, Donner, Lutter and Birge, 1972; Chang and Flaks, 1972), and neutron diffraction (Engelman and Moore, 1972).

In the present study, we have attempted to examine in vitro formation of ribonucleoprotein complexes from separated components of E. coli 50S subunit under various conditions. The groundwork laid down may ultimately lead to successful reconstitution of functional E. coli 50S subunits.

II. HISTORICAL

General Properties of Ribosomes

Ribosomes are ribonucleoprotein particles and the sites at which information in mRNA molecules is translated into polypeptides with specific amino acid sequences. There are 3 types of ribosomes depending on the biological source - those from procaryotic cells, from eucaryote cellular organelles, the mitochondrion and chloroplast, and from eucaryote cytoplasm (Spirin and Gavrilova, 1969). Regardless of origin, ribosomes are slightly prolate ellipsoids divided into 2 unequal subunits. A cleft between subunits was demonstrated in the electron microscope by means of negative staining techniques (Huxley and Zubay, 1960).

Reversible dissociation of the E. coli ribosome into 2 unequal subunits was first demonstrated in 1958 by Tissieres and Watson. At 1.0 mM magnesium, dissociation into 30S and 50S subunits occurred whereas at 10 mM magnesium, the subunits associated specifically at a 1:1 ratio. Mg^{++} could be replaced by Ca^{++} but not Be^{++}, Ba^{++}, Sr^{++},

Cd^{++}, Hg^{++} or Zn^{++} (Chao, 1957; Hamilton and Peterman, 1959; Roskoski, 1969; Walters and Van Os, 1970). Dissociation was also promoted by high concentrations of monovalent cations such as K^+ and NH_4^+. At 100 mM K^+, dissociation occurred (Watson, 1964) while at 50 mM K^+, nonspecific dimerization of ribosomes was inhibited (Watson, 1964; Dahlberg and Haselkorn, 1967). Other factors that promoted dissociation were elevated temperature (Zitomer and Flaks, 1972; Spirin, Sabo and Kovalenko, 1971; Tamaoki and Miyazawa, 1966), high pH (Spirin, Sabo and Kovalenko, 1971; Walters and Van Os, 1970), urea (Spirin and Lishnevskaya, 1971), and hydrostatic pressure such as that induced during ultracentrifugation (Infante and Krauss, 1971; Infante and Baierlein, 1971; Hauge, 1971).

The association of subunits to form ribosomes was induced by polyamines such as spermidine and putrescine in the presence of suitable concentrations of magnesium (Cohen and Lichtenstein, 1960; Martin and Ames, 1962; Silman, Artman and Engelberg, 1965; Pestka, 1966; Norton, Erdmann and Herbst, 1968). Factors that enhanced association included methanol (Spirin and Lishnevskaya, 1971; Voigt and Parmeggiani, 1973), ethanol, dimethyl sulphoxide, low ionic strength, low temperature and low pH (Spirin et al, 1971).

In general, subunits in the translating ribosome were firmly associated (Tissieres, Schlessinger and Gros, 1960) whereas uncharged subunits were loosely associated (Spirin, 1971). It has been demonstrated in vivo and in vitro that subunits tend to associate during initiation of translation and dissociate on termination of translation (Davis, 1971). As many factors which induced excessively stable association could inhibit elongation, it was suggested that lability of the association between subunits might be necessary during translation (Spirin, 1972). On this basis, a hypothesis was proposed in which subunits, that remained permanently joined by hinges, were locked and then unlocked in a cylical fashion during the process of translation (Spirin, 1969).

The average sedimentation coefficient for the procaryotic ribosome was about 70S (Tissieres and Watson, 1958; Tissieres, Schlessinger and Hollingworth, 1959), with a particle weight of 2.7×10^6 daltons with dimensions of $200 \times 170 \times 170$ Å in the dry state and $290 \times 210 \times 210$ Å in solution (Hall and Slayter, 1959). It is made up of a 30S subunit with a particle weight of 0.9×10^6 daltons, and a 50S subunit with a particle weight of 1.8×10^6 daltons.

The Structure of Ribosomes

(a) RNA Components

The 30S subunit of procaryotes contains a 16S RNA molecule and 21 proteins while the 50S subunit contains a 5S and 23S RNA molecules and 34 proteins (Traut, Moore, Delius, Noller and Tissieres, 1967; Kaltschmidt, Dzionara, Donner and Wittmann, 1967; Fogel and Sypherd, 1968; Kurland, 1972). The molecular weights of 5S, 16S and 23S RNA molecules were 4×10^4, 0.6×10^6 and 1.2×10^6 daltons respectively. The RNA made up about 2/3 of the weight of the procaryote ribosome (Pace, 1973).

The 70S ribosome from the mitochondrion and chloroplast of eucaryotic cells was very similar to the procaryotic 70S ribosome in physical and chemical properties. The eucaryotic 70S ribosome contained rRNA indistinguishable from the procaryotic rRNA (23S and 16S) both in sedimentation coefficient and in molecular weight (Stutz and Noll, 1967; Kuntzel and Noll, 1967).

The eucaryotic cytoplasmic ribosome has an S-value of about 80S (Kuff and Zeigel, 1960; Taylor and Storck, 1964; Dintzis, Borsook and Vinograd, 1958), and was made up of subunits larger than those of 70S ribosomes-a 40S subunit with a particle weight of 1.2×10^6 daltons, and a 60S subunit

with a particle weight of 2 x 10^6 daltons. The small subunit contained an 18S RNA molecule of 7 x 10^5 daltons whereas the large subunit contained a 28S RNA molecule of 1.5 x 10^6 daltons and a 5S RNA molecule of 4 x 10^4 daltons. Each subunit contained more proteins than its procaryote counterpart, although the amount of RNA and protein was approximately equal (Pace, 1973). In mammalian cells, 2 classes of ribosomes are known to be present, free and membrane-bound. It has been suggested that the free ribosomes synthesized proteins destined for intracellular use while membrane-bound ribosomes were involved in synthesis of membrane proteins as well as proteins for secretory purposes (Palade, 1966; Takagi and Ogata, 1968; Ikehara and Pitot, 1973).

(b) Protein Components

In contrast to rRNA, the protein composition of ribosomes is extremely complex, the total protein mass in procaryotic 70S ribosomes being about 10^6 daltons. Chemical analysis of the N-terminal groups indicated the average length of ribosomal polypeptide chains of E. coli to be about 230 amino acid residues (Waller and Harris, 1961). The average molecular weight was estimated to be about 26,000 daltons based on data from sedimentation and diffusion experiments (Moller and Chrambach, 1967). It was first demonstrated in 1964 (Waller) by means of electro-

phoretic and chromatographic fractionation that many different proteins were present in procaryotic ribosomes. Polyacrylamide gel electrophoresis of ribosomal proteins also revealed numerous protein bands (Traub, Hosokawa, Craven and Nomura, 1967; Gesteland and Staehelin, 1967). These and other data lent support for the view that the apparent electrophoretic and chromatographic complexity of ribosomal proteins reflected a real protein heterogeneity. The possibility of common sequences and homologies among the heterogeneous protein population was investigated by immunological methods (Fogel and Sypherd, 1968). It was found that the majority of proteins did not exhibit significant homologies and that only 2 pairs of proteins gave reactions of partial serological identity.

Several laboratories demonstrated that there were about 20-21 different proteins in the 30S subunit and as many as 34 in the 50S subunit (Traut, Moore, Delius, Noller, and Tissieres, 1967; Kaltschmidt, Dzionara, Donner and Wittmann, 1967; Fogel and Sypherd, 1968). Two-dimensional polyacrylamide gel electrophoretic analysis of the proteins of E. coli ribosomal subunits (Kaltschmidt and Wittmann, 1970a), in conjunction with the isolation and chemical, physical and immunological studies of the individual ribosomal proteins (Kurland et al, 1969; Traut, Delius, Ahmed-Zadeh, Bickle, Pearson and Tissieres, 1969)

unequivocally showed 21 distinct proteins in the small subunit and 34 in the large subunit (Kaltschmidt and Wittmann, 1970b). A numbering system was proposed based on the position of the protein spots on the two-dimensional electropherogram in which the spots from each ribosomal subunit reproducibly appearing in the same position were numbered from left to right and top to bottom of the electropherogram. In this way, the proteins in the 30S subunit were numbered from S1 to S21 while those in the 50S subunit, from L1 to L34.

The absence of common sequences among E. coli 30S ribosomal proteins was confirmed by elucidation of amino acid sequences of the N-terminal region of some 30S proteins (Yaguchi, Roy, Matheson and Visentin, 1973). However, when individual sequences of 30S proteins from E. coli were compared with that of corresponding proteins from Bacillus stearothermophilus, numerous structural homologies were observed (Yaguchi, Matheson and Visentin, 1974; Isono, Isono, Stoffler, Visentin, Yaguchi and Matheson, 1973). On the basis of comparative studies of ribosomal proteins from different procaryotic ribosomes, including those of organisms subjected to greater evolutionary pressures, it was suggested that the high degree of homology observed in some 30S ribosomal proteins might indicate a substantial level of evolutionary conservation in both structure and function of ribosomes (Chow, Visentin, Matheson and Yaguchi, 1972;

Visentin, Chow, Matheson, Yaguchi and Rollins, 1972).

Each 30S subunit from E. coli contains a total protein mass of about 300,000 daltons. A simple calculation revealed that if there was one copy of each of 21 different proteins in the subunit, the total protein mass would approach 500,000 daltons, and therefore, the sum of the molecular weights for twenty-one 30S proteins exceeded the total protein mass per 30S subunit by 200,000 daltons (Kurland et al, 1969). This showed that not only were ribosomal proteins heterogeneous but also that the ribosomes themselves were heterogeneous in terms of their protein complement. Stoichiometric studies revealed that some 30S proteins were present as unit proteins (in amounts close to one copy per 30S subunit), some as fractional proteins (in amounts much less than one copy per 30S subunit), and others as marginal proteins (in amounts a little less than one copy per 30S subunit) (Weber, 1972; Kurland, Voynow, Hardy, Randall and Lutter, 1969; Voynow and Kurland, 1971). A similar situation occurs with proteins in the 50S subunits. In Table I, U indicates unit protein; F, fractional protein; M, marginal protein; and FR, repeat copy of a fractional protein.

To account for the observed ribosome heterogeneity, 2 models have been proposed. In the static model, ribosomes belonged to functionally distinct groups based on specific

Table I. Molecular weight and stoichiometry of ribosomal proteins (Garrett and Wittmann, 1973).

30S Protein	Molecular weight	Stoichiometry	50S Protein	Molecular weight	Stoichiometry
S1	65,000	F	L1	22,000	U
S2	27,000	F	L2	28,000	U
S3	28,000	M	L3	23,000	FR
S4	25,000	U	L4	28,500	U
S5	21,000	M	L5	17,500	R
S6	17,000	M	L6	21,000	U
S7	26,000	U	L7	15,500	FR
S8	16,000	U	L8	19,000	F
S9	17,500	U	L9	-	F
S10	17,000	M	L10	21,000	U
S11	-	F	L11	19,000	U
S12	17,000	F	L12	15,500	F
S13	14,000		L13	20,000	FR
S14	15,000	F	L14	18,500	U
S15	13,000	U	L15	17,000	(U)
S16	13,000	U	L16	22,000	U
S17	10,000	U	L17	15,000	U
S18	12,000	M	L18	17,000	R
S19	14,000	F	L19	17,500	U
S20	13,000	M	L20	16,000	(F)
S21	13,000	F	L21	14,000	U
			L22	17,000	R
			L23	12,500	U
			L24	14,500	FR
			L25	12,500	FR
			L26	12,500	F
			L27	12,000	M

Table I. Continued.

30S Protein	Molecular weight	Stoichiometry	50S Protein	Molecular weight	Stoichiometry
			L28	15,000	F
			L29	12,000	U
			L30	10,000	U
			L31	-	(F)
			L32	-	F
			L33	9,000	F
			L34	-	(F)

U, unit protein

F, fractional protein

M, marginal protein

R, repeated protein

FR, fractional repeated protein

fractional proteins in stable association with a common core particle consisting of RNA and unit protein components. In the steady-state model, each ribosome readily exchanged, in an orderly fashion, its fractional proteins which determined its functional mode at any time during the process of translation.

To study heterogeneity of ribosomes in vivo, the protein composition of ribosomes synthesized by E. coli cultured in rich (yeast extract and peptone) or in minimal media, containing (^{14}C) or (^{3}H) labelled amino acid mixture, respectively, were compared (Deusser, 1972). The ribosomal proteins from (^{14}C) and (^{3}H) labelled cells were extracted together and separated by means of bidimensional gel electrophoresis. The ratio of (^{14}C) and (^{3}H) radioactivity associated with each protein spot showed that the majority of proteins exhibited ratios between 0.9 and 1.1. However, 3 proteins, S6, S21 and L12, showed ratios of 2.46, 2.36 and 3.10 respectively, indicating that they were synthesized in 2 to 3-fold greater amounts in cells grown in rich medium than in minimal medium. This suggested that heterogeneity in ribosomes could be brought about by variations in growth conditions. That growth rates could also alter the rate of synthesis of ribosomal macromolecules in cells, resulting in detectable heterogeneity in ribosomes was recently demonstrated by Milne, Mak and Wong (1975).

Many observations have also suggested heterogeneity of eucaryotic ribosomes (Friedlander and Wettstein, 1970; Burka and Bulova, 1971: MacInnes, 1972; Lambertsson, 1972; Rodgers, 1973; Borgese, Blobel and Sabatini, 1973), and recently, Sherton and Wool (1974) showed the existence of fractional proteins in eucaryotic ribosomes. McConkey and Hauber (1975) presented direct evidence for heterogeneity of Hela cell ribosomes by comparing the protein compositions of native subunits and derived subunits from single ribosomes, free polysomes and membrane-bound polysomes.

Dissembly of Ribosomal Subunits

Removal of magnesium by EDTA (Gesteland, 1966), or its replacement by monovalent ions (Gavrilova, Ivanov and Spirin, 1966), brought about unfolding of ribosomal particles. During unfolding, subunits became less compact although no proteins were lost, and the S-values of subunits, in their fully unfolded state, approached that of the RNA in their structures. The process of unfolding was characterized by decreases in sedimentation coefficient and by concomitant increases in specific viscosity. In the E. coli system, the transitional states of subunits during unfolding (Gavrilova et al, 1966) could be schematically represented

by:

50S ⟶ 35S ⟶ 22S

30S ⟶ 26S ⟶ 15S

The final products of unfolding was not due to fragmentation of unfolded particles but to the polyelectrolyte nature of unfolded ribonucleoprotein particles (Gavrilova et al, 1966).

Although there was no change in the protein content of unfolded subunits, the 5S RNA in the unfolded large subunit was readily exchangeable with exogenous 5S RNA (Hosokawa, 1970). The unfolded particles were very sensitive to nucleolytic attack (Natori, Maruta and Mizuno, 1968; Moller, Amons, Groene, Garrett and Terhorst, 1969). Unfolding was also induced by elevated temperature (Tal, 1969; Bodley, 1969). The polyamines, in the presence of magnesium, stabilized the associated state of ribosome but when they completely replaced magnesium, unfolding of subunits was induced (Weiss and Morris, 1973; Weiss, Kines and Morris, 1973).

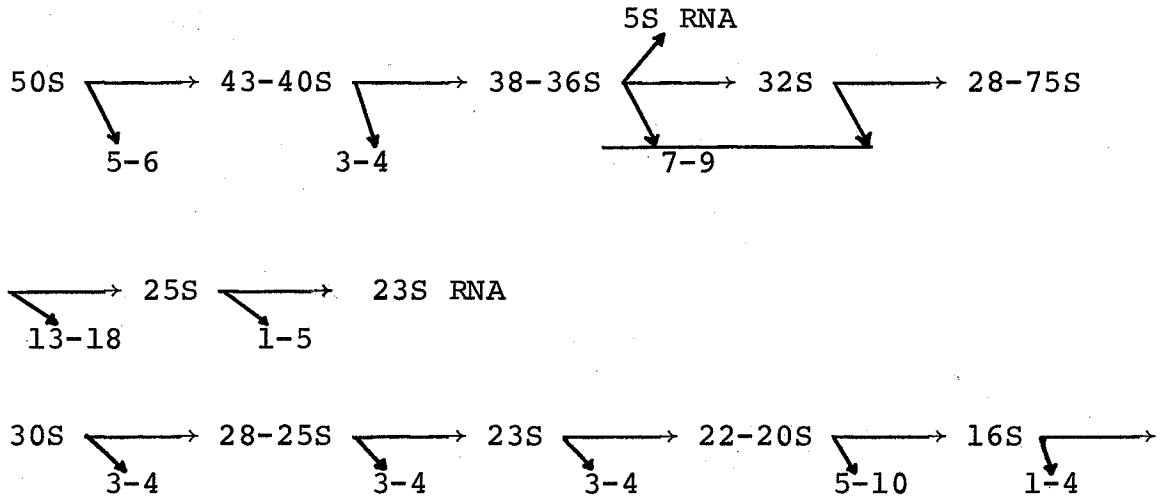
The transitions during unfolding were reversible in the early stages. On restoration of the magnesium content, particles with the original S-values and biological activity were recovered (Van Duin, Van Diejen, Van Knippenberg and Bosch, 1970; Gesteland, 1966; Miall and Walker, 1969). However, the final stages of unfolding could only be reversed by heat activation (Traub and Nomura, 1969). Presumably, the 5S RNA dissociated from the large subunit

during the final stages of unfolding but reassociated with the subunit upon heat activation. Unfolding studies have shown that rRNA has a definite secondary structure-short double helical regions punctuated by single-stranded regions. Proteins or groups of proteins are thought to bind specifically to single-stranded regions. The resulting ribonucleoprotein strand, with the participation of more proteins, folds back on itself to achieve the compact subunit structure (Spirin, 1974).

When ribosomes were centrifuged in 0.5 M CsCl, 23S and 42S particles which were protein-deficient were formed (Meselson, Nomura, Brenner, Davern and Schlessinger, 1964). The proteins removed from ribosomes were recovered from the tops of the CsCl gradients and referred to as "split proteins", and resulting protein-deficient particles were referred to as "core particles". It was later shown that proteins could be split off in a step-wise manner by incubation of ribosomes in varying high CsCl concentrations (Spirin, Belitsina and Lerman, 1965), and that the dissembly process could be reversed by replacing CsCl with magnesium. Since then, it has been shown that formation of split proteins and cores depended upon the concentrations of monovalent cations and magnesium to which ribosomes and subunits were exposed. Various reagents such as CsCl (Marcot-Queiroz and Monier, 1966; Atsmon, Spitnik-Elson and Elson, 1967;

Homann and Nierhaus, 1971), LiCl (Atsmon et al, 1967; Itoh, Otaka and Osawa, 1968), other monovalent salts (Spitnik-Elson and Atsmon, 1969; Atsmon, Spitnik-Elson and Elson, 1969), EDTA and urea (Kaltschmidt, Rudloff, Janda, Cech, Nierhaus and Wittmann, 1971) have been used to dissemble ribosomal subunits.

Dissembly took place in an orderly fashion, depending upon ionic conditions to yield a variety of core and split protein fractions. The process is summarized below (arrows orientated at diagonals indicating the number of proteins removed at each step):



Dissembly in varying CsCl concentrations took place in 4 steps. The proteins removed at each stage have been identified and are grouped in Table II according to the order in which they have been stripped from subunits (Traub et al, 1967; Homann and Nierhaus, 1971: Kaltschmidt et al,

Table II. Dissembly of ribosomal subunits : Order of release of ribosomal proteins during dissembly (Spirin, 1974).

Group[a] / subunit	I	II	III	IV
30S	S1,S2,S3, S5,S9,S10, S14	S11,S12,S18, S21	S4,S6, S16	S7,S8,S15, S17,S19
50S	L16,L26, L33	L7,L8,L10, L18	L6,L11, L12,L15, L25,L27, L31	L3,L4,L13, L17,L19, L21,L22, L23,L24, L29

[a]Group numbers are according to order of release.

1971).

The studies on dissembly led to the concept that ribosomal proteins were not bound equally in ribosomes. Some proteins were more accessible to the effects of monovalent cations suggesting these may be more exposed to the environment or that they may be bound weakly. Also, the arrangement of the proteins in ribosomes could be inferred from dissembly studies. In one case, S11, S18, S21 and S12 which were neighbouring proteins in the 30S subunit were detached as one group (Shin and Craven, 1973).

Assembly of Ribosomal Subunits

(a) Partial Reconstitution in vitro

The understanding that dissembly could be reversed led to studies on the possibility of self-assembly of ribosomes. Systems for the partial reconstitution of ribosomes were devised (Staehelin and Meselson, 1966; Hosokawa, Fujimura and Nomura, 1966). Ribosomal 30S subunits were partially dissembled in 5.0 M CsCl into core particles (α,β, γ) sedimenting slower than 30S, and split proteins (30 to 40% of total ribosomal proteins) and separated by CsCl gradient centrifugation. The inactive core particles were mixed with split proteins to produce functionally active ribosomes (Nomura and Traub, 1968). The partial reconstitution reaction was very rapid, being complete within a few minutes at 37^{o}.

With this system, attempts were made to determine the function of each of the split proteins. This could be done by "single omission" experiments in which the function of a particular split protein was studied by assaying for various biological activities of ribosomal particles reconstituted in the absence of the split protein to be tested. Thus, particles reconstituted in the absence of S3, S10 or S14 were inactive in polypeptide synthesis indicating these proteins were required for function. Such reconstituted particles sedimented around 30S. On the other hand, particles reconstituted in the absence of S9 were not only inactive, but also sedimented with S-values much less than 30S, indicating the protein was essential in assembly. (Traub et al, 1967; Wittmann, Stoffler, Hindennach, Kurland, Randall-Hazelbauer, Birge, Nomura, Kaltschmidt, Mizushima, Traut and Bickle, 1971). On this basis, proteins could be divided into 2 broad groups - those required essentially for activity, i.e., functional proteins, and those required for assembly, i.e., assembly proteins.

Ribosomal functions were similarly assigned to the rest of the 30S split proteins: S4 enhanced polypeptide synthesis activity; S1 enhanced mRNA binding (Van Duin and Kurland, 1970); and S2 facilitated fMet-tRNA binding (Van Duin et al, 1972). In order to study proteins other than the split proteins, a total reconstitution system was

developed, that is, reconstitution of functional ribosomal particles from separated RNA and protein components. As in the case of 30S subunits, the first partial reconstitution of E. coli 50S subunits made use of core particles and split proteins produced in CsCl (Traub and Nomura, 1968; Staehalin, Maglott and Munro, 1969). Although active particles were obtained, this system has not been extensively exploited for determination of the functions of the 50S split proteins.

Treatment of 50S subunits with 1.0 M NH_4Cl and 50% ethanol yields core particles and a split protein fraction containing L7, L12 and a few other 50S proteins (Hamel, Koka and Nakamoto, 1972; Brot, Yamasaki, Redfield and Weissbach, 1972). Use of these preparations in partial reconstitution showed that binding of proteins L7 and L12 to cores was dependent on the presence of L10 in particle. In turn, the binding of L10 in reconstituted particles was dependent on the previous binding of L11 (Highland and Howard, 1975). Proteins L7 and L12 were essential for EF-G and EF-T-dependent ribosomal functions in this system (Weissbach, Redfield, Yamasaki, Davis, Pestka and Brot, 1972). In addition, the removal of protein L11 resulted in loss of thiostrepton binding activity of the ribosome (Highland, Howard, Ochsner, Stoffler, Hasenbank and Gordon, 1975). Thiostrepton specifically inhibited IF-2-dependent GTP hydrolysis (Mazumder, 1973), both elongation reactions

involving EF-T_U and EF-G (Kurland, 1972), and RF-directed termination (Brot, Tate, Caskey and Weissbach, 1974).

(b) Total Reconstitution in vitro

The 30S subunit of E. coli was successfully reconstituted from 16S RNA and total 30S ribosomal proteins by Traub and Nomura (1968) and subsequently, with individually purified 30S proteins (Nomura, Mizushima, Ozaki, Traub and Lowry, 1969; Nomura, 1972; Held, Mizushima and Nomura, 1973). The reconstituted 30S subunit was as active as the original 30S subunit in the binding of aminoacyl-tRNA and in polypeptide synthesis. Reconstitution was carried out at 40° for 20 min in a standard reconstitution buffer made up of 30mM Tris, pH 7.4, 20mM $MgCl_2$, 300mM KCl and 6mM 2-mercaptoethanol.

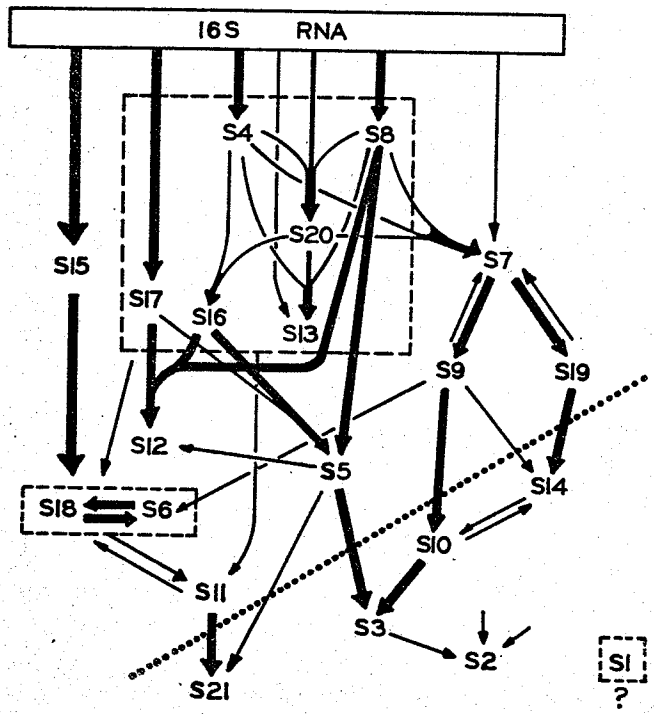
At 0°, reconstitution intermediate particles (RI particles) were formed which sedimented at 21S in low magnesium buffers. These RI particles were inactive, being deficient in several ribosomal proteins. At higher temperatures, the RI particles bound the remaining proteins and were transformed to functionally active 30S particles. It was suggested that the heat activation step represented a rate-limiting unimolecular reaction involving a conformational change in the structure of the RI particles that allowed other proteins to bind. A reaction scheme was proposed for the total reconstitution of 30S subunits (Traub and

Nomura, 1969) in which proteins were bound to 16S RNA in 2 stages. 16S RNA $\xrightarrow{\text{RI proteins}}$ RI particles $\xrightarrow{\text{heat}}$ RI^* particles $\xrightarrow{\text{S proteins}}$ 30S particles

RI proteins were a group of 15 proteins, that bound to 16S RNA to form a 21S reconstitution intermediate particle (RI). Seven of these proteins could bind independently to 16S RNA (see later). The S proteins are mainly split proteins that could bind only to heat-activated reconstitution intermediate particles (RI^*).

The sequence of binding of 30S ribosomal proteins during in vitro assembly of 30S subunit was determined, using separated and purified 30S ribosomal proteins. Under the conditions of reconstitution, 7 of the twenty-one 30S ribosomal proteins were found to bind directly to 16S RNA (Mizushima and Nomura, 1970) whereas the rest of the proteins did so cooperatively, i.e., in the presence of previously bound proteins. However, some workers reported only 5 of the 7 proteins as specific initial binding proteins (Schaup, Green and Kurland, 1971; Garrett, Rak, Daya and Stoffler, 1971). These studies have led to the construction of an assembly map (Fig. 1) depicting the order of addition of proteins in the course of reconstitution (Mizushima and Nomura, 1970; Schaup et al, 1971; Garrett et al, 1971; Nashimoto, Held, Kaltschmidt and Nomura, 1971; Nomura and Held, 1974).

Figure 1. Assembly map of E. coli 30S ribosomal proteins (taken from Nomura and Held in Ribosomes,1974).



In Figure 1, the thick and thin arrows between 16S RNA and 30S proteins indicate strong and weak direct binding, respectively, and those between 30S proteins indicate strong or weak facilitating effect respectively, of one protein on the binding of another. For example, the thick arrow from 16S RNA to S15 shows that S15 binds directly to 16S RNA in the absence of other proteins while the thin arrow from 16S RNA to S7 shows that S7 binds weakly to 16S RNA in the absence of other proteins. The thin arrows from S4 and S8 to S20 indicate that the binding of S20 to 16S RNA is enhanced by S4 and S8. The large box with dashed outlines include the proteins (S4, S8, S20, S13, S16 and S17) that facilitate the binding of S11. The dotted line divides the 30S proteins into 2 groups - proteins above the dotted line are required for the formation of 21S RI particles and proteins below it are bound to RI particles only after heat activation of the particles. Those below the dotted line are generally split proteins.

The complete reconstitution of 50S ribosomal subunits has not been as successful as that of 30S subunits. The difficulty in reconstituting 50S subunits may be due to the greater complexity of the large subunit since as mentioned earlier, it is composed of 34 proteins, 23S and 5S RNA's. The 50S subunits of the thermophilic bacterium Bacillus stearothermophilus was successfully reconstituted from 23S

RNA and 50S ribosomal proteins (Nomura and Erdmann, 1970; Fahnestock, Erdmann and Nomura, 1973). The ionic conditions for reconstitution of 50S subunits were essentially the same as that for 30S subunits, but reconstitution required incubation at 60° for at least 1 h. Presumably, the ribosomal components of the 50S subunits of B. stearothermophilus were heat-stable and could tolerate the conditions required for reconstitution. RNA extracted from 50S subunits with phenol was much less active in reconstitution than urea-LiCl extracted RNA from the same subunits (Nomura and Erdmann, 1970).

Although studies on the biosynthesis of E. coli ribosomes suggested a requirement of 30S subunits for assembly of 50S subunits (Nashimoto and Nomura, 1970), the presence of 30S subunits was not required in the reconstitution of B. stearothermophilus 50S subunits (Nomura and Erdmann, 1970). In the presence of exogenous 5S RNA, reconstituted 50S particles exhibited greater poly(U)-directed phenylalanine incorporation activity. The rate of in vitro assembly of E. coli 30S subunits at 40° was about 300 times faster than that of B. stearothermophilus 50S subunits at 50°. Since much more is known of E. coli ribosomes, it is not surprising that there have been many attempts at the total reconstitution of the 50S subunit of this organism. A report that functionally active E. coli 50S subunits were reconstructed from 50S proteins, 5S and

23S RNA's in the presence of 30S subunits (Maruta et al, 1971) could not be successfully repeated (Nomura, 1973). A second report of total reconstitution of functionally active E. coli 50S subunits has recently been described (Nierhaus and Dohme, 1974) in which reconstitution was achieved by a 2-step incubation procedure of 50S components. Incubation was carried out first at 40° in the presence of 4.0mM magnesium for 20 min, then at 50° in 20.0 mM magnesium for 90 min. No requirement for 30S subunits or polyamines was found. The reconstituted particles were reported to be highly active in polypeptide synthesis with R17 RNA or poly(U) as messengers, in peptidyltransferase reaction and in the binding of chloramphenicol.

Hosokawa, Kiho and Migita (1973) assembled inactive 48S particles from 5S and 23S RNA's, and 50S proteins under conditions which allowed formation of active 30S subunits. Reconstitution was carried out at 42° for 15 min in the presence of 14.0mM magnesium and 18.0mM spermine. Nikolaev and Schlessinger (1974) attempted reconstitution with 30S precursor rRNA in place of RNA extracted from mature 50S subunits. The 30S precursor rRNA, isolated from chloramphenicol-treated E. coli strain AB 105 cells, contained both 16S and 23S rRNA sequences (Nikolaev, Silengo and Schlessinger, 1973; Dunn and Studier, 1973). However, their reconstituted 46S particles were functionally inactive. Generally, therefore, total reconstitution of 50S subunits of

E. coli has not been successful and has hampered investigations in the function of the subunit.

The binding of 50S proteins to 23S RNA resembles, in some respects, the specific binding of 30S proteins to 16S RNA during reconstitution. For instance, when tested individually, only 10 (L1, L2, L3, L4, L6, L13, L16, L20, L23, L24) of 34 of the 50S proteins were bound specifically to 23S RNA, much like the binding of some of the RI proteins to 16S RNA. Specificity of binding was indicated by the exclusive binding of individual proteins to 23S RNA in the presence of 16S RNA and saturation of binding at a molar protein: RNA ratio of 1.4:1 or less (Garrett, Muller, Spierer and Zimmermann, 1974).

Several groups have shown that the 50S proteins, L18 and L25, could bind to 5S RNA, independently of other proteins, and that the 5S RNA-protein complex could then bind to 23S RNA in the presence of proteins L2 and L16 (Gray, Garrett, Stoffler and Monier, 1972; Horne and Erdmann, 1972; Gray and Monier, 1972). The 5S RNA-protein complex possessed GTPase, as well as ATPase, activities (Horne and Erdmann, 1973).

Although there is reason to believe that assembly of 50S subunits in vitro may proceed by mechanisms similar to that of 30S reassembly, there are some points of difference. The association of specific RNA-binding proteins in sub-

units differs in 30S and 50S subunits. In 30S subunits, the proteins were less accessible to antibody-binding and glutaraldehyde than non-specific binding proteins (Craven and Gupta, 1970; Chang and Flaks, 1970; Kahan and Kaltschmidt, 1972). However, in 50S subunits, the specific binding proteins were very accessible in the subunit structure to the same reagents.

(c) Biosynthesis of Ribosomal Subunits

The biosynthesis of ribosomal subunits proceeds in discrete steps to yield various precursor particles identified by different sedimentation coefficients. (Britten and McCarthy, 1962; Mangiarotti, Apirion, Schlessinger and Silengo, 1968; Osawa, 1968). In the case of 30S assembly, the participation of precursors was confirmed by the isolation of 21S precursor particles from mutant cells defective in the assembly process at cold temperatures (Nashimoto, Held, Kaltschmidt and Nomura 1971; Nashimoto and Nomura, 1970; Guthrie, Nashimoto and Nomura, 1969). Proteins associated with this precursor or intermediate particle were similar to those in the RI particle formed by reconstitution (Nashimoto et al, 1971; Homann and Nierhaus, 1971; Osawa, Otaka, Itoh and Fukui, 1969; Nierhaus, Bordasch and Homann, 1973). A comparison of the data, presented in Table III, indicates that formation of 30S subunits in vitro

Table III. Comparison of 30S proteins required for RI* particle formation *in vitro* with those found in 21S particles *in vivo* (Nomura and Held, 1974).

Protein	Required for RI* formation[a]	Present in *in vitro* 21S[b]	Present in *in vivo* 21S[c]
S4	++	++	+
S7	++	++	-
S8	++	++	+
S16	++	++	+
S19	++	+	-
S15	+	++	+
S17	+	++	+
S5	±	+	+
S9	±	++	-
S11	±	++	-
S12	±	-	-
S18	±	++	-
S6	-	++	-
S13	-	++	+
S20	-	++	+
S1	-	-	+
S2	-	-	-
S3	-	-	-
S10	-	-	-
S14	-	-	-
S21	-	-	-

a Held and Nomura, 1973. ++, strongly required; +, moderately required; ±, weakly required; -, not required.

b Traub and Nomura, 1969; Held and Nomura, 1973. ++, present in normal amounts; +, present in reduced amounts; -, absent or almost absent.

c Nierhaus, Bordasch and Homann, 1973. +, present in normal amounts; -, absent.

and *in vivo* proceeded in basically the same manner. However, in biosynthesis, 30S subunits were formed from larger undermethylated precursors of 16S RNA and unmodified proteins (Yuki, 1971; Corte, Schlessinger, Longo and Venkov, 1971; Terhorst, Wittmann-Liebold and Moller, 1972) whereas in cell-free reconstitution, mature 16S RNA and modified proteins were employed.

There have been numerous attempts at elucidating the biosynthetic assembly sequence of *E. coli* 50S subunits (Osawa *et al*, 1969; Nashimoto and Nomura, 1970; Pichon, Marvaldi and Marchis-Mouren, 1972; Nierhaus *et al*, 1973). It has been proposed that the 50S subunits were formed via precursor 30S and 40S ribosomal particles in the cell (Roberts, Britten and McCarthy, 1963; Mangiarotti *et al*, 1968). These precursors were demonstrated by sucrose gradient centrifugation of crude extracts from growing *E. coli* cells pulse-labelled with isotopic precursors. Similar particles containing undermethylated 23S RNA were detected in extracts from cells treated with low concentrations (less than 3.0 μg/ml) of chloramphenicol (Osawa, Otaka, Muto, Yoshida and Itoh, 1967). The precursor ribosomal particles from the 2 sources were found to be indistinguishable in their sedimentation coefficients, nature of 5S and 23S RNA's and protein compositions (Osawa *et al*, 1969).

Cold sensitive mutants of spectinomycin resistant mutants of E. coli, unable to assemble ribosomal particles at low temperatures (20°), and known as sad or "subunit assembly defective" mutants, accumulated 32S and 43S particles at 20° instead of 50S subunits. Some mutational alterations in the 30S ribosomal components impaired 30S as well as 50S subunit assembly at low temperatures (Guthrie et al, 1969; Nashimoto and Nomura, 1970). While all mutations inhibiting 30S subunit assembly also inhibited 50S subunit assembly, the reverse was not true (Guthrie et al, 1969). The data indicated that biosynthesis of 50S subunits was dependent on the simultaneous assembly of 30S subunits in E. coli.

More recently, 2 different approaches have been employed to elucidate the sequence of binding of proteins in the assembly of 50S subunits of E. coli in vivo. In the first approach, the rate of appearance of ribosomal proteins in mature 50S subunits was followed in E. coli strain 19 cells pulse-labelled for 3, 6 and 12 min (Pichon et al, 1972). Proteins from 50S subunits were separated into 2 groups, the split and core proteins, by incubation in 0.6 to 1.0M LiCl, and analyzed by means of bidimensional gel electrophoresis. On the basis of rate of increase in the specific radioactivity of individual separated proteins, proteins were assigned to 5 groups ordered from early to late protein

additions during assembly as follows:

Group I: L21, L22, L3, L4, L13, L23, L24, L18, L5

Group II: L32, L17, L19, L15, L29, L16, L30, L2

Group III: L25, L20

Group IV: L1, L6

Group V: L12, L10, L7, L9, L33.

In the second approach, ribosomal precursor particles from E. coli K12 were isolated and their RNA and protein compositions determined (Nierhaus et al, 1973). The 32S and 43S precursor particles of 50S subunits contained precursor 5S and precursor 23S RNA's in a 1:1 molar ratio. The 43S particles were deficient in only 7 proteins (L2, L6, L12, L16, L28, L31, L32) while the 32S lacked 8 additional proteins (L3, L7, L11, L14, L15, L19, L23, L33). The 43S precursor particles could be converted to 50S subunits at 42° in the presence of the missing ribosomal proteins and conditions promoting RNA methylation. The resulting 50S subunits were active in poly(U)-directed polyphenylalanine synthesis. On this basis, the following assembly scheme was proposed for the 50S subunit:

$$\rho 23S\ RNA + \rho 5S\ RNA + 16 \text{ or } 17 \text{ proteins} \longrightarrow 32S$$

$$32S \xrightarrow{8 \text{ proteins}} 43S$$

$$43S \xrightarrow{7 \text{ proteins}} 50S$$

Depending upon the method used, the order of protein addition during assembly and the protein composition of precursor particles differed considerably.

Functions of Ribosomes in Translation

The process of polypeptide synthesis occurs in 3 stages, namely initiation, elongation and termination. As well as ribosomes, the process requires mRNA, aminoacyl-tRNA's, GTP, Mg^{++}, K^{+}, NH_4^{+}, initiation factors (IF-1, IF-2, IF-3), elongation factors (EF-T_u, EF-T_s, EF-G), termination factors (RF-1, RF-2) and an energy generating system.

In the course of protein synthesis, a 70S initiation complex is formed from a 30S subunit, 50S subunit, mRNA and f-Met-$tRNA_F^{met}$ in the presence of initiation factors and GTP. The binding of single stranded phage RNA, a natural mRNA, to hybrid ribosomes formed from subunits of E. coli and B. stearothermophilus showed that the 30S subunit was responsible for recognition of specific initiation sequences in mRNA. The binding of 30S subunit to mRNA was mediated by the 30S subunit protein S1, a fractional protein, (Van Duin and Kurland, 1970). This protein alone could bind poly(U)and in so doing, was protected against trypsin digestion (Rummel and Noller, 1973). However, this function of S1 was in contradiction to the report that 30S particles reconstituted in the absence of S1 retained full activity in in vitro poly (U)-directed polyphenylalanine synthesis (Held, Mizushima and Nomura, 1973). The proteins, S12, has also been implicated in specific binding to mRNA (Pongs, Nierhaus, Erdmann and Wittmann, 1974).

IF-2 facilitated the binding of fMet-tRNA$_F^{met}$ to initiation complexes with concomitant hydrolysis of GTP. Six 30S proteins were involved in fMet-tRNA$_F^{met}$ binding. Addition of S2, S3 and S14 to protein-deficient cores stimulated IF-dependent fMet-tRNA$_F^{met}$ binding (Lelong, Gros, Gros, Bollen, Maschler and Stoffler, 1974) while specific antibody fragments (F ab) against S3, S10, S14, S19 and S21 strongly inhibited binding to 30S subunits (Van Duin, Van Knippenberg, Dieben and Kurland, 1972). GTP hydrolysis was catalyzed by IF-2 in the presence of 50S proteins, L7 and L12, which constituted part of the binding site for IF-2, and ribosomes deficient in L7-L12 (Fakunding, Traut and Hershey, 1973). Removal of L7 and L12 from ribosomes resulted in a reduction of IF-2 dependent activities (Kay, Sander and Grunberg-Monago, 1973).

IF-1 stabilized the initiation complex after its formation whereas IF-3 directed the 30S subunit specifically to the initiation region of mRNA during initiation complex formation (Revel, 1972; Yoshida and Rudland, 1972). The binding site for IF-3 on the 30S subunit was shown to be a segment of 16S RNA (Gualerzi and Pon, 1973). There is ample evidence showing that IF-3 was required in the ribosome cycle (Subramanian, Ron and Davis, 1968). The 30S and 50S subunits associate to form the 70S ribosome in the initiation complex in the presence of initiation factors. At the end

of a round of translation, the ribosome is released and spontaneously dissociates into subunits. IF-3 was found to promote dissociation of ribosome (Subramanian, Davis and Beller, 1969) but later work indicated that IF-3 was an anti-association factor that was bound to all free 30S subunits (Revel, 1972).

Recent studies of fMet-tRNA-directed binding of 30S subunits to the starting codon of coat protein of R17 RNA suggested the following sequence of assembly of an initiation complex between E. coli ribosome and R17 RNA (Noll and Noll, 1974):

(1) 70S + IF-1 ⟶ 30S.IF-1 + 50S (Noll and Noll,1972)

(2) 30S.IF-1 + IF-3 ⟶ 30S.IF-1, 3

(3) 30S.IF-1, 3 + fMet-tRNA.IF-2.GTP ⟶ 30S.IF-1, 2, 3. GTP.fMet-tRNA

(4) 30S.IF-1, 2, 3.GTP.fMet-tRNA + mRNA ⟶ $40S_i$ + IF-3

(5) $40S_i$ + 50S ⟶ $76S_i$ + IF-1, 2 + GDP

(S_i stands for the S value of the various initiation complexes)

From studies on the effect of antibodies specific for each ribosomal protein on reassociation of dissociated subunits, it was concluded that five 30S proteins (S9, S11, S12, S14, S20) and nine 50S proteins (L1, L6, L14, L15, L19, L20, L23, L26, L27) might be located at the subunit interface of E. coli 70S ribosome (Morrison, Garrett, Zeichhardt and Stoffler, 1973). However, investigation of the surface

topography of 50S subunits alone or in 70S ribosomes by lactoperoxidase-catalyzed iodination showed only 5 proteins, (L2, L13, L26, L27, L28), in 50S subunits were subunit interface proteins. These proteins were labelled substantially more in isolated 50S subunits than in the ribosomes (Litman and Cantor, 1974). Surprisingly, in similar studies, significant increases in iodination was noted in a completely different group of 50S proteins (L3, L8, L9, L18, L19) in ribosomes in which association of subunits was "loosened" by incubation at 37^{o} compared to "tight" ribosomes maintained at 0^{o} (Michalski and Sells, 1974).

