STUDIES ON THE STRUCTURE, FUNCTION AND REGULATION OF RABBIT RETICULOCYTE RIBOSOMES

by

Alphonse P. Krystosek, B. A.

A THESIS

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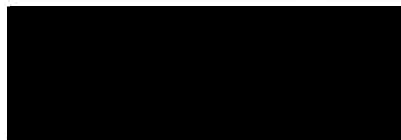
APPROVED: 

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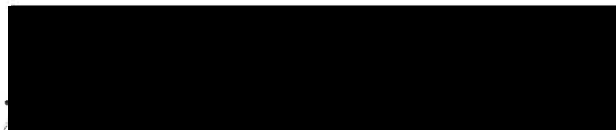

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I. Introduction

Ribosomes are the cellular organelles upon which protein biosynthesis takes place. Whether the ribosome is a passive component in this process, providing only the active site structure for peptide bond formation, or whether the ribosome itself is a regulated element in the control of protein synthesis has long been an unanswered question. As will be discussed later both active and inactive forms of ribosomes exist in cells, but the cause of the inactivation of ribosomes is not known. It could be due to either a modification of the ribosome, or inactive single ribosomes might accumulate secondarily to translational regulation of other components which are required for protein synthesis. This dissertation will, in part, be concerned with an examination of the nature of the inactivity of single ribosomes.

In this introduction I discuss in Part A the various forms and activity states of ribosomes and their relation to the normal ribosome cycle of protein synthesis. In B an evaluation of the possible role of ribosomes in translational regulation is given. It is emphasized that it has been difficult to localize regulatory events to the ribosome itself due to the complexities and several levels of translational regulation. In C, I discuss aspects of protein synthesis in reticulocytes which makes this cell a favorable model system for studying ribosome activity. In D, the possible relation of the phosphorylation of ribosomal proteins to ribosome activity is presented. An approach to the functional significance

of this ribosomal metabolism forms part of the experimental studies of this dissertation. Based on the above discussions, the specific aims of this thesis research are presented in E. And in F, the preparation of ribosomal subunits, which is of paramount importance in the research undertaken, is reviewed in terms of ribosome structure and function. This last discussion is based on my belief that the results of experiments on the function of ribosomal subunits must be interpreted in terms of their structural integrity in relation to the preparative methods employed.

Important aspects of ribosomes and protein synthesis not discussed here are given in recent papers, reviews, monographs, and symposia proceedings. These aspects include the protein composition of animal cell ribosomes (1-8), the biosynthesis and assembly of animal cell ribosomes (9), the mechanism of protein biosynthesis with emphasis on the role of nonribosomal protein factors (10-13), the general structural design principles of ribosomes and their functional interpretation (14-19), and a detailed discussion of the control of hemoglobin synthesis at several levels (20). Interesting historical accounts of the discovery of the mechanism of protein synthesis have appeared (21-23).

Although this dissertation is concerned with animal cell ribosomes, the following discussion will, when appropriate, incorporate results obtained from the more intensive investigations of bacterial

ribosomes. Such results often emphasize the universality of ribosome structure and function and give a reference point for evaluating new data when similar examples are not available in higher cell systems.

A. Different Ribosome Classes and the Ribosome Cycle of Protein Synthesis

Protein synthesis occurs on ribosomal aggregates, termed polyribosomes or polysomes by Rich (24). Such polysomes contain messenger RNA and have been shown to occur in a wide variety of higher cells (24-34). A distinct size class of active ribosomes also exists in bacteria (22,35-38), but due to the sensitivity of bacterial ribosomes to breakdown by the vigorous isolation methods used, polysomes were hard to detect in early studies. Their existence is now, however, well established (22,38).

Native ribosomal subunits in mammalian cells are termed 60S (large subunit) and 40S (small subunit), based on their sedimentation coefficients. (This common S value nomenclature is used in this dissertation, and in this field in general, without implying an exact determination of the sedimentation coefficients of the ribosomal particles.) Native subunits occur as a constant proportion, about 15-17 percent, of the cell's ribosomal population (39) and are implicated as the initiating ribosomal species in protein synthesis in both procaryotes and eukaryotes. The evidence for this

was at first indirect (40-47) but is amply confirmed by the direct demonstration of initiation complexes involving ribosomal subunits (48-54). The small native subunit is heterogeneous in size and protein content (55-61) due to its association with initiation factors (60) and/or initiator tRNA (56,62,63). Without discussing the mechanism of initiation of protein synthesis in detail, it is however important to note in this regard that the most recent evidence from both bacterial (64) and mammalian systems (54,62,65) indicates that the first initiation step is the binding of an initiation factor-initiator tRNA-GTP complex to the 40S subunit. This ribosomal species then selects the initiation region on a messenger RNA. These first interactions are a likely site for the regulation of protein synthesis initiation (63). All 40S subunits may also have at least one bound initiation factor to prevent their association to form single ribosomes (66,67). Messenger RNA does not occur on the small subunit (63,68,69).

A general outline of the ribosome cycle is shown in Fig. 1. The native subunits initiate on polysomal-bound messenger RNA and according to the tape theory (21,26,31,36) traverse the message reading the code and catalyzing the polymerization of amino acids. The early studies of Rich and coworkers (24-27) provide evidence for this model. All polysomes are apparently active and each ribosome

Figure 1. The ribosomal subunit-polyribosome cycle of protein synthesis. As described in the text, polyribosomes and native ribosomal subunits are active forms and are in equilibrium. Single ribosomes are inactive in protein synthesis.

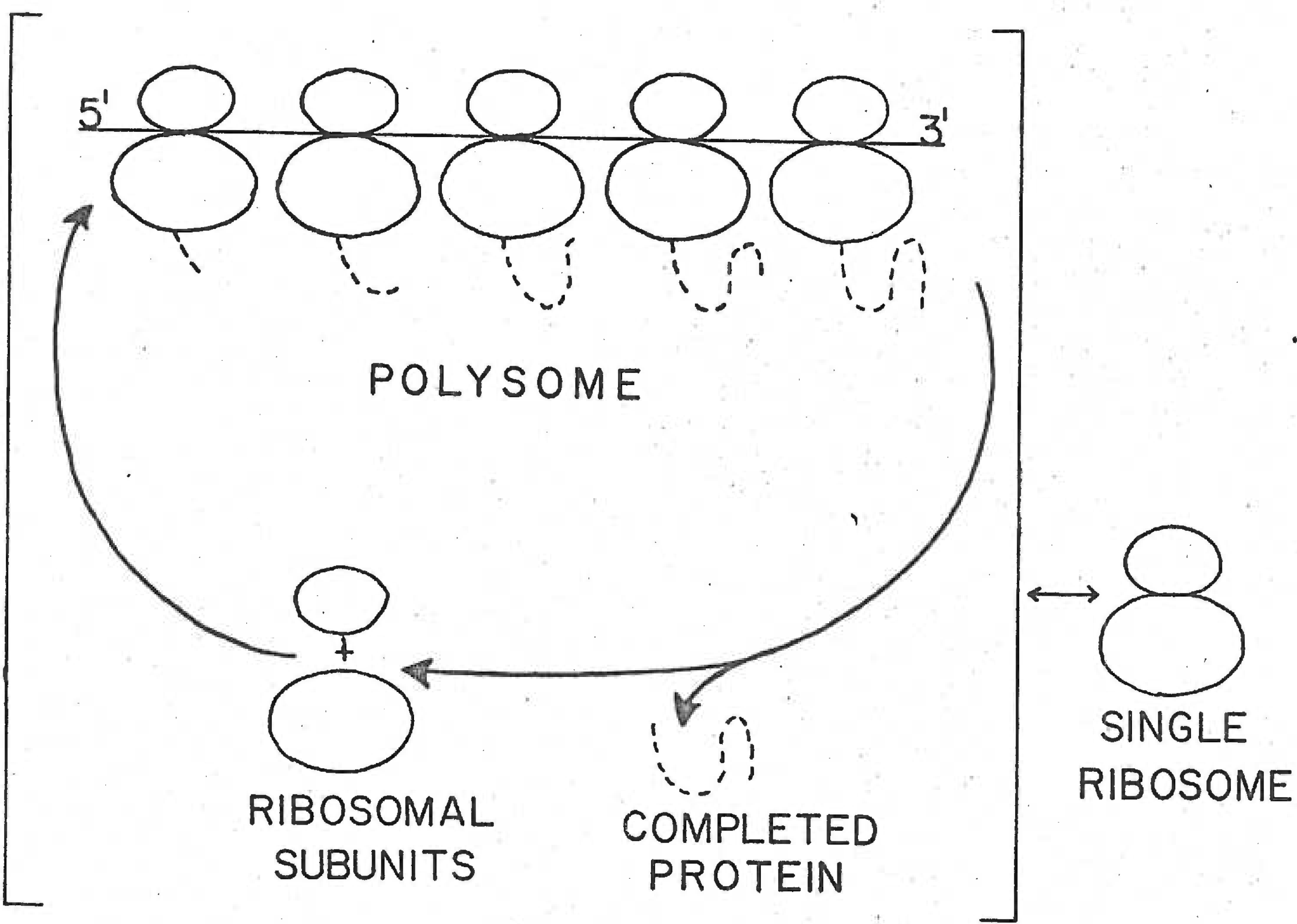


FIGURE 1

in a polysome contains one nascent polypeptide chain which is released upon termination of translation. Ribosomal subunits are released uncombined upon termination and are able to reinitiate (66,67), but this point was difficult to establish since single ribosomes accumulate from terminating subunits if reinitiation does not occur (66,67,70). The subunits of the ribosomal couple of polysomes are thus not obligatory partners when they are off the messenger (71,72). This model of the ribosome cycle derived from the early work on reticulocyte ribosomes also applies to bacterial protein synthesis (38).

The single ribosome[1] is thus not an obligatory intermediate in the subunit-polyribosome cycle of protein synthesis. Two important points bear on the nature of the inactivity of this ribosomal form in eukaryotic cells. Single ribosomes do not contain messenger RNA (68,73-76) and studies on the kinetics of entry of newly biosynthesized subunits into the active ribosome cycle show that the active ribosomal forms are not in rapid equilibrium with single ribosomes (42-47). Thus, single ribosomes are not simply inactive

[1] The following terminology is used to distinguish between different functional forms of the '80S' ribosomes. A single ribosome is an inactive ribosomal couple not containing mRNA. A monosome is a polysome containing one ribosome on the messenger. The term monomer is used here when the distinction cannot be made on the evidence available.

at a given instant (due to lack of mRNA) but rather are excluded from protein synthesis for long periods of time. They, therefore, represent a stagnant pool, the physiological significance of which is not understood. The amount of single ribosomes varies in different tissues and with cellular growth state (77,78) and differentiation (79-82); it, therefore, appears subject to physiological control. Protein factors capable of dissociating single ribosomes have been described in Escherichia coli (83), rabbit reticulocytes (84-86), rat liver (87), and yeast (88). However, their role, if any, in activating single ribosomes for protein synthesis remains uncertain. This account of the ribosome cycle and the inactive nature of single ribosomes has also been confirmed in studies with in vitro protein synthesizing systems (89-91).

B. Translational Regulation of Protein Synthesis

The existence of both active and inactive forms of ribosomes suggests that the ability to modulate ribosomal activity may be important in the regulation of protein synthesis. But this conjecture must be viewed in light of the extensive work on translational regulation. In particular whether the ribosomal activity state is an inherent property of ribosomal structure and/or metabolism or a secondary consequence of a more direct control point in translational regulation must be evaluated.

It is perhaps most important to realize that protein

synthesis may be regulated at several levels. Transcriptional control is of paramount importance in bacteria (21,92,93) due in part to the short half-life of bacterial messenger RNA. The longer half lifes of mammalian mRNAs (94,95) suggests that translational regulation will be important in higher cells, although transcriptional control may be of critical importance in the regulation of specific gene expression. Translational regulation in this discussion will generally refer to the cell's commitment to total protein synthesis rather than the regulation of the translation of specific messengers. A recent review by Pain and Clemens (96) discusses the manifold components of the translational system which have been implicated in regulation under various conditions.

As a general rule, macromolecular syntheses are regulated at the initiation step and many examples of the physiological regulation of protein synthesis have been shown to occur at the initiation steps (63,97-105). Several such studies included a demonstration that the observed decrease in protein synthesis [e.g., during amino acid starvation (105), mitosis (106), and transition from proliferating to resting phase of cultured cells (77)] was not the result of lack of mRNA. Ribosomal monomers accumulate in circumstances where initiation is inhibited but in most cases further localization of the inhibitory defect to the ribosome or initiation factors has not been accomplished.

Moreover, translational regulation is multifaceted and coordinate control of several components is often seen (96). The dramatic increase in the rate of protein synthesis seen in sea urchin eggs at the time of fertilization appears to be due to the coordinate activation of ribosomes, messenger RNA and elongation factors (107-109). Hormonal regulation of protein synthesis in chick oviduct appears to involve effects on the amount of ribosomes and specific mRNAs per cell as well as regulation of the rate of initiation and elongation (97,110). Studies on the role of nutritional effects on protein synthesis showed regulatory responses at both initiation and elongation phases (111,112).

Other suggested regulatory components in translation are transfer RNA and its synthetases (113-115) and messenger specific translation factors (116-121). Although some of the work on the latter point seems good, there is also an equally impressive body of evidence against eukaryotic messenger specific factors. This evidence is mainly the general ability to translate a given mRNA in a heterologous cell-free system. Extensive references to this work may be found in references 96 and 122. Finally, various workers have found inhibitors of protein synthesis in cell extracts (123,124) or associated with ribosomes (101,107,108,125-127) under various physiological conditions.

Thus, despite much evidence on translational regulation,

the role of the ribosome remains uncertain. Furthermore, there have been no unambiguous systems in which ribosome activity could be studied in the absence of effects on other components of the translational apparatus. Also, the relation of results obtained in one cell type or growth condition to other systems remains in doubt. For example, while inactive monomers accumulate in mature sea urchin eggs prior to fertilization (128-130) the turning on of protein synthesis involves coordinate activation of several components, as already noted. Furthermore, whether the monomers of sea urchin protein synthetic defect lies in mRNA "masking" has long been debated (see 109). The most recent evidence implicates a ribosomal bound inhibitor as being, at least in part, responsible for the inactivity of these monomers (107-109). An inhibitor may also be present on the inactive monomers which accumulate in mitotic cultured mammalian cells (101). Other experiments by other workers suggest that there is not an extractable inhibitor of protein synthesis on the single ribosomes which accumulate during reticulocyte maturation (131). The single ribosomes of different cell types have never been systematically investigated and whether they are inactivated by a common mechanism as it is attractive to think, remains open to investigation.

Several groups of workers have approached the study of single ribosomes by investigating the mechanism of the nutritional

dependent interconversion of single ribosomes and polysomes (103,111, 112,132-134). However, in these studies the "single ribosomes" capable of polysome formation under complete nutritional conditions were produced by polysome runoff during a previous inhibition of protein synthesis initiation by amino acid starvation. Such polysome-derived runoff ribosomes behave differently than the native pool of single ribosomes. In fact, Bitte has concluded that the amount of ribosomes capable of rapidly shifting into polysomes when mouse sarcoma-180 tumor cells are incubated in a complete nutritional medium is limited to those ribosomes which ran off polysomes during a previous incubation in a deficient medium (135). Moreover, the sedimentation characteristics of these runoff ribosomes suggested their nonequivalence to normal single ribosomes (135). Thus, such studies do not contribute to an understanding of the native pool of single ribosomes.

Early studies in which single ribosome activity was studied in in vitro cell-free protein synthesis systems are similarly flawed by uncertainty about the ribosomal species under consideration (i.e., does the single ribosome preparation also contain monosomes?) and by the possibility of reversal of ribosomal inactivation in the crude assay systems (26,136-138). Such studies were performed before the subunit nature of ribosome initiation was known and, therefore, contain incorrect interpretations of the data.

Some studies on single ribosome activation are further complicated by the several levels of ribosomal control. Thus, both the fraction of ribosomes existing in the active form and the rate of protein synthesis per active ribosome unit have been found to be simultaneously affected in various experimental systems (78,112).

Finally, another possible level of ribosomal control is the variation of the protein composition of ribosomes with cellular growth rate or ribosome activity state. Rodgers has reported such a variation in protein composition of ribosomes from mouse cells of different growth rates (139) and an abstract suggesting possible compositional differences of reticulocyte ribosome classes has appeared (5). Such studies are, however, complicated due to the difficulty of analyzing the complex protein complement of animal cell ribosomes. Furthermore, chemical modification (e.g., phosphorylation) rather than new distinct protein species might be responsible for such observations. It should be mentioned, however, that bacterial ribosomes are better characterized in this regard and appear to be heterogeneous in protein content (18,140) in a manner related to cellular growth rate (141) or ribosomal functional state (142,143).

However, two examples of translational defects have been traced to a ribosomal localization. After hypophysectomy in the rat, isolated liver ribosomes are less active in endogenous and poly(U)-directed protein synthesis and the defect resides in the 40S subunit

(144). Similarly, the defect in protein synthesis in muscle of experimentally diabetic rats was localized to the 60S subunit (145). The mechanism and generality of these ribosomal defects remain unknown. It is still attractive to think, however, that other specific ribosomal regulatory events will be found when appropriate systems are carefully studied. A favorable model system in which ribosome activity may be studied is now discussed.

C. Control of Protein Synthesis During Reticulocyte Maturation

As erythroid precursor cells differentiate and mature to circulating erythrocytes, they undergo characteristic changes in metabolism and cellular composition (146). Bone marrow erythroid cells are more active in hemoglobin synthesis than the later reticulocytes (147). But large amounts of circulating reticulocytes, still active in hemoglobin synthesis, can be obtained for experimental study by phenylhydrazine-induced anemia (148). The resulting reticulocytosis is due to a regenerative macrocytosis and the nature of these reticulocytes has been investigated (149,150).

The following aspects of protein synthesis during reticulocyte maturation makes these cells a favorable system for the study of ribosome activity.

1) Reticulocytes are enucleate and have lost the capacity for RNA synthesis (29,151).

2) Reticulocytes make greater than 90% of their protein as

the α and β globin chains (152). Such synthesis occurs on a distinctive size class of free polysomes (24,25,153) which are readily isolated for study.

3) The globin mRNAs of reticulocytes are stable during the period of protein synthesis (154).

4) The ribosomal changes accompanying maturation have been well studied. Maturation of reticulocytes in vivo and in vitro leads to a gradual shift from polysomes to inactive single ribosomes which is correlated with the declining rate of protein synthesis (79-82). Maturation is also accompanied by a decrease in the total amount of ribosomes per cell as a result of ribosomal degradation (155). It is not known if single ribosomes represent a direct precursor pool of this degradation. Electron microscopic autoradiography reveals that single ribosomes accumulate in cells which are inactive in protein synthesis (156); these cells presumably represent older reticulocytes. Some evidence also suggests that inactive (156) or less active (80) forms of polysomes may also exist in reticulocytes. This developmental decline in protein synthesis is not due to changes in RNAse activity, membrane permeability, diffusable activators or inhibitors of protein synthesis, ribosomal composition, or translation components in the supernatant (157). However, the amount, activity or composition of the initiation factor fraction recovered by salt washing of the ribosomes changes;

this fraction from older cells is less able to support in vitro initiation (150,159). Thus the correlates of the decline in protein synthesis during maturation are a decreased ribosome content of the cells, a shift from an active to an inactive ribosome form, and a decrease in the capacity for initiation.

5) The single ribosomes which accumulate during reticulocyte maturation are more well characterized than the monomers seen in various cells and conditions discussed in the previous section. For example, reticulocyte single ribosomes have been shown to lack globin mRNA (68,73-75) and appear to have the same protein composition as polysomes (160, but see also 5) and do not have bound inhibitors of protein synthesis (131). The inactivity of these single ribosomes and the existence of a subunit-polyribosome cycle of protein synthesis has been demonstrated in a reticulocyte lysate cell-free system (89,90). Possibly, the nature of single ribosomes in reticulocytes may be relevant to understanding polysome ↔ single ribosome interconversions that are correlated with the growth state in other cell types (77,78). Also the low levels of RNAse in reticulocytes and the gentle cell lysis and ribosome isolation procedures makes possible the isolation for experimental study of native single ribosomes free of contamination by polysomal breakdown monomers.

In summary, the reticulocyte represents a differentiating

mammalian cell in which ribosome activity can be studied independently of effects of transcription, messenger RNA stability, and activity changes in most of the other components of the translation system. Reticulocyte ribosomes exist in well characterized active and inactive forms which are related to the cell's developmental process. These ribosome classes are readily isolated for experimental study.

D. Possible Relation of the Phosphorylation of Ribosomal Proteins to Ribosome Activity

Several of the constituent proteins of the ribosomes of reticulocytes (160) as well as other eukaryotic cells (161-165) are phosphoproteins. Bacterial ribosomes are not phosphorylated in vivo. This ribosomal metabolism has recently been reviewed (166,167). Ribosomal protein phosphoryl groups turn over intracellularly (168) and protein kinase and phosphoprotein phosphatase activities have been detected on reticulocyte ribosomes (167,169,170) and in the supernatant fraction (171,172). This metabolism has also been studied intensively in vitro (167,170,173-176).

It is of particular interest that ribosome phosphorylation has been shown in intact cells and in cell-free systems to be stimulated by cyclic adenosine 3',5'-monophosphate or by hormones affecting the level of this regulatory nucleotide (135,162-165,175-177). This effect may be related to the cyclic AMP stimulation of protein synthesis which has been observed in various intact cells and

extracts (135,178,179), but inhibition has also been observed in some cases (179).

Kabat (160,168) and Bitte and Kabat (161) have shown that polysomes and single ribosomes in rabbit reticulocytes and mouse sarcoma-180 tumor cells differ quantitatively in their protein phosphorylation pattern. These differences appear to be due to conformational differences between these two ribosomal forms (168). Furthermore, Kabat has proposed that such phosphorylation differences may be related to the mechanism of single ribosome inactivation (160). This idea has, however, been difficult to test experimentally (135).

Moreover, few studies have been directed at looking for specific functions of ribosomal protein phosphoryl groups. The available evidence (167,180) has provided no support for a role of these protein phosphoryl groups for protein synthetic activity of ribosomes. But these studies must be considered inconclusive due to limitations in the experimental methods employed, as reviewed elsewhere (167). Indeed this ribosome metabolism is ubiquitous in eukaryotes (167) and the ribosomal phosphoproteins of different mammalian cells appear to be homologous proteins related by evolution (161). Furthermore, this metabolism is subject to physiological regulation. Therefore, ribosomal protein phosphorylation may have a functional significance which remains unknown.

E. Statement of the Objectives of the Thesis Research

The rationale of this thesis work can be seen by considering two proposed models for the inactivity of single ribosomes. In the first model the single ribosome is viewed as being inherently inactive due to a chemical or structural modification (e.g., phosphorylation of a specific protein site(s)). In another model, Kaempfer has proposed that in both bacterial (66) and mammalian (67) cells the entry of ribosomal subunits into active (polysomes) or inactive (single ribosomes) states is mediated by the availability of a specific initiation factor. Subunits binding this factor are recycled into polysomes, whereas subunits spontaneously reassociate to form single ribosomes in the absence of this factor. The availability of the factor is postulated to be limiting at times when initiation of protein synthesis is slow or inhibited for whatever reason; in such conditions single ribosomes would accumulate. Thus, according to this idea, formation of single ribosomes would be a secondary rather than primary event in translational regulation. Whereas, in the first model a specific ribosomal defect is postulated, the second model predicts that the subunits of single ribosomes are structurally and chemically equivalent to those of polyribosomes.

To study the nature of the inactivity of single ribosomes and to perhaps decide between these models, I have prepared from

rabbit reticulocytes subunits from single ribosomes and compared them to subunits derived from polyribosomes and to native subunits. I have asked whether functional differences exist between polysomal- and single ribosome-derived subunits. The advantages of comparing subunits from each source are the following. The inherent difference in mRNA content of the two ribosomal sources is eliminated. Subunits are the initiating ribosomal species in protein synthesis; therefore, assay of subunits for the ability to translate natural messenger RNA necessarily tests functional competence at the several distinct phases of translation. And such functional studies may allow localization of a defect in inactive ribosomes to one of the constituent subunits. I have also tried to evaluate the importance of ribosomal protein phosphoryl groups for protein synthetic activity. For all of the functional studies done in this research I have used two different in vitro protein synthesizing systems which utilize natural messenger RNA. These systems are characterized in Results.

At the time this work was begun, the sole criterion for the in vitro activity of mammalian ribosomal subunits prepared by dissociation methods was their ability to catalyze the polymerization of radioactive phenylalanine directed by a poly(U) template. However, the following characteristics of poly(U) assays suggested that an activity assay with a synthetic messenger template would not be

desirable for the proposed studies. Poly(U) has an abnormally high affinity for ribosomes (36,181) and does not require subunits as the ribosomal initiating species (182,183). Peptide synthesis on poly(U) does not utilize all of the natural initiation factors (184-188) and exhibits an unphysiological Mg^{++} ion optimum (186-188). Most importantly, ribosomes inactive in vivo may be active in a poly(U) assay (189-191). Also, the percentage of ribosomes active in poly(U) assays is usually not directly determinable. The consideration of the initiation pathway is important since regulation of protein synthesis is likely to occur at an initiation step and an inactive ribosome species may be specifically defective for this process. Therefore, a system capable of natural initiation may be imperative to detect such a defect.

Therefore, I have given priority to characterizing the ability of purified ribosomal subunits to translate natural mRNA and to finding preparative conditions which yield the highest ribosome activity. This last consideration is also important since functional differences between ribosome classes may not be detected if subunits become largely inactivated by the preparative methods.

I also wished to learn if subunits of polysomes and single ribosomes differed structurally in a way which could be detected by physical chemical criteria.

F. The Relation of Subunit Preparation to Ribosome Structure and Stability

It was found in the early studies on bacterial ribosomes that partial removal of ribosomal-bound Mg^{++} ions by chelation with EDTA or by dialysis of ribosomes against buffers of low Mg^{++} ion concentration or Mg^{++} ion free buffers resulted in the formation of ribosomal subunits (192,193). Such subunits are biologically active. However, the application of the EDTA method to the ribosomes of higher cells produces irreversibly inactivated subunits with lowered (as compared to native subunits) sedimentation values (194-196). Such subunits are unfolded and have lost ribosomal components (160,197,198). This difference in stability towards Mg^{++} ion removal emphasizes the important role of Mg^{++} ions in ribosome structure in eukaryotes (15). Monovalent cations have also been shown to be important for ribosome activity (199,200).

More recent studies by various workers have led to the development of procedures whereby active subunits can be produced from the ribosomes of higher cells by dissociation of ribosomes with high concentrations of KCl at $MgCl_2$ concentrations appropriate to prevent subunit inactivation (145,196,201-206). The criteria for activity of the subunits has usually been a poly(U) assay, but such subunits are also active with natural mRNAs (see Results and Discussion). In these procedures inactivation is obviated by allowing

the partial replacement of divalent cations by monovalent cations, thereby preventing electrostatic repulsion from causing the subunits to unfold.

The nature of ribosomal-bound Mg^{++} ions and its role in maintaining the stability of the ribosomal couple merits further discussion. Studies in which the quantity of bound Mg^{++} ion have been measured have yielded values approaching 0.5 for the ratio of bound Mg^{++} ion/RNA phosphate (207,208). The ribosomal Mg^{++} ions are almost entirely bound by the ribosomal RNA (207,208) and part of these ions can be exchanged with Mg^{++} ions in buffer solutions (209). The number of Mg^{++} ions stabilizing the inter subunit contacts is, however, small (210). It is not known if this class of Mg^{++} ion is bound to rRNA phosphates or participates in another form of salt linkage. Studies on the amount of Mg^{++} ion bound to the ribosome in buffers of different monovalent cation concentration support the idea that at high concentrations the monovalent cation physically displaces Mg^{++} ions from the ribosome (207,211). During ribosome dissociation by the high salt method the subunits undergo conformational changes which are due to conformational changes in their constituent RNAs caused by electrostatic repulsion as Mg^{++} ions are lost (212). "Swollen" ribosomes have been observed as intermediates in the dissociation process (213).

Another factor affecting the stability of the subunit

couple is hydrogen bonding; hydrophobic bonding seems to be relatively unimportant in maintaining ribosome structure (214). Thus Petermann et al. (214) concluded, "Ribosomal subunits appear to be springy structures whose shapes depend on a delicate balance between cohesion (due to hydrogen bonding in RNAs, proteins and associated water) and the electrostatic repulsion of RNA phosphates." Less well characterized, but suggested as being important in maintaining the associated form of subunits, are protein-protein (215,216) and RNA-protein interactions (211,217).

The high salt method is effective in dissociating only non-polysomal bacterial (193,218) and eukaryotic ribosomes (196,201,203, 205,206,219,220). This effect is due to the stabilization of subunit association in active ribosomes by peptidyl-tRNA (221,222). Recent modifications of the procedure allow preparation of active subunits from polysomes also. When polysomes are allowed to form runoff couples by an in vitro incubation (202,223) or are treated with puromycin to cause removal of the peptidyl group (196,201,203,204, 206) they also become susceptible to salt dissociation. In the experiments reported here a modification of the puromycin-high salt method of Blobel and Sabatini (206) was used. Its advantages were that it allowed me to prepare subunits from single ribosomes and from polyribosomes by equivalent methods and did not require an in vitro incubation in a cell extract which could cause modification

of the isolated ribosomes. As will be shown, such subunits are capable of translating natural mRNA.

II. Materials and Methods

A. Preparation of Reticulocytes

Rabbits were made anemic by seven daily injections of a neutralized phenylhydrazine solution (10 mg/kg body weight) (148). Blood was obtained by bleeding from a small incision in the ear vein and was collected into heparinized beakers and then chilled on ice. All subsequent procedures were done on ice. The cells were sedimented by centrifugation at 1900 x g for seven minutes and were further washed three times by resuspension in cold physiological salt solution (0.13M NaCl, 0.005M KCl, 0.0015M $MgCl_2$) followed by resedimentation. After each centrifugation step the buffy coat containing leukocytes was removed. To determine the percentage reticulocytes in the red blood cell preparation two drops of packed cells were suspended with two drops of physiological salt solution in a tube and three drops of 0.5% methylene blue solution was added. The tube was incubated for 1 hr at 37°C and a smear of cells was made on a slide and examined at 100X magnification under a microscope. Reticulocytes are seen as cells taking up the stain due to their cytoplasmic RNA content (i.e., ribosomes). The per cent reticulocytes was routinely greater than 90%. The distribution of staining intensities amongst the cells indicated a preparation of reticulocytes of different degrees of maturation, but with at least one-half of the cells very heavily stained (i.e., young reticulocytes).

The methylene blue solution was made by dissolving 0.5 g methylene blue chloride and 1.6 g potassium oxalate in 99.5 ml physiological salt solution, heating at 50° for 10 minutes followed by filtering.

B. Preparation of Ribosomes

1. General

The separation of the different size classes of ribosomes was achieved by velocity sedimentation on sucrose density gradients. This technique separates subcellular particles on the basis of size (224) and has thus proved useful in these studies for preparative procedures as well as for analysis of ribosome function in the cell-free protein synthesizing systems.

2. Polyribosomes and Single Ribosomes

Washed, packed cells were lysed by mixing with two volumes of a low ionic strength buffer, RSB (0.01M Tris-HCl pH 7.35, 0.01M KCl, 0.0015M $MgCl_2$). The lysate was centrifuged at 12,000 x g for 10 minutes and the supernatant carefully removed. This supernatant was adjusted to pH 5.0 by slowly adding with mixing dilute acetic acid to precipitate the ribosomes (225). The crude ribosome precipitate was collected by centrifugation at 12,000 x g for 10 minutes. The supernatant was removed and the pellet was resuspended in RSB with readjustment of the pH to 7.35 by addition of Tris buffer. Any unresuspended material was removed by centrifugation at 12,000 x g for five minutes. 1-2 ml of the ribosome

solution was then layered on 36 ml linear 15-30% sucrose gradients containing the RSB buffer; all gradients used to prepare ribosomes for activity studies contained 1 mM 2-mercaptoethanol. For preparative purposes, the amount of ribosomes added per gradient was the amount from 0.75 ml packed cells; thus, ribosomes could be obtained from 4.5 ml cells by one 6 gradient centrifugation. The gradients were centrifuged for 4 hr at 25,000 rpm at 2° in the Beckman SW27 rotor in an ultracentrifuge. Following centrifugation the gradients were pumped from the bottom through a Gilford spectrophotometer containing a 2 mm path-length flow cell. The absorbance at 260 nm was plotted on a chart recorder and the fractions corresponding to polysomes and to single ribosomes were collected into ice-cooled tubes; base line absorbance was determined by blanking with a solution of the heavier sucrose gradient buffer. The ribosomes were concentrated by centrifugation for 2-1/2 hr at 40,000 rpm at 2° in the Spinco 40 rotor. The ribosome pellets were rinsed with cold water and stored at -76°C until use. The centrifugation conditions described allowed clear resolution of polysomes and single ribosomes. Absorbance profiles and a discussion of the purity of the fractions is given in Results.

For preparation of ribosomes to be used in subunit preparation, a convenient modification of this procedure was found which gave ribosome pellets which were more easily resuspended for

subsequent procedures and which avoided subjecting the ribosomes to an elevated H^+ ion concentration. 1.8-2.0 ml of the cell-free lysate (volume equivalent to 0.75 ml packed cells) was layered onto gradients without a pH 5 precipitation step. In this case, the resolution of single ribosomes was less clear and care was taken in collecting the appropriate gradient fractions. The gradient-separated ribosomes of this method are more contaminated by adsorbed proteins, but the ribosomes become highly purified in the subsequent subunit preparation which involves treatment of the ribosomes with solutions having a high salt concentration.

