A RIBOSOME DISSOCIATING FACTOR FROM RAT LIVER

AN INVESTIGATION IN VITRO OF A RIBOSOME DISSOCIATING FACTOR FROM RAT LIVER

By

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SCOPE AND CONTENTS: Monomeric ribosomes and very few subunits are found in mammalian cells and because subunits are required for initiation of protein biosynthesis in both mammalian and bacterial systems, this implies that the dissociation step in the ribosome cycle does not occur spontaneously. Our attention was drawn to the possibility that the monomeric ribosomes in mammalian cells could complex with a dissociation factor. This factor would perhaps be present in the cell in limited supply and would, therefore have to recycle in the course of initiation, from a completed initiation complex to another free ribosome. An assay was set up whereby the existence of a dissociation factor in a subcellular fraction of rat liver could be determined. The perfecting of the assay system for the dissociation factor yielded much information on the ionic concentration necessary for both ribosome and subunit stability. The factor was found to be present in the fraction containing the "native" subunits. This is identical to the situation which exists in *E. coli*. The factor is capable of dissociating rat liver monomeric ribosomes into 60S and 40S subunits. The factor was found to act on ribosomes freed of both messenger RNA and nascent protein.

Purification of the crude dissociation factor preparation was achieved by obtaining at 4°C the 35-65% ammonium sulphate fraction. Purification was also achieved by means of an incubation of the preparation at 40°C for 30 minutes followed by a centrifugation to remove precipitated protein.

The DF was determined to have a molecular weight in excess of 85,000 by column chromatography.

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PART I

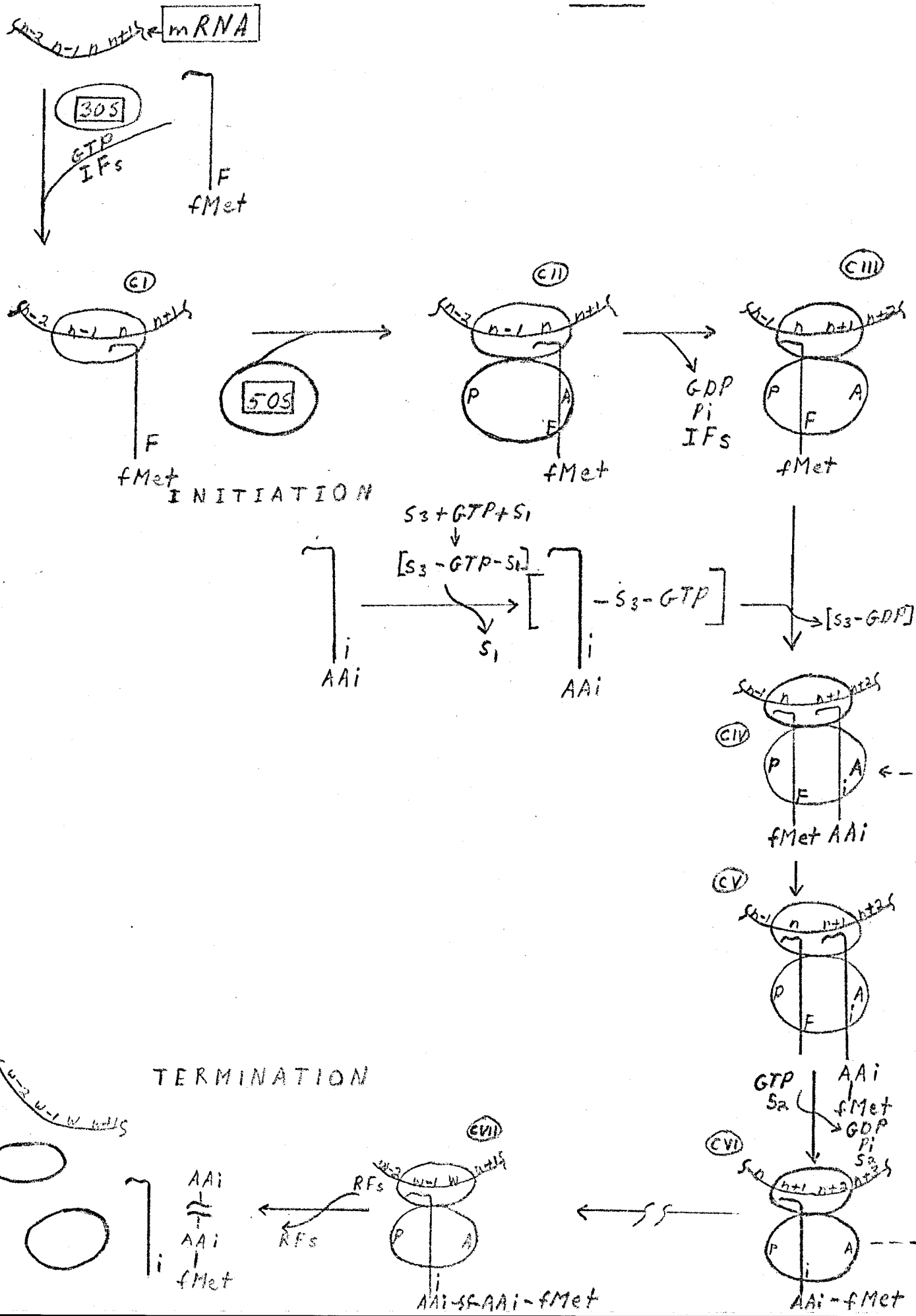
INTRODUCTION

An attempt is made to give a general summary of the mechanism of protein synthesis as a foundation for the present studies. Since most of our knowledge concerns the mechanism in prokarytes, this area will be reviewed first. This will give the reader an idea of the present state of knowledge as it exists for prokaryotes and serve as an introduction to the frontiers of this research. The mechanism in eukaryotes will be discussed in the light of this knowledge and this mechanism will be contrasted and compared to that which exists in prokaryotes. This summary will thus act as a background to a discussion of the specific research problem and reasons for attempting this problem.

(1) Protein Synthesis in Prokaryotes

When a discussion of the mechanism of protein synthesis is undertaken, the logical place to begin is the initiation of such a mechanism, and much of our knowledge about initiation has come from studies on Escherichia coli. The components involved are an initiator tRNA (fMet-$tRNA_F$) initiator codons, several initiation factors, ribosomal subunits, and GTP. The initiator tRNA (fMet-$tRNA_F$), can serve as peptide chain initiator for each of the proteins programmed by a polycistronic mRNA (1, 2, 3, 4, 5). FMet-$tRNA_F$ can also serve as a source of N-terminal methionine residues; whereas Met-$tRNA_M$ provides methionine residues for internal and C-terminal positions of the polypeptide chains (6, 7, 8). The codons specifying $tRNA_F$ are AUG and

Figure 1 The Mechanism of Protein Synthesis in E. coli



GUG; the codon specifying $tRNA_M$ is AUG (6, 7, 8, 9). This triplet, AUG, sets the phase of the reading and this "phasing" activity is maximal at low Mg^{++} concentration (4 to 9mM) (9, 10). It is known that an early intermediate in initiation is a complex which includes the 30S subunit, mRNA, and fmet-$tRNA_F$ (Complex I, see figure 1). Protein synthesis in vitro at low Mg^{++} concentration (4 to 9 mM) seems to require proper chain initiation (i.e. fMet-$tRNA_F$, initiation factors, and mRNA with properly located initiator codons). In the absence of any one of these, a higher Mg^{++} concentration is mandatory for protein biosynthesis. The formation of Complex I requires GTP and initiation factors (11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 8). Subsequently, a 50S subunit attaches to Complex I (Complex II). The fMet-$tRNA_F$ in Complex II is thought to be bound at the hypothetical site A of the ribosome (21, 22, 23, 19, 24, 25). In the next step, fMet-$tRNA_F$ is believed to be translocated to site P, a second hypothetical site on the ribosome (Complex III). GTP may be cleaved into GDP and Pi in the course of the transformation of Complex II or III (26). The formation of Complex III is the last step in chain initiation. The coupling of 30S and 50S subunits to form 70S ribosomes (i.e. Complexes II or III) at 5 mM of Mg^{++} depends on the presence of mRNA, fMet-$tRNA_F$, GTP and reportedly initiation factors (23). 5'-Guanylyl-methylene-diphosphonate (GMPPCP), an analogue of GTP, can substitute for the latter in the formation of Complex I (12, 19) and in the subsequent attachment of the 50S subunit to this complex (22, 23). GMPPCP has a methylene bridge between the β and γ phosphorus atoms

and thus, cannot undergo enzymatic cleavage into GDP and Pi (89). The fact that it can substitute for GTP indicates that GTP cleavage need not occur before or during the initial attachement of the 50S subunit to Complex I. The complex formed in the presence of GMPPCP and the complex formed in the presence of GTP behave similarly. However, the complex formed in the presence of GMPPCP largly dissociates in the course of centrifugation through a sucrose gradient (23) and does not react with puromycin (27), whereas the complex formed in the presence of GTP largely persists in the sucrose gradient test (23) and does react with puromycin, giving rise to the molecule fmet-puromycin (28, 29, 27, 8). Puromycin is an inhibitor of protein synthesis which may be considered an analogue of the terminal aminoacyladenosine portion of AA-tRNA (30). This inhibitor can react with peptidyl-tRNA (31) in the presence of ribosomes (32), giving rise to a peptidyl-puromycin molecule and the free tRNA (33). This reaction may serve as a basis for defining different tRNA binding sites on the ribosome (34). Ribosomes with bound peptidyl-tRNA can exist in two states: (i) In one state they can react with puromycin (forming peptidyl-puromycin) without further addition. It is customary to define that such ribosomes have the peptidyl-tRNA attached to the peptidyl tRNA donor site (P site). ii. Ribosomes with bound peptidyl tRNA in the other state require the addition of GTP and the high-speed supernatant fraction in order to react with puromycin. Such ribosomes are said to have the peptidyl-tRNA bound to the AA-tRNA receptor site (A site). The supernatant fraction and GTP are required for

catalyzing the translocation of the peptidyl-tRNA from site A to site P (33). In the course of this step GTP is cleaved into GDP and Pi.

FMet.-tRNA$_F$ is attached to the A site first (Complex II) and is subsequently translocated to the P site (Complex III). GTP is cleaved before or during this translocation (26).

At relatively high (presumably unphysiological) Mg^{++} concentration, incorporation of aminoacyl residues from AA-tRNA into polypeptidyl-tRNA can be directed by natural messengers or a synthetic messenger such as poly U (even if the latter lack initiator codons), and does not require either fmet-tRNA$_F$ or initiation factors (35, 36, 37). The need for "proper" chain initiation is obviated at high Mg^{++} concentration, probably because in such conditions AA-tRNA molecules may attach to both the P and A sites of ribosomes bound to mRNA. Dipeptidyl-tRNA may be formed since the enzyme for peptide bond formation is part of the ribosome.

A difficulty has been that the high nuclease activity in extracts from E. coli causes fast degradation of added synthetic mRNA such as poly U. A system of lower nuclease activity can be prepared by washing the ribosomal pellet with ammonium chloride (0.5 to 2M), purifying the washed ribosomes by chromatography on diethylaminoethyl cellulose, and mixing the purified ribosomes with the high-speed supernatant fraction (38). Such a system incorporates amino acids in response to synthetic and natural messengers with or without initiator codons, at high Mg^{++} concentration (10 mM or greater); it is not active, however, at low Mg^{++} concentrations (4-9 mM) with any kind of

messenger. Adding the ammonium chloride solution in which the ribosomal pellet was washed makes the system responsive at low Mg^{++} concentration to certain natural mRNA's or to synthetic mRNA's containing initiator codons. Fractionation of the ribosomal washing solution led to the the discovery of several complementary factors which are required at low Mg^{++} concentration for the translation of mRNA containing initiator codons, These factors are designated initiation factors of which there are three: F_1, F_2 and F_3 (38, 39, 40, 41). Some of the initiation factors can be found attached to native 30S subunits (13, 42, 118). The same factors do not occur on either 70S ribosomes or free 50S subunits (42). This suggests that the factors are released from the 30S subunit sometime after the 50S subunit becomes attached to the 30S subunit in the course of initiation. Since initiation factors were not found in the high-speed supernatant fraction of the cell extract, it may be assumed that they are present in short supply, and as soon as they are released from 70S ribosomes they become bound to 30S subunits.

The next step in protein synthesis after initiation is peptide elongation which also requires certain factors designated elongation factors. Elongation factor activity has been found in the high-speed supernatant fraction of microbial extracts (43). This is in marked contrast with the initiation factor activity which is exclusively located in the pellet obtained by centrifuging the extract at high speed.

The first step in peptide elongation is presumably the attachment (to the vacant A site in Complex III) of AA-tRNA specified by

the codon adjacent to the 3' side of the initiator codon (AA-tRNA binding (44, 45, 46). The product of this step will be designated as Complex IV. The formation of Complex IV requires GTP and involves the elongation factors S_3 and S_1 (47, 48, 49, 50). Results of in vitro experiments seem to indicate that GTP is cleaved to GDP and Pi in this step (51). S_1 and S_3 form a complex with GTP (Complex A). Complex A binds AA-tRNA in a subsequent step and is transformed into a GTP-AA-tRNA-S_3 complex; (Complex B) (52, 50). The GTP molecule in Complex B is cleaved to GDP and Pi after the binding of phe-tRNA to the poly U-ribosome complex, but before peptide bond formation (51). After peptide bond formation, the newly formed peptidyl-tRNA (fMet-AA-tRNA in the first cycle of chain elongation) is located in site A (53, 54, 55) and the discharged tRNA, which remains bound to the ribosome (56), is probably in site P. Translocation is a composite step catalyzed by the S_2 factor. Translocation requires GTP, which is cleaved to GDP and Pi (57, 58, 53, 54, 55, 59, 60, 61, 34). The action of S_2 results in (i) release of the discharged tRNA, (ii) translocation of the peptidyl-tRNA from A site to the P site, and (iii) movement of the ribosome along the mRNA the length of one codon (37, 68). During chain elongation, the growing polypeptide chain remains linked to tRNA and bound to the mRNA-ribosome complex. After completion the polypeptide is released from both of these bonds in the course of a composite process called peptide chain termination. Termination is triggered when in the course of movement of the ribosome along the mRNA a chain termination signal is reached at

the A site of the 30S subunit. Two release factors (R_1 and R_2) were found to be involved in this process. The release factors R_1 and R_2 catalyze chain termination in reponse to UAA or UAG, and UAA or UGA respectively (63).

(2) Frontiers in Studies on the Mechanism of Protein Synthesis in Prokaryotes

It is known that ribosomes exchange their subunits after each passage over asingle cistron of mRNA (64). As an explanation for the exchange, it was presumed that ribosomes dissociate into subunits after finishing the synthesis of a protein molecule and that they are reformed by the coupling of subunits when initiating the synthesis of a new protein molecule (64, 65). As a consequence, the hypothetical schemes concerning the fate of ribosomes in the chain termination complex after the release of the completed polypeptide can be divided arbitrarily into two classes: (i) ribosomes released as free subunits and (ii) ribosomes released as 70S particles which subsequently dissociate into subunits (66, 67, 68). If among the products of chain termination, free 70S ribosomes (i.e. free of mRNA, peptidyl-tRNA, and fMet-tRNA) could be demonstrated, this would rule out the first class and be consistent with the second. 70S particles (in addition to subunits and polyribosomes) have been detected in cell lysates (69, 67, 70, 65, 71, 72, 73, 68). However, although a 70S particle (at least in principle) may be a free ribosome, it may also be a monosome (i.e., a complex of mRNA with

a single ribosome and its attached peptidyl-tRNA) or an initiation complex (a complex of mRNA with a single ribosome with attached fMet-tRNA).

70S particles accumulate in the cells that are incubated without a carbon source or treated with actinomycin D (67). The latter treatment depletes the polysomes by blocking messenger RNA synthesis. The fact that such 70S particles dissociate fully into subunits at a Mg^{++} concentration of (1 mM), the same Mg^{++} concentration at which monosomes dissociate only partially was taken as support for the view that the 70S particles are free ribosomes (72). (Puromycin-treated 70S monosomes also dissociated at 1 mM Mg^{++} (72), and so they behave as free ribosomes and yet such ribosomes carry a tRNA which has discharged its peptide in the reaction with puromycin (50). Adding a protein fraction obtained from a 1M NH_4Cl wash of the native 30S subunits, causes rapid dissociation of the presumed free ribosomes (74). The active component of the protein fraction was designated as a dissociation factor and acts on the ribosomes in a stoichiometric rather than in a catalytic manner. The dissociation factor resembles initiation factors in at least one aspect: it is present on native 30S subunits but not on 70S ribosomes (74). On the basis of these results it was postulated that after chain termination ribosomes are released as free 70S particles (66, 74) which are dissociated to subunits after reacting with a dissociation factor which combines with the 30S subunit. The dissociation factor (hereinafter referred to as DF) is released from the 30S subunit some

time after the latter combines with a 50S subunit to form a 70S initiation complex (74). The amount of dissociation produced by a given amount of DF was found to fall off sharply with increasing Mg^{++} concentration, in the range of 5-20 mM.

The dissociation factor is a protein and does not require a source of energy for its dissociation activity. It seems the DF and 70S ribosomes are in an equilibrium with the subunits and this equilibrium is dependent on the concentration of Mg^{++} ions. Its reaction is temperature dependent in the 0-37°C range. Recently, the DF has been equated to the initiation factor F_3 by J. Albrecht, F. Stap, H. O. Voorma, P. H. Van Knippenberg and L. Bosch (75). They found that the ability of a more purified DF preparation to dissociate 70S ribosomes decreased with increasing Mg^{++} concentrations as reported for crude factors (74). DF is thermolabile. Preheating at 70°C for 5 minutes abolished the activity by about 90%. After 3 minutes incubation at various intermediate temperatures in the range of 0-37°C, the reaction had still not reached completion. The reaction was found to be temperature dependent in this range. Above 37°C the stoichiometric reaction may have reached completion or may have stopped due to inactivation of the factor or of some other component in the incubation system. Recently, a requirement for F_3 was found both for translation and formation of the initiation complex (76) when a natural mRNA (such as phage RNA) is used. The results of this work (76) suggest that formation of the initiation complex involves two steps: (a) the F_1 and F_3 dependent binding of mRNA to the ribosomes, and (b) the F_2 dependent binding of fMet-$tRNA_F$ to the mRNA-ribosome complex.

F_3 has an RNA binding activity, as judged by "Millipore" filtration assays; F_3 promotes dissociation of 70S ribosome to 30S and 50S ribosomes subunits (77); and F_3 is directly involved in the formation of the chain initiation complex (78). F_3 is a basic protein and has a M.W. of 21,000 as determined by SDS-gel electrophoresis (79). DF activity is found in crude preparations of initiation factors (74, 80, 75, 81). Fractionation of the factors by DEAE-cellulose chromatography (76) showed that DF is associated with F_3 (77), a finding that is confirmed with highly purified F_3 preparations (78, 82). No effect of GTP on the dissociation reaction was found (82). Similar results were reported from two other laboratories (75, 83).

Our understanding of the scheme of protein synthesis in prokaryotes is nearing completion. Only the exact details of the reactions remain to be elucidated. More information is necessary on the interaction of the three initiation factors and to discover just at what point the dissociation factor F_3 leaves the initiating complex as well as to determine its specificity of reaction with 70S ribosomes. Does it recognize a ribosome-mRNA complex free of tRNA? Would it recognize a ribosome-tRNA complex free of mRNA? We know it recognizes 70S ribosomes free of tRNA and mRNA. Does DF play any role in the termination process after release of the completed polypeptide? In the case of a polycistronic mRNA, just what would the role of DF be in the termination and release which occurs after the first cistron? Does reinitiation occur at this point with perhaps a whole new set of subunits; or is the same ribosome used for translating the second

cistron; or is only one of the ribosomes subunits used again? These are only a few of the questions which make up the frontier of protein biosynthesis in prokaryotes.

(3) Differences in Protein Biosynthesis Encountered in Eukaryotes

Much less information is known about protein biosynthesis in Eukaryotes. It is presumed that many steps will be the same, but it is generally expected that there will be differences. Eukaryotes like prokaryotes require the presence of ribosomal subunits for the initiation of protein synthesis (84). The work of Colombo and Baglioni (84) supports a model of ribosome function in which the 80S ribosome is an intermediate between polyribosomes and ribosomal subunits. When rat-liver ribosomes, free of peptidyl-tRNA and mRNA, are incubated with ^{14}C-phenylalanyl-tRNA in relatively low $MgCl_2$-containing solutions, polyphenylalanine is synthesized in the presence of purified elongation factors, transferase I (aminoacyl-tRNA binding factor) and transferase II (translocation factor), GTP, and poly U (85). The aminoacyl-tRNA binding is specific for the aminoacyl site; the peptidyl site can be filled only as the results of translocation which differs from the situation in prokaryotes. At higher Mg^{++} concentrations, binding requires poly U but not transferase I or GTP. Transferase II is required for translocation only. A single translocase catalyzes translocation on several ribosomes, the interaction with ribosomes being a reversible process and active translocase dissociates from the

complex on formation of the product (85).

Liver ribosomes differ from bacterial ribosomes in that the bound Mg^{++} must be reduced to a much lower level before subunits may be separated (86). Bacterial (70S) ribosomes may be dissociated by lowering the Mg^{++} concentration to 0.1 mM. This dissociation is reversible and the reformed 70S particle is active in amino acid incorporation. However, liver ribosomes dissociate only at a Mg^{++} concentration of 10^{-5} M or less forming subunits of 53S and 28S with the concomitant release of transfer RNA and 5SRNA (86). On restoration of the the Mg^{++} they reassociate mainly to random aggregates, inactive in amino acid incorporation (86). The major difference between 70S and 80S ribosomes may be related to the greater degree of stability conferred on the 80S subunit couple by the presence of the peptidyl-transfer RNA (87). In eukaryotes, as in prokaryotes, the smaller of the two ribosomal subunits binds mRNA during the initiation of protein synthesis. The formation of the complete initiation complex, sedimenting at 75S, requires GTP, tRNA and initiation factors (88).

(4) Frontiers in Studies on the Mechanism of Protein Synthesis in Eukaryotes

The mechanism for initiation of protein synthesis involving ribosomal subunits appears to be similar in both prokaryotes and eukaryotes. Further, the requirement of GTP, tRNA and initiation factors for the formation of the initiation complex is the same for both prokaryotes and eukaryotes. In eukaryotic systems the presence

of at least two $tRNA^{Met}$ species has been shown $tRNA_1^{Met}$ and $tRNA_2^{Met}$ (90, 91, 92, 93, 94, 95, 96). Recent studies suggest that $tRNA_1^{Met}$ may be the initiator tRNA in the eukaryotic system (90-95, 97, 98, 99, 100). Results obtained with mammalian systems have shown that a species of $tRNA^{Met}$ which can be both charged and formylated by E. coli enzyme is involved in initiation of polypeptide synthesis (90, 91, 92, 97, 98, 99). The initiator methionyl-residue, however, appeared to be unmodified in mammalian systems. An initiator $tRNA^{Met}$ species in higher plants has been confirmed which can be charged by E. coli enzyme but can not be formylated (100). The precise role of the initiation factors in the formation of the complex are still not clear.

Since ribosomal subunits exchange freely after completion of the polypeptide chain (64, 101), it is likely that at least part of the "native" subunits, which are found in the cytoplasm of eukaryotic cells as well as in bacterial extracts, are produced after complete translation of the messenger information. A specific protein factor dissociating Saccharomyces cerevisiae ribosomes has been characterized and some of its properties have been examined. The reaction gives 60S and 40S subunits. It is heat dependent. The factor will not dissociate Escherichia coli or rabbit reticulocyte ribosomes, and the factor-dependent dissociation reaches a plateau within 30 minutes of incubation, due either to the stoichiometric mode of action of the factor or to the inability of ribosomes to support complete dissociation under the ionic conditions of the experiments (102).

(5) The Specific Problem

When the products at the finish of peptide synthesis by

polysomes are caused to accumulate, the products are commonly referred to as "runoff" products or polysome "runoff". Runoff in the bacterial cell or *in vitro* results in the accumulation of free 70S ribosomes rather than subunits. Subunits are required for the initiation of protein synthesis. Therefore a factor involved in dissociation would be in a sense an initiation factor. Such a factor was found in *Escherichia coli* by Subramanian *et al.* (74). When the products of peptide synthesis by polysomes are caused to accumulate in *Escherichia coli* cells or extracts they appear as 70S ribosomes (67); and such "free" or "runoff" ribosomes differ from complexed monosomes carrying messenger RNA and nascent polypeptide, in being more easily dissociated by low Mg^{2+} concentrations (72). Mammalian ribosomes are present in living cells mainly as polysomes (103, 104). The incubation of polysomes in a system containing supernatant enzymes results in the formation of monomeric ribosomes (105, 106). Single ribosomes have been considered the active units in the formation of the ribosome-poly-U-phenylalanine-tRNA complex in bacterial as well as in mammalian systems (107, 108); recent evidence, however, has suggested that the subunits, rather than the monomeric ribosomes, interact with natural messengers (20, 109, 13, 21). On the other hand, G. Blobel and V. R. Potter (110), have found mainly monomeric ribosomes and very few subunits in mammalian cells and R. E. Kohler, E. Z. Ron and B. D. Davis (67) have found mainly monomeric ribosomes and very few subunits in bacterial cells as well (109). Because "runoff" causes no parallel increment of ribosomal subunits, and because subunits are required for the initiation of protein synthesis in both mammalian and bacterial

systems as already mentioned, this implies that the dissociation step in the ribosome cycle does not occur spontaneously. Moreover, a protein factor prepared from the "native" 30S subunits has been reported to bind to Escherichia coli ribosomes, causing dissociation into 30S and 50S subunits (74). So with all this preceding knowledge our attention was drawn to the possibility that "runoff" ribosomes could possibly complex with a dissociation factor in mammalian cells as well. This factor would perhaps be present in the cell in limited supply and would, therefore, have to recycle in the course of initiation, from a completed initiation complex to another free ribosome. So in consideration of this real possibility our problem was initially to set up an assay system whereby the existence of a DF in a subcellular fraction of rat liver could be determined. We expected the DF to be in the fraction containing the "native" subunits because that is where the corresponding factor from E. coli was found. So we looked to the "native" subunit fraction first.

SECTION A

STUDIES ON AN ASSAY PROCEDURE FOR DISSOCIATION FACTOR

INTRODUCTION

Our assay for a dissociation factor would consist of the following steps: (i) Monomeric ribosomes would be isolated in such a way that they could be incubated as substrate with a DF preparation. (ii) The mixture would be centrifuged through a sucrose gradient to separate the components. (iii) The gradient would then be monitored for the appearance of subunits (compared to controls) by pumping them through a Gilford flow cell system at a wavelength of 260 nm. The control would be a sample of monomeric ribosomes incubated with TKM buffer in place of the dissociation factor preparation. This would serve as a standard for the non-dissociated condition. The ratio of the total area of the subunit peaks to the monomer peak of the control would give the fraction of dissociation caused by the DF preparation.

We anticipated a "good" assay to be one in which the large and small subunit peaks are sharp, and well separated from each other as well as from the monomer (monomeric ribosome) peak. We expected the size of the large subunit peak as compared to the size of the small subunit peak to be in the ratio of approximately 2 to 1. We expected that we could integrate the area under the peaks in the control profile. In the experimental profiles (those due to incubation with DF preparation) we expected the area integrated under the monomer, large subunit and small subunit peak to equal that integrated under the control profile. This would provide a means of quantitating the amount of dissociation

caused by the addition of a given amount of DF preparation. From this quantitation the specific activity of the DF preparation could be determined. However, several restraints on our assay were encountered. The initial restraint was the degree of resolution and sharpness of the peaks. The peaks in the control initially lacked sharpness as a result of inappropriate conditions. The area under the peaks represent the amount of ribosomes used. This should be identical for both experimental and control profiles. This was often a problem because the profiles were not identical.