During elongation, 2 tRNA molecules are aligned side by side on the ribosome. The tRNA that carries the incomplete peptide chain (or $fMet\text{-}tRNA_F^{met}$) occupies the donor or P site while the tRNA that carries the incoming amino acid occupies the acceptor or A site. The enzyme, peptidyltransferase, located in the 50S subunit, catalyzes the formation of a peptide bond between the amino group of aminoacyl-tRNA and the carboxylic ester of peptidyl-tRNA by a nucleophilic reaction. The deacylated tRNA is then removed from the P site, vacating the site for translocation of the newly-formed peptidyl-tRNA. Simultaneously, mRNA slides with respect to the ribosome so that the next 3 nucleotides are at the A site. The translocation of peptidyl-tRNA and the binding of an incoming aminoacyl-tRNA require elongation

factors, GTP and the cooperation of various ribosomal proteins and RNA components.

Studies on the effect of streptomycin on misreading demonstrated enhancement of translational fidelity by 30S proteins S3, S4, S5 and S11, as well as reduction in translational fidelity by S12 suggesting that these 30S proteins may be located at the site of mRNA-tRNA interaction (Schreiner and Nierhaus, 1973; Pongs and Erdmann, 1973; Ozaki, Mizushima and Nomura, 1969; Birge and Kurland, 1969; Nomura, Mizushima, Ozaki, Traub and Lowry, 1969). Poly (U)-directed phe-tRNA binding experiments suggested that S2, S3, S10, S14, S19 and S21 were involved in aminoacyl-tRNA binding (Rummel and Noller, 1973). Using antibodies to these 30S proteins, their involvement in fMet-tRNA binding was implicated (Lelong, Gros, Gros, Bollen, Maschler and Stoffler, 1974). It was recently shown that S21 was required for full activity of ribosomes in initiation of polypeptide synthesis (Held, Nomura and Hershey, 1974). The inhibition of EF-T_U-dependent poly(U)-directed phe-tRNA binding by antibodies against S9, S11, and S19 ascribed the function of EF-T_U-dependent aminoacyl-tRNA binding to these proteins (Lelong et al, 1974).

The involvement of proteins of the 50S subunit in the elongation step was also demonstrated. Studies on the effect of binding of chloramphenicol and its analogues to

the A site of the peptidyltransferase centre showed that L6 and L16 were located in the A site (Nierhaus and Nierhaus, 1973; Stoffler, Daya, Rak and Garrett, 1971; Pongs, Bald and Erdmann, 1973), whereas L2, L4, L15 and L27 were located in the P site (Sonenberg, Wilchek and Zamir, 1973; Oen, Pellegrini, Eilat and Cantor, 1973; Czernilofsky, Collatz, Stoffler and Kuchler, 1974). It was demonstrated by means of affinity-labelling with peptidyl-tRNA analogues that proteins L24 and L33 were located in the P site (for ref., see Pongs et al, FEBS Letters 40 Supplement, S28, 1974).

Protein L11 was inferred to possess peptidyltransferase activity since 50S particles formed by partial reconstitution from 0.8M LiCl core particles and a split protein fraction lacking L11 exhibited no peptidyltransferase activity (Nierhaus and Montejo, 1973). However, it was shown recently that 50S core particles, prepared by ethanol-NH_4Cl treatment and lacking proteins L7, L10, L11 and L12, possessed enzyme activity (Howard and Gordon, 1974; Ballesta and Vazquez, 1974). It has been suggested that the 0.8M LiCl core particles lacking many proteins (Nierhaus and Montejo, 1973) required binding of L11 to assume a conformation necessary for expression of peptidyltransferase activity whereas the core particles lacking only 4 proteins (Howard and Gordon, 1974; Ballesta and Vazquez, 1974) possessed the necessary conformation for expression of the

activity, even in the absence of L11. Results of affinity-labelling experiments suggested that protein L11 was situated near the 3'-terminus of peptidyl-tRNA (Pongs et al, 1974). Results of chemical modification studies showed that protein L15 was near the peptidyltransferase centre (Ballesta, Montejo, Hernandez and Vazquez, 1974) - more specifically at the binding site of fMet-$tRNA_F^{met}$ (Czernilofsky, Stoffler and Kuchler, 1974). From partial reconstitution studies it was deduced that L11 was situated near proteins L16 and L6 which were located in the A site (Diedrich, Schrandt and Nierhaus, 1974). Proteins L6 and L16 stimulated peptidyltransferase activity of 50S particles reconstituted in the absence of either protein (Pongs et al, 1974). However, such stimulation was not observed with 50S particles deficient in both proteins.

There are 2 energy-requiring reactions in the elongation step-the translocation of peptidyl-tRNA from the A site to the P site catalyzed by EF-G, and the binding of incoming aminoacyl-tRNA to the A site catalyzed by $EF\text{-}T_U$. Ribosomal proteins involved in these steps of GTP hydrolysis were different from those involved in the peptidyltransferase centre (Modollel, Vazquez and Monro, 1971). EF-G and $EF\text{-}T_U$ probably bound to overlapping sites since binding of the ternary $EF\text{-}T_U$ ~ GTP ~ aminoacyl-tRNA complex to the A site

was inhibited by EF-G (Richman and Bodley, 1972; Richter, 1972; Miller, 1972; Modollel and Vazquez, 1973; Chinali and Parmeggiani, 1973). Cross-linking experiments indicated that the 50S proteins, L7 and L12, were located at the EF-G binding site (Acharya, Moore and Richards, 1973). Removal of L7 and L12 eliminated EF-G and EF-T_U-dependent GTP hydrolysis activities from 50S subunits although they could be restored by assaying in the presence of methanol (Ballesta and Vazquez, 1972). Antibodies against L7 and L12 inhibited EF-G and EF-T_U-dependent functions (Kischa, Moller and Stoffler, 1971; Hamel, Koka and Nakamoto, 1972; Brot, Yamasaki, Redfield and Weissbach, 1972; Ballesta and Vazquez, 1972; Sander, Marsh and Parmeggiani, 1972; Highland, Bodley, Gordon, Hasenbank and Stoffler, 1973; Highland, Ochsner, Gordon, Bodley, Hasenbank and Stoffler, 1974). Besides L7 and L12, L6 and L10 were involved in EF-G-dependent GTP hydrolysis (Schrier, Maasen and Moller, 1973; Stoffler, Hasenbank, Bodley and Highland, 1974).

The 5S RNA molecule in 50S subunit was important for ribosomal functions linked to the A site (Erdmann, Sprinzl and Pongs, 1973; Richter, Erdmann and Sprinzl, 1973). In oligonucleotide binding experiments, the CGAA sequence in 5S RNA stabilized the binding of aminoacyl-tRNA by base-pairing with the TΨCC sequence which is common to most tRNA (Schwarz, Luhrmann and Gassen, 1974). Affinity-labelling

experiments utilizing a fusidic acid stabilized complex of ribosomes, EF-G and the photo-affinity label APh-GDP suggested that 5S RNA binding proteins (L18, L25) were located at the functional site of EF-G and EF-T_U (Maasen and Moller, 1974), and that proteins L5, L18, L30 and L11 were involved in GDP binding.

Only a few 30S proteins were involved in formation of 70S ribosomes possessing EF-G and EF-T_U-dependent activities (Parmeggiani, Sander, Voigt and Marsh, 1973). Data from partial reconstitution experiments identified S2, S9 and S5 as the 30S proteins needed for EF-T_U-dependent GTP hydrolysis (Sander, Marsh and Parmeggiani, 1973; Marsh and Parmeggiani, 1973).

Not surprisingly, all subunit interface proteins (Morrison et al, 1973) were involved in some ribosomal function. The 30S protein, S9, was required in GTPase activity; S11 and $S1_2$ in translational fidelity; and S14 and S20 in tRNA binding. Similarly, the 50S subunit interface proteins, L1 and L6 participated in A site formation; L14, L15, L19 and L12 in 5S RNA binding; and L23, L26 and L27 in P site formation (Oen et al, 1973; Czernilofsky et al, 1974; Morrison et al, 1973).

When a termination signal is read on the mRNA, release factors become bound to the ribosome and trigger hydrolysis of the bond between tRNA and the finished peptide,

resulting in release of the peptide (Beaudet and Caskey, 1972; Caskey, 1973). The 50S proteins L7 and L12 were required in the termination step. Antibodies against L7 and L12 inhibited binding of release factors suggesting that the release factors might be bound at a site similar or close to the EF-G and EF-T_U binding site (Tate, Caskey and Stoffler, 1975). Antibodies against 2 other 50S proteins L11 and L16 inhibited peptidyl-tRNA hydrolysis (Tate et al, 1975).

III. MATERIALS AND METHODS

Chemicals

L-amino acids - (^{3}H) (1 mCi/ml), L-amino acids-(^{14}C) (0.1 mCi/ml), L-methionine-(methyl-^{3}H) (190 mCi/mM), L-phenylalanine-(^{14}C) (20 μCi/mM) and guanosine triphosphate-γ-(^{32}P) (16 Ci/mM) were obtained from New England Nuclear Corp.; adenosine triphosphate, guanosine triphosphate, phosphoenolpyruvate, pyruvic kinase, polyuridylic acid, pancreatic ribonuclease, spermidine-trihydrochloride, putrescine-dihydrochloride, spermine-tetrahydrochloride, 2-mercaptoethanol, d l, L-tetrahydrofolic acid, puromycin dihydrochloride, trizma base and the 20 L-amino acids from Sigma Chemical Co.; crystalline deoxyribonuclease from Worthington Biochemical Co., cesium chloride from Harshaw Chemical Co.; E. coli sRNA and adenylyl-uridylyl-guanylic acid (AUG) from General Biochemicals; sucrose (density gradient grade, ribonuclease free) and ammonium sulphate (special enzyme grade) from Mann Research Lab.; sodium dodecyl sulphate and phenol from Matheson Coleman and Bell;

phenol reagent (2 M) from Harleco; PPO and POPOP from Kent Lab. Ltd.; 1, 4-dioxane, toluene, orcinol and glycine-hydrochloride from Fisher Scientific Co.; acrylamide, bis-acrylamide, riboflavin and N, N, N', N'-tetramethyl-ethyl-enediamine from Eastman Kodak Co.; ammonium persulphate from EC-Apparatus Corp.

Buffers

The following buffers were used throughout this investigation: TM(10mM Tris-HCl, pH 7.4, 10mM $Mg(OAc)_2$, 6mM 2-mercaptoethanol); TKMI (10mM Tris-HCl, pH 7.4, 10mM $Mg(OAc)_2$, 50mM KCl, 6mM 2-mercaptoethanol); TKMS I (TKMI plus 5mM spermidine); TKM II (10mM Tris-HCl, pH 7.4, 1mM $Mg(OAc)_2$, 50mM KCl, 6mM 2-mercaptoethanol); TKMS II (TKM II plus 1mM spermidine); TMN I (10mM Tris-HCl, pH 7.4, 10mM $Mg(OAc)_2$, 500mM NH_4Cl, 6mM 2-mercaptoethanol); TMN II (10mM Tris-HCl, pH 7.4, 10mM $Mg(OAc)_2$, 10mM NH_4Cl, 6mM 2-mercaptoethanol); TMN III (10mM Tris-HCl, pH 7.4, 10mM $Mg(oAc)_2$, 60mM NH_4Cl, 6mM 2-mercapto-ethanol); SR (30mM Tris-HCl, pH 7.4, 20mM $Mg(OAc)_2$, 300mM KCl, 6mM 2-mercaptoethanol); SRS (SR plus 10mM spermidine).

Growth of Organism

Escherichia coli MRE600, a RNase I^- strain obtained from G. R. Craven (University of Wisconsin, Madison,

Wisconsin) was used in this investigation. The cells were grown at 37°C in 15-litre carboys in minimal salts-glucose medium (pH 7.4) supplemented with 0.2% casamino acids (Difco) and 0.2% yeast extract (Difco) (Kurland, 1966), aeration being provided by forcing air through bubbling tubes. The composition of the medium per litre was as follows:

3.0g KH_2PO_4

6.8g $Na_2HPO_4.H_2O$ or 6.0g Na_2HPO_4

2.0g NH_4Cl

2.0g casamino acids

0.2g $MgCl_2.6H_2O$

5.0g glucose

After the culture reached late logarithmic phase, the cells were harvested in a Sharples centrifuge and stored at -70°.

Labelled cells were obtained by growing cells in (^{14}C) amino acids (10μCi/ml) or (^{3}H) amino acids (4μCi/ml) in 400 ml of above medium in 1-litre Erlenmeyer flask on a rotary shaker. In this case, the casamino acid concentration was reduced to 0.02%.

Preparation of Ribosomes

Ribosomes were prepared from 50g frozen cells by a modification of the method of Kurland (1966). Cells were thawed and broken by one passage through an Aminco French

pressure cell at 20,000 p.s.i. in TKM I buffer containing 100µg deoxyribonuclease. Cell debris was removed by centrifugation at 10,000 x g for 30 min in a Sorvall RC-2B centrifuge. The supernatant was subjected to 3 successive $(NH_4)_2SO_4$ fractionations as described by Kurland (1966). The final pellet was dissolved in TM buffer containing 0.6M $(NH_4)_2SO_4$. The ribosomes were washed twice in this buffer by sedimentation at 105,000 x g for 3 h, and finally dissolved and dialyzed in TM buffer at a concentration of 600 A_{260} units/ml. One ml-portions were frozen in liquid nitrogen and stored at -70°.

Radioactivity labelled ribosomes were prepared by the same method from labelled cells (see above) except only two $(NH_4)_2SO_4$ fractionations were carried out.

Preparation of Ribosomal Subunits

The 30S and 50S ribosomal subunits were dissociated and isolated from 70S ribosomes by sucrose gradient centrifugation. Ribosomes in TM buffer were dialyzed in 2 changes of TKM II buffer (1mM magnesium) for 8 h at 4° prior to centrifugation. Samples containing a total of 15,000 - 20,000 A_{260} units of ribosomes were layered on a 5 - 20% convex sucrose density gradient in TKM II buffer and centrifuged in a Spinco Ti-15 zonal rotor at 34,000 r.p.m. for 12 h at 4°. Fractions (about 12 ml) were

collected and suitably diluted aliquots were assayed at 260 nm in a DB Beckman spectrophotometer. The peak fractions corresponding to 50S and 30S ribosomal subunits were pooled, made to 10mM $Mg(OAc)_2$, precipitated with 0.7 volume of ethanol (Staehelin et al, 1969) and allowed to stand at -20° for at least 2 h. The precipitated subunits were sedimented at 10,000 x g for 30 min in a Sorvall RC-2B centrifuge, dissolved in TKM I buffer and dialyzed in the same buffer for 6 h at 4°. The subunits (600 A_{260} units/ml) were frozen in liquid nitrogen in small aliquots and stored at -70°.

Labelled subunits were prepared from labelled ribosomes in the same way except that subunits were isolated by sucrose gradient centrifugation in a Beckman SW 25.2 rotor at 24,000 r.p.m. for 12 h at 4°.

Extraction of Ribosomal RNA and 50S Ribosomal Proteins

Ribosomal RNA was extracted from 70S ribosomes (600 A_{260} units/ml) with an equal volume of 8.0M urea-4.0M LiCl and 0.6 volume of 10M LiCl at 4° for 24 h as described by Nomura and Erdmann (1970). The precipitated RNA was pelleted by centrifugation at 10,000 x g for 10 min, dissolved in TKMS II buffer and reextracted for 6 h. The RNA was again pelleted, dissolved and dialyzed in TKMS II

buffer for 8 h at 4°. One ml-aliquots (350 A_{260} units/ml) were frozen in liquid nitrogen and stored at -70°. The preparation was free of proteins as judged by electrophoresis (Leboy et al, 1964) of acetic acid extracts of the RNA (Hardy et al, 1969).

The 16S RNA and a mixture of 23S and 5S RNAs were extracted from purified 30S and 50S subunits, respectively, by the same method. The 5S RNA was isolated from rRNA by elution through a G-100 Sephadex (Pharmacia) column equilibrated with buffer containing 100mM Na(OAc) and 1.0 mM Na_2-EDTA at pH 6.5.

Ribosomal proteins were extracted from purified 50S subunits, (less than 2% 30S subunit contamination), by the same method except the supernatant containing the proteins was kept after centrifugation. Proteins from the first extraction were used immediately in reconstitution. Protein concentration is given in terms of "equivalents/ml" where 1 equivalent of 50S proteins is the amount extracted from 1 A_{260} unit of 50S subunits.

Preparation of S-30 and S-100 Extracts

The procedure for preparation of cell-free extracts was basically that developed by Nirenberg (1964). All steps were carried out at 4°. Cells suspended in TMN III buffer were broken by one passage through the French press

at 20,000 p.s.i. and centrifuged at 10,000 x g for 30 min to sediment cell debris. The supernatant was centrifuged in a Beckman 60-Ti rotor at 30,000 x g for 30 min. The upper 4/5 of the supernatant was removed by a syringe and labelled S-30. After dialysis for 4-6 h in TMN III, portions (0.5ml) were frozen in liquid nitrogen and stored at -70^{o}.

The S-100 fraction was prepared from S-30 fraction by centrifugation in a Beckman 60-Ti rotor at 105,000 x g for 2 h. The upper 4/5 of the supernatant was aspirated, frozen in small aliquots (0.5ml) in liquid nitrogen and stored at -70^{o}. This was labelled S-100. High salt S-100 fraction was prepared by the same method except that TMN I buffer (500mM NH_4Cl) was used.

Preparation of Crude EF and Crude IF Fractions

The method of preparation was basically that of Revel, Greenshpan and Herzberg (1971). Crude EF was prepared from S-30 fraction by centrifugation at 150,000 x g for 2½ h at 4^{o}. The top 2/3 of the supernatant was aspirated as crude EF. Portions (0.5ml) were frozen in liquid nitrogen and stored at -70^{o}.

The ribosome pellet was resuspended in buffer containing 30mM Tris-HCl, pH 7.4, 10mM $Mg(OAc)_2$, 2.0M NH_4Cl and 12mM 2-mercaptoethanol and incubated with stirring in ice overnight. The resulting ribosome suspension was mixed with an equal volume of TMN II buffer and centrifuged at 150,000 x g for 2½ h. The top 2/3 of the supernatant was collected by aspiration and dialyzed in TMN III buffer for 2 h at 4°. The dialysate was made to 75% saturation with crystalline $(NH_4)_2SO_4$ and stirred for 1 h at 4°. The precipitate, collected by centrifugation at 10,000 x g for 10 min, was dissolved in buffer containing 10mM Tris-HCl, pH 7.4, 2.0mM $Mg(OAc)_2$, 60mM NH_4Cl and 6.0mM 2-mercaptoethanol, then dialyzed in the same buffer for 3 h. The dialysate was suitably diluted with buffer to a final concentration of 20 mg proteins/ml, quick-frozen in small aliquots (0.5ml) in liquid nitrogen and stored at -70°. This was labelled crude IF.

The ribosome pellet from high salt wash was washed again in the same high salt buffer, finally suspended in TMN II buffer at 400 A_{260} units/ml and labelled salt-washed ribosomes. Small portions (1ml) were quick-frozen and stored at -70°.

Preparation of Crude fMet-tRNA

A modification of the method of Miskin et al (1970) was employed. Crude fMet-tRNA was prepared by charging E. coli tRNA (General Biochemicals) at a concentration of 2.5 mg/ml with methionine (0.1mM) in the presence of dl, L-tetrahydrofolic acid (0.5 mg/ml) and ammonium formate (40mM) in 2.0ml of buffer containing 100mM Tris-HCl, pH 7.8, 15mM $Mg(OAc)_2$, 5.0mM ATP, 6.0mM 2-mercaptoethanol and concentrated high salt S-100 fraction. The charging mixture minus tRNA and methionine was incubated at 37^{o} for 5 min before tRNA and methionine were added. After 15 min, the mixture was chilled in ice. After addition of 0.1 volume of 20% K(OAc) (pH 5.0), the mixture was extracted with an equal volume of water-saturated phenol. The aqueous phase was mixed with 2 volumes of ethanol to precipitate tRNA which was washed once in ethanol and dissolved in water. The final concentration was about 200 A_{260} units/ml. To prepare crude f(^{3}H) Met-tRNA, 5μCi (^{3}H) methionine (190mCi/mM) was added to the charging mixture.

To determine the amount of f (^{3}H) Met-tRNA in the crude preparation, 0.025ml of crude f (^{3}H) Met-tRNA was hydrolyzed in 0.1 N NH_4OH at 37^{o} for 60 min. The hydrolysate was acidified to pH 1.0 with HCl and rapidly extracted

with 1.5 volume of ethyl acetate. The upper ethyl acetate phase was removed for counting (Leder and Bursztyn, 1966). The specific radioactivity of crude f (^{3}H) Met-tRNA was about 3.5 x 10^3 c.p.m./A_{260} unit, 35-40% of which represented f (^{3}H) Met-tRNA.

Preparation of "Split" Proteins

(a) $SP_{50-\gamma}$

The procedure of Maglott and Staehelin (1971) was used with slight modifications. E. coli 50S ribosomal subunits were centrifuged in a Beckman SW 50.1 rotor at 40,000 r.p.m. for 48 h at 4^o in a buffer containing 30mM Tris-HCl, pH 7.4, 4.0M CsCl, 10mM $Mg(OAc)_2$ and 0.5 mM Na_2-EDTA. Fractions were collected and those from the top 1/5 of the gradient were pooled and dialyzed in TMN II buffer for 6 h at 4^o with 2 changes of buffer. Portions (0.5ml) of the dialysate were quick-frozen in liquid nitrogen and stored at -70^o as $SP_{50-\gamma}$.

(b) PI and PI-II Proteins

A slight modification of the procedure developed by Hamel et al (1972) was used. For PI proteins, E. coli 50S subunits were extracted in buffer containing 10mM Tris-HCl, pH 7.4, 20mM $Mg(OAc)_2$, 1.0M NH_4Cl, 50% ethanol

and 6.0mM 2-mercaptoethanol at 0^{o} for 10 min. After extraction, ribosomal "core" particles were precipitated with 0.7 volume of ethanol. The precipitated ribosomal particles were removed by sedimentation at 10,000 x g for 20 min and the ethanolic supernatant containing the PI "split" proteins was dialyzed in TMN II buffer and concentrated by lyophilization. The PI proteins were dissolved in TMN II buffer, frozen in small amounts (0.3ml) in liquid nitrogen and stored at -70^{o}.

PI-II proteins were prepared by the same method from 50S subunits except that protein extraction with NH_4Cl-ethanol mixture was performed at 30^{o}. This procedure removes still more proteins from the subunits (Hamel et al, 1972).

Reconstitution

A modification of the method of Nomura and Erdmann (1970) was followed throughout. The 50S ribosomal proteins were added first to SR or SRS buffer at the temperature at which reconstitution was to be carried out. The reaction was initiated by addition of ribosomal RNA. After incubation, the mixture was chilled in ice, precipitated with 0.7 volume of ethanol (prechilled at -20^{o}) and then allowed to stand at -20^{o}. In some instances, the reaction was terminated by cooling to 0^{o}, and the reconstituted particles were isolated after sedimentation through 1 ml of 10%

sucrose in a Beckman 60-Ti rotor at 150,000 x g for 5 h.

(^{14}C) or (^{3}H) ribonucleoprotein complexes were reconstituted in the same way from (^{14}C) or (^{3}H) 50S ribosomal proteins, and rRNA.

Sedimentation Analysis

Analysis of reconstituted mixtures was carried out in 5-20% linear sucrose gradient in TKM buffer containing either 1.0mM or 10mM Mg^{++}, or in TMN I buffer with a Beckman SW 50.1 rotor at 45,000 r.p.m. for 105 min. The gradients were analyzed with a flow-through cuvette in a Gilford model 2000 recording spectrophotometer at 260 nm. The method described by O'Brien (1971) was used to obtain the S-values using E. coli 16S and 23S RNA's, 30S and 50S subunits, and 70S ribosomes as markers. The method provided a convenient system for identification of the reconstituted particles.

Isolation of Reconstituted Particles

Reconstituted particles were separated from 16S RNA and unbound proteins by centrifugation in 5-20% sucrose gradient in TKM I, TKM II or TMN I buffers in a Beckman SW 25.2 rotor at 24,000 r.p.m. for 12 h. Fractions were

collected and suitably diluted aliquots were assayed for absorbance at 260 nm. Fractions containing reconstituted particles were pooled and precipitated with 0.7 volume of ethanol. When the particles were isolated in TMN I buffer, pooled fractions were mixed with an equal volume of TM buffer to lower the ammonium chloride concentration to 0.25M before precipitation with ethanol. The precipitate was dissolved in a small amount of TKM I buffer to a final concentration of 400-450 A_{260} units/ml, frozen in 0.5 ml aliquots in liquid nitrogen and stored at -70°. The purity of isolated reconstituted particles was checked by centrifugation in 5-20% sucrose gradient in TKM I buffer with a Beckman SW 50.1 rotor as described in "Sedimentation Analysis".

Protein Determination

Protein content of reconstituted particles was determined by the phenol method of Lowry et al (1951) using crystalline bovine serum albumin (Sigma Chemical Co.) as standard.

RNA Determination

RNA content of reconstituted particles was estimated by the method of Schneider (1957) using yeast RNA (Sigma

Chemical Co.) as standard.

Protein Analysis by Disc-Gel Electrophoresis

Proteins were extracted from reconstituted particles or 50S subunits with 67% glacial acetic acid in 0.1M $Mg(OAc)_2$ (Hardy et al, 1969). The precipitated RNA was discarded and the supernatant containing extracted proteins were lyophilized. Prior to electrophoresis, the lyophilized powder was dissolved in 0.1 to 0.15ml sample gel solution. Disc-gel electrophoresis was performed as described by Leboy et al (1964) in glass tubes 10.5cm long with an inner diameter of 6mm. The separating gel contained 12.5% acrylamide and 0.75% bisacrylamide in 8.0M urea. The volume of lower (separating) gel was 2.0ml; the spacer gel, 0.1 to 0.2ml; and the sample gel, 0.15 to 0.2ml. Usually 50-100µg of ribosomal proteins was applied to each tube. Electrophoresis was carried out at 4^{o} in β-alanine-acetic acid buffer (pH 4.6) at a constant current of 3mA/tube. A trace of 0.1% pyronin red (British Drug Houses Ltd.) was used as tracking dye. At the completion of electrophoresis, gels were removed from glass tubes by rimming with water and fixed in 12.5% TCA at 37^{o} for 1 h, then stained for at least 1 h in 0.05% Coomassie brilliant blue (Sigma Chemical Co.) in 12.5% TCA.

Destaining was effected in 10.0% TCA at room temperature.

Protein Analysis by Bidimensional Electrophoresis

Bidimensional polyacrylamide gel electrophoresis system used was essentially that developed by Kaltschmidt and Wittmann (1970a) with minor modifications. In the first dimension, electrophoresis was carried out in 4% polyacrylamide gel in a glass tube (180 x 5 mm) at 3mA for 20 h in urea-EDTA-borate buffer at pH 8.6. About 1.5mg of ribosomal proteins was dissolved in 0.15 to 0.20 ml sample gel sandwiched between separation gels. After electrophoresis, the disc gel was removed from the glass tube by rinming with water, dialyzed in 100ml of 0.3M HCl at 4^{o} for 10 min (Avital and Elson, 1974), and used as sample for electrophoresis in the second dimension in a slab gel (200 x 180 x 3 mm) containing 17% polyacrylamide. The slab-gel was prerun for 2-3 h at 100mA in glycine-acetic acid buffer at pH 4.6 with a modified model 490 vertical gel electrophoresis cell (E-C Apparatus Corp). The dialyzed disc-gel was placed on the top of the slab and electrophoresis was continued for 20 h, with cooling by running tap water (approx. 10^{o}C). After electrophoresis, the slab was stained with 0.6% amido black (10 B, E. Merck AG Darmstadt) in 5% acetic acid for 15 min with occasional agitation and destained in 1% acetic

acid at room temperature. The stained protein spots were identified according to the numbering system of Kaltschmidt and Wittmann (1970b).

RNA Analysis by Disc-Gel Electrophoresis

RNA was extracted from samples of ribosomal particles with an equal volume of 8.0M urea-4.0M LiCl plus 0.6 volume of 10M LiCl and analyzed by electrophoresis in 2.65% polyacrylamide gels as described by Peacock and Dingman (1968) in glass tubes 12.0 cm long with an inner diameter of 6mm. Electrophoresis was carried out in Tris-EDTA-borate buffer at pH 8.4 at a constant current of 3mA/tube with a trace of 0.1% bromphenol blue (Matheson, Coleman and Bell) as tracking dye. Usually 0.5 - 1.0 A_{260} unit of RNA was applied to each tube. An initial current of 1 mA/tube was applied for 30 min to allow large RNA molecules to enter the separating gel. At the end of electrophoresis, gels were fixed in 1.0% acetic acid and scanned at 260 nm in a Joyce Loebl U.V. recording scanner Type D8 MK2 or a Shimadzu MPS 50L equipped with linear transport accessory. In some cases, samples were analyzed without prior extraction of RNA by electrophoresis into gels containing 0.2% sodium dodecyl sulphate (Bishop, Claybrook, and Spiegelman, 1967). In such cases, electrophoretic buffer was also made 0.2% with sodium dodecyl sulphate and electrophoresis was carried out at room temperature

without cooling. There were no differences in RNA profile when RNA was analyzed in this way.

To analyze for 5S RNA content, electrophoresis was carried out in "double" gels - 1 ml 2.65% polyacrylamide gel was polymerized on top of 2 ml 12.5% gel. About 35 - 40 A_{260} units of RNA was applied to each gel. In this way, only RNAs of low molecular weight were separated in the 12.5% gel whereas RNA's of high molecular weight were retained in the 2.65% polyacrylamide.

Measurement of Binding of Total 50S Proteins

Reconstitution was carried out as previously described except that (^{14}C) 50S proteins were used. After ethanol precipitation, reconstituted particles were isolated after 5 - 20% sucrose gradient centrifugation in TMN I buffer in a Beckman SW 41 rotor at 40,000 r.p.m. for 4 h at 4^{o} as described previously. The RNA content of reconstituted particles was estimated by the orcinol method and by measuring the absorbance at 260 nm. Their total protein content was estimated from measurement of radioactivity associated with the reconstituted particles. The amount of 50S protein binding was expressed as c.p.m. (^{14}C) 50S proteins per µg (or A_{260} unit) 23S RNA.

Measurement of Binding of Individual 50S Proteins

(^{14}C) or (^{3}H) 50S subunits were extracted and purified from cells grown in (^{14}C) or (^{3}H) amino acids as previously described. (^{3}H) reconstituted particles were obtained as above except (^{3}H) 50S proteins were used in reconstitution. To measure the extent of binding of individual 50S proteins, proteins extracted from a mixture of 8.0 A_{260} units of (^{3}H) 50S subunits or isolated (^{3}H) reconstituted particles and 10 A_{260} units of (^{14}C) 50S subunits were separated by bi-dimensional polyacrylamide gel electrophoresis and analyzed as described below. Each mixture from which protein was extracted also contained 32 A_{260} units of unlabelled 50S subunits so that after electrophoresis, each protein spot could be readily visualized and identified. The stained protein spots were punched out of the gel slab with a cork borer and extracted with 0.2 ml distilled water and 2.0 ml Protosol (New England Nuclear Corp.) in tightly capped vials at 60° for 4 h and at 40° for another 12 h. The samples were counted in 10 ml toluene containing 0.4% Omnifluor (New England Nuclear Corp.) in a Beckman LS-230 scintillation counter. Quenching was monitored with the external standard. The (^{3}H) and (^{14}C) counts were corrected for isotope spill-over and background. The (^{3}H)/(^{14}C) ratios for each protein from mixtures of (a) (^{3}H) reconstituted particles + (^{14}C)

50S subunits, and (b) (^3H) 50S subunits + (^{14}C) 50S subunits were calculated. Comparison of ratios (a) and (b) for each protein gave the normalized ratio which represented the amount of protein present in reconstituted particles as a fraction of that in 50S subunits. The calculation is summarized below.

(i) 'X' A_{260} (^3H) reconstituted particles
+ 'Y' A_{260} (^{14}C) 50S subunits ⟶ test $^3H/^{14}C$ ratio (a)

(ii) 'X' A_{260} (^3H) 50S subunits
+ 'Y' A_{260} (^{14}C) 50S subunits ⟶ control $^3H/^{14}C$ ratio (b)

(iii) $\frac{\text{test } ^3H/^{14}C \text{ ratio (a)}}{\text{control } ^3H/^{14}C \text{ ratio (b)}}$ = Normalized $^3H/^{14}C$ ratio

In Vitro Polypeptide Synthesis

The assay system developed by Nirenberg (1964) was used to measure the polyuridylic acid-directed incorporation of (^{14}C) phenylalanine into TCA-precipitable polyphenylalanine. Each reaction mixture contained 10mM Tris-HCl, pH 7.6, 60mM KCl, 50mM NH_4Cl, 20mM $Mg(OAc)_2$, 5mM spermidine, 6mM 2-mercaptoethanol, 2mM dithiothreitol, 2.5mM ATP, 0.25mM GTP, 20mM PEP, 40.0 µg pyruvic kinase, 0.05mM (^{14}C) phenylalanine (4 Ci/mole), 0.05mM 19 other unlabelled amino acids,

100 µg poly (U), 300 µg S-100 protein and 3.0 A_{260} units of ribosomes or a mixture of ribosomal subunits. The final volume of each assay was 0.2 ml. After incubation at 37° for 30 min, the reaction was terminated by addition of 2.0 ml cold 5% TCA and then boiled for 20 min. After cooling at 0°, the precipitate was collected by low-speed centrifugation, dissolved in a minimal amount of 0.1 N NaOH, and reprecipitated with 2 ml cold 10% TCA. The precipitate was collected on a membrane filter (HA, 0.45 µM, Millipore Corp.), washed twice with 3 ml cold 5% TCA, and counted in 10 ml Bray's scintillation liquid (Bray, 1960) in a vial. Radioactivity was measured in a Beckman LS-230 scintillation counter. Quenching was monitored with the external standard. All samples were assayed at least in duplicate.

Assay of Peptidyltransferase Activity

The method of Monro and Marcker (1967) as modified by Miskin et al (1970) was used. Each reaction mixture contained: 60mM Tris-HCl, pH 7.4, 400mM KCl, 20mM Mg $(OAc)_2$, 1.0mM neutralized puromycin, 4.0 A_{260} units of crude f (^3H) Met-tRNA, 2.0 A_{260} units of 50S subunits or reconstituted particles and 0.025 ml methanol in a volume of 0.1 ml. The reaction was started by addition of methanol and, after incubation at 0° for 15 min, was stopped by addition of 0.02 ml

5.0N KOH. After heating for 5 min at 40°, 1.0 ml of 1.0M Na_3PO_4 (pH 7.0) was added. The mixture was extracted with 2.0 ml ethyl acetate and centrifuged at low speed to accelerate phase separation and 1.5 ml of the upper ethyl acetate phase was removed for counting in 10 ml Bray's scintillation liquid. The counts were corrected to 2.0 ml ethyl acetate.

Assay of Ribosome Dependent IF- Catalyzed Uncoupled GTPase Activity

Uncoupled hydrolysis of GTP was measured by a modification of the method developed by Hershey, Remold-O'Donnell, Kolakofsky, Dewey and Thach (1971). Each reaction mixture contained in a total volume of 0.1 ml: 50mM Tris-HCl, pH 7.4, 100mM NH_4Cl, 10mM $Mg(OAc)_2$, 5.0mM 2-mercaptoethanol, 1.5 A_{260} units of 30S subunits, 2.0 A_{260} units of 50S subunits or reconstituted particles, 0.02mM GTP-γ-(^{32}P) (about 100 c.p.m./pmole) and 1.5µg crude IF. Reaction was started by addition of GTP and incubated at 30° for 15 min. The reaction was stopped by addition of 0.1 ml of 1mM K_3PO_4 (pH 7.0). To adsorb nucleotides, 0.1 ml of Norit-A (Fisher Scientific Co.) in water (50 mg/ml) was added followed by brief voltexing and low-speed centrifugation for 30 sec to sediment Norit-A.

To measure the released (^{32}P) phosphate, 0.15 ml of the supernatant was dispensed into a scintillation vial and counted in 10 ml Bray's scintillation liquid. Counts were corrected to 0.3 ml supernatant.

Assay of Ribosome Dependent IF-Catalyzed Coupled GTPase Activity

The reaction components were the same as that for assay of uncoupled GTPase activity except that 1.0μg AUG and 4.0 A_{260} units of crude fMet-tRNA were included (Hershey et al, 1971). Reaction was started by addition of a mixture of AUG, fMet-tRNA and GTP. The reaction conditions and subsequent extraction of released (^{32}P) phosphate were the same as those described for assay of uncoupled GTPase activity.

Assay of Ribosome Dependent EF-Catalyzed Uncoupled GTPase Activity

The reaction components and conditions were same as those for assay of uncoupled GTPase activity except that 12.7μg crude EF was used in place of crude IF in the reaction (Brot et al, 1973). Released (^{32}P) phosphate was processed and assayed in the same manner.

Assay of fMet-tRNA Binding Activity

The reaction components and conditions were same as those for assay of coupled GTPase activity except that crude f[^{3}H]Met-tRNA (about 7000 c.p.m./A_{260} unit) and unlabelled GTP were used (Brot et al, 1973). Reaction was stopped by dilution with 3.0 ml cold buffer containing 10mM Tris-HCl, pH 7.4, 100mM NH_4Cl and 5mM $Mg(OAc)_2$. The diluted reaction mixture was immediately filtered through Millipore filter. The filter was washed twice with 3.0 ml cold buffer, dried and then counted in scintillation vials containing 10 ml Bray's scintillation liquid.

Assay of Subunit Association Activity

Purified 50S subunits or reconstituted particles were mixed with 30S subunits previously activated at 37° for 45 min (Noll et al, 1973) at a molar ratio of 0.8 in TKMS I buffer at 37°. After 20 min, the mixture was chilled in ice and analyzed in the same buffer by sucrose gradient centrifugation in a Beckman SW 50.1 rotor at 45,000 r.p.m. for 90 min. The relative amounts of 70S complexes formed were estimated gravimetrically from tracings of sedimentation profiles.

When labelled subunits or reconstituted particles were used in the association reaction, 0.2 ml fractions were

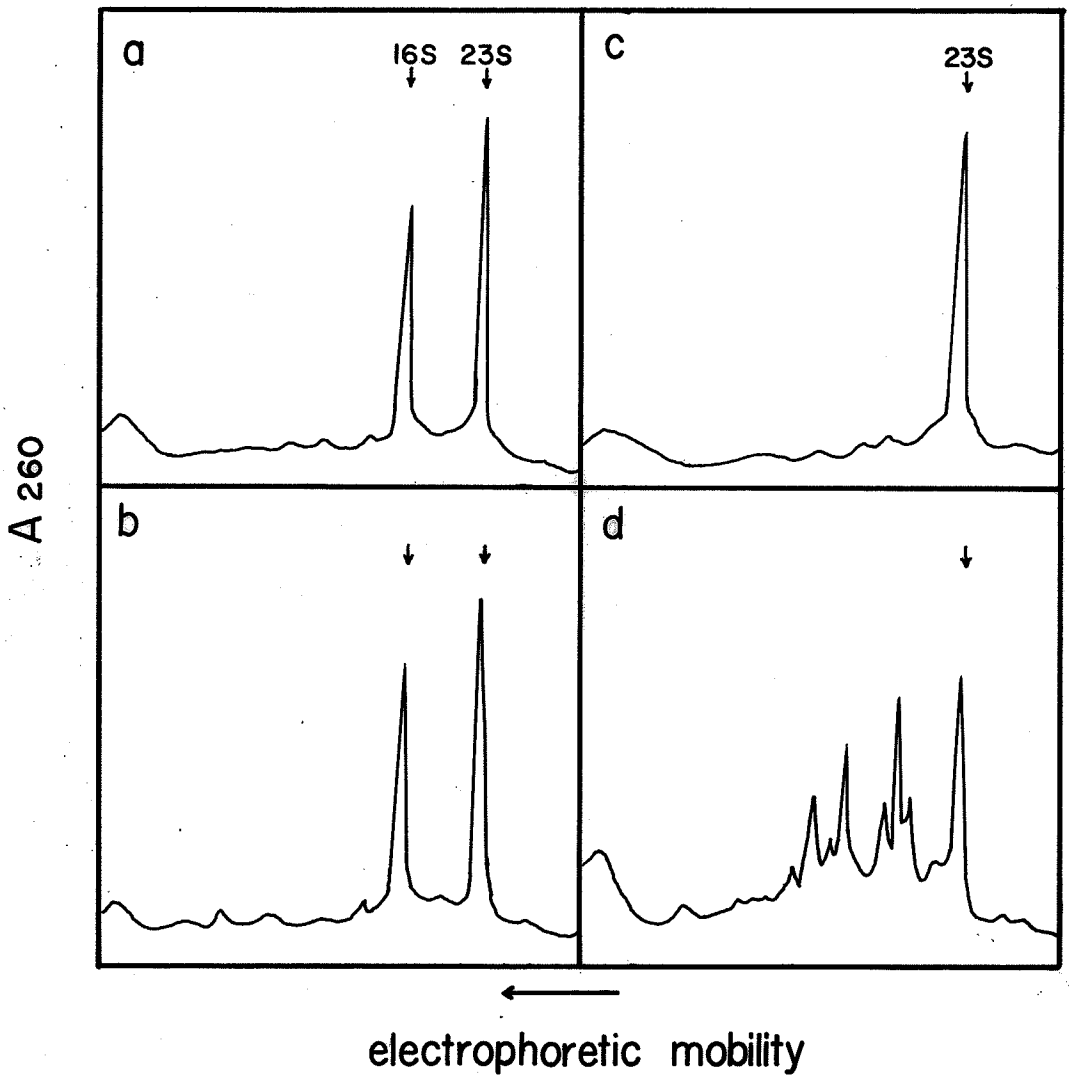
collected from the gradient and aliquots were assayed for absorbance at 260 nm and for radioactivity. Where necessary, the 70S peak fractions were pooled and precipitated with 0.7 volume of ethanol.

IV. RESULTS

RNA Components

Ribosomal RNA containing 5S, 16S and 23S RNAs was used for reconstitution rather than isolated 23S RNA. The 23S RNA extracted from 50S subunits or purified from rRNA by phenol or urea in the presence of LiCl (Methods), macaloid (Stanley and Bock, 1965) or diethyl pyrocarbonate (Solymosy, Fedorcsak, Gulyas, Farkas and Ehrenberg, 1968) appeared intact (Fig. 2c), but was extensively nicked as revealed by polyacrylamide gel electrophoresis of heat-denatured (75^{o} for 1 min) RNA (Fig. 2d). This degradation profile for 23S RNA was quite reproducible suggesting specific cleavages. However, all RNA species in rRNA (Fig. 2a) remained intact even after heat denaturation (Fig. 2b). Although the reason for stability of 23S RNA in the presence of 5S and 16S RNAs was not clear, some evidence has been presented indicating that the nucleolytic activity might be due to a 50S ribosomal protein (Ceri and Maeba, 1973). For this reason rRNA rather than isolated 23S RNA was used in attempts to reassemble 50S subunits in vitro.