3. Native Subunits

The term native subunit is used here to denote the large and small ribosomal subunits seen when crude ribosomes are resolved by centrifugation on gradients containing a low ionic strength (i.e., under conditions where subunits should not be formed by ribosome dissociation). Crude ribosomes from the pH 5 precipitation step were layered on 36 ml 15-30% gradients containing RSB buffer and 1 mM 2-mercaptoethanol and were centrifuged for 24 hr at 15,000 rpm at 2° in the SW27 rotor. The subunit peaks are identified from the plotted absorbance tracing, and the subunits are sedimented by high speed centrifugation for 2-3 hr at 65,000 rpm in the 65 rotor. Resolution and purity of the subunits is discussed in Results.

4. [^{32}P]-labelled Ribosomes

Ribosomes were biosynthetically labeled in their ribosomal RNA chains by providing [^{32}P]-orthophosphate to the reticulocyte precursors during their maturation in the bone marrow. A total of 10 or 20 mc of [^{32}P]-orthophosphate was injected into an ear vein of an anemic 2.5 kg rabbit in two injections 18 and 36 hr before collection of blood. Thirty-six hours is the optimum time for labeling of rRNA (74). One phenylhydrazine injection was given during the labeling period. Ribosomes were prepared by the methods already described. The specific activity of labeling was determined as the amount of trichloroacetic acid (TCA) precipitable radioactivity in a measured amount of gradient purified ribosomes. The highest specific activity obtained was 14,700 cpm/A_{260} unit ribosomes.

The in vivo labeling procedure required great care to be taken in containing the isotope and preventing contamination of the laboratory. For this purpose the rabbit was confined to a cage kept in an absorbant paper-lined hood. After bleeding, the rabbit was sacrificed and properly disposed of. All discarded components of the blood preparation were placed in a waste jar to allow radioactive decay. All glassware and equipment used were thoroughly washed and all working areas were monitored with a Geiger counter.

5. Subunits from Polysomes and Single Ribosomes

Method A. The method used for preparation of subunits

from both polysomes and single ribosomes was that of Blobel and Sabatini (206) with some modification. The method was used identically for preparation of subunits from unlabeled or from [^{32}P]-labeled ribosomes. Three dissociation conditions were utilized in these studies based on considerations discussed in Results. The dissociations were at the following ionic conditions: high salt buffer, 500 mM KCl, 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$; moderate salt buffers, 250 mM KCl, 50 mM Tris-HCl (pH 7.5), 2.5 mM $MgCl_2$; 250 mM KCl 50 mM Tris-HCl (pH 7.5), 1.5 mM $MgCl_2$. These buffers will occasionally be referred to by the following abbreviations: K_{500} T_{50} M_5; K_{250} T_{50} $M_{2.5}$; and K_{250} T_{50} $M_{1.5}$, the subscripts indicating the millimolar concentrations of K^+, Tris, and Mg^+ ions, respectively. Note that the high and moderate ionic strength buffers have similar monovalent to divalent cation ratios.

For the dissociation incubation, gradient purified polysomes were dissolved in ice-cold water (the ribosome pellets contained enough ions to maintain ribosome stability). A small aliquot of the solution was removed to obtain an absorbance measurement of the ribosome concentration, and the solutions could then be adjusted to the desired concentration. However, I have found that the ribosome concentration in either the dissociation mix or during gradient centrifugation did not influence the parameters of subunit dissociation (i.e., degree of dissociation, extent of dimerization

of the subunits, or resolution on the gradients). Therefore, 1-50 A_{260} units could be used per gradient. 1-5 A_{260} units were used for gradients, the purpose of which was solely to analyze the ribosomal subunit profile. For preparations in which the subunits were to be further analyzed for constituents or assayed for activity, 8-12 or more A_{260} units of ribosomes had to be layered per gradient to allow efficient recovery of the separated subunits by subsequent high speed centrifugation.

To 0.20 ml of ribosome solution was added 0.25 ml of a compensating buffer containing twice the ionic concentrations of the desired dissociation conditions. 0.05 ml of $1x10^{-2}M$ puromycin dihydrochloride (Nutritional Biochemicals Corporation, Cleveland, Ohio 44128) was added to give a final puromycin concentration of 1 mM. 20 mM 2-mercaptoethanol was present in the dissociation mix and in the gradients. For large amounts of ribosomes, dissociation mixes of 1.0 ml were made. Incubation of the mix was for 15 minutes on ice followed by 10 minutes at 37°. The mix was then chilled before layering on gradients. Single ribosomes were dissociated by the same procedure except that 0.05 ml water was added in place of puromycin.

Gradients used for separation of the dissociated subunits were 36 ml 10-30% sucrose containing the ionic composition of the dissociation incubation. Centrifugation was at 2° for 5-12

hr at 25,000 rpm in the SW27 rotor. The subunit peaks were collected by pumping the gradient through a flow cell in a spectrophotometer and monitoring the absorbance profile. Care was taken to eliminate leading and trailing edges of the peaks. Subunit fractions were either frozen directly or were concentrated by high speed centrifugation (3 hr 65,000 rpm in the 65 rotor at 2°), followed by rinsing of the pellet with cold water and storage at -76°C.

For experiments in which [^{32}P]-ribosomal subunits from the gradients were to be examined directly (i.e., without concentrating the subunits by high speed centrifugation) in the lysate cell-free protein synthesizing system, it was desirable to obtain 1000 cpm of ribosomal subunits in a gradient volume of 25-100 µl. For this purpose, separation of 16-18 A_{260} units of dissociated ribosomes on 36 ml (1" diam x 3-1/2" tube) gradients or 8-9 A_{260} ribosomes on 16.4 ml (5/8" diam x 4" tube) gradients was found to achieve the desired result. Use of the smaller tube allowed the 40S peak to be collected in a smaller volume of higher ribosome concentration. To obtain the highest specific activity per volume only the central area of the subunit peaks were collected. The collected fractions were assayed for radioactivity and ribosome concentration and were stored at -76° until use for assay in the lysate system.

The ratio of the large subunit peak to that of the small subunit was calculated by tracing the subunit peaks from the

recorded absorbance plot onto tracing paper and determining the weight ratio of the traced and cut peaks.

Method B. Ten A_{260} units of polysomes or single ribosomes were dissociated by the procedure described above in K_{250} T_{50} $M_{1.5}$ and where pelleted from the dissociation incubation mix through a 3 ml cushion of 25% sucrose containing low ionic strength buffer RSB and 1 mM dithiothreitol. The ribosomal pellet was rinsed with a 7% sucrose solution in RSB-1 mM dithiothreitol and was then redissolved in this solution, clarified from any undissolved material by centrifugation at 12,000 x g for 5 minutes and then frozen at -76°C until used for assay. This method does not fractionate the individual subunits but reduces the time ribosomes must be kept in high salt concentrations. Such subunits were used in some assays in the fractionated cell-free protein synthesizing system and were messenger- and initiation factor-dependent.

6. Lysate Ribosomes

Washed reticulocytes are lysed in freshly prepared 0.002 M $MgCl_2$, 0.0001 M EDTA (pH 7.2), 0.001 M dithiothreitol and the lysate is centrifuged at 10,000 x g for 20 minutes. The supernatant is then centrifuged for 50 minutes at 65,000 rpm in the 65 rotor at 2°. The resulting pellet is termed lysate ribosomes and contains the total ribosome population of the lysed cells.

7. Ribonuclease-treated Ribosomes

Mild RNAse treatment of ribosomes was according to the procedure of Crystal et al. (226). Lysate ribosomes or gradient purified polysomes or single ribosomes, 60-100 A_{260} units/ml dissolved in [0.25M sucrose, 0.01M KCl, 0.01M Tris-HCl (pH 7.35), 0.0015M $MgCl_2$, 0.001M dithiothreitol], were incubated at 37° for 10 minutes with one volume of 40 mM Tris-HCl (pH 7.4) containing 0.02 μg/ml ribonuclease A (bovine pancreatic, Worthington). This solution was then chilled to 0° and layered over 3 ml of 25% sucrose in RSB buffer containing 1 mM dithiothreitol in a centrifuge tube for the Beckman type 65 rotor. The tube was filled with RSB lacking sucrose and was centrifuged at 65,000 rpm for 3 hr. The ribosome pellets, which are freed of RNAse by the sedimentation through the sucrose cushion, were redissolved in the original buffer, clarified by centrifugation at 12,000 x g for 5 minutes and frozen at -76° until use for assay. The use of the low ionic strength buffer reported here for sedimentation of ribosomes away from RNAse yielded more active ribosome preparations than were obtained when the high ionic strength buffer of Crystal et al. was used.

8. Enzymatically Dephosphorylated Ribosomes

The following procedures were employed for partial removal of the protein phosphoryl groups of ribosomes. Dephosphorylated ribosomal subunits were prepared by dissociating lysate ribosomes by method B (see above), modified by including 470 μg/ml of

Escherichia coli alkaline phosphatase (orthophosphoric monoester phosphohydrolase E.C. 3.1.3.1; Worthington, type BAPF, electrophoretically purified) in the incubation and extending the incubation at 37° to 15 minutes. The control ribosome preparation was incubated in the absence of the phosphatase, and should be only slightly dephosphorylated by the endogenous ribosomal-bound phosphoprotein phosphatase (see Results). The characteristics of protein phosphoryl group removal is given in experiments reported in Results. The sedimentation of the treated ribosomes through the sucrose cushion removed the phosphatase to an undetectable level as tested by assay of the recovered ribosomes with a synthetic substrate for alkaline phosphatase (227). Resuspension of the ribosome pellet, clarification of the ribosome solution and storage was as described for method B.

Dephosphorylated RNAse-treated ribosomes were prepared by resuspending the once sedimented pellet of RNAse-treated lysate ribosomes in 0.25M KCl, 0.01M Tris-HCl (pH 7.5), 0.01M $MgCl_2$ and incubating 90 A_{260}/ml ribosomes with 470 μg/ml alkaline phosphatase for 15' at 37°. The control RNAse-treated ribosomes were resuspended in the buffer and incubated without phosphatase. The ribosomes were purified from the phosphatase by resedimentation at 65,000 rpm for 3 hr through a 3 ml cushion of 25% sucrose in RSB containing 1 mM dithiothreitol. Resuspension, clarification and storage was as

described above.

C. Preparation of Reticulocyte Lysate for Cell-free Protein Synthesis

The lysate was prepared as described by Adamson et al. (228). One vol. cold water was added to the thrice washed cells and osmotic lysis was achieved by drawing the cells up and down in a Pasteur pipette. The cellular debris was removed by centrifugation at 30,000 x g for 10 minutes. The supernatant was removed and recentrifuged. The supernatant from the second centrifugation served as the lysate. The lysate was immediately sealed in 1 or 2 ml vials and stored in liquid nitrogen without loss of activity for at least two months. A vial of each lysate preparation was tested for protein synthetic activity to assure the suitability of the preparation for further experiments.

D. Assay of Lysate Activity

Protein synthetic activity in the lysate was determined as described by Adamson et al. (89,228). The assay mix contained the following components:

(a) buffer and mono- and divalent cations

ammonium acetate	0.10M
Tris-HCl pH 7.8	0.01M
magnesium acetate	0.002M

(b)[2] energy source

ATP, sodium salt 0.001M

GTP, sodium salt 0.0002M

creatine phosphate (Calbiochem) 0.015M

creatine phosphokinase 60 enzyme units/ml

(c) amino acids (Schwartz Bioresearch Inc., Orangeburg, N.Y.), one-tenth the concentration used by Borsook et al. (229)

L-alanine	4.5 μg/ml
L-arginine	2.1 μg/ml
L-aspartic acid	9.5 μg/ml
L-cysteine	1.3 μg/ml
glycine	10.0 μg/ml
L-glutamine	7.0 μg/ml
L-histidine	9.0 μg/ml
L-isoleucine	1.0 μg/ml
L-lysine	6.5 μg/ml
L-methionine	1.3 μg/ml
L-phenylalanine	6.5 μg/ml
L-proline	4.0 μg/ml
L-serine	9.0 μg/ml

[2] The first three compounds were prepared together and neutralized.

L-threonine	5.0 µg/ml
L-tryptophan	1.5 µg/ml
L-tyrosine	3.8 µg/ml
L-glutamic acid	7.0 µg/ml
L-asparagine	4.0 µg/ml
L-valine	9.0 µg/ml

[^{14}C]-L-leucine, obtained from New England Nuclear Corp., Boston, Mass. in lots ranging in specific activity from 280-311 mc/mM, was used at 1 µc (3.6 µmoles)/0.2 ml assay and did not become rate-limiting during a 30 minute incubation.

(d) reticulocyte lysate 0.4 ml/ml assay

(e) antioxidant

2-mercaptoethanol 0.005M

(f) ± hemin 5.0 x 10^{-5}M, Eastman Kodak Co., Rochester, N.Y. 14650, prepared as described by Adamson et al. (228).

The assay mixtures (0.2 ml) were preincubated for three minutes at 28°C before addition of the [^{14}C]-L-leucine. The incubation was continued and the rate of leucine incorporation was determined by removing 10 µl aliquots at various times. The aliquots were first added to 1 ml of 0.1N NaOH for one-half hour at room temperature to hydrolyze RNA (i.e., [^{14}C]-L-leucyl-tRNA). (The use

of higher concentrations of NaOH or the presence of 1 mg/ml "cold" L-leucine in the hydrolysis mixture did not further lower the counts obtained.) The samples were then adjusted to 5% TCA to precipitate macromolecules, chilled, and filtered with washing onto 0.45μ millipore filters (Millipore Corp., Bedford, Mass. 01730). The filters were glued onto metal planchets, dried and counted in a Nuclear Chicago low background gas flow counter.

Lysate preparations which showed good protein synthetic activity and were responsive to hemin (see Results) were used for studies on ribosomal subunit activity. It was generally found that lysates from reticulocytes of blood having a hematocrit of 20-30% showed good activity.

E. Assay of Ribosomal Subunit Activity in the Lysate System

The assays measured the ability of [^{32}P]-ribosomal subunits to enter various ribosome classes upon incubation in the reticulocyte lysate. The assay mixtures were 1.0 ml containing 0.4 ml lysate and contained the components already described except that 9 μg "cold" L-leucine/ml assay was used instead of [^{14}C]-L-leucine; thus, the subunits were the only radioactive component in the system. Hemin was used in all assays at its optimally stimulating concentration, 5.0×10^{-5}M. The assay mixture was preincubated three minutes at 28° before addition of 500-1000 cpm of the subunit preparation under study. The incubation was continued for the desired period of

time. The assay tubes were then chilled by transfer to ice and by the addition of one volume cold RSB containing enough dilute acetic acid to bring the pH of the assay mix below 6.0. The assays were then adjusted to pH 5.0 as soon as possible. Centrifugation, resuspension and clarification of the pH 5 precipitated ribosomes was as already described.

The clarified solutions (1.0 ml) of ribosomes from the assay mixtures were then layered on sucrose gradients for centrifugation to resolve the desired ribosomal size classes. For example, centrifugation for 20 hr at 15,000 rpm, at 2° on 33 ml isokinetic 15-30% sucrose gradients over a 3 ml 60% sucrose cushion in an SW27 rotor allowed resolution of large polysomes, small polysomes, single ribosomes and both subunits. Ribosomal material was not pelleted through the sucrose cushion as shown by attempts to resuspend radioactivity or material absorbing at 260 nm from the bottom of the tube. The use of these conditions was especially important in studies with the 60S subunit to ensure that radioactivity in the subunit peak could be distinguished from that in the single ribosome peak.

Centrifugation for 3.25 hr at 2° at 25,000 rpm on 29 ml 15-30% linear gradients in the SW25.1 rotor allowed resolution of polysomes in the di- to heptameric size range, and of single ribosomes from the small subunit. In these experiments the gradient

buffer was the low ionic strength RSB unless indicated otherwise for special purposes. The absorbance profile of the gradients was recorded at 260 nm by pumping through the flow cell spectrophotometer as already described, and 25 drop (approx. 1 ml) fractions were collected. Fifty μg of carrier bovine serum albumin was added to the chilled tubes and the samples were adjusted to 5% TCA. Filtering of the samples and determination of radioactivity was as already described. Counting in the low background counter was for at least 10 minutes.

The data is analyzed by plotting the radioactivity in the gradient fractions superimposed on the A_{260} profile of the ribosomes. The radioactivity distribution can be seen by visual inspection of such graphs, and the fraction of added subunits which entered a given ribosome size class was determined quantitatively by totaling the cpms in that gradient region and dividing by the total cpms on the gradient.

Inhibitors of protein synthesis were occasionally utilized in the lysate system as follows. Cycloheximide (Acti-dione; Calbiochem) was used at a concentration of 30 μg/ml (1.1×10^{-4}M). Aurintricarboxylic acid (as ammonium salt, Aluminon; Matheson Coleman and Bell, Norwood, Ohio) was used at a concentration of 2×10^{-4}M (96 μg/ml). Inhibitors were present in the assay from the time of preincubation. The choice of concentrations used was based

on considerations and experiments described in Results.

F. Analysis of Ribosomal Subunit Activity in a Fractionated Reticulocyte Cell-free Protein Synthesizing System

1. General

Crystal et al. (226) have described a fractionated rabbit reticulocyte cell-free system that is useful in demonstrating a messenger function for various purified mRNAs (226,230,231). In this system globin mRNA, tRNA, initiation factors, elongation factors and synthetases, and ribosomes are prepared and added separately. Protein synthetic activity is measured as [^{14}C]-L-leucine incorporation. As described by Crystal et al. (226), RNAse-treated ribosomes are used as a source of messenger-depleted ribosomes for the translation of added mRNA. I have shown that purified subunits can also serve with comparable activity as the ribosome source. In adapting this system for the study of ribosome function, care has been taken in optimizing conditions to maximize the possibility of detecting functional differences between different ribosomal sources. For this purpose mRNA and initiation factors were used at saturating levels, whereas a rate-limiting amount of ribosomal subunits was assayed.

2. Preparation of Components

A detailed account of our preparation and assay methods

has been prepared[3] and the methodology will only be summarized here. Most components were prepared as described by Crystal et al. (226). Reticulocytes were lysed and centrifuged as in the preparation of lysate ribosomes (see above). The supernatant recovered after pelleting of lysate ribosomes was either used for extracting transfer RNA (232) or was recentrifuged for 50 minutes at 65,000 rpm and the resulting "supernatant-2X" was stored in 0.1 ml aliquots in liquid nitrogen for use in the cell-free system. It was the source of elongation factors and of aminoacyl tRNA synthetases. For studies comparing the protein synthetic activity of phosphorylated and dephosphorylated ribosomes, the supernatant was further fractionated without loss of activity by adjusting its pH to 5.0 with 0.1M acetic acid. The precipitate was sedimented at 12,000 x g for 10 minutes and was redissolved in 0.1M Tris-HCl pH 7.2. This preparation was greatly reduced in protein kinase and probably other enzyme activities which occur in the unfractionated supernatant-2X.

Initiation factors ("0.5M KCl wash fraction") were prepared from lysate ribosomes as described by Shafritz et al. (231). Lysate ribosomes were dissolved at a concentration of 150-300 A_{260} units/ml in standard sucrose solution (0.25M sucrose, 0.001M EDTA pH 7.2, 0.001M dithiothreitol) and the solution was then adjusted

[3] A. Krystosek, M. L. Cawthon, and D. Kabat, in preparation.

to 0.5M KCl by adding with stirring 1/7 volume of 4M KCl. After 10 minutes at 0°, the ribosomes were pelleted by centrifugation at 65,000 rpm for 50 minutes in the 65 rotor. The upper 90% of the supernatant ("crude FxI") is slowly adjusted to 70% ammonium sulfate. After 10 minutes at 0° the precipitate was sedimented at 10,000 x g and was then redissolved to one-half of its original volume in 0.100M KCl, 0.010 Tris-HCl (pH 7.3), 0.001M $MgCl_2$, 0.0001M EDTA 0.001 μ dithiothreitol. The resulting FxI was frozen in 0.1 ml aliquots in liquid nitrogen and was stable for at least one year.

Globin mRNA was prepared from sedimented polyribosomes (lysate ribosomes or the ribosomal pellet obtained in the initiation factor preparation). The procedure is our adaptation of the procedure of Aviv and Leder (233) in which polyadenylic acid containing-messenger RNA is purified on oligo(dT)-cellulose columns. Our modification is to detergent solubilize rather than phenol extract the polysomal RNAs; and this procedure results in more active mRNA.[3] The crude polysomes were dissolved at room temperature in 0.5M NaCl which had been adjusted to 0.5% sodium dodecyl sulfate shortly before use by addition of 1/20 volume of a 10% solution. The solution (25 A_{260} units/ml) was layered onto oligo(dT)-cellulose columns prepared by the method of Gilham (234) or type T-2 (Collaborative Research, Waltham, Mass.). After passing the solution through the column at a rate of 1 ml/min, the columns were washed

with 10 column volumes of 0.5M NaCl-0.5% sodium dodecyl sulfate, and 10 column volumes of 0.5M NaCl. The poly(A)-containing fraction was then eluted with water or with 0.01M Tris-HCl (pH 7.4). This fraction was adjusted to 0.2M NaCl and to at least 1 A_{260}/ml by addition of tRNA, and the RNA was then precipitated overnight at -5°C after adding 2 volumes of ethanol. After centrifugation at 10,000 x g for 20 minutes, the RNA was dissolved in 0.01M NaCl and was stored frozen at -70°.

3. Assay Method

The cell-free system contained 20 mM Tris-HCl (pH 7.4), 1 mM dithiothreitol, 4.5 mM $MgCl_2$, 80 mM KCl, 1 mM ATP, 0.2 mM GTP, 3 mM phosphoenolpyruvate, and 0.04 mM of each amino acid except leucine. Each 0.1 ml assay sample also contained 0.16 A_{260} units transfer RNA, 0.2 I.U. pyruvate kinase, 1 µl supernatant 2-X (75 µg protein), 0.04 mM of [^{14}C]-L-leucine (300 millicuries/mM) and an optimal amount of FxI (generally 15 µl containing 65 µg protein) and of globin mRNA (0.1 A_{260}). 0.1 A_{260} units of various ribosome and subunit preparations were assayed as described in the individual experiments. Incubations were preincubated at 37° for 2 min before addition of the ribosomes, and the incubations were continued. Aliquots were removed into 2 ml 10% TCA after various incubation times. The samples were heated at 80° for 15 min to hydrolyze RNA and chilled to 0° for 30 minutes. The precipitates were collected

onto 0.45 μ millipore filters and were assayed for radioactivity in a low background gas flow counter.

G. Other Methods

1. Analysis of Ribosome Profiles

The centrifugation of pH 5 precipitated ribosomes on sucrose gradients provides a convenient method for determining the percentage of ribosomes existing as polyribosomes and single ribosomes in a cell preparation, or for example, for checking the retention of polysomes during an incubation of the lysate system. For such analytical purposes the ribosomes from 0.2-0.3 ml packed cells were centrifuged for 3 hr at 25,000 rpm at 2° on 15-30% sucrose-RSB gradients in the SW27 rotor or 2-1/2 hr at 25,000 rpm in the SW25.1 rotor. The percentage of ribosomes existing as polysomes and single ribosomes was evaluated by visual inspection or more quantitatively by tracing the absorbance profile onto onionskin paper, cutting and weighing the polysome and single ribosome areas and expressing their relative proportion as % of total ribosomal area.

2. Electrophoresis of Ribosomal RNA

Analysis of the rRNA components of the isolated subunits by polyacrylamide gel electrophoresis provided a convenient method for determining the purity of the collected subunit fractions.

Ribosomes or subunits were suspended in [0.5M NaCl,

0.05M EDTA, 0.01M Tris, 0.5% sodium dodecyl sulfate (SDS) (pH 7.4)] and RNA was extracted by the phenol method of Penman (235). The extracted RNA was precipitated from the aqueous phase of the last extraction by the addition of 2 volumes of 95% ethanol. The solution was stored several hours at -20°, after which the RNA was collected by centrifugation at 12,000 x g for 30 minutes. The precipitated RNA was further washed with 90% ethanol, recollected by centrifugation and dried in a vacuum dessicator.

Eight cm, 2.4% acrylamide gels were made in 6mm (I.D.) plastic tubes as follows. 4.0 ml of stock acrylamide-bisacrylamide [15 g acrylamide and 0.75 g N,N'-methylenebisacrylamide in 100 g of solution][4] and 1.25 ml 1.6% ammonium persulfate were diluted to 25 ml with electrophoresis buffer (0.036M Tris, 0.030M NaH_2PO_4, 0.001M EDTA). Twenty µl N,N,N'-tetramethylethylene diamine (Eastman Organic Chemicals, Rochester, N.Y.) was added to catalyze polymerization. The solution was quickly mixed and added to the tubes. 100 µl water was added to flatten the gel menisci. Before use the gels were soaked overnight in electrophoresis buffer containing 0.6% SDS and prerun for 1 hr prior to RNA electrophoresis. Gels were used to analyze 35 µg RNA (=0.4 A_{260}). Extracted RNA was resuspended in electrophoresis buffer containing 0.6% SDS and 5% sucrose and

[4] Acrylamide and bisacrylamide are recrystallized before use (236).

layered on the gels in a volume of about 20 µl. Electrophoresis was at 5 ma/gel for 110 minutes in electrophoresis buffer containing 0.6% SDS. Following electrophoresis the gels were scanned for absorbance at 260 nm in a Gilford gel scanning attachment for the spectrophotometer. The RNA components of subunits were identified by the relation of the mobilities of the RNA peaks to those of the characteristic 18S and 28S RNAs seen when the RNAs of polysomes or single ribosomes were analyzed.

3. [^{32}P]-labeling of reticulocyte ribosomal phosphoproteins was achieved by incubation of intact reticulocytes in a nutrient medium with [^{32}P]-orthophosphate as described by Kabat (160).

4. Determination of protein concentration of components of the fractionated cell-free protein synthesis system was by the method of Lowry et al. (237).

III. Results

A. Preparation of Polysomes, Single Ribosomes and Native Ribosomal Subunits

Figure 2 shows the resolution obtained when pH 5-precipitated ribosomes or the crude reticulocyte lysate are fractionated by size on sucrose gradients. Reticulocyte preparations routinely contained about equal amounts of polysomes and of single ribosomes. The purity of the polysomes and single ribosomes recovered from gradient centrifugation of the crude lysate were analyzed by re-sedimentation of the collected fractions. Figure 3 shows a re-sedimentation of the polysomes. The collected fraction contains polysomes of the di- to hexamer size range, characteristic of reticulocyte polyribosomes synthesizing globin. The small amount of 80S peak seen could be due to a slight contamination of the polysome fraction with single ribosomes and/or a small amount of polysome breakdown during the handling of isolated polysomes for resedimentation.

The purity of isolated single ribosomes was analyzed by resedimentation as shown in Figure 4. The single ribosomes are somewhat contaminated with dimeric ribosomes and with ribosomal subunits. However, this amount of contamination will probably not affect the usefulness of this preparation for two reasons. The dimeric polysomes will not dissociate by the procedure used for

Figure 2. Fractionation of single ribosomes and polysomes on 36 ml 15-30% sucrose gradients containing the low ionic strength buffer RSB. Panel A is pH 5 precipitated ribosomes and panel B is the crude lysate. Centrifugation was for 4 hr at 25,000 rpm at 2° in the SW27 rotor. Sedimentation is to the left and 80S shows that position of sedimentation of single ribosomes. The abscissa in this and subsequent figures is distance sedimented down the tube.

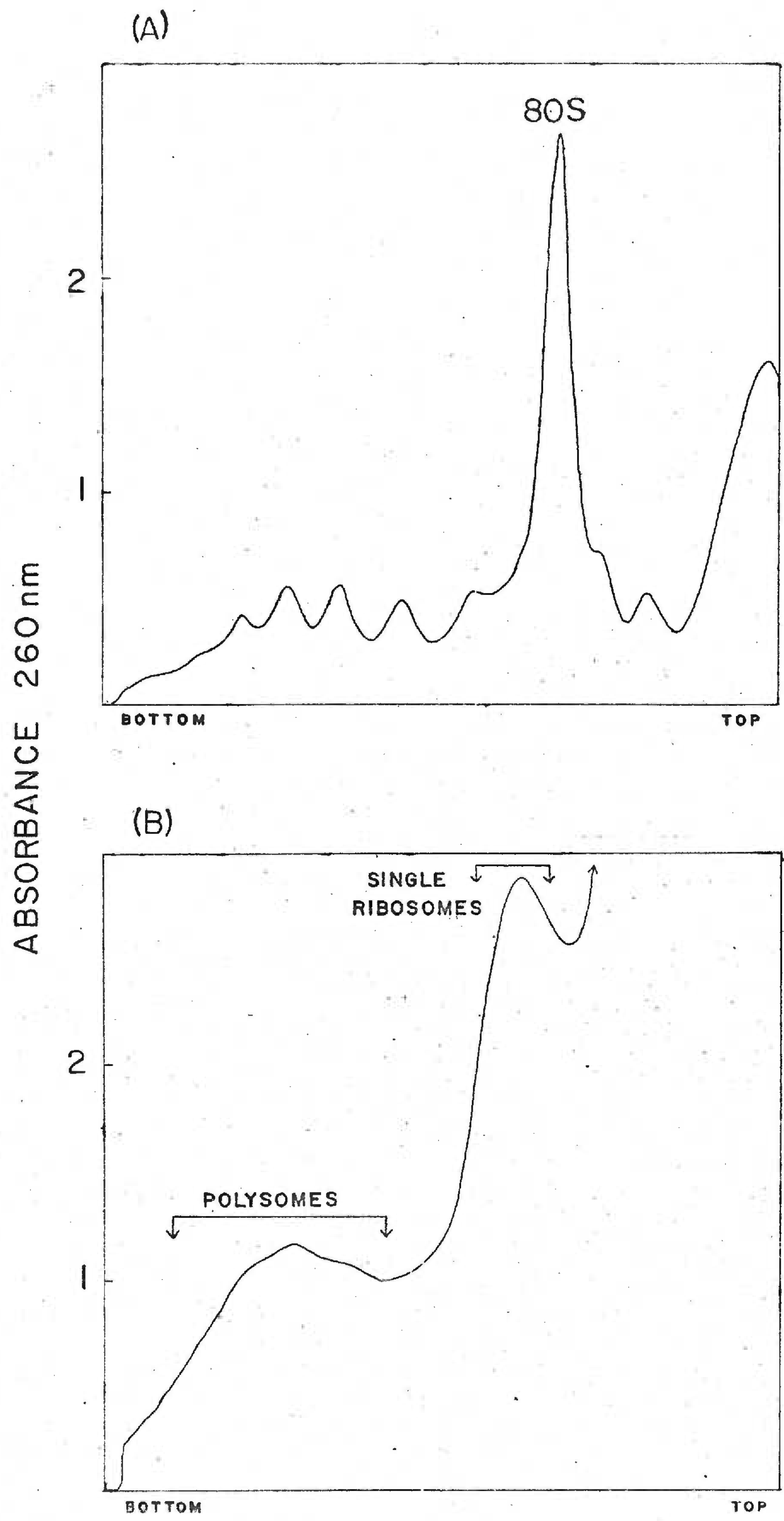
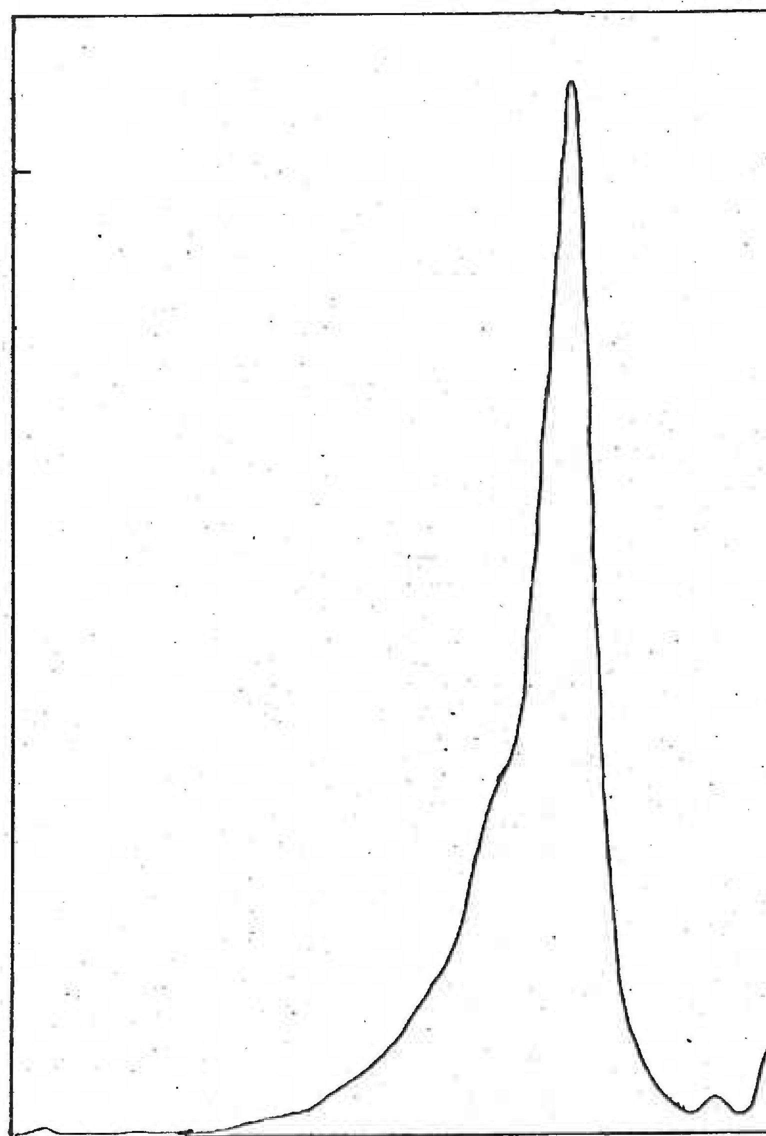


FIGURE 2

Figure 3. Resedimentation of polysomes. Polysomes prepared as shown in Figure 2B were resedimented on a 29 ml 15-30% sucrose-RSB gradient. Centrifugation was at 25,000 rpm at 2° for 3.25 hr in the SW25.1 rotor.

Figure 4. Resedimentation of single ribosomes. Single ribosomes prepared as shown in Figure 2B were resedimented on a 29 ml 15-30% sucrose-RSB gradient. Centrifugation was at 25,000 rpm at 2° for 3.25 hr in the SW25.1 rotor.