Another restraint on the assay was the need for the monomeric ribosomes to be of a type which could be recognized as substrate by the DF if such a factor existed. The question was whether or not the right type of ribosomes were being used as the substrate in the assay which was designed to detect the presence of a dissociation factor? If the correct type was not being used then there was no hope of detecting a dissociation factor. For this reason much work was devoted to the search for the correct substrate for the assay as well as conditions necessary for optimal resolution and sharpness of bands in the sucrose gradient analysis. Perfecting the assay was a necessary prerequisite to the search for the dissociation factor.

Ribosomes prepared by two different methods were used and these are designated Type I and Type II. The present work includes a study of the differences between these two types, as well as reasons why Type II was deemed to be the correct substrate for the dissociation factor assay. Another restraint on the assay was the necessity of having the correct ionic conditions. Although it was necessary to have the K^+

and Mg^{++} concentration of the assay such that optimal resolution and sharpness of the profiles were obtained, this in itself was not sufficient. These ionic conditions also had to be such that the DF could function in a normal way. It was expected that ionic conditions which would allow a DF to function normally would also give optimal resolution and sharpness to the profiles; however, it was not known whether or not the converse was also true. We, therefore, had to aim at conditions which would give optimal resolution and sharpness to the profiles and hope that the converse was true.

METHODS

(1) Preparation of Polyribosomes and Determination of Ribosomal Concentration

Conditions for preparation of polysomes were as described by G. R. Lawford (87), a modification of the method of F. O. Wettstein, T. Staehelin and H. Noll (104). Briefly, male hooded rats which had been starved 24 hours to reduce glycogen were stunned by a blow to the head. Their necks were broken and their livers quickly excised and washed in ice-cold 0.25M sucrose in TKM buffer (TKM is 50 mM Tris-HCl pH 7.8, 25 mM KCl and 5mM $MgSO_4$). All subsequent operations were performed in the cold (2-4°C). The livers were blotted, minced and then homogenized with 2-1/2 volumes of 0.25M sucrose in TKM in a Potter-Elvehjem homogenizer with a motor driven teflon pestle. The homogenate was centrifuged at 10,000 rev/min for 15 minutes in the A211 rotor of an International B-20 centrifuge. This yeilded the post-mitochondrial supernatant. To the post-mitochondrial supernatant was added 1/10 volume of 14.3% sodium deoxycholate (DOC). The supernatant was layered in

centributes containing two superimposed sucrose layers (104); 10 ml of 1.85M sucrose-TKM as the bottom layer and 4.0 ml of 0.5M sucrose-TKM as the top layer. The centritubes were then centrifuged at 45,000rev/min for 17 hours in the A211 rotor of an International B-50 centrifuge. The pellets were suspended in TKM Buffer and aggregates removed by centrifugation at 1,500 rev/min for 10 minutes. Samples (10 mg) were frozen in liquid nitrogen and stored at -2°C for up to one month (the assumption that a 1mg/ml solution of ribosomes gives an absorbance of 13.4 units at 260nm was used) (111). The purity of the polyribosome preparation was determined by the ratio of the absorbance at 260 nm to the absorbance at 280 nm. A reading of 1.8 or greater indicated a good preparation of polyribosomes. This technique of preparation of polyribosomes was used with only occasional minor modifications in the sucrose concentration of the super-imposed sucrose layers.

(2) Techniques for Centrifugation Analysis in Sucrose Density Gradients

In order to analyze a subcellular fraction containing polysomes, monomers, dimers, large and small subunits, these components were separated by centrifugation in a sucrose gradient. In this way the particles would centrifuge through the gradient at a rate which was proportional to the shape, density and size of the particles as well as to the viscosity of the sucrose at each point, the particles distance from the centrifugal axis and the angular speed of the rotor. Since ribosomal components absorb light of the wavelength 260 nm, because of their RNA content, each component could be resolved by means of a recording spectrophotemeter fitted with a flow cell, into a distinct absorbance peak characteristic of that component. If the separation

of these absorbance peaks is sufficient the amount of each component of the mixture can be accurately quantitated.

To separate monomeric ribosomes from subunits, extracts were usually placed on linear (29 ml) gradients containing 15-35% sucrose in TKM Buffer. If formaldehyde treatment was used (see appendix) the ionic concentration of the gradients was of no consequence because formaldehyde preserves the state of aggregation of ribosomes. However Tris-HCl must be replaced by triethanolamine since the former reacts with formaldehyde. The gradients, either cold (0°C) or warm (20°C), were spun 6 hours or 3 1/4 hours respectively at 25,000 rev/min in the SB110 rotor of an International B-50 centrifuge. The gradients were monitored for absorbance by pumping the gradients (at 1.5 ml/min) through a flow cell fitted in a Gilford 2400 recording spectrophotometer . When the gradients were pumped through the flow cell system, air had to be excluded from the line and a method was necessary to mark the beginning of the sucrose gradient. To satisfy these requirements, the line joining the gradient to the flow cell is previously filled with water. The starting point of the gradient is indicated by the increase in absorbance which occurs as a result of mixing at the sucrose-water interphase. With the use of this technique, the retention time, from the point of mixing to the end of the gradient, is identical for gradients of equal volume. In all the figures presented, the direction of sedimentation is from right to left. Positions of subunits, small and large, and of monomers and dimers are marked with S and L, 1 and 2, respectively.

(3) Preparation of Monomeric Ribosomes as Substrate for the Dissociation Factor Assay

The assay for dissociation factor was assumed to require monomeric ribosomes which are free of mRNA and peptidyl-tRNA.

(a) For this reason polysomes were incubated in the presence of cell sap, an energy generating system and puromycin (87). The incubation mixture contained in 12 ml; polysomes (1.6 mg/ml); ATP (2.0 mM); GTP (0.25 mM); phosphoenolpyruvate (10 mM); pyruvate kinase 50 mg/ml; puromycin (25.7 mM); Tris-HCl, pH 7.8 (0.05 M); KCl (0.025 M) and $MgSO_4$ (5.0 mM) and cell sap (2.4 ml). The mixture was incubated at 37°C for 45 min. It was centrifuged through 1M sucrose in TKM Buffer so that the free ribosomes were centrifuged to a pellet. This gave monomeric ribosomes (Type I) as substrate for the dissociation factor assay.

(b) As the result of a study presented here (see Section IA-6 of the methods) based on[a] communication with Blobel, G. and Sabatini, D. (recently published 112) a method was used for the preparation of monomeric ribosomes free of the nascent chain, mRNA and the tRNA moiety of peptidyl-tRNA. These monomeric ribosomes (Type II) were prepared as follows: The incubation mixture contained in 5.43 ml: 0.4 ml polysomes (25 mg/ml); 2.5 ml of a medium of composition Tris-HCl (0.1 M) KCl (1.0 M) and $MgSO_4$ (0.003 M); and 30µl puromycin (7.7 mM). The final concentrations were the following: Tris-HCl (0.05 M); KCl (464 mM); $MgSO_4$ (1.75 mM); puromycin dihydrochloride (0.41 mM) and ribosomes (1.84 mg/ml). The mixture was incubated at 0°C for 30 minutes. This was a modification of the method of G. Blobel and D. Sabatini (112). The temperature of

the incubation was then raised to 37°C for 10 minutes. All the subsequent steps were done at 20°C. Reassociation of subunits was accomplished by a dilution with 4 volumes of T-M* buffer [T-M* buffer is Tris-HCl (0.05 M) and $MgSO_4$ (1.5 mM)] to adjust the incubation mixture to the following approximate concentrations: Tris-HCl (0.05 M); KCl (0.1 M) and $MgSO_4$ (1.5 mM). The incubation mixture was then centrifuged through 1M sucrose in TKM* buffer [TKM* buffer is Tris-HCl (0.05 M) KCl (25mM) and $MgSO_4$ (1.5 mM)] to pellet the ribosomes and lower the KCl concentration to 25 mM. The ribosomes were suspended in TKM* buffer and centrifuged to remove aggregates. The ribosome concentration was then determined as previously described. The ribosomes were stored at room temperature for up to two days or at 4°C for up to a week.

(4) The Dissociation Factor Assay and Quantitation of Results

Monomeric ribosomes which were suspended in TKM* buffer (concentration 4 mg/ml) and which were prepared as previously described are used as substrate in the dissociation factor assay. Ribosomes (0.5 mg) in TKM* buffer were mixed with 0.1 - 0.5 ml of dissociation factor preparation (protein concentration 17-55 mg/ml) to give a total volume of 0.6 ml when diluted with TKM* buffer. A typical assay protocol is shown in Table 1. Assay samples were usually incubated for 15 minutes at 30°C and then layered on 15-30% (W/V) linear sucrose in TKM* gradient at 20°C, a modification of the method of A. R. Subramanian, Eliora Z. Ron and Bernard D. Davis (74). Any further modifications of this method will be described as they occur. Gradients were analyzed as previously described.

The results were quantitated by estimating the area under each peak. This was accomplished by multiplying the peak height by the peak width at half height. The percent dissociation was computed as follows:

$$\text{Percent dissociation} = \frac{\text{40S + 60S peak area}}{\text{40S + 60S + 80S peak area}} \times 100\%$$

This percent dissociation was converted to mg monomers dissociated per mg of protein present in the crude or purified dissociation factor preparation.

(5) Amino Acid Incorporation in vitro

Endogenous incorporation: The incorporation mixture contained in 6 ml: polysomes (10 mg); (^{14}C) L - leucine (1 μC); ATP (2.0 mM); GTP (0.25 mM); phosphonenolpyruvate (10 mM); pyruvate kinase (50 μg/ml); $MgSO_4$ (6.0 mM); KCl (22 mM); and 1.2 ml cell sap. The mixture was incubated 5 minutes at 37°C. The polysomes were pelleted by centrifugation through 1M sucrose in TKM buffer for 1.5 hours at 50,000rev/min in an International A321 rotor. The pellet was then suspended in TKM buffer and centrifuged to remove aggregates. The (^{14}C) counts were 2,060 cpm per mg of suspended pellet.

(6) Comparison of Sedimentation Profiles of Monomers and their Release of Nascent Labelled Polypeptides

Monomers were prepared from polysomes (labelled in vitro) by two different methods. One method was the use of puromycin, low salt, and an incubation with added energy (87). The other method was the use of puromycin, high salt and an incubation without added energy (87).

The effect, of different ionic concentrations in the gradients, on the sedimentation profiles and the release of nascent labelled polypeptides was studied.

The following six sucrose gradients were prepared at 0°C: # 1 and # 2 were 15-40% sucrose in TK·^{12}M buffer [TK·^{12}M is Tris-HCl (0.05M); KCl (0.12M) and $MgSO_4$ (5 mM)]; # 3 and # 4 were 15-40% sucrose in TK·^{15}M^{-3} buffer [TK·^{15}M^{-3} Is Tris-HCl (0.05 M); KCl (0.15 M) and $MgSO_4$ (1 mM)]; and # 5 and # 6 were 15-40% sucrose in TK$^{0.5}$M$^{0.012}$ buffer gradients [TK$^{0.5}$M$^{0.012}$ is Tris-HCl (0.05 M); KCl (0.5M) and $MgSO_4$ (12 mM)].

(i) The Puromycin-low salt-energy Method

The incubation mixture contained in 3 ml: same as previously described in Section IA-3 (a) of the methods with the following changes: polysomes (1.67 mg/ml labelled in vitro (8,230 cpm) as in Section IA-5 of the methods); $MgSO_4$ (6.0 mM); KCl (22 mM); and cell sap (0.6 ml). The mixture was incubated as previously described. Samples (0.9 ml) were layered on each of gradients # 2, # 4, and # 6.

(ii) The Puromycin-high salt-no energy Method

The incubation mixture contained in 3.0 ml: polysomes (1.67 mg/ml labelled in vitro (8,230 cpm) as in Section IA-5 of the methods); puromycin (25.7 μM); Tris-HCl (0.05 M); $MgSO_4$ (14 mM); and KCl (507 mM). This was a modification of the method of G. Blobel and D. Sabatini (112). The incubation mixture was incubated at 0°C for 30 minutes. The

temperature was then raised to 37°C for 10 minutes. Samples (0.9 ml) were layered on each of the gradients #1, #3, and #5. The gradients #1 through #6 were analyzed at 0°C as previously described.

(iii) Release of Nascent Labelled Polypeptides

Fractions (1.5 ml) were collected from each of the six gradients (#1 through #6). To each fraction an equal volume (1.5 ml) of 10% trichloroacetic acid was added. Cell sap (1 drop) was added to each tube to act as co-precipitant. The fractions were stored at 4°C for 15 minutes and then filtered on glass fibre filters using the millipore apparatus. The tubes were rinsed twice with 5% trichloroacetic acid. The filters and residue were then washed twice with chloroform-ether-methanol (2:1:1 by vol.). The filters were placed in counting vials and dried. To each vial 10 ml of scintillation fluid 2, 5, diphenyl-oxazole (4 g/l of toluene) and 1,4-bis-(5-phenyloxazol-2-yl) benzene (100 mg/l of toluene) was added. The (^{14}C) was counted on a Nuclear Chicago Unilux II with approximately 70% efficiency. The background (20 cpm) was subtracted and the results bar graphed on the appropriate absorbance profiles.

RESULTS

(1) Techniques for Centrifugation Analysis in Sucrose Density Gradients

The concentration of the sucrose used to make the gradients could be varied in order to separate the particles desired. To separate ribosomes from subunits a 15-30% sucrose gradient was found to be the best. If the sucrose gradients were centrifuged at 0°C, a centrifugation

Figure 2

Centrifugation Analysis in a Linear Sucrose Density Gradient of Ribosomes of Type I Prepared by the "Energy" Method

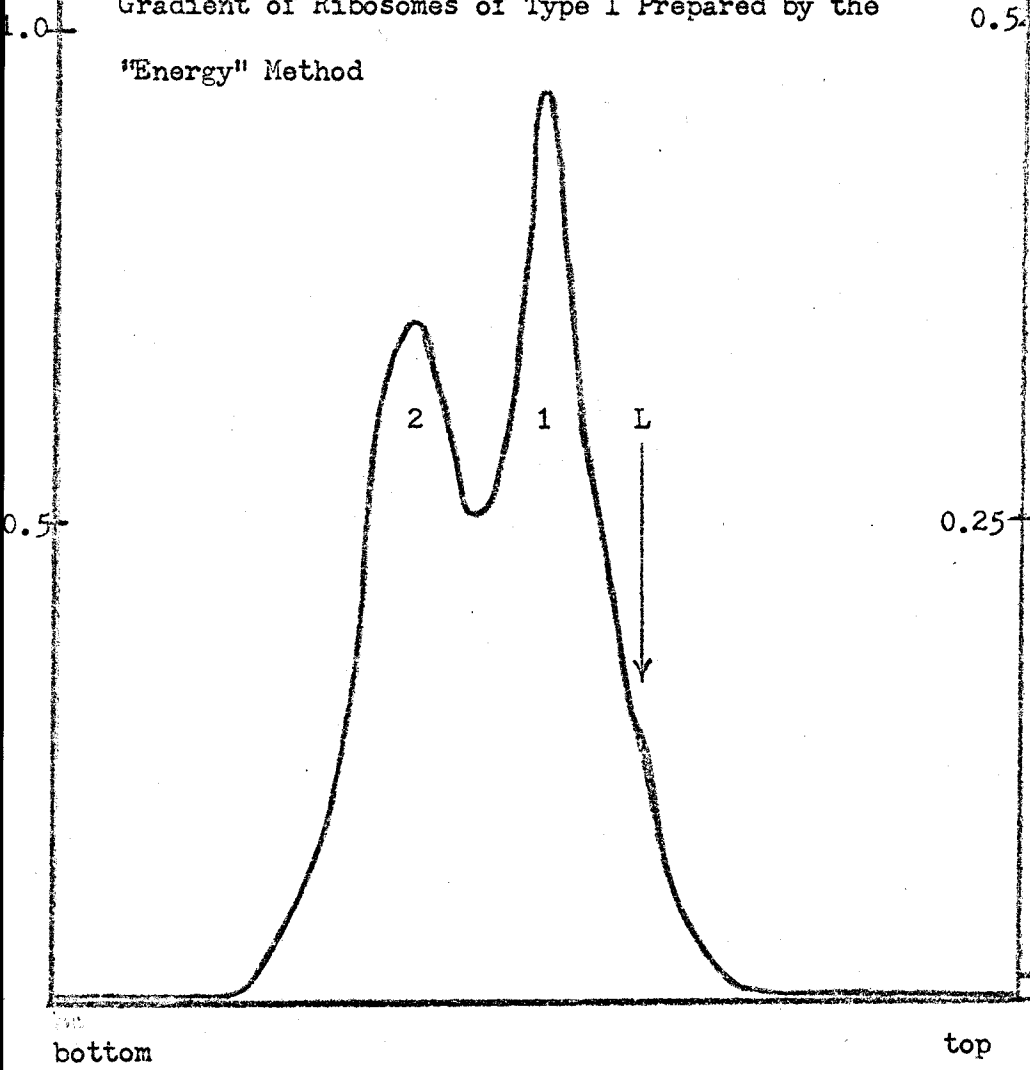
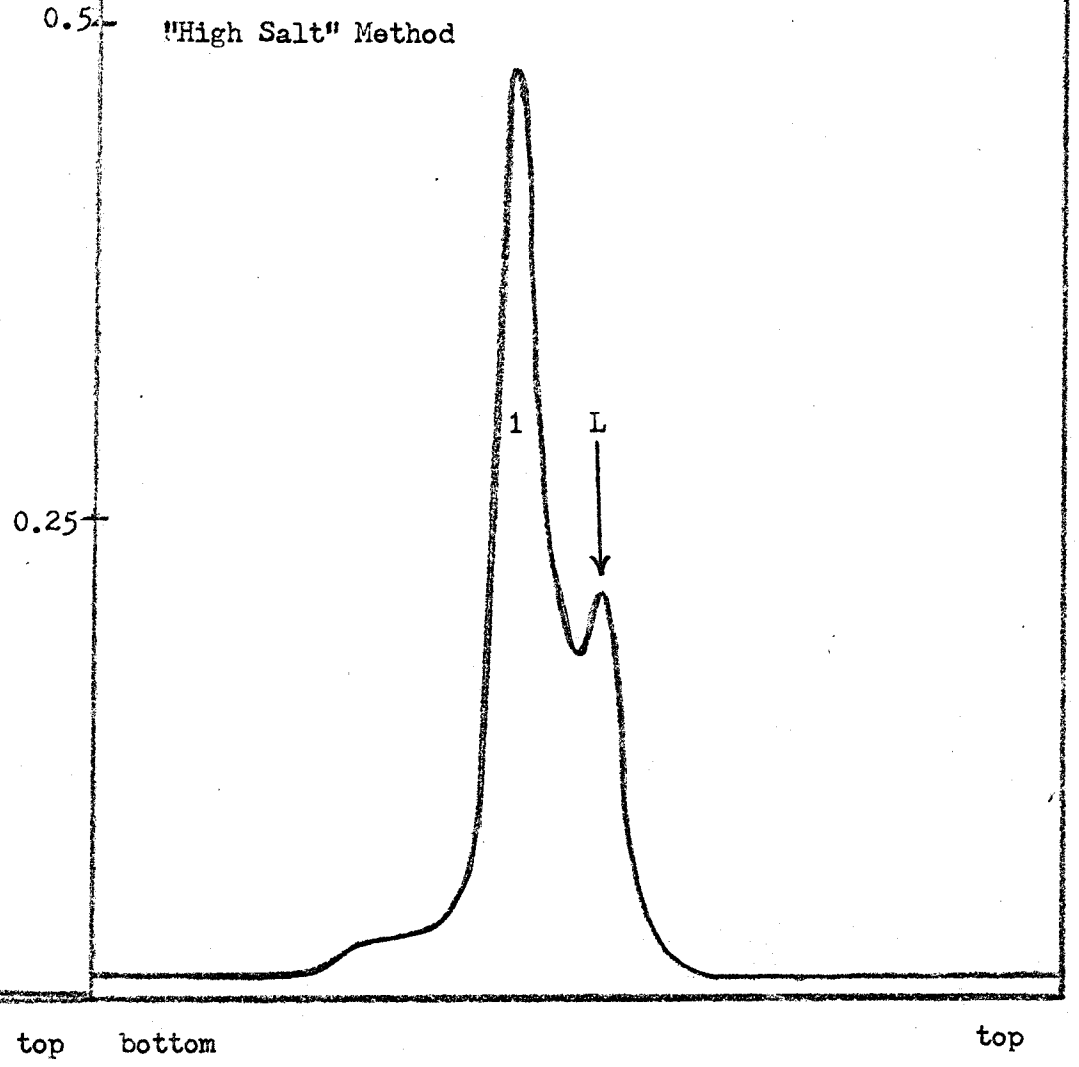


Figure 3

Centrifugation Analysis in a Linear Sucrose Density Gradient of Ribosomes of Type II Prepared by the "High Salt" Method



time of six hours was necessary to get adequate separation of the monomer, large and small subunit bands. Centrifugation at 20°C only required 3 1/4 hours to get a similar separation.

(2) The Dissociation Factor Assay and Quantitation of Results

The quantity of ribosomes used per assay was kept low (0.5 mg) purposely so that the presence of DF in a preparation would give the optimal effect. Thus the assay would have the greatest sensitivity while maintaining the maximum resolution. The protein concentration of the DF preparation differed depending on the method used to prepare it. The concentration usually fell within the range stated in Section IA-4 of the methods. Optimal conditions of incubation are as established in the Appendix. Proper assay results will be presented in Section IB-2 of the results.

(3) A Comparison of Release of Nascent Labelled Polypeptides Between Ribosomes of Type I and II

The monomeric ribosomes described in Section IA-3(a) of the methods are designated Type I because they will later be seen to be different from those prepared in Section IA-3(b) of the methods which are designated Type II. Centrifugation analyzes of these two Types, I and II, can be seen in Fig. 2 and Fig. 3 respectively. The difference will be seen in the results of the following experiment where the degree of release of the nascent labelled polypeptides is investigated. The nascent peptide was labelled as described in Section IA-5 of the methods. The experiment was performed as described in SectionIA-6(i),

(ii) and (iii) of the methods. The gradient analyses are as shown in Fig. 4. The profiles show that there is a great deal of similarity between ribosomes prepared by the two methods (compare Profile A with B: Profile C with D and Profile E with F). These comparisons show identical behaviour of the two types under identical ionic conditions. However at any constant K^+ and Mg^{++} concentrations, the release of labelled peptide always occurred to a greater extent for ribosomes of Type II than for those of Type I. This can be seen by comparing the total (^{14}C) counts left at the top of each gradient (approximately the top three fractions). When each gradient is compared for the total counts released (compare Profile A with B, Profile C with D and Profile E with F) the total is always found to be greater for gradients containing ribosomes of Type II (Profiles A, C and E). Therefore, more release of the nascent peptide occurs with ribosomes of Type II. Ribosomes of Type I are not free of peptidyl-tRNA whereas ribosomes of Type II are mainly free of the nascent peptide and perhaps also of the tRNA (the tRNA of the former peptidyl-tRNA). Therefore the ribosomes (Type I) prepared as described in Section IA-3 (a) of the methods were designated differently than those (Type II) prepared as described in Section IA-3(b) of the methods because they are different in regards to the extent of release of the nascent peptide.

The fact that little success had been achieved in locating ribosome dissociating activity using ribosomes of Type I as substrate together with the fact that these same ribosomes still contained much of the nascent peptide (presumably as peptidyl-tRNA) added support

Fig. 4 Sucrose density gradient analysis of monomeric ribosomes which have been prepared from [^{14}C] labelled polysomes.

Profiles A, C and E - Profiles of ribosomes which were prepared by the puromycin- high salt- no energy method (Type II ribosomes)

Profiles B, D and F - Profiles of ribosomes which were prepared by the puromycin - low salt - energy method (Type I ribosomes)

Profiles A and B - Profiles represent the centrifugation of the above mentioned ribosomes in a 15-40% sucrose gradient containing 120 mM K^+ and 5.0 mM Mg^{++}.

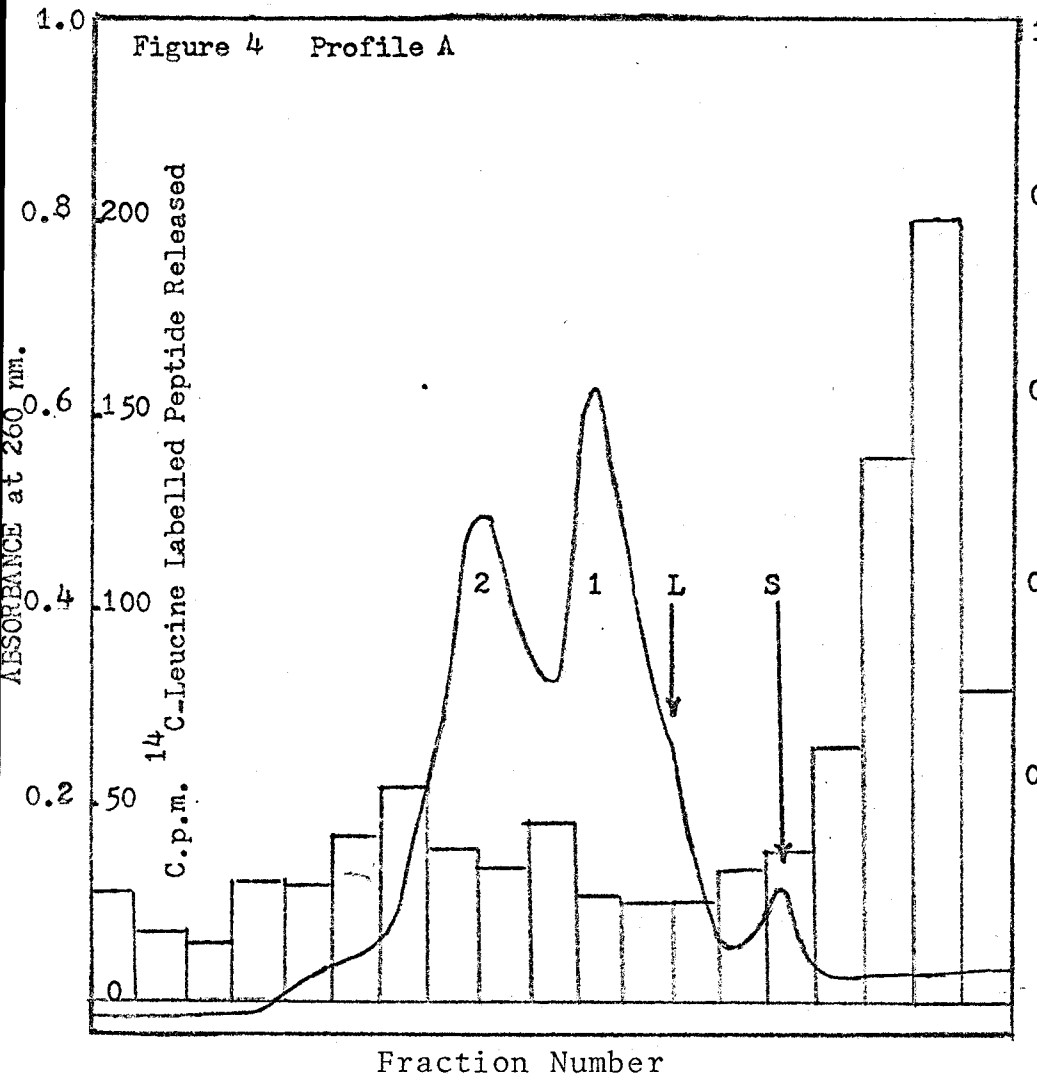
Profiles C and D - Profiles represent the centrifugation of the above mentioned ribosomes in a 15-40% sucrose gradient containing 150 mM K^+ and 1 mM Mg^{++}.