Figure 2. Electrophoresis of RNA. RNA samples were extracted with urea-LiCl from (a) 70S ribosomes and (c) 50S subunits, and electrophoresced in 2.65% polyacrylamide gels (Materials and Methods). Panel (b) shows the profile of heat-denatured (75^{o} for 1 min) RNA from 70S ribosomes and panel (d), from 50S subunits. Approximately 0.5 A_{260} unit RNA was applied to each gel which was scanned at 260 nm in a Shimadzu MPS 50L recording spectrophotometer equipped with a linear transport accessory.


a
16S
23S
c
23S
b
d
A 260
electrophoretic mobility

Incubation Temperature for Reconstitution

The 50S ribosomal proteins extracted from purified 50S subunits (Methods) and rRNA extracted from 70S ribosomes by urea-LiCl (Methods) were mixed at a ratio of 1.2 A_{260} equivalents of 50S proteins per A_{260} unit of 23S RNA in 1.0 ml of S R buffer at temperatures ranging from 0 to 45° for 30 min. The ethanol precipitated material was analyzed by 5-20% sucrose gradient centrifugation in TKM II buffer (1.0mM magnesium). As described in "Methods", the S-values of reconstructed particles were determined by the procedure of O'Brien (1971) by comparison with sedimentation rates of known markers. The S-values of markers are depicted in Figure 3. The standard curve of S-values obtained by this method was intended to identify the various particles obtained and served as a convenient system of classification. The relative distances of sedimentation of the markers in sucrose gradients in 1.0mM magnesium (TKM II buffer) or in 10mM magnesium (TKM I buffer) were not detectably different (data not shown).

Reconstitution at 0°, 10° or 28° produced material sedimenting as 2 peaks with S-values of 16 and 28 (Fig. 4b, c, d). Compared to rRNA centrifuged in a parallel gradient (Fig. 4a), the peak representing 23S RNA was replaced by 28S

Figure 3. Standard curve for determination of sedimentation coefficients. Two A_{260} units of 70S ribosomes or rRNA were centrifuged in 5-20% sucrose gradients in TKM II buffer with a Beckman SW 50.1 rotor at 45,000 r.p.m. for 105 min at 4^{o}. After centrifugation, gradients were analyzed with a flow-through cuvette in a Gilford model 2000 recording spectrophotometer at 260 nm. The relative sedimented distances of 5, 16 and 23S RNA's, and of 30 and 50S subunits were measured and plotted against their respective sedimentation coefficients in Svedberg units.

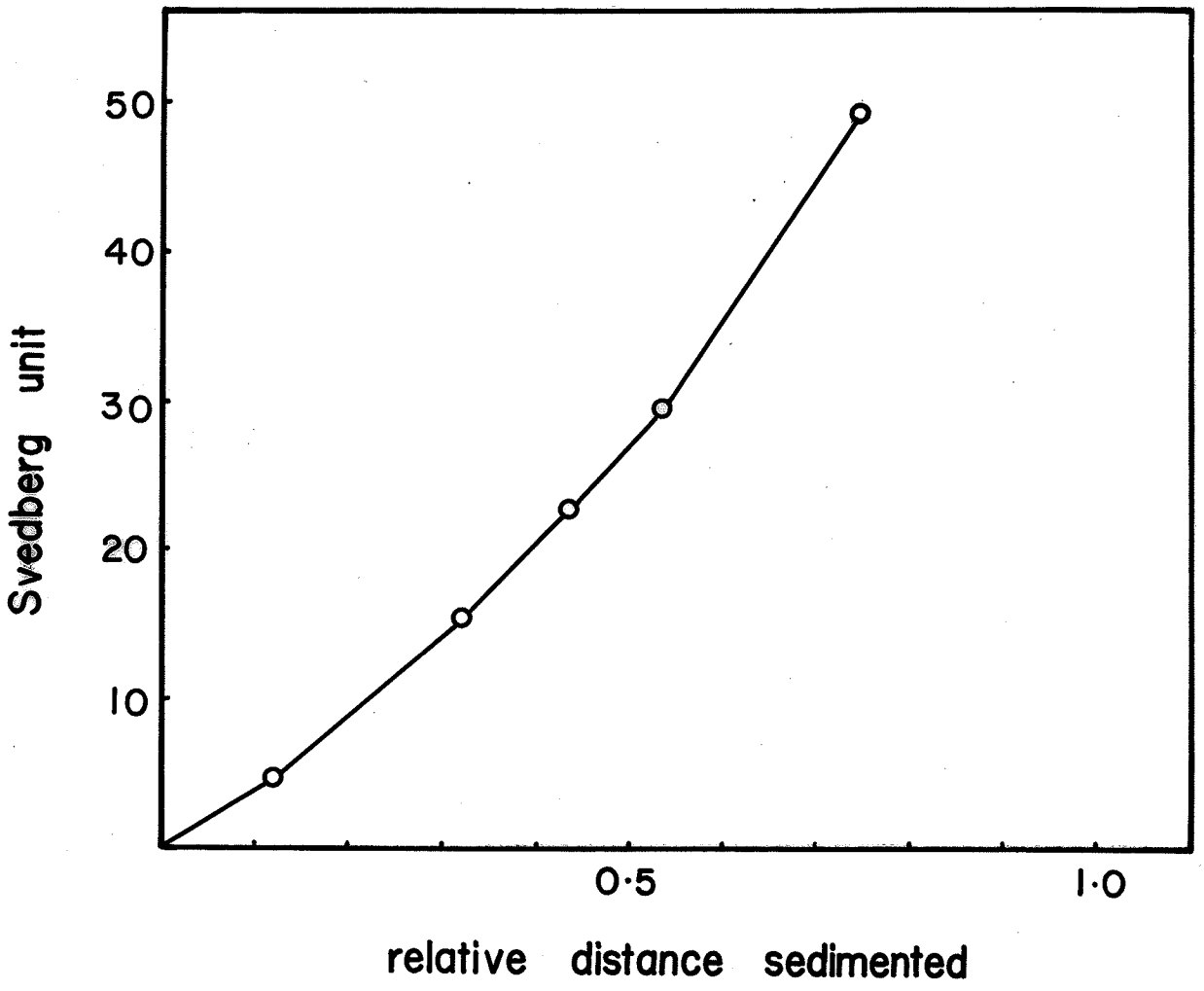
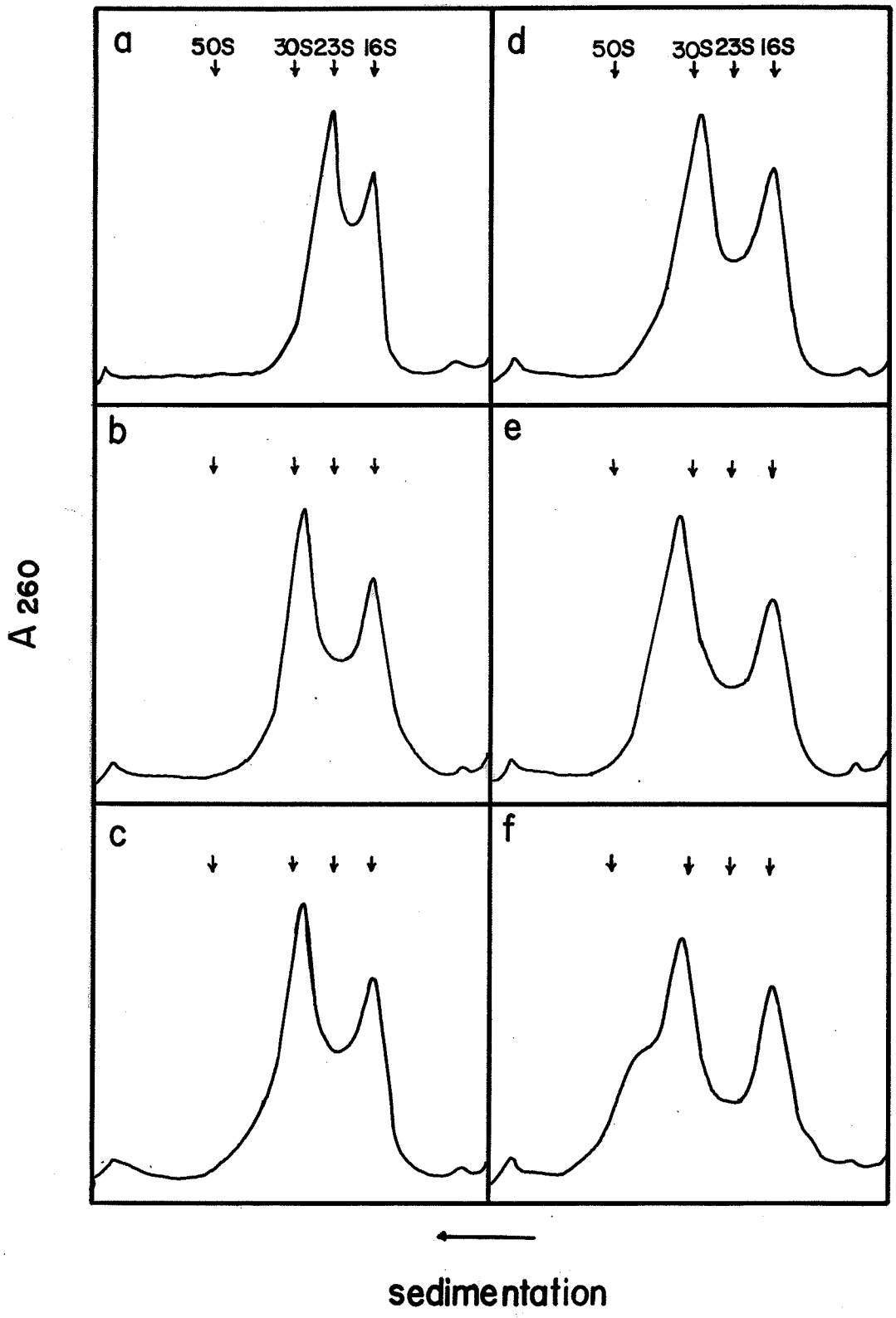

50
40
30
20
10
Svedberg unit
0·5
1·0
relative distance sedimented

Figure 4. Sedimentation analysis of reconstituted mixtures incubated at different temperatures. Panel (a) shows rRNA alone. Ribosomal RNA (3.6 A_{260} units) and 2.9 A_{260} equivalents of 50S proteins were incubated for 30 min in 1.0 ml SR buffer at (b) 0^{o}; (c) 10^{o}; (d) 28^{o}; (e) 37^{o}; and (f) 45^{o}. The ethanol precipitated material was dissolved in 0.1 ml TKM II buffer and centrifuged in 5-20% sucrose gradients in the same buffer in a Beckman SW 50.1 rotor at 45,000 r.p.m. for 105 min. Gradients were then analyzed as in the legend to figure 3. The arrows indicate positions of sedimentation of markers centrifuged in parallel gradients.

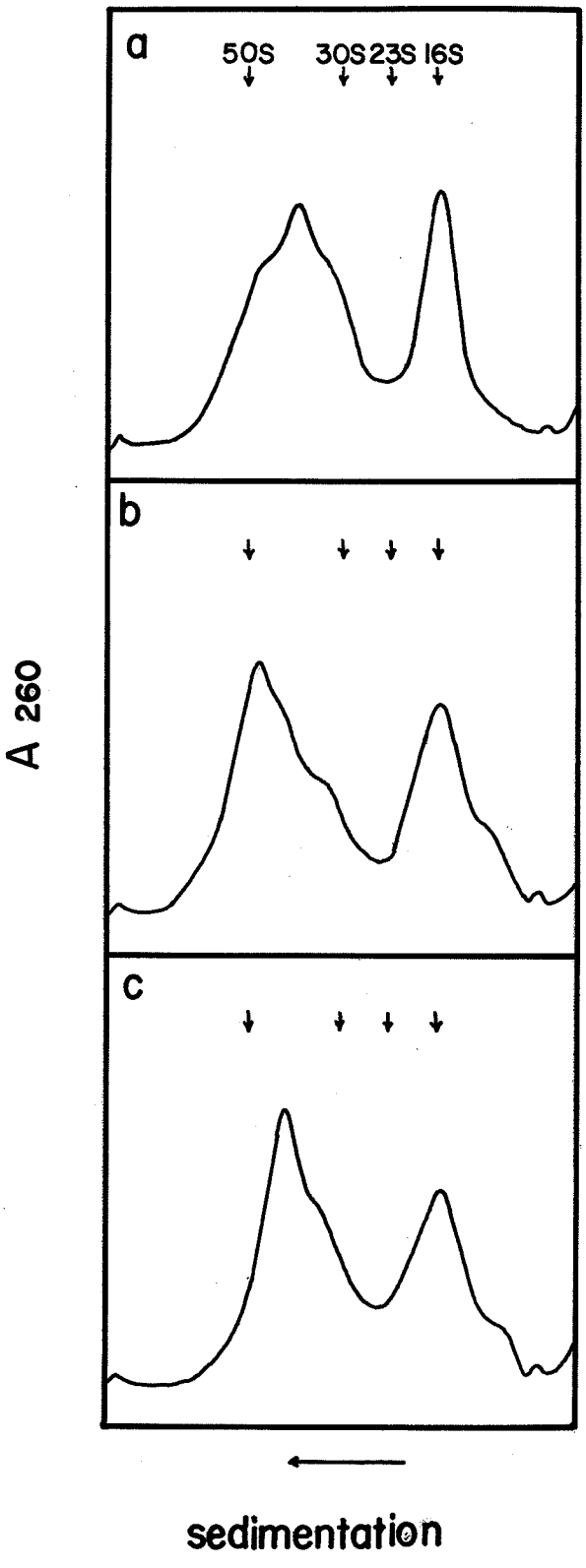

a
50S
30S
23S
16S
d
50S
30S
23S
16S
b
e
c
f
A 260
sedimentation

species while the peak representing 16S RNA was unchanged.

Reconstitution at 37° yielded particles sedimenting as a single peak at 32S (Fig. 4e) while at 45°, a faster migrating species sedimenting as a shoulder of the 32S peak was obtained (Fig. 4f). Therefore incubations at 0° and 37° were used in subsequent sequential reconstitutions. The formation of 28S particles was complete after 5 min at 0° and further incubation did not yield faster sedimenting particles. When temperature was shifted to 37°, 32S particles were formed at the expense of the 28S species. Transition to 32S particles was complete within 5 min and incubation periods of up to 60 min at 37° did not alter the sedimentation profile.

When temperature was further shifted to 55°, peaks sedimenting faster than 32S were detected (Fig. 5). After 5 min at 55°, the 32S peak was reduced to a shoulder on the light side of a broad profile representing 3 discernable peaks sedimenting at 32, 43 and 48S (Fig. 5a). After 15 min at 55°, the largest of the 3 peaks predominated (Fig. 5b), and after 45 min, a major 43S peak appeared with a 32S shoulder (Fig. 5c). Prolonged incubation led to degradation of RNA components as evidenced by formation of A_{260}-absorbing material at the top of the gradient and the broadening of 16S RNA peak.

Figure 5. Time course analysis of reconstituted mixtures. Incubation was carried out sequentially at 0^{o} for 15 min, 37^{o} for 15 min and 55^{o} for (a) 5 min; (b) 15 min; and (c) 45 min. The ethanol precipitated materials from reconstituted mixtures were analyzed by sucrose gradient centrifugation (Methods).


a
50S
30S
23S
16S
b
c
A 260
sedimentation

Direct incubation of the reconstitution mixture at 55° without prior incubation at the lower temperatures resulted in formation of 30-32S particles which could not be converted to particles with greater sedimentation rates (Fig. 6a). On prolonged incubation at 55°, these 30-32S particles were replaced by A_{260}-absorbing material at the top of the gradient (Fig. 6b), presumably due to degradation of RNA. Although initial incubation at 37° was essential for formation of larger complexes at 55°, incubation at 0° was not required for formation of 32S particles at 37° (Fig. 6c) nor for subsequent formation of 43-48S particles at 55° (Fig. 6d).

The S-values of particles formed at 55° varied. At times, 43S particles were replaced by a peak sedimenting between 36 and 43S, and 48S species by a peak sedimenting between 45 and 48S. However, for purposes of identification, S-values for particles formed at 55° were conveniently designated 43 and 48. It should be noted that complete conversion to 48S particles was not achieved under these conditions.

To test specificity of binding, particles were reconstituted with ribosomal proteins extracted from (^{14}C) 50S subunits. Approximately 85-90% of the bound radioactivity cosedimented with the peaks of reconstituted particles, formed at 0, 37, and 55° (Fig. 7a, b, c), indicating that 50S proteins were bound selectively to 23S RNA in the

Figure 6. Sedimentation analysis of reconstituted mixtures incubated at (a) 55^{o} for 20 min; (b) 55^{o} for 90 min; (c) 37^{o} for 15 min; and (d) 37^{o} for 15 min and 55^{o} for 20 min. The ethanol precipitated materials were analyzed as in the legend to figure 4.

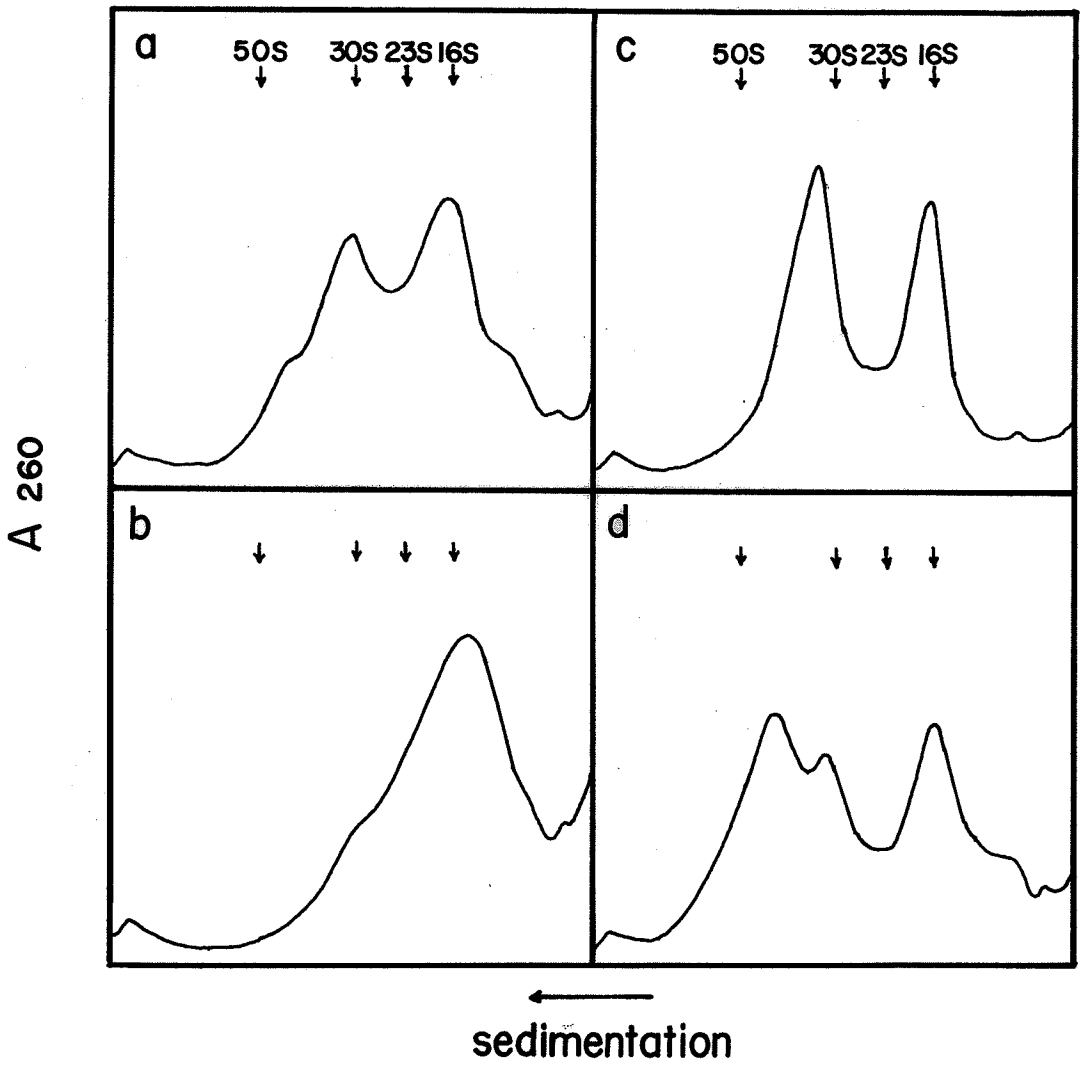

a
50S
30S
23S
16S
c
50S
30S
23S
16S
b
d
A 260
sedimentation

Figure 7. Sedimentation analysis of binding of (^{14}C) 50S proteins to rRNA.I. Reconstitution was performed at (a) 0^{o} for 15 min; (b) 37^{o} for 15 min; and (c) sequentially at 37^{o} for 15 min, 55^{o} for 20 min. The ethanol precipitated material was analyzed in 5-20% sucrose gradients in 1.0mM magnesium (TKM II buffer) as described in "Methods". Open circles (⊙) indicate (^{14}C) protein binding profiles. Solid lines represent A_{260} profile.

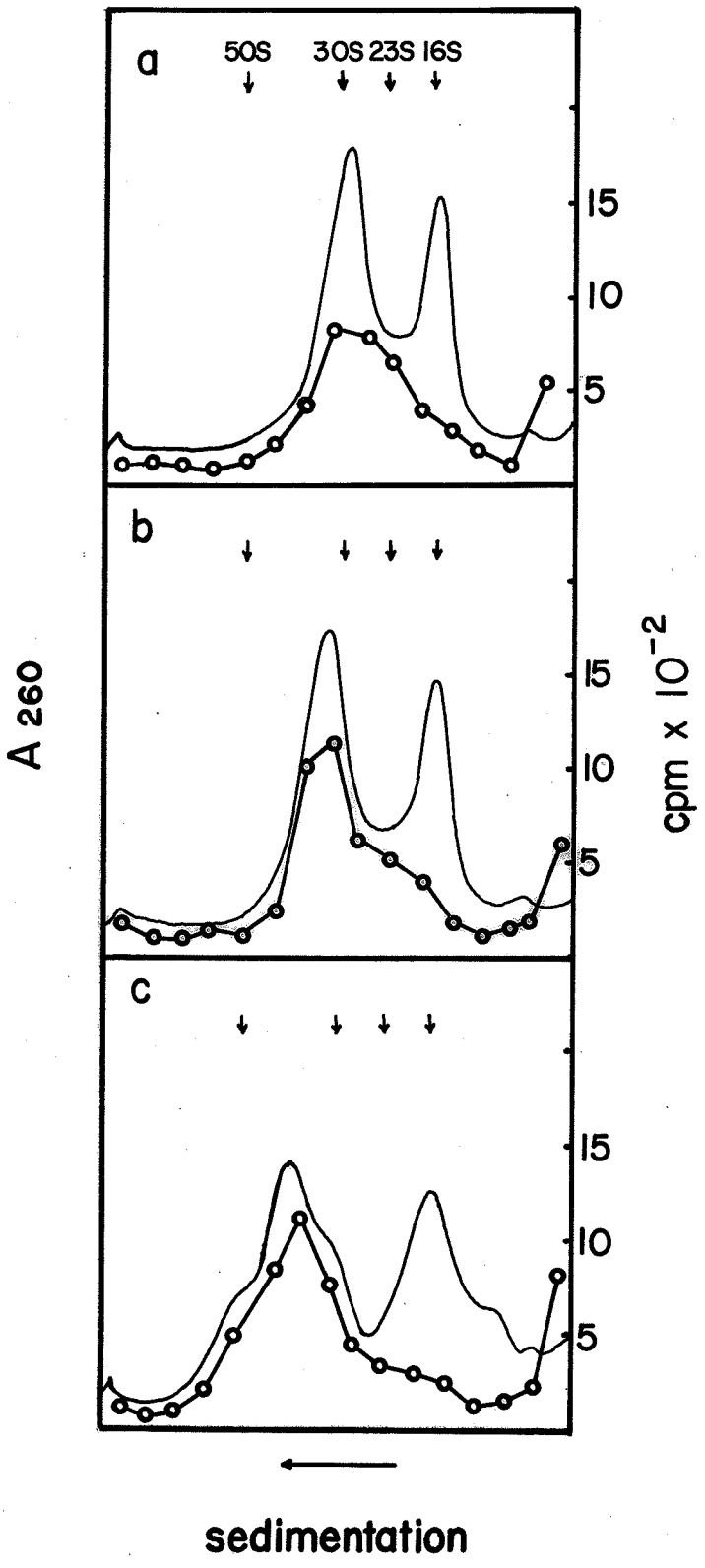
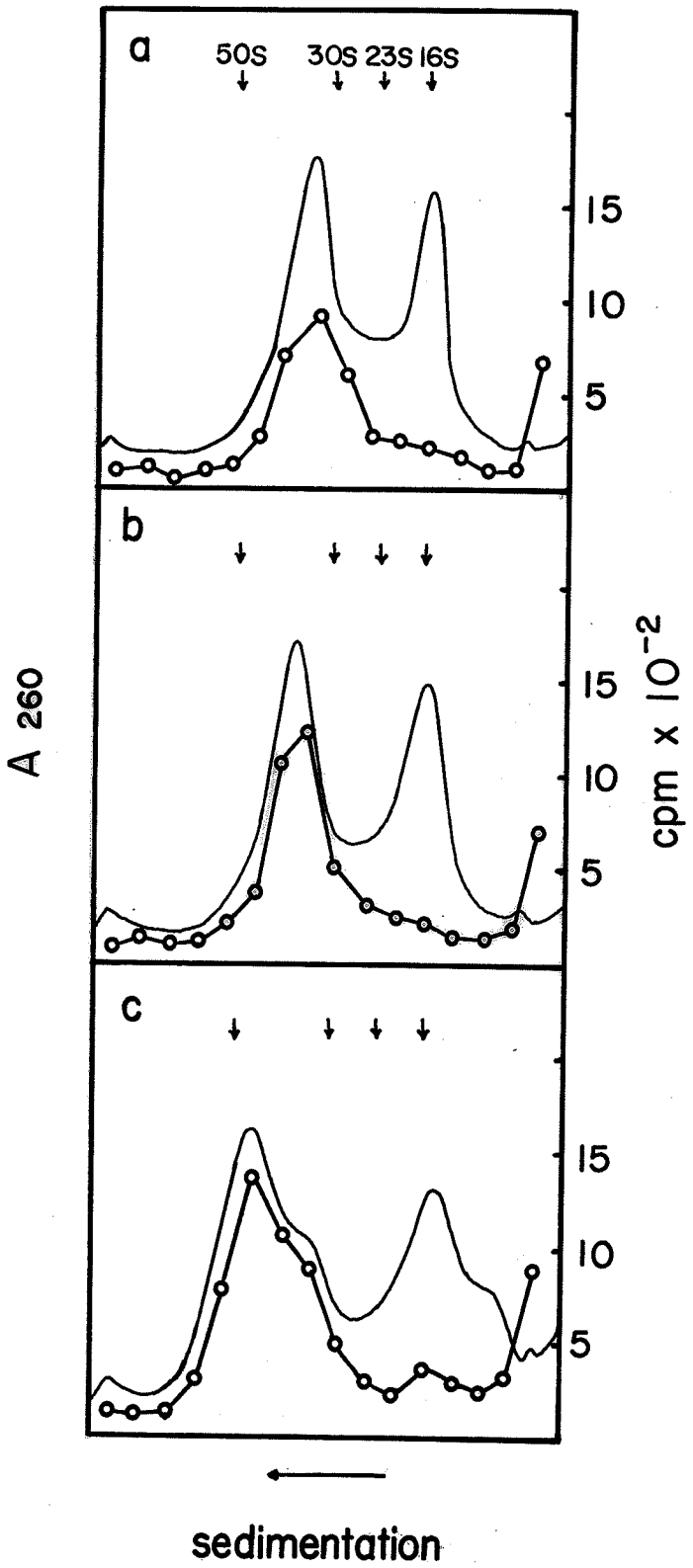

a
50S
30S
23S
16S
15
10
5
b
15
10
5
c
15
10
5
A 260
cpm x 10^{-2}
sedimentation

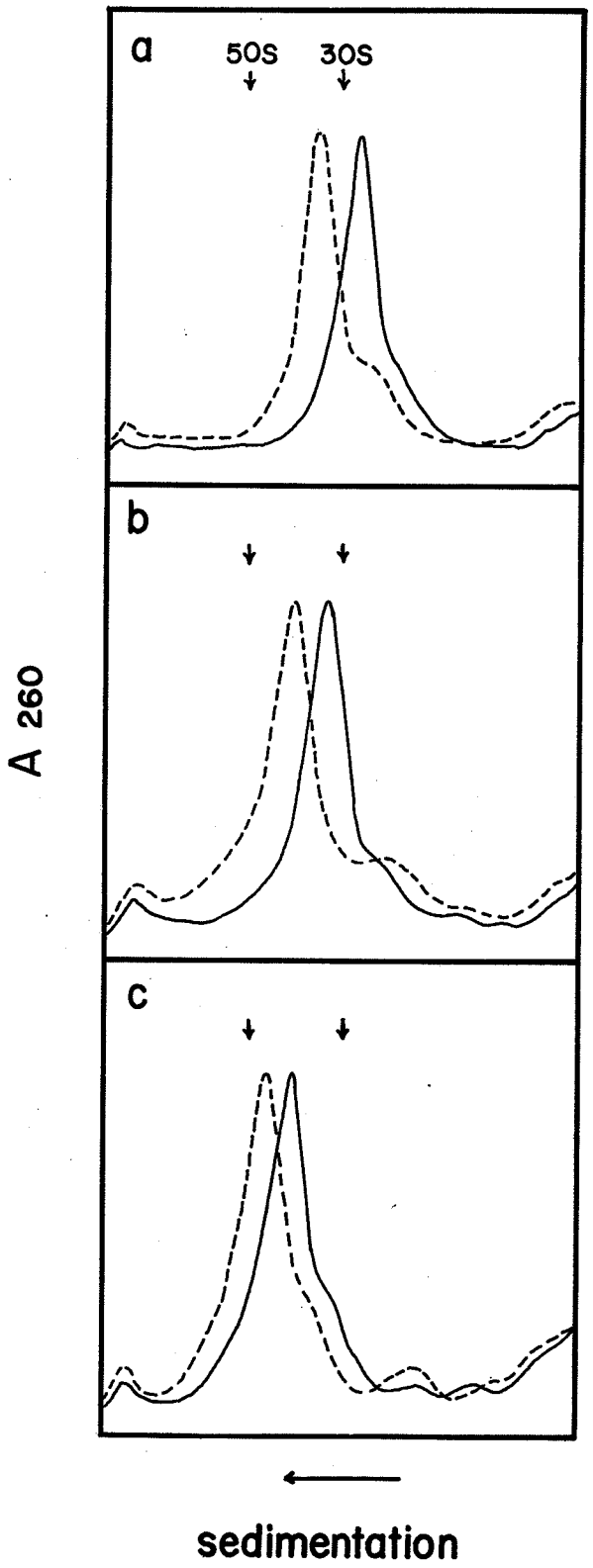
Figure 8. Sedimentation analysis of binding of (^{14}C) 50S proteins to rRNA.II. Reconstitution was carried out as in the legend to Figure 7. Analysis of reconstituted mixture was performed in 10mM magnesium (TKM I buffer). Open circles (o) indicate (^{14}C) protein binding profile. Solid lines represent A_{260} profile.


a
50S
30S
23S
16S
15
10
5
b
15
10
5
c
15
10
5
A 260
cpm x 10^{-2}
sedimentation

presence of 16S RNA.

When the same particles were analyzed in sucrose gradients in 10mM magnesium (TKM I buffer), sedimentation values of the particles increased to 32S, 37S and 48S from 28S, 32S and 43S, respectively, when analyzed in 1.0mM magnesium (compare Figs. 7 and 8). It was uncertain whether the heavier particles were products of conformational changes or resulted from binding of more proteins induced by presence of 10.0mM Mg^{++}. To distinguish between these alternatives, the largest particles reconstituted at each temperature were isolated by sucrose gradient centrifugation in TKM II buffer (1.0mM magnesium), then analyzed in 5-20% sucrose gradients in TKM I (10mM magnesium) and TKM II (1.0mM magnesium) buffers. The isolated particles sedimented at 32, 37 and 48S in TKM I buffer and at 28, 32 and 43S in TKM II buffer respectively (Fig. 9). This indicated that conversion of particles to faster-sedimenting species in high magnesium concentration was due to conformational changes rather than binding of additional proteins. As a less heterogeneous 48S peak was obtained in 10mM magnesium, all subsequent sedimentation analyses, except where stated, were performed in buffer containing 10mM magnesium.

Figure 9. Sedimentation analysis of the largest isolated reconstituted particles formed after (a) 15 min at 0°; (b) 15 min at 37°; and (c) 15 min at 37° and 20 min at 55°. Two A_{260} units of isolated particles were centrifuged in 5-20% sucrose gradients in TKM II (——) or TKM I (---) buffers and analyzed as in the legend to figure 4.


a
50S
30S
b
c
A 260
sedimentation

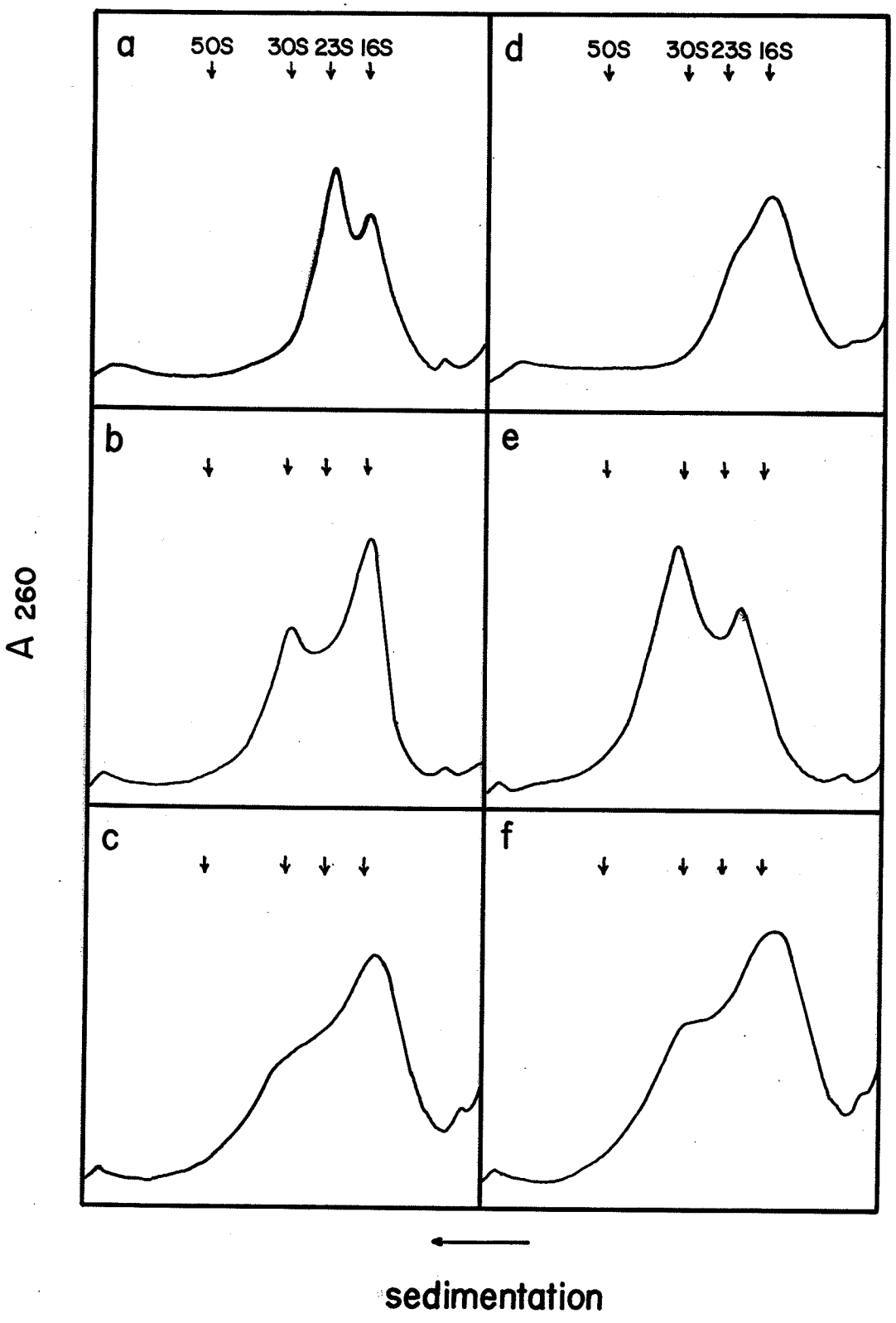
Role of RNA Components in Reconstitution

Intact RNA was required for formation of 50S protein - 23S RNA complexes. When rRNA was allowed to autodegrade by incubation at 37° for 48 h before reconstitution, particles sedimenting faster than 30S were not formed (Fig. 10 b, c). When used to construct 48S particles at 55°, sedimentation analysis revealed a broad peak of A_{260}-absorbing material at the top of the gradient (Fig. 10c). Since the RNA was probably extensively nicked, as revealed after heat denaturation (Fig. 10d), incubation at 55° probably led to melting (Tal, 1969) and liberation of small RNA fragments.

As stated earlier, attempts to isolate 23S RNA resulted in a product that contained many scissions (Fig. 2). When isolated 23S RNA was used in reconstitution, 30-32S particles were formed at 37° which could not be transformed to faster-sedimenting species at 55° (Fig. 10 e, f). The presence of a 16-20S peak was probably due to ribonucleoprotein complexes formed from protein binding to fragments of degraded 23S RNA (Fig. 10c). As in reconstitution with degraded rRNA, A_{260}-absorbing material appeared at the top of the gradient, presumably due to the melting of RNA at 55° (Fig. 10 f).

The state of RNA in 32S, 37S and 48S reconstituted complexes formed from undegraded rRNA was analyzed by gel

Figure 10. Sedimentation analysis of ribonucleoprotein complexes formed from 50S proteins and degraded rRNA (b and c), or 23S RNA (e and f). Incubation was at 37° for 15 min (b and e), or sequentially at 37° for 15 min and 55° for 20 min (c and f). The ethanol precipitated material was analyzed by sucrose gradient centrifugation in TKM I (Methods). Panel (a) shows sedimentation profile of degraded rRNA and (d), that of degraded rRNA heated at 75° for 1 min prior to sedimentation analysis.


a
50S
30S
23S
16S
d
50S
30S
23S
16S
b
e
c
f
A 260
sedimentation

electrophoresis (Methods). Particles were collected from reconstituted mixtures by ethanol precipitation and heated at 75° for 1 min in 0.2% SDS prior to electrophoresis, to disrupt secondary structure of RNA and reveal any "hidden scissions" (Bruening and Bock, 1967). Profiles obtained by scanning gels at 260 nm revealed intact 16S and 23S RNA peaks (Fig. 11) in all particles showing that rRNA was not degraded during reconstitution.

Protein Content in Reconstituted Particles

The binding of proteins in particles was measured as a function of incubation time. Particles were constructed by incubating unlabelled or (^{14}C) 50S proteins and rRNA for varying times under varying conditions and isolated by sucrose gradient centrifugation. The protein content of isolated particles was estimated colorimetrically by the Lowry method (1951) or by measuring radioactivity and were expressed as μg protein/A_{260} unit reconstituted particles or as c.p.m./A_{260} unit reconstituted particles when (^{14}C) 50S proteins were used.

The results are presented in Figure 12. At 0°, very little protein was bound in 32S particles and saturation was reached within 2 min. The amount of protein in 32S particles (6-7 μg/A_{260} unit reconstituted particles) was

Figure 11. Electrophoretic analysis of RNA associated with reconstituted mixtures incubated at (a) 0^{o} for 15 min; (b) 37^{o} for 15 min; and (c) 37^{o} for 15 min and 55^{o} for 20 min. Approximately 0.7 A_{260} unit of reconstituted mixture was applied to each gel containing 2.65% polyacrylamide and 0.2% SDS. Gels were electrophoresced at pH 8.4 and scanned in a Joyce Loebl U.V. recording scanner Type D8 MK2. All samples were heated at 75^{o} for 1 min in 0.2% SDS prior to electrophoresis.