ABSORBANCE 260 nm



BOTTOM TOP

FIGURE 4

the preparation of single ribosome-derived subunits; these subunits will therefore not be contaminated by polysomal subunits. As will be shown, the native subunits are not more active in the assay systems employed than the derived subunits; therefore, the activity studies will not be biased by a small amount of contamination by native subunits. Although the single ribosomes could be further purified by a second centrifugation, it was deemed more important in these studies to treat polysomes and single ribosomes by equivalent procedures and to minimize the number of purification steps in order to obtain maximum activity of the final ribosomal products -- subunits for activity assays.

A typical gradient used for the separation of native subunits is shown in Figure 5. The small subunit is well resolved. The large subunit is somewhat contaminated by a small ribosomal subunit species (presumably by contamination with either native small subunit or single ribosomes) as was shown by gel electrophoresis of the RNAs recovered from this peak and by activity assays (see later). The native reticulocyte ribosomal subunits were found, by analysis on other gradients, to represent about 15% of the ribosomal population -- a result consistent with studies on other cell types (39).

B. Preparation of Ribosomal Subunits : Differences between Polysomal- and Single Ribosome-Derived Subunits

Figure 5. Preparation of native ribosomal subunits. pH 5 precipitated ribosomes were layered on a 36 ml 15-30% sucrose-RSB gradient and centrifuged for 24 hr at 15,000 rpm at 2° in the SW27 rotor. Large polysomes were pelleted and the position of sedimentation of single ribosomes, large and small native subunits are indicated by 80S, 60S, and 40S, respectively. The subunit peak areas between the dashed lines were collected.

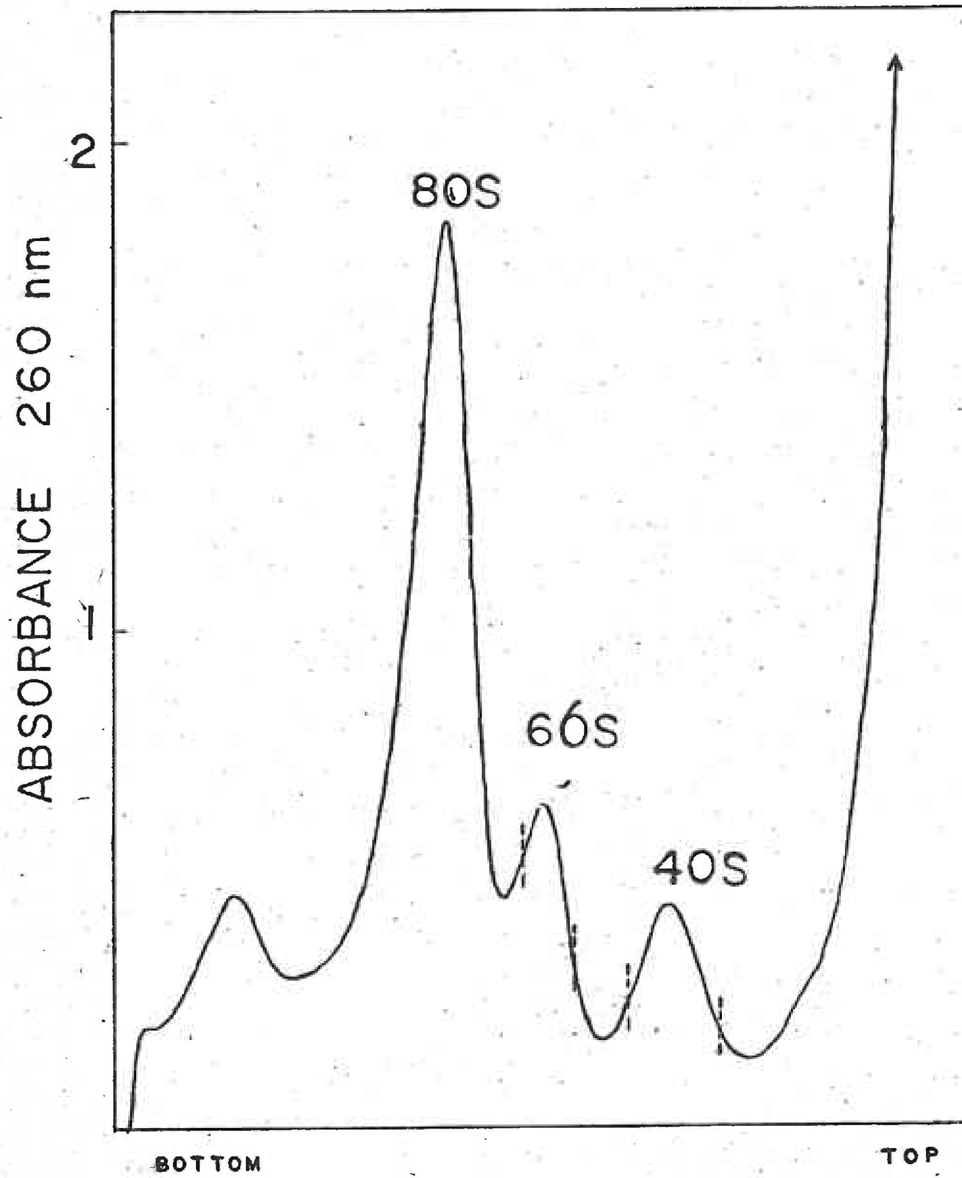
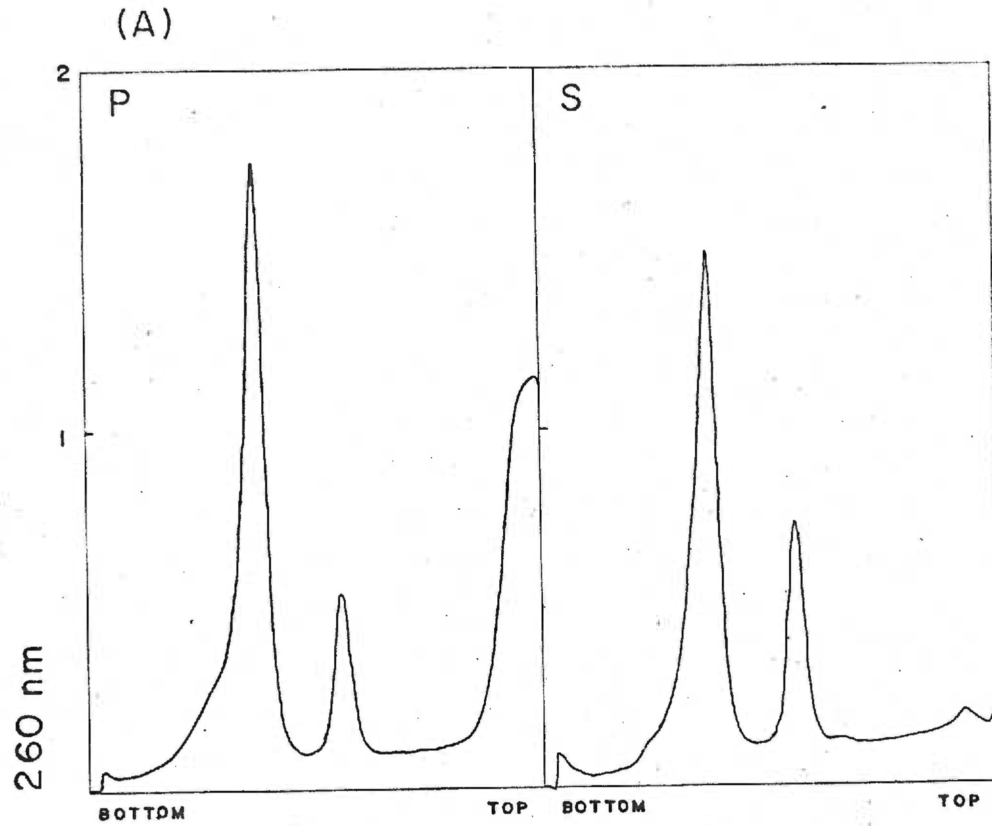


FIGURE 5

In my initial experiments I prepared an unfractionated ribosome pellet from reticulocytes by high speed centrifugation as described by Blobel and Sabatini for hepatic ribosomes (206). Dissociation of these unfractionated ribosomes showed the same ionic parameters of dissociation as reported for hepatic ribosomes. That is, dissociation at K_{500} T_{50} M_2 gave a good absorbance profile of resolved subunits on sucrose gradients. These authors had indicated that their procedure was applicable to other cell types. However, when I applied these conditions to gradient fractionated ribosomes, I found that dissociation of the more purified ribosomes yielded a derivatized form of the large subunit which sedimented with a lowered sedimentation coefficient. Such derivitization was observed by Blobel and Sabatini only at a low Mg^{++} ion concentration (0.5 mM) when crude ribosomes were dissociated. I have, therefore, concluded that the optimum dissociation conditions depend on the degree of purity of the starting ribosomes.

Therefore, the dissociation of polysomes and single ribosomes in high ionic strength buffer (Figure 6A) was performed at a $MgCl_2$ concentration of 5 mM. This value was chosen from an experiment in which ribosomes were dissociated at 500 mM KCl and various $MgCl_2$ concentrations ranging from 1-20 mM. The absorbance profiles (Figure 6A) show that derivatized subunits are not formed at K_{500} T_{50} M_5. At $MgCl_2$ concentrations above 5 mM dissociation was inhibited,

Figure 6. Dissociation of polysomes and single ribosomes at high and moderate ionic strengths. Polysomes (P) and single ribosomes (S) are dissociated in K_{500} T_{50} M_5 (panel A) and K_{250} T_{50} $M_{2.5}$ (panel B) and sedimented on 36 ml 10-30% sucrose gradients containing the ionic conditions of the dissociation incubation. Centrifugation was for 11 hr at 25,000 rpm at 2° in the SW27 rotor. The peak sedimenting about two-thirds the way down the tube is the large subunit and the slower sedimenting peak is the small subunit.



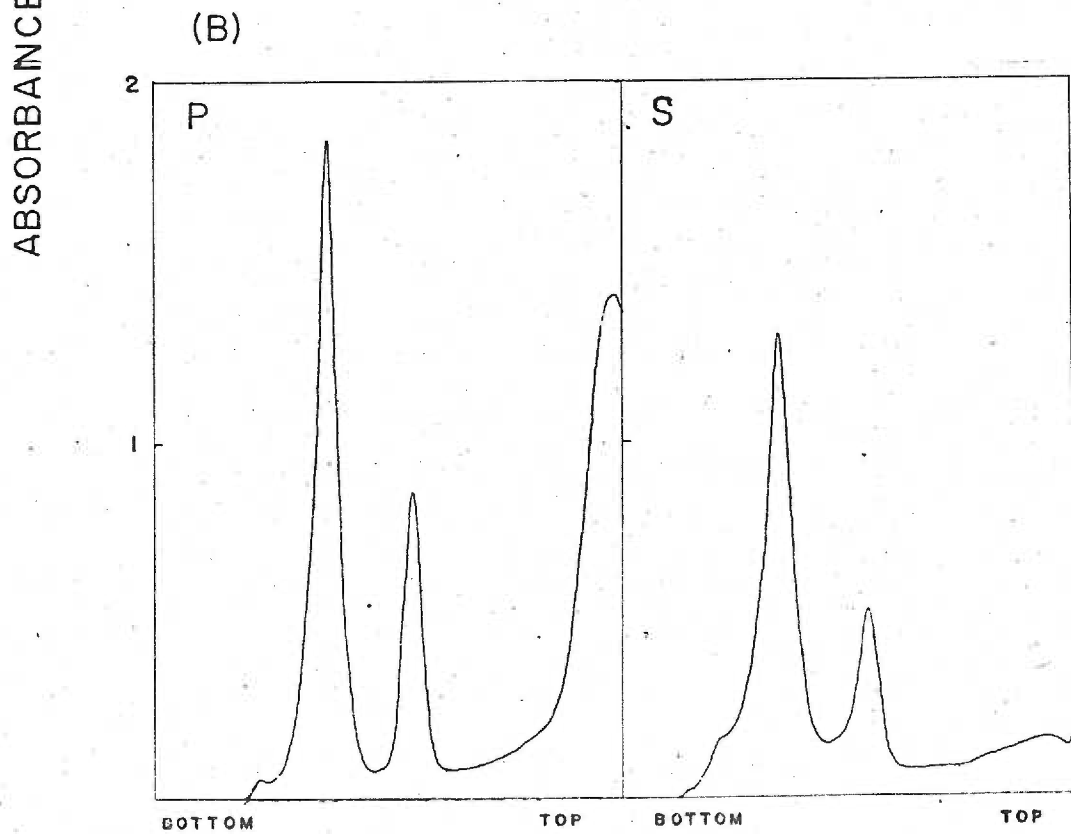


FIGURE 6

whereas derivitized particles occurred at lower $MgCl_2$ concentrations.

The dissociation reaction showed the following characteristics. Dissociation of polysomes required both puromycin treatment and an incubation at 37°. Single ribosomes were readily dissociated without puromycin and often exhibited a small 80S peak when their dissociation products were analyzed on gradients. This peak was diminished if the single ribosomes were puromycin treated and, therefore, represents messenger-containing ribosomes in the single ribosome fraction. Single ribosome-derived subunits are therefore not contaminated with subunits derived from messenger-containing ribosomes (i.e., polysomes or monoribosomes). The extent of dissociation of both polysomes and single ribosomes under best conditions was greater than 85%. High salt dissociated ribosomes largely reassociate when sedimented on a gradient containing a low ionic strength buffer. Other workers have suggested that the gradient centrifugation step itself influences the extent of dissociation (196).

In the experiments reported here I have utilized both high and moderate salt concentrations for ribosome dissociation, for reasons which will be explained later. Examples of the ribosomal subunit sucrose gradient profiles obtained by these dissociation conditions are shown in Figures 6A and 7. Extensive dissociation is achieved at both high and moderate ionic strengths.

Figure 7. Dissociation of ribosomes in moderate ionic strength buffer. Single ribosomes (S) and polysomes (P) are dissociated in K_{250} T_{50} $M_{2.5}$ (panel A) and K_{250} T_{50} $M_{1.5}$ (panel B) and are analyzed on 36 ml 10-30% sucrose gradients containing the same ionic conditions of the dissociation reaction. Centrifugation was for 10 hr at 25,000 rpm at 2° in the SW27 rotor. For subunit preparation for activity assays, narrow central portions of the subunit peaks were collected.

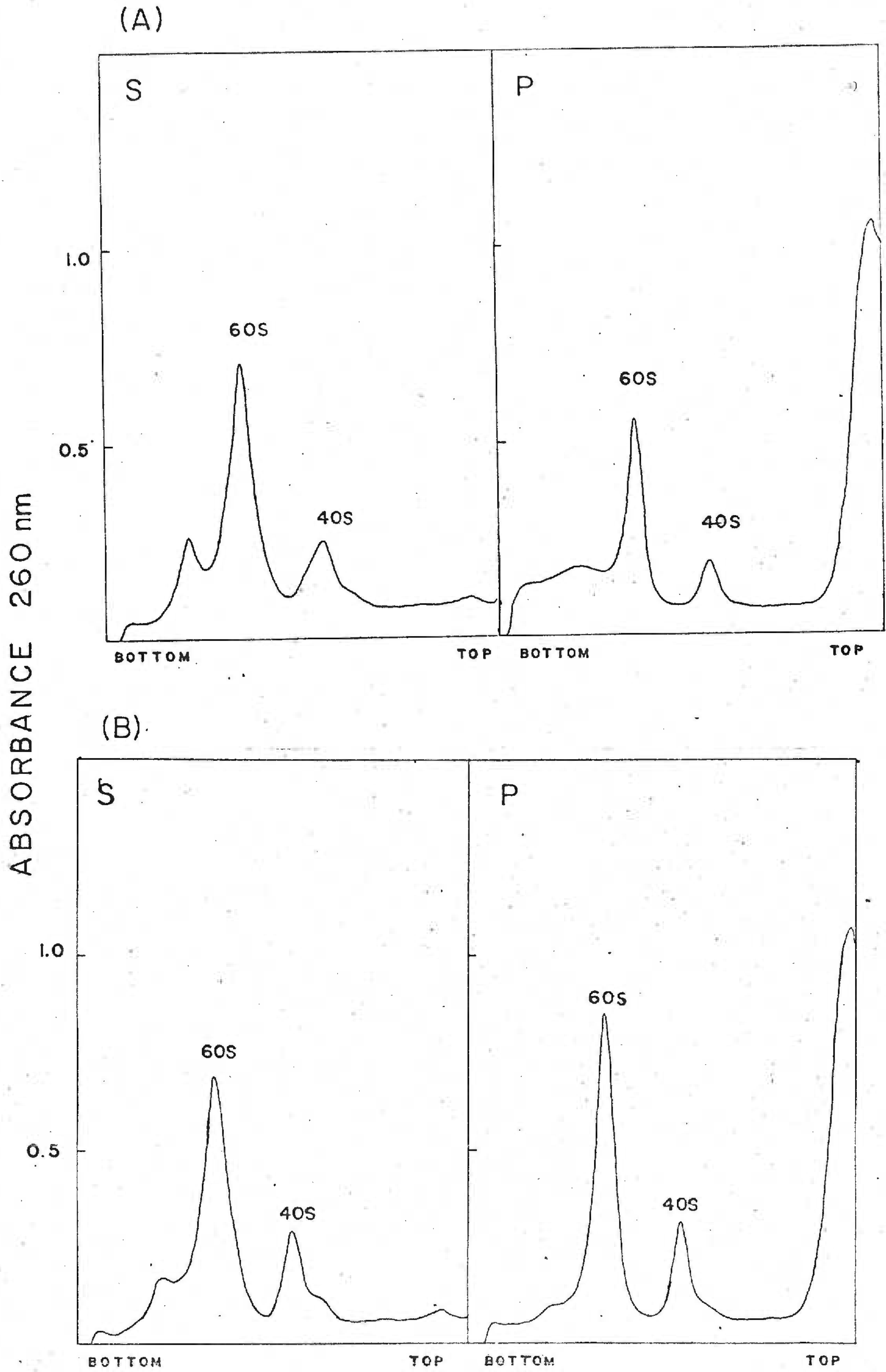


FIGURE 7

Polysome dissociation is only partial at K_{250} T_{50} $M_{2.5}$ but is largely complete at K_{250} T_{50} $M_{1.5}$ (Figure 7B), as well as at K_{500} T_{50} M_5 (Figure 6A). In all cases an underivatized large and small subunit peak could be collected.

Analysis of the 60S:40S peak area ratios gave values of 2.6:1 for both polysomes and single ribosomes for the best preparations. The expected 60S:40S absorbance ratio is 2.25:1 based on their rRNA content. The value I have obtained, therefore, is consistent with the idea that there is a small degree of contamination of the 60S peak with dimers of the small subunit. The extent of this contamination is further analyzed in Figure 8, which shows an electrophoretic analysis of the RNAs of the isolated ribosomal subunit peaks. The derived 60S subunits contain a small amount of RNA which has the same mobility as 18S rRNA, suggesting that there may be a small amount of contamination with small subunits. However, nucleolytic breakdown products of 28S RNA having this same mobility are sometimes observed (226). Therefore, the extent of small subunit contamination cannot be quantitatively measured from this experiment. This RNA analysis illustrates two further points. The RNA moieties of the subunits do not become extensively degraded during the preparative procedures used; furthermore, the RNA profiles of polysomes and single ribosomes are very similar. This latter point suggests that single ribosomes do not accumulate intracellularly

Figure 8. Electrophoresis of the RNA components of ribosomes and ribosome subunits. RNA was extracted and electrophoresed as described in Materials and Methods. Electrophoresis was for 110 minutes at 5 ma per gel. Electrophoretic mobility of RNA in the presence of sodium dodecyl sulfate is according to size. The mobilities of 18S and 28S ribosomal RNA is indicated by the arrows. The ribosomes analyzed are from top to bottom: native 40S subunit, single ribosome-derived 40S subunit, single ribosome-derived 60S subunit, single ribosomes, and polysomes.

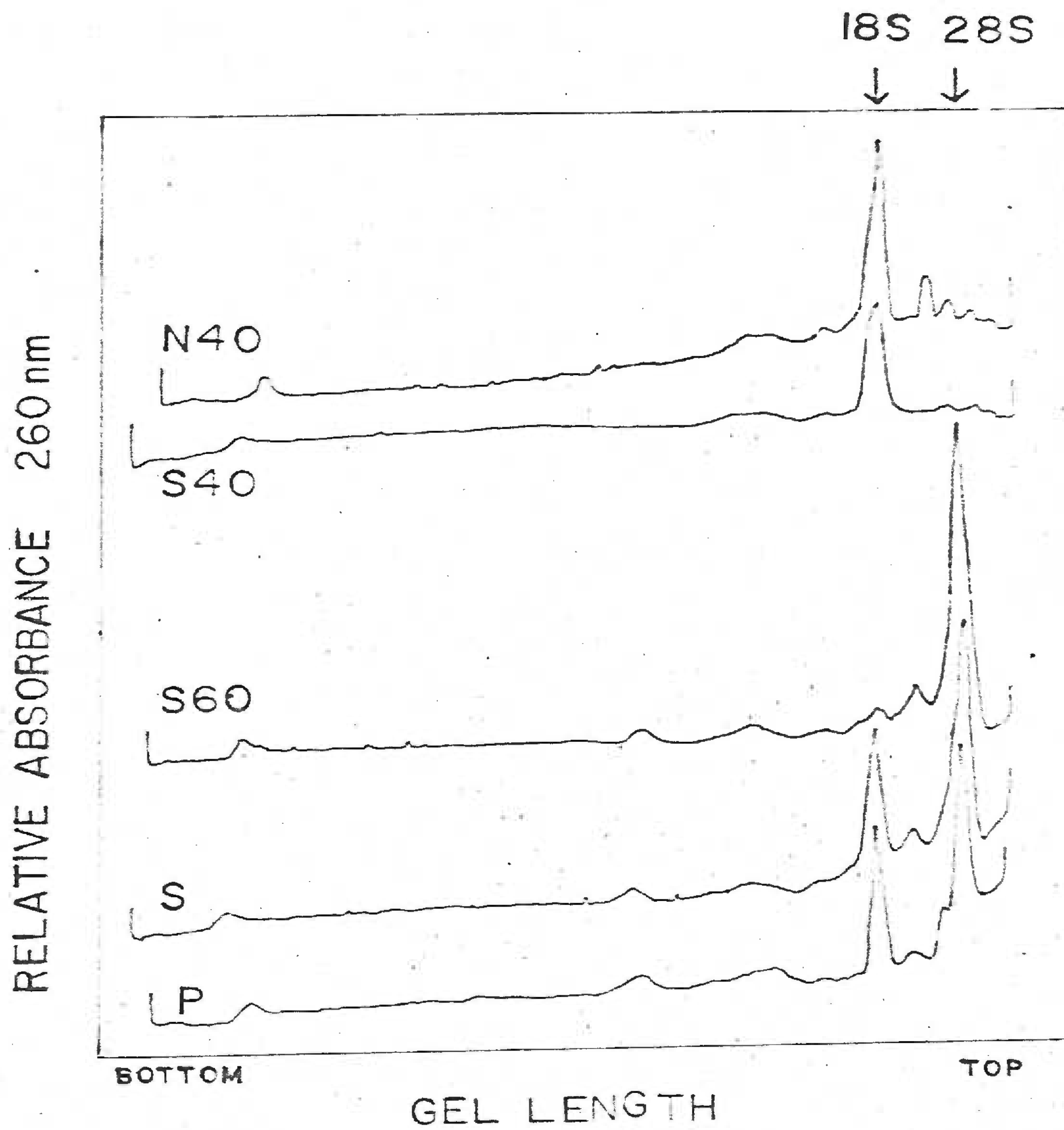


FIGURE 8.

as a degraded ribosomal form during reticulocyte maturation.

The procedure of Blobel and Sabatini employed a gradient centrifugation at 20°, presumably based on reports that centrifugation at higher temperatures avoided small subunit dimerization (196). However, I found in my early experiments that centrifugation at 20° inactivated ribosomal subunits to a large extent so that only 10-20% of the subunits could enter polysomes in the lysate protein synthesizing system. If the centrifugation was at 2° the subunits were much more active, as will be shown. Furthermore, I noted no difference in the extent of dimerization of the small subunit when centrifugation was at 20° or at 2°. I, therefore, routinely used a 2° centrifugation in these studies.

Although an aim of my studies on ribosomal subunits was to learn if structural differences existed between the subunits of polysomes and single ribosomes, the evidence for a difference came about in an unexpected way. It became obvious simply by comparing the sucrose gradient profiles of dissociated ribosomes that the subunits of these two ribosomal classes differed in several respects. First, as shown in Figure 6A, the small subunits derived from polysomes reproducibly tended to dimerize to a greater extent at high ionic strength than those from single ribosomes. This is shown by the relatively low yield of 40S subunits from polysomes and the high 60S:40S peak area ratio (Figure 6A). Consistent with this

conclusion, dissociation of polysomes at high ionic strength often resulted in a 60S subunit peak with a pronounced leading edge (e.g., Fig. 6A) and it is not clear whether this is due to an undissociated ribosomal form or to an aggregated form of small subunit. Dissociation of single ribosomes in the presence of puromycin did not increase the extent of small subunit dimerization. Panel B of Figure 6 shows that this dimerization of the polysomal small subunit is dependent upon ionic strength and/or monovalent to divalent cation ratio, because only minimal dimerization is observed at K_{250} T_{50} $M_{1.5}$. By contrast, analysis of single ribosome dissociation under several ionic conditions (e.g., Figure 6) shows that the small amount of small subunit dimerization is independent of ionic strength. Small subunit dimerization was independent of the amount of ribosomes dissociated and of the centrifugation temperature.

The width of the 60S subunit peak derived from single ribosomes, observed by an absorbance scan of the sucrose gradients, was reproducibly found to be wider than that of the polysomal 60S subunit (e.g., Figure 9). This effect does not seem to be due to contaminating components in the 60S peak (e.g., a leading edge of undissociated single ribosomes or a trailing edge of dimerized subunits) since it can be demonstrated by measurement of the 60S peak at above the one-half peak height. Furthermore, this peak widening is seen on both sides of a line drawn vertically through

Figure 9. Broadness of the large subunit peak of single ribosomes. Single ribosomes (S) and polysomes (P) are dissociated in K_{250} T_{50} $M_{1.5}$ and are sedimented on 36 ml 10-30% sucrose gradients containing the moderate ionic strength buffer. Centrifugation was for 11 hr at 25,000 rpm at 2° in the SW27 rotor.

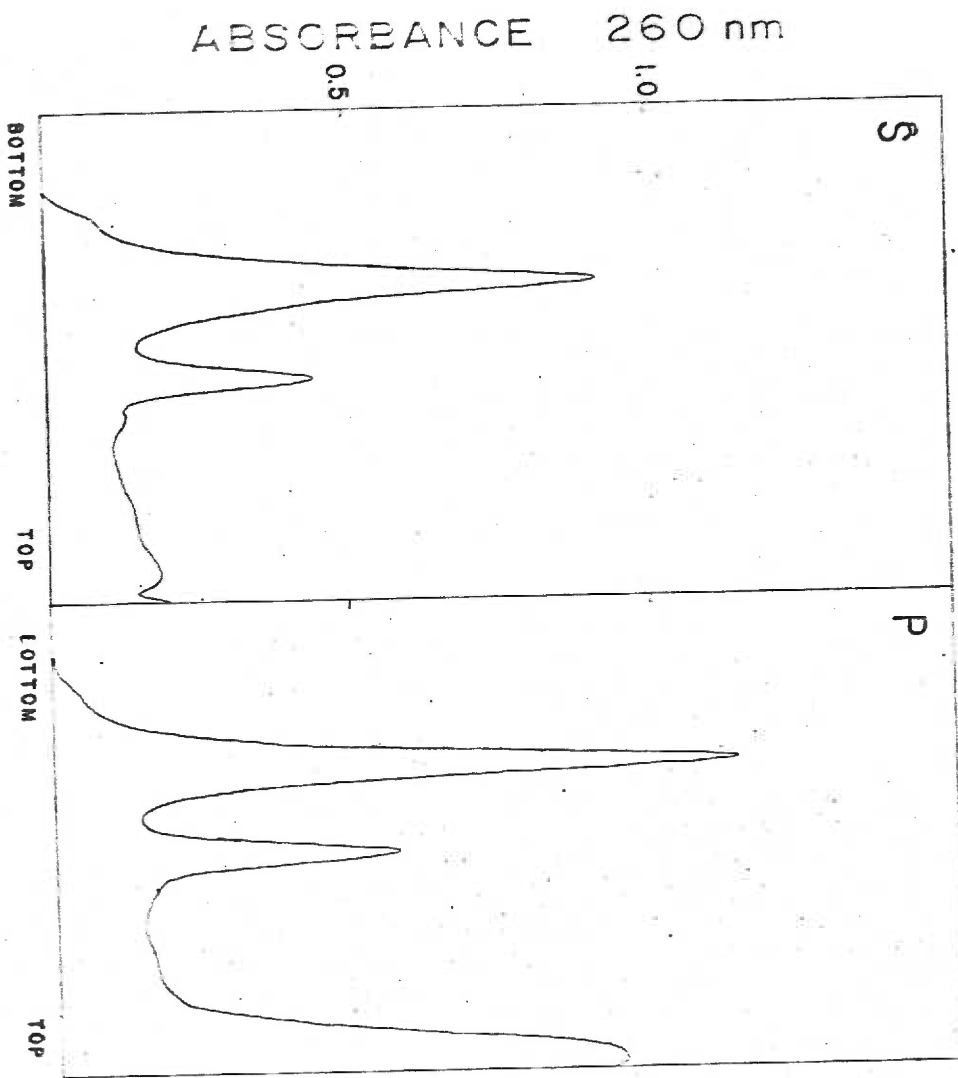


FIGURE 9

the center of the peak. This difference between polysomal and single ribosome-derived 60S subunits is observed independently of the ionic strength used for ribosome dissociation, the gradient sucrose concentration, the distance sedimented down the centrifuge tube, and the temperature of gradient centrifugation. I suggest that the most likely explanation for the broadness of the single ribosome-derived 60S peak is that the peak contains a structurally heterogeneous population of subunits, some of which may be on the verge of unfolding. Such subunits may exhibit only slightly altered sedimentation coefficients and thus sediment as one broad peak.

To test this idea I examined whether the subunits from single ribosomes are more susceptible to forming derivative particles than are those from polysomes. Figure 10 shows this experiment. Single ribosomes and polysomes were dissociated at 500 mM KCl at various $MgCl_2$ concentrations. At 5 mM $MgCl_2$ (which is routinely used for subunit preparation) both ribosome sources dissociate to structurally stable subunits. At 3 mM $MgCl_2$, dissociation of single ribosomes but not of polysomes results in the partial derivatization of the 60S peak to a subparticle, sedimenting ahead of the small subunit at about 50S. At 2 mM $MgCl_2$ this derivatization is extensive for single ribosome derived 60S subunits but is just beginning for the polysomal subunit. This difference in the broadness of the 60S subunit peak and the relative susceptibility of the single ribosome

Figure 10. Derivitization of the large ribosomal subunit at high monovalent:divalent cation ratios. pH 5 precipitated gradient fractionated single ribosomes and polysomes were the starting material for this dissociation experiment. Part A shows the dissociation of single ribosomes at K_{500} T_{50} and $MgCl_2$ concentrations of 5-1 mM as indicated in the individual panels. Part B shows the identical experiment for polysomes. The same amount of ribosomes (three A_{260} units) is dissociated in each case. The dissociated ribosomes are analyzed on 36 ml 10-30% sucrose gradients. Centrifugation was for 10 hr (A) and 11 hr (B) at 25,000 rpm at 2° in the SW27 rotor.