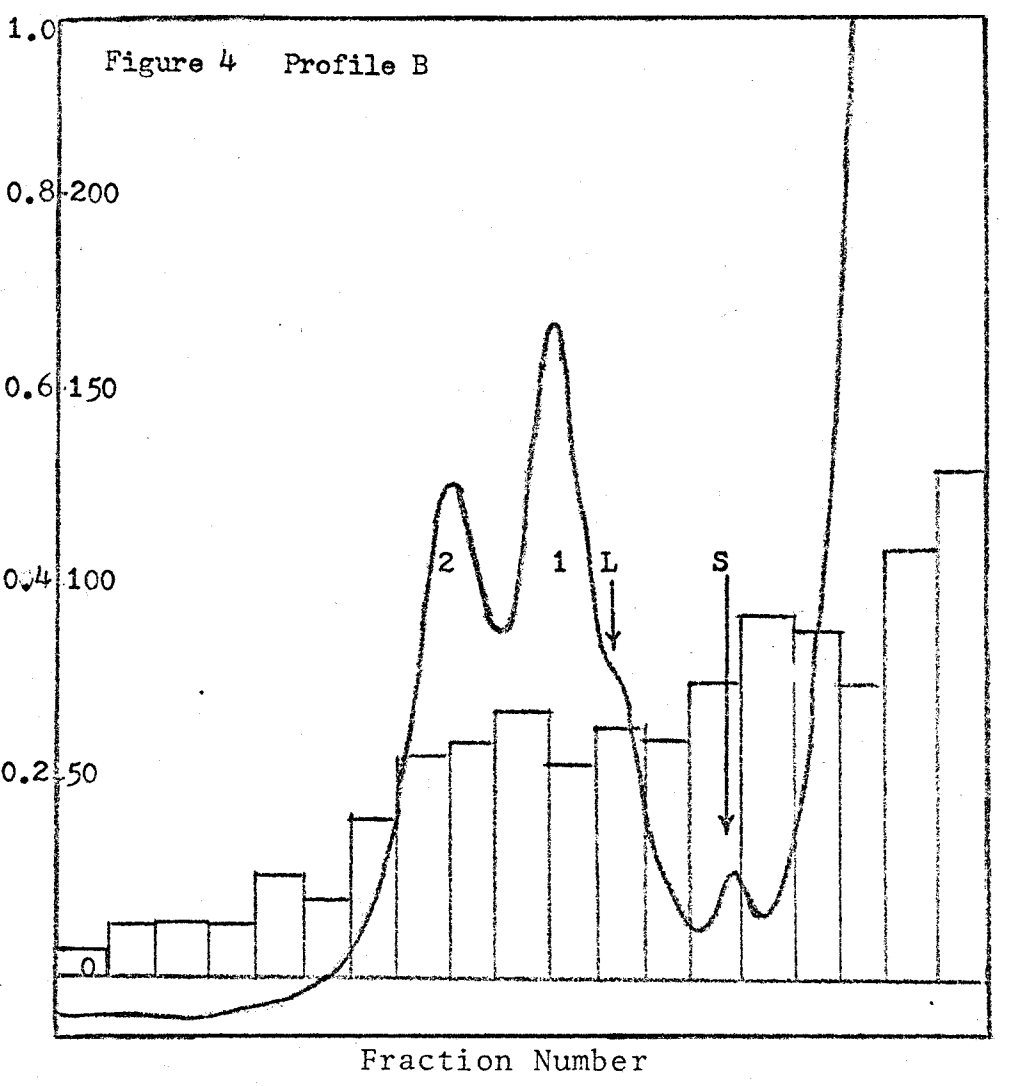
Profiles E and F - Profiles represent the centrifugation of the above mentioned ribosomes in a 15-40% sucrose gradient containing 500 mM K^+ and 12 mM Mg^{++}.

The bar graphs indicate the amount of labelled polypeptide found in that fraction.

A Comparison of Release of Nascent Labelled Polypeptides

Between Ribosomes of Type I and II





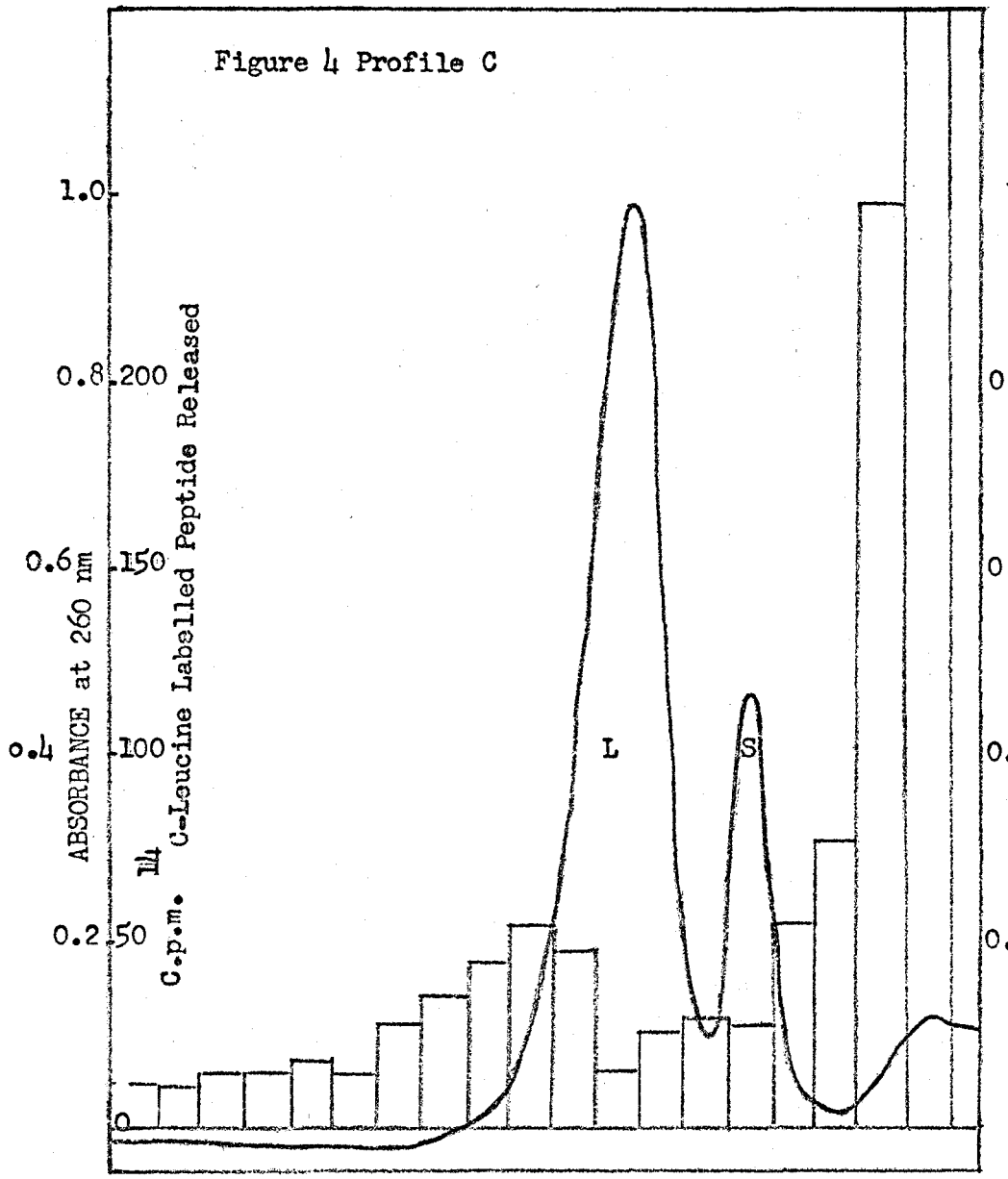


Figure 4 Profile C
1.0
0.8
0.6
0.4
0.2
0
200
150
100
50
L
S
ABSORBANCE at 260 nm
C.p.m. ^{14}C-Leucine Labelled Peptide Released
Fraction Number

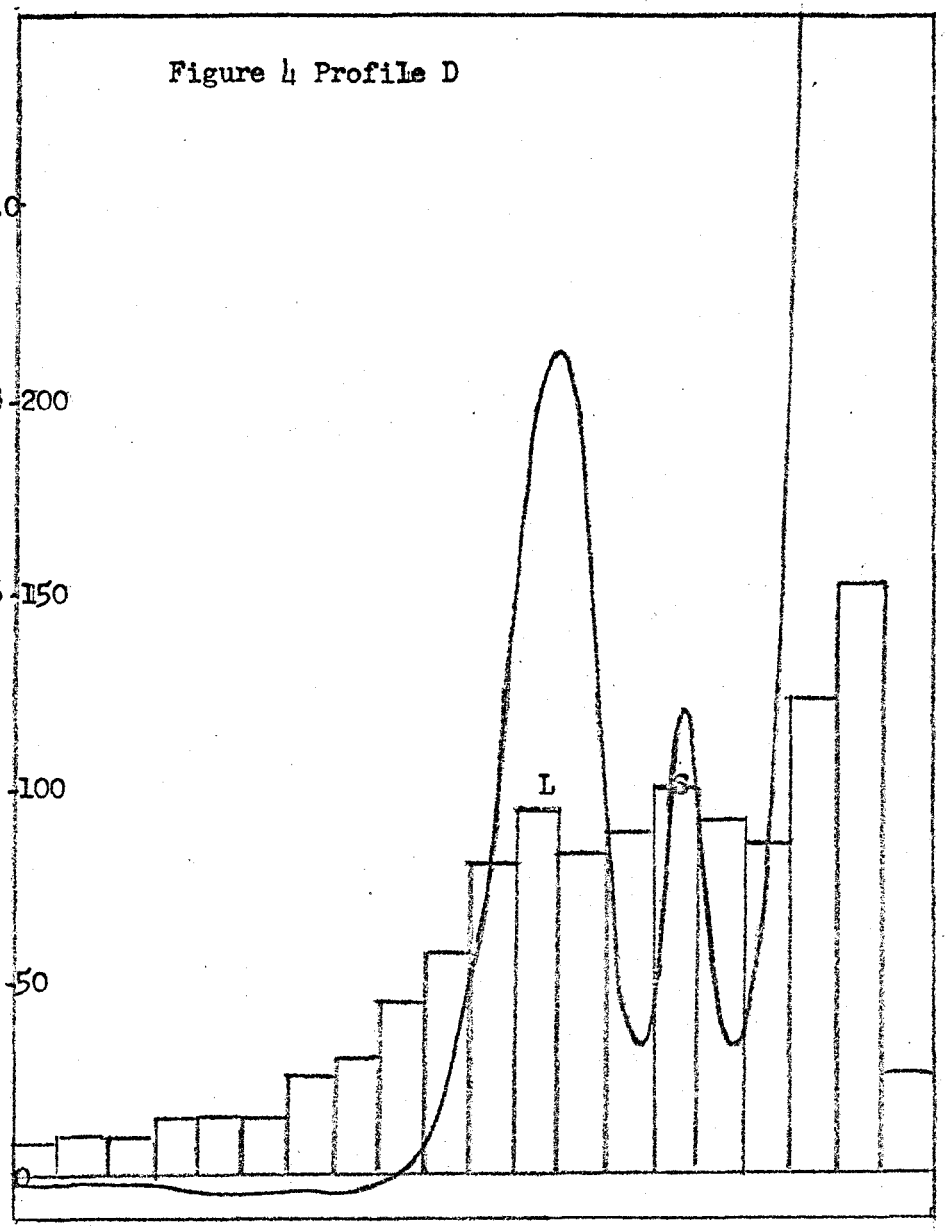


Figure 4 Profile D
1.0
0.8
0.6
0.4
0.2
0
200
150
100
50
L
S
Fraction Number

Figure 4 Profile E

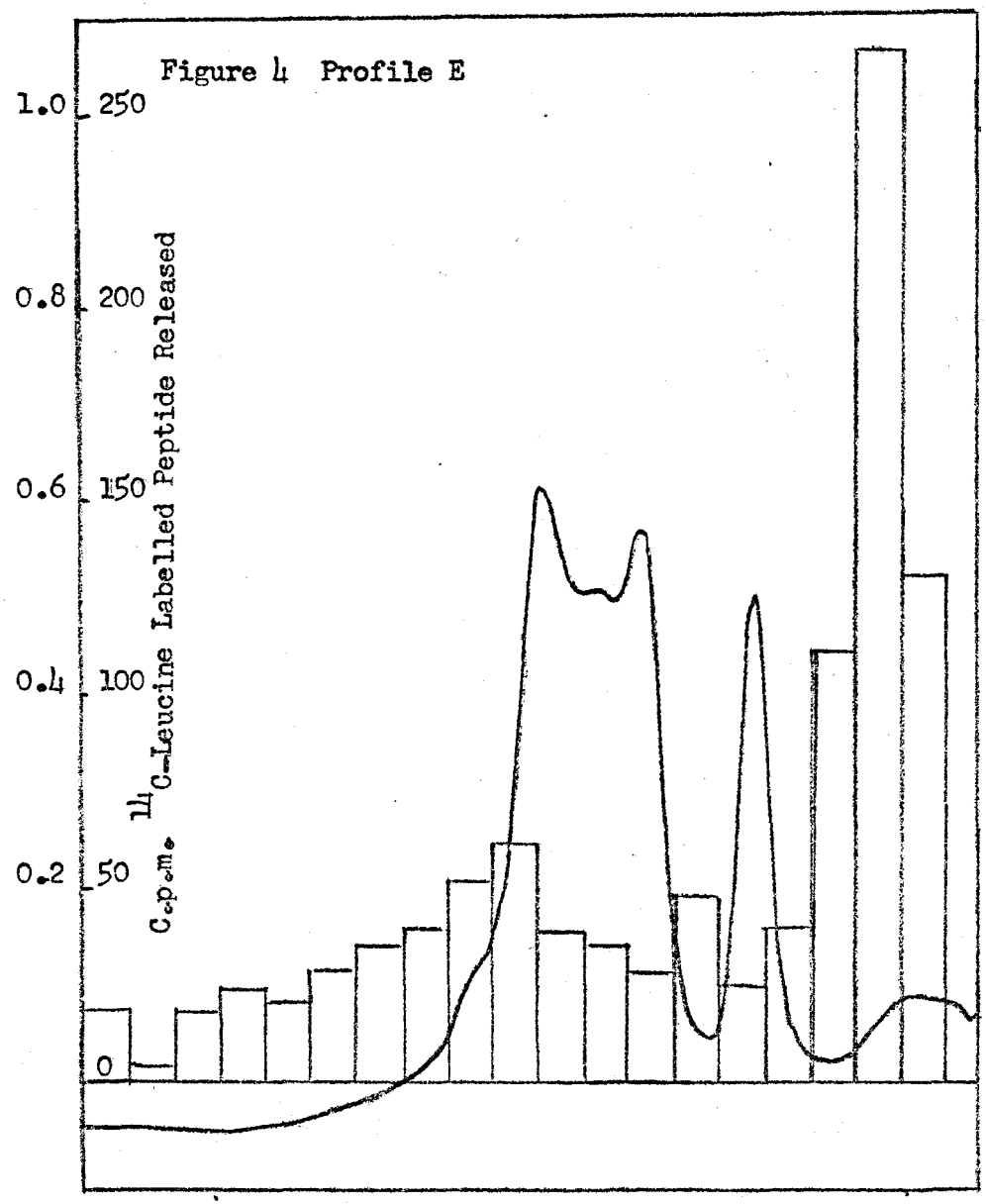
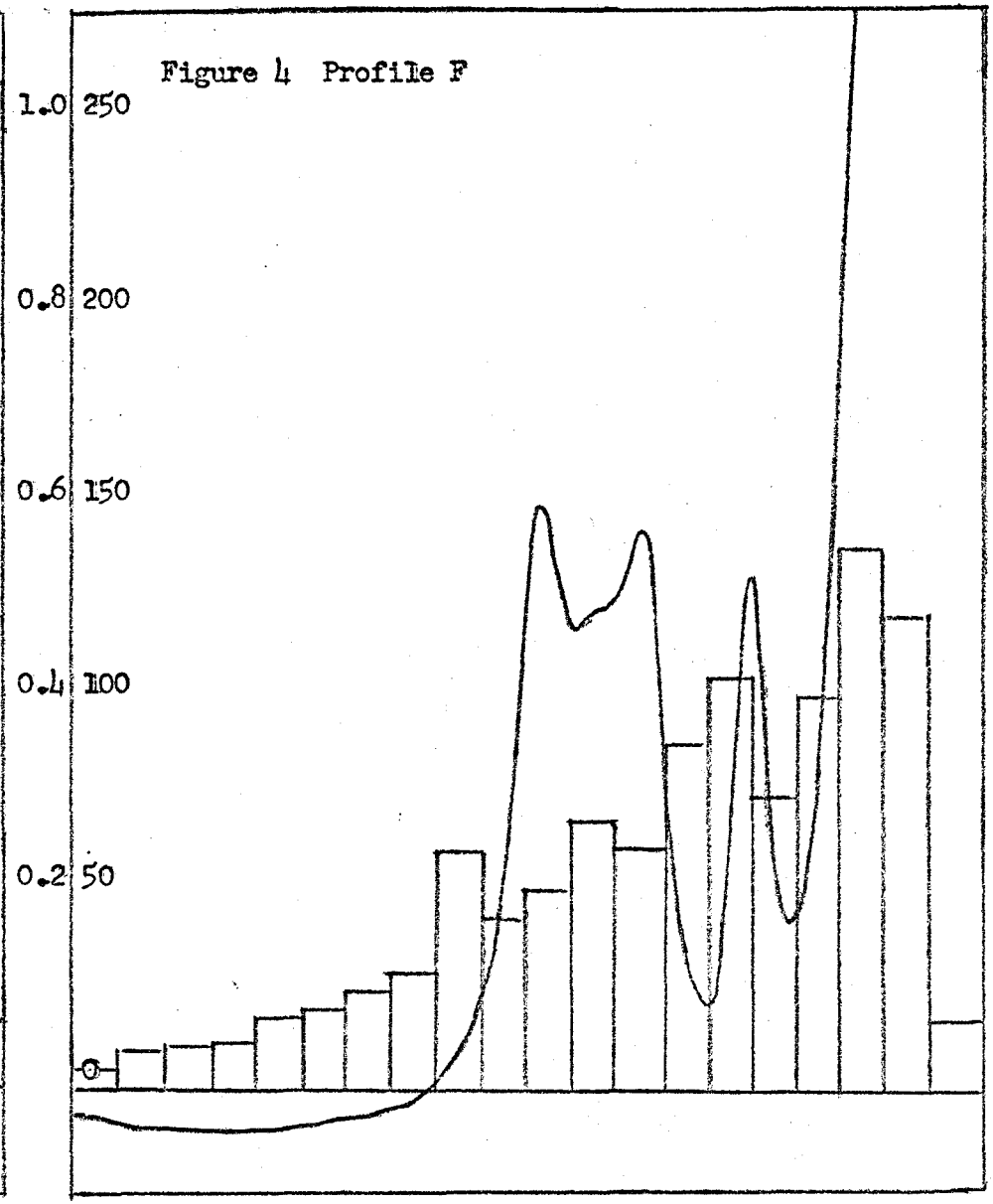


Figure 4 Profile F



to our assumption that the correct substrate for the assay would be ribosomes free of peptidyl-tRNA. This led to the choice of ribosomes of Type II prepared as described in Section IA-6(ii) of the methods as the substrate for the DF assay system. The method of preparation of these ribosomes finally adopted is described in Section IA-3(b) of the methods. (See Fig. 2 and 3 for a comparison of the gradient analysis of these ribosomes of Type II with those of Type I.

DISCUSSION

The method chosen for the preparation of polyribosomes is one which has already been well documented (see Section IA-1 of the methods).

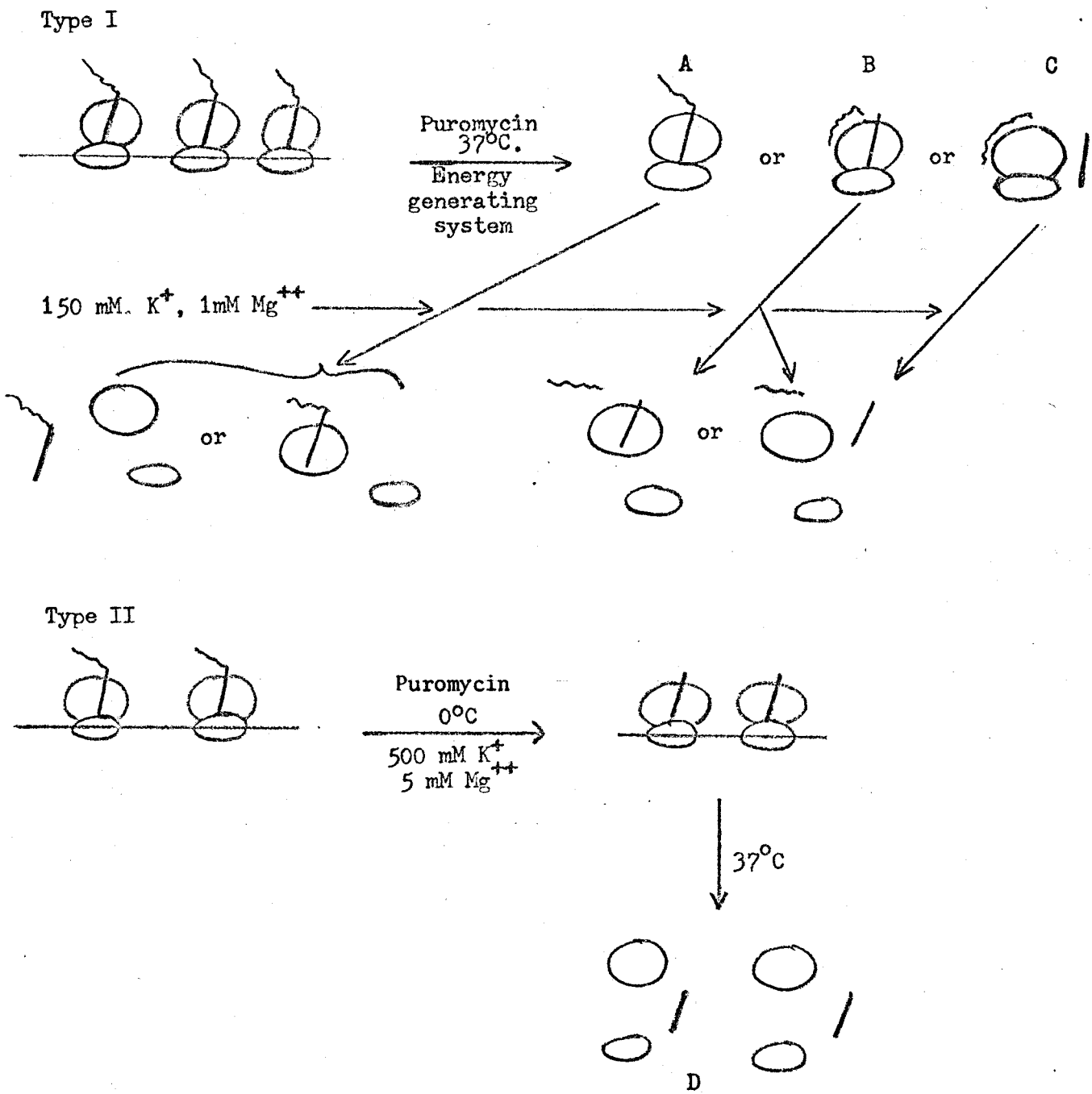
Monomeric ribosomes of Type I had been used as substrate in the assay system up to the time of the communication with G. Blobel and D. Sabatini. It was thought that these ribosomes were free of both mRNA and peptidyl-tRNA (87). This was the form of the substrate which was expected to be recognized by a dissociation factor. The information received via the communication indicated that the ribosomes used in our assay were, in fact, not free of peptidyl-tRNA. As a result of this information the release of polypeptides by the two methods was compared. The results showed clearly that there was a great deal of similarity between the ribosomes prepared by the two methods (their centrifugation analyses were almost identical), but irrespective of the concentrations of K^+ and Mg^{++} used in the gradients, the release of labelled peptide always occurred to a greater extent in a modified version of the new method communicated to us (hereinafter referred to as

the "high salt" method), than it had in our method (hereinafter referred to as the "energy" method). In fact our results showed that there was at best only 30% of the nascent polypeptides released in the "energy" method. The (^{14}C) counts, resulting from the "energy" method of preparation, were found spread throughout the gradient (see Fig. 4) indicating that the nascent polypeptide was being removed during its sedimentation through the sucrose density gradient. This showed that the release of polypeptide had only occurred to a limited extent, if at all, previous to the layering of the ribosomes on the gradient. The ribosomes prepared by the "high salt" method are freed of the nascent peptide previous to layering on sucrose gradients. This result led to the choice of ribosomes of Type II as the new substrate for the DF assay system.

This study leads to some interesting conclusions on the behaviour of ribosomes under various conditions. Ribosomes of Type I could have released at the most only 30% of their peptidyl-tRNA and yet the results show ribosomes of Type I and Type II behave identically during centrifugation through sucrose gradients which contain the same ionic concentrations. So the presence or absence of peptidyl-tRNA appears to make little difference in the interactions which occur between subunits at different ionic concentrations. Type I ribosomes prepared by the "energy" method have nascent peptides which have not been released but which may have either reacted or not reacted with puromycin. When ribosomes of Type I are adjusted to an ionic concentration of 150 mM K^+ and 1 mM Mg^{++} they dissociate to subunits active in amino acid in-

corporation (87). Therefore the mechanisms shown in Fig. 5 may be functioning in the two systems. Subunits prepared by both of these methods are active and interact identically on sucrose gradients depending on the ionic conditions. On a gradient of 1.5 mM Mg^{++} and 25 mM K^{+} they form largely monomeric ribosomes. It can therefore be proposed that if the ribosomes of Type I have peptide which has reacted with puromycin but is still attached (Cases B and C, Fig. 5) and if ribosomes of Type I are incubated at 37°C in the presence of 500 mM K^{+} and 5 mM Mg^{++}, they should form active subunits identical to those in the "high salt" method (D of Fig. 5). In any case, the attached component does not interfere in the reassociation or rearranging of the subunits which depends only on the K^{+} and Mg^{++} concentration of the sucrose gradients. Ribosomes of Type II are subunits when they are layered on the sucrose gradients. Ribosomes of Type I are presumably not. However they behave identically in the gradient. Ribosomes of Type II are subunits because the conditions at 500 mM K^{+} and a temperature of 37°C cause them to lose the tRNA moiety of the former peptidyl-tRNA (112). For use in the DF assay these subunits (Type II) are reformed to monomeric ribosomes by adjusting the K^{+} and Mg^{++} concentrations by a dilution step followed by a centrifugation through 1 M sucrose in TKM to pellet the monomers. At 1 mM Mg^{++} and 150 mM K^{+} both Types, I and II, form only large and small subunits. At 5 mM Mg^{++} and 25 mM K^{+} our results show that both types of ribosomes rearrange in the gradients to mostly dimers and monomers. This indicates that the correct Mg^{++} concentration in the

Figure 5 Probable Results of Puromycin Treatment



sucrose gradient for the existence of mainly monomers at 25 mM K^+ is between 1 mM and 5 mM. Our results indicate further that 1.5 or 2 mM is the correct Mg^{++} concentration to have in the gradients to get mostly monomeric ribosomes. In the "high salt" method of preparation (Section IA-3(b) of the methods), a Mg^{++} concentration of 2 mM was used. For analysis, a Mg^{++} concentration of 1.5 mM was used in the gradients.