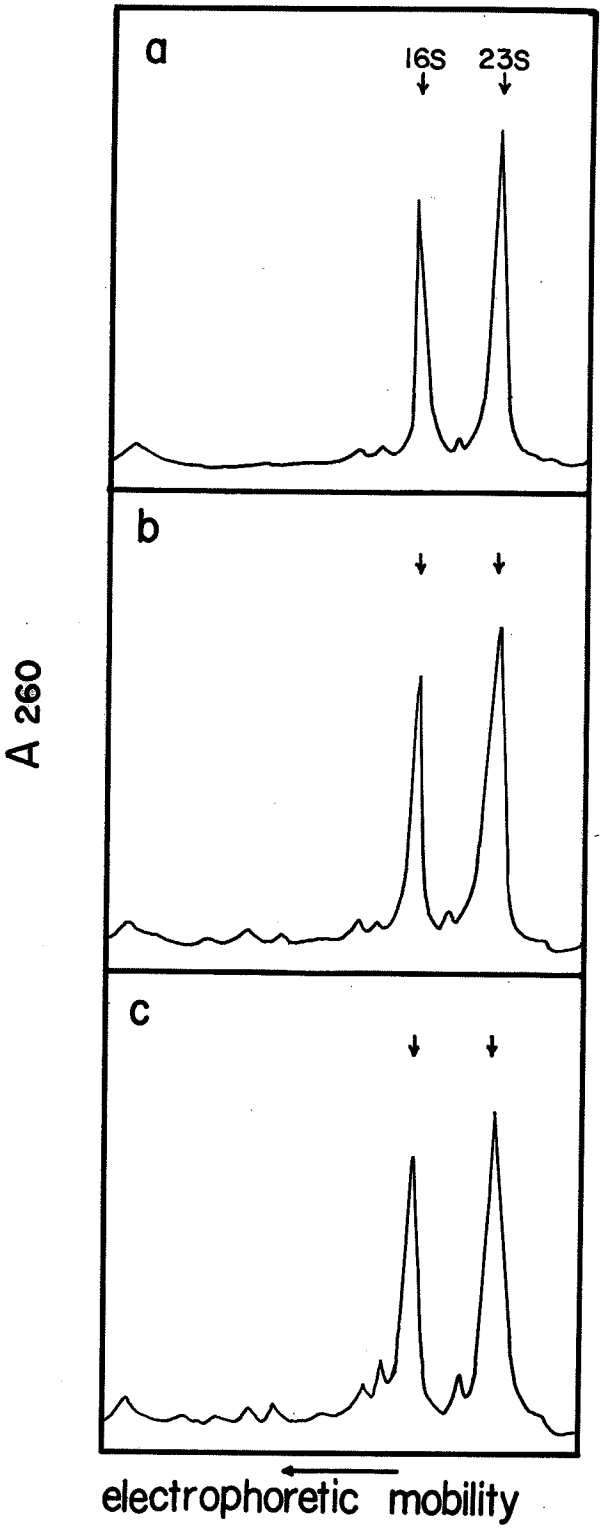
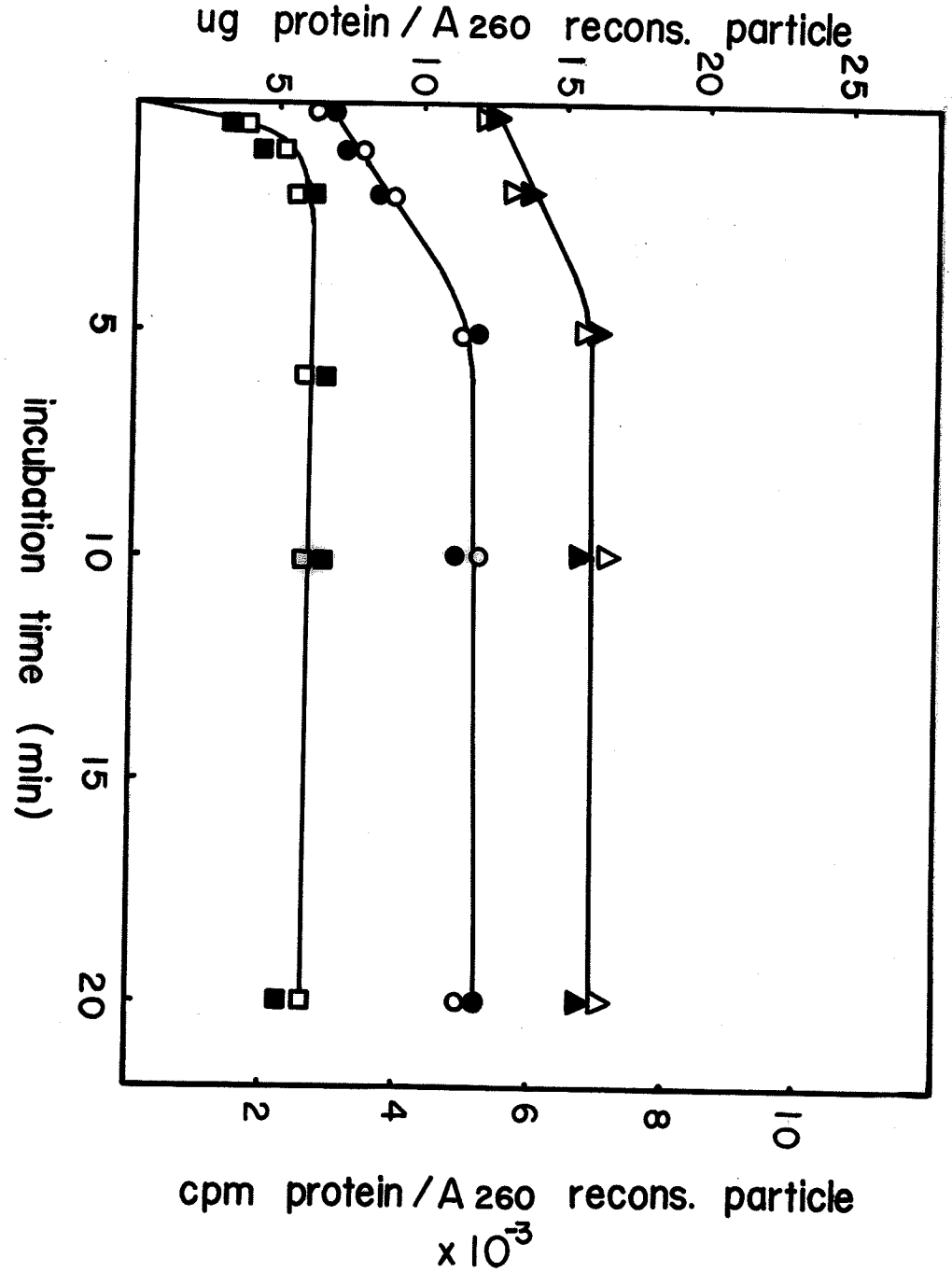

a
16S
23S
b
c
A 260
electrophoretic mobility

Figure 12. Time course analysis of binding of 50S proteins to 23S RNA. Ribosomal RNA (18.0 A_{260} units) and 15.0 A_{260} equivalents of 50S proteins or (^{14}C) 50S proteins were mixed at 0° (□), 37° (○) or 37 + 55° (△) in 5.0 ml SR buffer. Reactions were stopped at various times by addition of ethanol and reconstituted particles were isolated by centrifugation in 5-20% sucrose gradient in TKM II buffer in a Beckman SW 41 rotor at 40,000 r.p.m. for 4 h at 4°. RNA content of particles was assayed for absorbance at 260 nm while their protein content was assayed by Lowry's method (closed signs) or radioactivity in c.p.m. (open signs).


ug protein / A 260 recons. particle
5
10
15
20
25
incubation time (min)
5
10
15
20
2
4
6
8
10
cpm protein / A 260 recons. particle
x 10^3

about 30% of that in 50S subunits (22 μg/A_{260} unit ribosomal particles). At 37°, protein binding reached a maximum within 5 min of incubation, the amount of protein in 37S particles being about 50% of that in 50S subunits or roughly twice that in 32S particles formed at 0°. At 55°, the amount of bound protein in 43-48S particles increased to a maximum of 70% of that in 50S subunits within 5 min of incubation. The data showed that temperature dependent formation of ribonucleoprotein particles with increasing sedimentation coefficients was associated with increases in protein binding and that most of the proteins were bound within 5 min. There was no difference when protein was assayed in c.p.m. or in μg (Lowry's phenol method). Also, there was close correspondence in results when RNA was assayed in A_{260} unit and by the orcinol method (Schneider, 1957). Furthermore, as described earlier, the formation of 48S particles at 55° as revealed by sucrose gradient centrifugation in TKM II buffer (1.0mM magnesium) was variable.

Function of Temperature in Reconstitution

To determine whether temperature dependent conversion of particles to faster sedimenting species could be achieved in the absence of additional protein binding, reconstituted

particles, formed at 0^{o} and at 37^{o}, were freed of unbound proteins by sedimentation at 30,000 r.p.m. in a Beckman 60-Ti rotor for 12 h at 4^{o} through 2 ml of 10% sucrose cushion. The pellets were dissolved in S R buffer and sedimentation rates of particles were checked in TKM II buffer (1.0mM magnesium) before and after incubation at 37^{o} or 55^{o}. The results summarized in Table IV showed that increases in S-values of reconstituted particles were induced by incubation alone in the absence of exogenous 50S proteins, and suggested that the main function of temperature was to induce conformational changes in the particles, resulting in faster sedimenting particles. This aspect of the system will be dealt with in greater detail later.

Protein Analysis of Reconstituted Particles

Proteins were extracted with 67% acetic acid in 100mM magnesium (Hardy et al, 1969) from 28S, 32S, 43S and 48S particles isolated in TKM II (1.0mM magnesium) buffer and analyzed by electrophoresis at pH 4.5 in 12.5% polyacrylamide-urea gels (Leboy et al, 1964). The protein bands in the electropherograms shown in Plate I, along with that of control 50S subunits, were numbered according to the classification system of Kurland (1971). Only 14 of the 23 protein bands in 50S subunits were detected in 28S particles. The staining intensity of many of these bands indicated that

Table IV. Incubation of reconstituted particles isolated in TKM II buffer (1.0mM Mg^{++}).

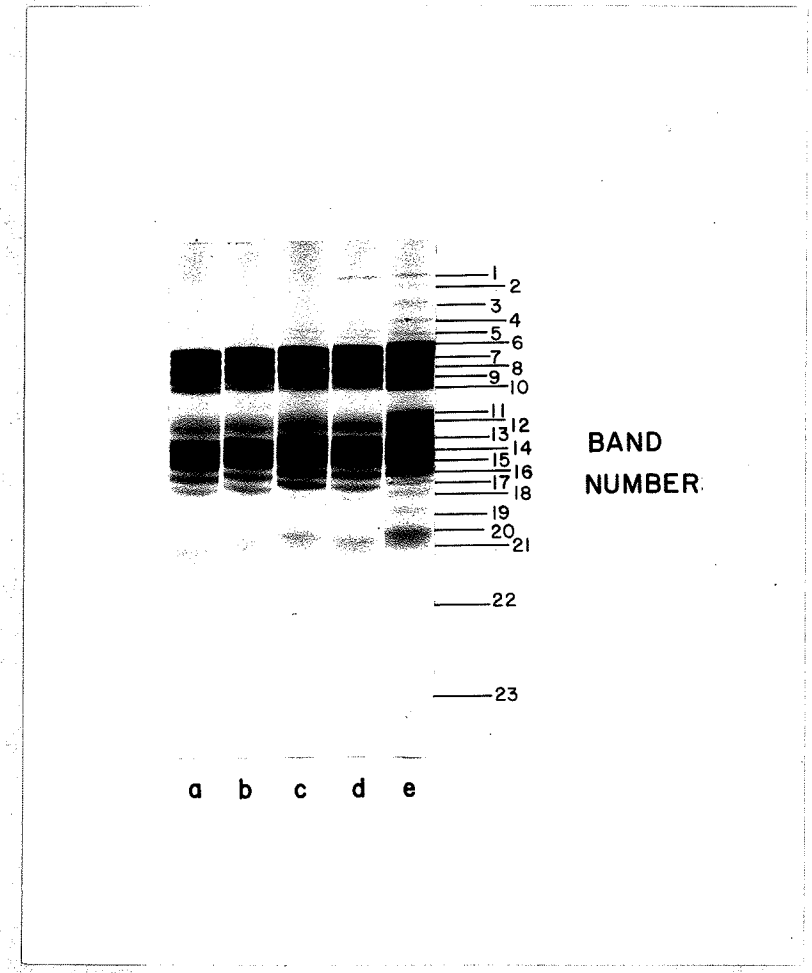
Original reconstituted particles	Incubation I (37°, 15 min)	Incubation II (55°, 15 min)
28S	30S	30S, 41S
32S	-	32S, 42S

Note: Reconstituted particles were formed at 0 and 37° and centrifuged free of unbound proteins (Methods). The pelleted particles were resuspended in SR buffer and further incubated in the absence of exogenous proteins as indicated above. The S-values were checked after each incubation by sucrose gradient centrifugation (Methods).

Legend. Proteins were extracted with 67% acetic acid in 100mM $Mg(OAc)_2$ and 100 µg were electrophoresed into urea-polyacrylamide gels at pH 4.5 as described in "Methods". Running time was 5 h at 10^{o} at a constant current of 3 ma/gel tube. Gels were stained with Coomassie Brilliant Blue and destained with 10% TCA (Methods). The proteins were extracted from 28S (a), 32S (b), 43S (c) and 48S (d) reconstituted particles, and E. coli 50S ribosomal subunits (e). The protein bands were numbered according to the classification system of Kurland (1971).

Plate I

Electrophoretic patterns of ribosomal proteins of reconstituted particles and E. coli 50S ribosomal subunits.



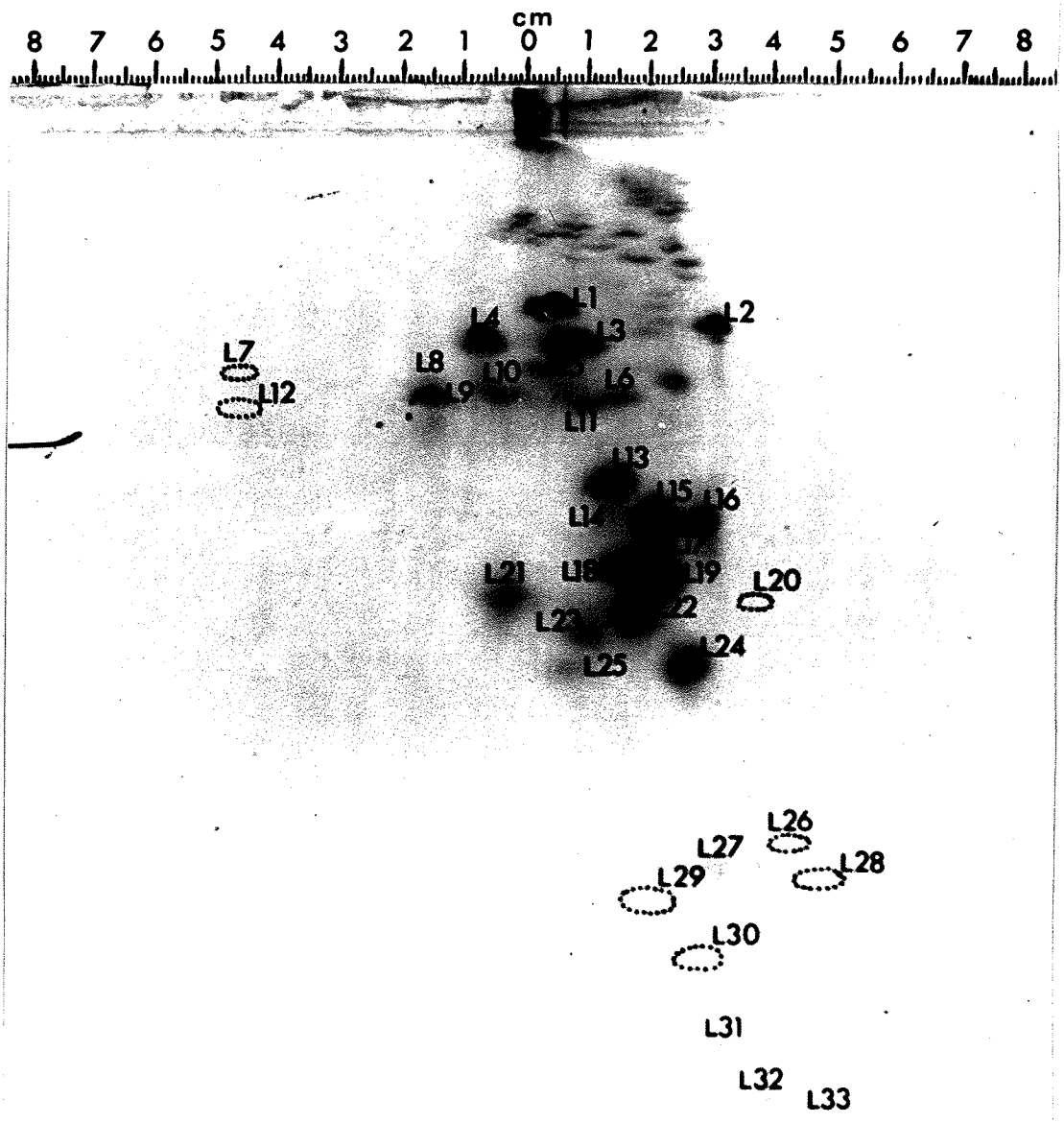
some proteins were absent or present in reduced amounts. As particles increased in S-value, additional protein bands were acquired. The 32S particles showed addition of bands 20 and 22. The 43S and 48S particles acquired bands 3, 4 and 23 in addition to bands found in 32S particles. Band 1, found in 48S particles, may not be a ribosomal protein and could be an artifact produced by oxidation of ribosomal proteins.

Analysis by one-dimensional electrophoresis gave only qualitative estimates of proteins since some bands may represent two or more different proteins. Therefore the bi-dimensional electrophoretic system of Kaltschmidt and Wittmann (1970a) was used to analyze and identify the 50S proteins in reconstituted particles. Proteins associated with each of the particles isolated in 1.0mM and 10.0mM magnesium were subjected to bidimensional polyacrylamide gel electrophoresis. The proteins associated with 28S, 32S (formed at 37^{o}), 43S particles isolated in 1.0mM magnesium sucrose gradients, and 48S particles isolated in 10mM magnesium gradient are shown in Plates II to V. Plate VI shows the protein pattern of control 50S subunits. As compared to the control gel of total 50S proteins showing 32 protein spots (L8 and L9 appeared as a composite spot and L34, the fastest running 50S protein, was not retained in the slab gel),

Legend. Proteins were extracted with 67% acetic acid in 100mM $Mg(OAc)_2$ from isolated 28S reconstituted particles and 1.5 mg were analyzed with the bidimensional polyacrylamide gel electrophoretic system (Kaltschmidt and Wittmann, 1970a; Methods). Running time was 20 h at 10^{o} at a constant current of 3 ma/gel tube at pH 8.6 in the first dimension and 20 h at 10^{o} at a constant current of 100 ma/gel slab at pH 4.5 in the second dimension. Gel slab was stained with Amido Black and destained with 1% acetic acid (Methods).

Plate II

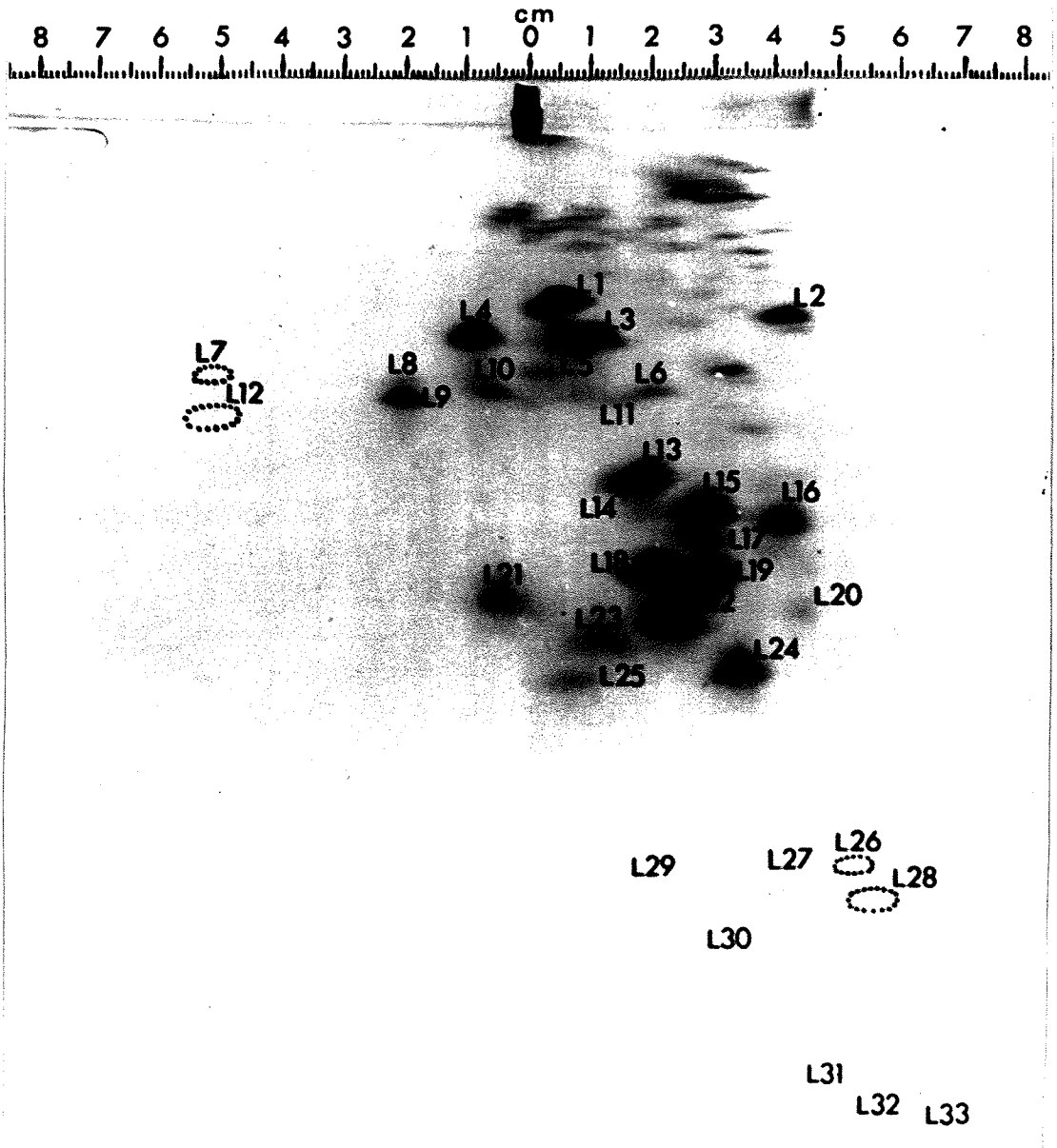
Two-dimensional electropherogram of ribosomal proteins of 28S reconstituted particles.



Legend. Proteins were extracted from isolated 32S reconstituted particles and electrophoresed as described in the legend to Plate II and in "Methods". Staining and destaining of gel slab was described in "Methods".

Plate III

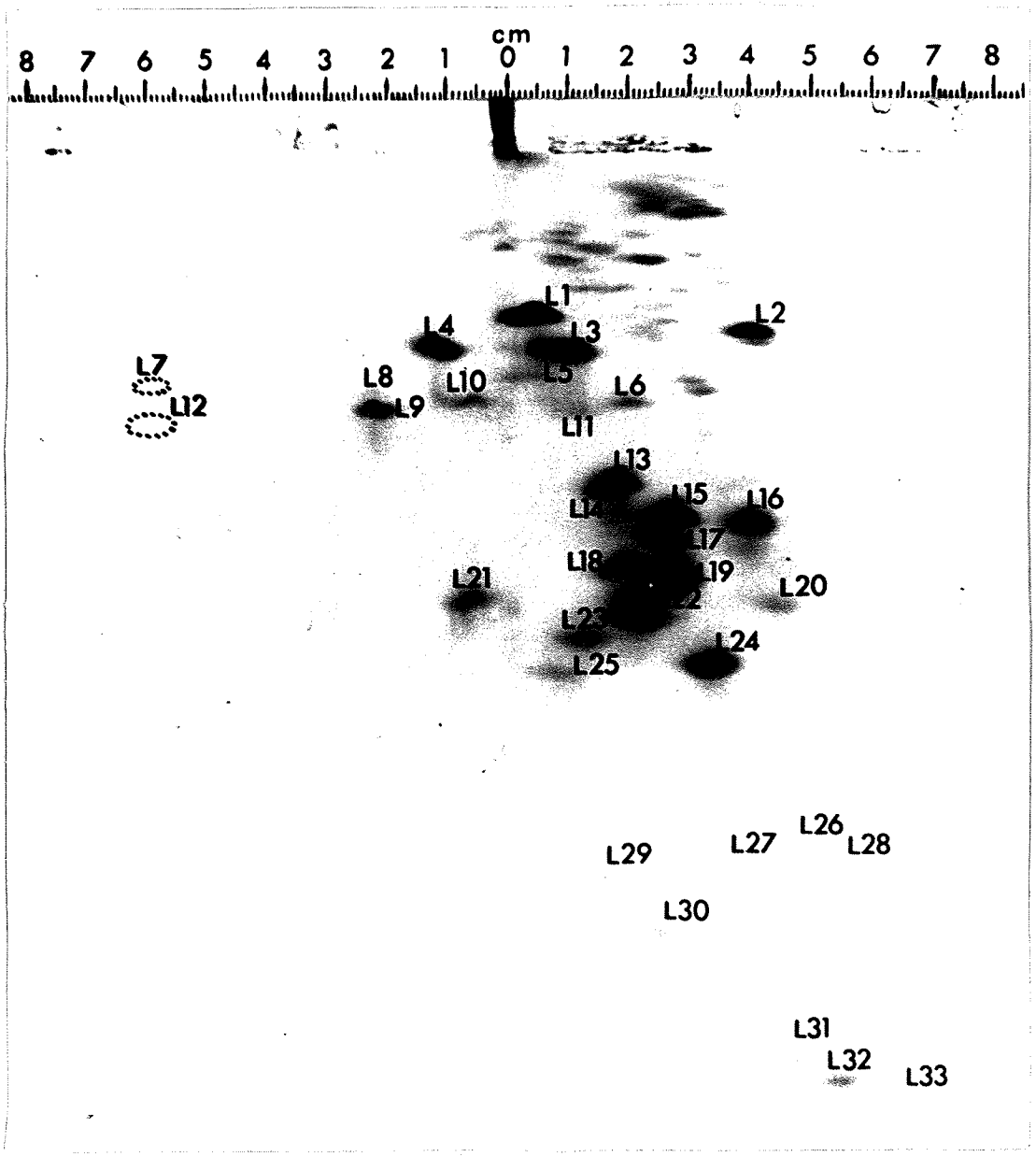
Two-dimensional electropherogram of ribosomal proteins of 32S reconstituted particles.



Legend. Proteins were extracted from isolated 43S reconstituted particles and electrophoresed as described in the legend to Plate II and in "Methods". Staining and destaining of gel slab was described in "Methods".

Plate IV

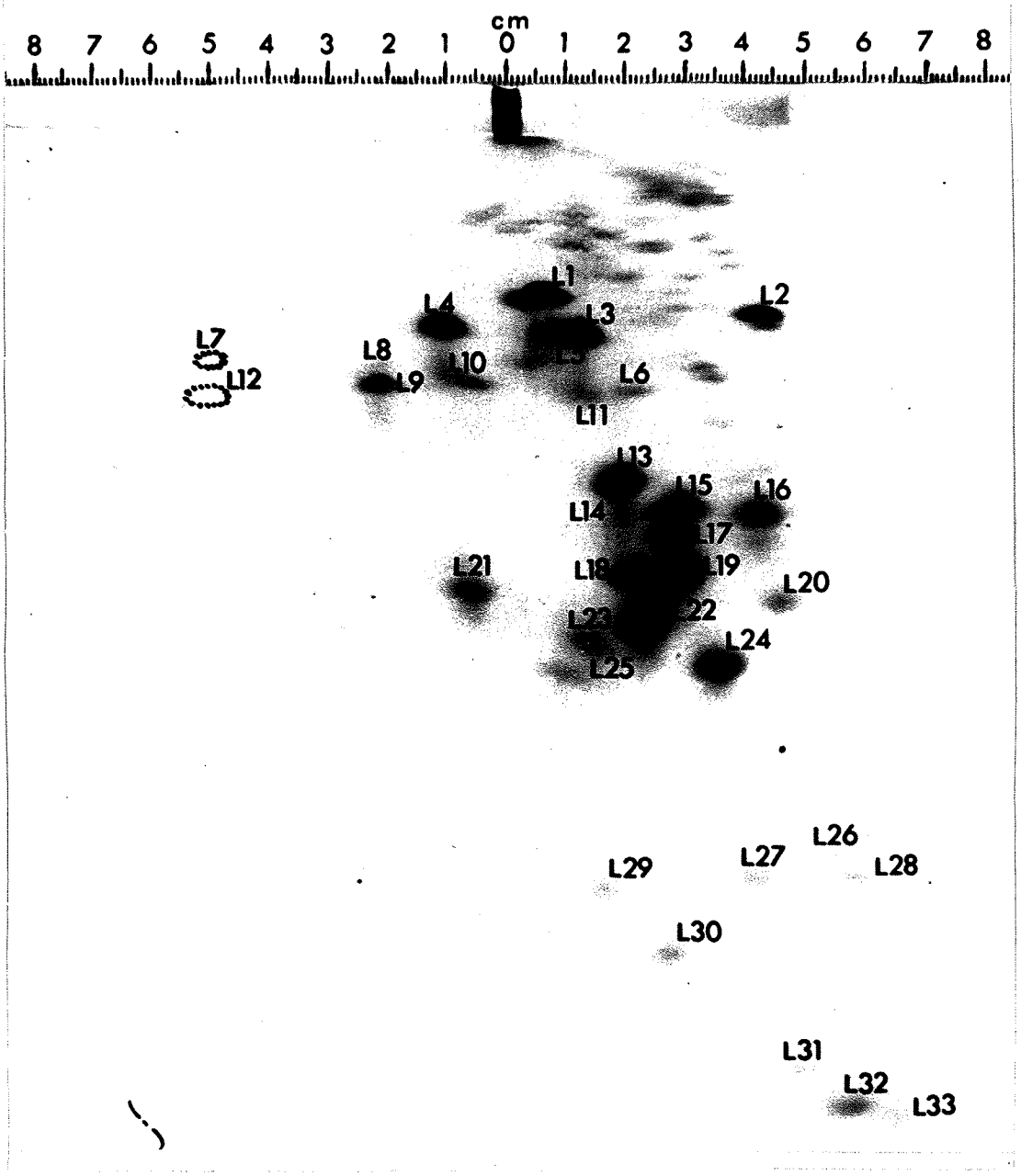
Two-dimensional electropherogram of ribosomal proteins of 43S reconstituted particles.



Legend. Proteins were extracted from isolated 48S reconstituted particles and electrophoresed as described in the legend to Plate II and in "Methods". Staining and destaining of gel slab was described in "Methods".

Plate V

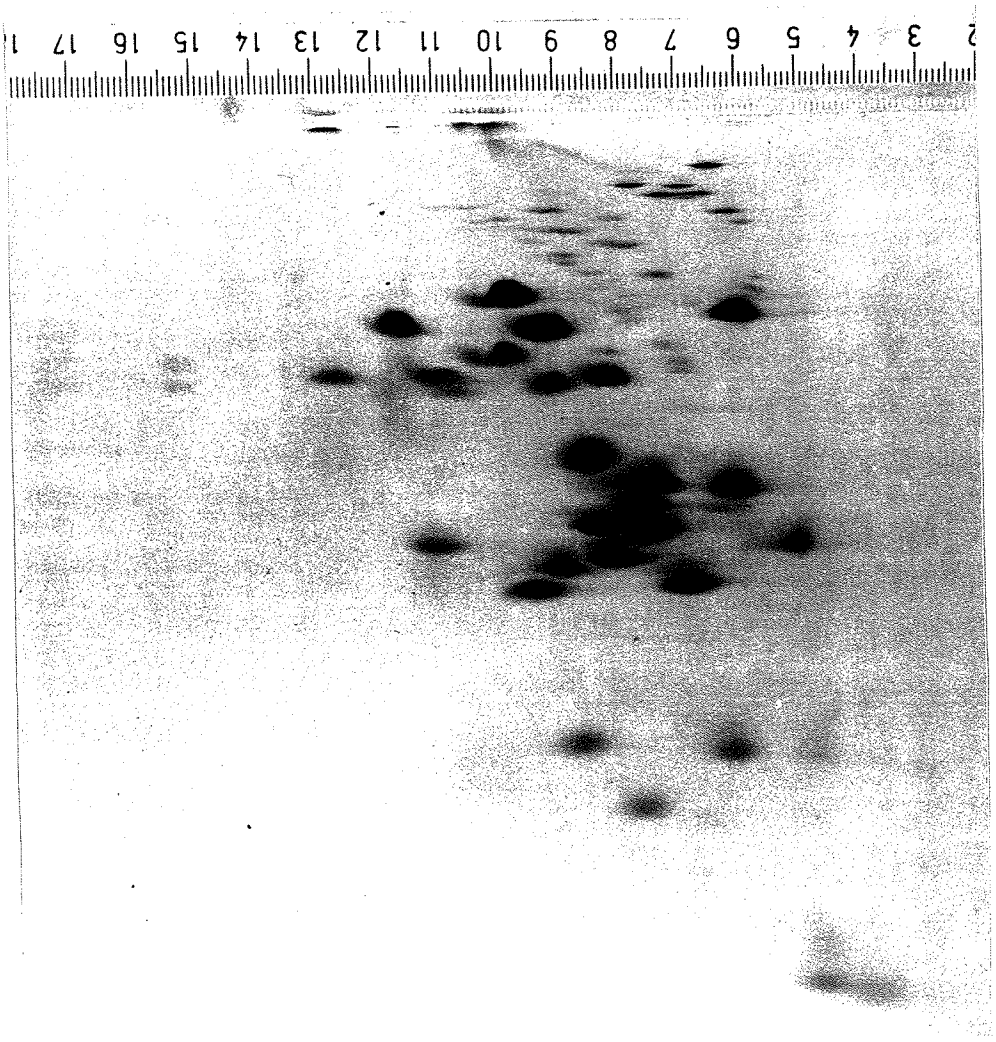
Two-dimensional electropherogram of ribosomal proteins of 48S reconstituted particles.



Legend. Proteins were extracted from 50S ribosomal subunits and electrophoresed as described in the legend to Plate II and in "Methods". Staining and destaining of gel slab was described in "Methods".

Plate VI

Two-dimensional electropherogram of ribosomal proteins of E. coli 50S ribosomal subunits.



those of the 28S, 32S and 43S reconstituted particles had 25, 28 and 30 spots respectively. The presence of L34 in reconstituted particles was confirmed in shorter electrophoretic runs (12h) of extracted proteins (data not shown). Proteins associated with the 28S particles appeared as relatively fainter spots than those in 50S control in the slab gel when compared to proteins of other reconstituted particles, suggesting that particles formed at 0^{o} could have arisen from weak interaction of RNA and protein components at the low temperature. The protein pattern and staining intensity of individual proteins in 32S particles formed at 0^{o}, 37S particles formed at 37^{o} and 48S particles formed at $37 + 55^{o}$ (37^{o} 15 min and 55^{o} 20 min) isolated in 10mM magnesium (data not shown) were identical to those of 28S (Plate II), 32S (Plate III) and 43S (Plate IV) particles respectively, isolated in 1.0mM magnesium, indicating that particles isolated in low and high magnesium concentrations had the same protein compositions despite the different S-values.

Seven 50S proteins, L7, L12, L26, L27, L28, L29, and L30 were absent from 28S particles. Of these, 3 (L27, L29, L30) were bound in reduced amounts to 32S particles formed at 37^{o}, as judged by the staining intensity of spots. The 43S and 48S particles formed at $37 + 55^{o}$ (37^{o} for 15 min and 55^{o} for 20 min) contained L26 and L28 in addition to the complement of proteins in 32S particles, and were deficient

in only two 50S proteins, L7 and L12. Proteins L29 and L30 which were in reduced amounts in 32S particles were found in appreciable amounts in 43S and 48S particles.

The results confirmed that binding took place sequentially so that more proteins were bound to complexes with greater S-values, and that proteins binding at higher temperatures did not displace previously bound proteins. Although the missing proteins could be identified by the absence of stained spots, quantitative determination of bound proteins by comparing the staining intensity of protein spots with controls was imprecise.

Effect of Various Factors on Reconstitution

The present reconstitution system yielded 28S, 32S and 43-48S particles on incubation of the reconstitution mixture consisting of 50S proteins and rRNA sequentially at 0, 37 and 55^{o} respectively. The sedimentation coefficient of the reconstituted particles increased to 32, 37 and 48S, respectively, when analyzed in 10mM magnesium. The conversion of 43S to 48S particles was incomplete (Fig. 8). Furthermore, all reconstituted particles, being protein deficient to different extents, were inactive in poly(U)-directed polyphenylalanine synthesis. Therefore the effects of different protein and RNA fractions were tested in the reconstitution system to see whether functional 50S subunits could be constructed.

During dissociation of 70S ribosomes to subunits, A_{260}-absorbing material appeared as a small peak at the top of the sucrose gradient in which dissociation was carried out (Fig. 22d). To determine whether this fraction contained factors required for subunit assembly, the fraction was isolated after 5-20% sucrose gradient centrifugation of 70S ribosomes in TKM II buffer (1.0mM magnesium) and dialysed in the same buffer. Inclusion of varying amounts of this fraction in the reconstitution mixture failed to induce formation of particles sedimenting faster than 48S at 55^{o}, or formation of particles heavier than 37S at 37^{o} when compared with 30S and 50S subunits centrifuged in parallel gradients (data not shown).

Comparison of the protein patterns of 48S particles with 50S subunits showed that proteins classified as split proteins were missing from the reconstituted particles. Consequently, excesses of split proteins produced by the method of Hamel et al (1972), and Maglott and Staehelin (1971) were added to the reconstitution system. The method of Hamel et al (1972) effected the selective release of proteins L7 and L12 from 50S subunits. These proteins have been shown to be involved in the expression of many ribosomal functions (Hamel et al, 1972; Brot et al, 1973; Lockwood et al, 1974). As 48S particles were deficient in these 2 proteins, the effect of excess amounts (3.0 A_{260}

equivalents/A_{260} unit 23S RNA) of L7 and L12 on reconstitution was tested. No difference was found in the S-values of particles reconstituted in the presence or absence of excess L7-L12 (data not shown). Profiles obtained by one-dimensional polyacrylamide gel electrophoresis with proteins extracted from 48S particles reconstituted in the presence of excess L7-L12 showed that no additional bands were acquired (Plate VII).

It did not appear likely that split proteins in addition to L7 and L12 were required for conversion of 48S to 50S particles since addition of a split protein fraction $SP_{50-\gamma}$ (Maglott & Staehelin,1971) to the reconstitution mixture also had no effect. This fraction contained more proteins and has been used for partial reconstitution of functional 50S subunits.

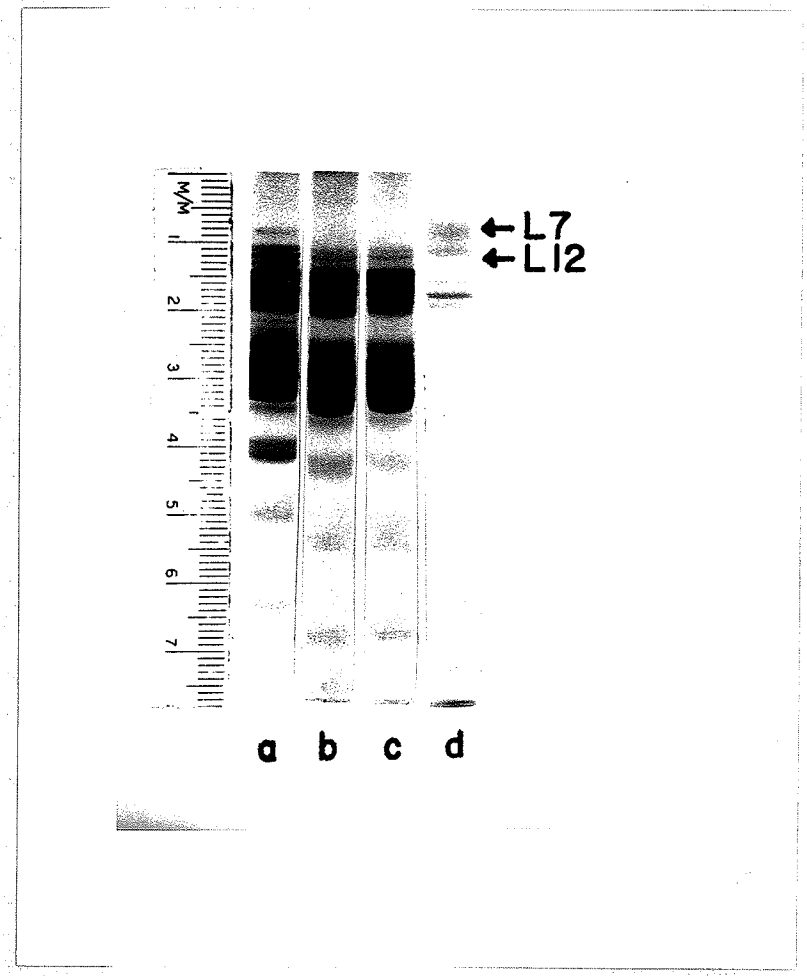
The effect of exogenous 5S RNA on reconstitution was tested in case the reconstitution mixture did not contain sufficient amounts of 5S RNA. Addition of stoichiometric amounts of purified 5S RNA (Methods) to the reconstitution mixture did not result in the formation of 50S complexes. It was unlikely that the reconstitution system should be deficient in 5S RNA since 5S RNA was present both in the protein and in the RNA fractions.

Requirement of simultaneous assembly of 30S subunits has been implicated in the assembly of E. coli 50S subunits

Legend. Proteins were extracted with 67% acetic acid in 100mM $Mg(OAc)_2$ and 100 µg were electrophoresed into disc gels as described in the legend to Plate I and in "Methods". Gels were stained with Coomassie Brilliant Blue and destained with 10% TCA (Methods). The proteins were extracted from 50S ribosomal subunits (a), 48S reconstituted particles (b), 48S particles reconstituted in the presence of PI-II proteins (c). Gel (d) showed the band pattern of PI-II proteins extracted from 4 A_{260} units of 50S ribosomal subunits with ethanol-NH_4Cl mixture.

Plate VII

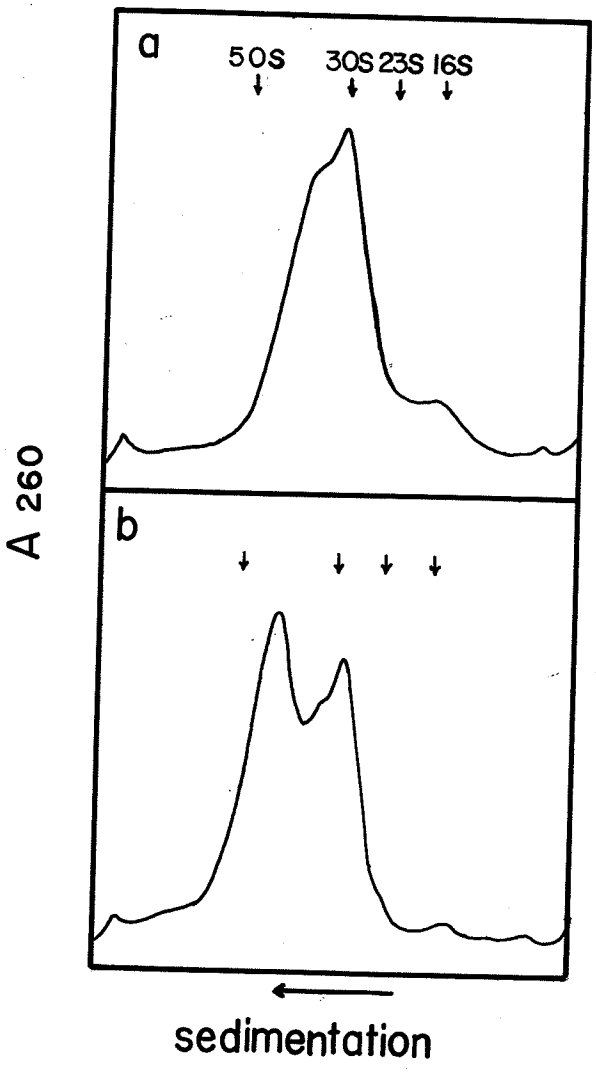
Electrophoretic patterns of ribosomal proteins of 48S reconstituted particles , E. coli 50S ribosomal subunits and PI-II proteins.



both *in vitro* (Maruta *et al*, 1971) and *in vivo* (Nashimoto and Nomura, 1970). To test if reconstitution of 50S subunits in the present system had a similar requirement, proteins extracted from 70S ribosomes were used in place of 50S proteins. Reconstituted particles were analyzed in sucrose gradients in TKM II buffer (1.0mM magnesium). Although 30S particles were formed both at 37° and at 37 + 55°, indicating 30S subunits were reconstituted under these conditions, largest particles formed at 37° were 32S and those at 37 + 55° were 43-48S (Fig. 13). The profiles were the same as in the absence of 30S proteins (Fig. 7). The data indicated that assembly of the smaller subunit during reconstitution did not facilitate formation of the 50S particles in the present system.

Ribonuclease activity associated with 50S subunit has been reported (Szer, 1969; Staehelin *et al*, 1969). Since the activity remained associated with rRNA and became pronounced during prolonged incubation at high temperatures (Ceri and Maeba, 1973), it was possible that 23S RNA degradation might account for inability to form 50S particles during reconstitution. It had been shown that 5-10% ethanol was a potent inhibitor of autodegradation. However, no detectable difference was observed in sedimentation profiles of reconstituted particles formed by incubation in the presence or absence of 5 and 10% ethanol.