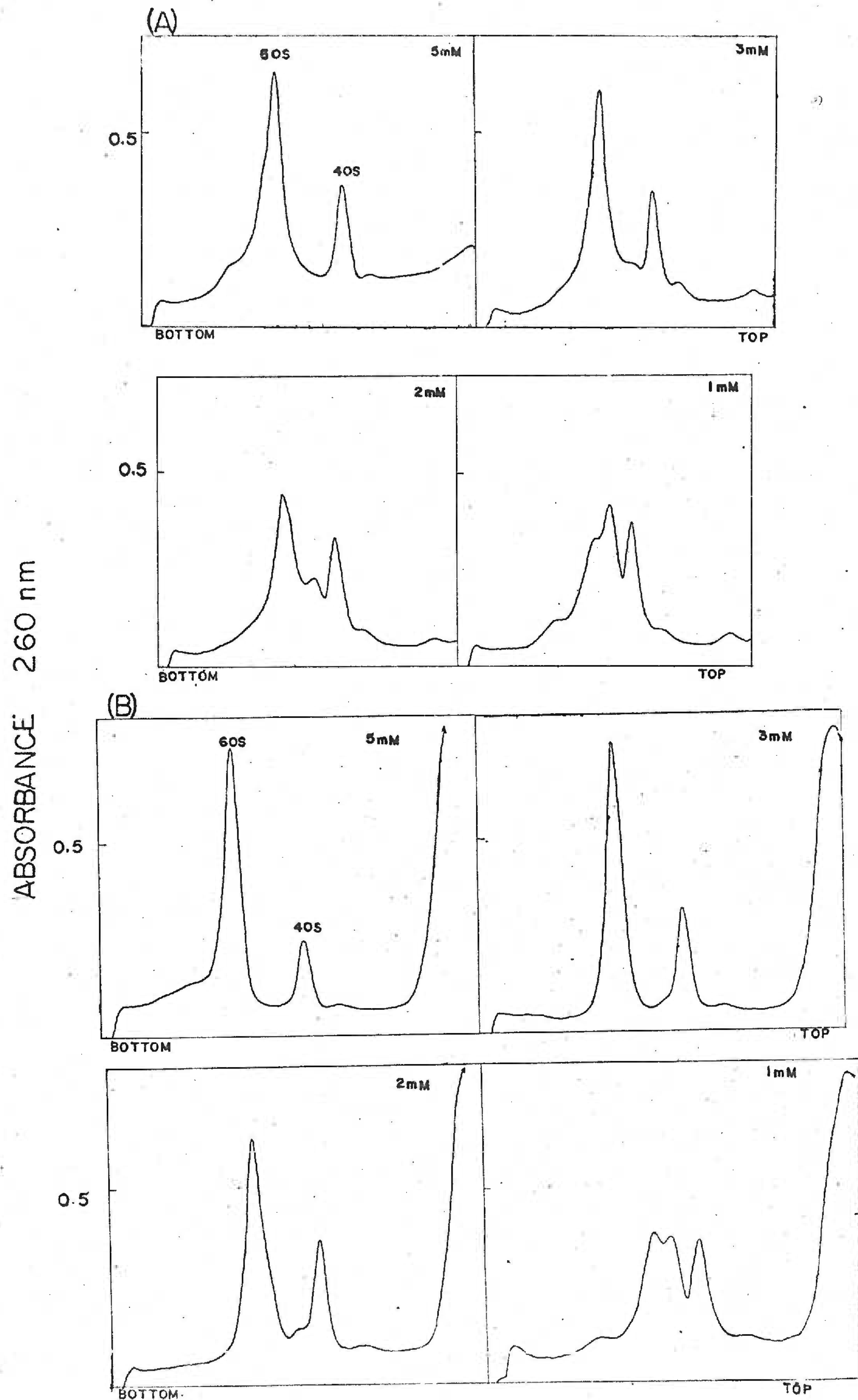


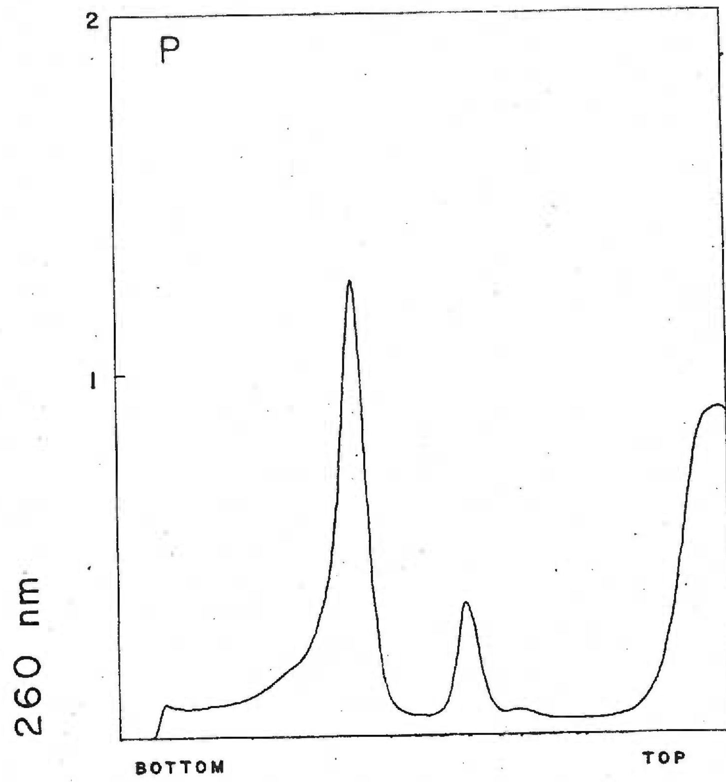
FIGURE 10

derived subunits to derivatize was observed at both moderate and high ionic strength dissociation conditions. Thus, I feel that the broadened peak and enhanced susceptibility to derivatize are related phenomena.

The 40S peak from dissociated single ribosomes did not appear to be broader than the polysomal 40S subunit, although this was difficult to compare since the small subunits from these two ribosome sources dimerized to different extents. However, a derivative particle sedimenting slower than the 40S peak was occasionally observed. As is shown in Figure 11 (see also Figures 9 and 10), this peak is produced to a greater extent from single ribosomes than from polysomes. This peak is more pronounced when dissociation is at high rather than moderate ionic strength. This peak is likely to be derived from the small subunit, since the derivative form of the large subunit never sediments slower than the 40S peak (see Figure 10).

Although I do not understand these dissociation differences between single ribosomes and polysomes, they seem to represent intrinsic structural differences rather than any preparative artifacts. For example, for the derivatization differences shown in Figure 10 pH 5-precipitated gradient purified ribosomes were used to eliminate any contamination present in the usual ribosome preparative method -- gradient centrifugation of the crude lysate.

Figure 11. A derivative peak from single ribosomes. Polysomes (P) and single ribosomes (S) are dissociated at K_{500} T_{50} M_5 and layered on 10-30% sucrose gradients containing the high ionic strength buffer. Centrifugation was for 11 hr at 25,000 rpm at 2° in the SW27 rotor.



ABSORBANCE

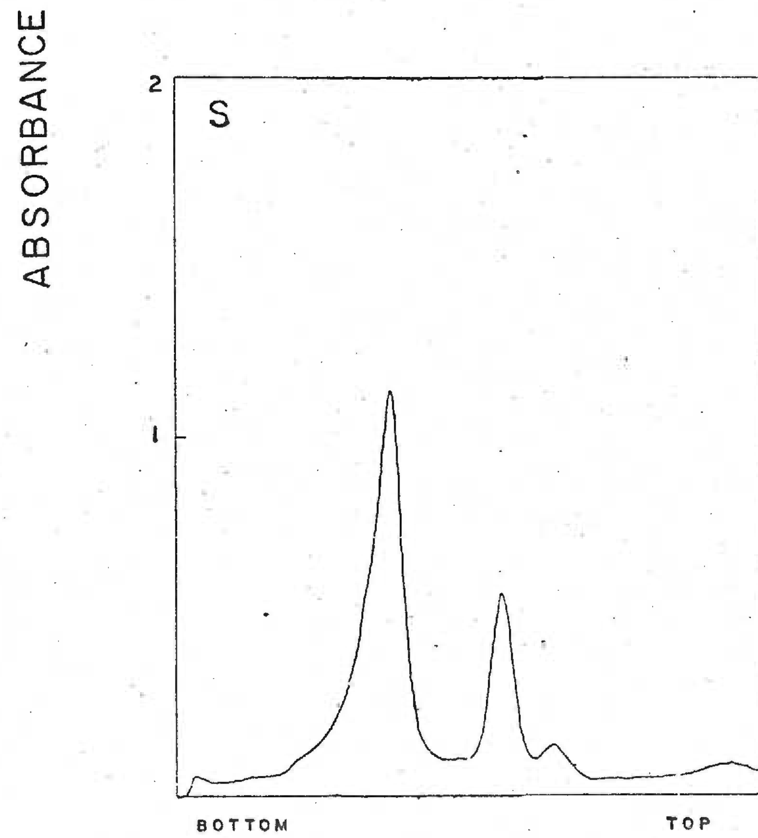


FIGURE 14

Furthermore, these differences were still observed when highly purified polysomes and single ribosomes (obtained by centrifugation on gradients containing a moderate ionic strength buffer [0.25M KCl; 0.01M Tris-HCl, pH 7.4; 0.01M $MgCl_2$] to remove contaminants) were used.

C. Assay of Ribosomal Subunit Activity in the Reticulocyte Lysate Cell-free Protein Synthesis System

1. Characterization of protein synthesis in the lysate system.

Figure 12 shows that the best lysates gave linear protein synthesis for 30 minutes or longer. The maintenance of this linear rate of synthesis is dependent upon the addition of hemin to the lysate. 5×10^{-5}M hemin was found to be optimally stimulating.

A short digression is needed to explain this well known effect of hemin, which is still not understood in molecular detail. The presence of hemin is needed for continuing initiation in the lysate system (238,239). In the absence of hemin the rate of protein synthesis rapidly decreases and polysomes disaggregate. A similar need for hemin is exhibited by intact reticulocytes (240, 241). In the absence of hemin a translation repressor is formed from a pro-inhibitor in the lysate (242,243). The pro-inhibitor form is stabilized by hemin. The inhibitor apparently interferes with the function of an initiation factor (244-247) and in the most

Figure 12. Rate of protein synthesis in the lysate system and the effect of hemin. Two different lysate preparations are incubated with $[^{14}C]$-L-leucine and $\pm$ $5x10^{-5}M$ hemin. Radioactivity incorporation is determined as described in Materials and Methods. The lysates shown are typical of the best preparations. They exhibit a linear rate of protein synthesis which is dependent upon the presence of hemin.

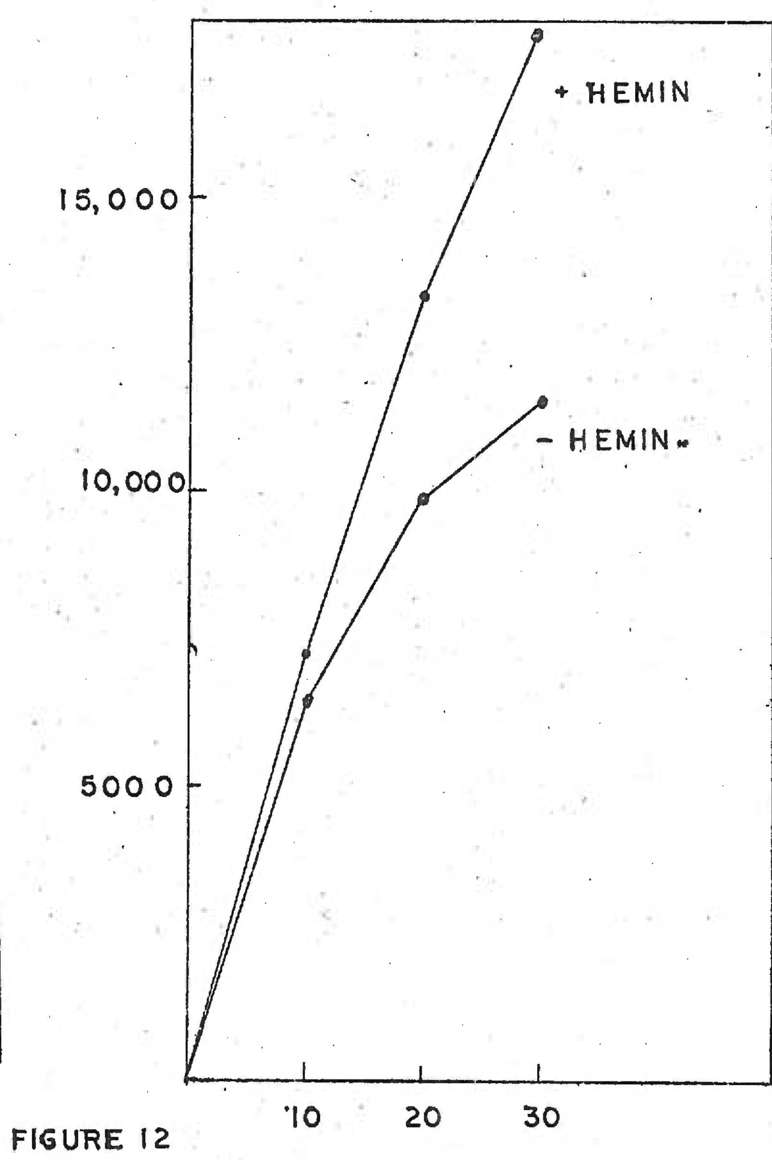
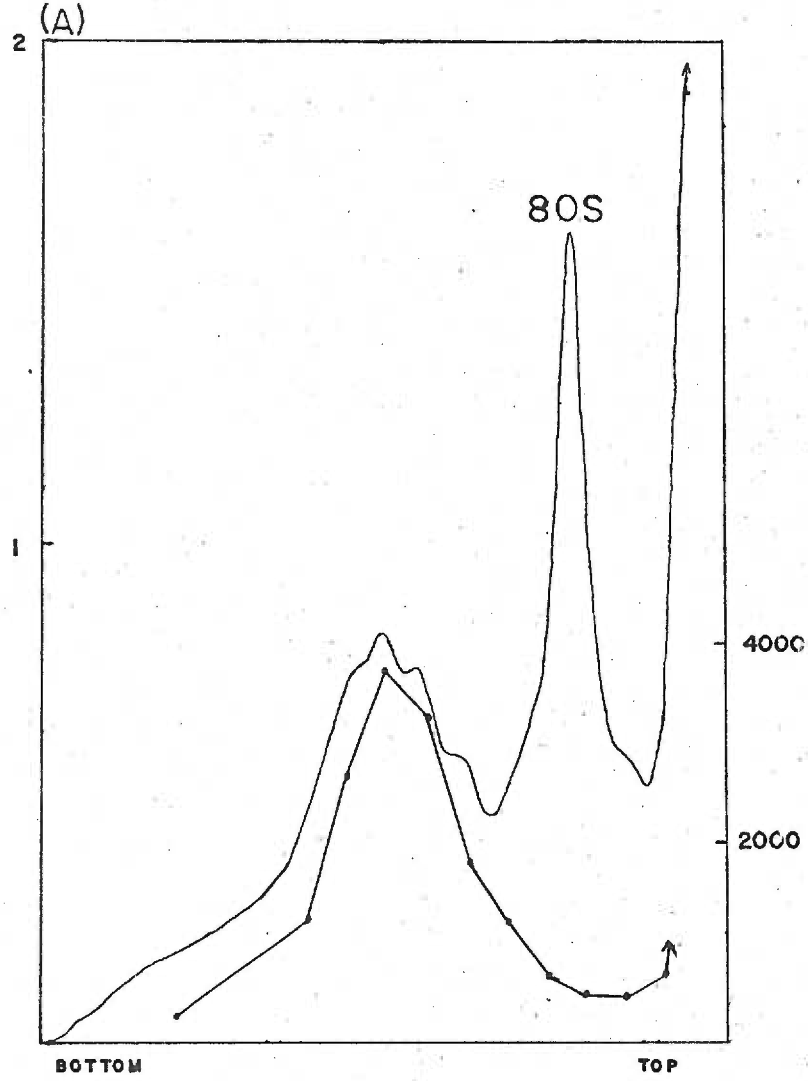


FIGURE 12

recent research this inhibition is localized to an early step in initiation complex formation (63,248,249). While this effect of hemin or reticulocyte protein synthesis was initially thought to be a manifestation of the coordinate regulation of heme and globin syntheses (250), this interpretation is no longer unambiguous, since the effects of hemin and of the inhibitor extend to the synthesis of nonglobin proteins (251-255) and have been demonstrated in protein synthesizing systems from other mammalian cells (247, 251,256).

The absorbance profiles in Figure 13 show that polyribosomes are maintained in the lysate cell-free system for at least 10 minutes during this period of linear synthesis at 28°. The radioactivity profiles suggest that the polysomes rapidly reach a steady state of nascent chain radioactivity and that ribonuclease activity does not cause accumulation of polysomal breakdown products in the 80S peak. The radioactivity profiles also illustrate the inactivity of single ribosomes in amino acid incorporation. By comparison with Figure 12 it can be seen that these steady state polysomes must be continuously releasing globin chains and that efficient ribosome re-initiation of protein synthesis on mRNA occurs in this system. In fact, Adamson et al. (89) showed that the lysate functions at close to the level of the intact cell for at least 15 minutes. However, as will be seen in other figures, earlier polysome

Figure 13. Polysome function in the reticulocyte lysate. 0.5 ml lysate incubations were incubated for 5 minutes (A) and 10 minutes (B) with [^{14}C]-L-leucine. The pH 5 precipitated ribosomes from the incubation mix were sedimented on 16 ml 15-30% sucrose-RSB gradients. Twenty-five drop fractions of interest were collected for radioactivity determination.



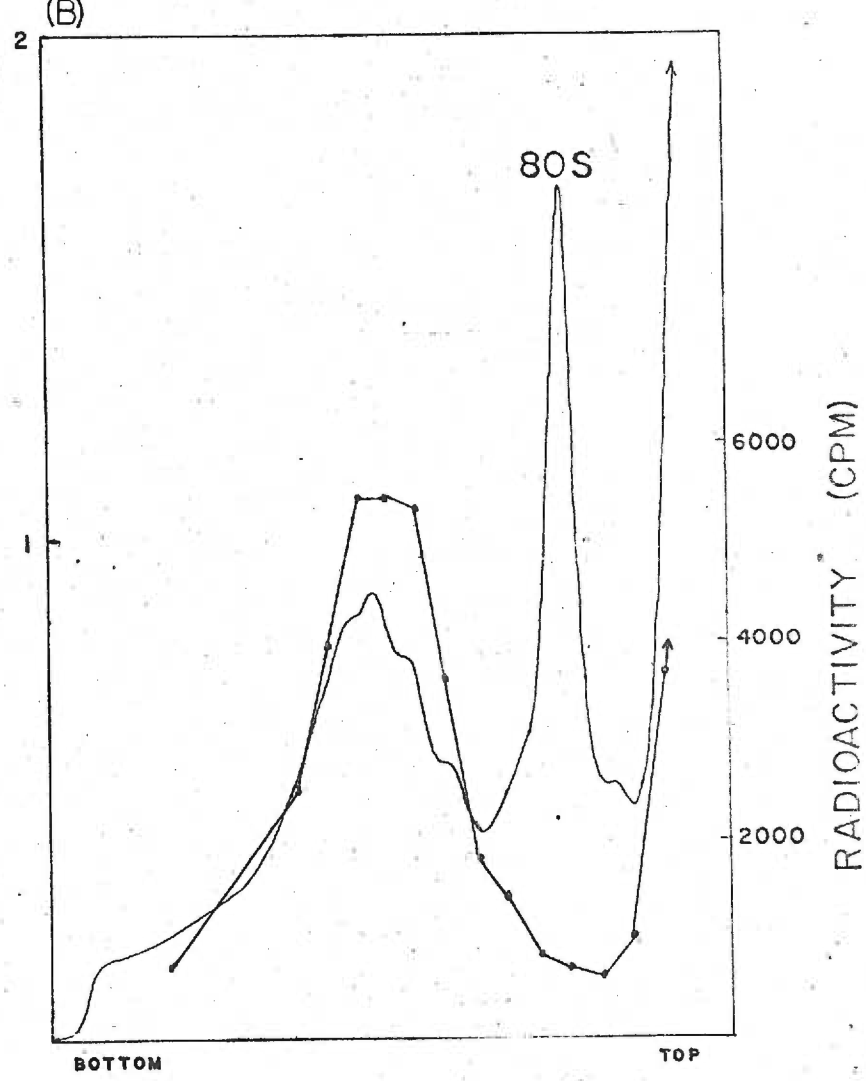


FIGURE 13

rundown is sometimes observed in some lysate preparations. The above characteristics suggest the utility of the lysate system for examining purified ribosomal subunits for activity and efficiency.

Figure 14 shows an analysis of the function of gradient purified [^{32}P]-labeled polysomes and single ribosomes in the lysate system. The added polysomes are seen to equilibrate with the ribosomal subunits since these fractions attain a similar specific radioactivity (cpm/A_{260}). The radioactivity in the 80S peak is less than would be expected if this ribosomal class had also equilibrated with the added radioactive ribosomes, and could be due to [^{32}P]-polysome breakdown or to runoff. It was experiments of this type that allowed Adamson et al. (89) to characterize a subunit-polyribosome cycle of protein synthesis in the reticulocyte lysate and to demonstrate the nonequilibration of single ribosomes with the active ribosomal forms. Added [^{32}P]-single ribosomes are seen to be largely inactive in protein synthesis in the lysate system (Figure 14). However, single ribosomes can be partially recruited into polyribosomes, a process which involves a preliminary dissociation step (257).

2. Rationale of ribosomal subunit assay in the lysate system.

As described, the lysate is highly efficient in protein synthesis -- multiple rounds of translation occurring by the normal

Figure 14. Analysis of polysome and single ribosome function in the lysate system. [^{32}P]-labeled polysomes (P) and [^{32}P]-labeled single ribosomes were incubated for 10 minutes in lysate incubations (1.0 ml). After incubation the ribosomes were obtained from the lysate by pH 5 precipitation. The redissolved ribosomes were layered on a 33 ml 15-30% isokinetic sucrose-RSB gradient over a 3 ml 60% sucrose-RSB cushion. Centrifugation was for 20 hr at 15,000 rpm at 2° in the SW27 rotor. Sedimentation is to the left and large polysomes collect at the sucrose cushion interface; and the absorbance at 260 nm is read manually in the collected fractions of this region. Single ribosomes (80S) sediment about half way down the gradient. The gradients are collected in 25 drop fractions for radioactivity determination by pumping from the bottom, as described in Materials and Methods.

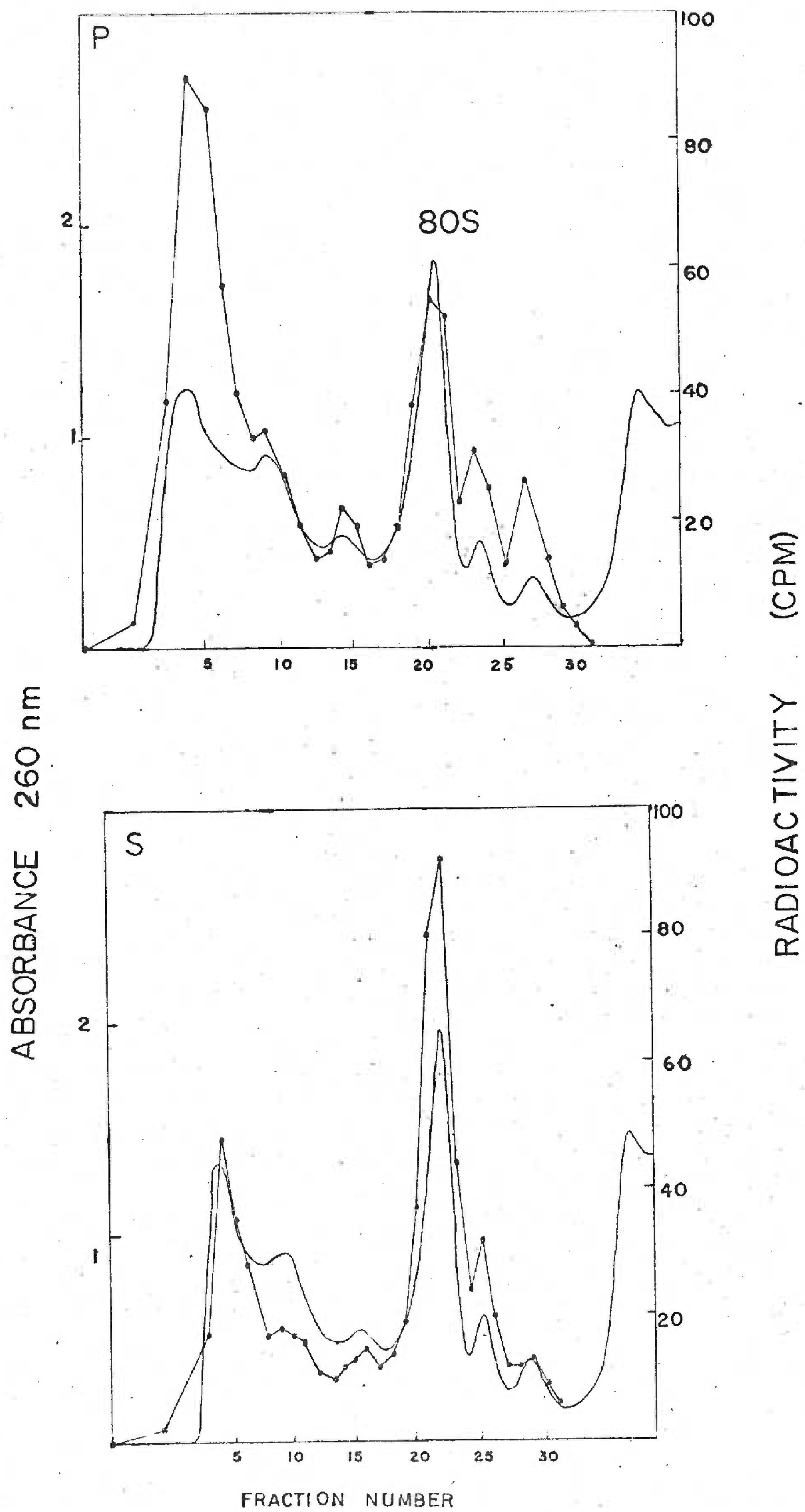


FIGURE 14

subunit-polyribosome cycle of protein synthesis. Subunit activity assay in the lysate system measures the ability of added purified subunits to become involved in this active cycle of protein synthesis. Specifically, the ability of subunits to enter polyribosomes is measured. For this purpose, subunits from various ribosome sources were incubated in the lysate and their entry into different ribosome classes was analyzed as described in Materials and Methods. As will be characterized here, the appearance of radioactivity in polysomes is due to the efficient participation in protein synthesis of the added [^{32}P]-ribosomal subunits.

The amount of subunits added per assay was generally 500-1000 cpm (approx. 0.15-0.34 A_{260} units ribosomes). I have estimated that this amount of added subunits represents about 25% of the native pool of the large or small subunits. Therefore, as an approximation, the added subunits should behave as a tracer amount.

3. Comparison of polysomal- and single ribosome-derived subunits in the lysate system

Figure 15 shows an assay of the activity of 60S subunits from polysomes and from single ribosomes which were prepared by dissociation in high ionic strength buffer. The comparison shows that the large subunits from these two ribosome sources show similar activity in the lysate system. The important point to note in this type of comparison is the percentage of added subunits which enter active polysomes. In both cases approximately 50% of the added

Figure 15. Comparison of polysomal- and single ribosome-derived large subunit activity in the lysate system. The 60S subunit was from the dissociation of [^{32}P]-labeled polysomal (P) and single ribosomes (S) in K_{500} T_{50} M_5. The incubations (1.0 ml) were for 10 minutes and the ribosomes from the incubations were analyzed on sucrose gradients as described in Figure 14 and Materials and Methods.

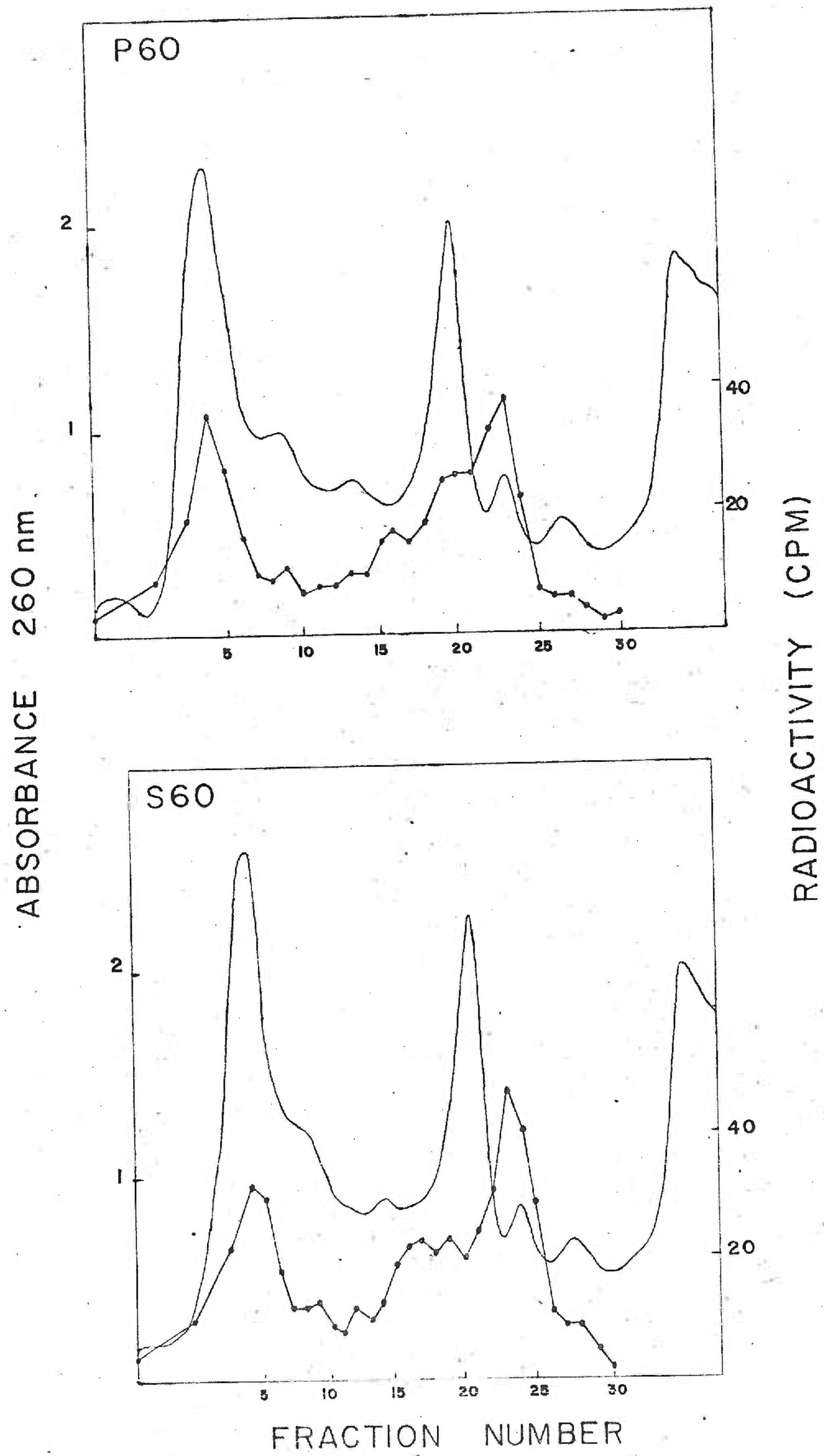


FIGURE 15

subunit radioactivity has entered polysomes during a 10 minute incubation at 28°. For the initial studies, this incubation time was chosen based on the observation of Adamson et al. (89) that added [^{32}P]-polysomes become equilibrated with the pool of native subunits within a 6 minute incubation at 37°. I found that incubations longer than 10 minutes at 28° do not result in a further increase in the amount of subunits entering polysomes. The radioactivity seen in the 80S peak could be due to polysome runoff and/or a fraction of the added subunits entering single ribosomes directly. These possibilities are distinguished in kinetics of entry studies presented next. Evidence that the radioactivity seen in polysomes is due to the participation of the added subunits in protein synthesis is shown in a subsequent section.

The type of experiment just presented cannot eliminate the possibility that single ribosome-derived subunits are initially inactive (or less active than polysomal subunits) in the lysate system but become activated during the incubation and become as active as polysomal-derived subunits. To test this possibility a kinetic study of the entry of subunits from both ribosome sources into the different ribosome classes in the lysate was performed. As seen in Figure 16A-C, large subunits from both ribosome sources enter into polysomes rapidly. Within one minute the amount of subunits from both ribosome sources which entered polysomes was

Figure 16. Kinetic study of the entry of large subunits into different ribosome size classes in the lysate system. Single ribosome-derived (S60) and polysomal-derived (P60) large subunits were prepared by dissociation of [^{32}P]-labeled ribosomes in K_{500} T_{50} M_5. Subunits were incubated in the lysate for one minute (panel A), three minutes (panel B) and 10 minutes (panel C). Ribosomes from the incubations were analyzed on sucrose gradients as described in Figure 14 and in Materials and Methods. Distance sedimented down the tube is shown on the abscissa. 1 ml fractions of interest along the gradient were collected and analyzed for radioactivity.