To illustrate that K^+ concentration is also important in relation to the Mg^{++} concentration, the results show that if the K^+ concentration is high enough subunits can still be obtained even if the Mg^{++} concentration is as high as 12 mM. Therefore the ratio between the two ionic concentrations is of importance. Raising Mg^{++} and keeping K^+ constant favours formation of monomers and dimers. Raising K^+ and keeping Mg^{++} constant favours formation of subunits. However, raising the Mg^{++} concentration as high as 12 mM causes unknown combinations of subunits to arise when the K^+ concentration is raised and some are caused to aggregate and to centrifuge to the bottom of the gradient.

It is interesting to note that there were always more (^{14}C) counts associated with the leading edge of each peak in the profile (Fig. 4). This is the position in which one would expect the ribosomes to band if some ribosome components still have the labelled peptide attached since the peptide might increase the density or alter the shape of the ribosome component so that it would sediment slightly faster than the ribosomes not possessing the peptide.

The results of Blobel and Sabatini and our results indicate that puromycin reacts with the nascent peptide and ribosomes at 0^oC

in a "high salt" concentration to form a (peptidyl-pur-rib) complex. The release of the peptidyl-puromycin molecule from the ribosomes to the extent of 80% (112) requires "high salt" (high K^+ concentration). Whether the reaction of the nascent peptide and ribosomes with puromycin at 0^oC requires a high salt concentration as well is not known, although Blobel and Sabatini (112) suggest that "high salt" is required. However, the high salt concentration does serve the purpose of releasing the peptidyl-puromycin molecule. If "high salt" is required for the reaction it may be proposed that puromycin has two reactions in which it may participate depending on the conditions. Our results show that ribosomes incubated with puromycin and an energy generating system yield monomeric ribosomes free of mRNA, but still containing most of the nascent peptide either as peptidyl-puromycin or as peptidyl-tRNA; whereas, we know that an incubation at 0^oC with no energy and a high K^+ concentration causes the release of peptidyl-puromycin only and yet the ribosomes are still attached to mRNA (112). Therefore, in summary, when an energy generating system is used, puromycin will cause the ribosomes to separate intact from the mRNA and when a high K^+ concentration and no energy is used, puromycin will react with the nascent peptide causing its release as a complex with puromycin.

The subunits formed as a result of the DF activity on ribosomes of Type II centrifuge at a faster rate than those produced as a result of lowering the Mg^{++} concentration to 0.5 mM and adjusting the K^+ concentration to 150 mM (designated "low Mg^{++} subunits"; see Fig. 7 for the comparison) which in turn centrifuge at a faster rate

than ribosome subunits formed from EDTA treated polysomes (87). It may be that the difference in sedimentation of Type II ribosomes from either of the others is the result of more attached proteins. Since they would be "more complete" subunits than the "low Mg^{++} subunits", which are active (87), they are expected to be active as well and indeed they must be if this is to be support for the natural function of the DF in vivo.

Ribosomes free of mRNA and peptidyl-tRNA depend critically on the correct Mg^{++} and K^{+} concentrations in the gradient to remain as monomers throughout centrifugation. As a result of this fact the assay system for DF would be improved if a method could be devised to stop the reaction of DF with ribosomes at a particular point previous to gradient analysis. Then it would be certain that no changes in the ribosomes were occurring as a result of either the action of the sucrose gradient or slight variations in the ionic conditions. In the Appendix such a method was used. Formaldehyde (4%) has been used to "fix" the ribosomes so that no other interactions can take place. In the reaction of DF with monomers the dependence of DF activity on the temperature and time duration of the incubation could then be determined. This method of "fixing" the ribosomes has also been used by G. Blobel and D. Sabatini (112). Fixation in formaldehyde preserves the state of aggregation of ribosomes and immediately prevents changes in ionic conditions from interconverting monomers and subunits.

SECTION B: STUDIES ON A FRACTIONATION SCHEME TO ISOLATE DISSOCIATION FACTOR

The following describes attempts to isolate a factor capable

of dissociating rat liver ribosomes which are free of mRNA and peptidyl-tRNA.

METHODS

(1) Preparation of Liver Subcellular Fractions

It was expected as a result of work done with E. coli (74) that the DF would be found associated with the native subunits of the cell. For this reason we isolated the subunit enriched fraction to look for DF.

Male hooded rats which had been starved 24 hours to reduce glycogen, were stunned by a blow to the head. Their necks were broken and their livers quickly excised and washed in ice-cold 0.25 M sucrose-TKM*. All subsequent operations were performed in the cold (2-4°C). The livers were blotted and minced and then homogenized with 2 1/2 volumes of 0.25 M sucrose-TKM* in a Potter-Elvehjem homogenizer with a motor driven teflon pestle. The homogenate was centrifuged at 15,000 rev/min for 15 minutes in the A211 rotor of an International B-20 centrifuge and yielded the post-mitochondrial supernatant. This post-mitochondrial supernatant was adjusted to 0.5 mM dithiothreitol and then centrifuged at 40,000 rev/min for 45 minutes in the A211 rotor. The supernatant was centrifuged 50,000 rev/min for 2 hours in the A321 rotor. The pellet was extracted by first suspending it in $TK^{1.0}{}_{M}{}^{\cdot 010}$ ($TK^{1.0}{}_{M}{}^{\cdot 010}$ is 50 mM Tris-HCl, pH 7.8; 1 M KCl; and 10 mM $MgSO_4$) and then stirring by means of a magnetic stirrer for half an hour. It was then centrifuged at 50,000 rev/min for 1 hour in the A321 rotor.

The supernatant was passed through a G-25 sephadex column equilibrated with TKM* to adjust the KCl and $MgSO_4$ concentrations to 25 mM and 1.5 mM respectively. The protein concentration was determined by the method of Warburg and Christian (113). In this method the extinction of an appropriately diluted protein solution was measured at 260 nm and 280 nm, and from this the ratio E_{280}/E_{260} was calculated. Using this ratio, the proportion of nucleic acid in the protein solution was determined and a factor for the calculation of the protein concentration was also determined from a standard curve (114). The protein concentration is given by the following:

Concentration (mg/ml) = Extinction at 280 nm X Factor X 1/d

where d=length of the light path in cm.

Since pure yeast nucleic acid and crystalline yeast enolase were used to prepare the standard curve, the method is liable to error in so far as other proteins and nucleic acids have different extinctions. However the method was found to be approximate and rapid and it could be used with small quantities of material.

(2) Assay of Liver Subcellular Fractions for Dissociating Activity

The preparation of DF, prepared as described in Section IB-1 of the methods, and other subcellular fractions were assayed for ribosome dissociating activity according to the scheme of Section IA-4 of the methods and the technique of centrifugation analysis as shown in Section IA-2 of the methods.

(a) The polysome subcellular fraction was explored for dissociating

activity. Polysomes (10 mg) as prepared in Section IA-1 of the methods were extracted by suspending in $TK^{1.0}{}_{M}{}^{.010}$ (previously defined) to a volume of 2 ml and stirring for 2 hours at 4°C. The supernatant was obtained by centrifugation in centritubes (2 ml. by volume) in the International A321 rotor fitted with centritube adaptors at 50,000 rev/min for 1 hour.

The supernatant was desalted by passage through a column of sephadex G-15 equilibrated with TKM. The substrate ribosomes were prepared as described in Section IA-3(b) of the methods except that the incubation mixture contained these final concentrations in 10.42 ml: polysomes (1 mg/ml); Tris-HCl (0.05 M); KCl (480 mM); $MgSO_4$ (1.15 mM) and puromycin (14.8 μM).

The preparation was analyzed for dissociating activity as described in Section IA-4 of methods except that the monomeric ribosomes were suspended in TKM buffer (concentration 5 mg/ml) and 1.0 mg samples in TKM buffer were mixed with dissociation factor preparation. The protocol was as shown in Table 2.

The centrifugation analysis was performed as in Section IA-2 of methods except that three 15-40% sucrose in TKM gradients and one 15-40% sucrose in $TK{\cdot}^{5}{}_{M}{}^{\cdot 002}$ gradient were used. The results can be seen in Fig. 6 in Section IB-2(a) of the results.

(b) The subunit enriched fraction was explored for dissociating activity. This experiment was identical to the previous except for the following differences: the DF was prepared as described in Section IB-1 of the methods substituting a 25,000 rev/min centrifugation for

Table 1 An Example of a Typical Assay Protocol

Component Added	Test Tube			
	A	B	C	D
Ribosomes(5mg/ml)	0.1 ml	0.1 ml	0.1 ml	0.1 ml
DF preparation (30mg/ml)	———	0.2 ml	0.4 ml	———
TKM*	0.5 ml	0.3 ml	0.1 ml	———
$TK \cdot 500_M$*	———	———	———	0.5 ml
Total Volume	0.6 ml	0.6 ml	0.6 ml	0.6 ml

$TK \cdot 500_M$* is a medium composed of Tris-HCl(0.05M); KCl(0.5M); and $MgSO_4$(1.5mM)

Table 2 Protocol for the DF Assay of the Polysome Subcellular Fraction

Addition	Assay Tube			
	A	B	C	D
Ribosomes(5mg/ml)	0.2 ml	0.2 ml	0.2 ml	0.2 ml
TKM	0.4 ml	0.2 ml	———	———
$TK \cdot 12_M 5 \times 10^{-4}$	———	———	———	0.4 ml
DF preparation	———	0.2 ml	0.4 ml	———
Total Volume	0.6 ml	0.6 ml	0.6 ml	0.6 ml

$TK \cdot 12_M 5 \times 10^{-4}$ is a medium composed of Tris-HCl(0.05M); KCl(0.12M); and $MgSO_4$(0.5mM)

45 minutes for the centrifugation at 40,000 rev/min for 45 minutes; and the subunit enriched pellet was extracted by first suspending it in $TK^{1.0}M^{0.010}$ to a total volume of 3 ml and then stirring for a period of 17 hours. In this case the protein concentration of the factor preparation was found to be 35 mg/ml. The protocol was shown in Table 3. The results are presented in Fig. 7 of Section IB-2(b) of the results.

If this same experiment was repeated except that 1/10th volume of 14.3% DOC was added after the 25,000 rev/min centrifugation, the results are as shown in Fig. 8 of Section IB-2(b) of the results.

(c) A brief KCl extraction (half an hour) was compared with a 16 hour KCl extraction. Monomeric ribosomes were prepared as in Section IA-3(b) of methods and DF was prepared as in Section IB-1 of the methods except that half of the 50,000 rev/min x 2 hrs. supernatant was extracted 16 hours instead of one half hour. Each of the two halves of the preparation were desalted separately on columns of G-25 Sephadex equilibrated with TKM*. The samples were treated as shown in Fig. 9.

The dissociating factor assay was done similarly to Section IA-4 of methods but the protocol was as shown in Table 4.

The incubation was the same and the gradient analysis was the same as for 20°C gradients in Section IA-2 of the methods. The results are presented in Fig. 10 of Section IB-2(d) of the results.

(3) Specific Activity of Dissociation Factor

A quantitation of the peak areas was often difficult because of the incomplete separation of the peaks. However where separtion was

Table 3 Protocol for the DF Assay of the Subunit Enriched Fraction

Component Added	Assay Tube				
	A	B	C	D	E
Ribosomes(2mg/ml)	0.5 ml	0.5 ml	0.5 ml	0.5 ml	Ribosome Component *
TKM	0.5	0.2	———	———	———
TK $\cdot 12_M 5X10^{-4}$	———	———	———	0.5	———
DF preparation	———	0.3	0.5	———	———
Total Volume	1.0	1.0	1.0	1.0	

* A sample of ribosome components suspended in TKM. These components were subsequently extracted to yield the DF preparation shown above.

Figure 9 Treatment of the DF Extract for the Protocol of Table 4

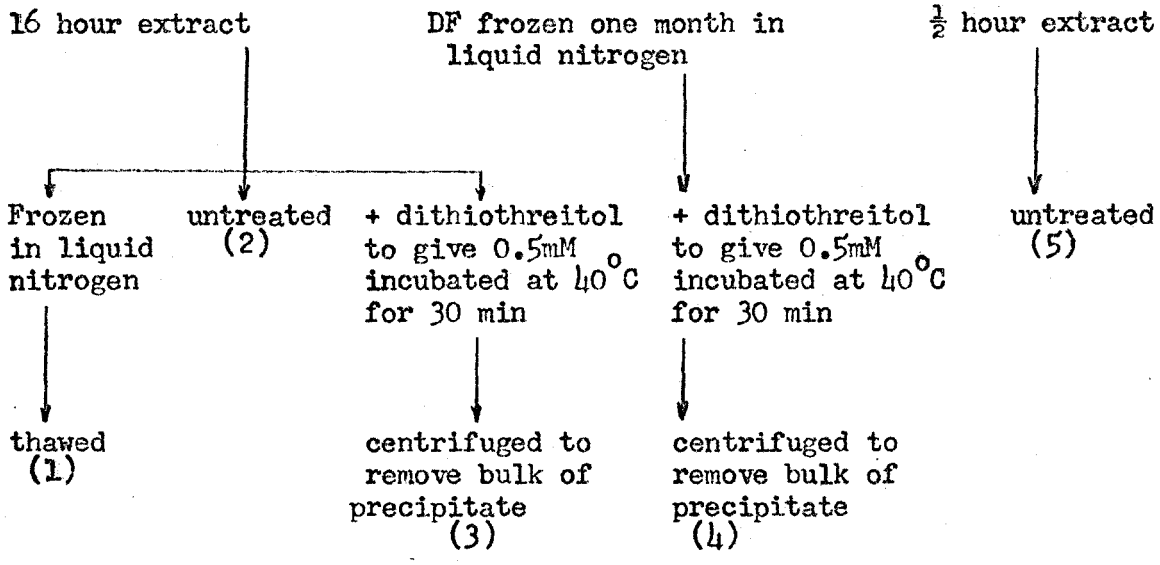


Table 4 Protocol for the DF Assay which Shows a Comparison between DF Preparations which have been Extracted and Treated as Shown in Figure 9

Component Added	Assay Tube A (ml)	B (ml)	C (ml)	D (ml)	E (ml)	F (ml)
Ribosomes(4mg/ml)	0.15	0.15	0.15	0.15	0.15	0.10
TKM*	0.5	—	—	—	—	—
DF(55mg/ml)	—	0.5	0.5	0.5	0.5	0.4
Source	—	(3)	(4)	(2)	(1)	(5)
Total Volume	0.65	0.65	0.65	0.65	0.65	0.50

For the Source refer to numbers in Fig. 9
Each tube contained 0.5mM Dithiothreitol.

incomplete the peaks were extrapolated and the percent dissociation calculated as in Section IA-4 of methods. The specific activity of the preparation was calculated in terms of mg of ribosomes dissociated per mg of protein in the preparation. This was calculated in the following way: The difference in the percent dissociation of the assay and that of the monomer control was multiplied as a fraction of 100% dissociation by the number of mg of ribosomes used for the assay. This gave the number of mg of ribosomes dissociated per x ml of the dissociation factor used in that assay. Since the protein concentration of the DF preparation is known (it was calculated by the method of Warburg and Christian) then the mg of ribosomes dissociated per mg of total protein in the DF preparation can be calculated.

RESULTS

1 Choice of Liver Subcellular Fraction for Analysis

The native subunit enriched fraction was chosen as the most likely subcellular fraction to contain dissociating activity for reasons previously stated. This fraction was isolated in various ways, but the method of isolation shown in Section IB-1 of the methods was found to yield a subunit enriched fraction which yielded, on extraction with KCl, the greatest dissociating activity and the purest DF preparation.

2(a) The results of an exploration of the polysome subcellular fraction for the ribosome dissociating activity is shown in the profiles of Fig.6.

Fig. 6 Sucrose density gradient analysis of ribosomes incubated with a dissociation factor preparation.

A Profile of a preparation of ribosomes (Type II) incubated 15 min at 30°C without addition of dissociation factor.

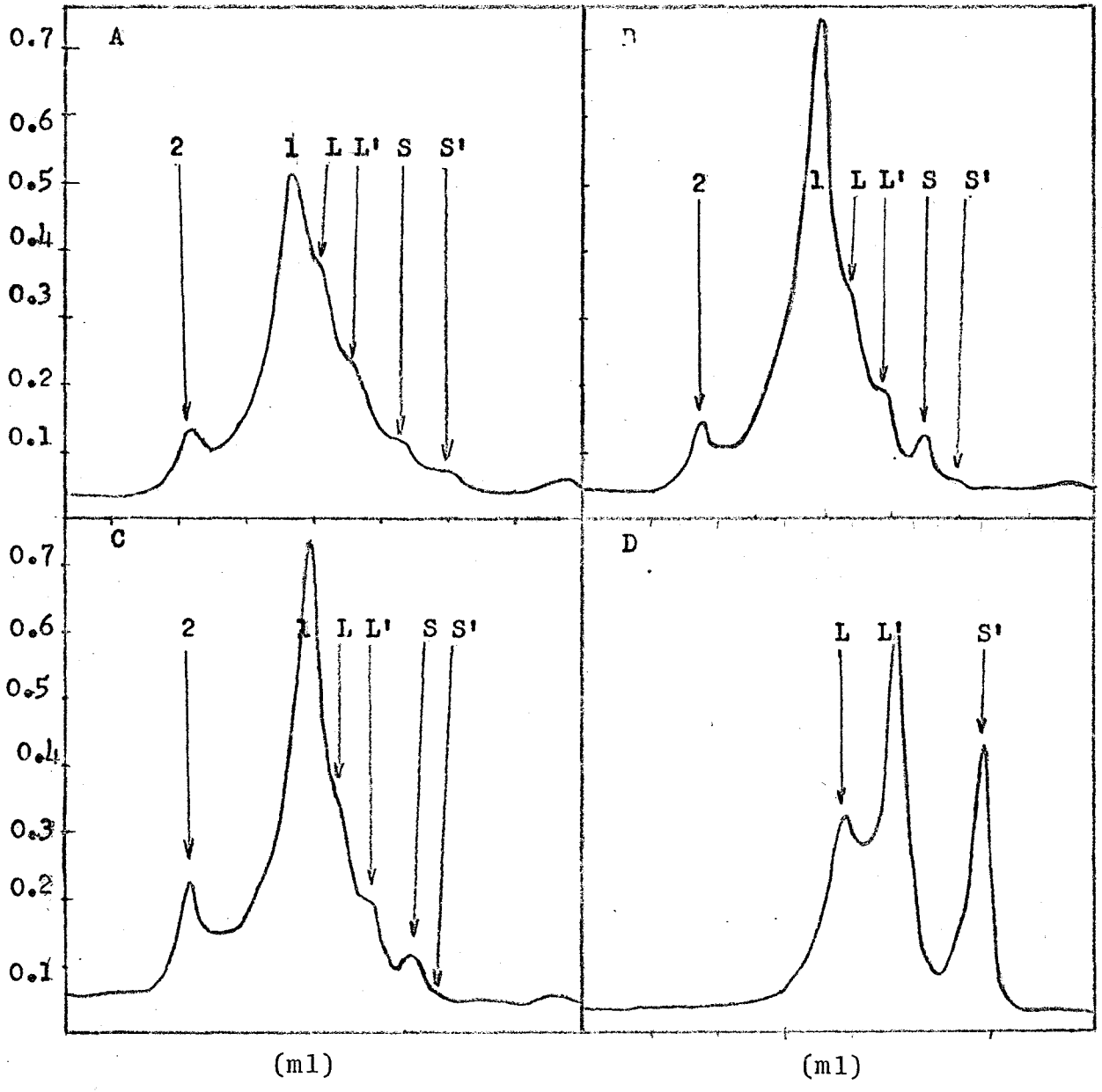
B Profiles of a preparation of ribosomes (Type II) incubated 15 min at 30°C with a preparation obtained by extraction of the polysome fraction.

C Same as B except that the ribosomes were incubated with twice as much of the preparation.

D Profile of a preparation of ribosomes (Type II) incubated 15 min at 30°C without the addition of dissociation factor. Centrifugation was for 6 hours at 25,000 rev/min at 0°C in a linear gradient of 15-40% sucrose containing 500 mM K^+ and 2 mM Mg^{++}.

The other centrifugations were for 6 hours at 25,000 rev/min at 0°C in a linear gradient of 15-40% sucrose in TKM buffer.

Figure 6 The DF Assay of the Polysome Subcellular Fraction
(See Table 2 for the Protocol)



There is no significant ribosome dissociating activity in the polysome subcellular fraction. The "high salt" gradient which was used for the centrifugation of the subunit control (Fig. 6D) possessed the following ionic conditions: KCl (500 mM) and $MgSO_4$ (2 mM). This gradient caused the breakdown of the large and small subunits to slower sedimenting components. These components are definitely different from those produced by the DF. The breakdown of the large subunits can be seen in Fig. 6D. The small subunit has also been broken to a slower sedimenting component, but this is more difficult to observe.

2(b) The results of the assay when DF is prepared by the KCl extraction of the "native" subunit enriched fraction is shown in Fig. 7. Zonal sedimentation showed that increasing amounts of the DF preparation caused increasing dissociation of the ribosomes into their subunits (see Fig. 7A, B and C). KCl (500 mM) and $MgSO_4$ (1 mM) were used in the preparation of monomeric ribosomes, but otherwise the procedure was as described in Section IA-3(b) of the methods. These conditions caused some rearrangement of the ribosome components. This is shown in the centrifugation profile of Fig. 7D. This rearrangement is attributable to the Mg^{++} concentration being less than the minimum required for stable ribosome and subunit conformations as mentioned previously.

If 1/10th volume of 14.3% NaDOC was added after the 25,000 rev/min centrifugation, all the ribosome dissociating activity was either released from the subunits or destroyed, so that it could not be obtained by the subsequent extraction with 1M KCl. This result is shown in Fig. 8.

Fig. 7 Sucrose density gradient analysis of ribosomes incubated with a dissociation factor preparation.

A Profile of a preparation of ribosomes (Type II) after incubation at 30°C for 15 min without the addition of dissociation factor.

B Profile of preparation of ribosomes (Type II) incubated at 30°C for 15 min with 0.3 ml of a preparation obtained by extraction of the subunit enriched fraction.

C Same as B except that the ribosomes were incubated with 0.5 ml of factor preparation.

D Profile of a preparation of ribosomes (Type II) incubated at 30°C for 15 min without the addition of dissociation factor. Centrifugation was for 6 hours at 25,000 rev/min at 0°C in a linear gradient of 15-40% sucrose containing 500 mM K^+ and 2 mM Mg^{++}.

E Profile of the components in the subunit enriched fraction which were extracted to yield the factor preparation used above.

The other centrifugations were for 6 hours at 25,000 rev/min at 0°C in a linear gradient of 15-40% sucrose in TKM buffer.

The numbers above the peaks refer to the distance of the peak from the top of the gradient. This enables the peaks in the different profiles to be compared.

Figure 7 The DF Assay of the Subunit Enriched Fraction (See Table 3 for the Protocol)

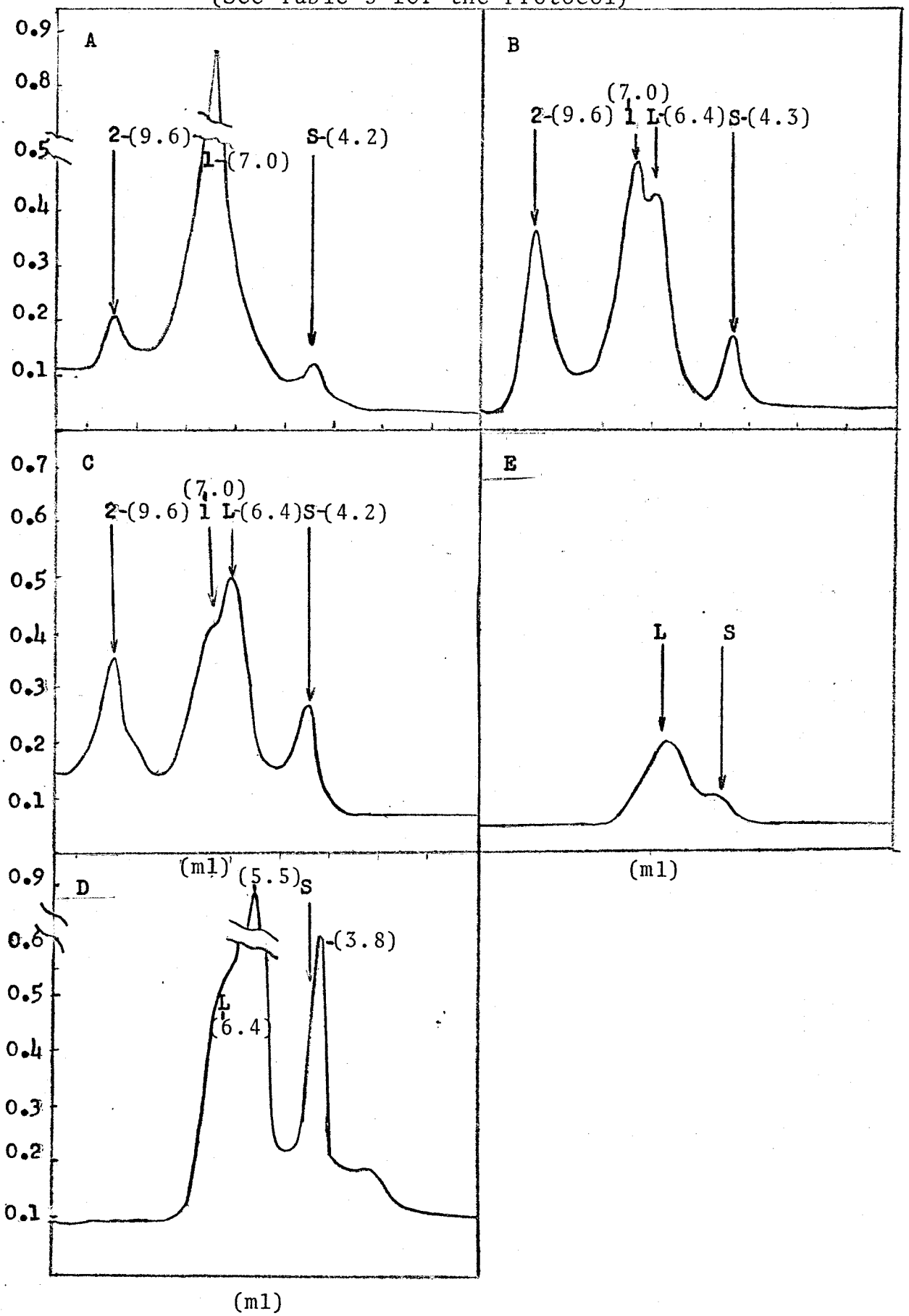


Fig. 8 Sucrose density gradient analysis of ribosomes incubated with a dissociation factor preparation.

A Profile of a preparation of ribosomes (Type II) after incubation at 30°C for 15 min without the addition of dissociation factor.