Figure 13. Sedimentation analysis of ribonucleoprotein complexes formed from 70S proteins (4.5 A_{260} equivalents) and rRNA (3.6 A_{260} units). Incubation was at (a) 37° for 15 min; and (b) 37° for 15 min and 55° for 20 min. Sucrose gradient analysis was performed in TKM II buffer (Methods).


a
50S
30S
23S
16S
b
A 260
sedimentation

Effect of Excess 50S Proteins on Reconstitution

Up to this point, 37S and 48S particles were obtained by incubating 50S proteins and rRNA at a ratio of 1.2 A_{260} equivalents of 50S proteins to 1.0 A_{260} unit of 23S RNA. As shown in Figure 14, when the amount of 50S proteins in reconstitution at 37 + 55° was increased to ratios of 2.4 (Fig. 14b) and 3.0 (Fig. 14c), increases in the yield of 48S particles at the expense of 37 S particles were observed. At the highest ratio of 3.0, complete conversion of 37S to 48S particles was effected (Fig. 14c). The data indicated that the 37S particles formed at 37° were precursors of the 48S species formed at 37 + 55°. The inclusion of excess proteins in reconstitution mixtures incubated at 37° had no effect on particle formation and particles sedimenting at only 37S were formed (Fig. 15). The results suggested that some 50S proteins essential for 48S particle formation, were present in reduced amounts in the 50S protein preparation, or were inactive and therefore unable to bind in 48S particles. Inactivation of proteins may have been brought about during urea-LiCl extraction of 50S proteins or by incubation of the reconstitution mixture at 55°. In this study, reconstituted mixtures were analyzed in sucrose gradients in TMN I buffer containing 10mM $Mg(OAc)_2$ and 500mM NH_4Cl. The high mag-

Figure 14. Sedimentation analysis of ribonucleoprotein complexes formed at 50S protein/23SRRNA ratios of (a) 1.2; (b) 2.4; and (c) 3.0. Ribosomal RNA (3.6 A_{260} units) and indicated A_{260} equivalents of 50S proteins were incubated in 1.0 ml SR buffer sequentially at 37^{o} for 15 min and 55^{o} for 20 min. The ethanol precipitated material was dissolved in 0.1 ml TMN I buffer and analyzed in the same buffer by sucrose gradient centrifugation (Methods).

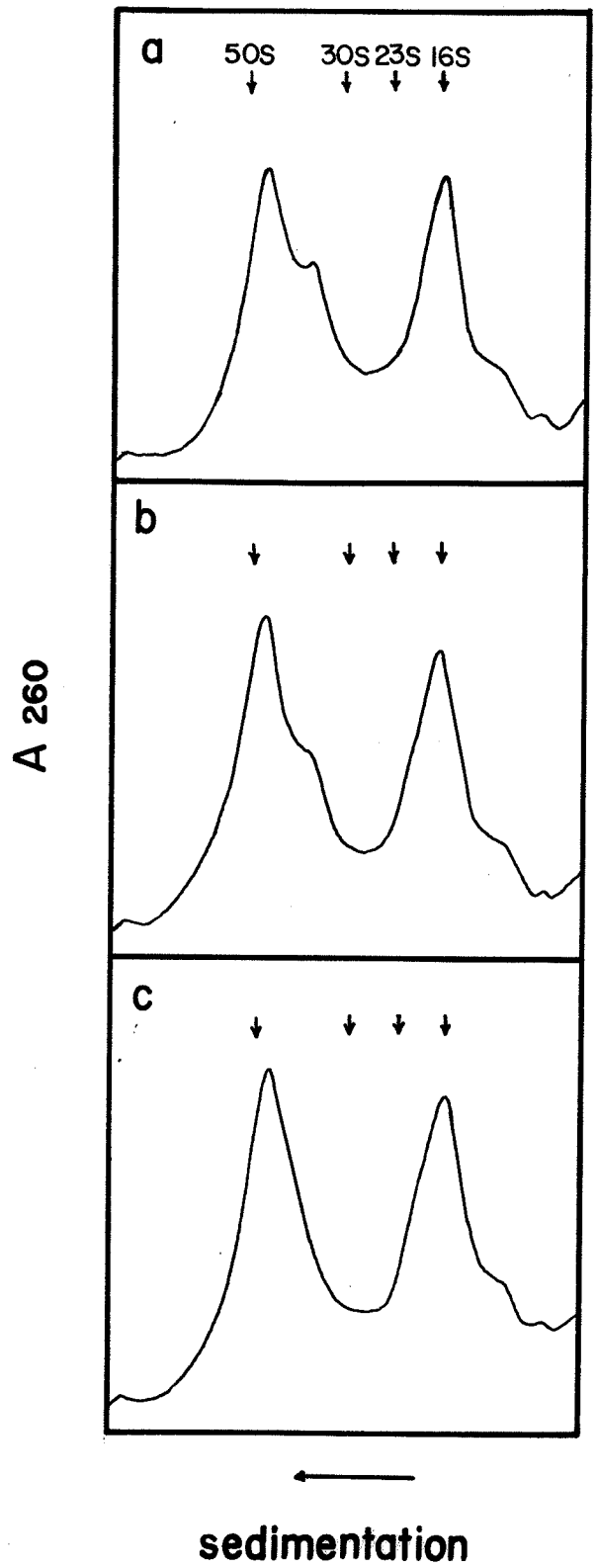
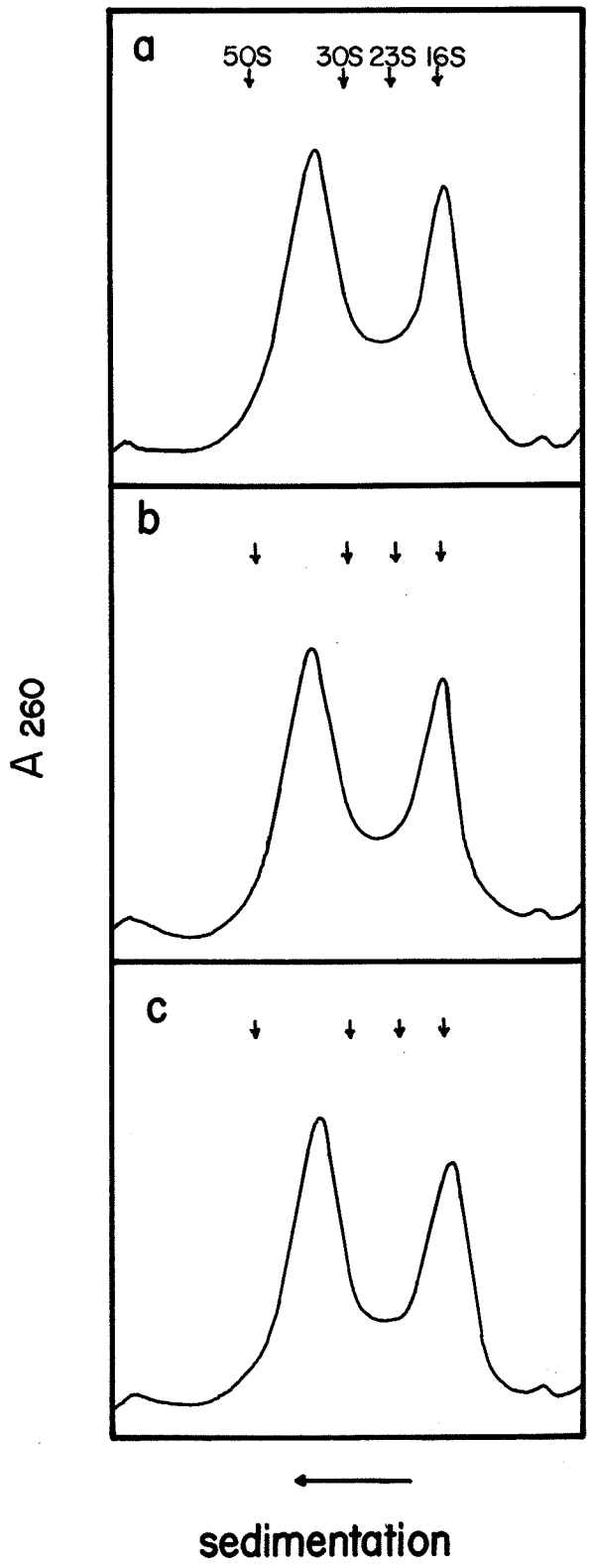

a
50S
30S
23S
16S
b
c
A 260
sedimentation

Figure 15. Sedimentation analysis of ribonucleoprotein complexes formed at 50S protein/23S RNA ratios of (a) 1.2; (b) 2.4; and (c) 3.0. Reconstitution was carried out at 37^{o} for 15 min. Analysis of reconstituted mixture was performed as in "Methods".

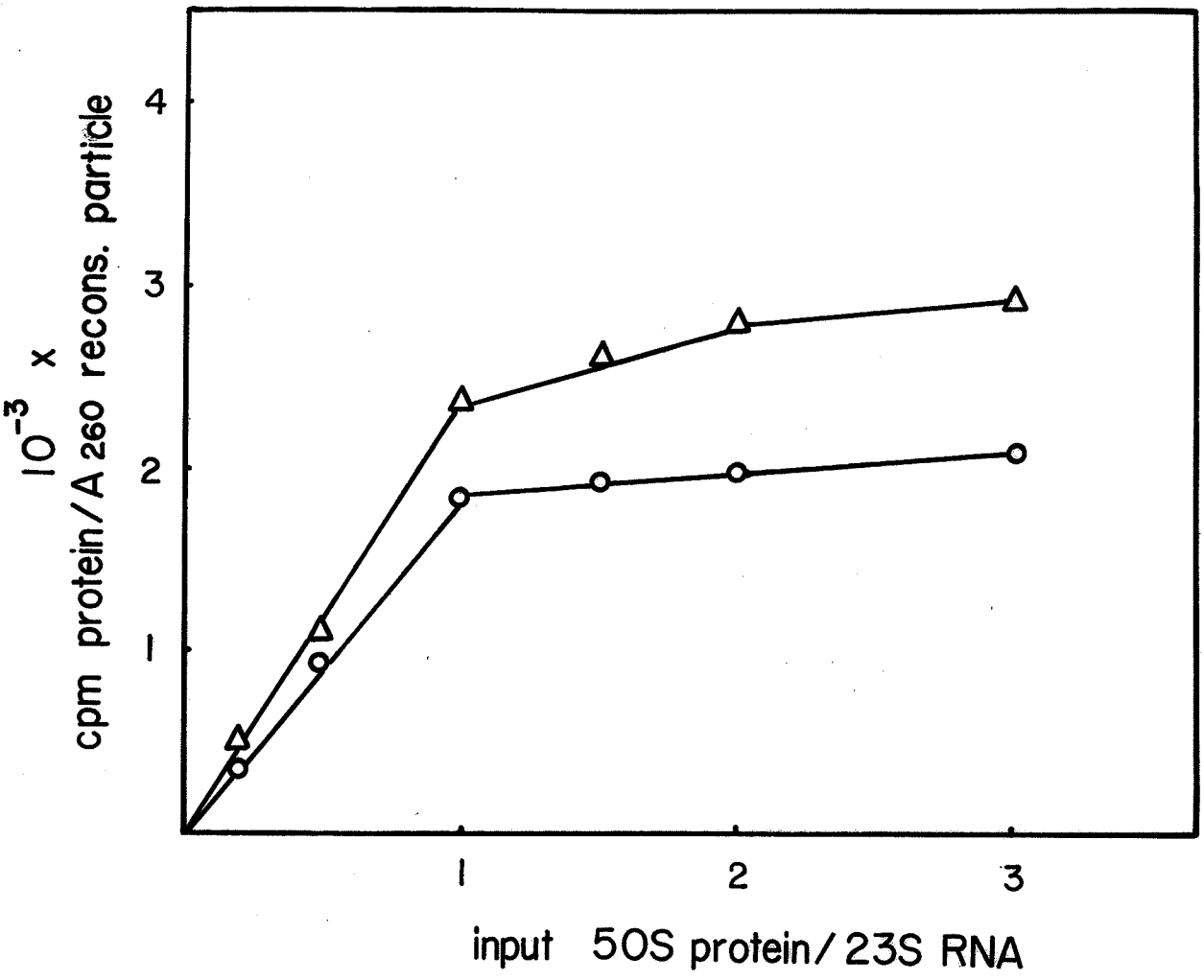

a
50S
30S
23S
16S
b
c
A 260
sedimentation

nesium concentration yielded reconstituted particles of higher S-values while the high monovalent ion concentration minimized nonspecific binding of proteins in the presence of excess 50S proteins.

To determine whether more 50S proteins were bound in the presence of excess proteins, particles were reconstituted from rRNA and varying amounts of (^{14}C) 50S proteins at 37^{o} and at 37 + 55^{o}. (^{14}C) labelled reconstituted particles were isolated by sucrose gradient centrifugation in TMN I buffer and their specific radioactivities were measured. The curves obtained by plotting specific radioactivities of particles against the ratios of 50S protein/23S RNA used in reconstituting the particles are shown in Figure 16. At 37^{o}, saturation was reached at a ratio of 1.0 and binding of proteins did not significantly increase at higher ratios. However, at 55^{o}, the specific activity of the particles increased gradually at 50S protein/23S RNA ratios greater than 1.0. This suggested that one group, consisting of the majority of 50S proteins, was readily bound at 50S protein/23S RNA ratios less than 1.0 whereas some proteins became bound at ratios greater than 1.0.

The influence of incubation temperature on the protein content of reconstituted particles was analyzed. Reconstituted 32S, 37S and 48S particles were constructed by incubating (^{14}C) 50S proteins and rRNA at 0^{o}, 37^{o} and

Figure 16. Binding of 50S proteins in reconstituted particles. Ribosomal RNA (18.0 A_{260} units) and indicated A_{260} equivalents of (^{14}C) 50S proteins were mixed at 37° for 15 min (○) or sequentially at 37° for 15 min and 55° for 20 min (△) in 5.0 ml SR buffer. Ribonucleoprotein particles containing 23S RNA were isolated by sucrose gradient centrifugation in TMN I buffer and their specific radioactivities in c.p.m./A_{260} were estimated (Methods).


10^{-3} x
cpm protein/A 260 recons. particle
4
3
2
1
1
2
3
input 50S protein/23S RNA

37 + 55°, respectively, at 50S protein/23S RNA ratios of 1.2 and 3.0. The specific radioactivities of the particles were plotted in Figure 17. The data showed that the amount of protein in 32S particles formed at 0° increased by less than 5%, that in 37S particles formed at 37° by about 5%, and that in 48S particles formed at 37 + 55° by about 20% respectively, when 50S protein/23S RNA ratios were increased from 1.2 to 3.0. The results suggested that many proteins were readily bound at 37° whereas others became bound only at elevated temperatures and in the presence of excess proteins.

Effect of Polyamines on Reconstitution

It It had been shown previously that polyamines stimulated *in vitro* reconstruction of subparticles of *E. coli* 50S subunits (Hosokawa *et al*, 1973). Polyamines were included in the reconstitution mixture and their effect on the formation of 48S particles at 55° was followed. The results obtained with spermidine are shown in Figure 18. In the absence of spermidine, incubation at 55° following incubation at 0° for 15 min and 37° for 15 min led to the formation of only small amounts of 48S particles (Fig. 18a, b, c). However, in the presence of 10mM spermidine, similar periods of incubation produced larger amounts of 48S particles (Fig. 18d, e, f) so that maximal formation of 48S particles was complete after 15 min at 55°. Spermidine increased the rate at which

Figure 17. Temperature dependent binding of 50S proteins to 23S RNA at 50S protein/23S RNA ratios of 1.2 (o) and 3.0 (•). Ribosomal RNA (18.0 A_{260} units) and indicated A_{260} equivalents of (^{14}C) 50S proteins were mixed at 0^{o} for 15 min, 37^{o} for 15 min, or sequentially at 37^{o} for 15 min and 55^{o} for 20 min in 5.0 ml SR buffer. Isolation of 23S RNA-containing ribonucleoprotein particles and assay of their specific radioactivities in c.p.m./A_{260} were performed as described in "Methods".

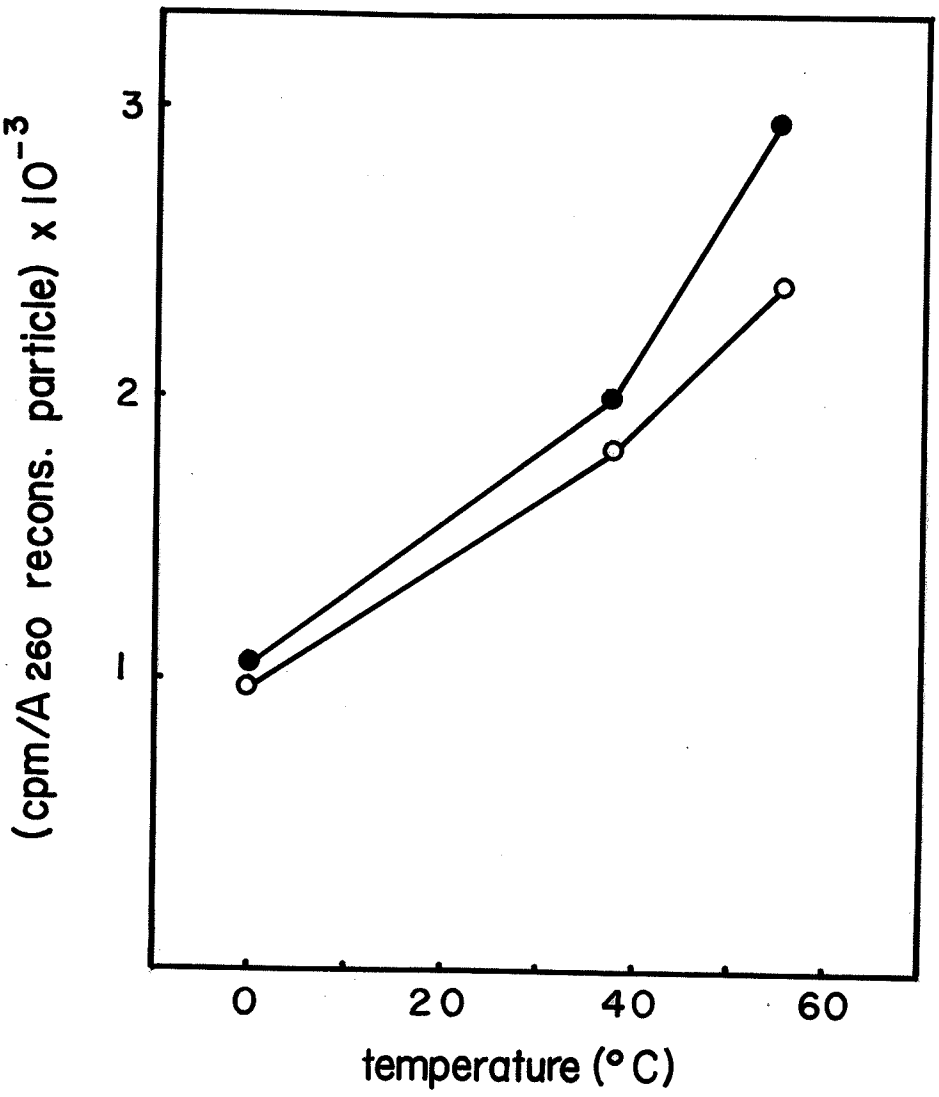
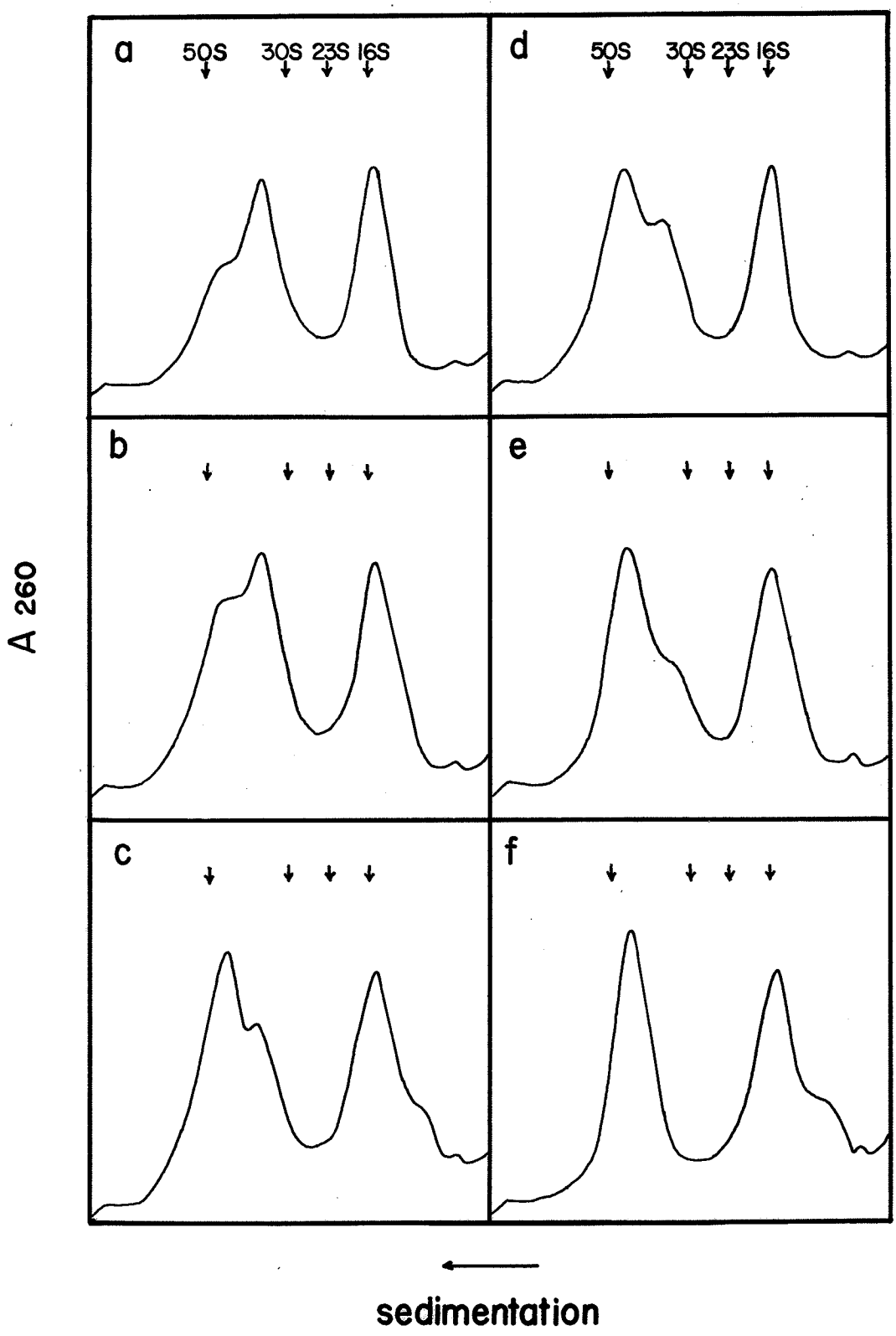

(cpm/A 260 recons. particle) x 10^{-3}
3
2
1
0
20
40
60
temperature (°C)

Figure 18. Sedimentation analysis of ribonucleoprotein complexes formed at 50S protein/23S RNA ratio of 3.0 in the presence of 0mM spermidine (a, b and c) or 10.0mM spermidine (d, e and f). Reconstitution mixtures were incubated sequentially at 0^{o} for 15 min, 37^{o} for 15 min and 55^{o} for 2 min (a and d); 5 min (b and e); and 15 min (c and f). The ethanol precipitated materials were analyzed in TMN I buffer (Methods).


a
50S
30S
23S
16S
d
50S
30S
23S
16S
b
e
c
f
A 260
sedimentation

48S particles were formed, but its presence did not lead to the formation of particles sedimenting faster than 48S.

It was previously shown that prior incubation at 37° for 15 min was essential when 48S particles were formed at 55°. The inclusion of 10mM spermidine in the reconstitution mixture abolished this requirement so that 48S particles were formed on direct incubation at 55° (Figure 19).

Spermine, but not putrescine, had a similar effect on the kinetics of particle formation although various combinations of the 3 polyamines were no more effective than when added singly (data not shown).

RNA Analysis of Reconstituted Particles

RNA analysis of particles reconstituted at a 50S protein/23S RNA ratio of 3.0 and in the presence of 10mM spermidine at 0, 37 and 55° in a sequential manner was carried out. Reconstituted particles and material sedimenting at 16S were isolated by sucrose gradient centrifugation in TMN I buffer. RNA was extracted from each of the particles in urea-LiCl (Methods) and examined by 2.65% polyacrylamide gel electrophoresis. Figure 20 showed that reconstituted particles formed at all temperatures contained 23S RNA exclusively which remained relatively intact even after heat denaturation (Fig. 20a, b, c). The 16S

Figure 19. Sedimentation analysis of ribonucleoprotein complexes formed at 50S protein/23S RNA ratio of 3.0 in the presence of varying spermidine concentrations: (a) 0mM; (b) 5.0mM; (c) 10.0mM; and (d) 20.0mM. Incubation was at 55^{o} for 20 min. After incubation, the material recovered by ethanol precipitation was analyzed in TMN I buffer (Methods).

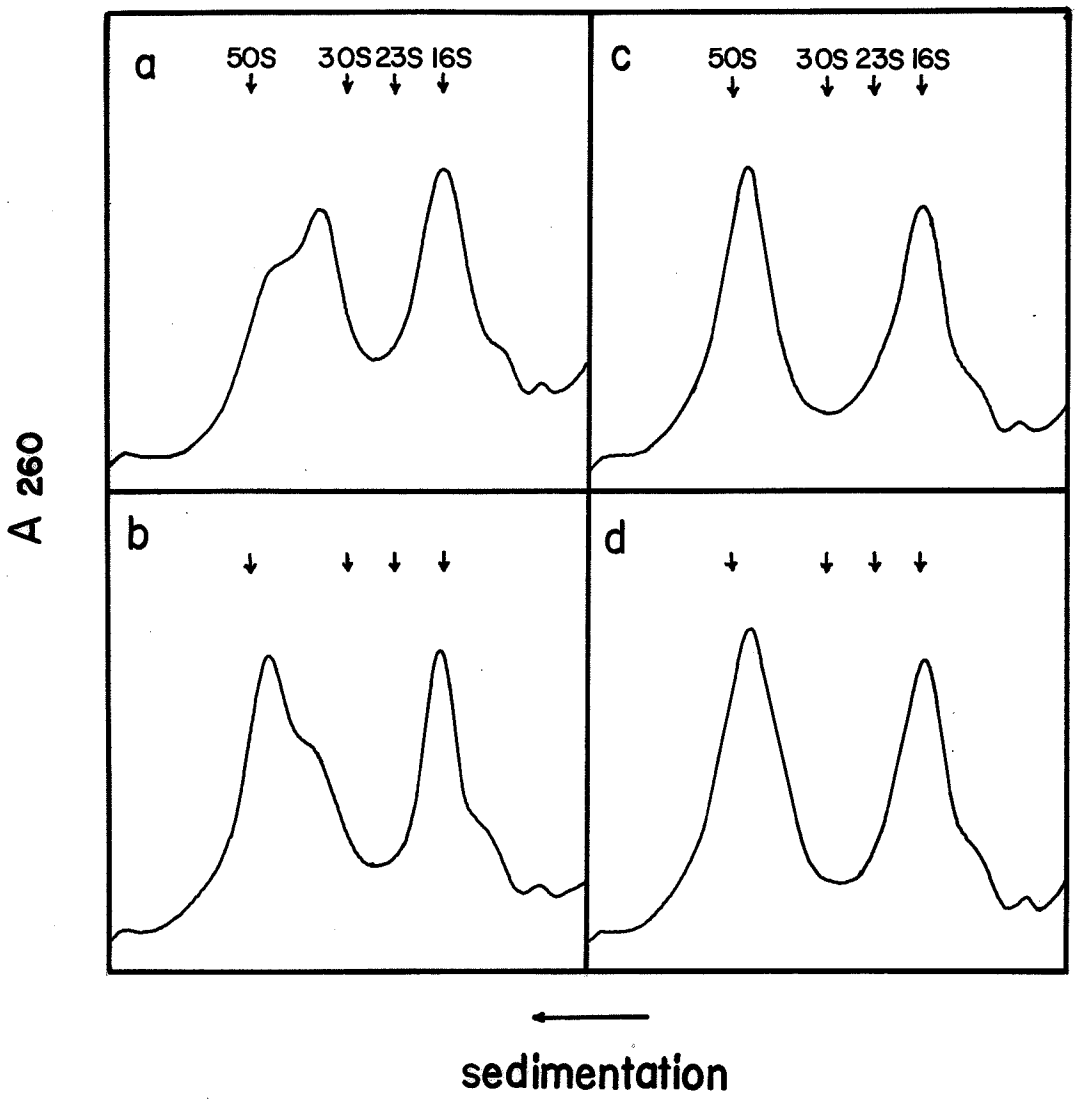
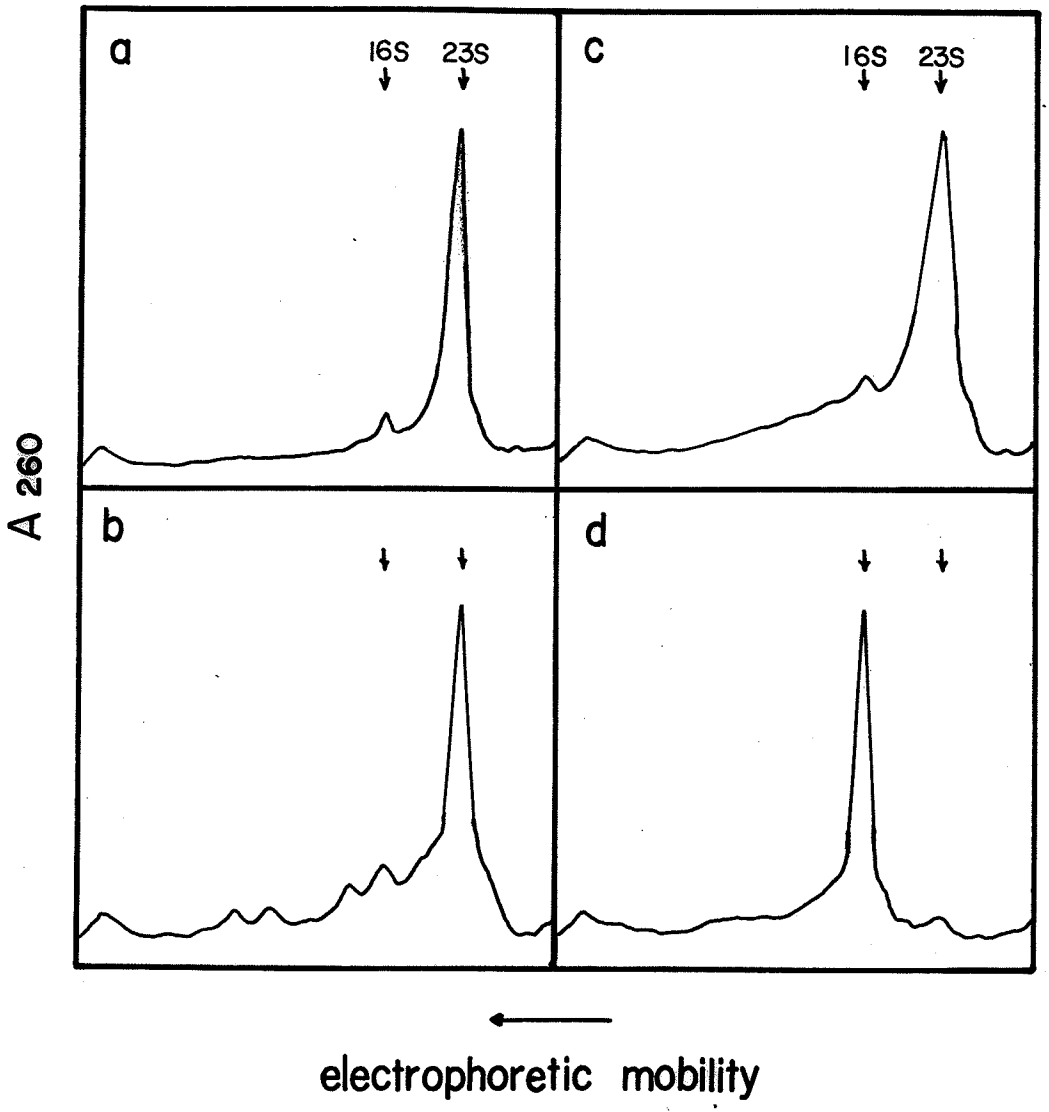

a
50S
30S
23S
16S
c
50S
30S
23S
16S
b
d
A 260
sedimentation

Figure 20. Electrophoretic profiles of RNA associated with isolated reconstituted particles: (a) 32S formed at 0^{o}; (b) 37S formed at 37^{o}; and (c) 48S formed at $37+55^{o}$. Panel (d) shows the profile of RNA associated with 16S material in reconstituted mixtures. RNA was extracted from reconstituted particles (0.5 A_{260} unit) with urea-LiCl and electrophoresed in 2.65% polyacrylamide and 0.1% SDS (Methods) and the gels were scanned at 260 nm (Methods).


a
16S
23S
c
16S
23S
b
d
A 260
electrophoretic mobility

material present in reconstituted mixtures was 16S RNA originally present in rRNA (Fig. 20d).

To analyze 5S RNA, RNA extracted from reconstituted particles by urea-LiCl (Methods) were applied to 2.65% polyacrylamide which overlay 12.5% polyacrylamide (Methods). After electrophoresis, gels were scanned at 260 nm. The A_{260} profiles of RNA extracted from isolated reconstituted particles are shown in Figure 21, along with that of RNA extracted from 50S subunits. All reconstituted particles possessed 5S RNA. Gravimetric quantitation of areas under the 5S RNA peaks in the RNA profiles revealed that 37S and 48S particles reconstituted at 37^{o} and at $37 + 55^{o}$ respectively contained 5S RNA in amounts equivalent to that in 50S subunits. However, the 5S RNA content in 32S particles reconstituted at 0^{o} was only 60% of the equivalent amount (Table V).

Assay of Subunit Association Activity of Reconstituted Particles

The ability of particles reconstituted at a 50S protein/23S RNA ratio of 3.0 in the presence of 10mM spermidine to associate with 30S subunits was tested. The requirements for optimal subunit association were first determined from the association of 30S and 50S subunits.

Figure 21. Electrophoretic analysis of RNA associated with isolated reconstituted particles in double gels: (b) 32S formed at 0^{o}; (c) 37S formed at 37^{o}; and (d) 48S formed at 37 + 55^{o}. Panel (a) shows the profile of RNA from 50S subunits. Each gel contained 1.0 ml of 2.65% polyacrylamide at the top and 2.0 ml of 12.5% polyacrylamide in the bottom. RNA was extracted from reconstituted particles (35.0 A_{260} units) with urea-LiCl, electrophoresed and scanned at 260 nm (Methods). Unmarked arrows indicate the beginning of 12.5% gel.

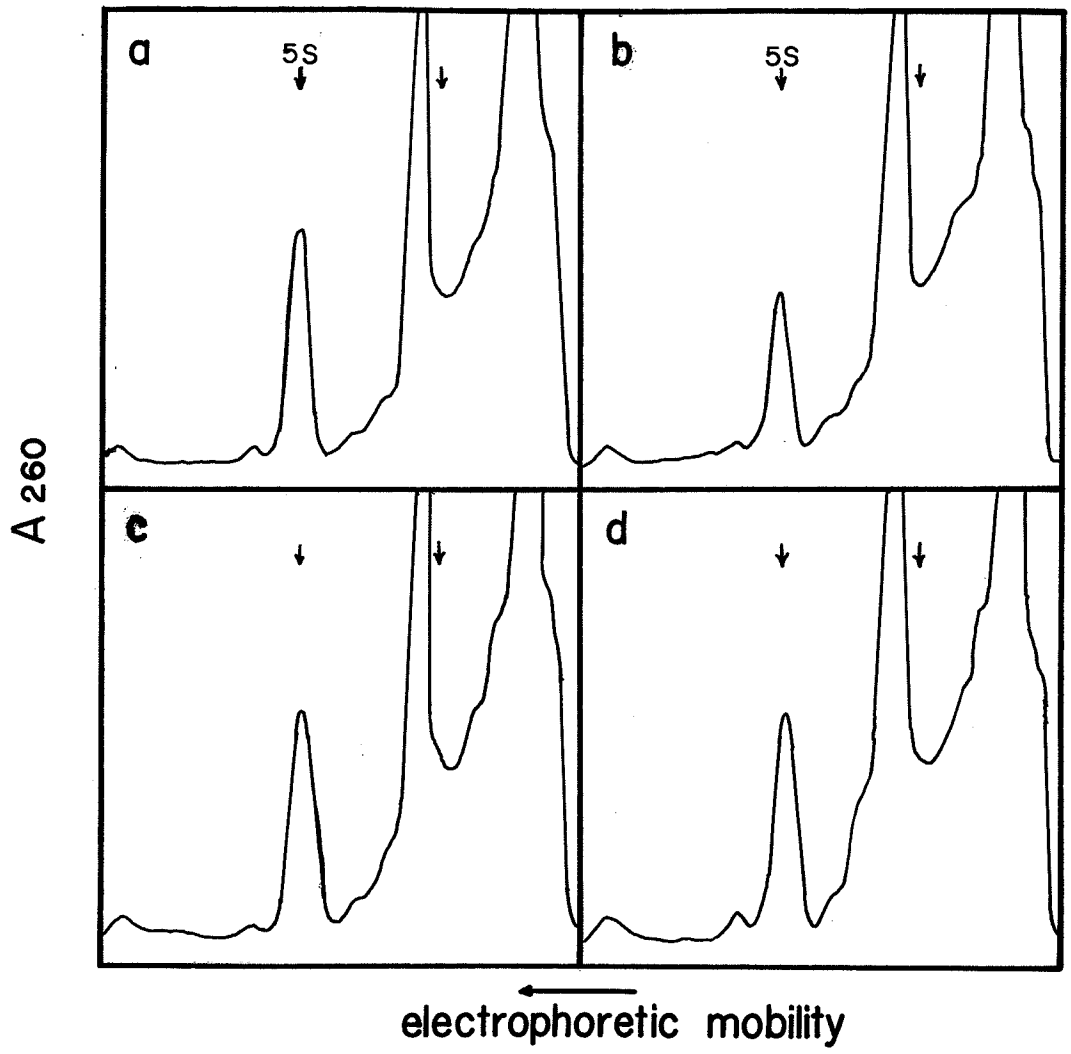

a
5S
b
5S
c
d
A 260
electrophoretic mobility

Table V. Content of 5S RNA in reconstituted particles.

Reconstituted particles	% 5S RNA as compared to that in 50S subunits
32S	60%
37S	92%
48S	98%

The results, summarized in Figure 22, showed that activation of 30S subunits at 37° for 45 min prior to association and the presence of 5.0mM spermidine during centrifugation optimized the formation of 70S complexes.

To test the association activity of particles, 1.0 A_{260} unit of reconstituted particles was mixed with 0.6 A_{260} unit of heat-activated 30S subunits in 0.1 ml of TKMS I buffer at 37° for 20 min. The mixtures were centrifuged in 5-20% sucrose gradients in a Beckman SW 50.1 rotor at 45,000 r.p.m. for 90 min at 4°. Sedimentation profiles (Fig. 23a, b, c) showed the state of 32S, 37S and 48S particles reconstituted at 0°, 37° and 37 + 55°, respectively. With these, little or no 70S complexes were formed by particles reconstituted at 0° and 37° (Fig. 23d,e). However, some 48S particles reconstituted at 37 + 55° associated with 30S subunits to form 70S complexes (Fig. 23f).

Reconstituted 48S particles were allowed to associate with excess (^{3}H) 30S subunits and the resulting labelled 70S complexes were isolated by sucrose gradient centrifugation and recentrifuged in sucrose gradients in TKMS I (10mM magnesium) or TKM II (1.0mM magnesium) buffers. Sedimentation profiles (Fig. 24) showed that the 70S complexes remained intact in 10mM magnesium and 5mM spermidine (Fig. 24a) but dissociated to (^{3}H) 30S subunits and unlabelled 48S particles in 1.0mM magnesium (Fig. 24b). The results indicated that the 70S peak was not an artifact arising from dimerization of 48S particles. When (^{3}H) 30S

Figure 22. Requirements of optimal subunit association. One A_{260} unit of 50S subunits was mixed with 0.6 A_{260} unit of 30S subunits in 0.1 ml TKMS I buffer at 37° for 20 min. After incubation, associated mixtures were chilled in ice and subjected to 5-20% sucrose gradient centrifugation in the same buffer in a Beckman SW 50.1 rotor at 45,000 r.p.m. for 90 min. The gradients were scanned at 260 nm as described in "Methods". The sedimentation profiles are of mixtures of: (a) 50S + 30S subunits; (b) 50S + heat-activated 30S (37° for 45 min); (c) same as (b) except that sedimentation analysis was performed in buffer lacking spermidine and (d) 70S ribosomes analyzed in TKM II buffer (containing 1.0mM magnesium).

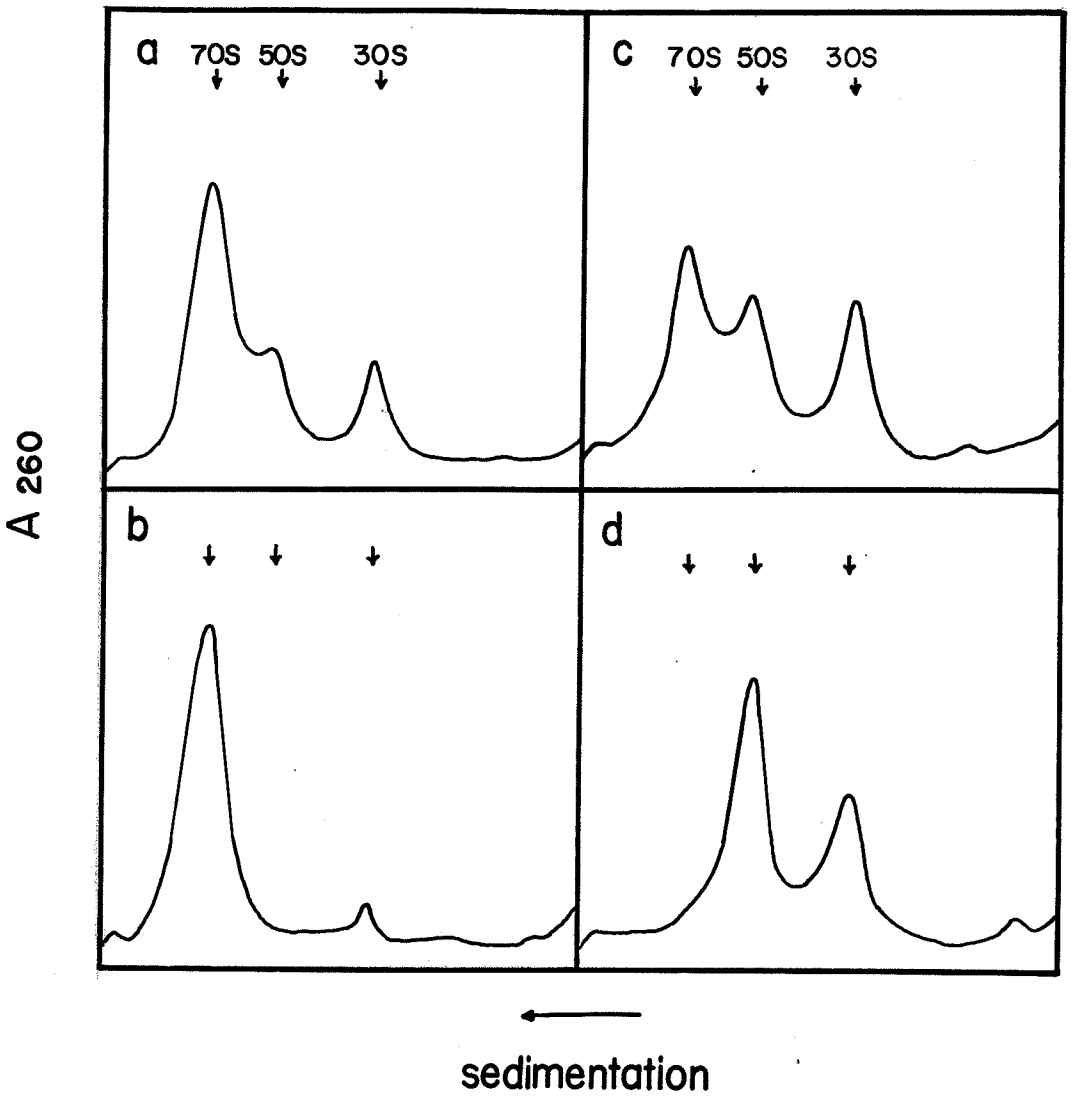

a
70S
50S
30S
c
70S
50S
30S
b
d
A 260
sedimentation

Figure 23. Sedimentation analysis of isolated reconstituted particles in TMN I buffer: (a) 32S; (b) 37S; and (c) 48S. Right panels (d, e and f) show complexes formed when reconstituted particles shown in panels (a), (b) and (c) respectively were mixed with 30S subunits. One A_{260} unit of particles was incubated with 0.6 A_{260} unit of heat-activated 30S subunits in 0.1 ml TKMS I buffer at 37^{o} for 20 min. After incubation, associated mixtures were analyzed by sucrose gradient centrifugation (Methods).