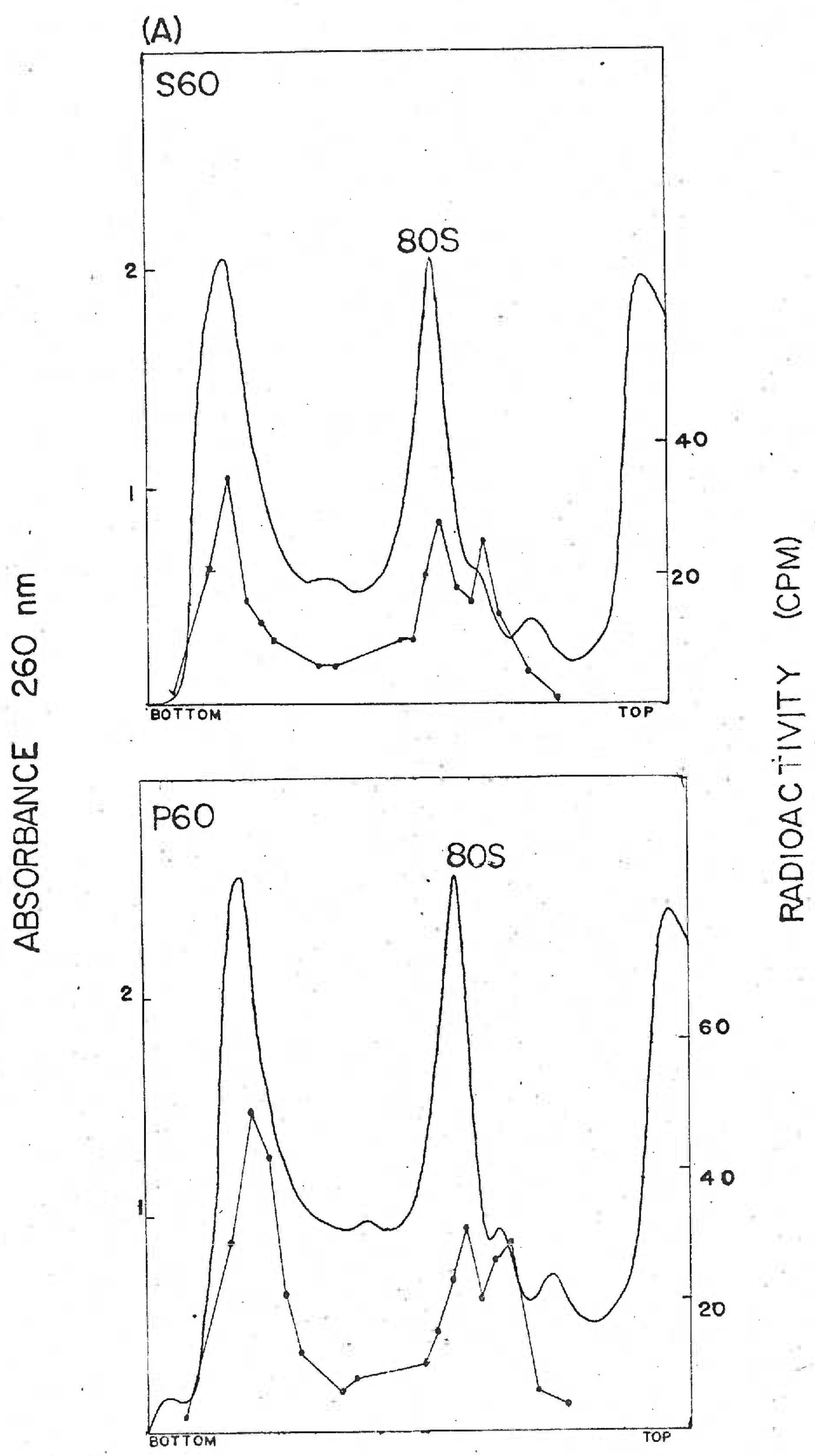
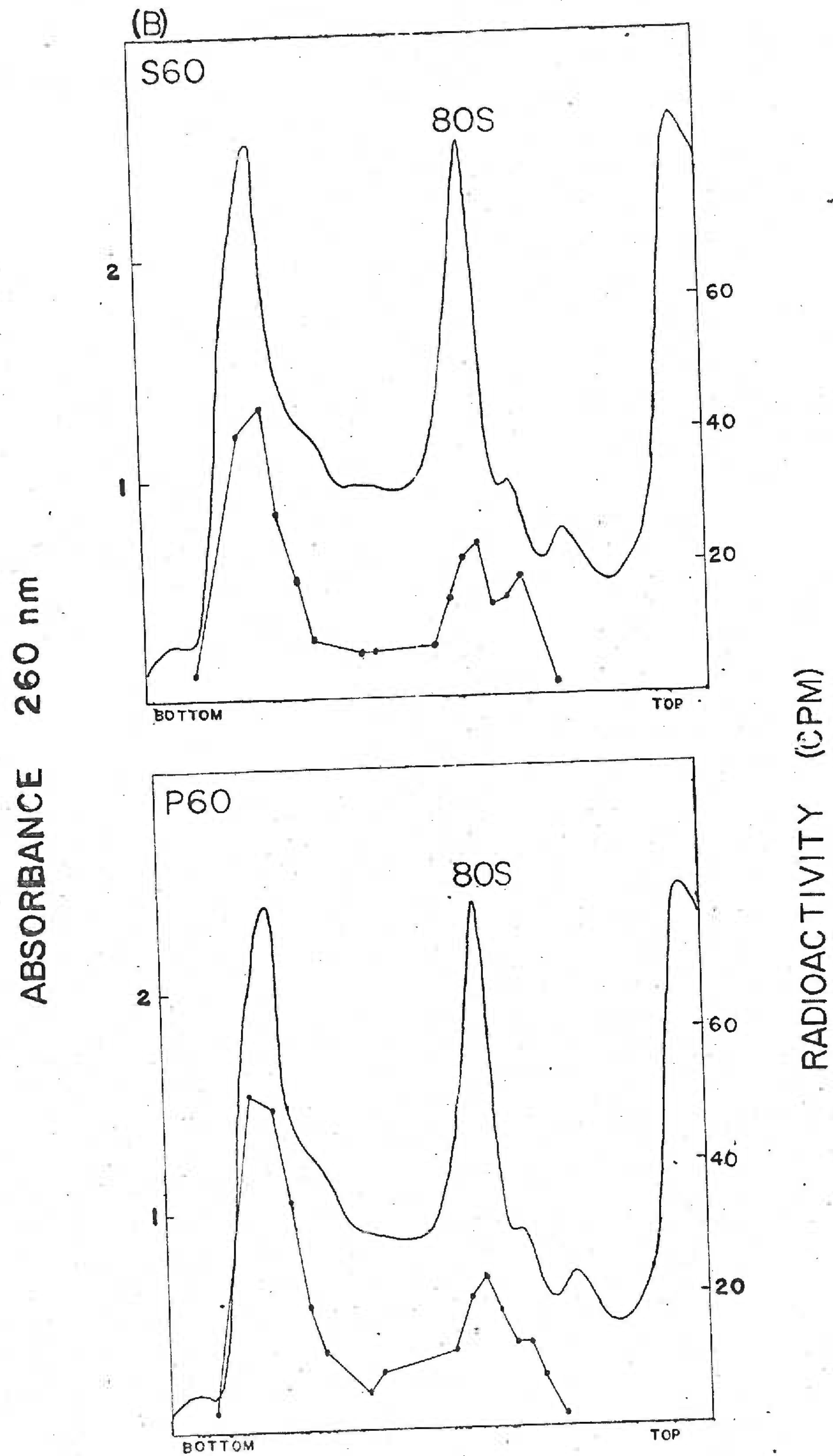
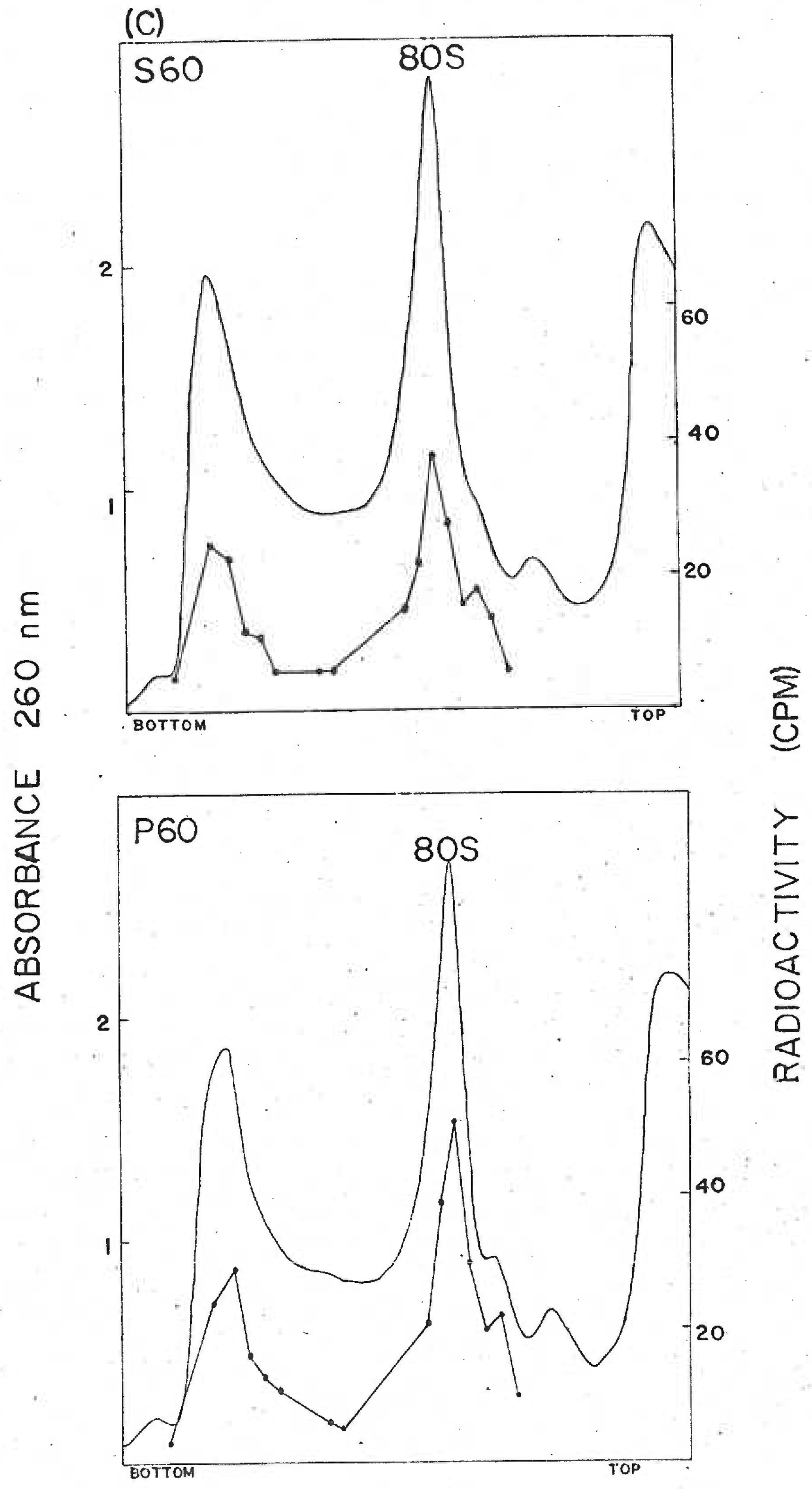


FIGURE 16



(B)
S60
80S
2
1
60
40
20
BOTTOM
TOP
P60
80S
2
1
60
40
20
BOTTOM
TOP
ABSORBANCE 260 nm
RADIOACTIVITY (CPM)


(C)
S60
80S
2
1
60
40
20
BOTTOM
TOP
P60
80S
2
1
60
40
20
BOTTOM
TOP
ABSORBANCE 260 nm
RADIOACTIVITY (CPM)

similar, but perhaps quantitatively greater for the polysomal-derived subunit. By three minutes the maximum amount of subunits had entered polysomes and this amount was similar (greater than 50% of the added subunits) for both ribosome sources. Further interpretations of this experiment are given after presentation of a similar experiment with the 40S subunits.

Figure 17 shows an experiment in which the kinetics of entry of the small subunit from polysomes and from single ribosomes into different ribosome classes in the lysate are compared. A small amount of subunits from each ribosome source enters polysomes by one minute and this amount increases and is maximal by three minutes. The rate of entry and the maximum amount of entry into polysomes seems similar for both ribosomal sources. The small subunits from both ribosome sources showed less radioactivity in polysomes at one minute of incubation than was seen in the experiments with the large subunits. This slower entry of small subunits may be related to a need to generate intermediate initiation forms of the small subunits before they become polysomal-bound. For example, as discussed in the Introduction, initiating small subunits contain bound initiation factors and initiator-tRNA (59-63). The generation of an initiating subunit from added salt dissociated small subunits is presumably similar to the mechanism by which normal small subunits, terminating synthesis on a polysome, are processed

Figure 17. Kinetic study of the entry of small subunits into different ribosome size classes in the lysate system. 40S subunits were prepared from [^{32}P]-labeled single ribosomes and polysomes dissociated in K_{500} T_{50} M_5. These subunits were from the same dissociation reaction as the large subunits assayed in Figure 16. Small subunits were incubated for 1, 3 and 10 minutes in the lysate system and ribosomes from the incubations were analyzed on 29 ml 15-30% linear sucrose-RSB gradients. Centrifugation was for 3.25 hr at 25,000 rpm at 2° in the SW25.1 rotor. Gradient fractions were collected and analyzed for radioactivity as in the previous figures.

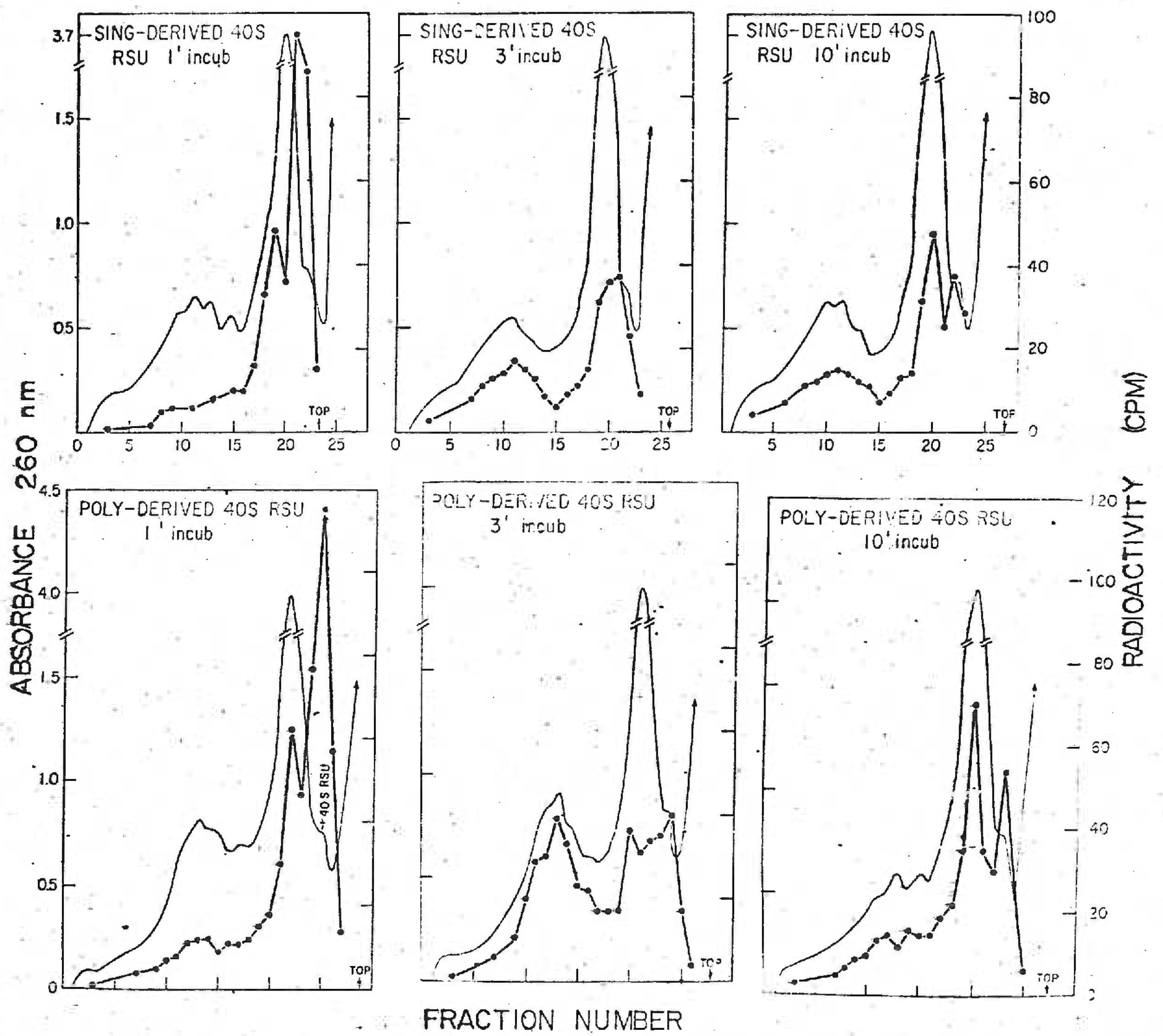


FIGURE 17

to reinitiate (91). By contrast, large subunits do not require activation before initiation.

In the kinetic studies with the small and large subunits a small proportion (20-30%) of the added subunits immediately (within one minute) entered the pool of single ribosomes. This initial entry into the inactive pool occurs without the subunits participating in a round of translation. The proportion of large or small subunits initially entering single ribosomes was similar for subunits derived from single ribosomes and from polyribosomes. In other words, there is no preferential tendency for subunits derived from single ribosomes to re-enter this inactive pool. Further evidence bearing on the nature of the subunits immediately entering single ribosomes is presented later. Besides the subunits initially entering single ribosomes, additional radioactivity is seen in the 80S peak at 10 minutes of incubation (Figures 16 and 17). This is apparently due to polysomal runoff during the incubation and is not limited to those polysomes containing radioactive subunits, since the total polysomal population, as evidenced by the absorbance profile, shows noticeable runoff. Thus, the quantity of active subunits in a preparation as judged by entry into polysomes during a 10 minute incubation (about 50%, e.g., Figure 15) provides a minimum estimate of the fraction of added subunits which has participated in protein synthesis.

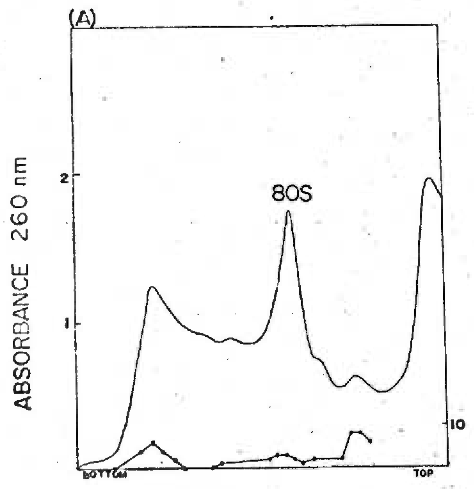
The kinetic studies illustrate a further important point about subunit function in the lysate system. As seen in Figures 16 and 17, and found also in other experiments, the size distribution of radioactive polysomes is identical at all times to the size distribution of unlabeled polysomes which are responsible for the absorbance at 260 nm. If the radioactive subunits were inactive or sluggish in protein synthesis after initiating on polysomal structures, the radioactivity distribution would not coincide with the absorbance profile. I, therefore, conclude that radioactive subunits in polysomes are as efficient in protein synthetic rate as are the nonradioactive endogenous ones. This conclusion is true regardless of whether the large or small subunit is assayed or whether the subunits were from polysomes or inactive single ribosomes.

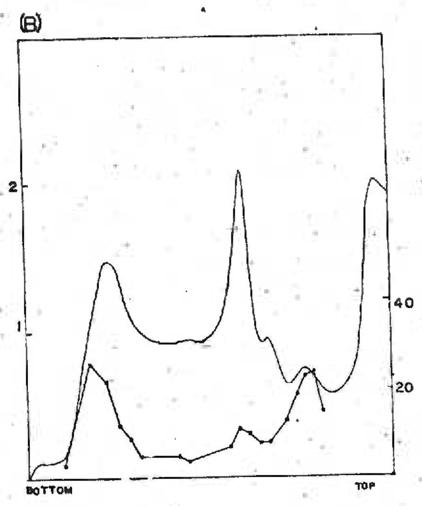
A series of experiments was designed to further investigate the nature of the subunits which immediately enter single ribosomes when incubated in the lysate. According to the model of Kaempfer (66,67) subunits will form single ribosomes upon termination of synthesis on polysomes if they interact with a complementary subunit before binding an initiation factor. Lubsen and Davis (86) have further reported the presence of distinct dissociation factors on both native reticulocyte ribosomal subunits. Thus, a random fraction of added subunits in the lysate assay system could initially enter single ribosomes by this mechanism; if this were the case, it

would be possible that such subunits were not inherently inactive when added to the lysate, but rather entered the inactive pool as a chance event.

This model predicts that the proportion of subunits immediately entering single ribosomes would depend on the amount of subunits added. For example, added subunits in excess of available initiation factors would spontaneously form single ribosomes. I, therefore, tested this model by incubating different amounts of subunits in the lysate and analyzing the proportion of subunits that entered single ribosomes. Figure 18 shows such an analysis for the small subunit. The proportion of subunits entering single ribosomes and polysomes is independent of the amount assayed. A similar experiment with the identical result is shown for the large subunit in Figure 19. These data do not support the model discussed, but rather suggest that a constant proportion of the purified subunits enter single ribosomes. As already described (Figures 16 and 17), this fraction of inactive subunits is similar for each subunit regardless of whether prepared from polysomes or single ribosomes. This fraction of inactive subunits is presumably different from the inactive fraction of subunits which still sediment in the subunit region of the gradients after incubation in the lysate and have apparently not participated in either protein synthesis or single ribosome formation.

Figure 18. Analysis of small subunit activity in the lysate system at three amounts of added subunits. The 40S subunit was from [^{32}P]-labeled single ribosomes dissociated in K_{500} T_{50} M_5 (same preparation as used in the experiment of Figure 17). The incubation in the lysate was for three minutes. Panel A shows the assay of a barely detectable amount of subunits. Panel B shows the normal amount of assay radioactivity. Panel C shows the assay of a larger than normal assay amount of radioactivity. Sucrose gradient analysis of the ribosomes from the incubations was as described in Figure 14 and Materials and Methods. Distance sedimented down the tube is shown on the abscissa. 1 ml fractions of interest along the gradient were collected and analyzed for radioactivity.





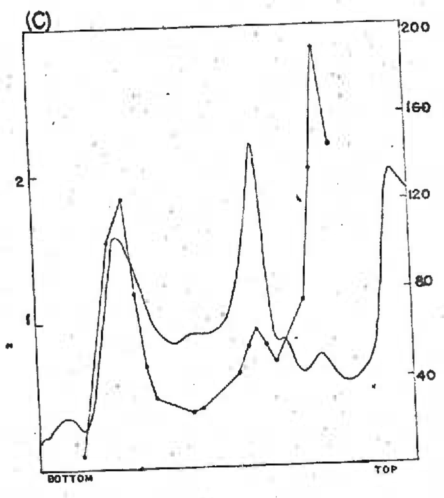
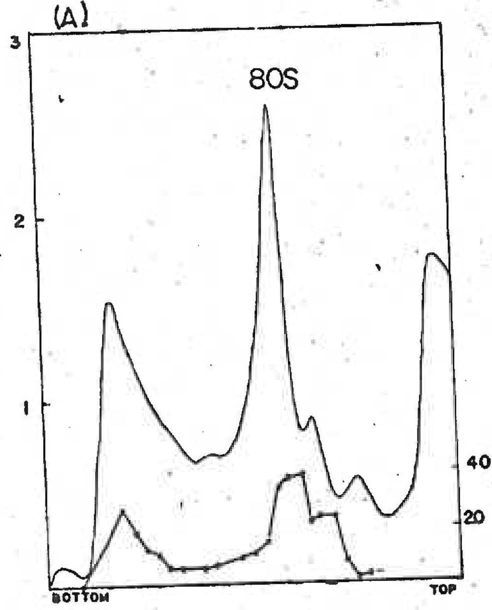
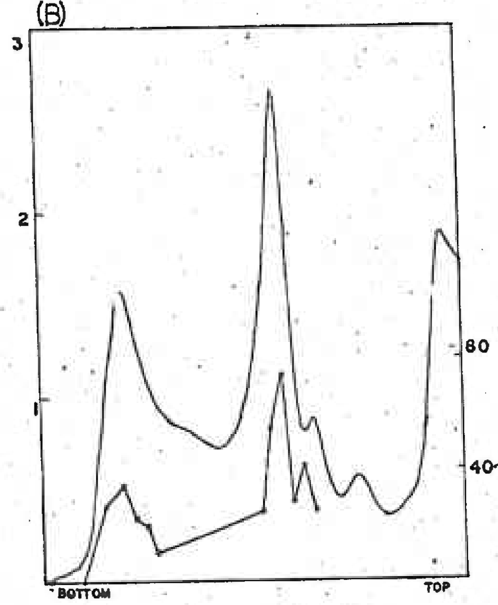


FIGURE 18

Figure 19. Analysis of large subunit activity in the lysate system at three amounts of added subunits. The 60S subunit was from [^{32}P]-labeled polysomes dissociated in K_{500} T_{50} M_5 (same preparation as used in Figure 16). The incubations in the lysate were for 10 minutes. Panel B shows an assay with the normal amount of radioactive subunit. Panel A shows an assay with one-half this amount and Panel C shows an assay with twice this amount of radioactivity. Sucrose gradient analysis of the ribosomes from the incubations was as described in Figure 14 and Materials and Methods. Distance sedimented down the tube is shown on the abscissa. 1 ml fractions of interest along the gradient were collected and analyzed for radioactivity.





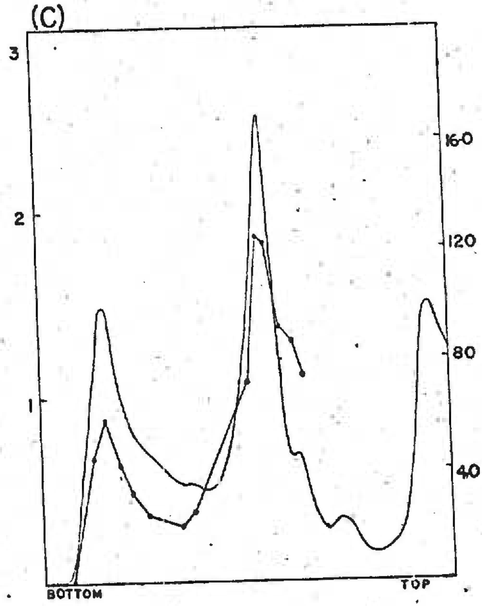
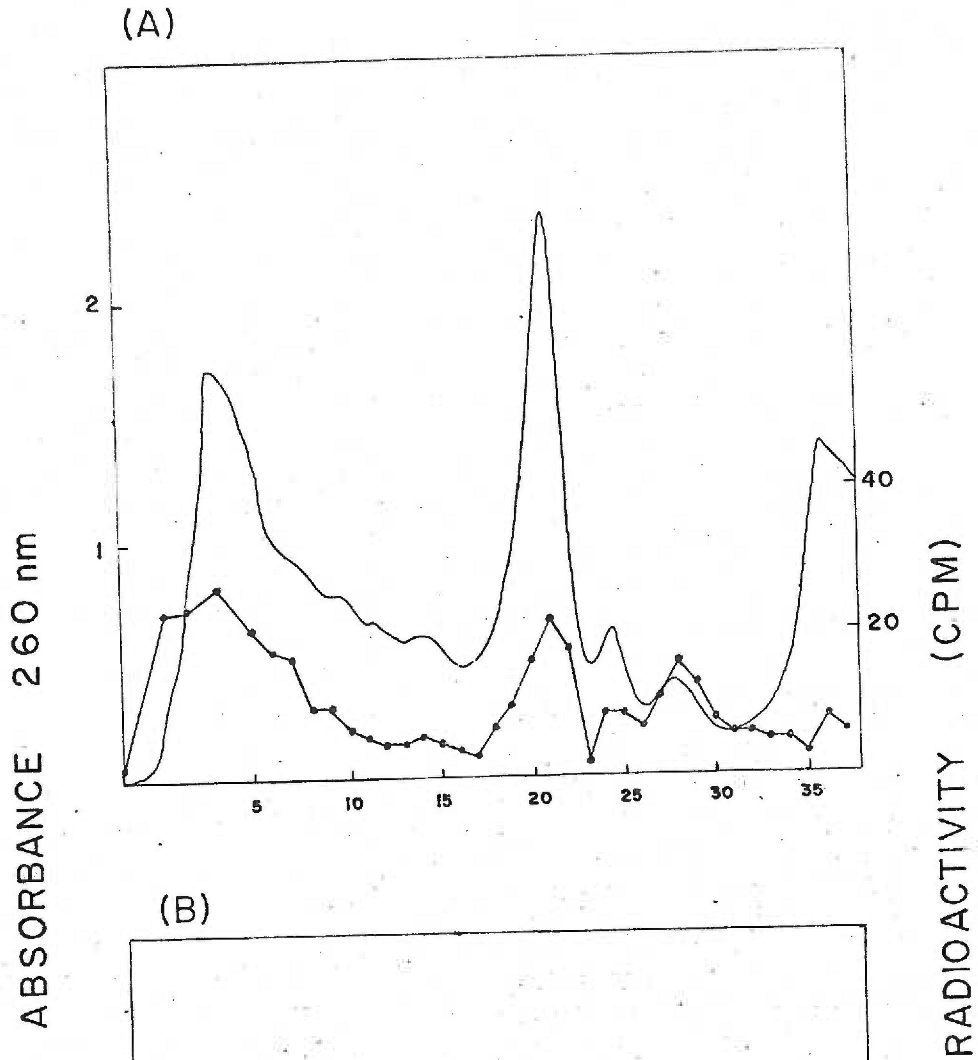


FIGURE 19

During the course of this work a report appeared showing that sea urchin ribosomal subunits were three times as active in a poly(U) assay if they were prepared by dissociation at 0.24M KCl rather than 0.50M KCl (258). Another report showed that dissociation of murine myeloma cell ribosomes with either high or moderate KCl concentrations (at appropriate $MgCl_2$ concentrations) produced subunits of similar activity in a poly(U) assay (204). Since a portion of subunits from the high ionic strength dissociation procedure appeared unable to participate in protein synthesis in the lysate system (Figures 15, 16 and 17), I wished to learn whether preparations with a larger proportion of active subunits could be obtained by dissociation of reticulocyte ribosomes by moderate rather than high ionic strengths. For this purpose I simply modified the procedure of Blobel and Sabatini to carry out dissociation of ribosomes and gradient centrifugation at K_{250} T_{50} $M_{2.5}$ and K_{250} T_{50} $M_{1.5}$. These subunit preparations have already been discussed.

Figure 20 shows a comparison of the activity in the lysate system of polysomal small subunits dissociated in high and moderate ionic strengths. The small subunits were not more active when prepared at the moderate ionic strength. In fact in the assay shown, the subunits prepared at the higher ionic strength appeared slightly more active (55% of added subunits in polysomes) than the subunits prepared at moderate ionic strength (50% of added subunits

Figure 20. Analysis in the lysate system of the activity of polysome-derived small subunits prepared in high and moderate ionic strengths. The polysomal-derived large subunits were prepared in K_{500} T_{50} M_5 (for the experiment shown in panel A) and K_{250} T_{50} $M_{1.5}$ (for the experiment shown in panel B). Incubation of the subunits in the lysate system was for 10 minutes. Ribosomes from the incubations were analyzed on sucrose gradients as described in Figure 14 and Materials and Methods.



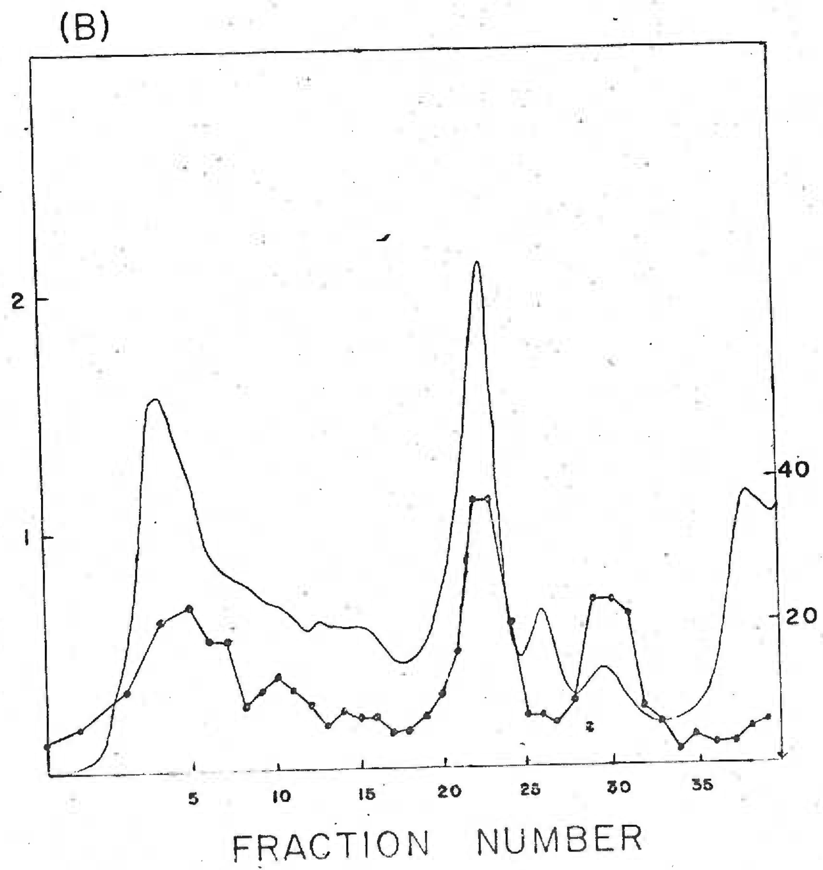


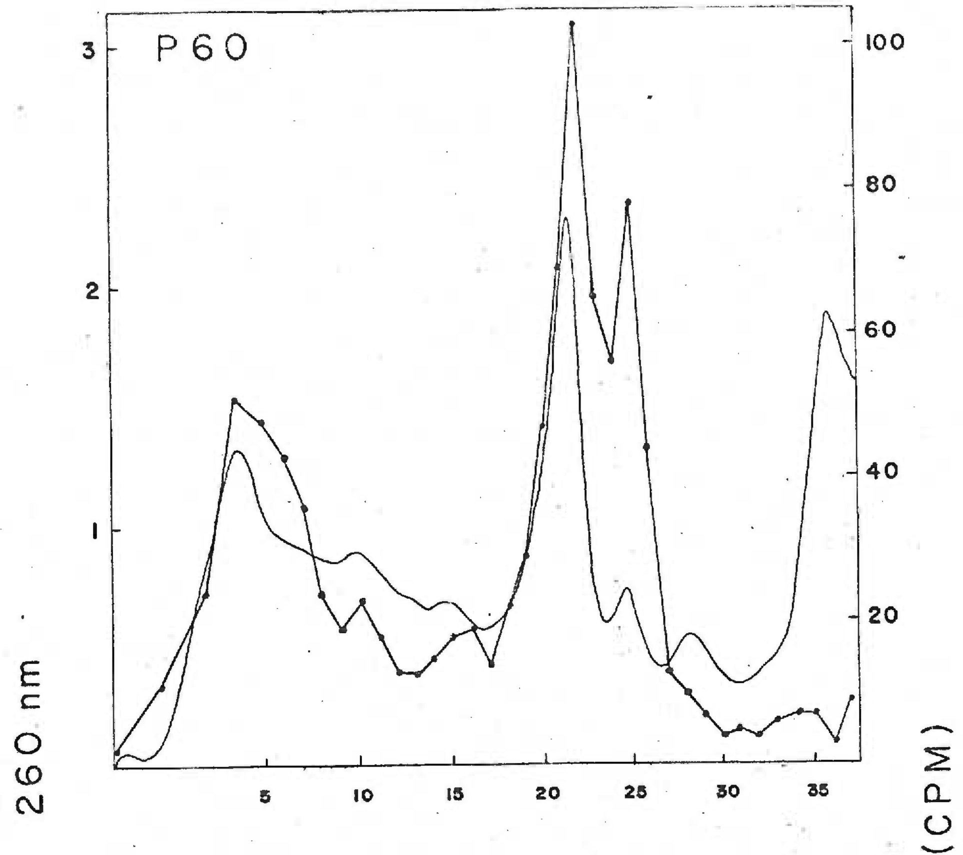
FIGURE 20

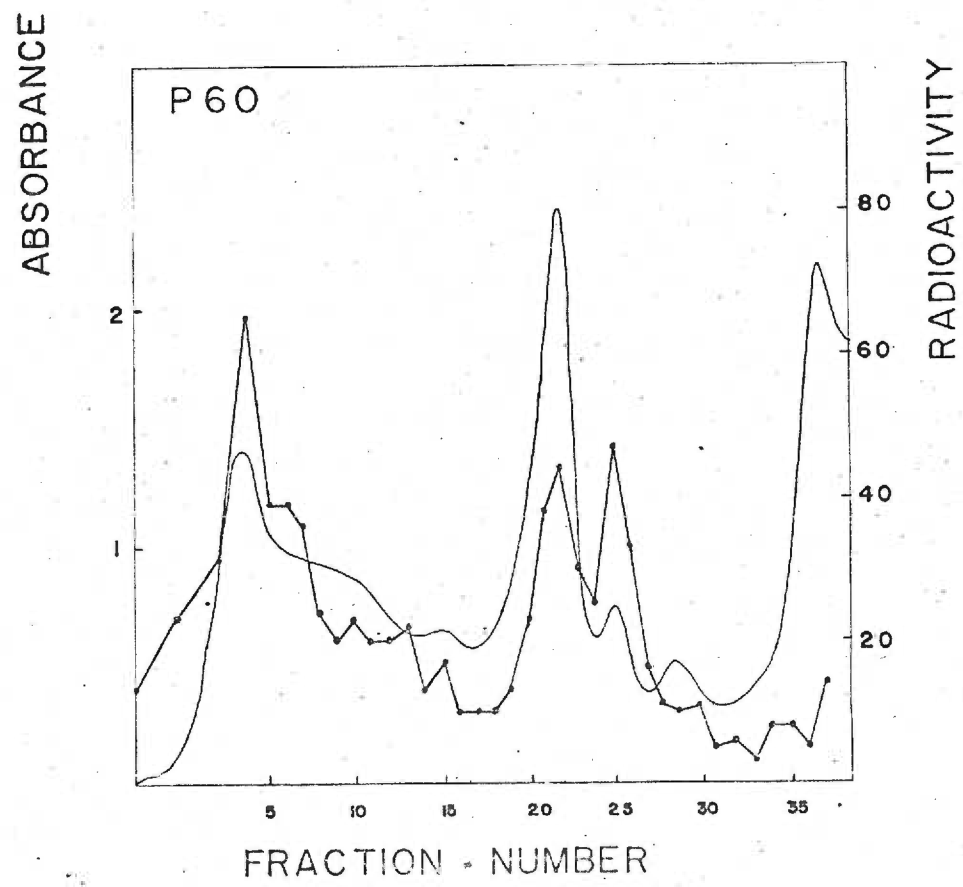
in polysomes). Figure 21 shows a similar experiment with the polysomal large subunit. In this case the 60S subunits prepared by moderate ionic strength dissociation appeared more active (61% of added subunits in polysomes) than the subunits obtained by dissociation at high ionic strength (43% of added subunits in polysomes). Figure 22 shows an assay of the 60S subunit from single ribosomes dissociated at moderate ionic strength. By comparison with the polysomal-derived subunit (Figure 21B), the single derived subunits (Figure 22) appeared slightly less active.