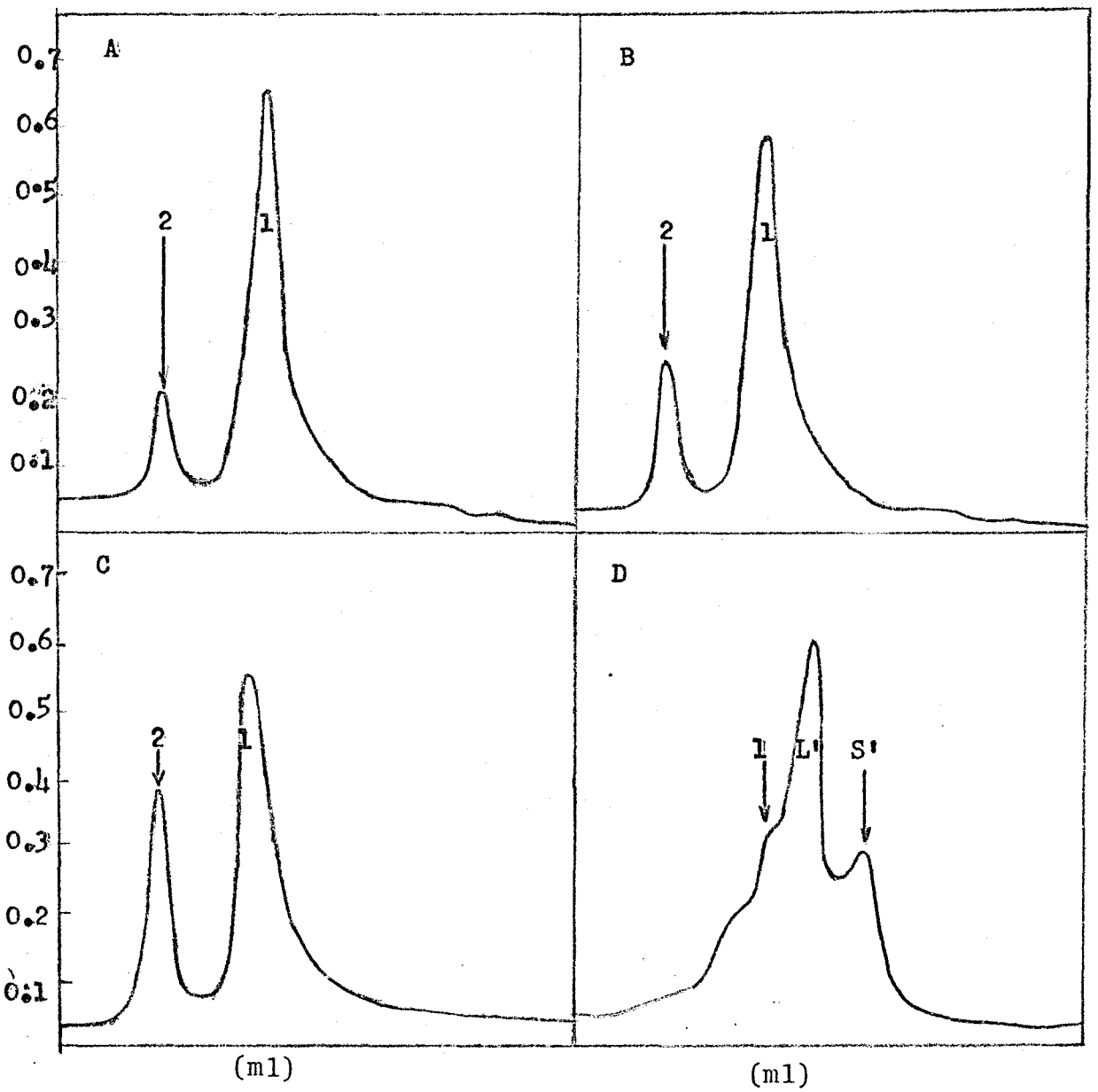
B Profile of a preparation of ribosomes (Type II) after incubation at 30°C for 15 min with a preparation obtained by extraction of the subunit enriched fraction. This preparation prior to extraction was treated with deoxycholate.

C Profile same as B except that 0.5 ml of factor preparation was incubated with ribosomes.

D Profile of a preparation of ribosomes (Type II) incubated at 30°C for 15 min without the addition of dissociation factor. Centrifugation was for 6 hours at 25,000 rev/min at 0°C in a linear gradient of 15-40% sucrose containing 500 mM K^+ and 2 mM Mg^{++}.

The other centrifugations were for 6 hours at 25,000 rev/min at 0°C in a linear gradient of 15-40% sucrose in TKM buffer.

Figure 8 The DF Assay of the Effect of Deoxycholate on the Subunit Enriched Fraction



2(c) The result of using a higher speed centrifugation, 40,000 rev/min and a 1/2 hour KCl extraction can be seen in Figures 10, 12 and 14. The increase in the centrifugation speed from 25,000 rev/min to 40,000 rev/min yields a DF preparation which when assayed gives sharper profiles because the preparation contains less of the material which causes ribosomes to aggregate. This is the result of an increase in purity of the subunit fraction and is due to the exclusion of monomers and larger aggregates by the 40,000 rev/min centrifugation. Also the 1/2 hour KCl extraction extracts only proteins which are loosely associated with the ribosomes (Fig. 10F). Since DF is suspected to be loosely associated with the ribosomes, this short extraction procedure preferentially excludes much of the extraneous protein, which is undoubtedly more tightly bound to the subunits than the DF is bound.

3 The specific activities of the DF preparation described in Section IB-2(b) of both the methods and results are shown in Table 5. This table refers to Fig. 7. The specific activities agree well, which is a good check on their method of calculation and indicates that twice as much DF causes the dissociation of twice the amount of substrate ribosomes. The specific activities as determined for the results in Section IB-2(c) of the methods are presented in Table 6.

DISCUSSION

Factor activity was measured by sucrose gradient analysis of ribosomes incubated with the protein extract. We must note that ribosomes used for dissociation tests contained a small amount of

Fig. 10 Sucrose density gradient analysis of ribosomes incubated with a dissociation factor preparation.

A Profile of a preparation of ribosomes (Type II) after incubation at 30°C for 15 min without the addition of dissociation factor.

B Profile of a preparation of ribosomes (Type II) after incubation at 30°C for 15 min with dissociation factor. The factor was prepared by extracting the subunit enriched fraction for 16 hours with KCl, followed by a 40°C incubation for 30 min. The precipitate formed was removed by centrifugation.

C Profile same as B except the factor was prepared from a factor preparation which had been frozen in liquid nitrogen for 1 month. This preparation was incubated the same as B and centrifuged to remove the precipitate.

D Profile same as B and C except the factor was prepared by extracting the subunit enriched fraction for 16 hours with KCl but was otherwise left untreated.

E Profile same as B, C and D except the factor was prepared by extracting the subunit enriched fraction for 16 hours with KCl followed by freezing in liquid nitrogen. The factor was thawed and used in the assay.

F Profile same as B, C, D, and E except that the factor was prepared by extracting the subunit enriched fraction for half an hour. Centrifugation was for 3 1/4 hours at 25,000 rev/min at 20°C in linear gradients of 15-40% sucrose in TKM* buffer.

In this experiment, unlike the previous experiments, an increase in centrifugation speed from 25,000rev/min to 40,000 rev/min was used in the factor preparation.

Figure 10 The DF Assay using DF Extracted and Treated as in Figure 9 (See Table 4 for the Protocol)

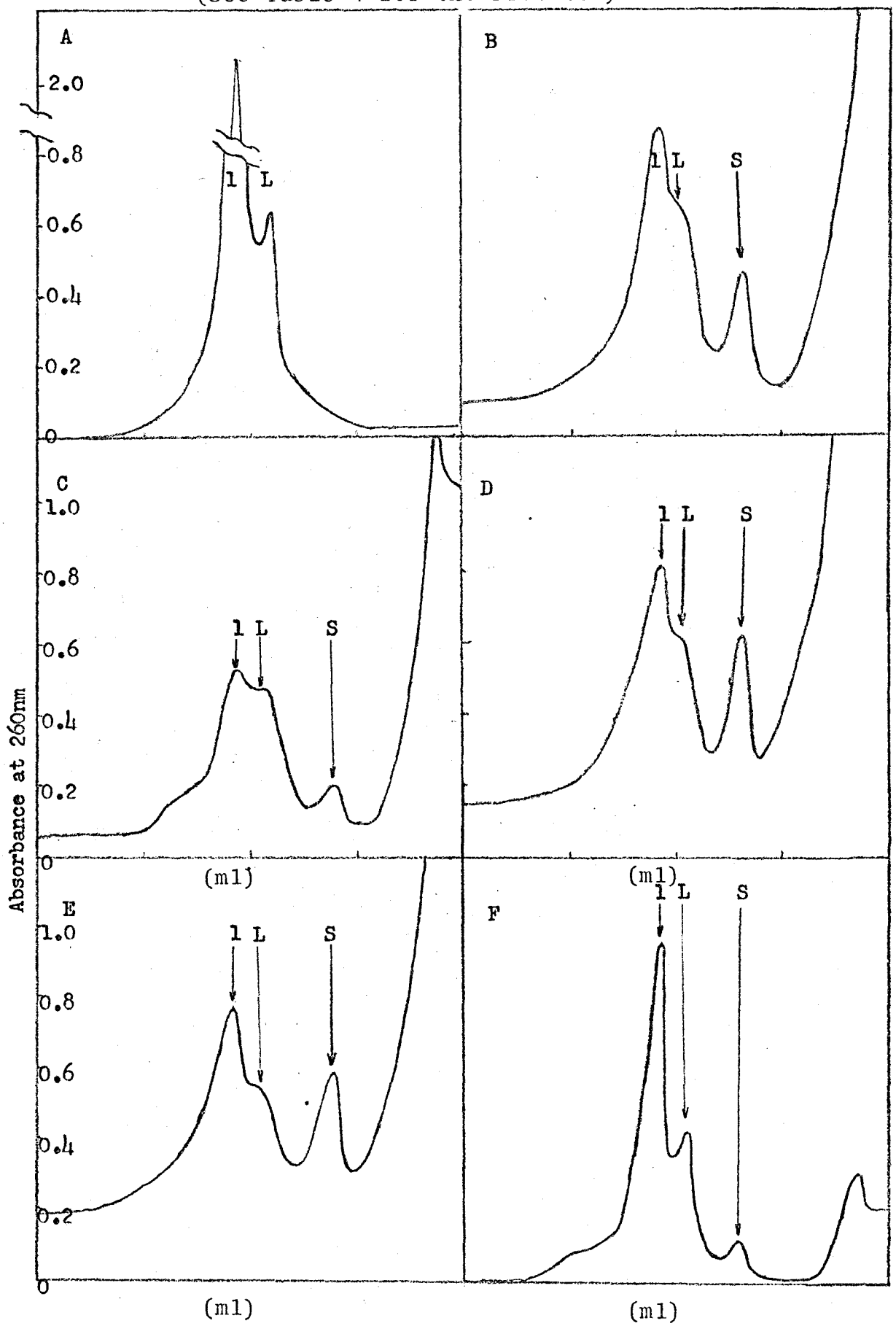


Table 5 Specific Activities of DF Prepared from the Subunit Enriched Fraction (Refers to Fig. 7)

Profile	Peak Areas (units)					Total % Dissociation	% Dissociation due to DF	mg of ribosomes dissociated	mg of protein in the assay	Specific Activity
	2	1	L	S	Total Area					
A	4.68	18.9	7.42	1.28	32.28	26.9	—	—	—	—
B	8.5	8.72	9.85	2.52	29.59	41.7	14.8	0.148	10.4	0.0142
C	7.15	4.74	10.0	2.87	24.76	52.0	25.1	0.251	17.4	0.0144
D	—	—	25.48	7.28	32.76	100.0	—	—	—	—

Table 6 Specific Activities of the DF Preparation which was Extracted and Treated as in Fig. 9 (refers to Fig. 10)

Profile	Peak Areas (units)					Total % Dissociation	% Dissociation due to DF	mg of ribosomes dissociated	mg of protein in the assay	Specific Activity
	2	1	L	S	Total Area					
A	—	18.1	5.25	—	23.35	22.5	—	—	—	—
B	—	9.0	7.7	4.0	20.70	56.6	34.1	0.204	<27.5	>0.00745
C	1.0	5.7	5.3	1.62	13.62	50.9	28.4	0.170	—	—
D	—	9.35	6.05	4.8	20.20	53.7	31.2	0.187	27.5	0.00681
E	—	10.14	4.68	4.68	19.50	48.0	25.5	0.153	27.5	0.00556
F	0.40	8.61	4.08	1.12	14.21	36.6	14.1	0.056	22.0	0.00256

Note: < Means "less than"
> means "greater than"

large subunits. These were large subunits which had not been completely separated from monomers during the final centrifugation into a pellet through 1 M sucrose-TKM* as described in Section IA-3(b) of the methods. The amount of subunits in the ribosome preparation did not vary with the length of storage for up to a week at 0°C and for up to two days at 30°C. Because the factor dependent dissociation is strictly temperature dependent (see Appendix), it is felt that the process involves a specific interaction mechanism. Much of the success of the assay method used appears to depend on the substrate monomers being completely free of peptidyl-tRNA and messenger RNA. The use of monomers reformed from the products of complete dissociation ensures their suitability as a substrate. The results indicate that there is a component with ribosome dissociating activity in rat liver cells and that it can be obtained from the subunit enriched fraction as described in Section IB-1 of the methods. No significant ribosome dissociating activity was isolated from the polysome subcellular fraction.

The centrifugation analyses in sucrose gradients depend critically on the Mg^{++} and K^{+} concentrations. It was found that the added affect of sedimenting through a gradient caused the breakdown of large and small subunits even though the same ionic conditions were used as those which in the presence of puromycin produced stable large and small subunits. The conditions KCl (500 mM) and $MgSO_4$ (2 mM) are therefore probably at the outer limit of "native" subunit stability and any further stress such as that imposed by a gradient will cause the subunits to break down. The temperature undoubtedly has an affect on

this stability as well. The subunits produced by the activity of DF have characteristic sedimentation rates in sucrose gradients, which are unlike those produced as a result of altering ionic conditions.

The effect of DOC action on the native subunits is either to release the DF from the subunit possessing it or to alter the DF in some way so that it is no longer active.

Some alterations in the method of preparation of DF such as a higher speed of centrifugation and a shorter extraction time yielded an increase in purity of the preparation, as judged by sucrose density gradient analyses of the dissociating activity.

In the preparation of monomeric ribosomes it was found that 2 mM Mg^{++} in the centrifugation through 1M sucrose gave more monomers and less dimers and subunits. However, when every step in the preparation was adjusted to 1.5 mM Mg^{++} and the ribosomes were maintained at 24°C after the rise to 37°C, the best monomers were obtained. Dithiothreitol had very little affect on sustaining the DF activity during its preparation or its assay and the DF seemed to be quite stable to freezing in liquid nitrogen.

A preliminary incubation at 40°C for 30 minutes was beneficial because it caused an increase in the specific activity of the preparation (Table 6). This indicated that much extraneous protein was removed by this procedure. However, the total dissociation decreased slightly, which indicates that some dissociation factor may also be inactivated at this temperature. In the preparation of DF, the short extraction period gives a much sharper sedimentation profile indi-

cating that less extraneous protein is present in the preparation. This can be seen in Fig. 10F of Section IB-2(c) of the results. This extraneous material causes aggregation of ribosomes and/or subunits. The effect of the short extraction and the preliminary incubation will be investigated more fully in Section II.

The calculation of the specific activity of the DF is useful for comparing assay results and for the determination of the usefulness of purification procedures which will be looked at in Part II.

The dependency of ribosome dissociation as a function of time and as a function of temperature, are shown in the Appendix. The percent dissociation reaches a plateau after 30 minutes incubation at 30°C.

The findings presented here confirm that a dissociation factor similar to that found in E. coli (74) exists in cells of rat liver. This factor extracted by 1 M KCl from the subunit enriched fraction, causes rapid dissociation of monomeric 80S ribosomes, but not complexed ribosomes. The DF was both obtained from and tested with ribosomes components that had been separated from supernatant proteins and metabolites by sedimentation through sucrose-buffer and so the dissociation reaction does not appear to require a source of energy. Since no increase of dissociation occurred on further incubation after the plateau was reached at 30°C for 30 minutes even when excess substrate ribosomes were present, then DF cannot act on ribosomes in a catalytic manner. If twice as much DF is added under the optimal conditions, twice as much dissociation occurs. This certainly seems to argue for a stoichiometric mode of action for the DF. A Mg^{++}

concentration of 5 mM was found to decrease the response of 80S ribosomes to DF. It therefore seems that DF and 80S ribosomes are in an equilibrium with the subunits; and the equilibrium is dependent on the concentration of Mg^{++} ions. When the subunits formed by DF were re-extracted separately with 1M KCl, the DF activity was found to reside exclusively with the small subunit (please see Appendix).

PART II

PARTIAL PURIFICATION
AND AN ATTEMPT AT MOLECULAR WEIGHT CHARACTERIZATION
OF THE DISSOCIATION FACTOR

INTRODUCTION

The presence in a crude dissociation factor preparation of substances which caused aggregation of ribosomes made impossible the calculation of meaningful specific activities of the factor at various stages in the purification. The effect of this aggregation can be seen by the decrease in "Total Areas" which occurs when DF is added to the assay as illustrated in Table 5 and 6 of Section IB-3 of the results. An increase in the quantity of DF preparation in the assay resulted in a decrease in the "Total Area" units. Since this indicates that a component in the DF preparation is causing ribosomes to precipitate, then some form of purification of the preparation is necessary.

Observations showed that the precipitation occurred during the assay incubation and that it was the result of some thermo-labile protein which caused co-precipitation of ribosomes. To solve the problem of co-precipitation an attempt was made to remove the thermo-labile component by a preliminary incubation of the DF preparation. It was thus hoped to improve the quantitation of the dissociating activity by this and other purification procedures.

The DF in E. coli was known to possess a molecular weight of 21,200 (115). It was of interest to determine how the mammalian

enzyme would compare as far as its molecular weight was concerned.

METHODS

Partial Purification of the Dissociation Factor

Three possible methods of purification were attempted. They were the following:

(i) Ammonium sulphate fractionation.

(ii) Differential inactivation and/or precipitation by heat.

(iii) Column chromatography using Sephadex gels.

It must be noted that prior to heat treatment and ammonium sulphate fractionation the crude factor was passed through a column of Sephadex G-25 equilibrated with TKM* in order to reduce the salt concentration.

1(a) Heat Inactivation

The shorter KCl extraction of the ribosome subunits produced sharper sedimentation profiles in the assay indicating that less extraneous protein was present in the preparation. After equilibration with TKM* we tried to further purify the factor preparation by a preliminary incubation at 40°C for 30 minutes. It was expected that this procedure would precipitate the substance which was causing the aggregation.

Ribosomes were prepared according to Section IA-3(b) of the methods and DF was prepared as in Section IB-1 of the methods. The protocol is as shown in Table 7. The remainder of the experiment was performed identically to that in Section IB-2(c) of the methods. The results are presented in Fig. 12 of Section II-1(a) of the results.

1(b) Ammonium Sulphate Fractionation

(i) The dissociation factor preparation was fractionated by ammonium sulphate at 20%, 35%, 50% and 70% saturation and the fractions analyzed to compare them for dissociating activity. Ribosomes were prepared as described in Section IA-3(b) of the methods. Prior to the assay, the ammonium sulphate fractions were suspended in TKM* and dialyzed against TKM*. The protocol was as shown in Table 8.

The assay and incubation were performed as previously described. The results are shown in Fig. 13 of Section II-1(b) (i) of the results.

(ii) The DF preparation was concentrated in order to obtain optimal dissociating activity and to aid in the correlation of the amount of dissociation to expect from a given concentration of factor preparation.

Factor was concentrated by obtaining the material precipitating between 0 and 70% $(NH_4)_2SO_4$ saturation. The precipitate was then dissolved in a small volume of TKM*. This solution was then dialyzed against TKM*. Half of this preparation and an equal amount of a different DF preparation which had been frozen in liquid nitrogen for one month were preincubated separately at 40°C for 30 minutes. They were centrifuged at 20,000 g for 10 minutes to remove the precipitate. The protocol is shown in Table 9. The assay was performed as previously described. The profiles are presented in Fig. 14 of Section II-1(b) (ii) of the results and in Table 12 of Section II-2 of the results.

Table 7 Protocol for the DF Assay using Heat Inactivation as a Method of Purification

Component Added	Assay Tube					
	A	B	C	D	E	F
Ribosomes(4.43 mg/ml)	0.13	0.13	0.13	0.13	0.13	—
TKM*	0.5	0.4	0.2	0.4	0.2	0.43
TK. 12_M $5X10^{-4}$	—	—	—	—	—	—
DF(55mg/ml)	—	0.1	0.3	0.1	0.3	0.2
Source(Note)		(1)	(1)	(2)	(2)	(2)
Total Volume	0.63	0.63	0.63	0.63	0.63	0.63

(Note) (1) Preincubated at 40°C for 30 minutes in the presence of 0.5mM Dithiothreitol and centrifuged to remove any precipitate.
(2) Untreated.

Table 8 Protocol for the DF Assay using Ammonium Sulphate Fractionation as a Method of Purification

Component Added	Assay Tube					
	A	B	C	D	E	F
Ribosomes(5.71 mg/ml)	0.09	0.09	0.09	0.09	0.09	0.09
TKM*	0.45	0.3	0.3	0.3	0.3	—
DF	—	0.15	0.15	0.15	0.15	0.45
Dithiothreitol (0.1 M)	5 µl	5 µl	5 µl	5 µl	5 µl	5 µl
Source of DF	(a)	(b)	(c)	(d)	(e)	(f)
Total Volume(ml)	0.545	0.545	0.545	0.545	0.545	0.545

(a)untreated (b) 20% ammonium sulphate cut. (c) 35% ammonium sulphate cut. (d) 50% ammonium sulphate cut. (e)70% ammonium sulphate cut. (f) supernatant of 70% ammonium sulphate cut.

1(c) Column Chromatography

An attempt at purification of the DF was made on a column of G-75 Sephadex. The object was to separate the dissociating activity from the component which was causing aggregation in our assay.

TKM* Buffer and G-75 Sephadex were degassed. The Sephadex was used to make a column (inside diameter - 1 cm) of G-75 Sephadex with a bed height of 25 cm. The column was equilibrated with degassed TKM* at 4°C. The void volume was determined with Blue Dextran 2000. A 1 ml sample of crude DF preparation was layered on the column and eluted with TKM*. Fractions (0.5 ml) were collected and assayed for dissociating activity as previously described. The protocol was as shown in Table 10. The assay was performed as previously described. The results are discussed in Section II-1(c) of the results.

2 Specific Activity of the Dissociation Factor as a Test for Purification

The specific activities were calculated for each assay where possible as a test for purification. In most cases the extent of purification could be obtained from the sharpness of the profiles and the comparison between "Total Areas" of the assays and the "Total Area" of the control. The "Total Area" for each assay should remain the same as that of the control whenever the "% Dissociation due to DF" increases. For the experiments of Section II-1(a) and Section II-1(b)(ii) of the methods the data and some specific activities are shown in Table 11 and 12 respectively of Section II -2 of the results.

Table 9 Protocol for the DF Assay Studying the Effect of Preincubation on a Concentrated DF Preparation

Component Added	Assay Tube					
	A	B	C	D	E	F
Ribosomes(4.41 mg/ml)	0.11	0.11	0.11	0.11	0.11	0.11
TKM*	0.50	0.30	—	0.30	—	0.30
DF(54.8mg/ml)	—	0.25	0.55	0.25	0.55	0.25
Dithiothreitol (0.1M)	5µl	5µl	5µl	5µl	5µl	5µl
Source of DF	—	(1)	(1)	(2)	(3)	(4)
Total Volume(ml)	0.615	0.615	0.615	0.615	0.615	0.615

(1)DF preparation which had been preincubated and concentrated by ammonium sulphate precipitation.
(2)DF preparation untreated but concentrated by ammonium sulphate precipitation.
(3)DF preparation which had been frozen one month in liquid nitrogen followed by preincubation.
(4)DF preparation which had been frozen one month in liquid nitrogen but left untreated.

Table 10 Protocol for the DF Assay Using Column Chromatography as a Method of Purification

Component Added	Assay Tube					
	A	B	C	D	E	F
Ribosomes(7.1 mg/ml)	0.08	0.08	0.08	0.08	0.08	0.08
TKM*	0.4	—	—	—	—	—
DF	—	0.4	0.4	0.4	0.4	0.4
Dithiothreitol (0.1M)	5µl	5µl	5µl	5µl	5µl	5µl
Fraction No.		1	2	3	4	5
Total Volume	0.485	0.485	0.485	0.485	0.485	0.485

3 Standardization of Sephadex Columns for the Molecular Weight Determination of the Dissociation Factor

The use of gel filtration for the determination of molecular weights offers several advantages.

(i) The substance of unknown molecular weight need not be pure if its elution volume can be determined selectively, as by enzyme activity.

(ii) Only very small amounts of substances are required.

(iii) The sample can almost always be recovered with little or no denaturation.

(iv) The molecular weight determination can be combined with a gel filtration purification step.

(v) The procedure is highly reproducible and rapid.

A column of Sephadex G-100 was prepared in a pyrex column (diameter: 29 mm; height: 42 cm) to a height of 32 cm. The G-100 was equilibrated with TKM*; the void volume was determined and the column packing checked with Blue Dextran 2000. Since the DF was expected to have a molecular weight of approximately 21,200 similar to that in bacterial cells (115), the column was calibrated for a molecular weight determination with cytochrome C (horse heart-M.W. 12,384) and bovine serum albumin (M.W. 67,000). Cytochrome C was located by its colour and its absorbance at 280 nm and bovine serum albumin was located by its absorbance at 280 nm. A sample (0.5 ml) of bovine serum albumin (2 mg/ml) and cytochrome C (2 mg/ml) both prepared in TKM* was layered on the column and eluted with TKM*. Fractions (1 ml) were collected. The elution curves are as shown

in Fig. 15 of Section II-3 of the results.

The relationship between elution volume and the logarithm of the molecular weight is linear over a wide range and it is thus fairly simple to obtain a calibration curve as shown in Fig. 16 of the results (116, 117).

4 Molecular Weight Determination

The DF was prepared as previously described and a 35-65% $(NH_4)_2SO_4$ cut was taken as a preliminary purification. A sample (0.5 ml) of DF preparation (2 mg/ml) was layered on the column of G-100 Sephadex and eluted with TKM*. Fractions (1 ml) were collected from the column. The elution curve is presented in terms of "total protein concentration" and "nucleic acid percent" as shown in Fig. 17 of Section II-4 of the results. The fractions were assayed for DF activity as previously described and the results are shown in Fig. 18 of Section II-4 of the results. The elution volume of the DF was determined by assaying the fractions for ribosome dissociating activity and the molecular weight was estimated from the elution volume using the calibration curve in Fig. 16.

RESULTS

1(a) The effect of a preliminary incubation at 40°C for 30 minutes on the ribosome-dissociating activity of crude DF preparation was investigated. The results are shown in Fig. 12. Profiles 2 and 3 which represent DF which has been treated with a preliminary incubation

Fig. 12 Sucrose density gradient analysis of monomeric ribosomes incubated with the dissociating factor preparation which had given a preliminary incubation.

1 Profile of a preparation of ribosomes (Type II) after incubation at 30°C for 15 min without the addition of dissociation factor.

2 Profile of ribosomes as in 1 incubated at 30°C for 15 min with 0.1 ml of factorpreparation which had been given a preliminary incubation at 40°C for 30 min. The factor preparation was cleared of precipitate by centrifugation prior to use in the assay.