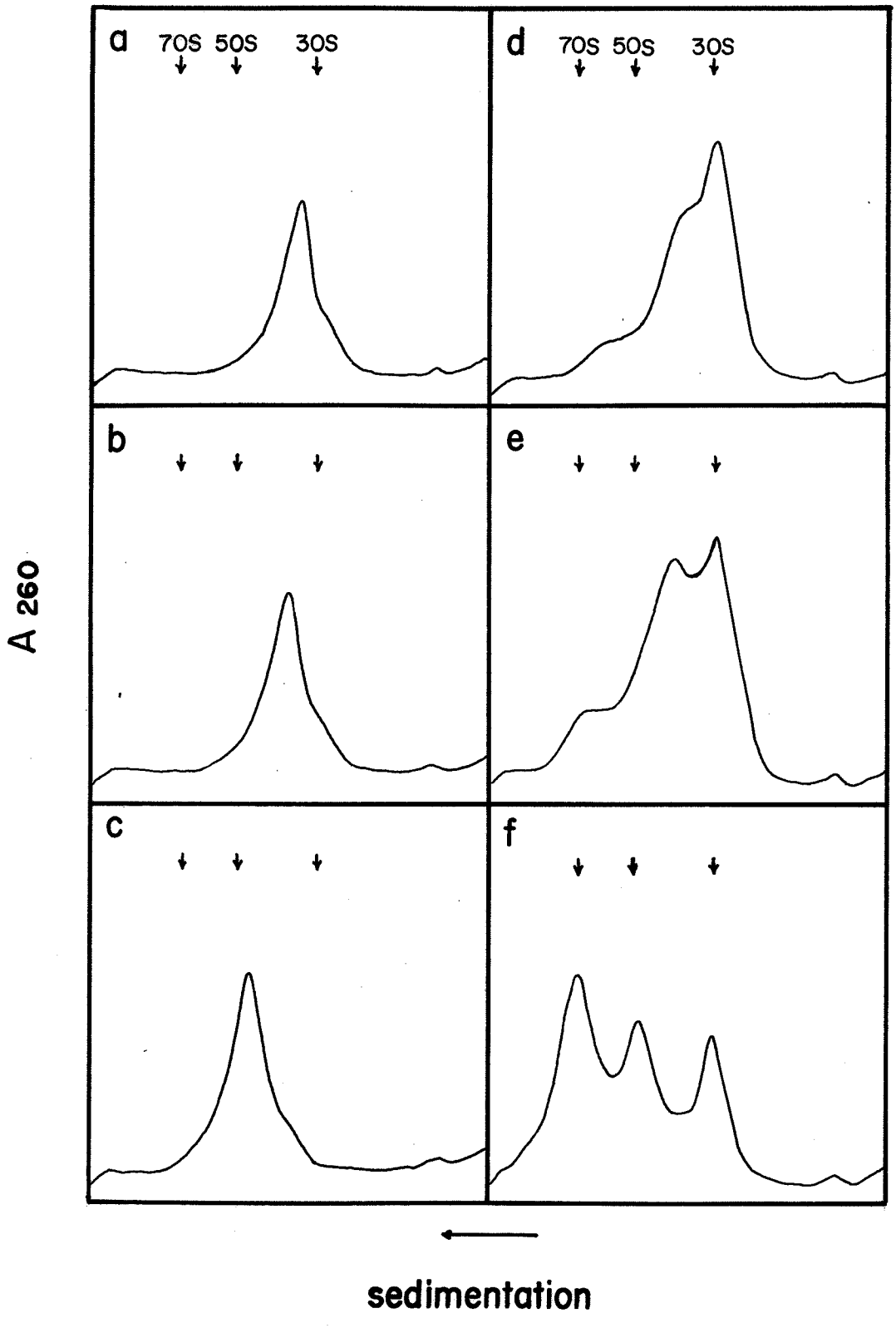
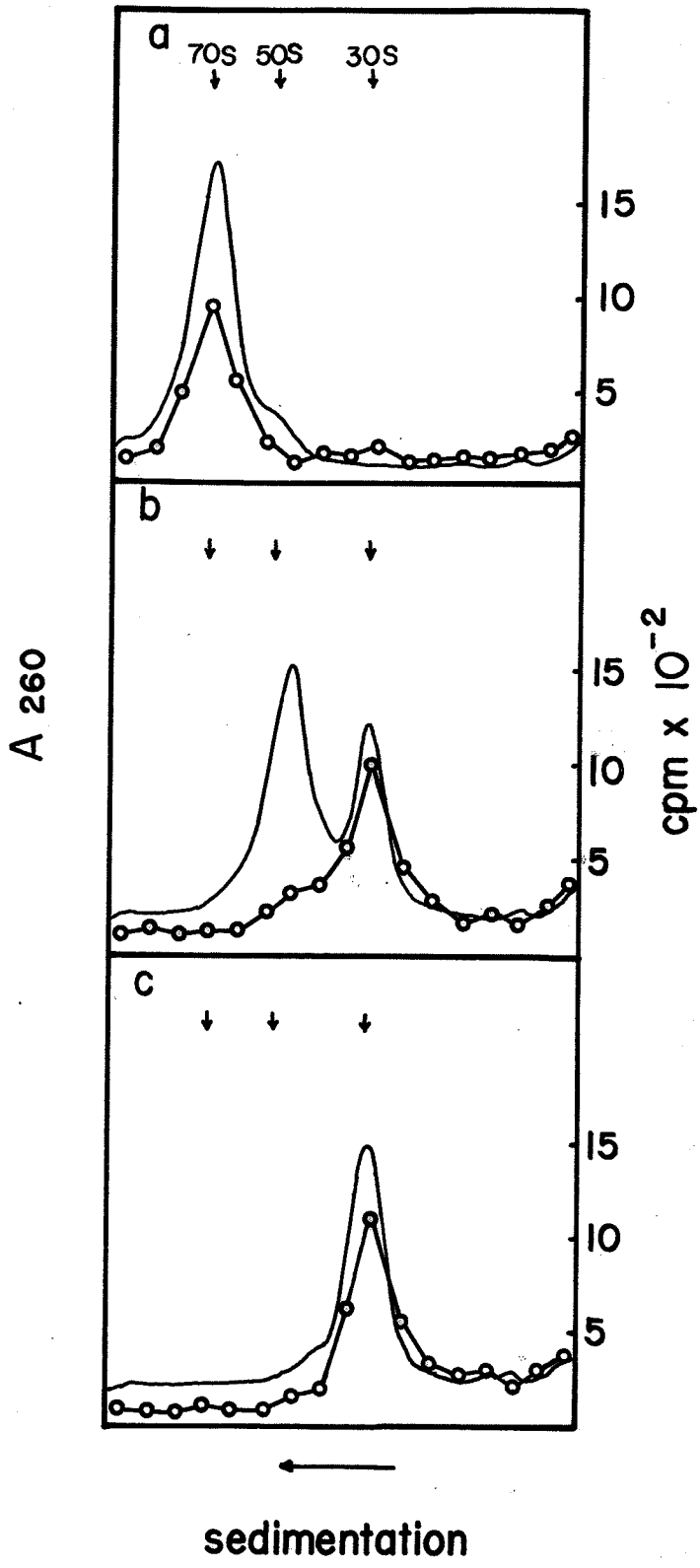

a
70S
50S
30S
d
70S
50S
30S
b
e
c
f
A 260
sedimentation

Figure 24. Association of 48S reconstituted particles with (^3H) 30S subunits. The 48S particles (2.5 A_{260} units) were incubated with heat-activated (^3H) 30S subunits (1.8 A_{260} units) in 0.2 ml TKMS I (10mM magnesium) buffer at 37° for 20 min. After incubation, (^3H) 70S complexes were isolated by 5-20% sucrose gradient centrifugation in the same buffer and recentrifuged in 5-20% sucrose gradients in (a) TKMS I and (b) TKM II (1.0mM magnesium) buffers. Panel (c) shows sedimentation profile of (^3H) 30S subunits analyzed alone in TKMS I buffer. After centrifugation, 0.25 ml fractions were collected from gradients and assayed for radioactivity (Methods).

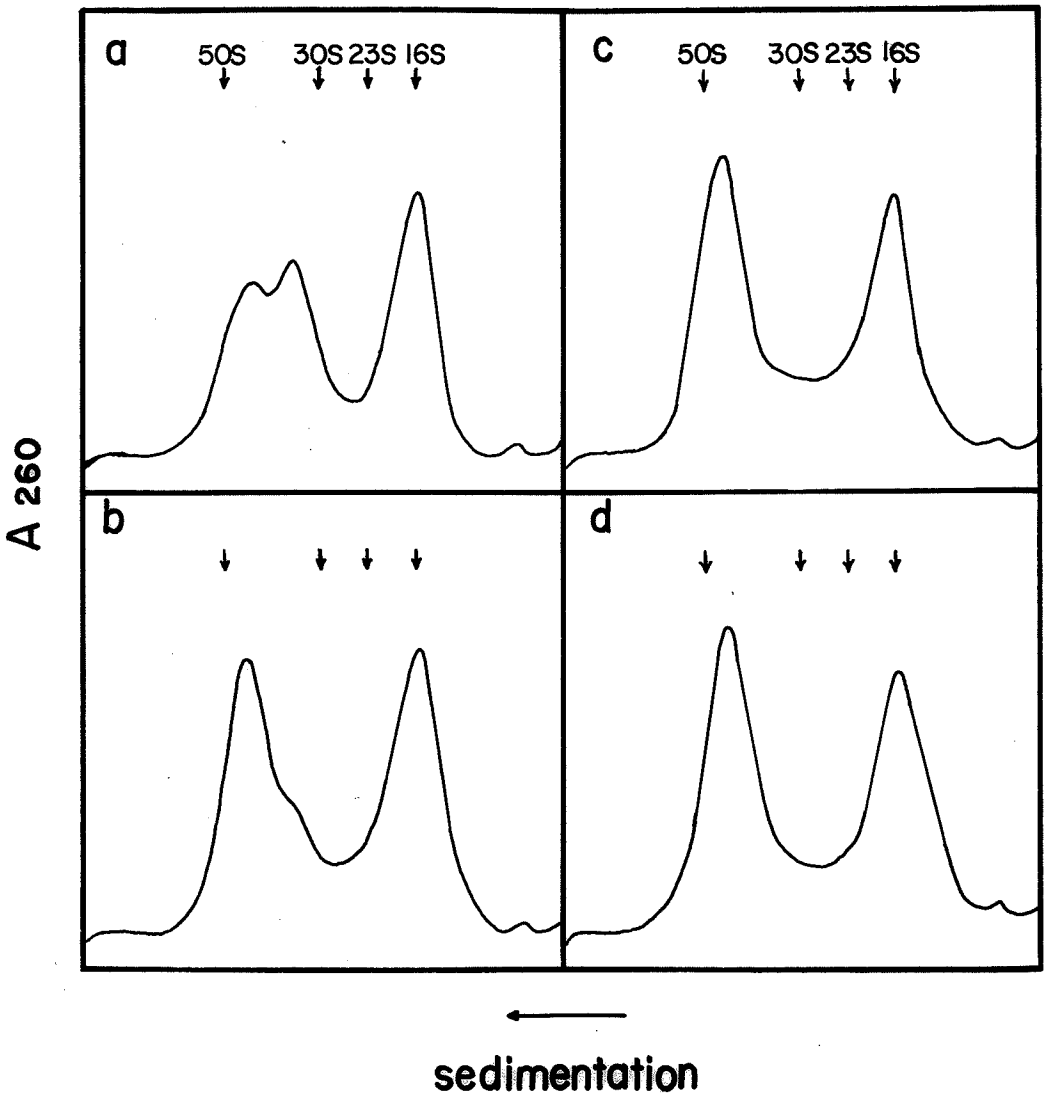

a
70S
50S
30S
15
10
5
b
15
10
5
c
15
10
5
A 260
cpm x 10^{-2}
sedimentation

subunits were incubated alone in TKMS I buffer, all the radioactivity and A_{260} absorbance were associated with a single 30S peak (Fig. 24c) indicating that 70S complexes were not aggregates of 30S subunits.

Reconstitution of 48S Particles at 50°

Reconstitution at 37 + 55° yielded 48S particles not all of which could associate with 30S subunits (Fig. 23f). It was suspected that high incubation temperatures (55°) might deleteriously affect components in the reconstitution mixture, resulting in formation of particles sedimenting at 48S but lacking subunit association activity. Consequently, reconstitution of 48S particles at 50° was attempted. The formation of 48S particles in the presence of 10mM spermidine was followed as a function of time of incubation of the reconstitution mixture at 50° (Fig. 25). After 5 min at 50°, a small amount of 48S particles as well as some lighter particles sedimenting at 37S were observed as double peaks in the sedimentation profile (Fig. 25a). After 15 min at 50°, the 48S peak predominated and the 37S particles appeared as a slight shoulder (Fig. 25b). After 30 min at 50°, only a single 48S peak was observed indicating that maximal amounts of 48S particles were formed (Fig. 25c). Prolonged incubation of up to 45 min at 50° did not alter the sedimentation profile (Fig. 25d).

Figure 25. Time course analysis of reconstituted mixtures incubated at 50° in the presence of 10mM spermidine for varying times: (a) 5 min; (b) 15 min; (c) 30 min; and (d) 45 min. The material recovered by ethanol precipitation wase analyzed in TMN I buffer by sucrose gradient centrifugation (Methods).


a
50S
30S
23S
16S
c
50S
30S
23S
16S
b
d
A 260
sedimentation

A comparison with the time course of reconstitution at 37 + 55° in the presence of 10mM spermidine and excess 50S proteins (Fig. 18f) showed that the time taken to form maximal amounts of 48S particles was nearly doubled although the sequence of assembly of 48S particles via intermediate 37S particles remained unchanged. However, 48S particles formed at 50° were more active in subunit association than those made at 37 + 55° (see later).

As in the reconstitution of 48S particles at 37 + 55°, the effect of spermidine on 48S particle formation at 50° was also stimulatory in nature (data not shown). In the absence of 10mM spermidine, maximal formation of 48S particles at 50° occurred after 45 min of incubation, compared to only 30 min of incubation in its presence (Fig. 25c). Also, there was no difference in the sedimentation profiles of reconstituted mixtures regardless of prior incubation of the reconstitution mixtures at 37° (data not shown).

Effect of Cations on Reconstitution at 50°

The role of spermidine in reconstitution and its requirement with respect to magnesium was studied. To test whether spermidine could completely replace magnesium, reconstitution was carried out at 50° without magnesium

in the presence of varying spermidine concentrations (5-40mM). Sedimentation profiles of reconstituted mixtures showed that virtually no reconstitution occurred in the absence of magnesium (Fig. 26). Even at 40mM spermidine, A_{260}-absorbing material all sedimented ss free rRNA (Fig. 26d). At low spermidine concentrations (lower than 20mM), degradation of RNA became evident (Fig. 26a, b, c) as judged by A_{260}-absorbing material sedimenting slower than 16S. The data indicated that spermidine could not replace magnesium in reconstitution and supports the contention made previously that it plays a stimulatory role.

On the other hand, in the absence of spermidine, maximal amounts of 48S particles were formed only at higher magnesium concentrations (40mM) as shown in Figure 27d. At magnesium concentrations of 15mM or less, very little or no 48S particles were formed (Fig. 27a, b, c). In fact, extensive RNA degradation occurred in 15mM magnesium or less resulting in the absence of any particles sedimenting faster than 23S (Fig. 27a, b). In the presence of spermidine, small amounts of reconstituted particles, though reduced in S-value, were formed at 10mM magnesium (Fig. 27e). At 10mM spermidine, maximum reconstitution was achieved at 20mM magnesium (Fig. 27f). Again, the data confirmed that spermidine lowered the requirement for magnesium in reconstitution, but could not replace the cation.

Figure 26. Sedimentation analysis of ribonucleoprotein complexes formed in the absence of magnesium at varying spermidine concentrations: (a) 5.0mM; (b) 10.0mM; (c) 20.0mM; and (d) 40.0mM. Incubation was at 50^{o} for 45 min. Analysis of ethanol-precipitated material was as described in "Methods".

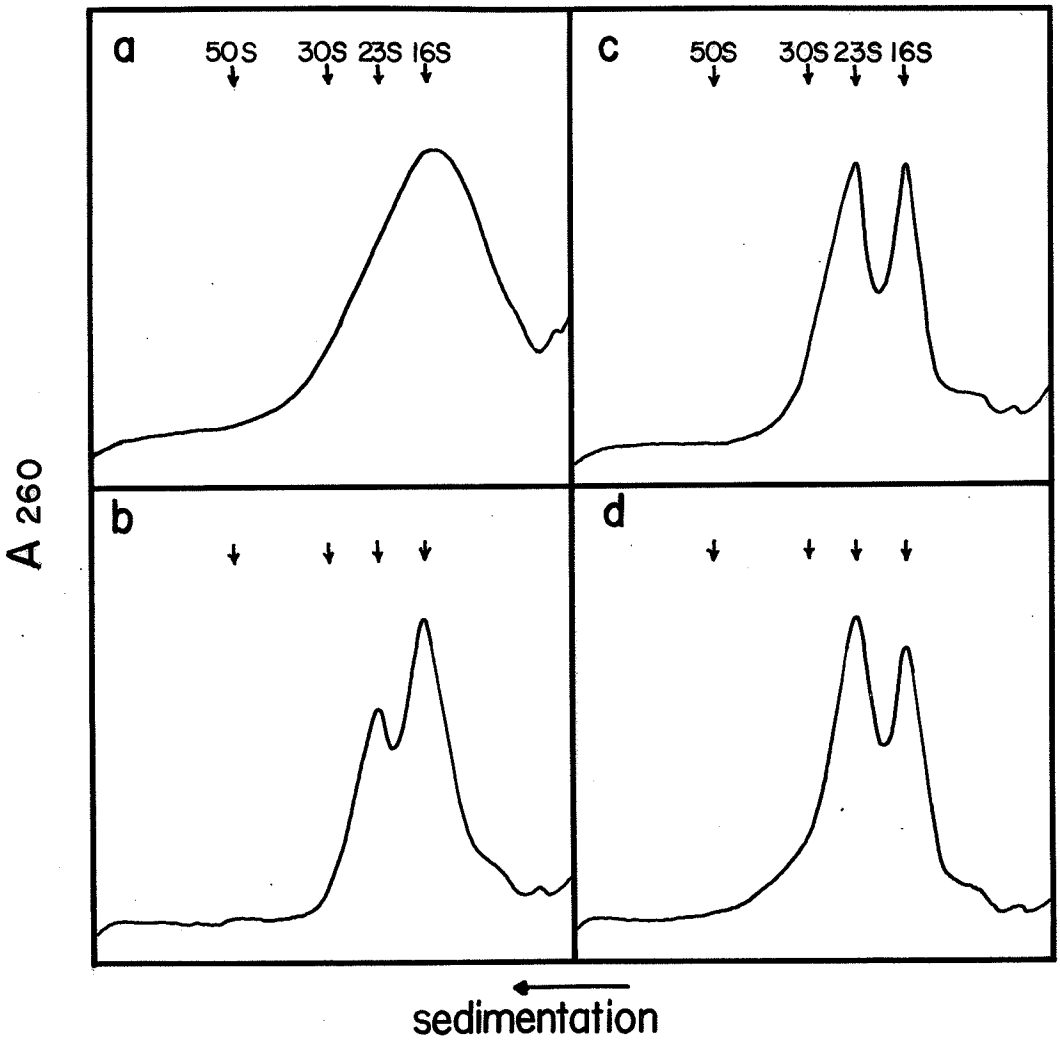
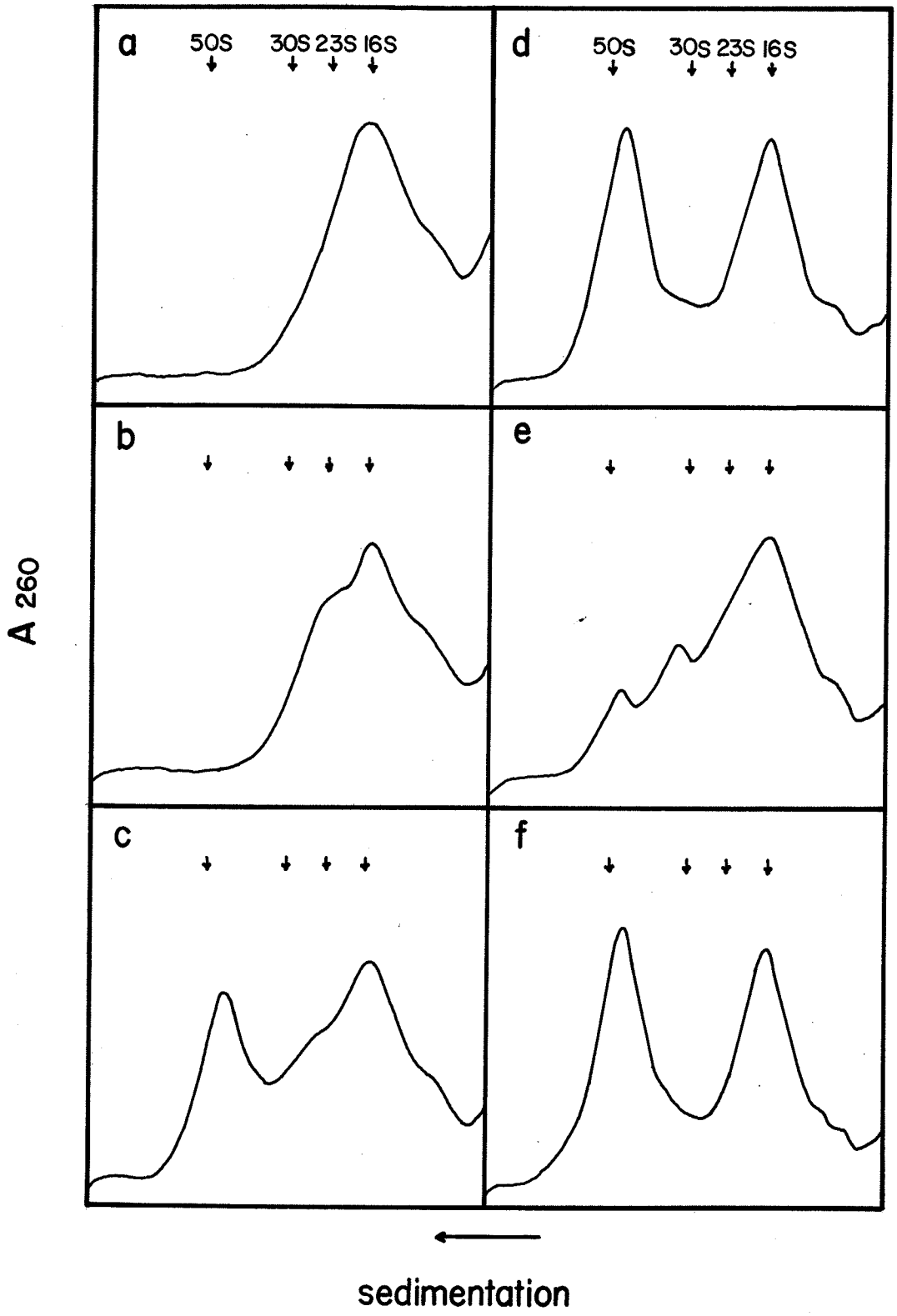

a
50S
30S
23S
16S
c
50S
30S
23S
16S
b
d
A 260
sedimentation

Figure 27. Sedimentation analysis of ribonucleoprotein complexes formed in the absence of spermidine at varying magnesium concentrations: (a) 5.0 mM; (b) 10.0mM; (c) 15.0mM; and (d) 40.0mM; in the presence of 10.0mM spermidine at (e) 10.0mM or (f) 20.0mM magnesium. Incubation was at 50^{o} for 45 min and analysis of reconstituted material was performed as described in "Methods".


a
50S
30S 23S 16S
d
50S
30S 23S 16S
b
e
c
f
A 260
sedimentation

To determine whether RNA degradation observed at low magnesium concentration (Figs. 26 and 27) was due to ribonuclease activity associated with a 50S protein, rRNA was incubated at varying magnesium concentrations (1.0 - 50mM) under the conditions of reconstitution at 50^{o} in the absence of 50S proteins. Sedimentation profiles of incubated RNA samples revealed extensive RNA degradation at 1.0mM magnesium (Fig. 28a). RNA degradation was gradually reduced with increasing magnesium concentration (Fig. 28b) and at 20mM magnesium or higher, more than 80% of RNA remained intact (Fig. 28c, d). The results indicated that ribonuclease activity was tightly bound to rRNA and, judging from the profiles (Fig. 28), was more effective in degrading 23S than 16S RNA. Even after repeated urea-LiCl extractions of RNA, the activity could not be removed. These characteristics are similar to those of a 23S RNA-associated ribonuclease previously observed by Ceri and Maeba (1973).

Although magnesium was shown to exert a profound effect on reconstitution, KCl (50-500mM) did not affect formation of 48S particles under the same reconstitution conditions (Fig. 29) and complete formation of 48S particles took place at all KCl concentrations tested.

Figure 28. Sedimentation analysis of rRNA incubated at 50° for 45 min in the presence of 10.0 mM spermidine at varying magnesium concentrations: (a) 1.0mM; (b) 5.0mM; (c) 20.0mM; and (d) 50.0mM in a total volume of 0.1 ml. The incubated samples were analyzed by sucrose gradient centrifugation as outlined in "Methods".

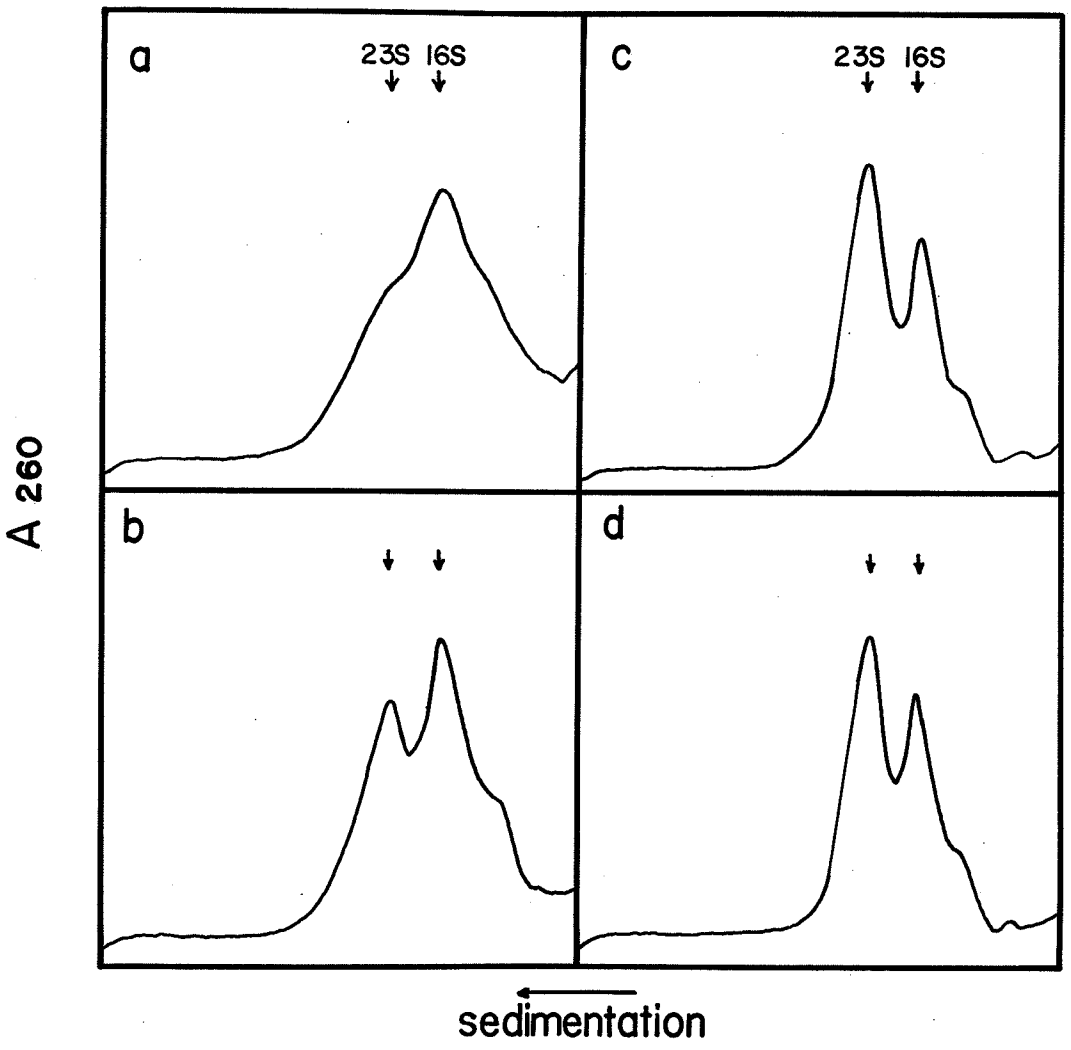
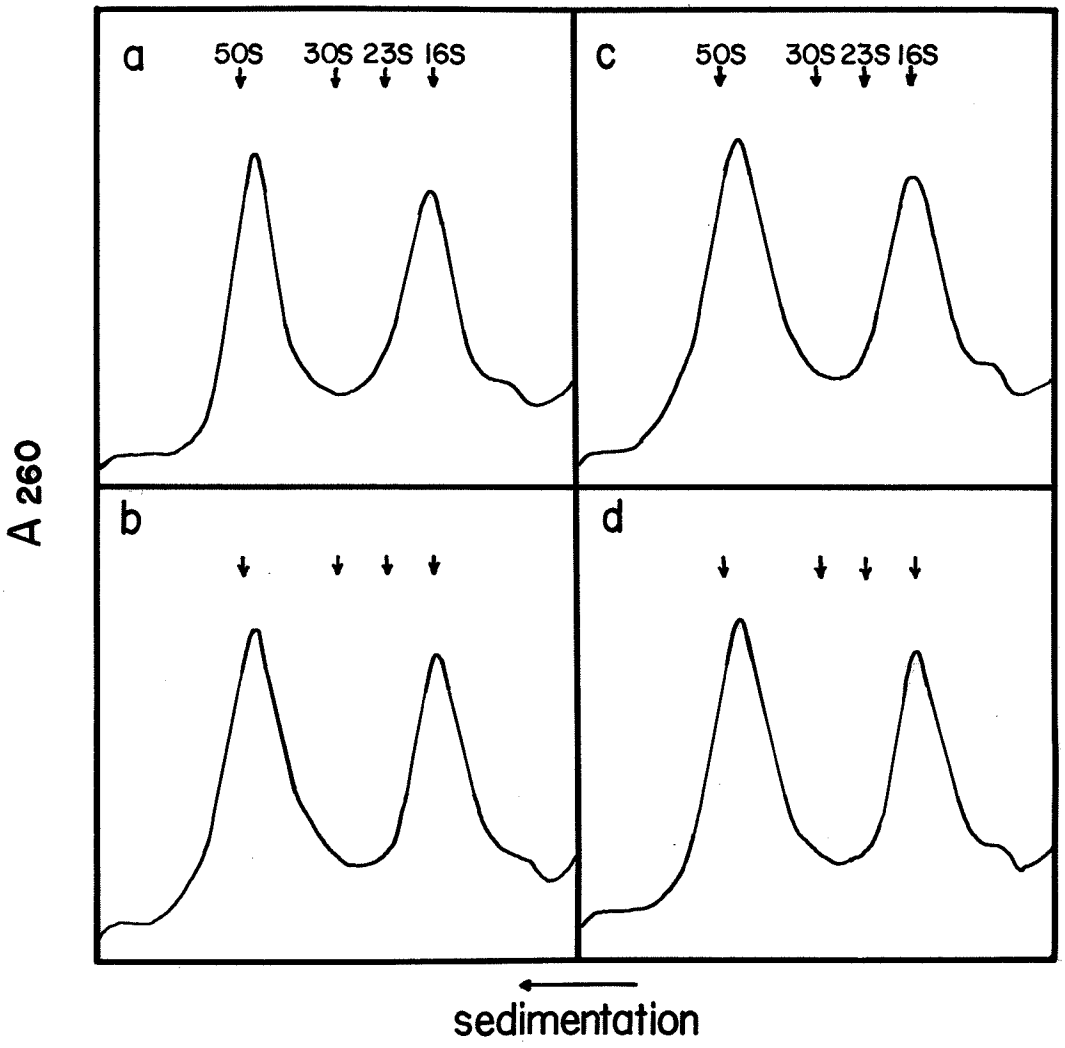

a
23S
16S
c
23S
16S
b
d
A 260
sedimentation

Figure 29. Sedimentation analysis of ribonucleoprotein complexes formed in the presence of 10mM spermidine at varying KCl concentrations: (a) 50mM; (b) 150mM; (c) 300mM; and (d) 500mM. Incubation was at 50^{o} for 45 min. The ethanol precipitated material was analyzed as described in "Methods".

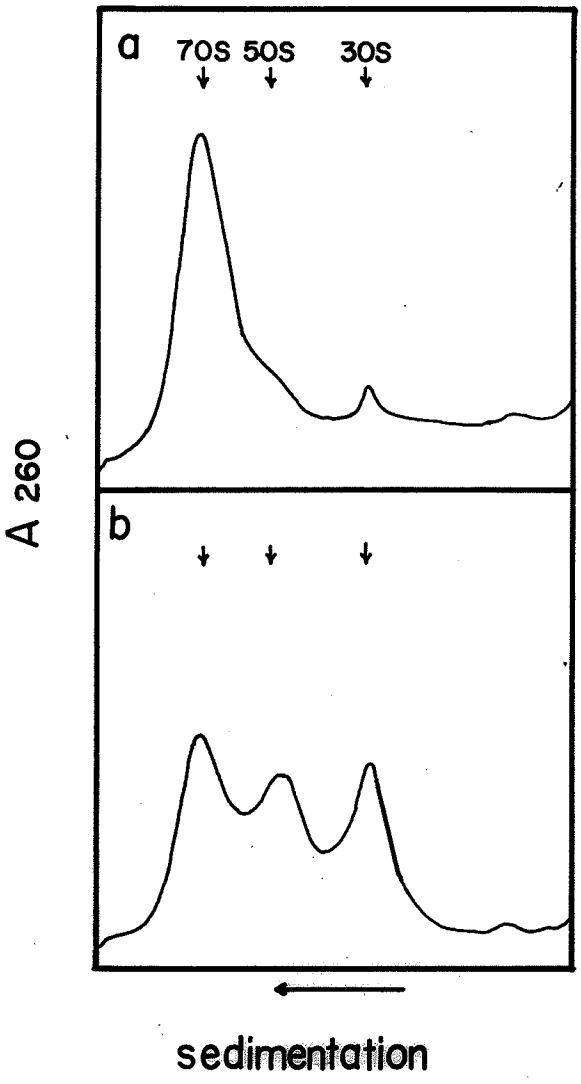

a
50S
30S
23S
16S
c
50S
30S
23S
16S
b
d
A 260
sedimentation

Assay of Subunit Association Activity of 48S Particles Reconstituted at 50°

Ability of 48S particles reconstituted at 50° to associate with 30S subunits was tested and compared with that of 48S particles reconstituted at 37 + 55°. One A_{260} unit of 48S particles, reconstituted at 50° for 30 min or at 37 + 55°, was incubated with 0.6 A_{260} unit of heat-activated 30S subunits in 0.1 ml TKMS I buffer at 37° for 20 min. Analysis of sedimentation profiles of these mixtures after sucrose gradient centrifugation showed that a greater proportion of 48S particles reconstituted at 50° was able to complex with 30S subunits to form 70S particles, as compared to those formed at 37 + 55° (Fig. 30). The data suggested that partial inactivation of some ribosomal components essential in subunit association might have occurred during incubation at 55°. Therefore, 48S particles reconstituted at 50° were used in subsequent association studies.

The formation of 48S particles at 50° was studied by sampling reconstitution mixtures at different times for particles active in subunit association. The 48S particles capable of associating with 30S subunits were determined by sucrose gradient centrifugation (Methods). The relative amounts of 70S complexes formed were obtained by gravimetric analysis of areas under the 70S peaks in sedimentation profiles and plotted against time of incubation of the re-

Figure 30. Sedimentation analysis of ribonucleoprotein complexes formed from association of 30S subunits with 48S particles reconstituted after (a) 45 min at 50° and (b) 15 min at 37° and 20 min at 55°. Subunit association was performed as in the legend to figure 23 and the associated mixtures were analyzed as in "Methods".


a
70S
50S
30S
b
A 260
sedimentation

constitution mixture at 50° (Fig. 31). The Figure showed that maximal amounts of 70S complexes were formed by particles reconstituted after 30 min of incubation at 50°. Comparison of these results with those in Figure 25 indicated that the rate of formation of 48S particles and their ability to associate with 30S subunits proceeded concomitantly.

Stoichiometry of Subunit Association

To determine whether association of 48S particles with 30S subunits occurred stoichiometrically, association of 1.2 A_{260} units of 48S particles with increasing amounts of (^{3}H) 30S subunits (0.2 - 2.4 A_{260} units) was measured. The relative amounts of 70S particles formed were calculated by gravimetric analysis of sedimentation profiles as described in the previous experiment, and plotted against A_{260} units of (^{3}H) 30S subunits used in the association assay (Fig. 32 bottom curve). A saturation curve was obtained which plateaued at 0.6 A_{260} unit of (^{3}H) 30S subunits added. Despite the large excesses of 30S subunits used in association, the Figure showed that 1.2 A_{260} units of 48S particles could only associate with approximately 0.6 A_{260} units of 30S subunits. Assuming that the absorption coefficient of 48S particles at 260 nm was twice that of 30S subunits, then

Figure 31. Time course analysis of formation of 48S particles by subunit association. Reconstitution was carried out at 50° for varying times in 1.0 ml SRS buffer. The reconstituted mixtures were chilled at various times and 1.0 A_{260} unit of heat-activated 30S subunits was added. Incubation was continued for 20 min at 37°. The ethanol precipitated materials were analyzed by sucrose gradient centrifugation as described in "Methods". Relative amounts of 70S complexes formed were determined gravimetrically from tracings of sedimentation profiles (Methods).