In the experiments just described [^{32}P]-subunits were frozen directly from gradient fractions, whereas in the experiment of Figure 15 the subunits were concentrated from the gradient fractions by high speed centrifugation. Both methods gave comparable ribosomal subunit activity. In the first case, control experiments showed that 50-100 μl of the sucrose-high ionic strength buffer could be added to the lysate system without inhibiting the rate of protein synthesis. Such an addition changes only slightly the Mg^{++} ion concentration in the assay mix.

Since two types of inactive subunits occur in the purified subunit preparations (i.e., those that enter single ribosomes and those that stay as subunits), I tried to learn if these inactive forms were related to the ribosome dissociation procedure. I, therefore, prepared native ribosomal subunits by a method which

Figure 21. Analysis in the lysate system of the activity of polysome-derived large subunits prepared in high and moderate ionic strengths. The polysomal-derived large subunit was prepared in K_{500} T_{50} M_5 (for the experiment shown in panel A) and K_{250} T_{50} $M_{2.5}$ (for the experiment shown in panel B). Incubations of the subunits in the lysate system was for 10 minutes. Ribosomes from the incubations were analyzed on sucrose gradients as described in Figure 14 and Materials and Methods.





ABSORBANCE 260 nm

RADIOACTIVITY (CPM)

FRACTION - NUMBER

FIGURE 21

Figure 22. Analysis in the lysate system of the activity of single ribosome-derived subunits prepared in moderate ionic strength. This is a companion experiment to the one shown in Figure 20B. The polysomes and single ribosomes used in preparing subunits for these assays were from the same preparation. The single ribosome-derived large subunit was prepared in K_{250} T_{50} $M_{2.5}$. Incubation of the subunits in the lysate system was for 10 minutes. Ribosomes from the incubations were analyzed on sucrose gradients as described in Figure 14 and Materials and Methods.

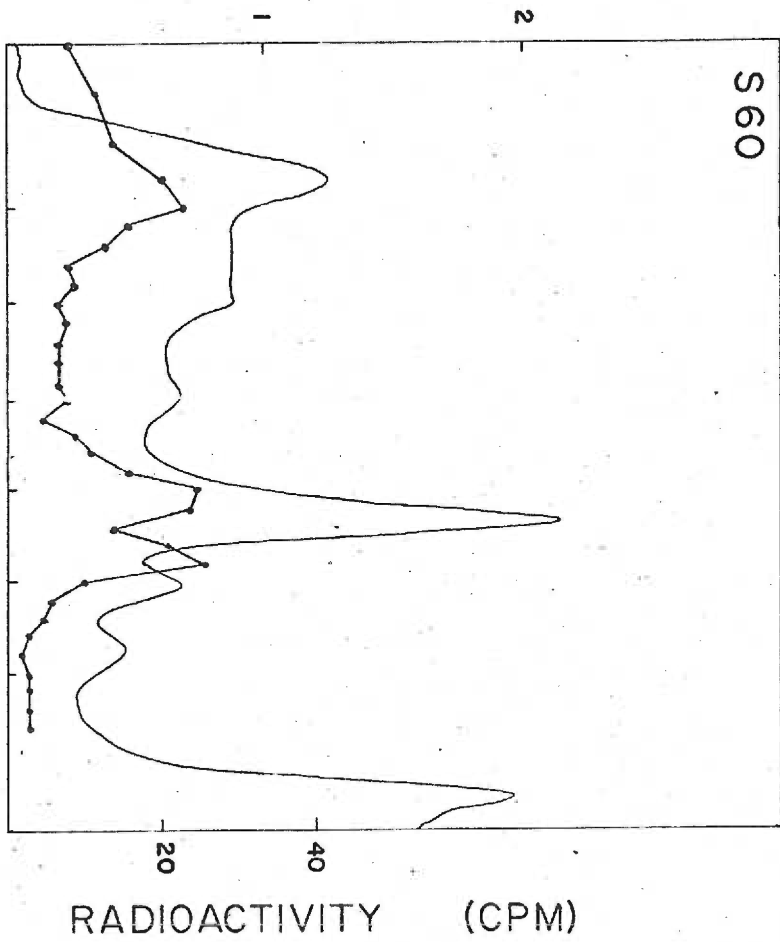


FIGURE 22

avoids treatment of ribosomes with high or moderate salt concentrations (see Materials and Methods). Figure 23 shows an analysis of the function of [^{32}P]-labeled native subunits in the lysate system. These subunits were not more active than the derived subunits in this assay system. As also seen with dissociated subunits, the native subunits showed fractions entering polysomes, single ribosomes and a sizeable fraction still sedimenting as subunits after incubation in the lysate. Thus, it seems that ribosome purification, in general, results in unavoidable inactivation of a fraction of the ribosomes.

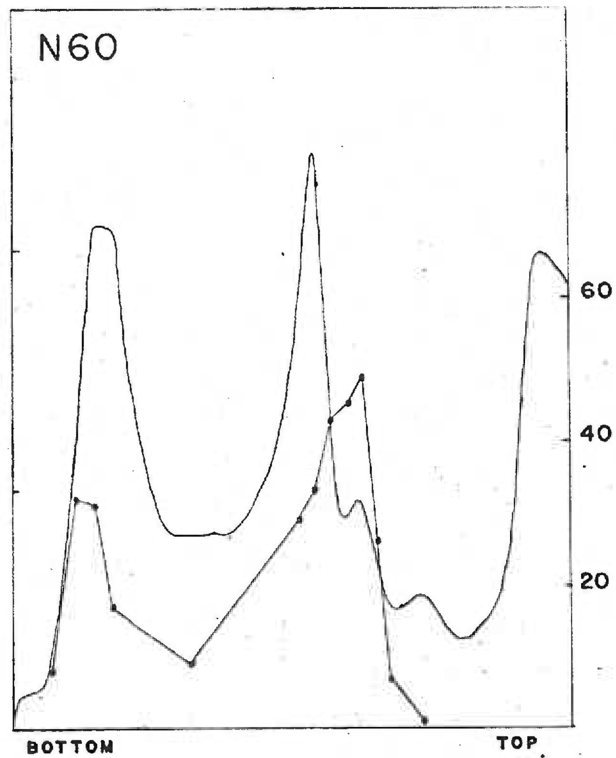
4. Evidence that entry of [^{32}P]-labeled ribosomal subunits into polysomes is the result of normal protein synthesis

I initially examined the relationship between the appearance of radioactive subunits in polysomes and the process of protein synthesis by testing whether radioactive subunits entered polysomes in the presence of cycloheximide, an inhibitor of protein synthesis elongation. In preliminary experiments I showed that either incubation of the lysate on ice or incubation at 28° in the presence of 30 μg/ml cycloheximide inhibited the rate of protein synthesis by greater than 98%. Yet I found that in either of these conditions about 20% of the added subunits were able to enter polysomes. I did not, at first, understand this observation but the recent work of Baglioni (259,260) suggests that ribosomal initiation

Figure 23. Assay of native ribosomal subunit activity in the lysate system. The native large subunit (N60) and the native small subunit (N40) were prepared by sucrose gradient centrifugation as described in Materials and Methods and as shown in Figure 5. Incubations were for three minutes. Ribosomes from the incubations were analyzed on sucrose gradients as described in Figure 14 and Materials and Methods. Distance sedimented down the tube is shown on the abscissa. 1 ml fractions of interest along the gradient were collected and analyzed for radioactivity.

ABSORBANCE 260 nm

RADIOACTIVITY (CPM)



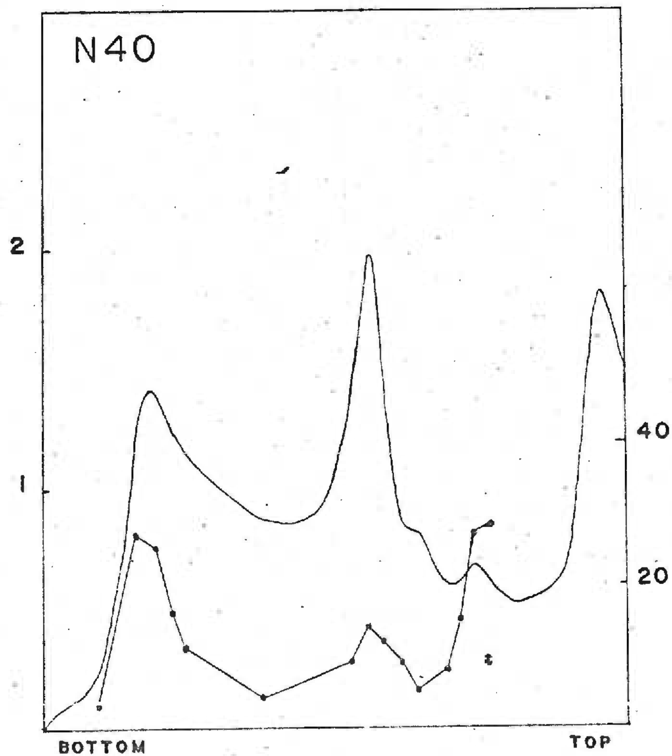
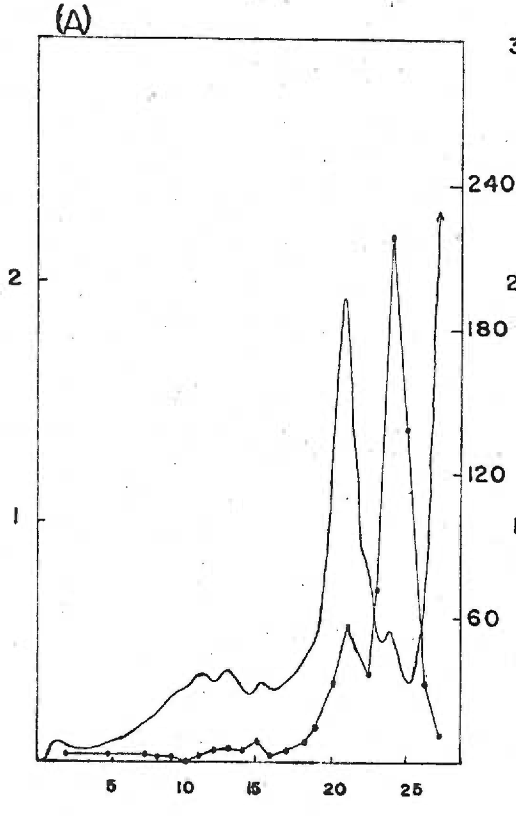


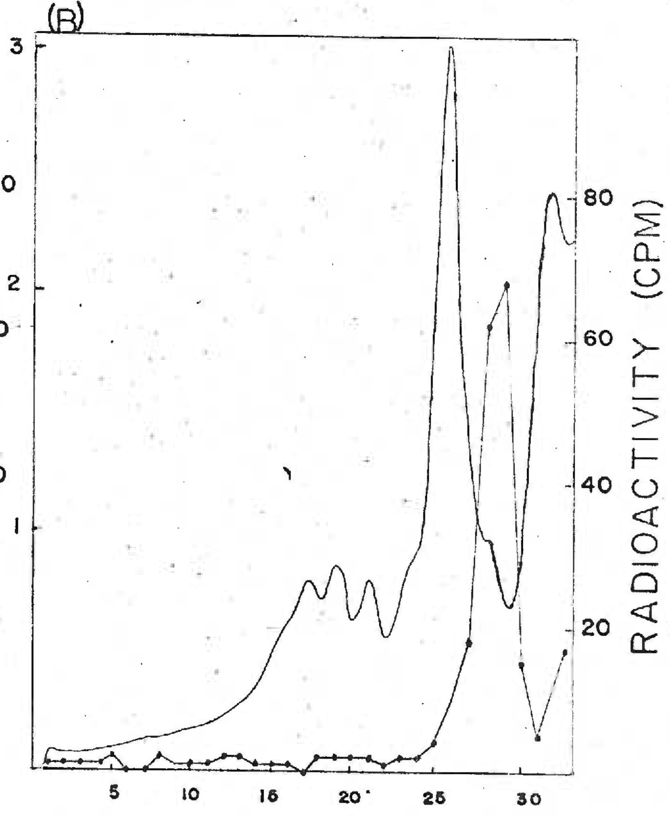
FIGURE 23

complexes can be formed in the presence of cycloheximide and therefore in the absence of peptide bond formation. Arnstein has shown that a small amount of radioactive rat liver ribosomal subunits can enter the polysomes in a reticulocyte cell-free lysate and that globin messenger ribonucleoprotein can be incorporated into ribosomes, both in the presence of cycloheximide (261). He, therefore, also suggested that initiation complexes can form in the absence of peptide bond formation. Thus, in my control experiments a small amount of both the large and small subunits may become polysomal-bound as a result of initiation complex formation in the presence of cycloheximide.

A more appropriate condition for examining the dependence on protein synthesis of subunit entry into polysomes was obtained by the use of a specific inhibitor of the initiation of protein synthesis, aurintricarboxylic acid. Preliminary gradient analyses showed that incubation of lysates with this inhibitor led to a rapid disaggregation of polysomes. However, polysomes are maintained in the presence of cycloheximide (262,263) and I was able to show that in the presence of both inhibitors polysomes are maintained fully at 0° and break down only slightly during a five minute incubation at 28°. I used aurintricarboxylic acid at a concentration (2×10^{-4}M) shown by other workers to inhibit initiation in a reticulocyte lysate (264). Figures 24 and 25 show analyses

Figure 24. Dependence on protein synthesis of [^{32}P]-labeled small ribosomal subunit entry into polysomes. Polysomal-derived small subunits for these assays were prepared by dissociation in K_{500} T_{50} M_5. The radioactive subunits were incubated in lysates for five minutes at 28° (panel A) or briefly on ice (panel B) in the presence of 1.1×10^{-4}M cycloheximide and 2×10^{-4}M aurintricarboxylic acid in both cases. The ribosomes from the incubations were analyzed on 20 ml linear 15-30% sucrose-RSB gradients. Centrifugation was for 3.25 hr (panel A) and 2.5 hr (panel B) at 25,000 rpm at 2° in the SW25.1 rotor. 1 ml fractions were assayed for radioactivity as already described.





FRACTION NUMBER

FIGURE 24

Figure 25. Dependence on protein synthesis of [^{32}P]-labeled large ribosomal subunit entry into polysomes. Polysomal-derived large subunits for this assay were prepared by dissociation in K_{500} T_{50} M_5. The radioactive subunits were incubated in the lysate system for five minutes at 28° in the presence of $1.1x10^{-4}$M cycloheximide and $2x10^{-4}$M aurintricarboxylic acid. The ribosomes from the incubation were analyzed on a sucrose gradient as described in Figure 14 and Materials and Methods. 1 ml fractions of interest were collected for radioactivity determination. Distance sedimented down the tube is shown on the abscissa.

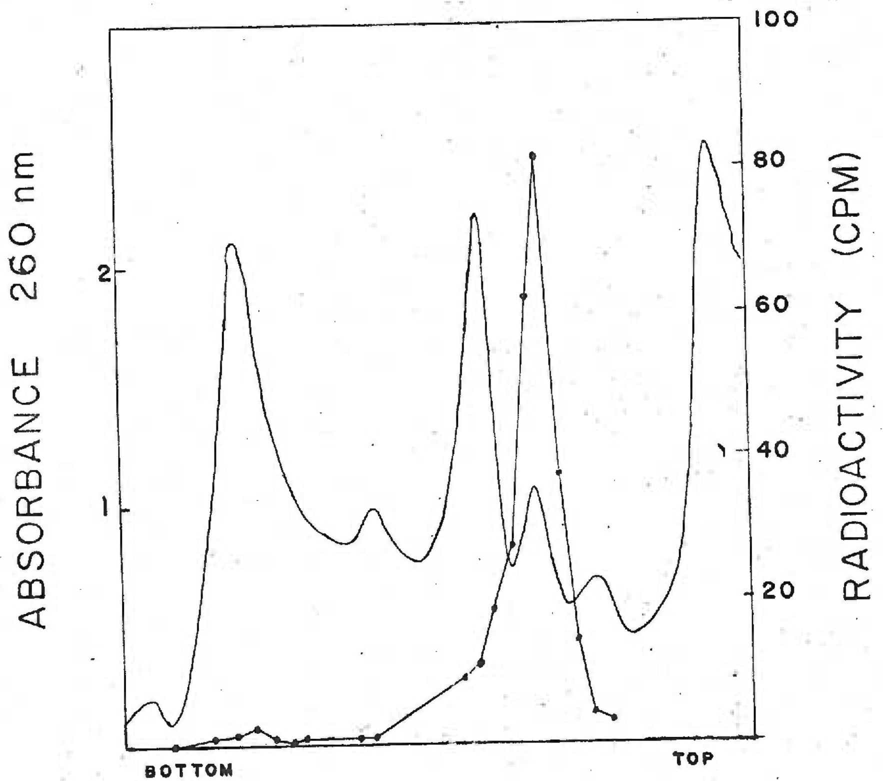
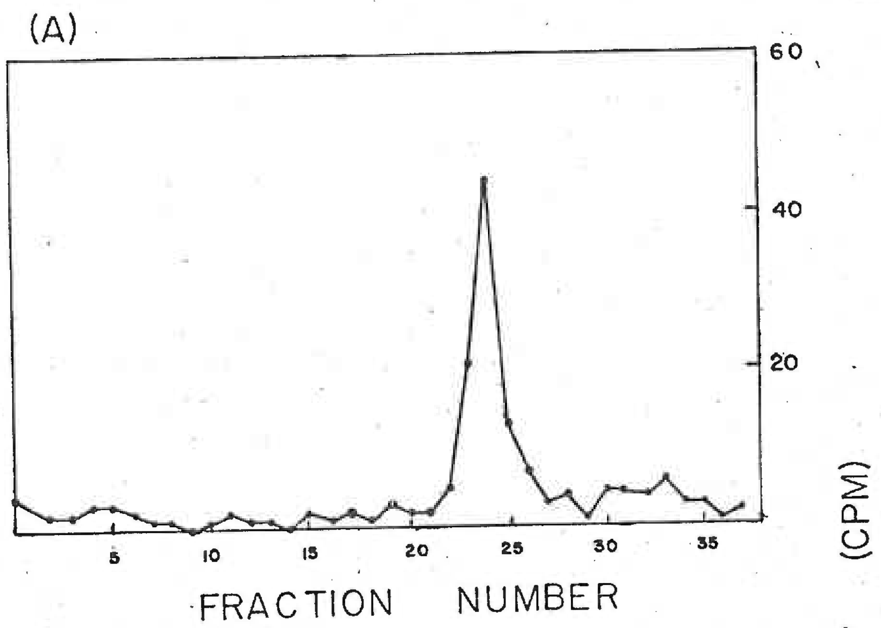


FIGURE 25

of radioactive subunit sedimentation after incubation in a lysate containing cycloheximide and aurintricarboxylic acid. The entry of either the large or small subunit into polysomes is blocked in the presence of both inhibitors. This data strongly suggests that the subunits entering polysomes in the usual assay conditions (i.e., no inhibitors present) are participating in protein synthesis in a manner requiring normal initiation.

Several additional types of experiments were designed to show that the subunit radioactivity appearing in the polysome gradient regions is not due to aggregation of subunits or their adsorption onto polysomes. Figure 26A shows that sedimentation of the large subunit alone on a gradient of the type used for analysis of the ribosomes from lysate incubations results in almost all of the radioactivity sedimenting in the large subunit region. In another experiment the same result was obtained if the large subunit was first incubated with a post-ribosomal supernatant fraction prior to centrifugation (not shown). Panel B of this figure shows that the radioactive small subunits do not sediment in the polysome region if they are layered on a gradient together with unlabeled ribosomes and centrifuged. Thus, the appearance of radioactive subunits in polysomes during a lysate incubation is not an artifact of gradient centrifugation or of a spurious interaction of the subunits with components of the lysate.

Figure 26. Evidence that ribosomal subunit radioactivity seen in polysomes is not due to artifacts. The single ribosome-derived subunits used in the analyses shown were obtained by dissociation in K_{500} T_{50} M_5. Panel A shows a sedimentation of radioactive large subunits alone on the type of gradient used for analysis of ribosomes from lysate incubations (see Figure 14). Panel B shows the sedimentation of radioactive small subunits when they were added to nonradioactive ribosomes from the reticulocyte lysate and centrifuged together (for gradient and centrifugation conditions see Figure 14). In neither case does the subunit radioactivity sediment in the polysomal region. See text for further explanation.



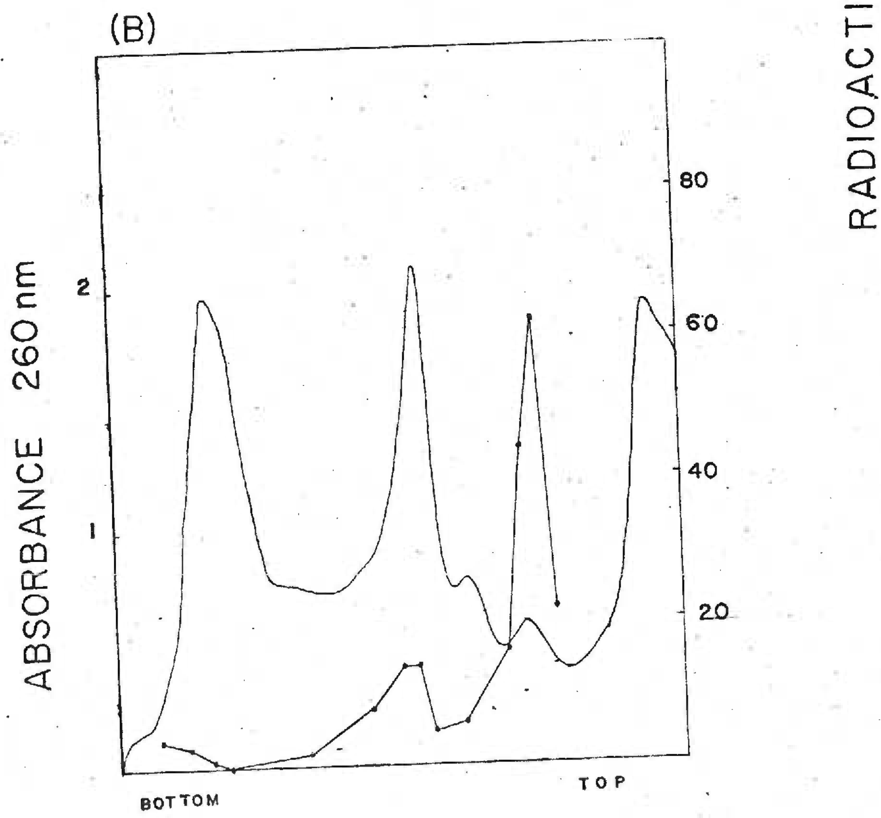


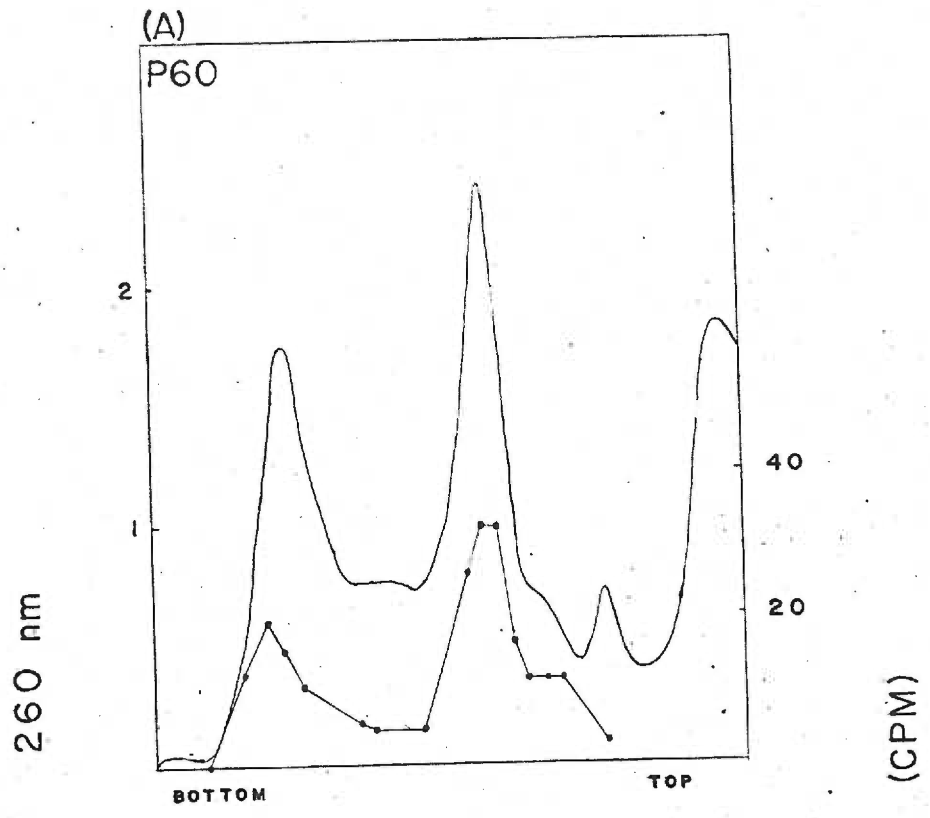
FIGURE 26

While the kinetics of entry experiments already described suggested that the radioactive subunits in polysomes are functioning normally, I have also examined this point by physical criteria. Figure 27 shows that the radioactive large and small subunits remain associated with polyribosomes when the ribosomes from lysate incubations are sedimented on gradients containing a moderate ionic strength buffer, which is routinely used for removing contaminants adsorbed to polysomes. This experiment therefore suggests that the radioactive subunits are not merely adsorbed to polysomes. In another experiment it was shown that release of the radioactive subunits from polysomes by the high salt dissociation method required an incubation with puromycin (not shown). This suggests that the radioactive subunits are in polysomal couples containing nascent polypeptide chains rather than merely having formed nonproductive complexes on the polysome. These control experiments strongly suggest that the radioactive subunits which are seen in the polysome gradient regions after incubation in the lysate system are participating in normal protein synthesis.

D. Assay of Ribosomal Subunit Activity in the Fractionated Reticulocyte Cell-free Protein Synthesis System

As discussed, I have shown that purified ribosomal subunits can function efficiently in the crude lysate protein synthesis system and that the large and small subunits from either polysomes or single

Figure 27. Retention of [^{32}P]-subunits in polysomes in the presence of moderate ionic strength. Polysome-derived large and small subunits were prepared by dissociation at K_{500} T_{50} M_5. Panel A shows an analysis in which the large subunit was incubated in the lysate for 10 minutes; the ribosomes from the incubation were sedimented on a 33 ml 15-30% isokinetic sucrose gradient over a 3 ml 60% sucrose cushion. The sucrose solutions contained a moderate ionic strength buffer [0.25M KCl; 0.01M Tris HCl (pH 7.4); 0.01M $MgCl_2$]. Panel B is an identical analysis for the small subunit. 1 ml fractions of interest were assayed for radioactivity. Distance sedimented down the tube is shown on the abscissa.



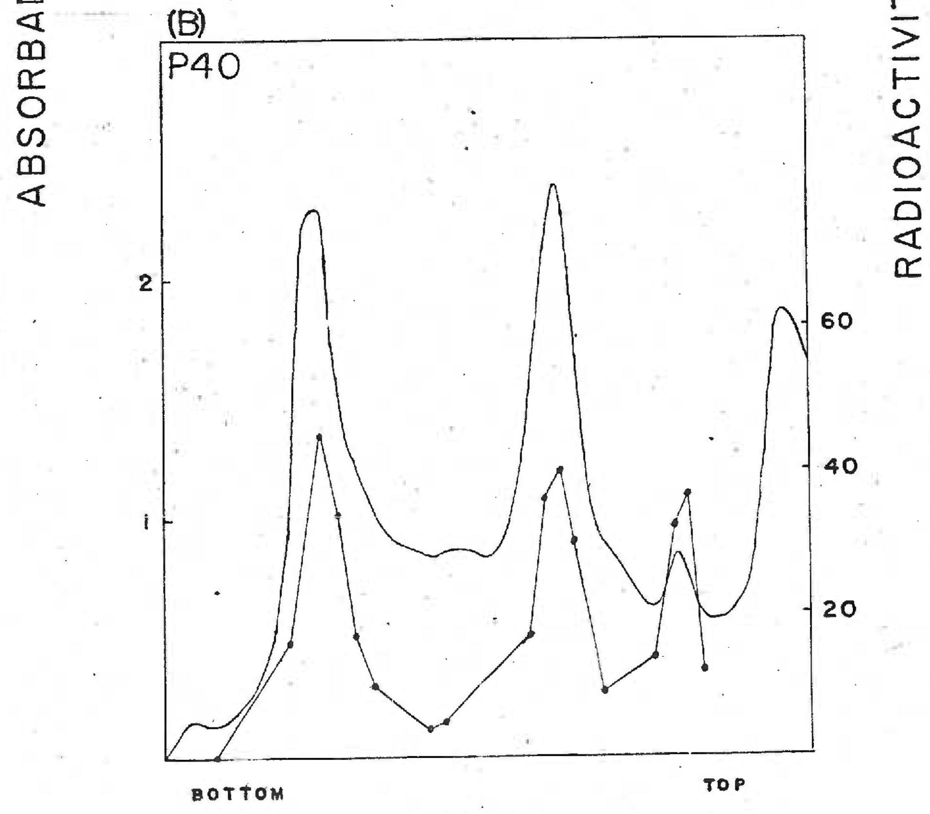


FIGURE 27

ribosomes seem to function with similar efficiency, with occasional minor differences observed. A conceivable disadvantage of the lysate system which is difficult to rule out is the possibility that the components of this crude system rapidly modify added subunits to equivalent forms, thereby making the detection of functional differences difficult. The presence of protein kinase and phosphoprotein phosphatase in the lysate would cause a similar uncertainty about the comparative assay of phosphorylated and dephosphorylated ribosomes in this system.

For these reasons, I have utilized a fractionated reticulocyte cell-free protein synthesis system. This system was developed by Crystal et al. (226) and was used by that group to study messenger RNA and initiation factor function. These workers used ribonuclease-treated ribosomes as the ribosomal source for the translation of added mRNA. Schrier and Staehelin have recently reported similar mammalian fractionated systems which use subunits as the ribosomal source for translation (265,266).

In my initial experiment I tested the ability of ribosomal subunits, prepared by the dissociation of unfractionated lysate ribosomes, to function in the reticulocyte fractionated system. Figure 28 shows this experiment in which the kinetics of [^{14}C]-L-leucine incorporation by the ribosomal subunits is measured. The incorporation is dependent upon ribosomes; messenger and initiation

Figure 28. Protein synthesis in the fractionated cell-free system by subunits prepared from lysate ribosomes. 0.25 A_{260} ribosomal subunits (60S and 40S subunits added in 2.5:1 ratio) were assayed. These incubations and those in subsequent figures contained saturating amounts of mRNA and initiation factor fraction. The assays shown were with subunits dissociated in K_{500} T_{50} M_5 (H) and K_{250} T_{50} $M_{1.5}$ (M). (-) indicates the control assay without ribosomal subunits. 20 μl aliquots of a 100 μl incubation were taken at the times indicated for determination of acid precipitable [^{14}C]-L-leucine as described in Materials and Methods.