3 Same as 2 except that 0.3 ml of factor preparation was used.

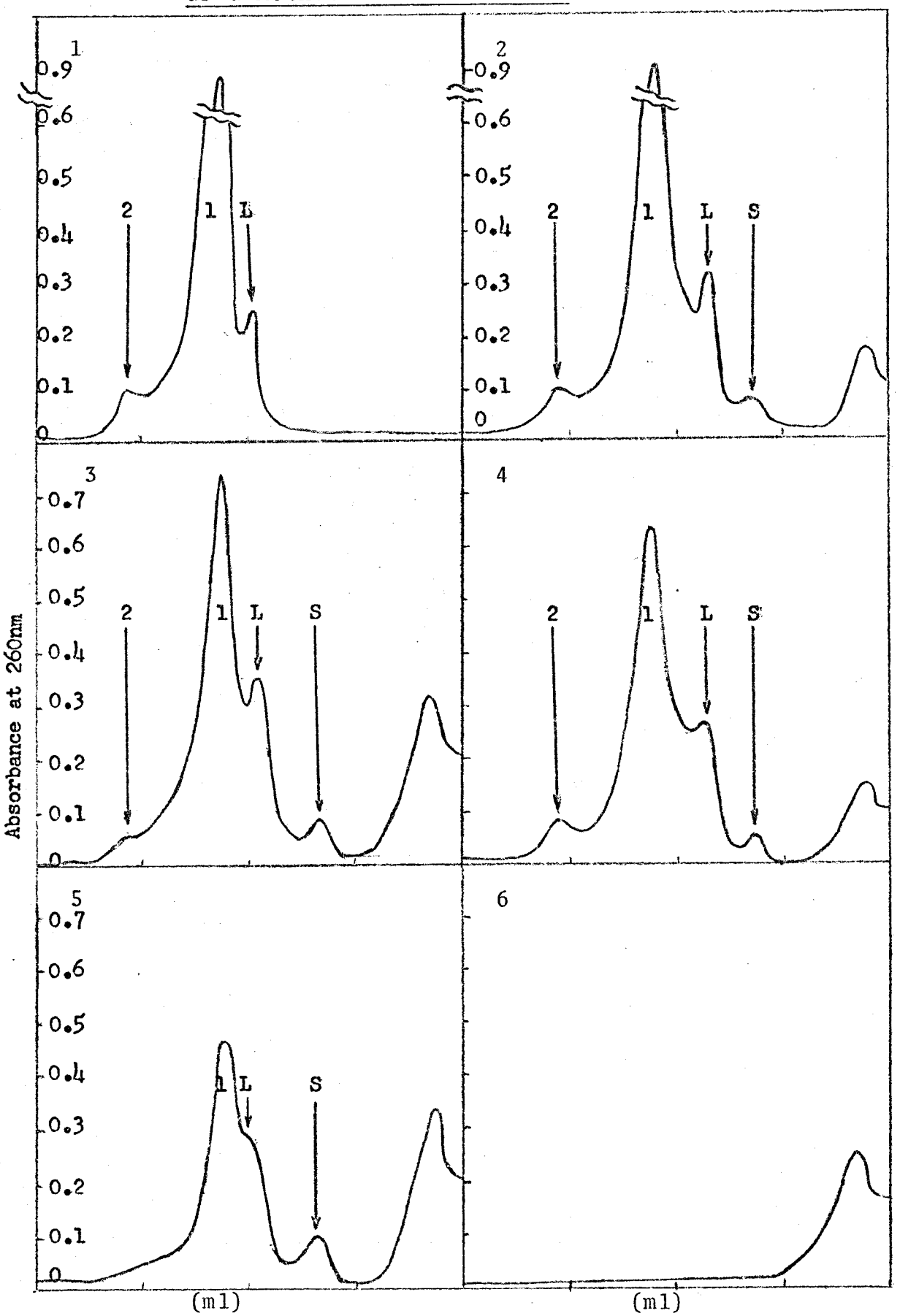
4 Profile of ribosomes as in 1 incubated at 30°C for 15 min with 0.1 ml of factor preparation which had been left untreated.

5 The same as 4 except that 0.3 ml of factor preparation was used.

6 Profile of the dissociating factor preparation which was left untreated.

Centrifugation was for 3 1/4 hours at 25,000 rev/min at 20°C in linear gradients of 15-40% sucrose inTKM* buffer.

Figure 12 The DF Assay using Heat Inactivation as a Method of Purification



show sharp peaks. This indicates that no aggregation has occurred. Profiles 4 and 5 which represent DF which has been left untreated are broad and the total area of the peaks ("Total Area" in Table 11) is much less than the total area of the control (Profile 1). This indicates that some aggregation of ribosomes has occurred. Since the preliminary incubation removes aggregating material it is a valuable aid in purification. Profile 6 of Fig. 12 indicates that there are no ribosomal particles present in the DF preparation. This verifies that the subunits arise from the substrate monomers themselves and not from the DF preparation assayed. Control experiments in which the factor was boiled before the incubation indicate that dissociation was due to the presence of a factor and not the ionic concentration of the preparation. Table 11 in Section II-2 of the results shows the quantitation of these profiles.

1(b)(i) The DF preparation was fractionated by ammonium sulphate and the results are shown in Fig. 13. The 20-35% fraction contained the bulk of the aggregating component (Profile 3). The 35-50% fraction contained the bulk of DF activity, but also some aggregating activity (Profile 4). Slight DF activity was found in the 50-70% fraction (Profile 5) and no activity in the 70% supernatant (Profile 6).

(b)(ii) The result of the incubation in Section II-1(b)(ii) of the methods is shown in Fig. 14 and in Table 12 of Section II-2 of the results. Profiles 2 and 3 represent assays in which DF has been treated with a preliminary incubation. The profiles have the same "Total Area"

Fig. 13 Sucrose density gradient analysis of monomeric ribosomes incubated with ammonium sulphate fractions of the dissociation factor preparation:

1 Profile of a preparation of ribosomes (Type II) after incubation at 30°C for 15 min without the addition of the dissociation factor.

2 Profile of a preparation of ribosomes after incubation at 30°C for 15 min with a fraction of dissociation factor preparation which was obtained from the material precipitating at 20% ammonium sulphate saturation.

3 Same as 2 except that the fraction was obtained from the material precipitating in the range 20% to 35% ammonium sulphate saturation.

4 Same as 2 except that the fraction was obtained from the material precipitating in the range 35% to 50% ammonium sulphate saturation.

5 Same as 2 except that the fraction was obtained from the materialprecipitating in the range 50% to 70% ammonium sulphate saturation.

6 Same as 2 except that the fraction was obtained from the material remaining in the supernatant after ammonium sulphate saturation.

Centrifugation was for 3 1/4 hours at 25,000 rev/min at 20°C in linear gradients of 15%-40% sucrose in TKM* buffer.

Figure 13 The DF Assay using Ammonium Sulphate Fractionation as a Method of Purification

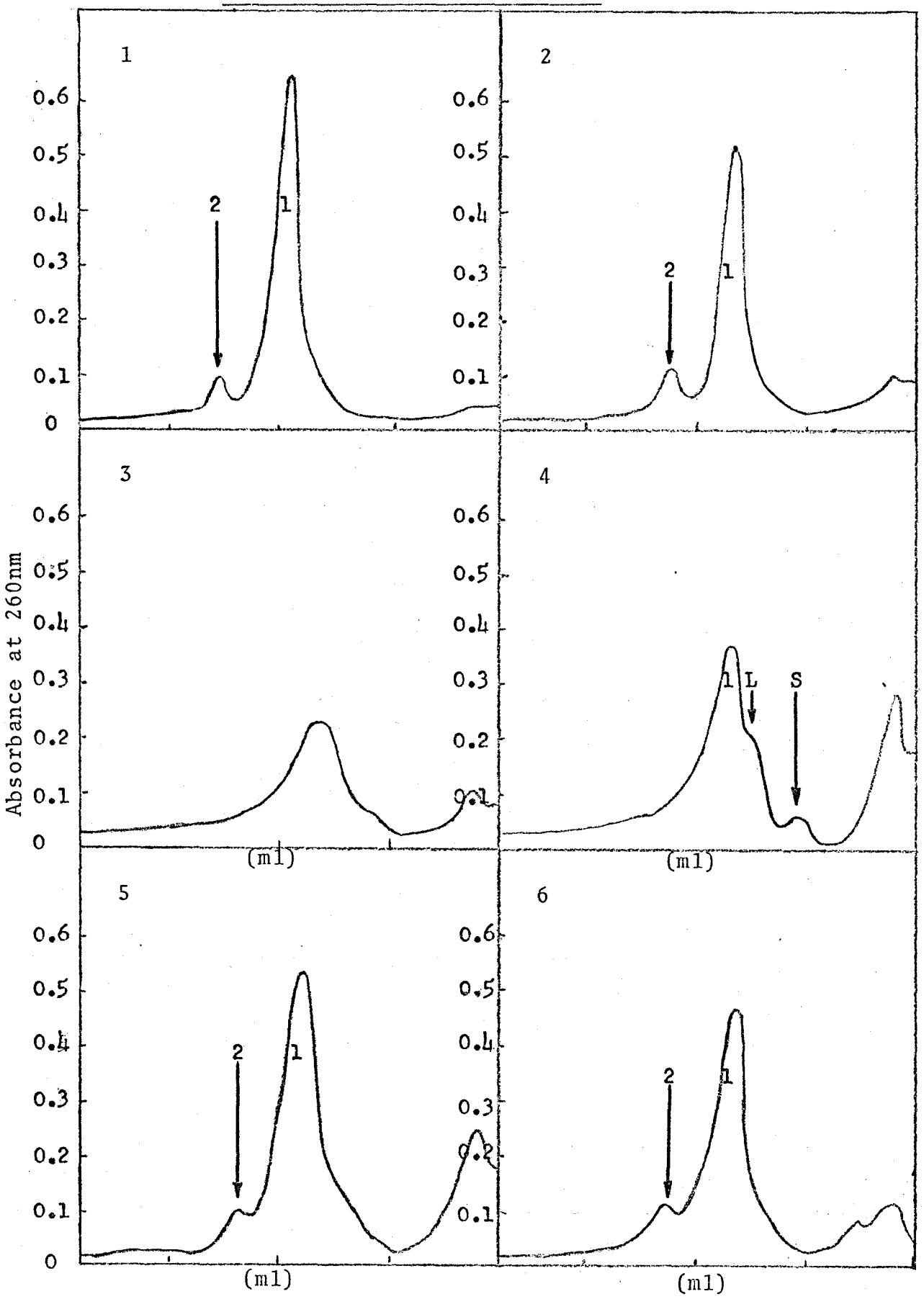


Fig. 14 Sucrose density gradient analysis of monomeric ribosomes incubated with dissociation factor.

1 Profile of a preparation of ribosomes (Type II) after incubation at 30°C for 15 min without the addition of dissociation factor.

2 Profile of a preparation of ribosomes (Type II) after incubation at 30°C for 15 min with 0.25 ml of a dissociation factor preparation which has been preincubated at 40°C for 30 min and concentrated by ammonium sulphate precipitation.

3 Same as 2 except that 0.55 ml of the factor preparation was used.

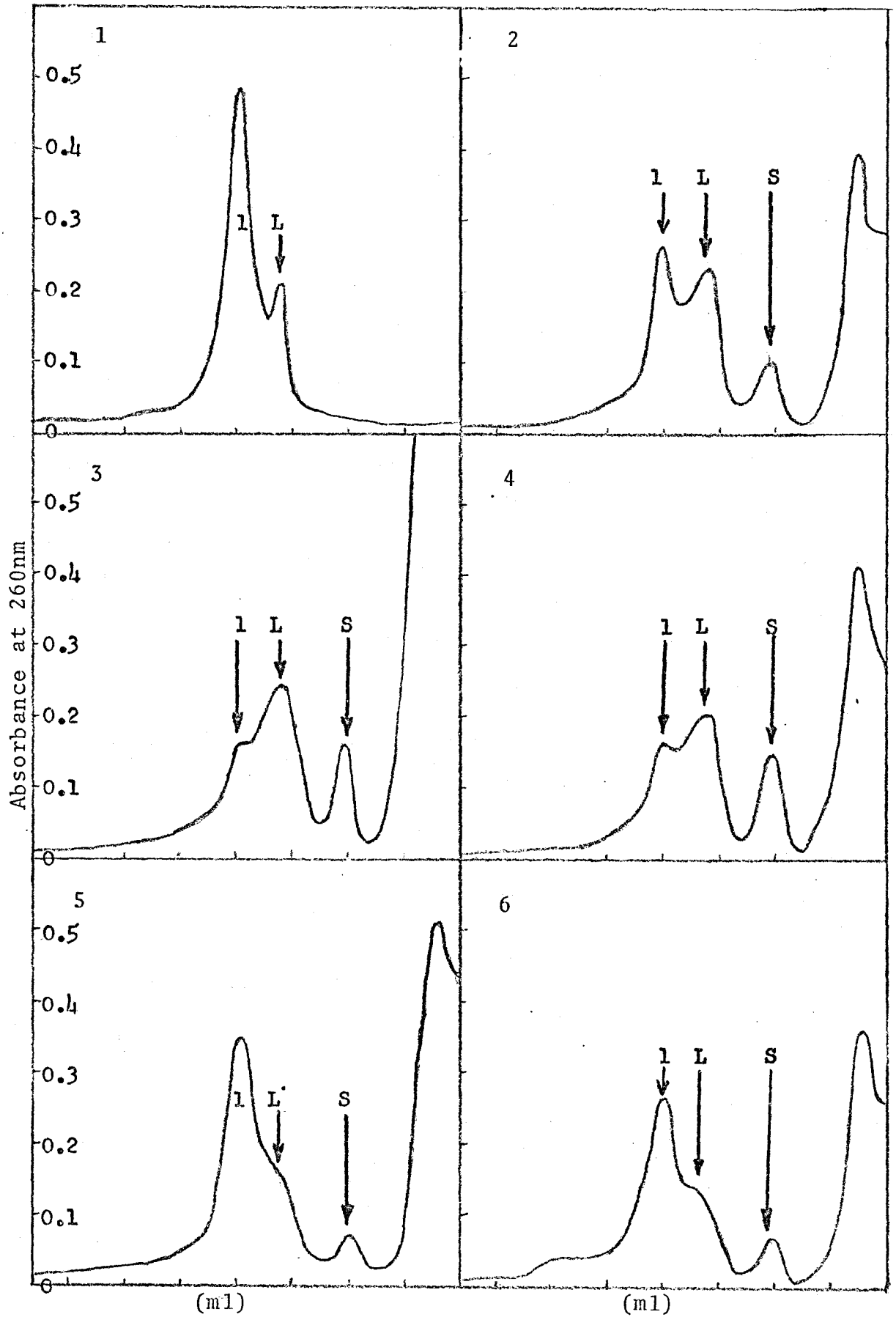
4 Same as 2 except that a dissociation factor preparation was used which was concentrated by ammonium sulphate precipitation. This factor preparation was otherwise untreated.

5 Same as 4 except that the factor preparation had been frozen one month in liquid nitrogen. The preparation was then incubated at 40°C for 30 min and the precipitate removed by centrifugation.

6 Profile of a preparation of ribosomes after incubation at 30°C for 15 min with 0.25 ml of a dissociation factor preparation which has been frozen one month in liquid nitrogen but otherwise left untreated.

Centrifugation was for 3 1/4 hours at 25,000 rev/min at 20°C in linear gradients of 15-40% sucrose in TKM* buffer.

Figure 14 The DF Assay of the Effect of Preincubation on a Concentrated DF Preparation



(Table 12) as the profile representing untreated DF (Profile 4). The "Total Area" of each of the three assays (Profiles 2, 3, and 4) is similar to that of the control (Profile 1). The similarity of the "Total Area" of each assay to the others is a good indication that the preparation is reasonably free of the material which had been causing ribosomes and/or subunits to aggregate. The increased purity of the preparation is the result of the exclusion of much extraneous protein by the 40,000 rpm centrifugation and the half hour KCl extraction.

The "% Dissociation" (Table 12) due to DF was equal in the assays represented by Profiles 3 and 4. However the assay represented by Profile 3 was an assay of twice the amount of DF preparation possessed by the assay represented by Profile 4. This indicates that the preliminary incubation (represented by Profile 3) must have destroyed some of the ribosome dissociating activity or else it would be expected to have twice the ribosome dissociating activity as the assay represented by Profile 4. The results (represented in Profiles 5 and 6) indicate that the preparation which had been frozen in liquid nitrogen for 1 month possessed much less of the ribosome dissociating activity than the fresh preparation and also possessed much more of the aggregating component (see Profile 6). This aggregating component was largely removed by the preliminary incubation treatment (Profile 5).

The specific activity in Table 12 as calculated for Profile 4 indicates that this preparation is purer than any previous preparation.

(c) When an attempt was made to separate the DF from the aggregating component using a column of G-75 Sephadex as in Section II-1(c) of the

methods no separation was achieved. Wherever dissociation was maximal as judged from the area of the small subunit peak, the "Total Area" was minimal indicating aggregation. The two components could not be separated on G-75 Sephadex and both were eluted just after the void volume indicating the DF may have a higher molecular weight than was anticipated.

2 Specific Activity of the Dissociating Factor as a Test for Partial Purification

Table 11 shows the results and specific activities for the experiment on Heat Inactivation as described in Section II-1(a) of both the methods and results. It can be seen from the close agreement of the "Total Areas" that DF treated by a preliminary incubation has most of its aggregating component removed in the precipitate whereas assay number 5 which contained a large quantity of untreated DF preparation has a large decrease in "Total Area", a result of the presence of aggregation in the assay. A comparison of the "% Dissociation due to DF" for assay numbers 2 and 3 indicate that a quantity of DF preparation three times greater causes three times the amount of dissociation when the aggregating component is removed by a preliminary incubation. Table 12 shows the results and specific activities for the experiment in Section II-1(b)(ii) of both methods and results. In this experiment the DF was concentrated by ammonium sulphate precipitation. In these two tables the "Total % dissociation" was calculated as previously described. "% Disscoiation due to DF" was calculated by subtracting

Table 11 Data for the DF Assay Using Heat Inactivation as a Method of Purification

Profile Number	Peak Areas (units)					Total % Dissociation	% Dissociation due to DF	mg of ribosomes dissociated	mg of protein in the assay	Specific Activity
	2	1	L	S	Total Area					
1	1.82	17.44	4.62	—	23.88	19.3	—	—	—	—
2	2.2	15.1	4.56	0.9	22.76	24.0	4.7	0.027	<5.5	—
3	0.66	16.38	7.56	1.52	26.12	34.8	15.5	0.090	<16.5	—
4	1.9	15.4	4.96	0.85	24.12	26.7	6.4	0.037	5.5	0.00675
5	—	12.1	5.36	1.26	18.72	35.3	16.0	0.093	16.5	0.00564
6	—	—	—	—	—	—	—	—	—	—

Table 12 Data for the DF Assay Studying the Effect of Preincubation on Concentrated DF Preparation

Profile Number	Peak Areas (units)					Total % Dissociation	% Dissociation due to DF	mg of ribosomes dissociated	mg of protein in the assay	Specific Activity
	2	1	L	S	Total Areas					
1	—	9.22	3.78	—	13.00	29.1	—	—	—	—
2	—	4.55	4.48	1.38	10.41	56.2	27.1	0.136	*<13.75	—
3	—	3.04	4.86	2.16	10.06	70.0	40.9	0.205	<30.2	—
4	—	3.6	4.7	1.92	10.22	64.6	35.5	0.178	13.75	0.0129
5	—	6.96	3.08	1.1	11.14	37.5	8.4	0.042	<30.2	—
6	0.42	4.65	1.96	0.50	7.53	32.7	3.6	0.018	13.75	0.0013

*<"less than"

the "Total % Dissociation" of the control from the "Total % Dissociation" of the assay. This corrects for subunits still present from the monomeric ribosome preparation. From the "% Dissociation due to DF" and the quantity of ribosomes available for dissociation, the "mg of Ribosomes Dissociated" is calculated. In cases where the DF preparation was not treated with a preliminary incubation, the mg of protein in the preparation was known and this is shown in the tables as "mg of Protein in the Assay". Specific activities were expressed in mg of ribosomes dissociated per mg of total protein.

3 The elution curves for the calibration of the Sephadex columns are shown in Fig. 15 as well as the elution curve for the void volume determination. The elution volume for bovine serum albumin is 25.3 ml and for cyt C is 44.3 ml. The void volume is 20.2 ml.

4 The molecular weight of DF is determined from its elution volume and the calibration curve. The elution volume of the DF preparation as determined in Fig. 17 from the protein absorbance at 280 nm is 20.2 ml, the same value as the void volume. Fig. 18 shows the results when fractions were assayed for DF activity. Maximal activity was obtained in fraction #5 indicating that the protein peak in Fig. 17 corresponds to the DF activity peak. The elution volume for DF is therefore 20.2 ml. The minimum molecular weight of DF is shown in Fig. 16 and is determined to be 85,000.

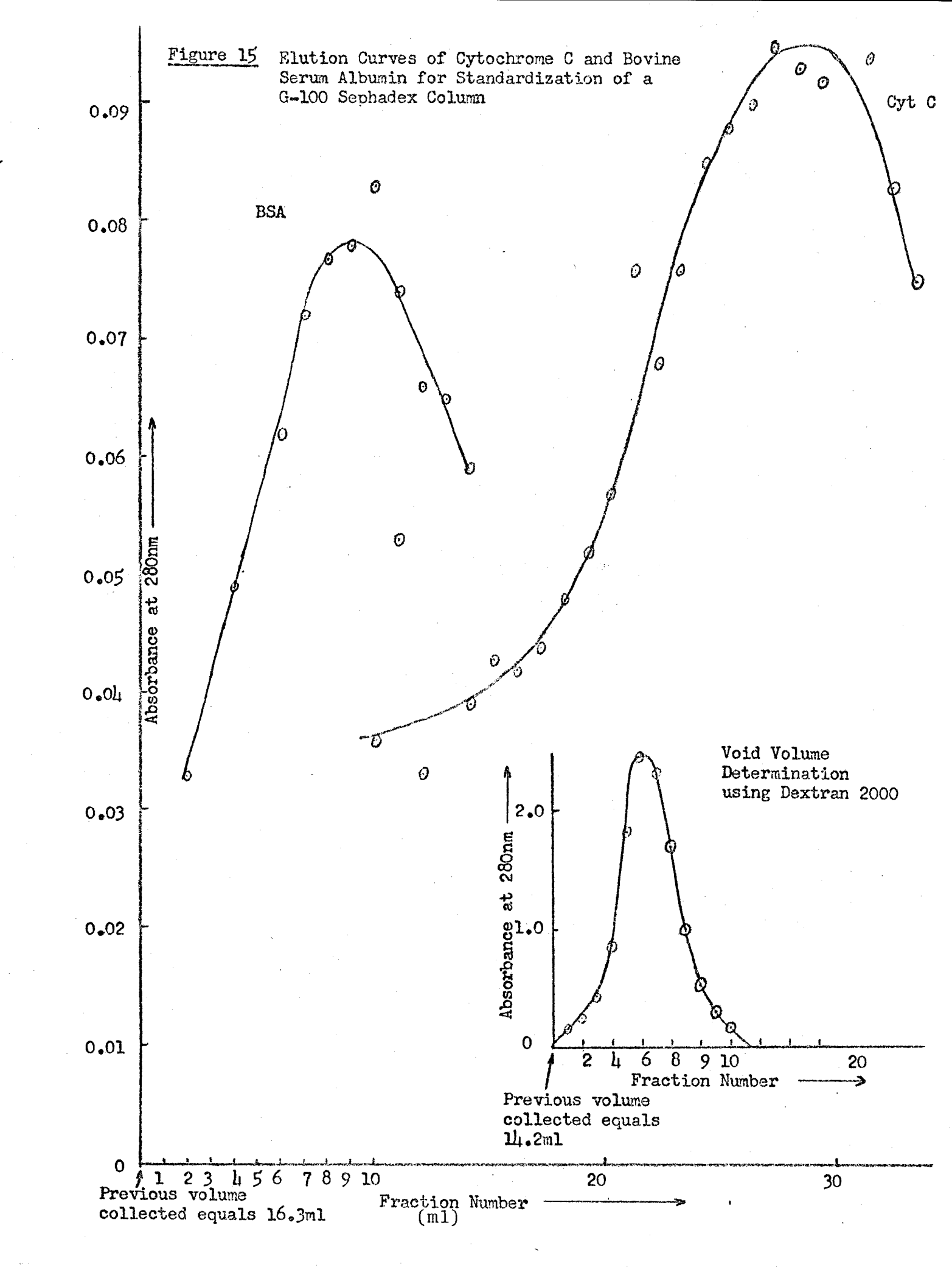


Figure 15 Elution Curves of Cytochrome C and Bovine Serum Albumin for Standardization of a G-100 Sephadex Column
BSA
Cyt C
Absorbance at 280nm
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
1 2 3 4 5 6 7 8 9 10
20
30
Previous volume collected equals 16.3ml
Fraction Number (ml)
Void Volume Determination using Dextran 2000
Absorbance at 280nm
2.0
1.0
0
2 4 6 8 9 10
20
Fraction Number
Previous volume collected equals 14.2ml