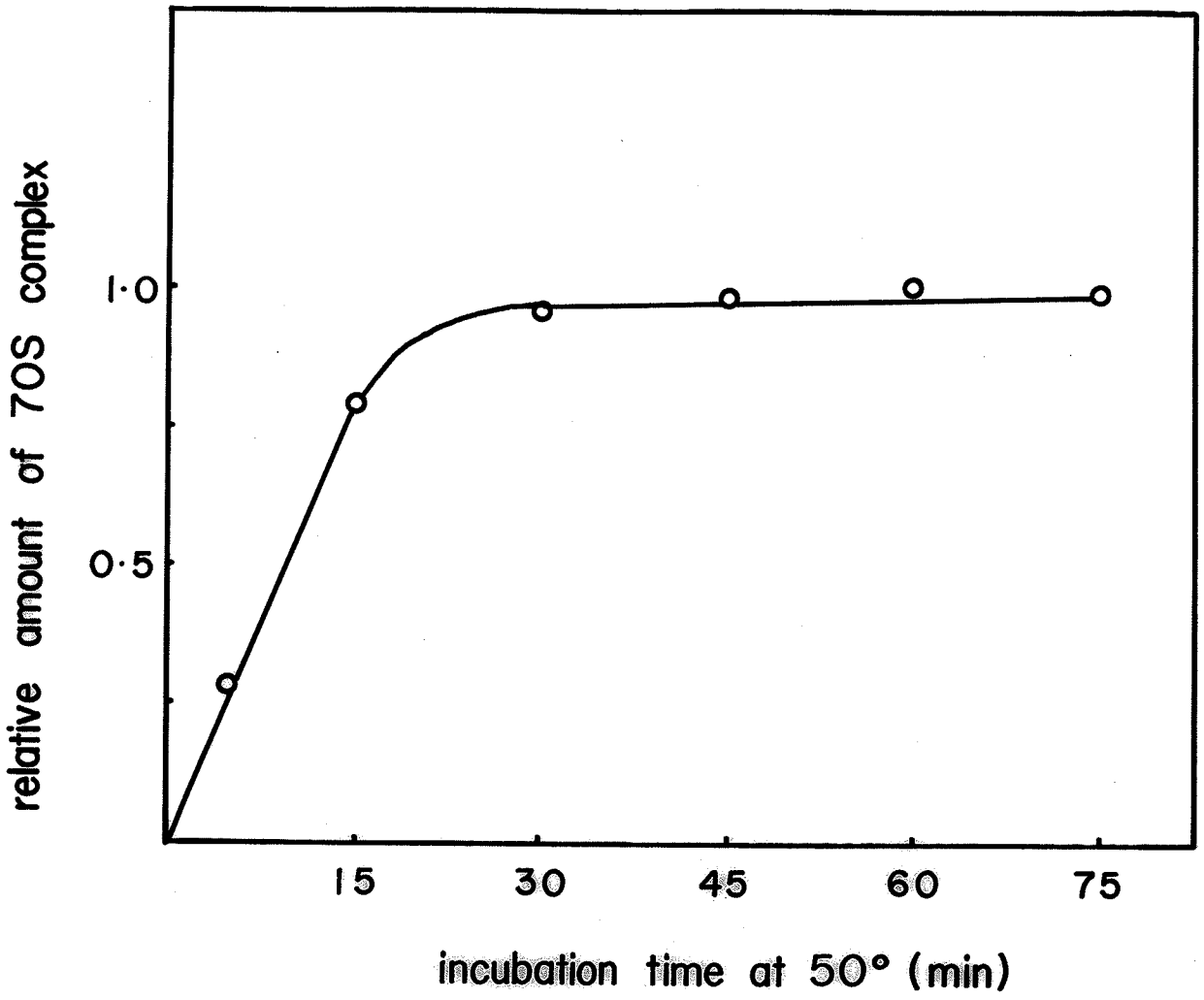
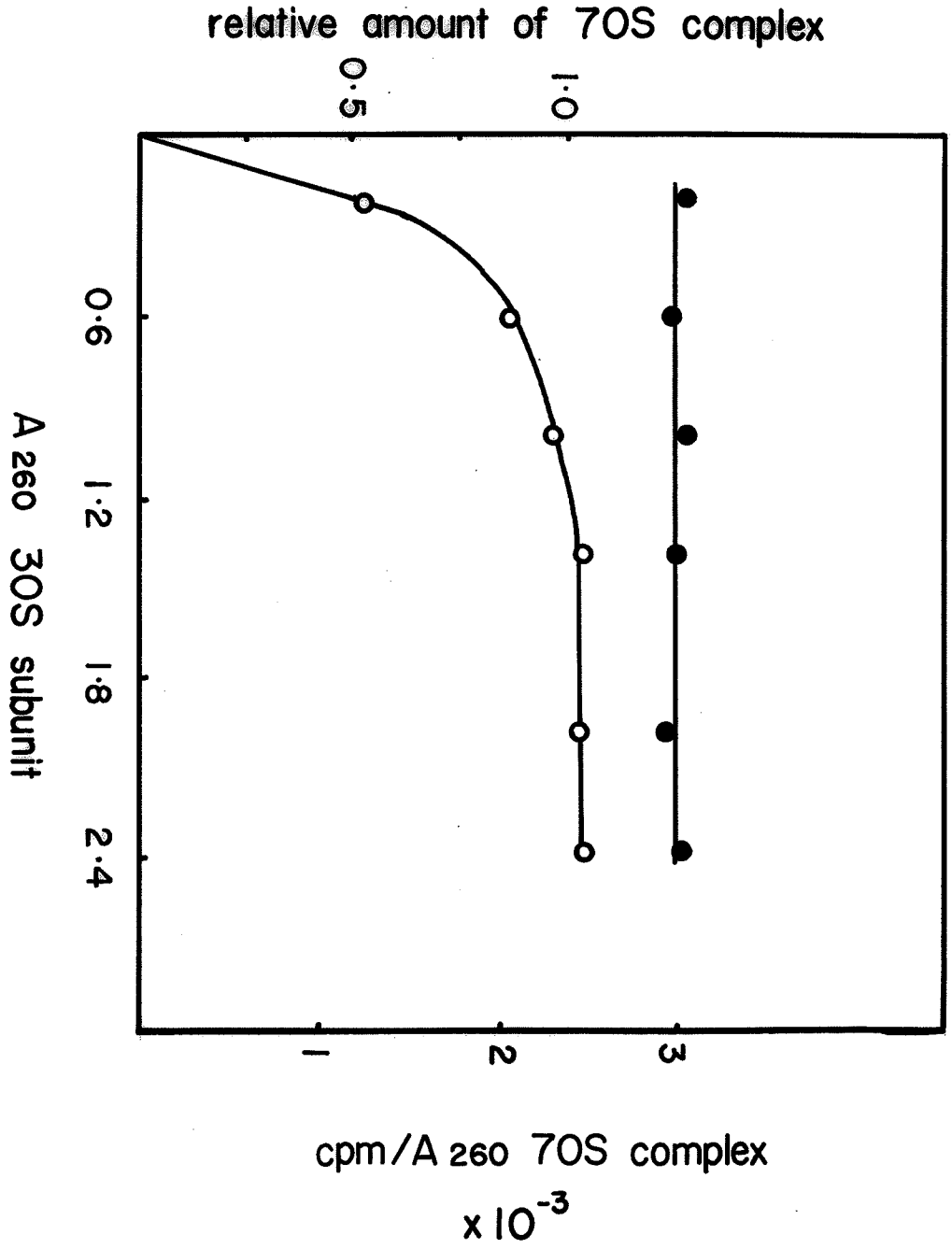

relative amount of 70S complex
1·0
0·5
15
30
45
60
75
incubation time at 50° (min)

Figure 32. Stoichiometry of association between 48S particles reconstituted at 50° and (^{3}H) 30S subunits. Reconstituted particles were associated with varying amounts of heat-activated (^{3}H) 30S subunits (0.2 - 2.4 A_{260}) and the associated 70S complexes were isolated by sucrose gradient centrifugation (Methods). The relative amounts of 70S complexes (o) were determined as in the legend to figure 31. Specific radioactivities (•) of these complexes were determined from pooled fractions containing them (Methods).


relative amount of 70S complex
0·5
1·0
0·6
1·2
1·8
2·4
A 260 30S subunit
1
2
3
cpm/A 260 70S complex
x 10-3

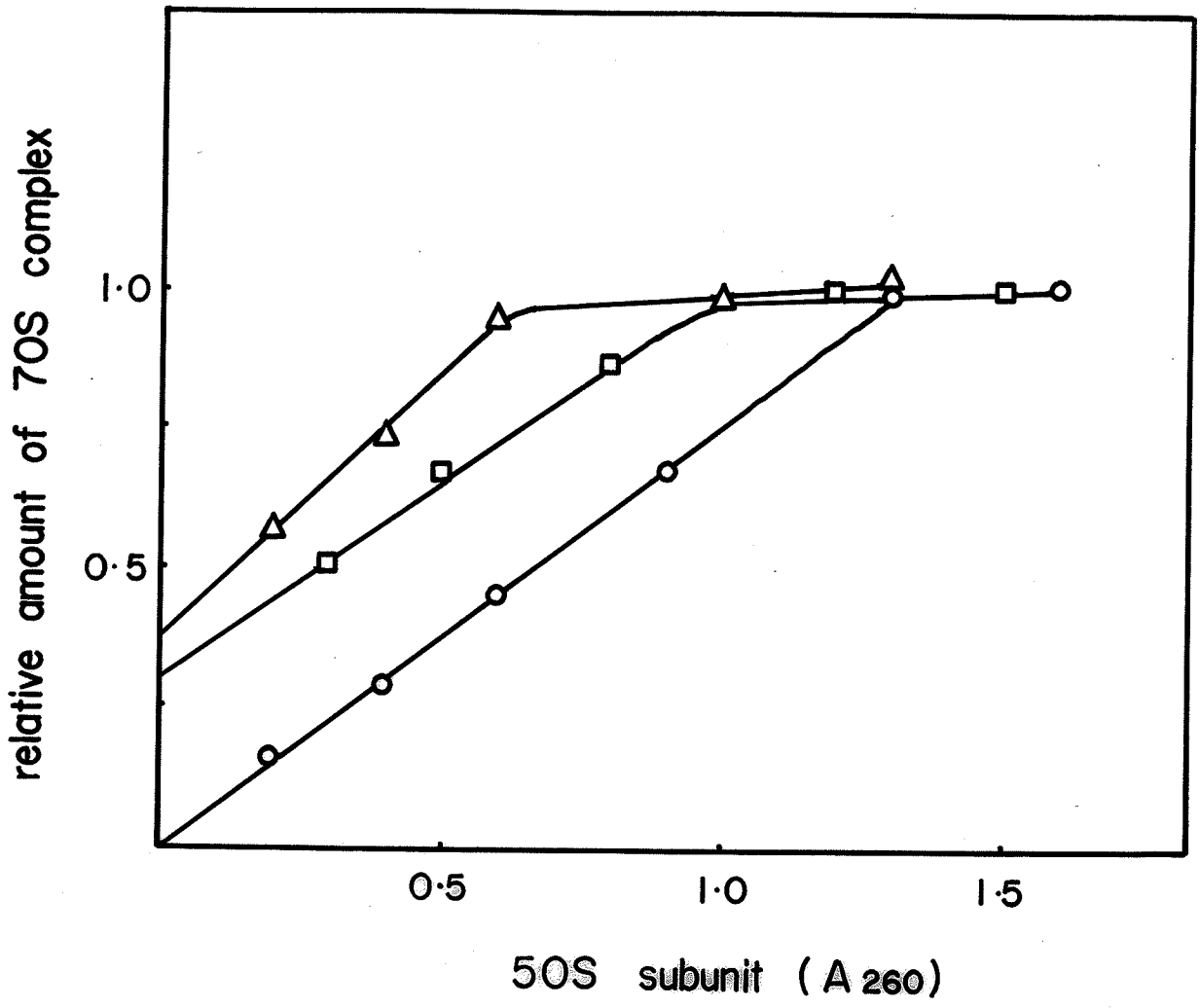
association between the 48S particles and 30S subunits occurred stoichiometrically at a ratio of 1:1.

To further examine the specificity of association, (^{3}H) 70S complexes formed from 48S particles with varying amounts of (^{3}H) 30S subunits in the previous experiment were isolated (Methods), and assayed for absorbance at 260 nm and for radioactivity to determine their specific radioactivity in c.p.m./A_{260}. The specific radioactivity of 70S particles formed over a wide range of added (^{3}H) 30S subunits remained constant (Fig. 32 top curve), indicating that the ratio of 48S particles to 30S subunits in the 70S complexes was constant.

Specificity of Subunit Association - Competition Studies

To determine whether 48S particles could compete effectively with 50S subunits for 30S subunits in association, 0.7 A_{260} unit of 30S subunits and varying amounts of 50S subunits (0.2 - 1.6 A_{260} units) were allowed to associate in the presence of nonsaturating amounts of 48S particles (0.33, 0.53 and 0.80 A_{260} unit). The relative yields of 70S particles calculated gravimetrically from sedimentation profiles after sucrose gradient centrifugation were plotted against A_{260} units of added 50S subunits (Fig. 33). As more 48S particles were added to the association mixture, smaller amounts of 50S subunits were required for saturation

Figure 33. Competitive subunit association. Heat-activated 30S subunits (0.7 A_{260} unit) were incubated with varying amounts of 50S subunits (0.2 - 1.6 A_{260} units) in the presence of 0.33 (○); 0.53 (□); and 0.8 (△) A_{260} units of 48S reconstituted particles at 37° for 20 min in 0.1 ml TKMS I buffer. The relative amounts of 70S complexes formed were estimated as in the legend to figure 32.

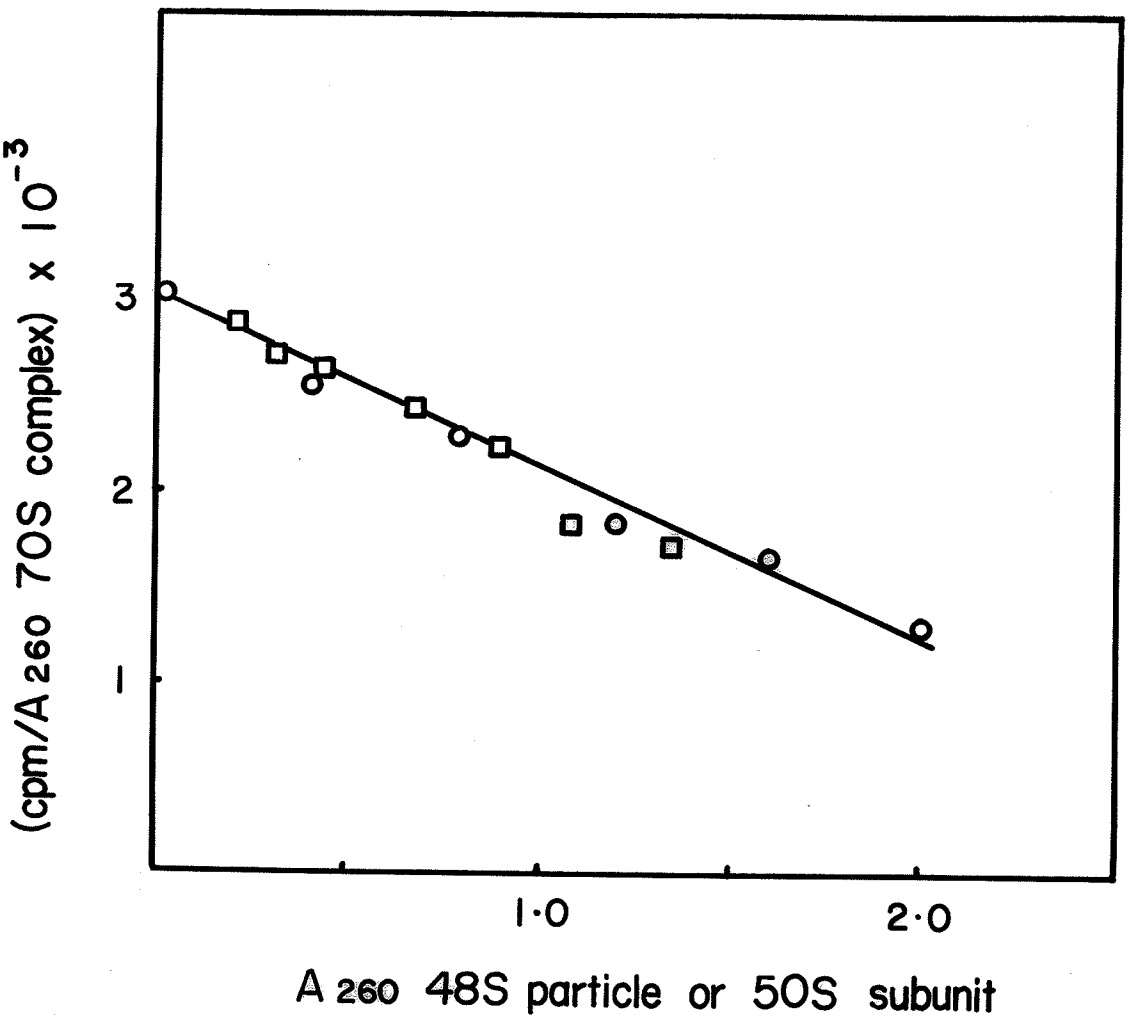

relative amount of 70S complex
1·0
0·5
0·5
1·0
1·5
50S subunit (A 260)

of 30S subunits, indicating that both 48S particles and 50S subunits were competing for 30S subunits.

To determine how effectively 48S particles competed with 50S subunits for 30S subunits, an isotope dilution technique was employed. Varying amounts of 48S particles or 50S subunits (0.3 - 2.0 A_{260} units) were allowed to associate with a constant amount of 30S subunits (0.7 A_{260} unit) in the presence of saturating amount of (^{3}H) 50S subunits (1.5 A_{260} units). The resulting 70S complexes were isolated and their specific radioactivities were calculated as previously described. As expected, the specific radioactivity of 70S complexes decreased whether 48S particles or 50S subunits were used for competing with (^{3}H) 50S subunits. Surprisingly, when specific radioactivities of 70S complexes were plotted against A_{260} units of 48S particles or 50S subunits used, the 2 sets of points could be represented by a single straight line (Fig. 34) indicating that 48S particles and 50S subunits could compete equally well with labelled 50S subunits for 30S subunits during association.

In another experiment, a fixed amount of 30S subunits (0.7 A_{260} unit) was allowed to associate with increasing amounts of 48S particles or 50S subunits (0.33 - 2.0 A_{260} units). The 70S complexes formed were quantitated gravimetrically from sedimentation profiles and plotted against A_{260} units of 48S particles or 50S subunits used. The 2

Figure 34. Competitive subunit association. Varying amounts (0 - 2.0 A_{260} units) of 50S subunits (O), or 48S reconstituted particles (□), were mixed with 0.7 A_{260} unit of heat-activated 30S subunits in the presence of 1.5 A_{260} units of (^3H) 50S subunits. The 70S complexes formed were isolated and the amounts of (^3H) 50S subunits present in them were determined in terms of c.p.m./A_{260} unit of 70S complexes (Methods).

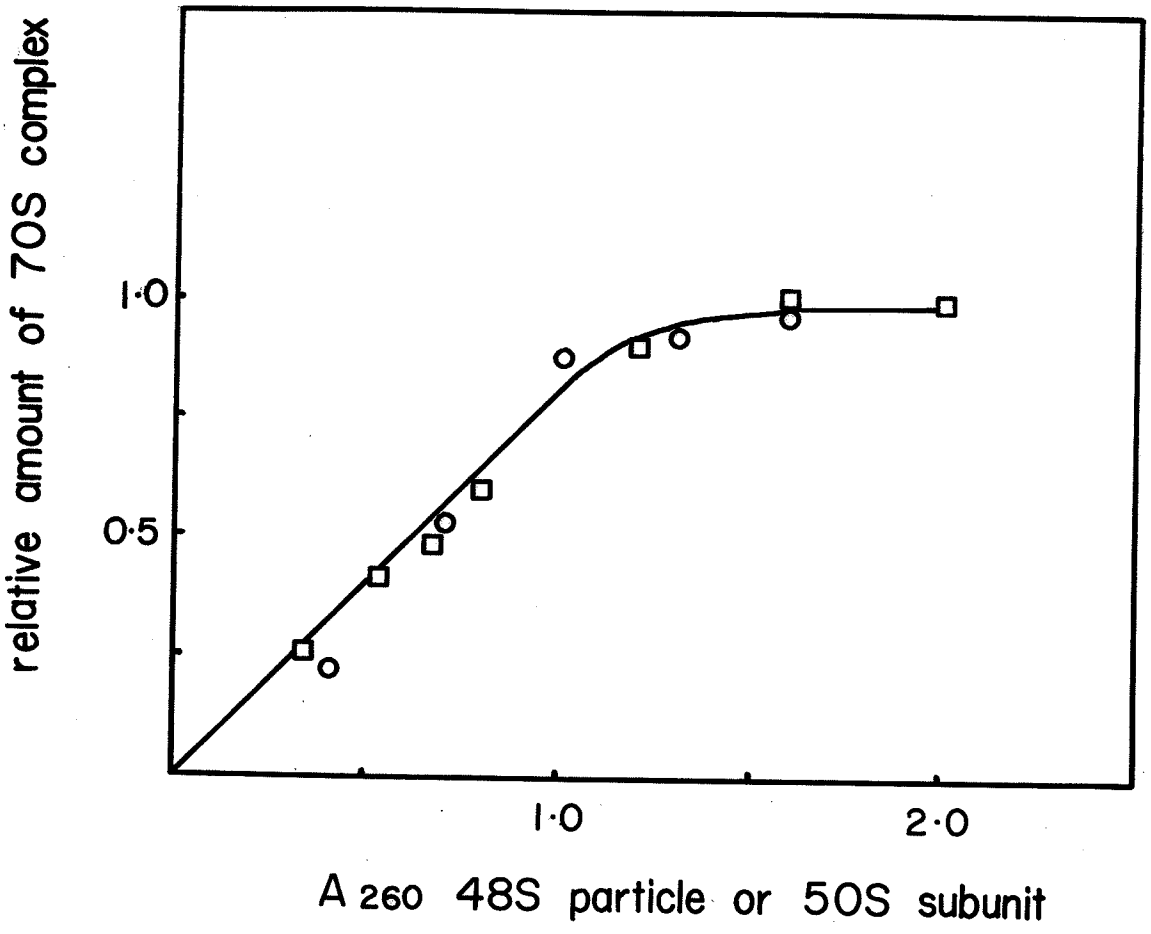

(cpm/A 260 70S complex) x 10⁻³
3
2
1
1·0
2·0
A 260 48S particle or 50S subunit

sets of points could be approximated by a single curve which levelled off at about 1.4 A_{260} units of 48S particles or 50S subunits added (Fig. 35). Again, the data suggested equal tendency of 48S particles and 50S subunits to associate with 30S subunits.

Assay of Ribosome Dependent GTPase Activities

Since the 48S reconstituted particles were active in subunit association, they were tested for other ribosomal functions. Crude preparations of IF (initiation factors) or EF (elongation factors) were used in GTPase assays (Methods). Protein concentrations in these crude preparations and in PI-II proteins (Hamel et al, 1972; Methods) as estimated by Lowry's phenol method (Lowry et al, 1951) were as follows: crude IF, 26.8 mg/ml; crude EF, 12.7 mg/ml; and PI-II proteins, 2.4 mg/ml. The effect of PI-II proteins extracted from 50S subunits was tested because they contained proteins L7 and L12 which were essential for expression of ribosome dependent GTPase activities (Hamel et al, 1972; Brot et al, 1973; Lockwood et al, 1974). Hydrolysis of GTP was measured by the amount of (^{32}P) phosphate released from 0.02mM GTP-γ-(^{32}P) (100 c.p.m./pmole) in 15 min at 30° in a total volume of 0.1 ml (Methods).

Figure 35. Subunit association stoichiometry. Varying amounts (0.33 - 2.0 A_{260} units) of 50S subunits (○) or 48S reconstituted particles (□), were mixed with 0.7 A_{260} unit of heat-activated 30S subunits. The relative amounts of 70S complexes formed were determined as described in the legend to figure 31.


relative amount of 70S complex
1·0
0·5
1·0
2·0
A 260 48S particle or 50S subunit

(a) IF-Catalyzed Uncoupled GTPase

Uncoupled GTPase activity is the hydrolysis of GTP by ribosomes and IF in the absence of AUG and fMet-tRNA. As crude IF exhibited GTPase activity even in the absence of salt-washed ribosomes, controls without ribosomes were included in all assays. The dependence of ribosome associated GTPase activity on crude IF is presented in Figure 36. In the presence of 2.0 A_{260} units of salt-washed ribosomes, maximum activity occurred at about 1.0 µg crude IF. To find the amount of 50S subunits required for optimal activity, duplicate assays were carried out using varying amounts of 50S subunits in the presence of 1.5 A_{260} units of 30S subunits and 1.5 µg crude IF. Assays containing all components except 50S subunits were included as controls. Saturation was reached at 1.0 A_{260} unit of 50S subunits (Fig. 37). Under the conditions of assay, 2.0 A_{260} units of 50S subunits hydrolyzed 75 pmoles of GTP in 15 min at 30°. The 37S and 48S reconstituted particles (2.0 A_{260} units) were tested for uncoupled GTPase activity with or without 24 µg PI-II proteins and were found to be only partially active (Table VI, experiment a). When compared to 50S subunits assayed under identical conditions, 37S particles exhibited about 40% activity and 48S particles about 54%. Inclusion of PI-II proteins did not enhance

Figure 36. Ribosome dependent uncoupled GTPase activity in crude IF. Reaction mixtures (0.1 ml) contained 50mM Tris-HCl, pH 7.4, 100mM NH_4Cl, 10mM magnesium acetate, 5mM 2-mercaptoethanol, 0.02mM GTP-γ-(^{32}P) (100 c.p.m./pmole), 2.0 A_{260} units of salt-washed ribosomes and varying amounts of crude IF as indicated. Reactions were initiated by addition of GTP-γ-(^{32}P). Incubation was for 15 min at 30°. (^{32}P) phosphate released was determined as in "Methods".

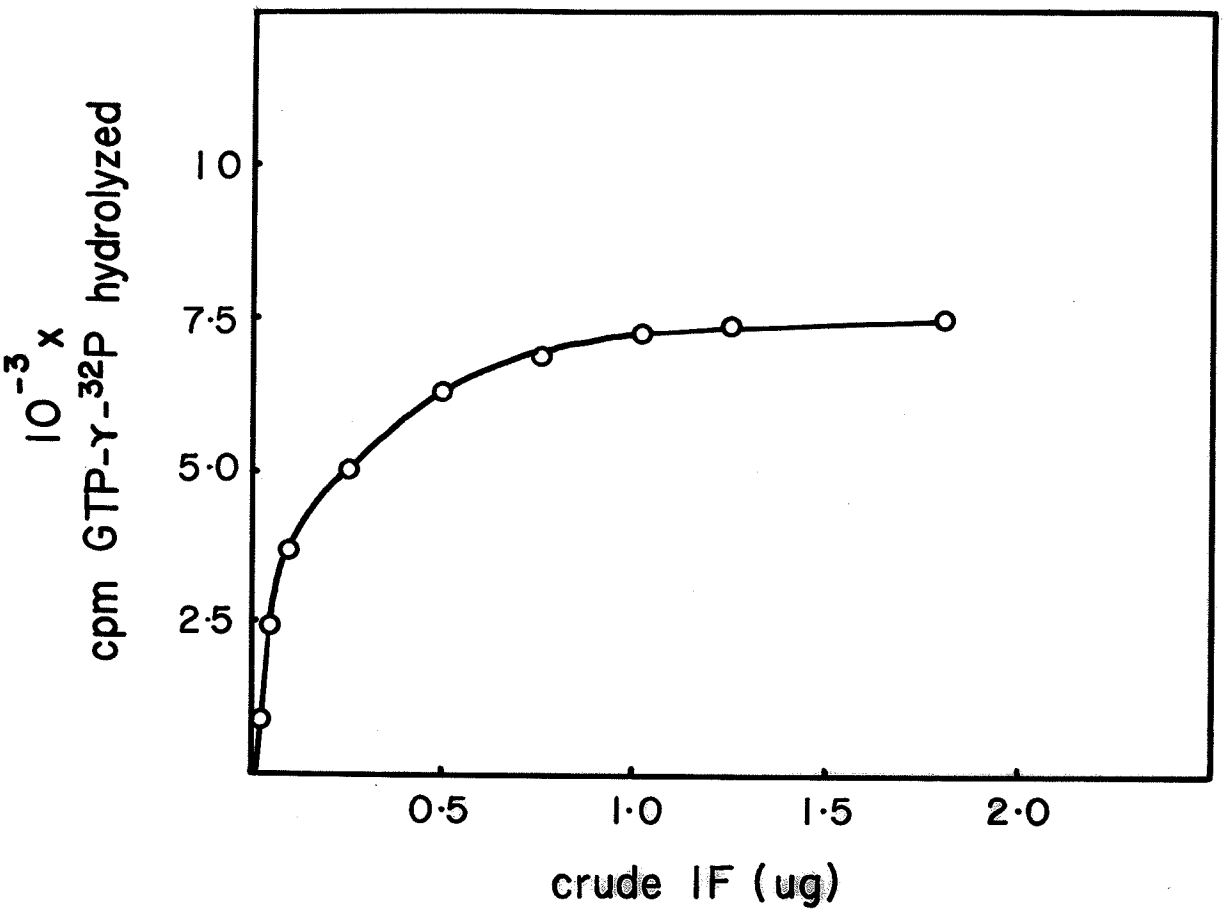
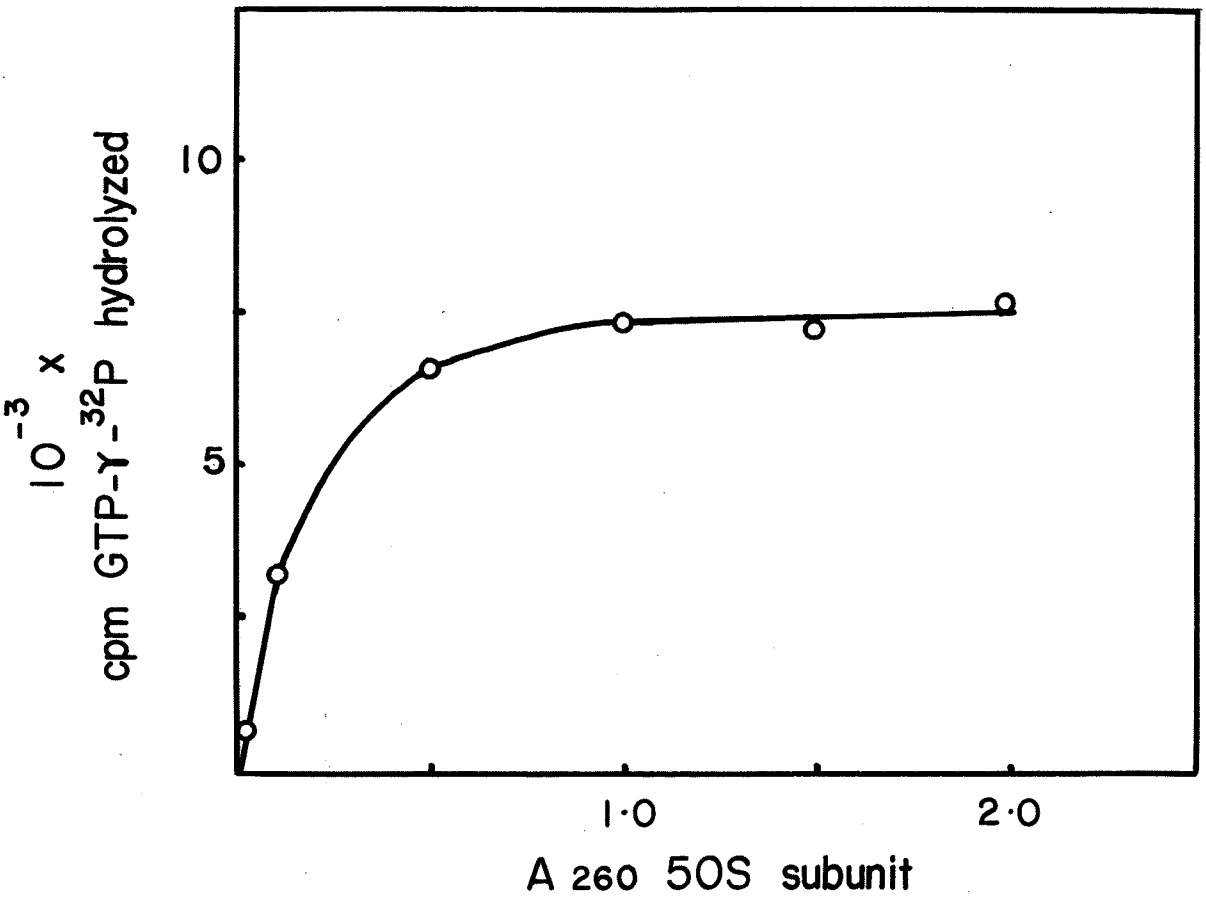

10
7·5
5·0
2·5
10^{-3} x
cpm GTP-γ-^{32}P hydrolyzed
0·5
1·0
1·5
2·0
crude IF (ug)

Figure 37. Titration of ribosome dependent uncoupled GTPase activity. Reaction conditions were same as described in the legend to figure 36 except that a mixture of 30S and 50S subunits was used instead of salt-washed ribosomes. Amount of 50S subunits was varied (0 - 2.0 A_{260} units) as indicated at fixed amounts of 30S subunits (1.5 A_{260} units) and crude IF (1.5 µg).


10
5
10^{-3} x
cpm GTP-γ-^{32}P hydrolyzed
1·0
2·0
A 260 50S subunit

GTPase activity of either species of particles.

(b) IF-Catalyzed Coupled GTPase

Coupled GTPase activity was assayed in the presence of 0.1 A_{260} unit of AUG and 4.0 A_{260} units of crude fMet-tRNA (containing 35% fMet-tRNA). Otherwise, assay conditions were the same as those for uncoupled GTPase assay. Compared to 50S subunits (40 pmoles GTP hydrolysed in 15 min at 30°), activities of 37S and 48S particles were 45% and 62% respectively (Table VI, experiment b). However, in the presence of PI-II proteins, activity of 37S particles was depressed to 27% while that of 48S particles was increased to 99%. The reason for the different responses of 37S and 48S particles to PI-II proteins in coupled GTP hydrolysis was not clear.

(c) EF-Catalyzed Uncoupled GTPase

All conditions in this assay were the same as those for IF-catalyzed uncoupled GTPase assay except that crude IF was replaced by 12.7 µg crude EF. Under the conditions of assay, 2.0 A_{260} units of 50S subunits hydrolyzed 65 pmoles of GTP in 15 min at 30°. Activities of 2.0 A_{260} units of 37S and 48S particles were found to be 42% and 81% respectively of that of 50S subunits (Table VI, experiment c). In the presence of PI-II proteins, the

activity of 37S particles was depressed to 5% whereas that of 48S particles remained unchanged. The reason for the inhibition of activity of 37S particles by PI-II proteins was unknown.

Assay of fMet-tRNA Binding Activity

The assay conditions were identical to those for coupled GTPase assay except that unlabelled GTP and f(^{3}H)Met-tRNA were used. Binding was measured as described in Methods. When compared to 50S subunits (2.0 A_{260} units) which bound about 20 pmoles of f(^{3}H)Met-tRNA under the conditions of assay (Methods), both 37S and 48S particles were inactive in binding fMet-tRNA, even in the presence of PI-II proteins (Table VI, experiment d).

Assay of Peptidyltransferase Activity

The peptidyltransferase activities of 37S and 48S particles, and of 50S subunits were assayed by the "alcohol reaction" which is a modification of the "fragment reaction" developed by Monro and Marcker (1967). Instead of using f(^{3}H)Met-oligonucleotide fragment produced by RNase T_1 digestion of f(^{3}H)Met-tRNA, whole f(^{3}H)Met-tRNA in crude form was used (Miskin et al, 1970). Neither 30S subunits

nor supernatant factors are required in this reaction in which formation of f(^{3}H)Met-puromycin was measured. The peptidyltransferase activity of 2.0 A_{260} units of 50S subunits under the reaction conditions (Methods) was 1930 c.p.m. as extracted by 2.0 ml of ethyl acetate. Compared to the control value, both 37S and 48S particles were inactive in fMet-puromycin formation (Table VI, experiment e). This was expected as both species of reconstituted particles were shown to be incapable of fMet-tRNA binding.

Assay of Polypeptide Synthesis Activity

Partial ribosomal functions were tested for in the previous assays. To test for activity of reconstituted particles in the overall translational process, poly(U)-directed polyphenylalanine synthesis activity was assayed (Methods). As expected, all species of reconstituted particles were found to be inactive in polypeptide synthesis when compared to the activity of 50S subunits (9250 c.p.m. of TCA-precipitable polyphenylalanine). This was not surprising as the particles were shown to be inert in some of the partial ribosomal functions tested, namely the binding of fMet-tRNA and the peptidyltransferase reaction (Table VI, experiment f).

Table VI. Activity of reconstituted particles in some ribosomal functions.

Experiment	Ribosomal function [a]	Reconstituted particles		% activity of 50S subunits
a	IF-catalyzed uncoupled	37S	-PI-II	40
			+PI-II	20
	GTPase	48S	-PI-II	54
			+PI-II	52
b	IF-catalyzed coupled	37S	-PI-II	45
			+PI-II	27
	GTPase	48S	-PI-II	62
			+PI-II	99
c	EF-catalyzed uncoupled	37S	-PI-II	42
			+PI-II	5
	GTPase	48S	-PI-II	81
			+PI-II	80
d	fMet-tRNA binding	37S	-PI-II	0
			+PI-II	0
		48S	-PI-II	<2
			+PI-II	0
e	peptidyltransferase	37S	-PI-II	NT
			+PI-II	NT
		48S	-PI-II	<2
			+PI-II	<2

Table VI. Continued.

Experiment	Ribosomal function [a]	Reconstituted particles		% activity of 50S subunits
f	poly(U)-directed polyphenylalanine synthesis	37S	-PI-II	0
			+PI-II	NT
		48S	-PI-II	<2
			+PI-II	0

NT : not tested

a Reaction conditions for assay of various ribosomal functions were as described in "Methods".

Protein Analysis of Reconstituted Particles

The 50S proteins bound in each species of reconstituted particles were quantitated by bidimensional polyacrylamide gel electrophoresis of proteins extracted from a mixture of (^{3}H) reconstituted particles (or (^{3}H) 50S subunits) and (^{14}C) 50S subunits. After electrophoresis, the (^{3}H) and (^{14}C) radioactivities associated with each protein spot was determined and a normalized ratio that indicated the amount of protein present in reconstituted particles as a fraction of that in 50S subunits was calculated (Methods). The (^{3}H)/(^{14}C) ratios of proteins of 32S, 37S and 48S particles reconstituted with excess proteins in the presence of spermidine, as well as those of 50S subunits, are shown in Table VII and the normalized ratios of the reconstituted particles in Table VIII. Proteins L8 and L9 could not be resolved by bidimensional electrophoresis so that ratios shown in the tables are for the composite spot. The unexpectedly high ratio for protein L3 in the 48S particles could not be explained - in 3 separate runs, ratios were 1.04, 1.67 and 1.37. Ratios obtained in separate analyses of other proteins were very close (10-15%).

From Table VIII, it is apparent that all proteins were bound to some extent in all reconstituted particles. In the 32S particles formed at 0°, only 4 (L1, L21, L24, L26) out of thirty-four 50S proteins were bound in significant amounts (a ratio of 0.5 or over) whereas the rest

Table VII. $^{3}H/^{14}C$ ratios [c] of 50S proteins in reconstituted particles.

50S protein	Reconstituted particles 50S	32S	37S	48S	48S [a]	50S protein	Reconstituted particles 50S	32S	37S	48S	48S [a]
L1	0.62	0.47	0.77	0.61	0.60	L18	0.79	0.17	0.44	0.60	0.48
L2	0.95	0.13	0.46	0.71	0.79	L19	0.88	0.15	0.30	0.65	0.72
L3	0.71	0.20	0.40	0.99	0.59	L20	0.87	0.32	0.99	0.64	0.77
L4	0.82	0.40	0.75	0.75	0.66	L21	0.71	0.52	0.89	0.77	0.74
L5	0.73	0.21	0.43	0.51	0.47	L22	0.81	0.12	0.58	0.71	0.59
L6	0.71	0.13	0.27	0.28	0.23	L23	0.74	0.23	0.39	0.63	0.77
L7	0.43	0.11	0.11	0.12	0.07	L24	0.72	0.46	0.61	0.65	0.67
L8 }[b]	0.66	0.17	0.31	0.40	0.38	L25	0.79	0.14	0.16	0.42	0.27
L9 }[b]						L26	0.84	0.60	0.42	0.34	0.34
L10	0.68	0.34	0.49	0.54	0.39	L27	0.68	0.06	0.17	0.40	0.41
L11	0.66	0.30	0.42	0.50	0.41	L28	0.63	0.11	0.09	0.38	0.33
L12	0.87	0.11	0.25	0.14	0.13	L29	0.56	0.18	0.23	0.50	0.57
L13	0.82	0.16	0.46	0.65	0.84	L30	0.65	0.08	0.14	0.38	0.32
L14	0.66	0.10	0.32	0.52	0.48	L31	0.85	0.10	0.13	0.05	0.14
L15	0.72	0.15	0.44	0.69	0.69	L32	0.78	0.04	0.09	0.52	0.39
L16	0.68	0.10	0.21	0.19	0.23	L33	0.71	0.06	0.12	0.10	0.16
L17	0.70	0.12	0.36	0.59	0.53	L34	0.82	0.11	0.62	0.80	1.20

a gravitational sedimentation used during isolation.

b proteins L8 and L9 could not be resolved so that ratio obtained is for the composite spot.

c $^{3}H/^{14}C$ ratios represent averages of at least 3 trials. Analysis of individual proteins generally agreed within 10-15%.

Table VIII. Normalized $^{3}H/14_{C}$ ratios [c] of 50S proteins in reconstituted particles.

50S protein	Reconstituted particles 32S	37S	48S	48S [a]
L1	0.75	1.23	1.00	0.96
L2	0.14	0.48	0.74	0.83
L3	0.28	0.56	1.36	0.83
L4	0.48	0.91	0.84	0.80
L5	0.29	0.59	0.70	0.65
L6	0.19	0.38	0.39	0.33
L7	0.26	0.25	0.25	0.16
L8, L9 [b]	0.25	0.47	0.61	0.58
L10	0.50	0.72	0.77	0.58
L11	0.45	0.64	0.83	0.63
L12	0.13	0.29	0.19	0.15
L13	0.20	0.56	0.84	1.03
L14	0.15	0.49	0.85	0.71
L15	0.21	0.61	0.96	0.96
L16	0.14	0.28	0.30	0.34
L17	0.16	0.51	0.83	0.75

50S protein	Reconstituted particles 32S	37S	48S	48S [a]
L18	0.21	0.56	0.76	0.61
L19	0.17	0.34	1.00	0.82
L20	0.37	1.14	0.74	0.89
L21	0.72	1.25	1.07	1.04
L22	0.15	0.72	0.97	0.72
L23	0.32	0.53	0.86	1.05
L24	0.64	0.85	0.92	0.94
L25	0.18	0.21	0.57	0.34
L26	0.71	0.50	0.40	0.40
L27	0.09	0.25	0.52	0.60
L28	0.17	0.14	0.58	0.52
L29	0.32	0.42	0.90	1.03
L30	0.12	0.22	0.58	0.49
L31	0.11	0.15	0.07	0.16
L32	0.05	0.12	0.64	0.50
L33	0.23	0.16	0.15	0.23
L34	0.13	0.75	0.98	1.47

For footnotes, see Table VII.

of the proteins were bound in very reduced amounts. This shows that the majority of the proteins were bound very weakly at the low temperature. In the 37S particles formed at 37°, as many as 17 proteins were bound in substantial amounts. In the 48S particles, 19 proteins were bound in nearly normal amounts (a ratio of 0.7 or greater), 9 in slightly reduced amounts (a ratio between 0.4 and 0.7) and 6 in reduced amounts (a ratio lower than 0.4). Comparisons of the normalized ratios for each protein in each species of reconstituted particle showed that increases in the S-value of the particles from 32S to 37S to 48S were accompanied by increased binding of proteins. The fact that 6 proteins -L6, L7, L12, L16, L31 and L33, were in markedly reduced amounts in 48S particles probably explained why these particles were inactive in many ribosomal functions (see later for a more detailed discussion of the protein data).

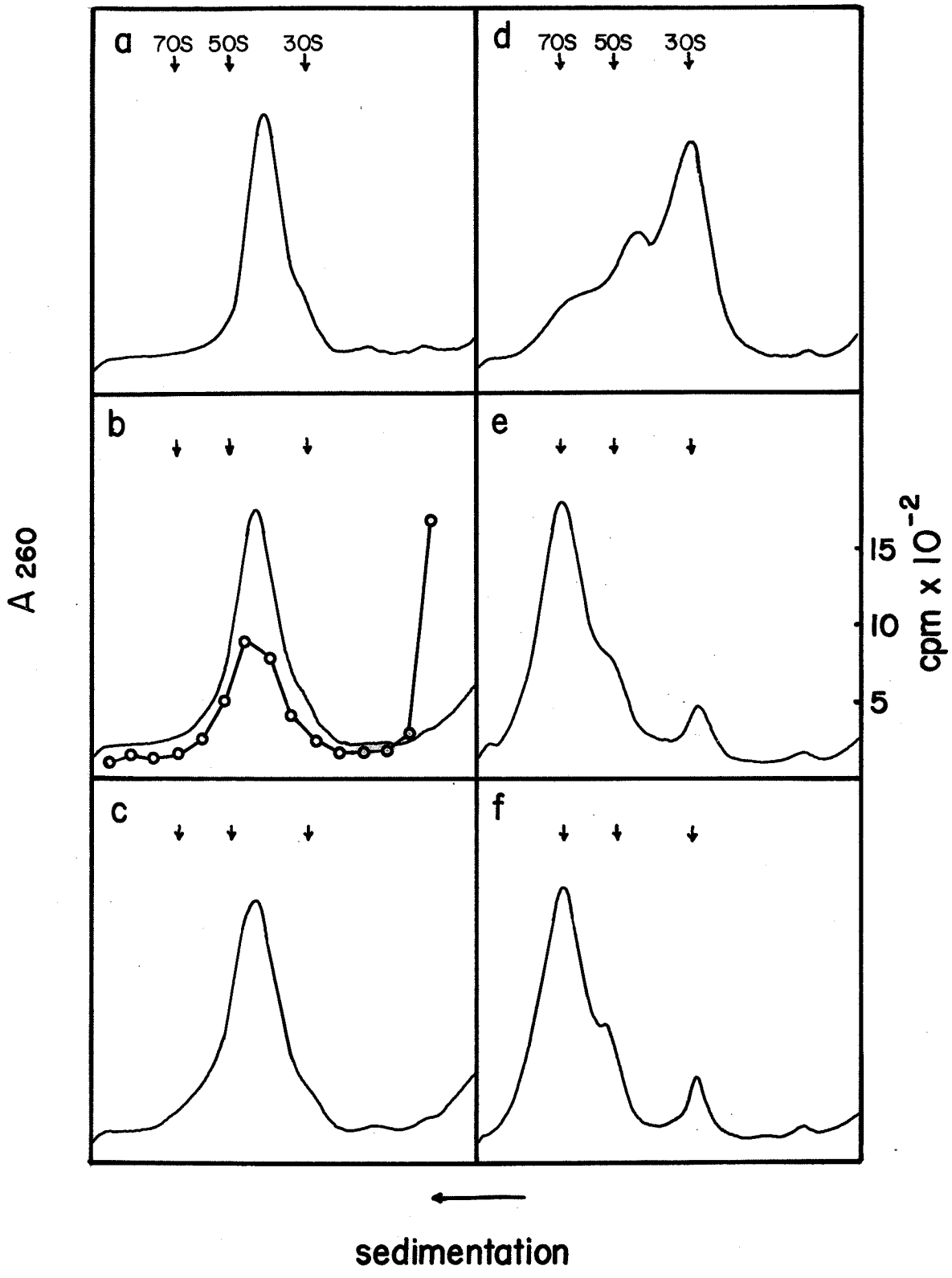
The results in Table VIII were obtained from reconstituted particles isolated on sucrose gradients and precipitated by ethanol (Methods). To show that ethanol precipitation did not induce loss of proteins, 48S particles were recovered by ultracentrifugation instead of ethanol precipitation. As shown in Table VIII column "48S^{a}", protein analysis of such particles showed no significant differences in protein composition or quantity from ethanol precipitated 48S particles indicating that ethanol precipi-

tation of particles, as such, did not introduce artifacts.