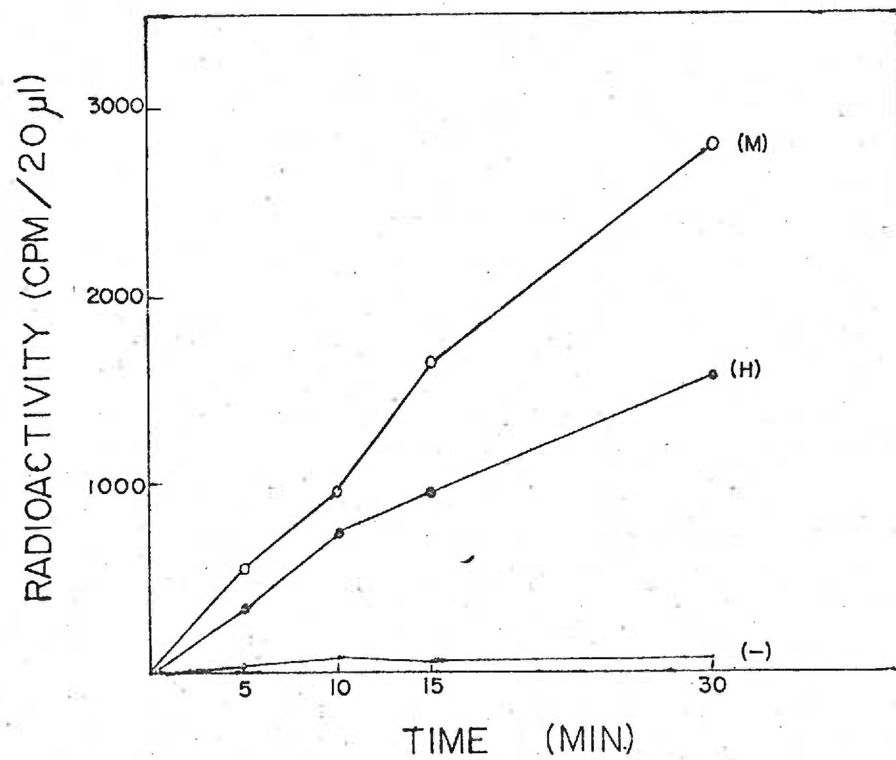


FIGURE 28

factor dependency is shown in subsequent experiments. Subunits prepared at a moderate ionic strength were more active than those prepared at a high ionic strength. The activity shown here for ribosomal subunit protein synthetic activity is very similar to that obtained in an identical assay with ribonuclease-treated ribosomes.

This approach, therefore, looked promising and I assayed subunits from single ribosomes and from polysomes in this system. Figure 29 shows such an experiment in which it is seen that polysomal-derived subunits had about 1.5 times the rate of protein synthesis as those derived from single ribosomes. This difference is seen at the several time points examined. For both polysomal- and single ribosome-derived subunits, about one-half of the total amount of incorporation seen with both subunit fractions is observed with the 60S fraction alone. In other subunit preparations synthesis was also observed with the 60S fraction alone; addition of the 40S fraction was not further stimulatory and was occasionally inhibitory. The native 60S subunit also showed synthesis without the addition of the native small subunit; the native subunits were not more active than derived subunits in this assay system. These results suggest that the 60S fraction is contaminated with dimers of the 40S subunit and that these small subunits are able to form productive ribosome couples with the large subunit when incubated in the fractionated cell-free system.

Figure 29. Protein synthesis in the fractionated cell-free system by subunits prepared from polysomes (P) and single ribosomes (S). The assay is as described in Figure 28 and Materials and Methods. 0.72 A_{260} large subunit (60) is assayed $\pm$ 0.28 A_{260} small subunit (40).

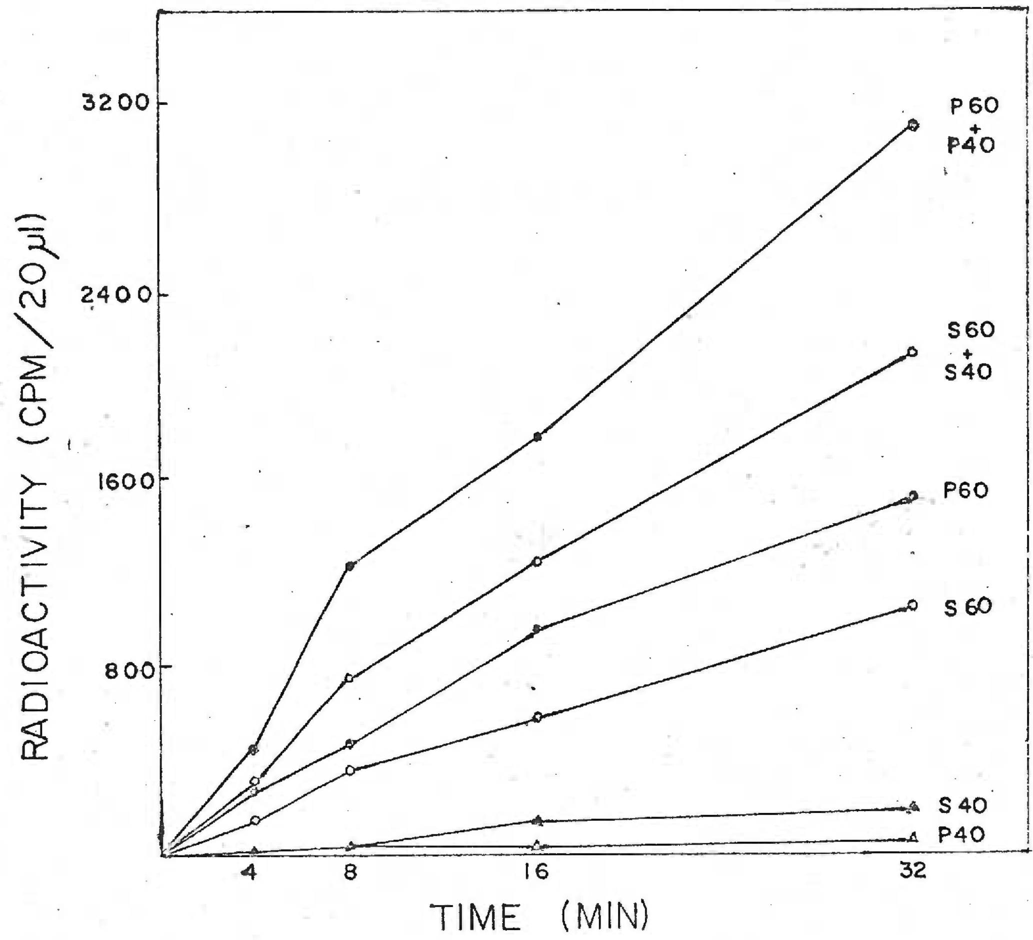
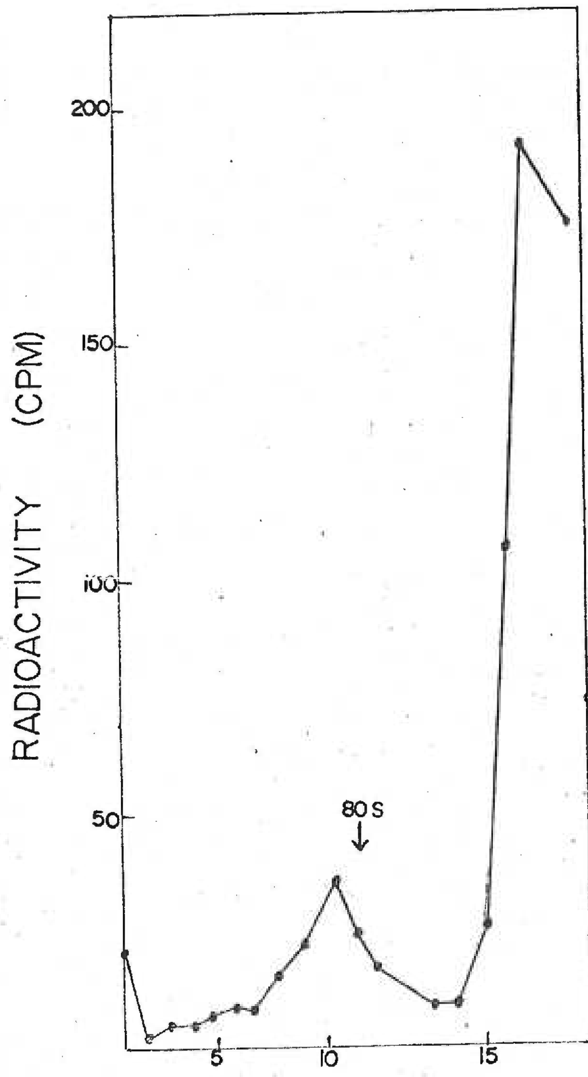


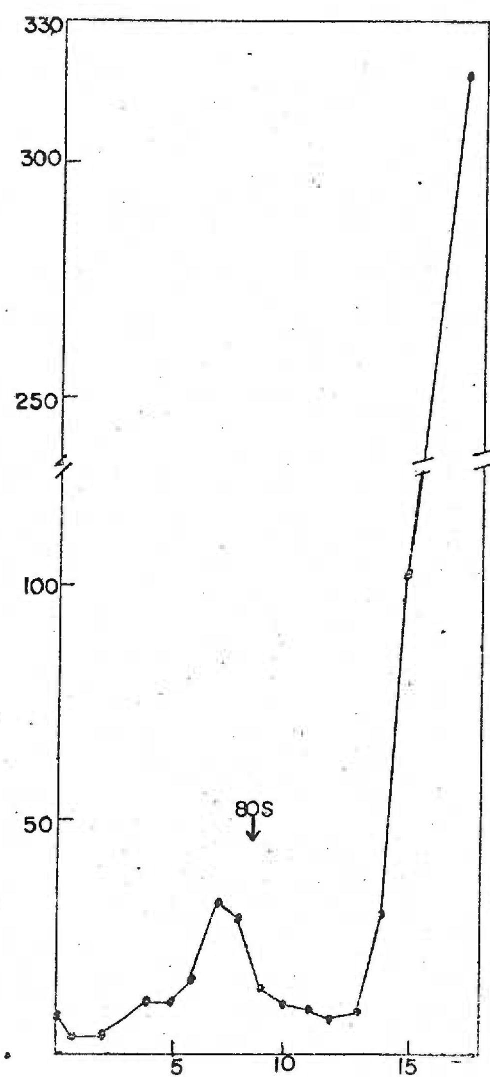
FIGURE 29

That this effect is due to cross-contamination of the large subunit peak is consistent with the 60S:40S peak area ratios obtained in the dissociation experiments and discussed previously. To test this interpretation of protein synthesis by the 60S fraction alone, I performed an experiment to determine the approximate sedimentation position of the active ribosomal species in the 60S fraction. As shown in Figure 30, gradient analysis of protein synthesis by the 60S ribosomal subunit fraction allows detection of the active ribosome species containing radioactive nascent polypeptide chains. This species sediments slightly faster than the 80S marker and is, therefore, probably a monoribosome (i.e., both ribosomal subunits on a globin mRNA molecule). This evidence supports the idea that the 60S subunit fraction is contaminated by active small ribosomal subunits.

Before discussing my attempts to resolve this preparative problem, the implications of the data already presented merits further discussion. The data from the nascent chain radioactivity experiment of Figure 30 can be treated quantitatively to calculate the percentage of ribosomes which are active at a given instant in the cell-free system. Since I have shown by an isotope dilution experiment that there is no endogenous pool of L-leucine in the fractionated system, the cpm's of nascent chain radioactivity can be converted directly to pmol nascent chain leucine per pmol ribosome

Figure 30. Position of sedimentation of the active ribosomal species in the 60S subunit peak. The large subunit peak used in this experiment was from the dissociation of polysomes at K_{500} T_{50} M_5. 0.1 A_{260} unit 60S fraction was assayed in a standard 100 µl assay in the fractionated cell-free system. At 10 minutes (panel A) and 20 minutes (panel B) of incubation 40 µl fractions were removed and layered on 4.8 ml 10-30% sucrose-RSB gradients. The gradients were centrifuged for 2 hr at 39,000 rpm at 2° in the SW39 rotor. The gradients were pumped from the bottom and collected in 8 drop fractions. Acid precipitable [^{14}C]-L-leucine was determined for the fractions. 80S indicates the sedimentation position of single ribosomes centrifuged on a separate gradient. The nascent chain radioactivity peak sediments slightly faster than 80S; released chain radioactivity is seen at the top of the gradient.





FRACTION NUMBER

FIGURE 30

added from the isotope specific activity, counting efficiency and amount of ribosomes assayed. The calculation yields a result of one nascent leucine/ribosome added. Each ribosome, in the steady state, which is actively synthesizing a globin chain should contain on the average one-half of a completed chain (8-9 nascent leucines). The fraction of added ribosomes which are active in this system is, therefore, 1 observed leucine/ribosome ÷ 8-9 expected leucines/ribosome = 11-13%. A comparison of the nascent chain radioactivity at 10 minutes and at 20 minutes (Figure 30) shows that the fraction of active ribosomes reaches a steady state by 10 minutes in this system and that the amount of released completed chains (radioactivity at top of gradient) increases linearly with time throughout the incubation.

That the fraction of active subunits is small in this system is surprising for several reasons. Both large and small subunits prepared by the same methods were shown to function efficiently in the lysate protein synthesis system. Since maximum synthesis in the fractionated system is sometimes observed with the 60S fraction alone, the small subunit dimers which contaminate the 60S fraction must represent an active ribosomal form, perhaps structurally protected against inactivation. Furthermore, one of the following possibilities must also be true. Either the small subunit fraction (40S) is largely or completely inactivated for assay in this system. Or, the large subunits are largely inactivated

and the active fraction of large subunits is already maximally stimulated by the small amount of contaminating active small subunits. Whether it is the large or small subunit which is inactive has not been revealed by further experiments.

As already mentioned, my initial experiment showed that purified subunits function as well as the ribonuclease-treated ribosome preparation of Crystal et al. (226). Since the subunits are shown here to be largely inactive, the ribonuclease-treated ribosomes must be similarly inactive. Indeed analysis of the percentage of active ribosomes in a ribonuclease-treated lysate ribosome preparation by an experiment similar to that shown in Figure 30 gave a value of less than 10% active ribosomes. This value can be raised to 15-19% by our improved preparative methods, which avoid treatment of the ribosomes with high salt concentrations.[3] I have also found a low efficiency of function in this system for intact polysomes prepared by very gentle methods. Thus, low efficiency of ribosome utilization appears to be a general feature of this highly fractionated cell-free protein synthesis system; and this is not solely due to ribosome inactivation by the preparative methods employed.

That a cross-contamination of the purified subunits would be problematic in these studies is surprising since, as already shown (Figure 8), the purified 60S subunit fraction is only minimally contaminated with small subunits as judged by analysis of the

ribosomal RNA content of the subunit fraction.

Several approaches were taken to try to eliminate or minimize the problem of small subunit dimerization. Martin et al. (196) have reported that dimerization of small subunits is dependent upon the temperature of gradient centrifugation, being less at higher temperatures. I have prepared ribosomes by the usual dissociation method, using either high or moderate ionic strength buffers, and have found no reduction in the extent of small subunit dimerization, as judged by the 60S:40S peak ratios in the sucrose gradients, when the centrifugation was at 20° as compared to 2°. Furthermore, the purified subunits from a 20° centrifugation were largely inactive in the fractionated system; subunits prepared at the higher temperature also function less well in the lysate system.

As I have noted above, the extent of small subunit dimerization does not depend on the amount of dissociated ribosomes separated on a gradient. Thus, I was unable to obtain a less contaminated large subunit peak by using small amounts (three A_{260} units) of ribosomes for dissociation. Furthermore, cross contamination occurred at both moderate and high ionic strength dissociation conditions.

Gradient resedimentation of the ribosomes in the 60S fraction in either high or low ionic strength buffers (e.g., Figure 26A) does not seem to cause the release of the small subunits from

this fraction, although I have not tested such resedimented fractions for activity. Furthermore, I have in several preparations collected a very narrow central portion of the 60S peak and assayed its activity with and without added small subunits. This preparative procedure restored somewhat the dependency of the 60S fraction on added 40S subunits for synthesis, but the overall activity of the subunits was greatly diminished. It thus appeared that highly purified subunits became unstable, at least with respect to this assay system. This effect has also been noted by Schrier and Staehelin (265) who find that the ribosome is the most easily inactivated component of fractionated protein synthesis systems. These workers found it necessary to freeze both subunits together with the supernatant enzyme fraction in order to maintain ribosome stability during storage before assay.

Since the problem with the subunit assay in the fractionated system seemed to be, at least in part, the result of the inactivation of a large fraction of the isolated subunits, I utilized another approach for subunit preparation. This was the preparation of subunits by the usual dissociation method followed by pelleting of the subunits together through a sucrose cushion as described in Materials and Methods. Table I shows the results of an initial comparison of polysomal- and single ribosome-derived subunits prepared by this method, as well as some characteristics of the subunit

Table I

Protein Synthesis by Ribosomal Subunits in the

Fractionated Cell-Free System

Subunit Source	Amount	mRNA	FX-I	cpm/40 µl[i] 15'	cpm/40 µl[i] 30'
polysomes	0.05 A_{260}	+[ii]	+	177	359
polysomes	0.1	+	+	335	638
polysomes	0.2	+	+	682	1185
polysomes	0.1	-	+	14	22
polysomes	0.1	+	-	21	44
single ribosomes	0.1	+	+	279	584
single ribosomes	0.1	-	+	10	14
ribonuclease-treated lysate ribosomes[iii]	0.1	+	+	230	428
ribonuclease-treated lysate ribosome[iv]	0.1	+	+	177	277

i standard assays were 100 µl and contained saturating amounts of mRNA and initiation factors.

ii + and - indicate the presence or absence of messenger RNA or initiation factors (FX-I).

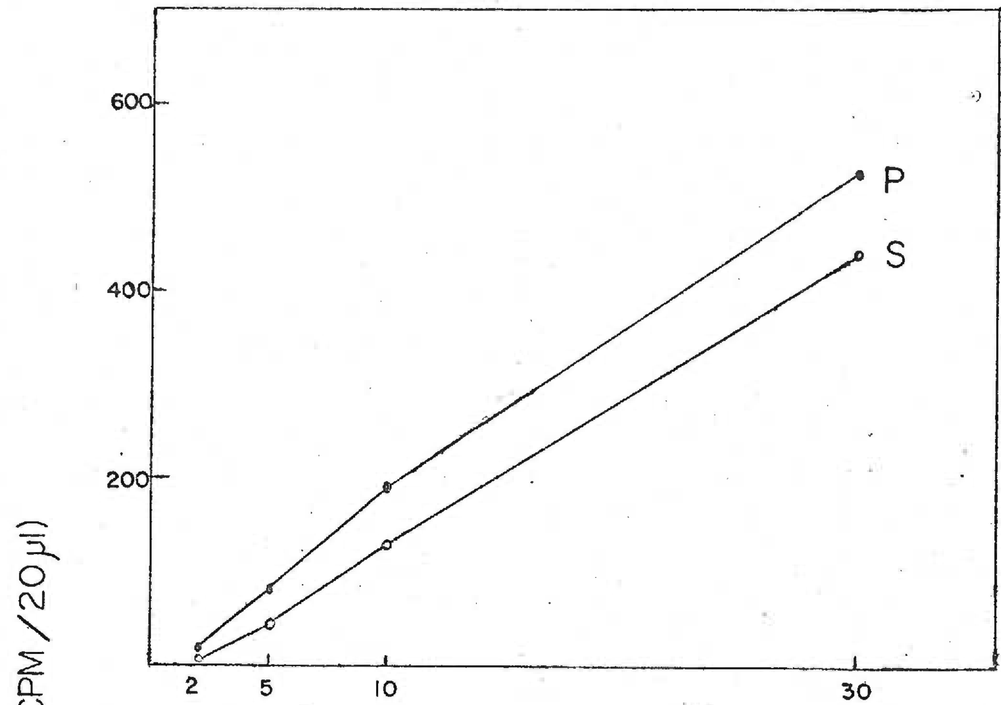
iii ribonuclease-treated ribosomes were sedimented away from ribonuclease in a moderate ionic strength buffer [0.25M KCl; 0.01M Tris-HCl (pH 7.4), 0.01M $MgCl_2$].

iv ribonuclease-treated ribosomes were sedimented away from ribonuclease in a high ionic strength buffer, K_{500} T_{50} M_5.

assay. The polysomal-derived subunits are seen to be somewhat more active than the subunits prepared from single ribosomes. The amount of [^{14}C]-L-leucine incorporated is linearly proportional to the amount of ribosomal subunits assayed. The subunits are messenger- and initiation factor-dependent even when prepared without exposure to high (0.5M KCl) salt concentrations. For comparison, the table includes assays performed with ribonuclease-treated lysate ribosomes prepared from the same reticulocyte preparation. Subunits and ribonuclease-treated ribosomes were generally of comparable activity in the fractionated cell-free system. Note again, as already described for ribosomal subunits, washing of ribonuclease-treated ribosomes with high salt concentrations decreases their activity in this assay system.

Further analyses of protein synthesis in the fractionated system by ribosomal subunits prepared by this method are shown in Figure 31. The subunits display good activity and again the polysomal-derived subunits are somewhat more active than the single ribosome-derived subunits. In both experiments in Figure 31 this difference is greatest at early time points; in the period 2-5 minutes the polysomal subunits are over twice as active as those from single ribosomes. The single ribosome-derived subunits appear to become activated during the incubation (5-10 minutes) and in the period 10-30 minutes they exhibit an identical rate of protein synthesis to the polysomal subunits. Thus, the difference seen in the amount

Figure 31. Protein synthesis in the fractionated system by subunits from polysomes (P) and single ribosomes (S). Ribosomes were dissociated by Method B (see Materials and Methods) in K_{500} T_{50} $M_{1.5}$. 0.1 A_{260} units ribosomal subunits were assayed. The assay was as described in Figure 28 and Materials and Methods. The two panels show identical experiments with two different subunit preparations.



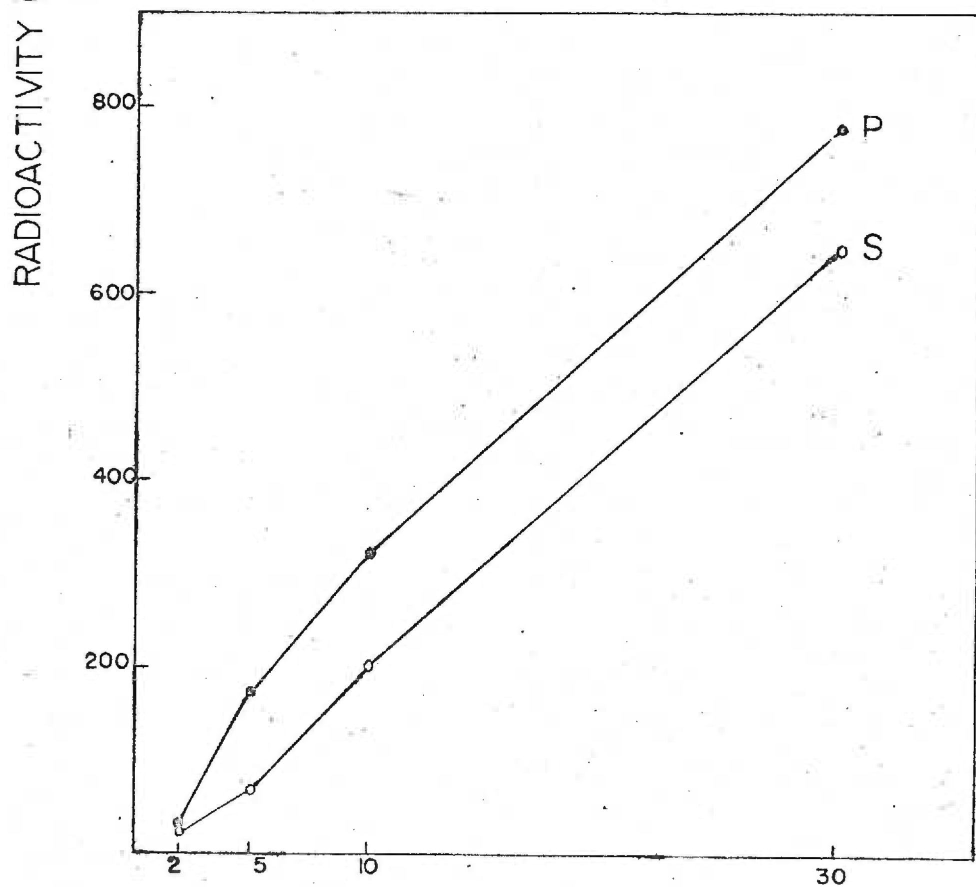


FIGURE 31

of incorporation by polysomal- and single ribosome-derived subunits during a 30 minute incubation is due almost entirely to their differing rates of protein synthesis at early time points.

Subunits from both ribosome sources, as well as other messenger-depleted ribosome forms (e.g., ribonuclease-treated ribosomes, single ribosomes), exhibit a lag of at least one minute before synthesis is detected (Figure 31). As shown below, intact polysomes do not exhibit a lag. These observations are consistent with a detailed study showing a rate-limitation of this fractionated system at the messenger selection step in protein synthesis initiation.[3]

Another approach I have used to study the protein synthetic ability of messenger-depleted polysomes and single ribosomes is to prepare ribonuclease-treated polysomes and ribonuclease-treated single ribosomes and to compare their abilities to translate added globin mRNA in the fractionated cell-free system. These experiments are shown in Table II. Ribonuclease-treated polysomes are about twice as active as ribonuclease-treated single ribosomes in globin mRNA-directed [^{14}C]-L-leucine incorporation (assays b and c). Assays a and e show that the ribonuclease-treated ribosomes are both messenger- and initiation factor-dependent, even though these ribosomes were prepared without a high salt concentration. Assay d shows that high salt treatment greatly inactivates ribosomes for assay in this system

Table II

Protein Synthetic Ability of Ribonuclease-treated Single Ribosomes and Ribonuclease-treated Polysomes in the Fractionated Cell-Free System

	Ribosome Source	Sediment. Buffer	mRNA	FX-I	cpm/40 µl/30'[i]
a	polysomes	L[ii]	-	+	55
b	polysomes	L	+	+	1292
c	single ribosomes	L	+	+	635
d	polysomes	H	+	+	341
e	polysomes	L	+	-	68

i standard assays contained 0.1 A_{260} mRNA, 0.1 A_{260} ribosomes and an optimal concentration of initiation factors in 0.1 ml.

ii the ribosomes were sedimented away from ribonuclease in sucrose containing either low ionic strength RSB (L) or high ionic strength K_{500} T_{50} M_5 (H).

(compare with assay b).

Another comparison of single and polysomal ribosomes is shown in Figure 32 in which the kinetics of endogenous and exogenous messenger translation are measured. The polysomes are more active than the single ribosomes and this difference is not just due to the endogenous messenger content of the polysomes. The data at early time points for synthesis by single ribosomes suggests, as was found for subunits derived from this ribosome class, that this ribosomal form undergoes an activation in the cell-free system. Synthesis by polysomes on endogenous messenger, in contrast to all other ribosomal forms tested, does not exhibit a lag phase. This further suggests that an initiation step rather than the elongation phase of protein synthesis is rate limiting in the fractionated system.

E. Assay of Phosphorylated and Enzymatically Dephosphorylated Ribosomes in the Fractionated Reticulocyte Cell-free Protein Synthesis System

As just described, the fractionated system has allowed the detection of a functional difference between the subunits of polysomes and of single ribosomes. This system, therefore, seemed appropriate for an analysis of the need for protein phosphoryl groups for the protein synthetic activity of ribosomes. Our preliminary comparison of control and dephosphorylated intact polysomes in this system failed to detect an effect of phosphoryl group removal on protein synthesis by these ribosomes (167). However, it seemed

Figure 32. Protein synthesis in the fractionated system by polysomes (P) and single ribosomes (S). 0.1 A_{260} of each ribosome source was assayed $\pm$ a saturating concentration of added mRNA. The assay was as described in Figure 28 and Materials and Methods. Polysomes contain more endogenous messenger activity, as expected. Polysomes are seen to also be more active with exogenous messenger, even when the data is corrected for their endogenous synthesis.

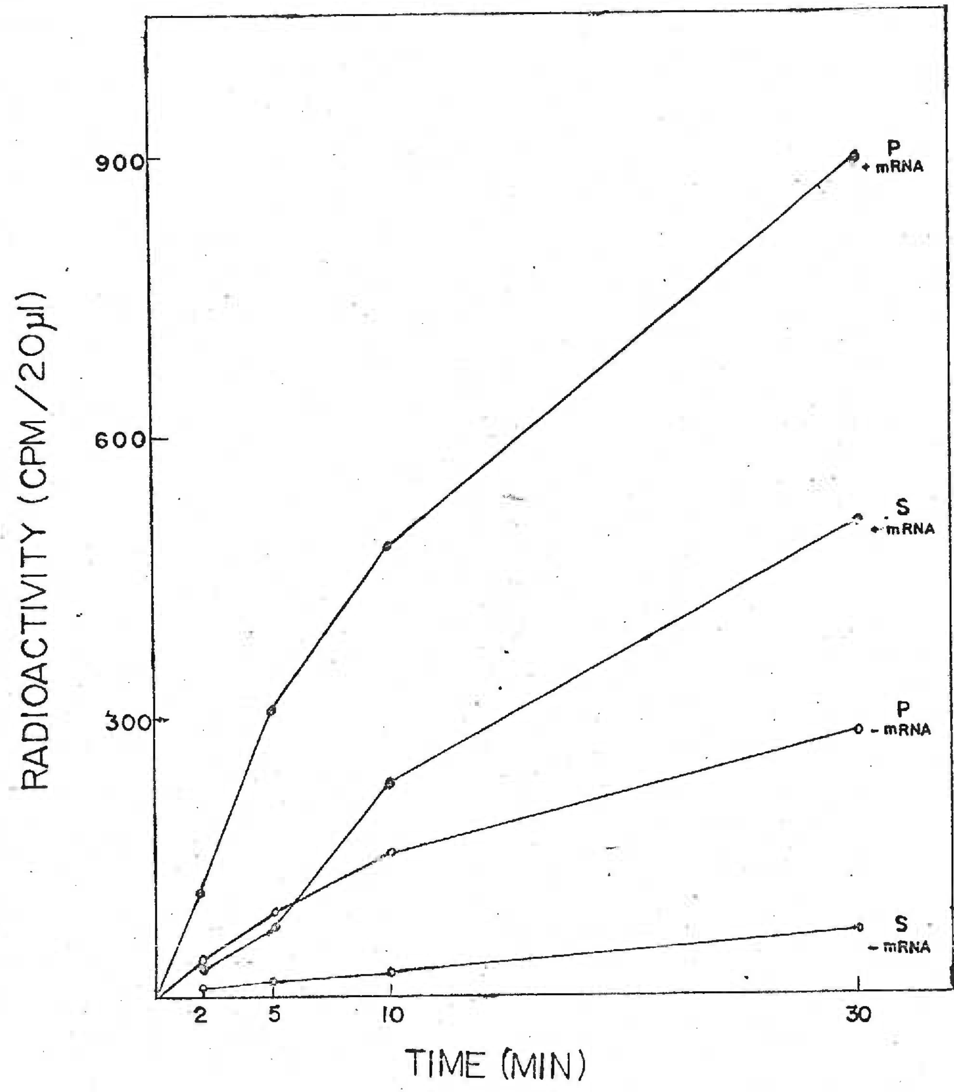


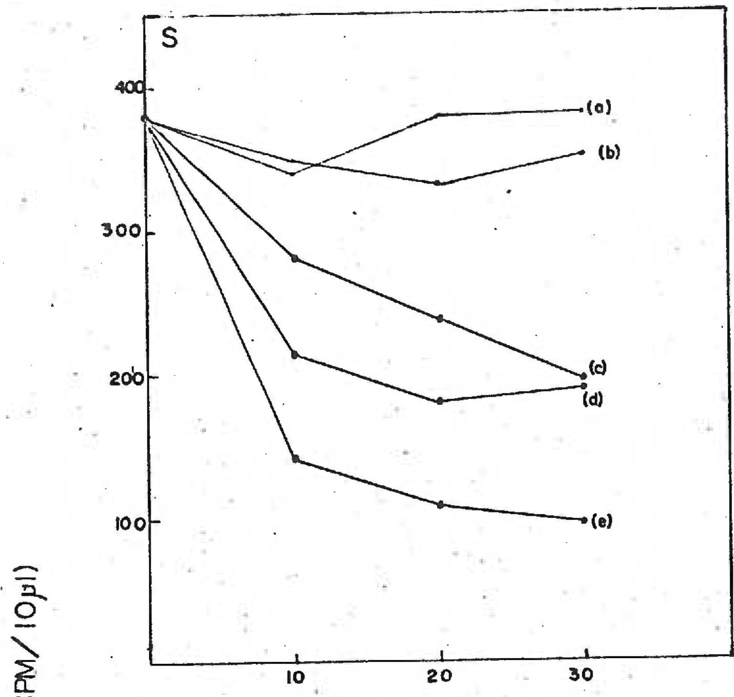
FIGURE 32

possible that such an effect might be demonstrated if other phosphorylated and dephosphorylated ribosomal forms were tested.

Figure 33 shows some characteristics of phosphoryl group removal from reticulocyte ribosomes by alkaline phosphatase. The ribosomal proteins were [^{32}P]-labeled by incubation of reticulocytes with ^{32}P-orthophosphate (160). The extent of reaction is dependent upon enzyme concentration and a maximum of 75% of the phosphoryl groups can be removed by this treatment. The resistant phosphoryl groups do not occur on a single protein but rather represent a resistant fraction on the several phosphoproteins (167). The control ribosome incubation shows only slight (5-10%) phosphoryl group removal by a phosphoprotein phosphatase in the ribosome preparation. Polysomes and single ribosomes appeared to differ in the kinetics of dephosphorylation, but I do not know if this result is reproducible.