Figure 16

Calibration Curve for the G-100 Sephadex Column

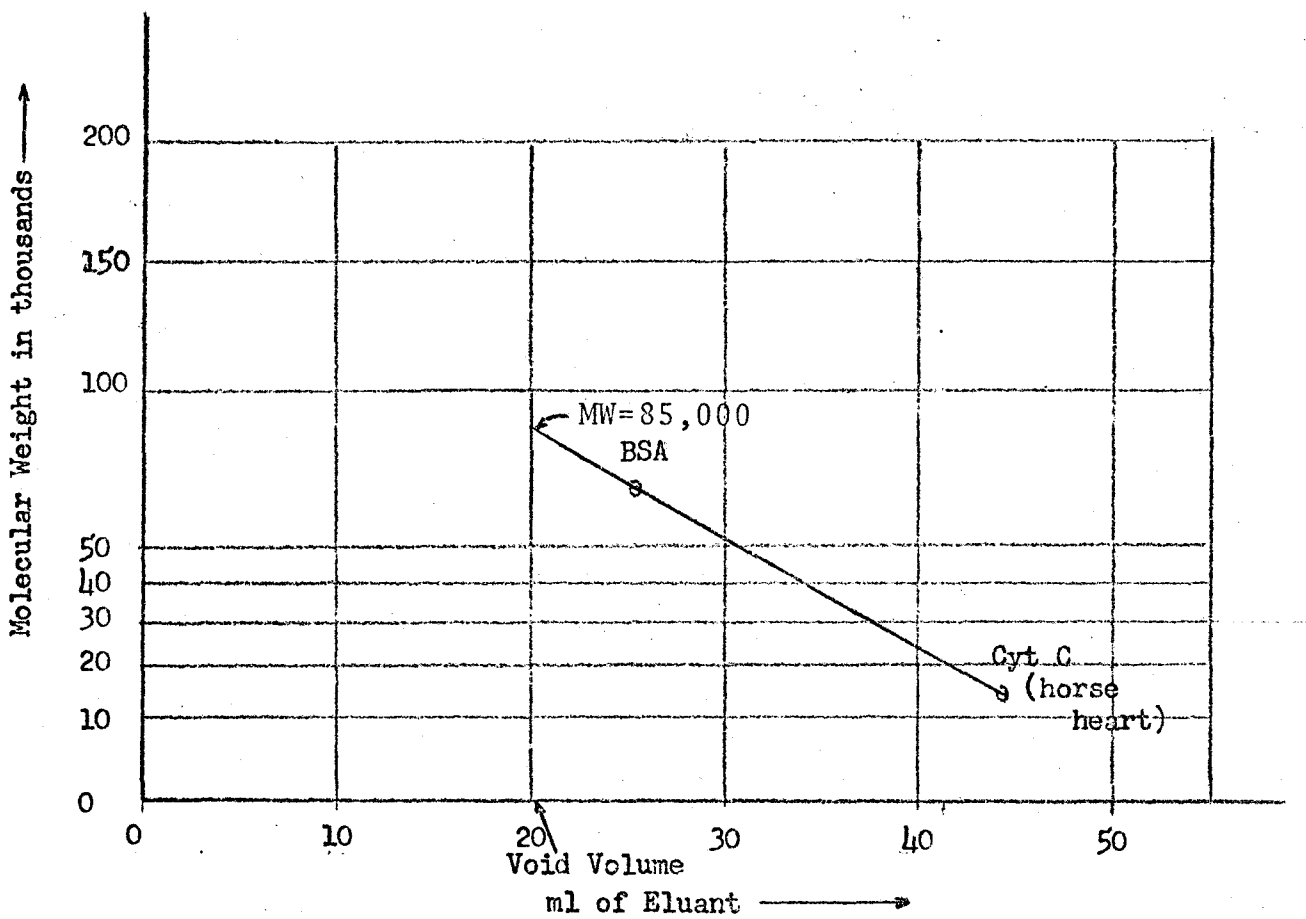


Figure 17 Elution Curve for DF on a G-100 Sephadex Column Equilibrated with TKM*

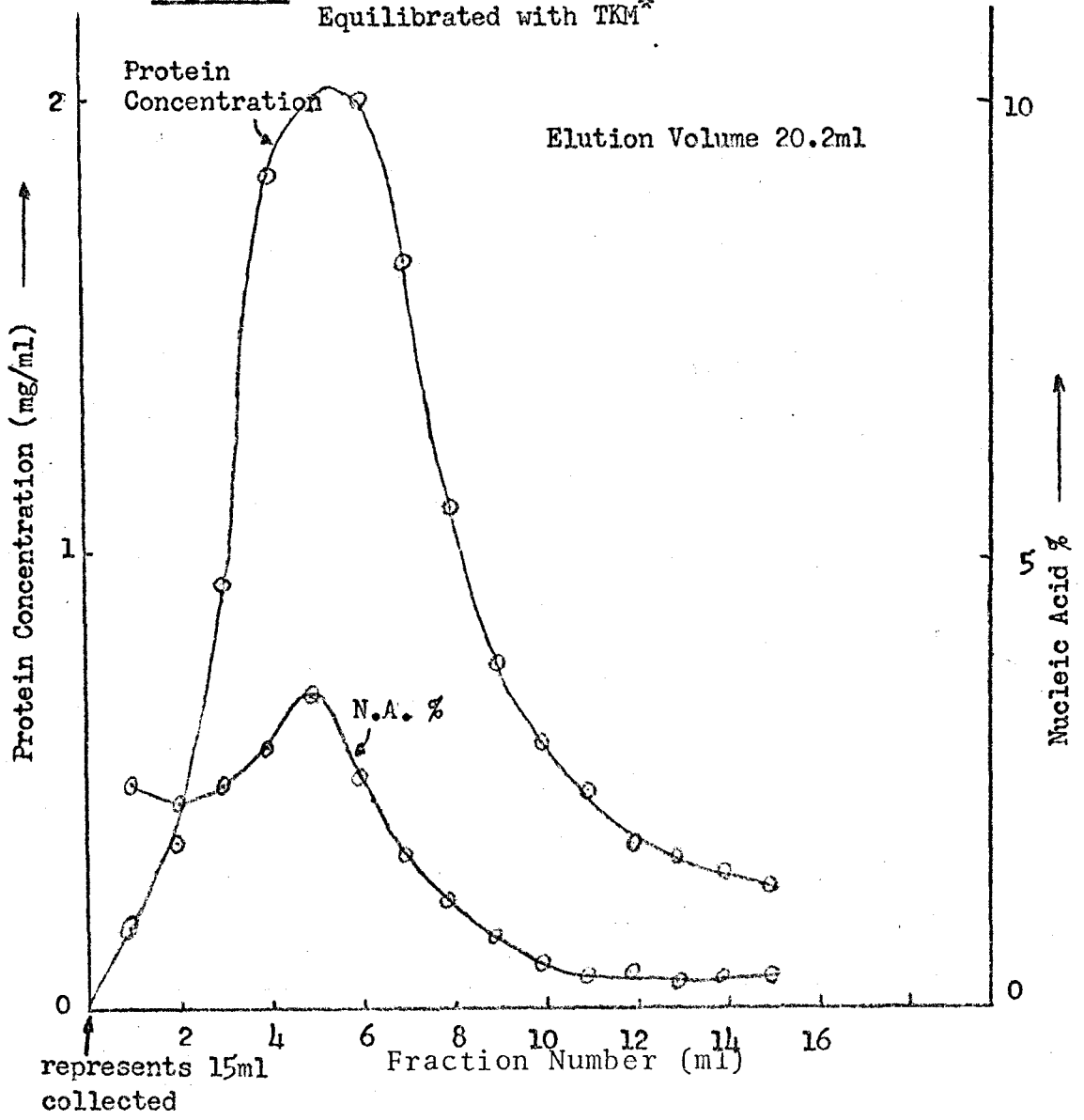
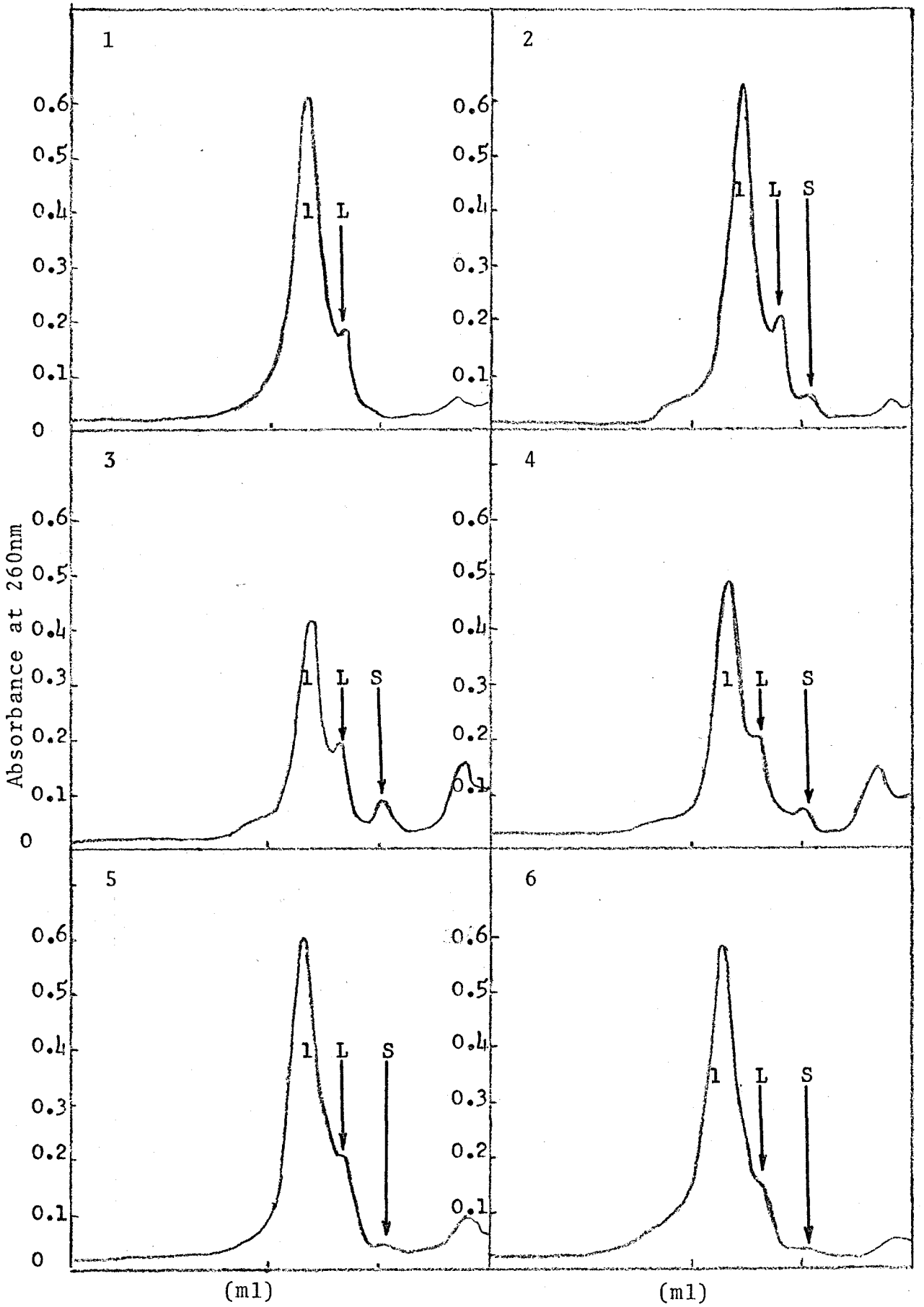


Fig. 18 Sucrose density gradient analysis of monomeric ribosomes incubated with the dissociation factor preparation. The factor preparation was layered on the column of G- 100 Sephadex and eluted with TKM*. Fractions (1 ml) were collected from the column. Profiles 1 through 6 represent the six fractions which were assayed for dissociating activity.

Figure 18 The DF Activity of Fractions from the G-100 Sephadex Column



DISCUSSION

A dissociation factor exists in rat liver cells and it dissociates uncomplexed ribosomes into their constituent subunits. The dissociation occurs at 1.5 mM Mg^{++}, but this does not exclude 2 mM Mg^{++} and also the range between 1.5 mM and 2 mM Mg^{++} which was not tested for dissociating activity. A Mg^{++} concentration of 5 mM inhibits DF activity. 1.5 mM to 2 mM Mg^{++} is the concentration range in which ribosomes exist predominantly as monomers. Dissociation by DF occurs at 1 mM Mg^{++} as well, but the dissociation yields an increase in dimers as well as subunits (Fig. 7) because monomers require 1.5 mM to 2 mM Mg^{++} to remain as such.

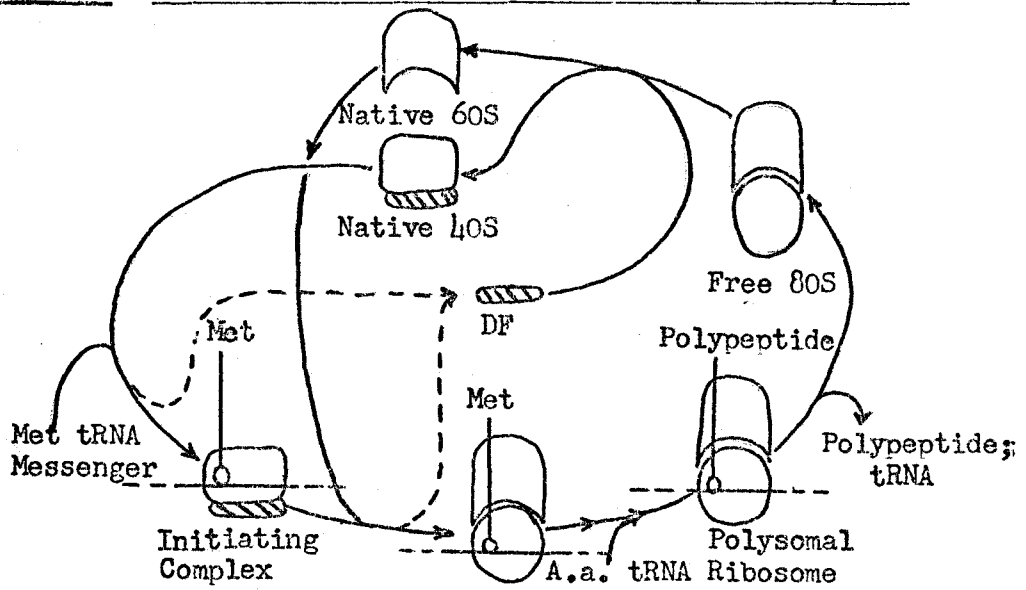
The critical step in monomer preparation is the suspension of polyribosomes in the incubation mix for the puromycin reaction (Section IA-3(b) of the methods). This dilution must be such that the Mg^{++} concentration does not fall below 1.75mM. Concentrations of 1 mM and 0.5 mM were tried without success, but it should be noted that 1.5 mM Mg^{++} is also a possibility. Dissociation factor prepared by means of a 125,000 X g[1] for 45 minutes centrifugation (the 40,000 rpm for 45 minutes centrifugation) and a half hour KCl extraction gave a preparation possessing the minimum aggregating components and the maximum ribosome-dissociating activity. This crude dissociation factor preparation can be further purified by means of a preliminary incubation at 40°C for 30 minutes, followed by a centrifugation to remove the precipitate. Some ribosome-

[1] g is always expressed as the average force exerted on the centrifuge tube.

dissociating activity is lost by this treatment, but the quantity lost relative to the purification achieved is small. Purification of the crude DF preparation can also be achieved by obtaining at 4°C the 35-65% ammonium sulphate fraction. Most of the aggregating material remains in the 0-35% fraction. Purification on a column of G-75 Sephadex was found to be impossible because the DF and the aggregating material are eluted from the column immediately after the void volume.

The free 80S ribosome is not as stable as the polysomal ribosome and its dissociation can be promoted by various nonspecific factors, including a low Mg^{++} concentration and a high K^{+} concentration. It is highly relevant that DF is recovered from the native 40S particle (see Appendix) and not from the 80S particles, even though the latter contain a 40S moiety. This finding points strongly to a cycle very similar to that which exists for DF in E. coli (74). DF attaches to and stabilizes a 40S subunit and then becomes detached as this subunit is converted into a 80S unit on the polysome. The demonstration of the distribution and the action of DF in vitro, and the demonstration that eukaryotes require the presence of ribosomal subunits for the initiation of protein synthesis (84), provides a coherent model for the ribosome polysome cycle. In this cycle a small DF cycle engages with a larger ribosome cycle (Fig. 11). It is uncertain whether or not the release of free DF occurs when the 40S particle forms an initiating complex with mRNA and met-$tRNA_1$, or only later, when the addition of a 60S subunit converts the initiating complex into a polysomal ribosome.

Figure 11 A Model for the Ribosome-Polysome Cycle



Since an increase in Mg^{++} concentration above 2 mM inhibits the formation of the initiating 40S-DF particle, it probably has an opposite affect on the subsequent release of DF, which is equally required for the DF cycle. It therefore seems possible that variability of the Mg^{++} optimum in experiments on polypeptide initiation (84) can be explained in terms of variation in the concentration of DF present.

The column chromatographic determination of the molecular weight of the DF indicates it has a value of at least 85,000. Further work (see the Appendix) showed that the molecular weight is in fact greater than 100,000. In this regard the DF of rat liver differs from the smaller DF in E. Coli.

SUMMARY

A factor which is capable of dissociating rat liver monomeric ribosomes into 60S and 40S subunits has been partially characterized and purified.

The factor was prepared by extracting a fraction of rat liver enriched in its content of native subunits, with 0.05 M Tris-HCl, 1.0 M KCl, 0.01 M $MgSO_4$ and 0.5 mM dithiothreitol. The activity of the preparation was assayed by testing its ability to dissociate monomeric ribosomes into subunits which were detected by sucrose density gradient analysis. The ribosomes (Type II) used as substrate were prepared by dissociating polysomes in the presence of puromycin, 0.464 M KCl and 1.75 mM $MgSO_4$ and subsequently reassociating the subunits into

monomers by lowering the ionic strength.

Ribosomes prepared by the incubation of polysomes with puromycin at ordinary K^+ and Mg^{++} concentrations still possess their nascent peptide, whereas those treated with puromycin at 0.464 M KCl and 1.75 mM $MgSO_4$ do not.

The presence or absence of peptidyl-tRNA appears to have no affect on the interactions between 60S and 40S subunits, all conditions being constant. The interactions between subunits depend critically on the Mg^{++} and K^+ concentrations. At a K^+ concentration of 25 mM, 1.5 to 2 mM Mg^{++} is the in vitro concentration range at which uncomplexed rat liver ribosomes exist largely as monomers.

Puromycin reacts with the ribosomes of rat liver in two ways. When an energy generating system at 37°C is used, puromycin will cause the ribosomes to separate largely as complexed monomers from the mRNA and when a high K^+ concentration at 0°C is used, puromycin will react with the nascent peptide causing its release as a complex with puromycin.

The free 80S ribosome is less stable than the polysomal ribosome, and its dissociation can be promoted by various nonspecific factors, including a low Mg^{++} concentration and a high K^+ concentration.

The factor acts on ribosomes freed of both messenger RNA and nascent protein. DF activity could not be assayed for with ribosomes (Type I) which still contained the nascent peptide.

DF is not found in the polysome fraction or in the monomeric ribosome fraction, but only in the native subunit enriched fraction.

The subunits produced as a result of DF activity are unique. They have a higher sedimentation rate in sucrose density gradients than subunits produced as a result of altering the ionic conditions of ribosomes. The DF activity extracted from the native subunit enriched fraction is DOC sensitive. Treatment with this detergent in the fractionation scheme prior to obtaining the native subunit enriched fraction effectively removes the DF from this fraction or destroys its activity. DF is loosely associated with the native subunits and it is easily extracted. Other ribosomal proteins which also may be extracted cause aggregation of ribosomes and/or subunits and so interfere in the assay.

The reaction of DF with monomeric ribosomes does not require a source of energy. There is much to argue for a stoichiometric mode of action of the DF as well, although this is not certain.

A Mg^{++} concentration of 5 mM was found to be antagonistic to the reaction of 80S ribosomes with the DF. The 80S ribosomes and the DF seem to be in an equilibrium with the subunits; and this equilibrium is dependent on the concentration of Mg^{++} ions.

Purification of the crude DF preparation can be achieved by obtaining at 4°C the 35-65% ammonium sulphate fraction. Purification may also be achieved by means of an incubation of the DF preparation at 40°C for 30 minutes followed by a centrifugation to remove precipitated protein.

A cycle is proposed similar to that which exists for DF in E. coli (74). DF attaches to and stabilizes a 40S subunit and then

becomes detached as this subunit is converted into an 80S unit on the polysome. The demonstration of the distribution and the action of DF *in vitro* and the demonstration that eukaryotes require the presence of ribosomal subunits for the initiation of protein synthesis (84) provides a coherent model for the ribosome-polysome cycle. In this cycle a small DF cycle engages with the larger ribosome cycle (Fig. 11). The variability of the Mg^{++} optimum in experiments on polypeptide initiation (84) may be explained in terms of a variation in the concentration of DF present.

The DF was determined to have a molecular weight in excess of 85,000 by column chromatography. Therefore the DF of rat liver differs at least in size from the much smaller DF of *E. coli*.

APPENDIX

A Factor Capable of Dissociating Rat Liver Ribosomes[1]

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1. Supported by a grant from the Medical Research Council of Canada (Grant #MA 3300).

Abstract

A factor capable of dissociating rat liver monomeric ribosomes into 60S and 40S sub-units has been partially purified and characterized.

The factor was prepared by extracting a fraction of rat liver enriched in its content of native sub-units with 0.05 M triethanolamine-HCl, 1.0 M KCl, 0.01 M MgSO4 and 2 mM dithiothreitol. The activity of the preparation was assayed by testing its ability to dissociate monomeric ribosomes into sub-units which were detected by sucrose density gradient analysis. The ribosomes used as substrate were prepared by dissociating polysomes in the presence of puromycin, 0.5 M KCl and 3 mM $MgSO_4$ and subsequently re-associating the sub-units into monomers by lowering the ionic strength. The factor acts only on ribosomes freed of both messenger RNA and nascent protein by associating with the small sub-unit. The activity was time and temperature dependent, reaching a plateau after 30 min at 30 °C.

The factor has been partially purified by ammonium sulfate fractionation between 35% and 65% saturation and by treatment at 40 °C for 15 min to precipitate ribosome aggregating substances.

Introduction

Since Subramanian et al (1) first reported the discovery of a factor capable of dissociating E. coli ribosomes into sub-units in 1968, much progress has been made in purifying this factor and in studying its mechanism of action, including its role as an initiation factor (2,3). Later, evidence began to accumulate indicating that the conditions required for the dissociation of ribosomes from eukaryotes were similar to those required for ribosomes from prokaryotes provided that the former were free of nascent polypeptides (4-7). We set out to determine, therefore, whether rat liver contained a factor capable of dissociating rat liver ribosomes from which the nascent proteins had been released by treatment with puromycin. Recently, a protein factor, capable of dissociating ribosomes has also been discovered in yeast (8). In this paper we describe the isolation of a ribosome dissociation factor from a homogenate of rat liver, some properties of this factor, and some observations concerning its mechanism of action.

Materials and Methods

Chemicals

Sodium desoxycholate was purchased from Schwarz-Mann and puromycin dihydrochloride was purchased from Nutritional Biochemicals Corporation. Other reagents were "Certified" grade chemicals from Fisher Scientific Company.

Subcellular Fractionation and Preparation of Crude Dissociation Factor

Male, Hooded or Charles River rats, weighing 300-400 g were fasted overnight before being killed. The rats were stunned by a blow to the head, decapitated and their livers quickly transferred to ice-cold 0.25 M sucrose in medium A (0.05 M triethanolamine (pH 7.5); 0.025 M KCl; 1.5mM

$MgSO_4$). The livers were blotted with filter paper and passed through a tissue press, homogenized in 2.5 volumes of 0.25 M sucrose in medium A, and the homogenate was centrifuged at 17 000 x g[2] for 15 min. The supernatant fraction was then centrifuged at 125 000 x g for 45 min. The pellets

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2. In all cases the relative gravitational force (g) is expressed as the average force exerted on the centrifuge tube.

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formed as a result of this 45 min centrifugation were set aside for the preparation of polysomes; the supernatant was centrifuged at 160 000 x g for 2 h.

For the preparation of polysomes, the supernatant from this 2 h centrifugation was re-homogenized with the pellets from the previous centrifugation, adjusted to 1.3% sodium desoxycholate, 20 ml samples layered over 8.5 ml of 2.0 M sucrose in medium A, and centrifuged at 160 000 x g for 4 h. The polysome pellets were rinsed with medium A and stored at -20 °C.

For the preparation of crude dissociation factor, the pellets from the 2 h centrifugation at 160 000 x g were homogenized in medium B (0.05 M triethanolamine-HCl (pH 7.5); 1.0 M KCl; 0.01 M $MgSO_4$; 2 mM dithiothreitol). The resulting mixture was stirred for ½ h at 4 °C and then re-centrifuged at 160 000 x g for 2 h. The supernatant (crude dissociation factor) was stored at 4 °C.

Purification of Dissociation Factor:

Prior to ammonium sulfate fractionation or heat treatment, the crude factor was passed through a column of Sephadex G-25 equilibrated with medium A in order to reduce the salt concentration. Ribosome-dissociating activity was found to be concentrated in the fraction precipitating between

35% and 65% saturation with ammonium sulfate at 4 °C. Ammonium sulfate was removed from the sample by passing it through a column of Sephadex G-25 which had been equilibrated with medium A.

Heat treatment consisted of incubating the factor preparation (equilibrated with medium A) at 40 °C for 15 min. At this stage the protein concentration should be 15 mg/ml or less. The resulting precipitate was removed by centrifuging at 20 000 x g for 10 min.

Preparation of Monomeric Ribosomes

Ribosomes completely free of messenger RNA and nascent protein were obtained by dissociating polysomes into 60S and 40S ribosomal sub-units and then re-associating the sub-units (7). Nascent protein was released from polysomes by incubation at 4 °C for 30 min. The incubation mixture contained:polysomes,2.5 mg/ml; triethanolamine-HCl (pH 7.5), 0.05 M; KCl, 0.5M; $MgSO_4$, 1.5 mM; and puromycin, 1.1×10^{-4} M. The mixture was then incubated at 37 °C for 10 min to dissociate the ribosomes into sub-units. Re-association of the sub-units was accomplished by diluting the mixture with 4 volumes of triethanolamine-HCl (pH 7.5), 0.05 M; $MgSO_4$, 1.5 mM at room temperature. Centrifugation of 8 ml aliquots over 2 ml of 1 M sucrose in medium A, at 160 000 x g for 2 h yielded pellets of monomeric ribosomes which were stored at 4 °C. For use in the assay of dissociation factor activity, these ribosomes were suspended in medium A to a concentration of approximately 5 mg/ml. Concentrations of ribosomes were calculated from the absorbance at 260 nm, assuming an absorbance of 134 for a 1% solution.

Preparation of Pseudopolysomes

Polysomes were treated according to the method of Blobel et al (7) to remove the nascent proteins but leaving the messenger RNA-ribosomes

complex intact (so-called pseudopolysomes). This was accomplished by incubating the following mixture at 4 °C for 30 min: polysomes (2.5 mg/ml); triethanolamine-HCl (pH 7.5), 0.05 M; KCl, 0.5 M; $MgSO_4$, 3 mM; puromycin, 1.1×10^{-4} M. To re-equilibrate the ribosomes with medium A, they were passed through a column of Sephadex G-25 at 4 °C. For purposes of comparison, samples of polysomes were treated exactly as above except that puromycin was omitted.

Assay of Dissociation Factor Activity

Samples to be tested for ribosome-dissociating activity were incubated with approximately 0.5 mg monomeric ribosomes at 30 °C for 30 min. The incubation also contained: 0.05 M triethanolamine-HCl, pH 7.5; 0.025M KCl; and 1.5 mM $MgSO_4$. To terminate the reaction and "fix" the ribosomes, formaldehyde was added to a final concentration of 4%. Analysis of the extent of dissociation involved layering the "fixed" ribosomes over 33 ml linear sucrose gradients of 15% to 35% sucrose in medium A; centrifuging at 75 000 x g for 6.5 h at 5 °C (IEC SB110 rotor); and measuring the absorbance at 260 nm while pumping the gradient through the flow cell of a Gilford model 2400 recording spectrophotometer. The quantity of the various ribosomal species present was estimated by calculating the area under each of the peaks from their heights and widths at half the height.

Results

A direct assay for dissociating activity in the crude factor preparation was made impossible by the presence in the preparation of substances which caused monomeric ribosomes to aggregate and sediment to the bottom of the sucrose gradient. It was found, however, that treatment of the preparation at 40 °C for 15 min caused much of the material responsible for the ribosome aggregation to precipitate out. A similar observation has been reported for a dissociation factor isolated from yeast (8). The factor was further purified by ammonium sulfate fractionation. Fig. 1 shows the absorbance profile of the monomeric ribosomes incubated (a) without and (b) with dissociation factor. There are some sub-units present in the monomer preparation but when the factor was present during the incubation the amount of sub-units increases at the expense of monomers. Control experiments in which the factor was boiled before the incubation or in which factor alone was applied to the gradient, indicate that dissociation was due to the presence of a factor and not the salt concentration of the preparation or contamination of the factor with ribosomal sub-units.

The dissociating activity of the factor was temperature dependent. In Fig. 2 the activity is expressed in terms of the amount of sub-units present as a percentage of all the ribosome species. It was observed that as the temperature was lowered from 30 °C the activity of the factor decreased but the amount of temperature-dependent aggregation of ribosomes (eg dimerization) increased, making dissociation harder to quantitate at these temperatures. For this reason the activity at 10 °C was not given much weight in drawing the graph.

The results depicted in Fig. 3 indicate that the dissociation reaction is complete after incubation at 30 °C for 30 min.

The extent of dissociation increased linearly with the amount of factor added over a wide range (see Fig. 4). Althoughnot a part of the experiment presented in Fig. 4, it was possible, by adding sufficient factor, to attain complete dissociation of the monomers.

The substrate specificity of the factor was tested by comparing the effectiveness of the following as substrates: intact polysomes; pseudopolysomes (ie polysomes devoid of nascent protein); and "reconstituted" monomers (ie ribosomes devoid of nascent protein, transfer RNA and messenger RNA). The appearance on the gradient of pseudopolysomes, and control polysomes (ie polysomes submitted to the same procedure as in the preparation of pseudopolysomes but omitting puromycin), was similar (see Fig. 5 (a) and 5 (b)). The remainder of Fig. 5 shows the sucrose density gradient profiles of the three classes of ribosomes (ie pseudopolysomes, polysomes, and monomers) after incubation with and without the addition of dissociation factor. To aid comparison, the same quantities of ribosomes and factor were used throughout this experiment. The addition of factor to the incubation of monomers resulted in considerable dissociation of the monomers into sub-units (compare Fig. 5 (e) and 5 (h)). Incubation of the pseudopolysomes and control polysomes, even in the absence of factor caused a significant increase in the proportion of monomeric ribosomes, presumably due to ribonuclease action (see Fig. 5 (c) and 5 (d)). Including cell sap as a source of ribonuclease inhibitor in this incubation had no apparent effect. In contrast to the monomers in Fig. 5 (h), neither the pseudopolysomes (Fig. 5 (f)) nor the polysomes (Fig. 5 (g)) were significantly dissociated into sub-units when incubated in the presence of dissociation factor. Because of the possibility that dissociation of pseudopolysomes involved the cooperative action of the dissociation factor and some other factor(s) similar to the tRNA releasing factor found in extracts of E. coli by Ishitsuka and Kaji (9), the incubation was also

performed with cell sap in addition to crude dissociation factor, but the result was identical to Fig. 5 (f). Since the results of Blobel et al (1) indicate that tRNA remains associated with pseudopolysomes, it is possible that the presence of the tRNA is responsible for the resistance of pseudopolysomes to dissociation by factor. Attempts were made, therefore, to remove the tRNA from the pseudopolysomes by incubating them at 37 ^{o}C for 5 min in the presence of cell sap, GTP, and an energy generating system (4). After incubation the pseudopolysomes remained resistant to dissociation by the factor but studies in which the transfer RNA was labelled with radioactive tracers indicated that it had not been released from the ribosomes by the incubation procedure.