Binding of Proteins to 37S Particles at 50°

The ability of 48S particles to associate with 30S subunits may be due to temperature-induced changes in the 37S complexes, per se, or to temperature dependent binding of proteins to 37S complexes. To distinguish between these alternatives, 1.5 A_{260} units of isolated 37S reconstituted particles were incubated at 50° for 30 min in the absence of exogenous 50S proteins or with 50S proteins (3.0 A_{260} equivalents) added either during or after incubation. The particles were freed of unbound proteins by sucrose gradient centrifugation and were then tested for subunit association ability. The results in Figure 38 showed that incubation of 37S particles alone resulted in particles that were inactive in subunit association, although the S-value of particles increased to 43S (Fig. 38a). However, addition of 50S proteins either during or after incubation at 50° resulted in the formation of 45S particles that could associate with 30S subunits (Fig. 38b, c). It was therefore likely that incubation at 50° induced changes in 37S particles that facilitated binding of additional 50S proteins, and that these proteins were related to the ability to associate with 30S subunits.

Figure 38. Subunit association of isolated 37S reconstituted particles incubated with or without exogenous 50S proteins. Before association, 37S particles (1.5 A_{260} units) were incubated at 50° for 30 min in (a) the absence or (b) presence of exogenous (^3H) 50S proteins (3.0 A_{260} equivalents) in 0.1 ml SRS buffer. In (c), the particles were cooled to 37°, then incubated for 20 min at this temperature with exogenous 50S proteins. The open circles in panel (b) represent the (^3H) protein binding profile. Panels (d, e and f) show complexes formed when particles shown in (a), (b) and (c) respectively were mixed with heat-activated 30S subunits (0.8 A_{260} unit). After incubation at 37° for 20 min, the associated mixtures were chilled in ice and analyzed by sucrose gradient centrifugation (Methods).


a
70S
50S
30S
d
70S
50S
30S
b
e
c
f
A 260
15
10
5
cpm x 10⁻²
sedimentation

To find the amount of 50S proteins required for maximal yield of 45S particles, 37S particles were incubated at 50° in the presence of varying amounts of 50S proteins and the resulting particles were tested for subunit association activity. Areas under the 70S peaks in sedimentation profiles were measured and plotted as relative yields of 70S particles against A_{260} equivalents of 50S proteins added per A_{260} unit of 37S particles (Fig. 39). Maximal amounts of 30S subunit associating particles was formed at a 50S protein/37S particle ratio of 1.5.

To identify the additional proteins that were bound to 37S particles at 50°, (^{14}C) 50S proteins were incubated with 37S particles under conditions optimal for formation of 45S particles. Labelled 45S reconstituted particles (Fig. 38b -o-) were freed of unbound proteins by sucrose gradient centrifugation and proteins extracted from them were resolved by bidimensional gel electrophoresis. Radioactivity associated with each protein spot was assayed (Table IX) as described previously (Methods). In 3 trials, (^{3}H) label was found associated with only eight 50S proteins -L2, L5, L8-9, L10, L11, L15, L18 and L25. Reconstituted 48S particles were therefore different from the 45S particles not only in S-value, but also in protein composition. Under these conditions, only a few proteins showed increased

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Figure 39. Titration of formation of 45S particles by subunit association. Isolated 37S reconstituted particles (1.5 A_{260} units) were incubated with indicated amounts of exogenous 50S proteins at 50^{o} for 30 min. The resulting particles were tested for association with 0.8 A_{260} unit of heat-activated 30S subunits as in the legend to figure 38. The relative amounts of 70S couples formed were determined as described in "Methods".

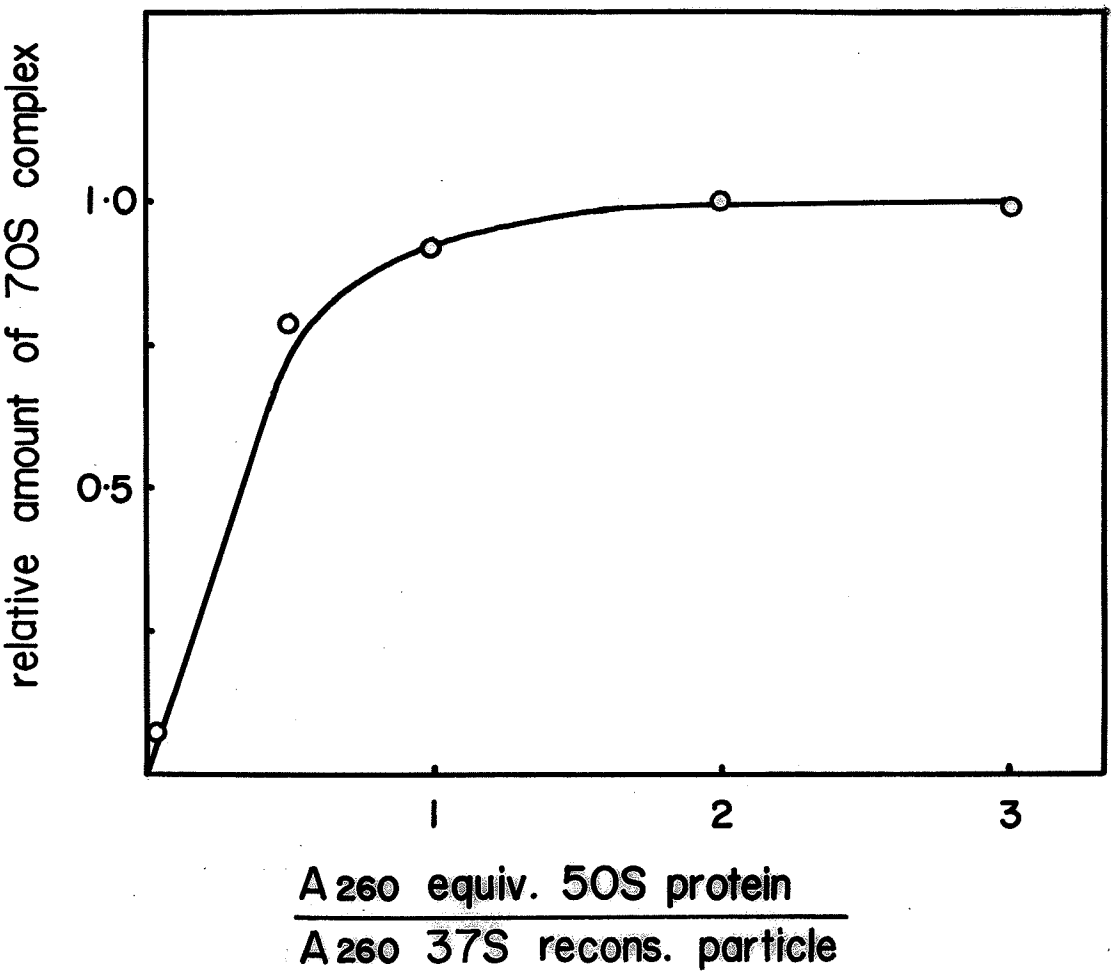
relative amount of 70S complex
1·0
0·5
1
2
3
A260 equiv. 50S protein
A260 37S recons. particle

Table IX. Binding of (^{14}C) 50S proteins to 37S reconstituted particles at 50°.

50S proteins	(^{14}C) label in c.p.m. [a] (corrected for background)
L1, 3, 4, 6, 7, 12, 13, 14, 16, 17, 19, 20, 21, 22, 23, 24, 26, 27, 28, 29, 30, 31, 32, 33, 34	0-35
L2	186
L5	180
L8-9	284
L10	378
L11	150
L15	330
L18	346
L25	146

[a] radioactivity represents average of 3 trials.

binding to 37S particles. However, both 48S and 45S particles were able to associate with 30S subunits. The differences in protein binding could probably be attributed to differences in the conditions of reconstitution and to possible changes in 37S particles brought about by the isolation process.

V. DISCUSSION

Although self-assembly of 30S ribosomal subunits from 16S RNA and 30S ribosomal proteins derived from many species of bacteria proceeds readily (Nomura et al, 1968; Traub and Nomura, 1969), that of the 50S subunits has proven more refractile. Possibly this reflects a more complex assembly reaction and the presence of greater kinetic energy barriers. This is not surprising in view of the complex structure of the large ribosomal subunit. Nonetheless, reconstitution of functional Bacillus stearothermophilus 50S subunits was demonstrated by Nomura and Erdmann (1970). Two reports of successful reconstitution of E. coli 50S subunits have since appeared. Maruta et al (1971) described the reassembly of functional E. coli 50S ribosomal particles from rRNA and a 50S protein fraction that contained RNase II, bovine serum albumin and various oligonucleotides. The system required concomitant reassembly of E. coli 30S subunits. Unfortunately, the work could not be verified in this or other laboratories (Chu and Maeba, 1973; Nomura, 1973). Very recently, Nierhaus and Dohme (1974) reported total reconstitution of functionally active E. coli 50S subunits from 23S RNA and total 50S

proteins by a two-step incubation procedure; first at 40° for 20 min in the presence of 4mM magnesium and 400mM NH_4Cl, then at 50° for 90 min in 20mM magnesium. However, in our hands, their procedure has not yielded the expected results, possibly due to differences in the strain of organisms used as well as to variations in the methods of preparation of ribosomal components (Chu and Maeba, In Press).

The system described in this thesis exploits the fact that 23S RNA extracted from 70S ribosomes was intact whereas that extracted from 50S ribosomal subunits or isolated from rRNA was extensively nicked (Fig. 2; Ceri & Maeba, 1973). Since formation of reconstituted particles with S-values approaching 50S required incubation temperatures of 50° or greater (Results), it was essential that breakdown of RNA (Tal, 1969) should be prevented during reconstitution. This is borne out by the fact that intact 23S RNA was essential for formation of 48S particles at 50° or 55°, but not for formation of the smaller particles at 0° and 37° (Fig. 10). Fahnestock et al (1972) also observed that Bacillus stearothermophilus 50S subunits reconstituted from RNA and protein derived from 70S ribosomes were more active then those formed from RNA and proteins derived from 50S subunits.

It was of interest to note that fragments of 23S RNA were able to bind 50S proteins (Fig. 10 e,f). If the fragments were produced by specific cleavages, as the electrophoretic scans suggested (Fig. 2d), these facts might be exploited to determine the sequence of 50S proteins along portions of the 23S RNA molecule. Similar lines of research are already in progress in a number of laboratories (see Schendel et al, 1972). While isolated 23S RNA was extensively nicked so that it could not effectively participate in reconstitution, especially in the formation of 48S particles, integrity of 23S RNA was preserved when 16S RNA was present (Fig. 2b).

If reconstitution of 50S subunit required concomitant assembly of 30S subunits as others have suggested (Maruta et al, 1971; Nashimoto and Nomura, 1970), the requirement may have been partially filled by the presence of 16S RNA in the system. If such were the case, it participated after formation of the 32S (or 37S) complexes at 37^{o} as these complexes were formed in the absence of 16S RNA (Fig.10e). Also, when reconstitution was carried out with simultaneous 30S subunit assembly, there was no stimulation of 48S particle formation (Fig. 13b) so that in this system, the requirement for 30S components was minimal.

The formation of progressively larger ribonucleoprotein particles composed of E. coli 50S ribosomal components was obtained by incubating rRNA and 50S proteins in sequence at 0^{o}, 37^{o} and 55^{o}. Two criteria were used to assess the specificity of protein binding. Firstly, 50S proteins were bound to 23S RNA in the presence of 16S RNA (Figs. 7 and 8). Secondly, binding was associated with increases in S-value of ribonucleoprotein particles (Figs. 4-8). Incubation of reconstitution mixture at 0^{o} led to the formation of particles that sedimented at 28S in 1.0mM magnesium (TKM II) buffer and 32S in 10mM magnesium (TKM I) buffer (Figs. 7a and 8a). Incubation at 37^{o} yielded particles sedimenting at 32 or 37S in low or high magnesium buffers, respectively, (Figs. 7b and 8b). When temperature of incubation was shifted from 37^{o} to 50 or 55^{o}, particles sedimenting at 43 or 48S, in low or high magnesium, respectively, were formed (Figs. 7c and 8c). The protein and RNA compositions of particles formed under each condition were identical (Plates II - V) and the differences in their S-values could be attributed to the magnesium concentration used for the determination - in 10mM magnesium, identical particles sedimented faster than in 1.0mM magnesium (Fig. 9). That S-values of ribosomes and subunits can be altered by different magnesium concentrations without loss of proteins has been well documented (Gesteland,

1966). For purposes of simplicity and identification, the S-values obtained in 10mM magnesium, i.e., the higher values, will be used throughout the "Discussion". The formation of reconstituted particles can be summarized as follows:

$$\text{rRNA} \xrightarrow{0^\circ} 32S \xrightarrow{37^\circ} 37S \xrightarrow{50\text{-}55^\circ} 48S$$

+ 50S proteins

The formation of particles with successively greater S-values was accompanied by the binding of increasing amounts of 50S proteins, such that 48S particles contained almost as much protein (> 90%) as in 50S subunits (Fig. 17). The specificity of binding of proteins in particles was supported by the fact that particles bound equivalent amounts of proteins even in the presence of excess proteins (Fig. 16). Also, the proteins bound preferentially to 23S RNA in the presence of 16S RNA (Figs. 7 and 8).

Particles of similar sizes were formed in the assembly of E. coli 50S subunits in vivo (Osawa et al, 1969; Mangiarotti et al, 1968). Also, in studies of temperature-sensitive mutants (Guthrie et al, 1969; Nashimoto and Nomura, 1970), 32S and 43S particles accumulated at restrictive temperatures which could be converted to 50S subunits upon temperature shift up to 42°. These findings suggest that similar particles may participate in in vivo and in vitro assembly of 50S subunits.

In the absence of spermidine, the formation of 48S particles at 55° depended upon incubation of the reconstitution mixture at 37°, indicating that its formation depended upon the formation of 37S particles. The formation of 37S particles, on the other hand, did not require preincubation at 0°. This suggests that 32S particles formed at 0° might not be important in the reconstitution process, or that they might be transitory intermediates that could be formed at 0 or 37°. Since both types of particles were formed rapidly, i.e., within 5 min (Fig. 4), it may be that at 37° incubation, the conversion of 32S particles, that were initially formed, to 37S particles took place within 5 min.

The rate-limiting step in the process was the conversion of 37S particles to 48S at 50° or 55°. Under conditions initially employed, i.e., SR buffer with 1.2 fold equivalent excess of 50S proteins, a maximum of approximately 70% of the 37S particles were converted to 48S particles in 20 min. Longer periods of incubation led to breakdown of RNA, probably due to the RNase activity mentioned previously. Two modifications to the system greatly improved the yield of partially reconstituted particles - the inclusion of spermidine (Fig. 18) and the use of excess amounts of 50S proteins in the incubation mixture (Fig. 14).

When 3-fold equivalent excess of 50S proteins were used, all 37S particles were converted to 48S and the breakdown of RNA was prevented as judged by the absence of A_{260}-absorbing material at the top of sucrose gradients. Possibly, slow inactivation of some 50S proteins required for formation of 48S particles took place at higher temperatures which was offset by increased amounts of those proteins. Similar results would also be expected if extraction of 50S proteins yielded a preparation that was deficient in proteins required for 48S particle formation.

The absence of RNA breakdown under these conditions indicated that 23S RNA in 37S particles was more susceptible to autodegradation at high temperatures than in 48S particles - once the latter were formed, degradation was prevented. Most likely, conversion to 48S particles led to an RNA conformation more resistant to autodegradation. This is supported by the fact that magnesium, which plays an important role in maintaining different conformational states of ribosomes and subunits (Gesteland, 1966), also played a critical role in preventing autodegradation during reconstitution (Fig. 27).

The inclusion of the polyamines, spermidine or spermine, in the reconstitution mixture increased the rate of formation of 48S particles at 50^{o} or 37 + 55^{o}, although they were not absolutely required for particle formation (Fig. 18) and could not replace magnesium in reconstitution (Fig. 26). In the presence of spermidine, the time for

maximal formation of 48S particles was almost halved (Fig. 18). Although the role of polyamines in ribosome structure is not well understood (Cohen, 1971), they may help to stabilize rRNA in a conformation that allows the binding of 50S proteins, particularly at high temperatures (Fig. 19). Evidence presented here indicates that polyamines maintained rRNA in a configuration that resisted nucleolytic attack. The agent responsible for autodegradation appeared to be tightly bound to 23S RNA (Fig. 28).

The stimulatory role of spermidine in reconstitution was different from that described by Hosokawa et al (1973) who reported that polyamines were essential for the formation of faster-sedimenting particles in a similar reconstitution system at 42°. Perhaps the requirement for polyamines in reconstitution at 42° could be substituted by heat so that 48S particles were formed at 50-55° even in the absence of polyamines.

Cohen and Lichtenstein (1960) observed that ribosomal fractions contained significant amounts (about 1.5%) of polyamines, putrescine and spermidine and that certain concentrations of spermidine preserved the integrity of large ribosomal particles even in the absence of magnesium. Furthermore, they found that magnesium and spermidine exerted a cooperative effect in the stabilization of ribosomal structure. This lends support to the finding (Results)

that spermidine could not replace magnesium in reconstitution and that the relationship between the two was a cooperative one.

The proteins associated with each species of reconstituted particles were identified and their quantities, relative to 50S subunits, were presented in Table VII & VIII. Although reconstituted particles sedimented as single peaks in sucrose gradients (Fig. 23a, b, c), the data show that they were quite heterogeneous with respect to protein composition. The observed protein heterogeneity in reconstituted particles suggests that binding of 50S proteins to 23S RNA might proceed by alternative routes as in the assembly of E. coli 30S subunits (Nomura, 1973; Historical).

The 32S particles formed at 0^{o} contained only 4 proteins (L1, L21, L24, L26) in substantial amounts (normalized ratios greater than 0.5). The rest of the proteins were all bound to some extent, but in very reduced amounts. The reduced binding of most proteins, coupled with the fact that 5S RNA was found in less than stoichiometric amounts (60%) in these particles (Table V), indicates that formation of ribonucleoprotein particles at 0^{o} may not be as specific as that at higher temperatures (37^{o} and 50 or 55^{o}). The 5S RNA was present in 37S and 48S particles in stoichiometric amounts. However, the fact that 2 (L1, L24) of the 4 proteins were among the ten 50S proteins known to bind directly to 23S RNA (Stoffler et al, 1971; Garrett et al,

1974) seems to imply some specificity in the binding process at 0^{o}.

A clearer picture of the proteins in the 37S and 48S reconstituted particles, and of the process of binding, is obtained by reorganizing the data of Table VIII as in Table X.

In Table X, 50S proteins were divided into 2 major classes on the basis of their quantities in 37S particles compared to their amounts in 48S particles. Class I proteins were bound in essentially the same amounts in both 37S and 48S particles whereas Class II proteins were bound in greater amounts in 48S particles. The subclasses a, b and c indicate whether the increase resulted in stoichiometric binding to 48S particles. In 37S particles, only 8 Class Ia proteins were bound in stoichiometric amounts (normalized ratios greater than 0.7) whereas 15 proteins in Classes Ib and IIa were found in slightly reduced amounts (normalized ratios between 0.4 and 0.7), and 11 proteins of Classes Ic and IIb were in reduced amounts (normalized ratios less than 0.4). The 48S particles, on the other hand, bound 19 proteins in almost stoichiometric amounts (Classes Ia and IIa); 9 in slightly reduced amounts (Classes Ib and IIb); and 6 in reduced amounts (Class Ic).

The formation of 48S complexes involved binding of greater amounts of proteins to 37S particles. Proteins showing the greatest increase in binding were the Class II

Table X. Classification of 50S proteins in reconstituted 37S and 48S particles.

50S protein	Normalized ratio		50S protein	Normalized ratio	
	37S	48S		37S	48S
Class I. Approximately same amounts in both particles.			**Class II. Increased binding to 48S particles.**		
(a) Stoichiometric amounts in both			(a) Stoichiometric amounts in 48S		
L1	1.23	1.00	L2	0.48	0.74
L4	0.91	0.84	L3	0.56	1.36
L10	0.72	0.77	L11	0.64	0.83
L20	1.14	0.74	L13	0.56	0.84
L21	1.25	1.07	L14	0.49	0.85
L22	0.72	0.97	L15	0.61	0.96
L24	0.85	0.92	L17	0.51	0.83
L34	0.75	0.98	L18	0.56	0.76
(b) slightly reduced amounts in both			L19	0.34	1.00
L5	0.59	0.70	L23	0.53	0.86
L8-9	0.47	0.61	L29	0.42	0.90
L26	0.50	0.40	(b) slightly reduced amounts in 48S		
(c) Reduced amounts in both			L25	0.21	0.57
L6	0.38	0.39	L27	0.25	0.52
L7	0.25	0.25	L28	0.14	0.58
L12	0.29	0.19	L30	0.22	0.58
L16	0.28	0.30	L32	0.12	0.64
L31	0.15	0.07			
L33	0.16	0.15			

proteins - of these, 11 proteins (Class IIa), that were bound in reduced or slightly reduced amounts in 37S particles, became bound in nearly stoichiometric amounts in 48S particles. Five proteins (Class II b), that were bound in reduced amounts in 37S particles, became bound in only slightly reduced amounts in 48S particles. In forming 48S particles, the normalized ratios for Class II proteins increased on the average by 0.39 indicating a substantial increase in these proteins. Some proteins in Class Ia also increased in their binding to 48S complexes, e.g. L22 and L34, but since they were already present in almost stoichiometric amounts in 37S particles, they were grouped as Class I proteins.

The differential binding of 50S proteins to 37S and 48S reconstituted particles strongly resembled protein binding to biosynthetic 50S precursors isolated by Nierhaus et al (1973). Of the nineteen 50S proteins bound in almost stoichiometric amounts in 48S reconstituted particles, 13 were found in the 50S subunit precursor particles (Table XI). Furthermore, of the six 50S proteins that were bound in very reduced amounts in the reconstituted particles, 5 were absent and 1 was present in reduced amount in the in vivo 50S precursor particles (Table XI). There was some resemblance to the order of binding of 50S proteins during 50S biosynthesis as determined by Pichon et al (1972), but due to

Table XI. Comparison of protein composition of 48S particles with that of other complexes derived from 50S ribosomal components.

50SP	amount bound	(a) p_o50S	(b) $p_1$50S	(c) 0.6C	(d) 23S RNA binding proteins	50SP	amount bound	(a) p_o50S	(b) $p_1$50S	(c) 0.6C	(d) 23S RNA binding proteins
L1	S	+	+	+	*	L18	S	+	+	+	
L2	S	+	-	+	*	L19	S	+	-	+	*(?)
L3	S	+	+	+	*	L20	S	-	+	-	*
L4	S	±	+	+	*	L21	S	±	+	+	
L5	SR	+	+	+		L22	S	+	+	+	
L6	R	+	-	±	*	L23	S	+	+	+	*
L7	R	+	±	-		L24	S	+	+	+	*
L8	SR	+	+	±		L25	SR	+	+	+	
L9	SR	+	+	+		L26	SR	±			
L10	S	+	+	+		L27	SR	+	+	±	
L11	S	+	±	+		L28	SR	-	-	-	
L12	R	+	±	-		L29	S	±	+	+	
L13	S	+	+	+	*	L30	SR	±	+	+	
L14	S	+	-	±		L31	R	-	-	-	
L15	S	+	-	±		L32	SR	±	-	+	
L16	R	+	-	-	*	L33	R	-	-	-	
L17	S	+	+	+		L34	S				

S Bound in stoichiometric amounts (normalized ratios greater than 0.7)
SR Bound in slightly reduced amounts (normalized ratios between 0.4 and 0.7)
R Bound in reduced amounts (normalized ratios less than 0.4)
(a) p_o50S particle from precursor rRNA and 50S proteins (Nikolaev and Schlessinger, 1974)
(b) de novo 50S precursors (Nierhaus et al, 1973)
(c) Cores from 0.6M LiCl treatment (Homann and Nierhaus, 1971)
(d) 23S RNA binding proteins (Garrett et al, 1974)
+, present in normal amount, ±, present in reduced amount,
-, present in traces or absent, * 23S RNA binding protein

differences in methodology, which was to follow the rate of appearance of labelled ribosomal proteins on to the mature 50S subunit, direct comparisons cannot be made. These available data suggest that the order of protein binding during *in vitro* assembly may be similar to that *in vivo*. The protein composition of 48S particles reconstituted in the present system as compared to those of other complexes derived from 50S ribosomal components is presented in Table XI.

Nikolaev and Schlessinger (1974) obtained ρo 50S particles sedimenting at 46S on mixing precursor rRNA with 50S proteins. The protein composition of ρo 50S closely resembled that of 48S particles (Table XI). For example, proteins that were absent or reduced in ρo 50S particles appeared as Class Ic (L31, L33) or Class IIb (L28, L30, L32) proteins found in fractional amounts less than 0.5 in 48S particles. However, there were some discrepancies, the most obvious being that L7 and L12 present in ρo 50S particles were almost absent from 48S particles. Within limits, it appeared that reconstitution led to formation of complexes with similar protein compositions in both systems. These observations further strengthen the argument that reconstitution proceeds as *in vivo*.

Of the ten 50S proteins which are known to bind specifically and independently to 23S RNA (Stoffler *et al*,

1971; Garrett et al, 1974), 8 were present in 48S particles in approximately stoichiometric amounts (Classes Ia and IIa). This was to be expected if formation of 48S particles occurred in a sequential manner. The other 2 proteins, L6 and L16 (Class Ic), though capable of direct binding to 23S RNA, did so poorly (at a molar protein/RNA ratio of 0.3) and only at high protein/RNA ratios (Garrett et al, 1974).

Fifteen proteins were bound in fractional amounts less than 0.7 in 48S particles (Classes Ib, Ic and IIb). A comparison of protein composition with other complexes derived from E. coli 50S subunits components shows that these proteins also were not readily bound (Table XI). For instance, these proteins were the easiest to remove from 50S subunits with LiCl (Homann and Nierhaus, 1971; Kaltschmidt et al, 1971), and were the last proteins to be added to the 50S subunit during biogenesis (Pichon et al, 1972). The data summarized in Table XI indicate that reconstituted 48S particles were similar in structure to other 50S complexes obtained in vitro and in vivo and suggest that reconstitution in the present system was proceeding in the correct manner.

It was not clear why 48S particles failed to bind proteins L7 and L12 as both L10 and L11, which have been shown to facilitate the binding of L7 and L12 (Schrier et al, 1973; Stoffler et al, 1974; Highland and Howard,

1975), were present in nearly stoichiometric amounts. Incubation of 48S particles with exogenous L7 and L12 extracted by alcohol-NH_4Cl treatment of 50S subunits (Hamel et al, 1972; Brot et al, 1973) had no effect upon either the S-value or protein composition of reconstituted particles (Plate VII). The discrepancy may lie in the fact that 48S particles had very reduced amounts of L6, L16, L33 and L31 all of which were present in particles employed in partial reconstitution systems. Therefore some or possibly all of these 4 proteins may be required, in addition to L10 and L11, for the binding of L7 and L12 in 50S subunits.

Although reconstituted particles were protein deficient, they contained 5S RNA. The 32S particles had 60% of the amount of 5S RNA in 50S subunits whereas the 37S and 48S particles possessed stoichiometric amounts of 5S RNA. Proteins L18 and L25 were bound directly to 5S RNA to form ribonucleoprotein complexes (Gray et al, 1972; Horne and Erdmann, 1972) that could bind to 23S RNA in the presence of proteins L2 and L6. A stable complex was formed which was isolated by sucrose gradient centrifugation (Gray and Monier, 1972). The fact that 37S particles deficient in L2, L6, L18 and L25 (normalized ratios of 0.48, 0.38, 0.56 and 0.21 respectively) and 48S particles deficient in L6 and L25 (normalized ratios of 0.39 and 0.57 respectively) both contained equivalent amounts of 5S RNA shows that

other proteins might be involved in the incorporation of 5S RNA into the 50S subunit structure. However, an alternative explanation that L2, L6, L18 and L25 could substitute for each other should not be ruled out. The accumulated evidence shows that 5S RNA was incorporated at an early stage in reassembly.

Since the largest reconstituted particles contained 6 proteins in reduced amounts, it was not surprising that they were inactive in poly(U)-directed polyphenylalanine synthesis (Table VI). The fact that 48S particles, though possessing L11 in almost stoichiometric amount, were also inactive in the peptidyltransferase reaction agrees with the observation that protein L11 by itself was neither the peptidyltransferase nor the peptidyltransferase centre (Ballesta and Vazquez, 1974; Howard and Gordon, 1974), contrary to the original suggestion by Nierhaus and Montejo (1973). The inability of these particles to bind fMet-t RNA_F^{met} may also account, in part, for the observed absence of peptidyltransferase activity (Table VI).

Both 37S and 48S reconstituted particles were partially active with respect to IF-catalyzed and EF-catalyzed GTPase activities (Table VI). The observation that 48S particles exhibited higher activities in IF-catalyzed uncoupled, IF-catalyzed coupled and EF-catalyzed uncoupled GTP hydrolysis (54, 62 and 81%)of activity of 50S controls respectively) than 37S particles (40, 45 and

42% of activity of 50S controls respectively) indicates that GTPase activities associated with the reconstituted particles were related to the amount of proteins bound in the particles and that the activities were quite specific.

Kolakofsky et al (1968) first made the observations that GTP was hydrolyzed concomitantly with IF-promoted binding of fMet-tRNA to ribosomes and that IF-2 possessed a ribosome-dependent GTPase activity independent of the other components of initiation and distinct from the ribosome dependent GTPase activity of polypeptide chain elongation factor G, originally described by Nishizuka and Lipmann (1966). The observation that the reconstituted particles were partially active in GTP hydrolysis was unexpected because the particles lacked proteins L7 and L12 (Table X) both of which were shown to be essential for the expression of ribosome dependent GTPase activity (Hamel et al, 1972; Brot et al, 1973; Lockwood et al, 1974). Two possibilities existed: either that L7 and L12 were not absolutely required for ribosome dependent GTPase activity, or that the crude supernatant fractions, containing IF and EF respectively, were contaminated by L7 and L12 which could bind in the reconstituted particles under the conditions of assay. The second possibility was more plausible as proteins L7 and L12 have been detected in the high speed supernatant of E. coli (Brot, personal communication).

However, for a more accurate assessment of the GTPase activities of reconstituted particles, purified protein factors (IF-1, 2, 3, and EF-T_u, T_s, G) from the respective supernatant fractions would have to be used. As the hydrolysis of GTP by IF and EF were essential energy-yielding reactions in the process of translation, and more specifically, in the initiation and elongation steps, the 37S and 48S particles which were partially active in GTP hydrolysis must possess many of the characteristics of the 50S subunits.

The 48S particles, but not 37S particles, were able to associate with 30S subunits to form 70S complexes (Fig. 23). The association of the reconstituted particles with 30S subunits occurred stoichiometrically at a 1:1 ratio (Fig. 32). The 48S particles reconstituted at 50° competed with 50S subunits for 30S subunits during association (Fig. 33) and even exhibited subunit association activity equal to that of 50S subunits (Figs. 34 and 35).

Noll et al (1973) stated that the ability of 30S and 50S subunits to form 70S couples was a stringent test for activity and that the structures necessary for subunit association were critical for the overall biological activity of the ribosomes. Therefore, the specificity with which association took place indicates that 48S reconstituted particles must have many of the structural features of 50S subunits. However, since these particles were inactive in poly(U)-directed polypeptide synthesis and in the peptidyl-

transferase reaction, the ability to form 70S couples must be distinct from these 2 functions and could represent one of the many reactions involved in these functions.

From the protein composition of 48S particles (Table X), it could be inferred that proteins present in very reduced amounts Class Ic) were not involved in subunit association. As expected, proteins that were implicated in subunit association or at the subunit interface (Morrison et al, 1973; Michalski and Sells, 1974; Litman and Cantor, 1974) appeared in Classes Ia and IIa, i.e., proteins present in stoichiometric amounts in 48S reconstituted particles. However, 3 of the subunit interface proteins, namely L6, L26 and L27, were present in fractional amounts of 0.39, 0.40 and 0.52 respectively. Since inactivation of these 3 proteins by specific antibodies only partially inhibited association (Morrison et al, 1973), their requirement in subunit association might not be critical.

Although only 48S particles were able to associate with 30S subunits, 37S particles could do so if incubated at 50° in the presence of exogenous 50S proteins (Fig. 38 e, f). Incubation of 37S particles at 50° in the absence of proteins resulted in increase in S-value to 43S which were inactive in subunit association (Fig. 38d). The proteins added on to 37S particles at 50° were L2, L5, L8-9, L10, L11, L15, L18 and L25 and conferred upon the particles the ability to associate with 30S subunits.

Although the binding of these proteins could not be readily quantitated, their requirement in subunit association could be inferred by comparing their differential amounts in 37S and 48S particles (Table VIII). For instance, the increases in amounts of L5, L8-9 and L10 in 45S particles were probably only marginal since they appeared in essentially the same amounts in 37S and 48S particles. This indicates that the apparent binding of these proteins to 43S particles might have resulted from exchange rather than additional binding, and that their requirement in subunit association might be minimal. The remaining 5 proteins, L2, L11, L15, L18 and L25 increased substantially in 48S particles and their presence in 45S particles, therefore, was likely the result of increased binding rather than exchange. Furthermore, these 5 proteins were likely to be involved directly or indirectly in subunit association. Since they were already present in fairly high amounts in 37S particles, it was unlikely that increases in any single one of these proteins was responsible for the property of subunit association. Also, since 37S complexes were highly heterogeneous with respect to protein composition, increased binding of exogenous 50S proteins would reduce protein heterogeneity of reconstituted particles. Possibly the 45S particles were homogeneous with respect to a complement of proteins that were required for subunit association. If

such were the case, the protein complement required for subunit association would include a few or all of the 5 proteins.

The available data concerning 50S proteins present at the subunit interface varied considerably depending on the method of analysis. These are presented in Table XII. The proteins (L2, L11, L15, L18, L25) identified here as required for subunit association agreed to some extent with all analyses - L15 of Morrison et al (1973); L2 and L18 of Litman and Cantor (1974); and L18 of Michalski and Sells (1974), but bore little resemblance to any one analysis. The differences may be due to the fact that the method described here identified proteins that were required for activity rather than those that physically participated in association. It may be that some proteins exert long range effects possibly through conformational alterations that allow subunits to associate, and that these proteins need not be directly involved at the interface. More work is required to further characterize the 5 proteins into either of these categories.

Since the conversion of 37S to 45S particles could be achieved whether 50S proteins were added during or after incubation at 50^{o} (Fig. 38 b, c), incubation at 50^{o} probably induced conformational changes in 37S particles

Table XII. Comparison of 50S proteins implicated in subunit association in different systems.

Method of approach	50S proteins
(1) I_gG antibodies to 50S proteins (Morrison et al, 1973)	L1,6,14,15,19,20,23, 26, 27
(2) Lactoperoxidase-catalyzed iodination (^{126}I) of 70S and 50S (Litman and Cantor, 1974)	L2,18,26,28
(3) Lactoperoxidase-catalyzed iodination (^{131}I) of tight and relaxed 70S (Michalski and Sells, 1974)	L3,8,9,18,19
(4) Present reconstitution system	L2,11,15,18,25

that would allow proteins to bind. Similar results were obtained from studies of the function of temperature on reconstitution in which the effect of temperature on isolated reconstituted particles was to convert them to faster sedimenting species (Table IV).

The manner of heat-activation of 37S particles to 43S particles in the absence of 50S proteins was probably analogous to the mechanism of formation of RI* (heat-activated reconstruction intermediate) particles which participated in the assembly of E. coli 30S subunits (Nomura 1973; Historical). The formation of RI complexes from 30S proteins and 16S RNA proceeded readily at 0° but activation to RI*, a form that could bind the remaining 30S proteins, required heat (Traub and Nomura, 1969). In an analogous way, the binding of 50S proteins to 23S RNA to form 37S particles was complete at 37° within 5 min but activation to 43S particles, a form that could bind more 50S proteins to produce 45S particles which associated with 30S subunits, required heating at 50-55° (Fig. 38).

The work presented in this investigation outlined the approaches used tosstudy the assembly of the 50S ribosomal subunit. The reconstitution reaction, though differing from biosynthetic assembly in some details (Historical), reflects the in vivo process in important ways - the sizes of subparticles formed (Mangiarotti et al,

1968; Osawa et al, 1969; Nashimoto and Nomura, 1970) and their protein compositions (Pichon et al, 1972; Nierhaus et al, 1973). Various reconstitution techniques, which have facilitated the elucidation of structure-function relationships in the E. coli 30S subunit, were employed in the work here. The present study was hampered by the failure to achieve conversion to fully functional 50S particles. One reason for this may be the RNase activity associated with the 50S subunits (Szer, 1969; Staehelin and Maglott, 1969; Ceri and Maeba, 1973). An alternative explanation may be the lability of the protein fraction which was extracted and maintained in high urea and LiCl concentrations before reconstitution. Furthermore, inactivation of 50S proteins during reconstitution when high temperature was employed has been reported (Nomura and Erdmann, 1970; Maruta et al, 1971) and may have occurred here.

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APPENDIX I

Attempts at Reproducing Nierhaus and Dohme's Results

Nierhaus and Dohme (1974) reported successful reconstitution of functionally active 50S ribosomal subunits from E. coli K12 strain A19. To verify their results, their reconstitution procedure was attempted as follows: 23S RNA was extracted from 50S ribosomal subunits of E. coli MRE 600 by the urea-LiCl method (Nomura and Erdmann, 1970) and 50S proteins were prepared in two ways: the urea-LiCl method (Nomura and Erdmann, 1970); and the acetic acid method (Hardy et al, 1969, Nierhaus and Dohme, 1974). Reconstitution of ribonucleoprotein particles from 23S RNA (10 A_{260} units) and 50S proteins (20 A_{260} equivalents) was carried out by the two-step incubation procedure as documented by Nierhaus and Dohme (1974) : first at 40^{o} for 20 min in 20 mM Tris-HCl (pH 7.2), 4.0 mM Mg^{++}, 400 mM NH_4Cl and 2.0 mM 2-mercaptoethanol, then at 50^{o} for 90 min in the same buffer except that the Mg^{++} concentration was raised to 20 mM. The final volume was 200 µl. After incubation, 50 µl of reconstitution mixture were analyzed by sucrose gradient centrifugation (Methods) for S value of reconstituted particles, 50 µl were tested in the subunit association system (Methods) and 50 µl in the poly(U)-directed

(^{14}C)polyphenylalanine synthesis system (Methods).

Compared to external markers, the reconstituted particles sedimented as a 43-45S peak (Fig. 40a). When the reconstituted mixture was tested for association with 30S ribosomal subunits, about 35% of the particles exhibited subunit association activity, giving rise to 70S couples (Fig. 40b). However, the reconstituted mixture was virtually inactive in poly(U)-directed polypeptide synthesis (Table XIII).

As evident from the results, Nierhaus and Dohme's procedure for total reconstitution of 50S ribosomal subunits of E. coli K12 strain 19 yielded only 43-45S ribonucleoprotein particles in the case of E. coli MRE 600. Furthermore, the 43-45S particles were only partially active in subunit association and totally inert in poly(U)-directed polyphenylalanine synthesis. If Nierhaus and Dohme's results should prove to be reproducible, the discrepancy might reside in the difference of the E. coli strains used.

Figure 40. Sedimentation analysis of ribonucleoprotein particles reconstituted according to the procedure of Nierhaus and Dohme (1974) : (a) reconstituted particles alone in a buffer containing 20 mM Tris-HCl (pH 7.3), 400 mM NH_4Cl and 20 mM $Mg(OAc)_2$; (b) reconstituted particles with 30S ribosomal subunits in TKMS I buffer (Methods).

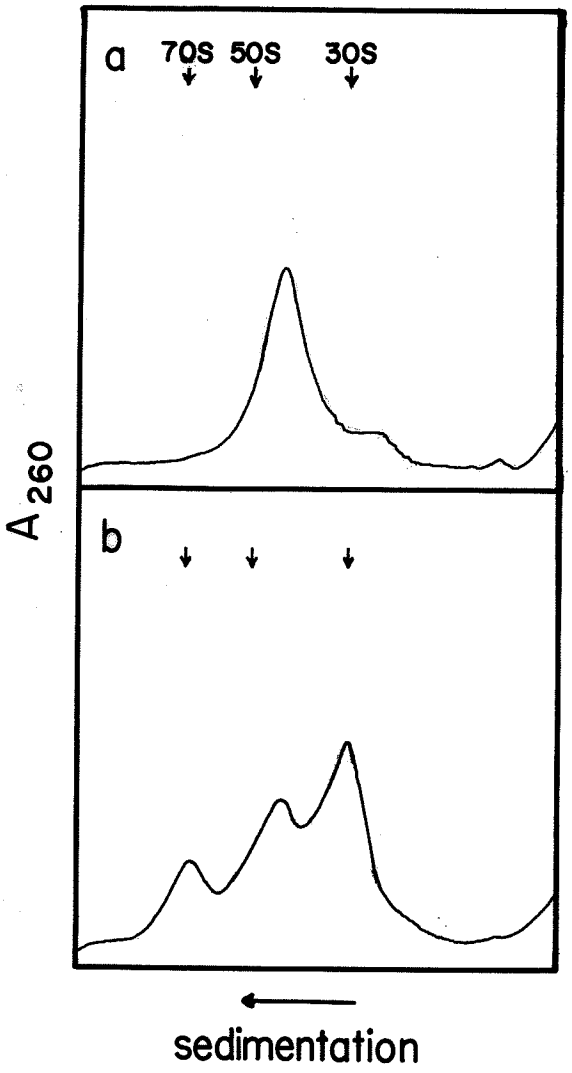
a
70S
50S
30S
b
A260
sedimentation

Table XIII. Activity of 43-45S reconstituted particles in poly(U)-directed polyphenylalanine synthesis.

Ribosomal particles	TCA precipitable (^{14}C) polyphenyl-alanine in c.p.m. over background	% activity
50S	9556	100
43-45S	109	1

APPENDIX II

Detection of Protein Contamination in Ribosomal RNA

Fahnestock, Erdmann and Nomura (1973) found that 23S RNA extracted from B. stearothermophilus 50S ribosomal subunits with 4M urea-2M LiCl still contained large amounts of protein L3 which could be removed from 23S RNA at pH 2.0 in the presence of 4M urea and 0.5 M Mg^{++}. Nierhaus and Dohme (1974), using the same method, detected L3 as well as small amounts of L13, L17 and L22 in purified 23S RNA extracted from E. coli 50S ribosomal subunits. In the present investigation, RNA was extracted twice from 70S ribosomes with 3.2 M urea-3.6 M LiCl (Methods). The RNA (200 A_{260} units) thus obtained was tested for undetached proteins by bidimensional polyacrylamide gel electrophoresis (Kaltschmidt and Wittmann, 1970a; Methods). No stained spots were observed in the slab gel indicating negligible protein contamination. Analysis of rRNA samples for protein by the phenol method of Lowry et al (1951), using crystalline bovine serum albumin as standard, yielded a protein concentration of 0.4 $\mu g/A_{260}$ unit rRNA. Assuming a ratio of 20 μg protein/A_{260} unit RNA in the ribosome, protein con-

tamination of rRNA was about 2%. Also, bidimensional electrophoretic analysis of the supernatant obtained from further extraction of rRNA at pH 2.0 in the presence of 4M urea and 0.5 M Mg^{++} (Fahnestock et al, 1973) revealed no stained spot indicating that if there were ribosomal proteins bound to rRNA after extraction with 3.2 M urea-3.6 M LiCl, the proteins were present in much less than stoichiometric amounts.