The presence of ferritin and other polydisperse contaminants sedimenting in the same area as ribosome monomers and sub-units made it impossible to analyze the precise components of the subcellular fraction from which the crude dissociation factor was extracted. The fractionation procedure, however, was developed in an attempt to prepare a fraction which would be enriched in its content of "native" ribosomal sub-units. Similar extraction procedures applied to preparations ofpolysomes prepared by detergent treatment or of membrane-free polysomes (see (9) for details of preparations) did not result in the release of any detectable dissociation activity, even at similar protein concentrations.

In order to confirm that this factor acted by combining with the small ribosomal sub-unit, in a manner analogous to that suggested for the factor isolated from E. coli by Subramanian et al (1), the following experiment was performed. Large and small ribosomal sub-units, formed from monomers by action of the factor, were isolated separately by centrifuging the appropriate fractions from sucrose density gradients; the sub-units were re-extracted

in medium B; and the extracts were re-assayed on monomeric ribosomes. Only the extract of the small sub-units (Fig. 5a) caused dissociation of monomeric ribosomes whereas the extract of the large sub-units (Fig. 5b) showed no dissociating activity.

Based on the suggestion of Colombo et al (10,11) that NaF may act on the mammalian protein synthesizing mechanism by inhibiting the ribosome dissociating process, an experiment was performed in which 0.01N NaF was present during incubation of factor with monomeric ribosomes. In contrast to the results reported by Colombo et al, the presence of NaF led to an increase in the extent of dissociation, presumably due to a reduction of the Mg^{2+} concentration.

A preliminary experiment in which gel filtration of the factor preparation on a column of Sephadex G-100 retarded the dissociation factor only slightly indicated an approximate molecular weight of 1×10^5 daltons but an accurate molecular weight determination was not possible using Sephadex G-100.

Discussion

Much of the success of the assay method used appears to depend on the substrate monomers' being completely free of peptidyl-tRNA and messenger RNA. The use of monomers reformed from the products of complete dissociation (7) ensure their suitability as substrate and make it possible to attain 100% dissociation when sufficient factor is used.

The presence in the crude dissociation factor preparation of substances which caused monomeric ribosomes to aggregate and sediment to the bottom of the sucrose gradient, made impossible the calculation of meaningful specific activities of the factor at various stages in the purification.

Pseudopolysomes not only provided a useful tool with which to test the specificity of the dissociation factor, but also served as a model system in which to study any possible involvement of the factor in the termination process after hydrolysis of the peptide-transfer RNA bond. The observations

reported, suggest that the factor is specific for monomeric ribosomes lacking nascent protein, messenger RNA and transfer RNA although it was not possible to test the case where messenger RNA alone remained attached to the ribosomes. The results do not support a role for this factor in releasing ribosomes from messenger RNA directly as sub-units although they do not rule out this possibility.

With the exception of its apparent molecular weight, many of the properties of this dissociation factor are similar to those of the dissociation factor from E. coli. Since the bacterial factor also has a role in initiation (2,3), we are presently examining the initiation of protein synthesis in rat liver and the possible involvement of the dissociation factor in this process.

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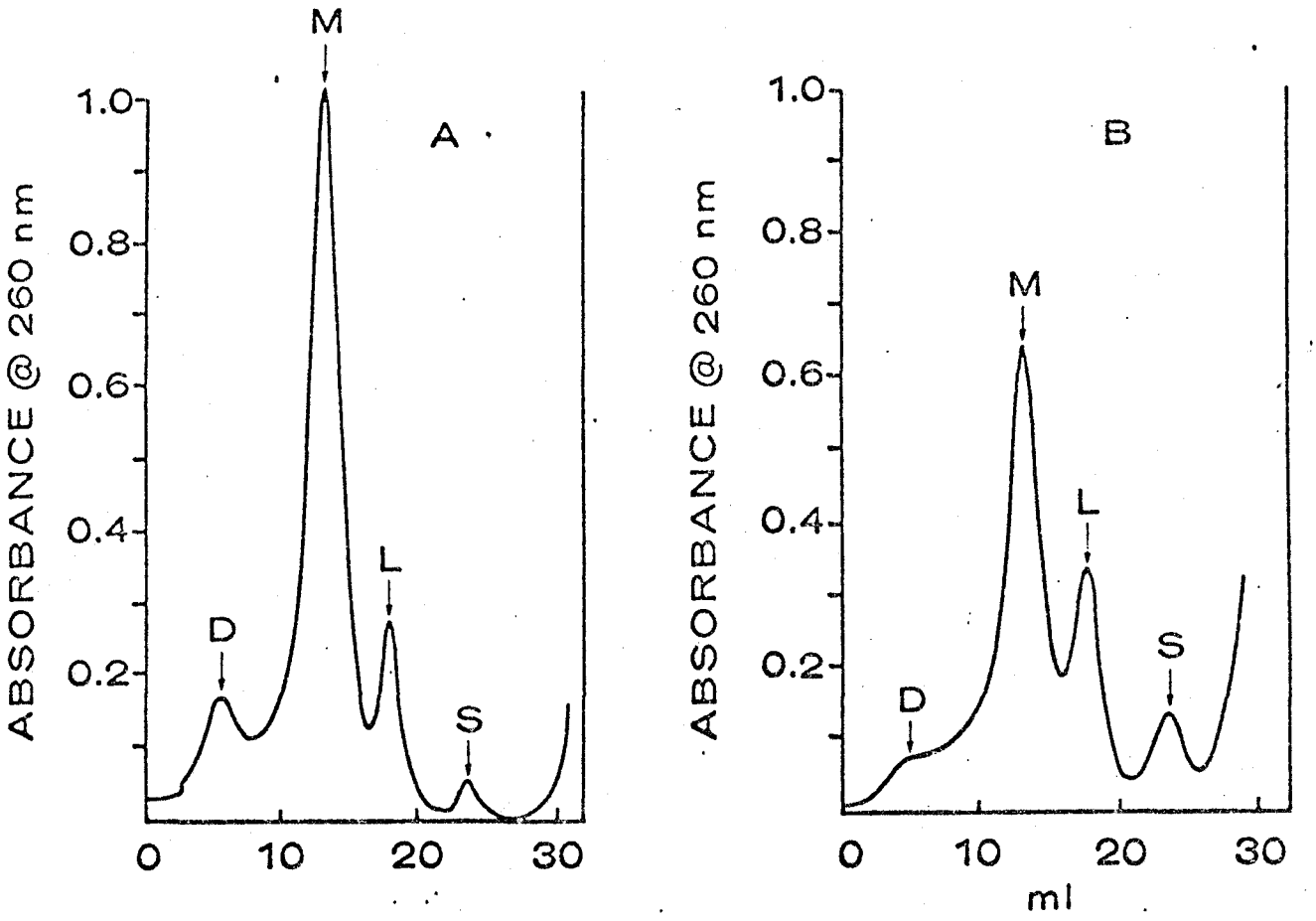


Fig. 1 Sucrose density gradient analysis of monomeric ribosomes (0.5 mg) incubated at 30 °C for 30 min.

A. without addition of dissociation factor

B. with addition of 9 mg of dissociation factor

The peaks are identified as: dimers, D; monomers, M; large sub-units, L; small sub-units, S.

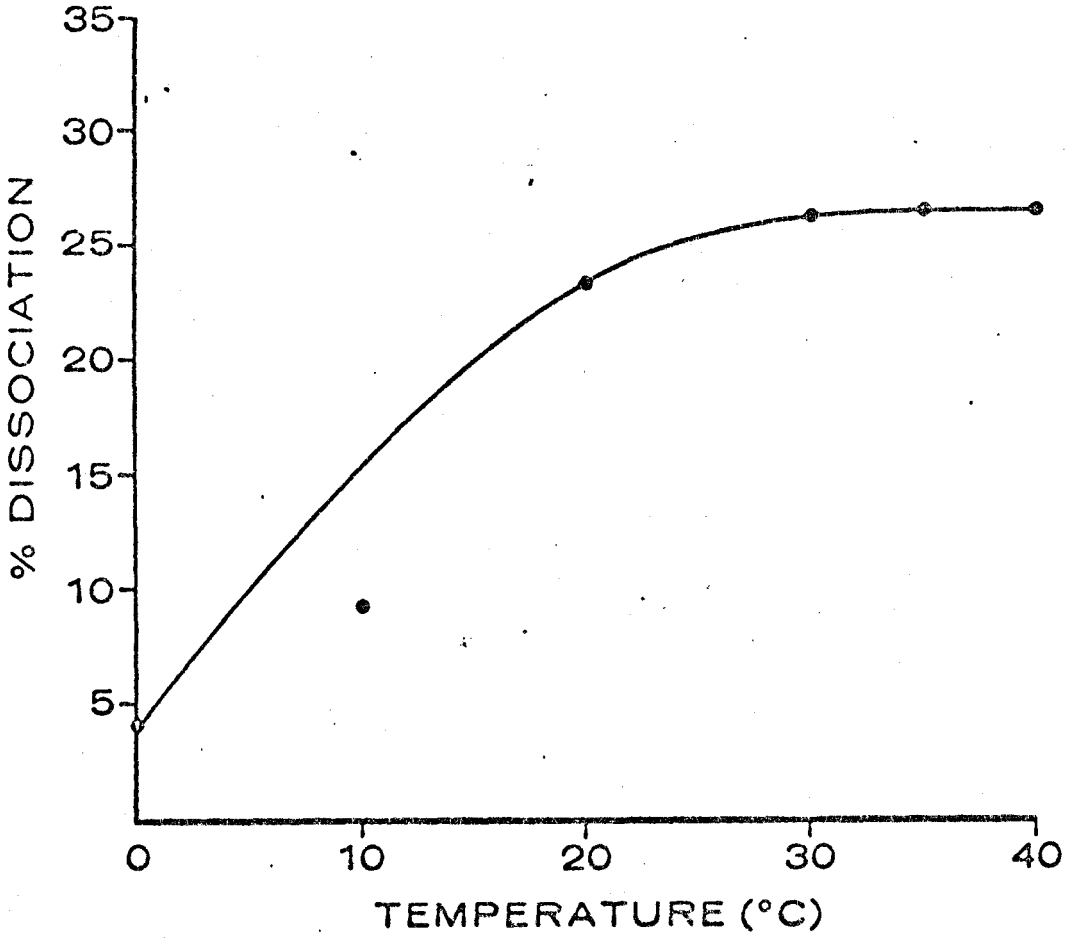


Fig. 2 The effect of the temperature of incubation on the activity of the dissociation factor. The activity was calculated from the areas under the peaks on sucrose gradient analysis and expressed as the percentage of the total absorbance which was present as sub-units. The total absorbance was the same for each. The incubations contained 4.3 mg of dissociation factor preparation each.

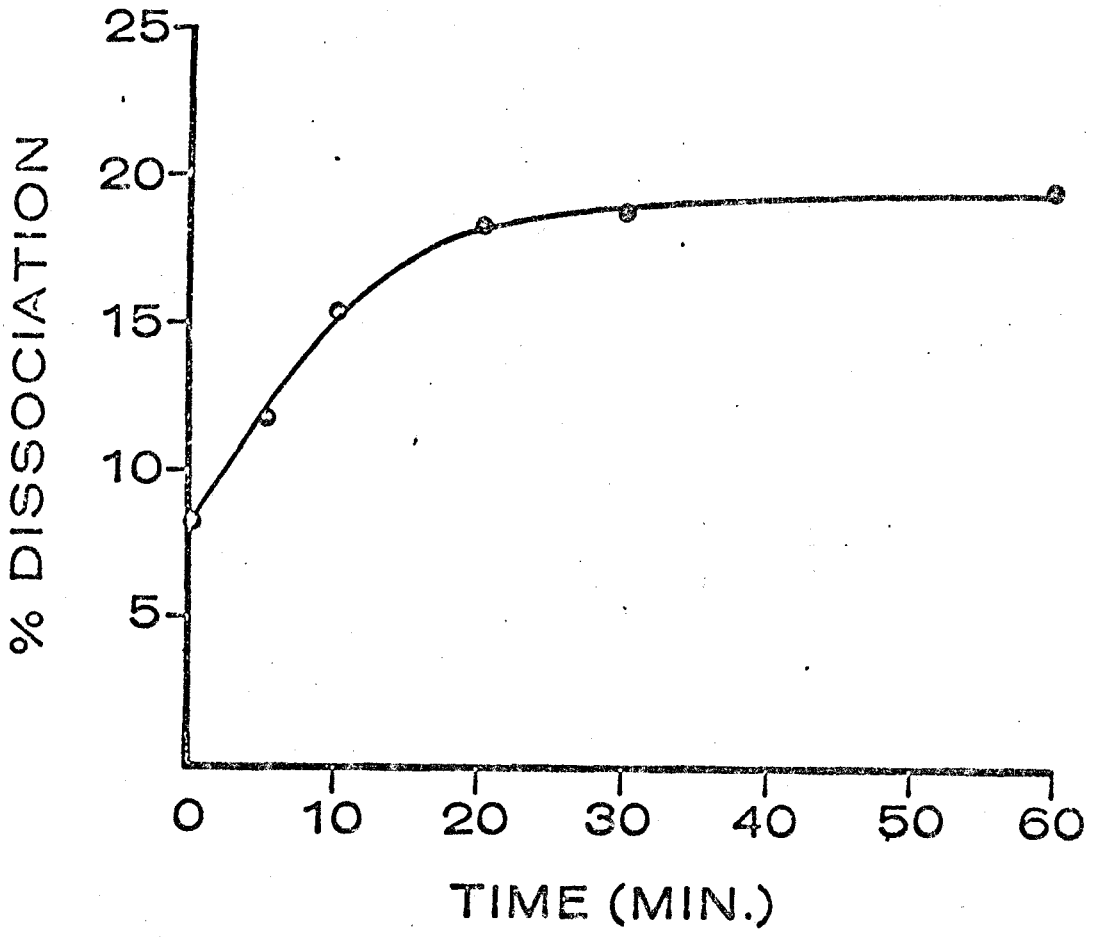


Fig. 3 The effect of the duration of the incubation on the extent of ribosome dissociation by factor. Incubations were performed at 30 °C. Quantitation of dissociation was as described for Fig. 2. Each incubation contained 3 mg of the dissociation factor preparation.

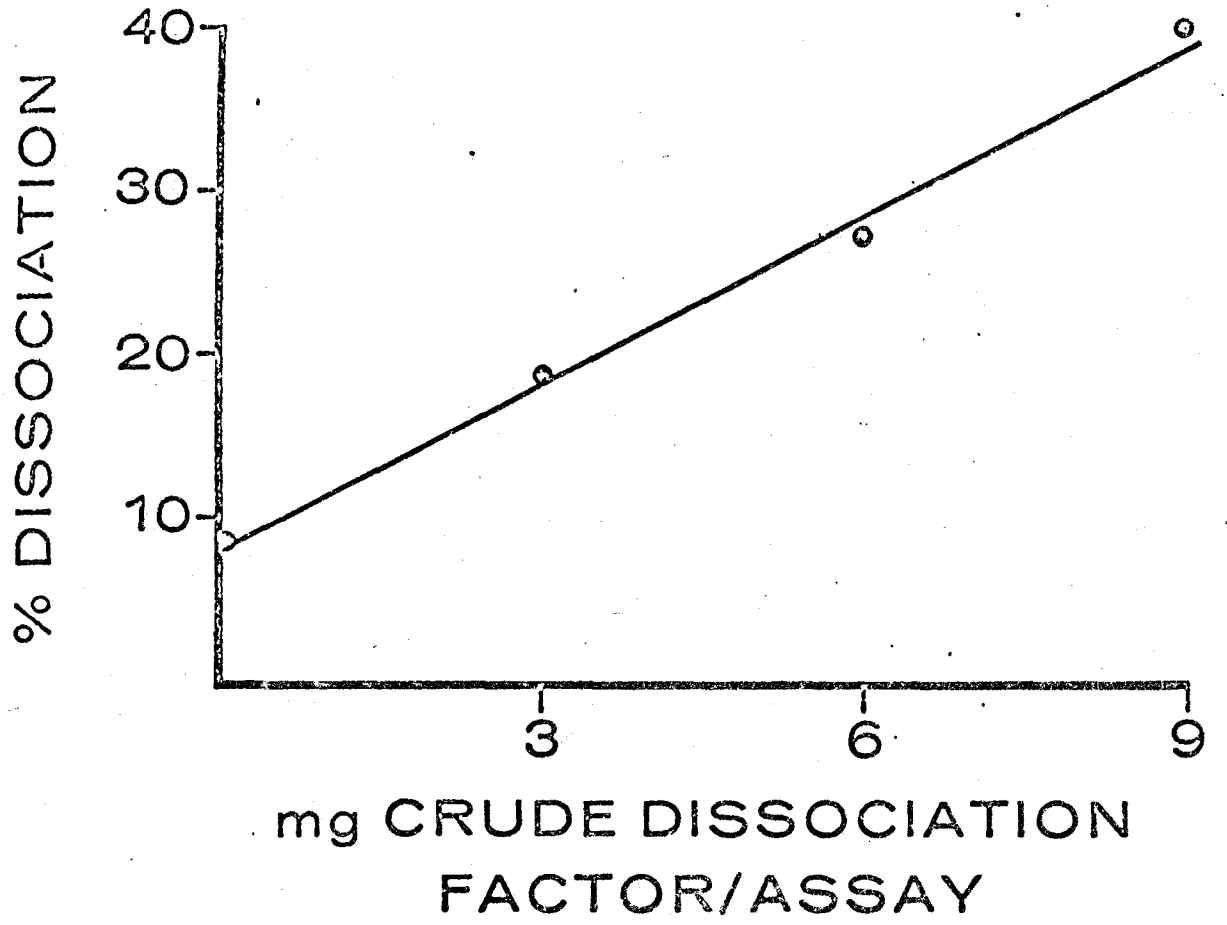


Fig. 4 Relationships between the extent of ribosome dissociation and the amount of factor added. Incubations were at 30 °C for 30 min. Quantitation of dissociation was as described for Fig. 2.

Fig. 5

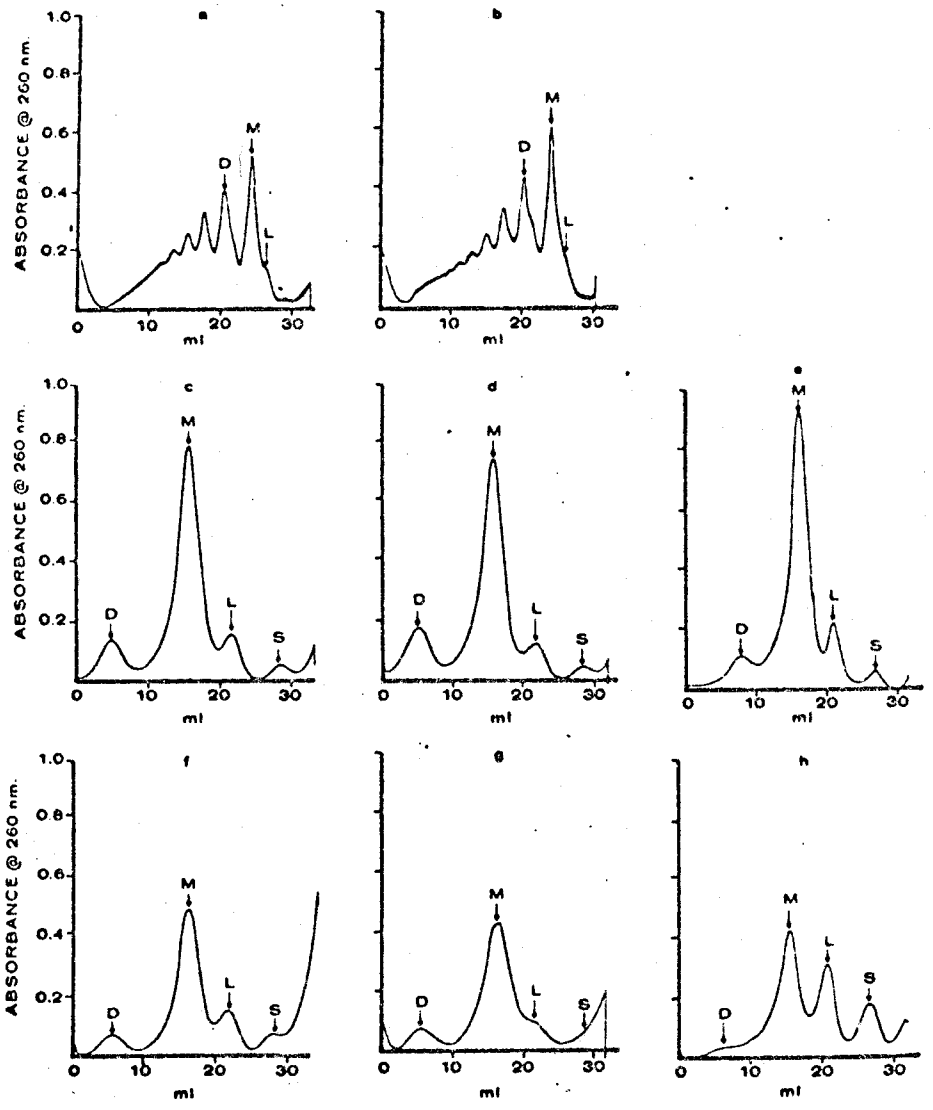


Fig. 5 Sucrose density gradient analysis of various ribosome preparations. The peaks are identified as: dimers, D; monomers, M; large sub-units, L; small sub-units, S.

(a) Profile of a preparation of pseudopolysomes. Centrifugation was for 3.5 h at 75 000 x g in a linear gradient of 15% sucrose in medium A.

(b) Profile of a preparation of polysomes. Centrifugation was as described in (a) above.

(c) Profile of a preparation of pseudopolysomes after incubation at 30 °C for 30 min without addition of dissociation factor. (This is the control for (f)).

(d) Profile of a preparation of polysomes after incubation at 30 °C for 30 min without addition of dissociation factor. (This is the control for (g)).

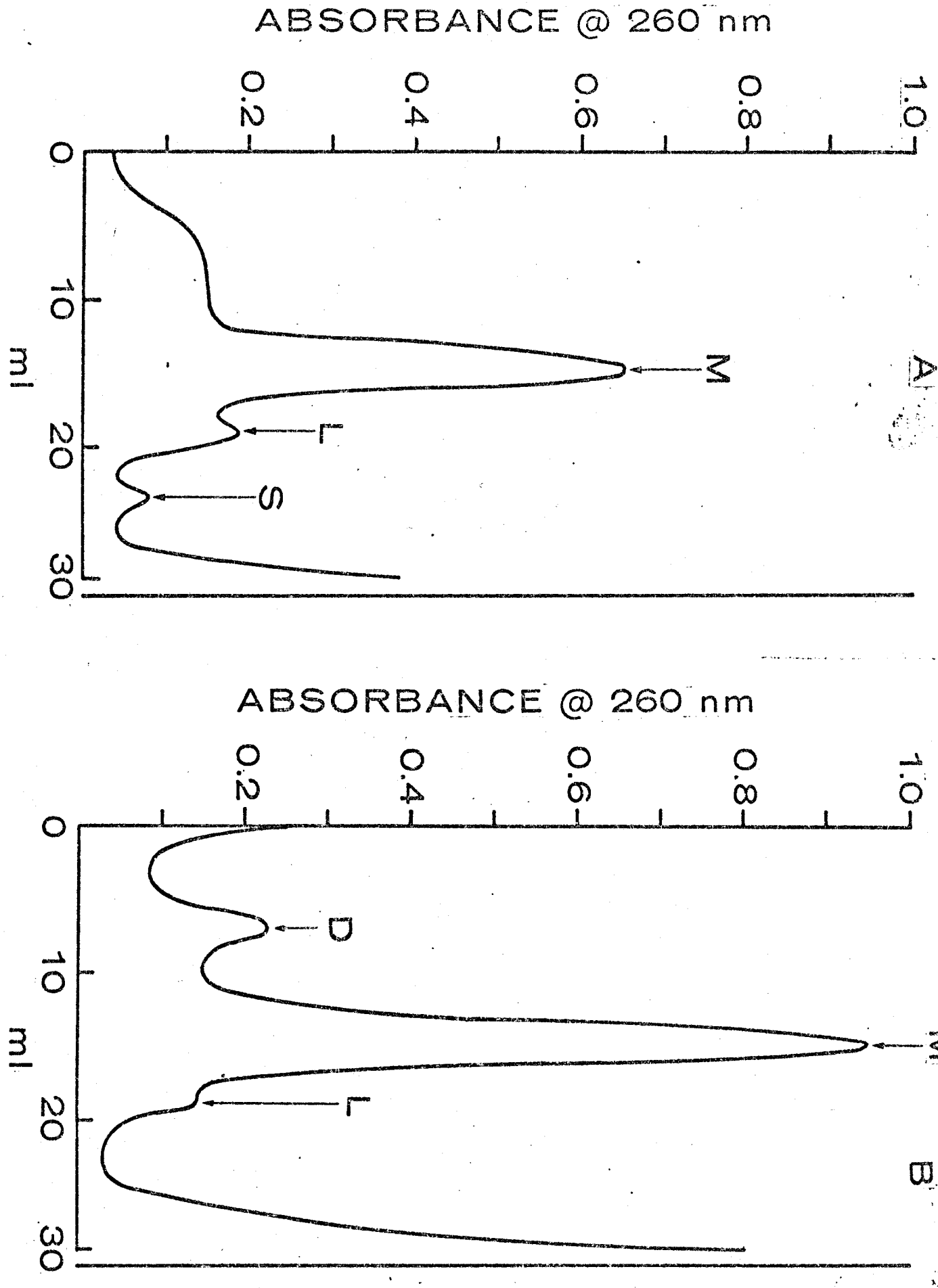
(e) Profile of monomeric ribosomes after incubation at 30 °C for 30 min without addition of dissociation factor. (This is the control for (h)).

(f) Profile of a preparation of pseudopolysomes after incubation at 30 °C for 30 min with dissociation factor.

(g) Profile of a preparation of polysomes after incubation at 30 °C for 30 min with dissociation factor.

(h) Profile of a preparation of monomeric ribosomes after incubation at 30 °C for 30 min with dissociation factor.

Fig. 6


ABSORBANCE @ 260 nm
0.2
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ABSORBANCE @ 260 nm
0.2
0.4
0.6
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1.0
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ml
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B

Fig. 6 Sucrose density gradient analysis of monomeric ribosomes (0.5 mg) incubated at 30 °C for 30 min.

A. with an extract from small sub-units formed by treating monomeric ribosomes with dissociation factor.

B. with an extract from large sub-units formed by treating monomeric ribosomes with dissociation factor.

The peaks are identified as: dimers, D; monomers, M; large sub-units, L; small sub-units, S.

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