I have prepared phosphatase-treated ribosomal subunits and lysate ribosomes treated with both phosphatase and ribonuclease as described in Materials and Methods. Although these preparations used different incubation conditions than the standard phosphatase assay, I have shown in control experiments that the extent of phosphoryl group removal is not greatly affected by such differences in ionic conditions. Therefore, I estimate that 50-75% of the protein phosphoryl groups were removed in the dephosphorylated ribosome

Figure 33. Phosphoryl group removal from [^{32}P]-labeled ribosomes by E. coli alkaline phosphatase. Ribosomes labeled with ^{32}P in their protein moieties were prepared as described in Materials and Methods. In panel A 50 µl aliquot of single ribosomes (S) (40 A_{260}/ml) was incubated at 37° and 10 µl samples were removed at the indicated times for acid precipitable ^{32}P determination. The incubations were (a) control, 0°; (b) control, 37°; (c) 9.4 µg/ml alkaline phosphatase; (d) 94 µg/ml alkaline phosphatase; (e) 470 µg/ml alkaline phosphatase. Panel B shows similar incubations with polysomes (P) (33 A_{260}/ml).



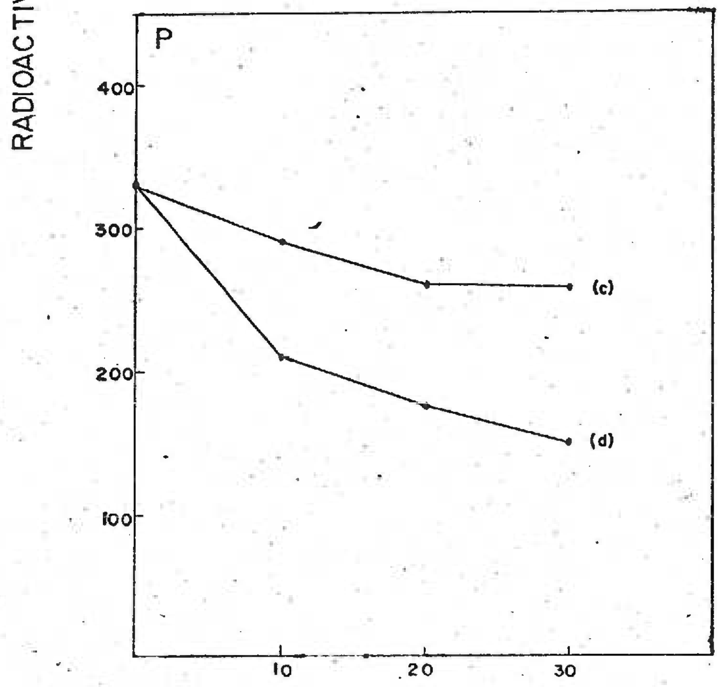


FIGURE 33

preparations used for assay. The assays to be described utilized a fractionated supernatant fraction (see Materials and Methods). This supernatant preparation, which still served efficiently as the elongation factor and synthetase source, had a reduced protein kinase activity as assayed by its ability to transfer phosphoryl groups from γ-[^{32}P]-ATP to histone and ribosome substrates. However, active kinases are also present in the initiation factor preparation.

Figure 34 shows an analyses of protein synthesis in the fractionated cell-free system by control and dephosphorylated ribonuclease-treated ribosomes and ribosomal subunits. In each case phosphoryl group removal diminished the rate of protein synthesis. In panel B this effect is seen to be greatest at early time points where the control preparation appeared twice as active. These experiments must be considered preliminary but suggest that the ribosome protein phosphoryl groups may be necessary for protein synthetic activity.

Figure 34. Protein synthesis in the fractionated cell-free system by control (c) and alkaline phosphatase treated ribosomes (P). The ribosome preparations used were ribosomal subunits (panel A) and ribonuclease-treated lysate ribosomes (panel B). The preparations are described in Materials and Methods. Alkaline phosphatase treatment to achieve dephosphorylation was with 470 μg/ml E. coli alkaline phosphatase, which should remove greater than 50% of the ribosomal protein phosphoryl groups. Assay of 0.1 A_{260} units ribosomes was as described in Figure 28 and Materials and Methods.

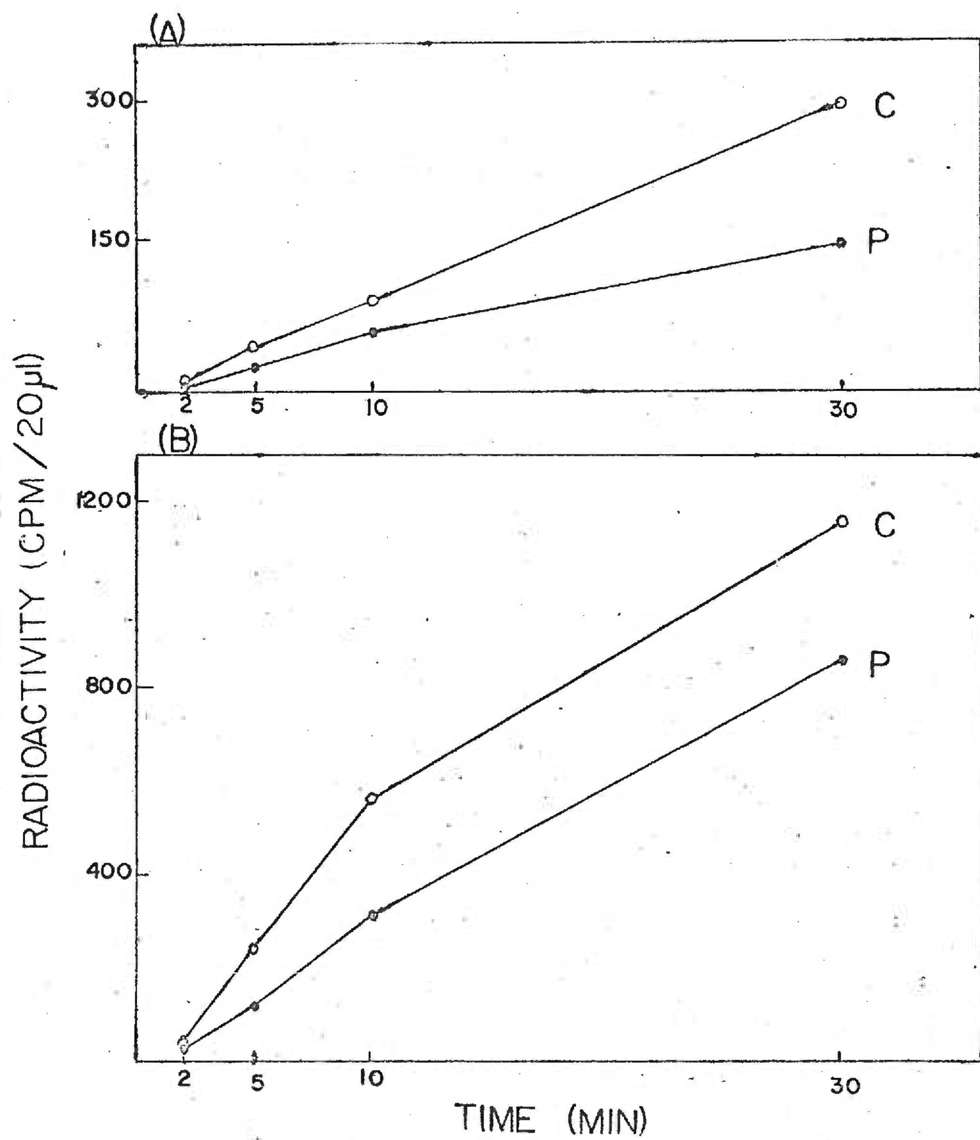


FIGURE 34

IV. Discussion

A. Structural Differences between the Subunits of Single Ribosomes and of Polysomes

I have prepared ribosomal subunits from rabbit reticulocyte ribosomes by a puromycin-salt dissociation method which has been used with success by other workers using several other cell types (196,202-206). Previous studies have used unfractionated ribosomes and an important feature of the present studies was the preliminary fractionation of ribosomes into active (polysomes) and inactive forms (single ribosomes). Dissociation of these two ribosome forms has revealed previously uncharacterized differences between their subunits. Specifically, I have obtained data showing that the large subunit of single ribosomes is probably structurally heterogeneous, and is more susceptible to forming unfolded derivative particles under ribosome destabilizing conditions than is the polysomal-derived large subunit. Another derivative particle, which may originate from the small subunit, forms to a greater extent from dissociated single ribosomes than from dissociated polysomes. These differences did not appear to be the result of a partial intracellular degradation of single ribosomes, since analysis of the RNAs of single ribosomes and polysomes gave a similar RNA profile (Figure 8).

Polysomal-derived small subunits aggregated to a greater

extent in high ionic strength than did the small subunits of single ribosomes. This subunit dimerization caused problems for the assay in the fractionated cell-free protein synthesis system. This small subunit dimerization problem has generally been encountered in studies on ribosomal subunits and was not overcome in the present studies.

Vournakis and Rich (267) have previously reported ultra-centrifigal studies showing size differences between single ribosomes and messenger-containing ribosomes. These differences may have been due to different configurations of ribosomes containing and lacking mRNA. However, the structural differences reported here are properties of the individual subunits rather than of the configuration of the ribosome couple.

B. Ribosomal Subunit Function in Cell-free Protein Synthesizing Systems

I have found that purified ribosomal subunits are active in two different cell-free protein synthesizing systems which use different criteria for activity. I have found the chief advantages of the lysate system to be the ability to directly observe the amount of added subunits which enter active polysomes and to compare the efficiency of these subunits to the endogenous pool of ribosomes participating in the subunit-polyribosome cycle of protein synthesis. Greater than 50% of ribosomal subunits prepared by dissociation at

either high or moderate ionic strength were able to enter polysomes and this entry showed the characteristics of normal and efficient protein synthesis.

Other workers have used this experimental design -- the entry of added radioactive subunits into ribosome classes in a protein synthesizing lysate in order to study aspects of ribosome function (55,91,257). However, the percentage of active subunits in the present investigation was greater than that of these previous studies. Also, the subunits of single ribosomes and of polysomes have not previously been compared in cell-free protein synthesizing systems.

Assay of ribosomal subunit protein synthetic activity in the fractionated system measures the ability of subunits to catalyze [^{14}C]-L-leucine incorporation programmed by purified globin mRNA. Protein synthesis by the ribosomal subunits is initiation factor- and messenger-dependent. Thus, both assay systems measure the ability of ribosomal subunits to translate natural mRNA, albeit by different criteria. Previous studies on the activity of purified ribosomal subunits have used poly(U) assays. The present work and recent studies by other workers (265,266,268) extend the criterion for ribosomal subunits to the ability to translate natural messenger -- a process which requires normal initiation of protein synthesis.

Only a small proportion of subunits were active in the

fractionated cell-free system. This differential utilization of ribosomes by crude and by fractionated protein synthesizing systems is an important point to emerge from these studies. Either the large or small subunit becomes inactivated in the fractionated system. Melcher and Mach (223) have reported data suggesting that small subunits are inactivated by the subunit preparative procedure. My data is consistent with this possibility. For example, the small subunit fraction often did not stimulate the extent of synthesis seen with the 60S fraction alone. Furthermore, the kinetics of entry experiments in the lysate system showed that added small subunits did not enter polysomes as fast as the endogenous pool of native subunits which rapidly reinitiate on polysomes (Figure 17). This may be indicative of an activation phase. Thus, ribosomes inactive in the fractionated system may become activated in the lysate system. The differing efficiencies of ribosome utilization in these two assay systems may be related to differing abilities of the components of these systems to repair "damaged" or inactivated ribosomes.

The nature of this ribosomal damage and possible repair mechanisms will now be considered. It seems clear that salt dissociation of ribosomes results in the loss of protein (204). However, the ribosomal nature of the lost protein has not been characterized, nor is data available on the stoichiometry of ribosomal proteins on

salt dissociated animal cell ribosomal subunits. The amount of protein removed from ribosomes by salt washing increases with increasing salt concentration; at a given salt concentration fractional release of specific proteins may occur (269,270). Dissociation procedures may yield a heterogeneous population of subunits deficient in different proteins. If certain of these fractional proteins are important for ribosome function with natural mRNA, the subunits would also be functionally heterogeneous.

My results in the fractionated system show that several ribosomal species are inactivated by salt treatment and that ribosomal subunits were more active if prepared at moderate rather than high ionic strength. This latter result is consistent with the work of Infante and Kraus (258) and of Mechler and Mach (223) who also showed similar effects of high salt treatment on ribosomal subunit activity. This activity effect may be due to the protein loss described above. However, Leader et al. have shown the efficient translation of viral mRNA by ribosomal subunits dissociated by a high (0.88M) KCl concentration (268). And Faust and Matthaei have shown equivalent activities in a poly(U) assay for ribosomal subunits dissociated by high and moderate ionic strengths (204).

Perhaps the crude lysate and the highly fractionated cell-free systems differ in the ability to reconstitute active subunits

from protein-deficient particles by a process of ribosomal protein exchange (271,272) between supernatant ribosomal proteins and the added ribosomal subunits. However, the existence of ribosomal protein exchange has been questioned (273) and this interpretation of ribosomal repair must be considered speculative. Possibly relevant to a consideration of ribosomal repair is the existence in the reticulocyte supernatant of protein kinase and perhaps other enzymes capable of modifying ribosomes; such activities are present in only small amounts in the fractionated cell-free system as compared to the crude lysate system.

C. Functional Differences between Polysomal- and Single Ribosome-derived Subunits

Assay of ribosomal subunits in the fractionated cell-free system has allowed me to show that polysomal derived subunits are more active than those derived from single ribosomes. This activity difference between polysomes and single ribosomes was also seen when ribonuclease-treated polysomes and ribonuclease-treated single ribosomes were assayed and when intact polysomes and single ribosomes were assayed in the presence of a saturating amount of added globin mRNA. The fractionated cell-free system is rate-limited at an initiation step in protein synthesis. The activity difference observed between the subunits of single ribosomes and polyribosomes must, therefore, be related to differing abilities to initiate

protein synthesis on mRNA. Even though the fractionated system is less efficient in ribosomal subunit utilization, I believe it is of greater discriminating power in detecting functional differences between different ribosome classes. By contrast the lysate system may modify (by the repair mechanisms discussed) added ribosomes, making difficult the detection of functional differences.

This demonstration of functional differences between polysomal- and single ribosome-derived subunits, together with the demonstration of structural differences between these subunits do not support the model of Kaempfer (67), which suggests that the subunits of single ribosomes are equivalent to those of polysomes. Rather, my results show that the subunits of these two ribosome classes are different in several ways and suggest that the regulation of ribosome activity may involve chemical and/or structural changes in the ribosome. Sundkvist and Howard (274) have recently reported comparative assays of ribosomal subunits prepared from different reticulocyte ribosome preparations. They used the fractionated protein synthesizing system of Schrier and Staehelin (265) and did not detect a functional difference between polysomal- and single ribosome-derived subunits. However, their methodology was different than that reported here and they did not report kinetic comparisons of the rate of protein synthesis by their subunit preparations.

Remaining questions concern the subunit localization of the defect in single ribosomes, the relation of the structural and functional differences between polysomes and single ribosomes, the mechanism of activation and inactivation of single ribosomes, the relation of the observed activity differences to the quantitative difference between polysomes and single ribosomes in their complement of protein phosphoryl groups, and the relevance of these studies on reticulocyte single ribosomes to the single ribosomes of other cells.

D. Function of Ribosomal Protein Phosphorylation

The preliminary experiments reported here suggest that ribosomal phosphoryl groups are needed for protein synthetic activity. Previous studies have failed to detect a need for the protein phosphoryl groups (167,180), but these studies were subject to experimental limitations as discussed elsewhere (167). In the present study I could not eliminate the possibility that the dephosphorylated ribosomes had been damaged (e.g., proteolytically) by incubation with the alkaline phosphatase preparation. To examine further the functional aspects of the phosphorylation of ribosomes will require improvements in the methodology for ribosome dephosphorylation and for ribosome assay in highly purified protein synthesizing systems.

References

1. Sherton, C. C. and Wool, I. G. Determination of the Number of Proteins in Liver Ribosomes and Ribosomal Subunits by Two-Dimensional Polyacrylamide Gel Electrophoresis. J. Biol. Chem., 1972. 247, 4460-4467.

2. Sherton, C. C. and Wool, I. G. A Comparison of the Proteins of Rat Skeletal Muscle and Liver Ribosomes by Two-Dimensional Polyacrylamide Gel Electrophoresis. Observations on the Partition of Proteins between Ribosomal Subunits and a Description of Two Acidic Proteins in the Large Subunit. J. Biol. Chem., 1974. 249, 2258-2267.

3. King, H. W. S., Gould, H. J. and Sherman, J. J. Molecular Weight Distribution of Proteins in Rabbit Reticulocyte Subunits. J. Mol. Biol., 1971. 61, 143-156.

4. Martini, O. W. and Gould, H. J. Enumeration of Rabbit Reticulocyte Ribosomal Proteins. J. Mol. Biol., 1971. 62, 403-405.

5. Gould, H. J., Martini, O. H. W. and King, H. S. W. 80S Ribosomal Proteins. Biochem. J., 1972. 129, 31P-32P (Abstract).

6. Delauney, J., Mathieu, C. and Shapira, G. Eukaryotic Ribosomal Proteins. Interspecific and Intraspecific Comparisons by Two-Dimensional Polyacrylamide Gel Electrophoresis. Eur. J. Biochem., 1972. 31, 561-564.

7. Welfe, H., Stahl, J. and Bielka, H. Studies on Proteins of Animal Ribosomes XIII. Enumeration of Ribosomal Proteins of Rat Liver. Fed. Eur. Biochem. Soc. Lett., 1972. 26, 228-232.

8. Delauney, J. and Shapira, G. Rat Liver and Hepatoma Ribosomal Proteins. Two Dimensional Polyacrylamide Gel Electrophoresis. Biochim. Biophys. Acta, 1972. 259, 243-246.

9. Maden, B. E. H. The Structure and Formation of Ribosomes in Animal Cells. Prog. Biophys. & Mol. Biol., 1971. 22, 129-177.

[illegible]cas-Lenard, J. and Lipmann, F. Protein Biosynthesis. Ann. [illegible]chem., 1971. 40, 409-448.

[illegible] and Rothman-Denes, L. B. Protein Synthesis. Ann. [illegible]., 1973. 42, 397-438.

12. Lipmann, F. Polypeptide Chain Elongation in Protein Biosynthesis. Science, 1969. 164, 1024-1031.

13. The Mechanism of Protein Synthesis. Cold Spring Harbor Symp. Quant. Biol., 1969, 34.

14. Spirin, A. S. and Gavrilova, L. P. The Ribosomes. Berlin, New York: Springer, 1969.

15. Petermann, M. L. The Physical and Chemical Properties of Ribosomes. Amsterdam, New York: Elsevier, 1964.

16. Spirin, A. S. Structural Transformations of Ribosomes (Dissociation, Unfolding and Dissassembly). Fed. Eur. Biochem. Soc. Lett., 1974. 40, S38-S47.

17. Pongs, O., Nierhaus, K. H., Erdmann, V. A. and Wittmann, H. G. Active Sites in Escherichia Coli Ribosomes. Fed. Eur. Biochem. Soc. Lett., 1974. 40, S28-S37.

18. Kurland, C. G. Ribosome Structure and Function Emergent. Science, 1970. 169, 1171-1177.

19. Kurland, C. G. Structure and Function of the Bacterial Ribosome. Ann. Rev. Biochem., 1972. 41, 377-408.

20. Maclean, N. and Jurd, R. D. The Control of Hemoglobin Synthesis. Biol. Rev., 1972. 47, 393-437.

21. Watson, J. D. Involvement of RNA in the Synthesis of Proteins. Science, 1963. 140, 17-26.

22. Schlessinger, D. Ribosomes: Development of Some Current Ideas. Bacteriol. Rev., 1969. 33, 445-453.

23. Zamecnik, P. C. An Historical Account of Protein Synthesis, with Current Overtones -- A Personalized View. Cold Spring Harbor Symp. Quant. Biol., 1969. 34, 1-16.

24. Warner, J. R., Knopf, P. M. and Rich, A. A Multiple Ribosomal Structure in Protein Synthesis. Proc. Nat. Acad. Sci. USA, 1963. 49, 122-129.

25. Warner, J. R., Rich, A. and Hall, C. E. Electron Electroscope Studies of Ribosomal Clusters Synthesizing Hemoglobin. Science, 1962. 138, 1399-1403.

26. Goodman, H. M. and Rich, A. Mechanism of Polyribosome Action during Protein Synthesis. Nature, 1963. 199, 318-322.

27. Rich, A., Warner, J. R. and Goodman, H. M. The Structure and Function of Polyribosomes. Cold Spring Harbor Symp. Quant. Biol., 1963. 28, 269-285.

28. Penman, S., Scherrer, K., Becker, Y. and Darnell, J. E. Polyribosomes in Normal and Poliovirus HeLa Cells and Their Relationship to Messenger-RNA. Proc. Nat. Acad. Sci. USA, 1963. 49, 654-662.

29. Marks, P. A., Burka, E. R. and Schlessinger, D. Protein Synthesis in Erythroid Cells I. Reticulocyte Ribosomes Active in Stimulating Amino Acid Incorporation. Proc. Nat. Acad. Sci. USA, 1962. 48, 2163-2171.

30. Staehelin, T., Brinton, C. C., Wettstein, F. O. and Noll, H. Nature, 1963. 199, 865-870.

31. Gierer, A. Function of Aggregated Reticulocyte Ribosomes in Protein Synthesis. J. Mol. Biol., 1963. 6, 148-157.

32. Mathias, A. P., Williamson, R., Huxley, H. E. and Page, S. Occurrence and Function of Polysomes in Rabbit Reticulocytes. J. Mol. Biol., 1964. 9, 154-167.

33. Wettstein, F. O., Staehelin, T. and Noll, H. Ribosomal Aggregate Engaged in Protein Synthesis: Characterization of the Ergosome. Nature, 1963. 197, 430-435.

34. Clark, M. F., Matthews, R. E. F. and Ralph, R. K. Polyribosomes in Leaves. Biochem. Biophys. Res. Commun., 1963. 13, 505-509.

35. Risebrough, R. W., Tissières, A. and Watson, J. D. Messenger-RNA Attachment to Active Ribosomes. Proc. Nat. Acad. Sci. USA, 1962. 48, 430-436.

36. Gilbert, W. Polypeptide Synthesis in Escherichia coli I. Ribosomes and the Active Complex. J. Mol. Biol., 1963. 6, 374-388.

37. Mangiarotti, G. and Schlessinger, D. Polyribosome Metabolism in Escherichia coli II. Formation and Lifetime of Messenger RNA Molecules, Ribosomal Subunit Couples and Polyribosomes. J. Mol. Biol., 1967. 29, 395-418.

38. Subramanian, A. R., Davis, B. D. and Beller, R. J. The Ribosome Dissociation Factor and the Ribosome-Polysome Cycle. Cold Spring Harbor Symp. Quant. Biol., 1969. 34, 223-230.

39. Hogan, B. L. M. and Korner, A. Ribosomal Subunits of Landshütz Ascites Cells during Changes in Polysome Distribution. Biochim. Biophys. Acta, 1968. 169, 129-138.

40. Bishop, J. O. Initiation of Polypeptide Chains. Biochim. Biophys. Acta, 1966. 119, 130-145.

41. Colombo, G., Vesco, C. and Baglioni, C. Role of Ribosomal Subunits in Protein Synthesis in Mammalian Cells. Proc. Nat. Acad. Sci. USA, 1968. 61, 651-658.

42. Joklik, W. K. and Becker, Y. Studies on the Genesis of Polyribosomes I. Origin and Significance of the Subribosomal Particle. J. Mol. Biol., 1965. 13, 496-510.

43. Girard, M., Latham, H., Penman, S. and Darnell, J. E. Entrance of Newly Formed Messenger RNA and Ribosomes into HeLa Cell Cytoplasm. J. Mol. Biol., 1965. 11, 187-201.

44. Ristow, H. and Köhler, K. Polysomal Precursors in KB Cells. Biochim. Biophys. Acta, 1966. 123, 265-273.

45. Hogan, B. L. M. and Korner, A. The Role of Ribosomal Subunits and 80-S Monomers in Polysome Formation in an Ascites Tumor Cell. Biochim. Biophys. Acta, 1968. 169, 139-149.

46. Chaudhuri, S. and Liberman, I. Time Required by the Normal and Regenerating Rat Liver Cell to Make a Ribosome. J. Mol. Biol., 1968. 33, 323-326.

47. Kabat, D. and Rich, A. The Ribosomal Subunit-Polyribosome Cycle in Protein Synthesis of Embryonic Skeletal Muscle. Biochemistry, 1969. 8, 3742-3749.

48. Nomura, M. and Lowry, C. V. Phage F2 RNA-Directed Binding of Formylmethionyl-tRNA to Ribosomes and the Role of 30S Ribosomal Subunits in Initiation of Protein Synthesis. Proc. Nat. Acad. Sci. USA, 1967. 58, 946-953.

49. Nomura, M., Lowry, C. V. and Guthrie, C. The Initiation of Protein Synthesis: Joining of the 50S Ribosomal Subunit to the Initiation Complex. Proc. Nat. Acad. Sci. USA, 1967. 58, 1487-1493.

50. Guthrie, C. and Nomura, M. Initiation of Protein Synthesis: A Critical Test of the 30S Subunit Model. Nature, 1968. 219, 232-235.

51. Grubman, M. J. and Nakada, D. Ribosomal Subunits and MS2 Phage RNA-directed Protein Synthesis. Nature, 1969. 223, 1242-1247.

52. Heywood, S. M. Formation of the Initiation Complex Using Muscle Messenger RNAs. Nature, 1970. 225, 696-698.

53. Heywood, S. M. and Thompson, W. C. Studies on the Formation of the Initiation Complex in Eucaryotes. Biochem. Biophys. Res. Commun., 1971. 43, 470-475.

54. Cashion, L. M. and Stanley, W. M. jr. Two Eukaryotic Initiation Factors (IF-I and IF-II) of Protein Synthesis that are Required to Form an Initiation Complex with Rabbit Reticulocyte Ribosomes. Proc. Nat. Acad. Sci. USA, 1974. 71, 436-440.

55. Baglioni, C., Vesco, C. and Jacobs-Lorena, M. The Role of Ribosomal Subunits in Mammalian Cells. Cold Spring Harbor Symp. Quant. Biol., 1969. 34, 555-565.

56. Baglioni, C. Heterogeneity of the Small Ribosomal Subunit and Mechanism of Chain Initiation in Eukaryotes. Biochim. Biophys. Acta, 1972. 287, 189-193.

57. Mullock, B. M., Hinton, R. H., Dobrota, M., Froomberg, D. and Reid, E. Subribosomal Particles Separated from Liver and Hepatoma Homogenates with a Zonal Rotor. Eur. J. Biochem., 1971. 18, 485-495.

58. Henshaw, E. C. and Lobenstein, J. Rapidly Labeled Polydisperse RNA in Rat-Liver Cytoplasm: Evidence That It Is Contained in Ribonucleoprotein Particles. Biochim. Biophys. Acta, 1970. 199, 405-420.

59. Hirsch, C. A., Cox, M. A., van Venrooij, W. J. W. and Henshaw, E. C. The Ribosome Cycle in Mammalian Protein Synthesis II. Association of the Native Smaller Subunit with Protein Factors. J. Biol. Chem., 1973. 248, 4377-4385.

60. Ayuso-Parilla, M., Henshaw, E. C. and Hirsch, C. A. The Ribosome Cycle in Mammalian Protein Synthesis III. Evidence that the Nonribosomal Proteins Bound to the Native Smaller Subunit are Initiation Factors. J. Biol. Chem., 1973. 248, 4386-4393.

61. Zehavi-Willner, T. and Danon, D. Complexes of Messenger RNA and 40-S Subunit in Rabbit Reticulocytes. Eur. J. Biochem., 1973. 33, 258-264.

62. Darnbrough, C., Hunt, T. and Jackson, R. J. A Complex between Met-tRNA$_f$ and Native 40S Subunits in Reticulocyte Lysates and its Disappearance during Incubation with Double-Stranded RNA. Biochem. Biophys. Res. Commun., 1972. 48, 1556-1564.

63. Legon, S., Jackson, R. J. and Hunt, T. Control of Protein Synthesis in Reticulocyte Lysates by Hemin. Nature New Biol., 1973. 241, 150-152.

64. Noll, M. and Noll, H. Mechanism and Control of Initiation in the Translation of R17 RNA. Nature New Biol., 1972. 238, 225-228.

65. Schreier, M. H. and Staehelin, T. Initiation of Eukaryotic Protein Synthesis: [Met-tRNA$_f$·40S Ribosome] Initiation Complex Catalysed by Purified Initiation Factors in the Absence of mRNA. Nature New Biol., 1973. 242, 35-38.

66. Kaempfer, R. Control of Single Ribosome Formation by an Initiation Factor for Protein Synthesis. Proc. Nat. Acad. Sci. USA, 1971. 68, 2458-2462.

67. Kaempfer, R. and Kaufman, J. Translational Control of Hemoglobin Synthesis by an Initiation Factor Required for Recycling of Ribosomes and for their Binding to Messenger RNA. Proc. Nat. Acad. Sci. USA, 1972. 69, 3317-3321.

68. Olsen, G. D., Gaskill, P. and Kabat, D. Presence of Hemoglobin Messenger Ribonucleoprotein in a Reticulocyte Supernatant Fraction. Biochim. Biophys. Acta, 1972. 272, 297-304.

69. Terada, M., Metafora, S., Banks, J., Dow, L. W., Bank, A. and Marks, P. A. Conservation of Globin Messenger RNA in Rabbit Reticulocyte Monoribosomes after Sodium Fluoride Treatment. Biochem. Biophys. Res. Commun., 1972. 47, 766-774.

70. Davis, B. D. Role of Subunits in the Ribosome Cycle. Nature, 1971. 231, 153-157.

71. Kaempfer, R. O. R., Meselson, M. and Raskas, H. J. Cyclic Dissociation into Stable Subunits and Re-formation of Ribosomes during Bacterial Growth. J. Mol. Biol., 1968. 31, 277-289.

72. Kaempfer, R. Ribosomal Exchange in the Cytoplasm of a Eukaryote. Nature, 1969. 222, 950-953.

73. Gaskill, P. and Kabat, D. Unexpectedly Large Size of Globin Messenger Ribonucleic Acid. Proc. Nat. Acad. Sci. USA, 1971. 68, 72-75.

74. Evans, M. J. and Lingrel, J. B. Hemoglobin Messenger Ribonucleic Acid. Synthesis of 9S and Ribosomal Ribonucleic Acid during Erythroid Cell Development. Biochemistry, 1969. 8, 3000-3005.

75. Laycock, D. G. and Hunt, J. A. Synthesis of Rabbit Globin by a Bacterial Cell Free System. Nature, 1969. 221, 1118-1122.

76. Heywood, S. M. and Nwagwu, M. DeNovo Synthesis of Myosin in a Cell-Free System. Proc. Nat. Acad. Sci. USA, 1968. 60, 229-234.

77. Stanners, C. P. and Becker, H. Control of Macromolecular Synthesis in Proliferating and Resting Syrian Hamster Cells in Monolayer Culture I. Ribosome Function. J. Cell. Physiol., 1971. 77, 31-42.

78. Kay, J. E., Ahern, T. and Atkins, M. Control of Protein Synthesis during the Activation of Lymphocytes by Phytohaemagglutinin. Biochim. Biophys. Acta, 1971. 247, 322-334.

79. Danon, D., Zehavi-Willner, T. and Berman, G. R. Alterations in Polyribosomes of Reticulocytes Maturing <u>In Vivo</u>. Proc. Nat. Acad. Sci. USA, 1965. 54, 873-879.

80. Marks, P. A., Rifkind, R. A. and Danon, D. Polyribosomes and Protein Synthesis during Reticulocyte Maturation <u>In Vitro</u>. Proc. Nat. Acad. Sci. USA, 1963. 50, 336-342.

81. Marbaix, G., Burny, A., Huez, G., Lebleu, B. and Temmerman, J. Evolution of the Polyribosome Distribution during <u>In Vivo</u> Reticulocyte Maturation. Eur. J. Biochem., 1970. 13, 322-325.

82. Rifkind, R. A., Danon, D. and Marks, P. A. Alterations in Polyribosomes during Erythroid Cell Maturation. J. Cell Biol., 1964. 22, 599-611.

83. Subramanian, A. R., Ron, E. Z. and Davis, B. D. A Factor Required for Ribosome Dissociation in Escherichia Coli. Proc. Nat. Acad. Sci. USA, 1968. 61, 761-767.

84. Lubsen, N. H. and Davis, B. D. A Ribosome Dissociation Factor from Rabbit Reticulocytes. Proc. Nat. Acad. Sci. USA, 1972. 69, 353-357.

85. Merrick, W. C., Lubsen, N. H. and Anderson, W. F. A Ribosome Dissociation Factor from Rabbit Reticulocytes Distinct from Initiation Factor M_3. Proc. Nat. Acad. Sci. USA, 1973. 70, 2220-2223.

86. Lubsen, L. H. and Davis, B. D. A Ribosome Dissociation Factor on Both Native Subunits in Rabbit Reticulocytes. Biochim. Biophys. Acta, 1974. 335, 196-200.

87. Lawford, C. R., Kaiser, J. and Hey, W. C. A Factor Capable of Dissociating Rat Liver Ribosomes. Can. J. Biochem., 1971. 49, 1301-1306.

88. Petre, J. Specific Dissociation of Ribosomes from Saccharomyces Cerevisiae by a Protein Factor. Eur. J. Biochem., 1970. 14, 399-405.

89. Adamson, S. D., Howard, G. A. and Herbert, E. The Ribosome Cycle in a Reconstituted Cell-Free System from Reticulocytes. Cold Spring Harbor Symp. Quant. Biol., 1969. 34, 547-554.

90. Howard, G. A., Adamson, S. D. and Herbert, E. Subunit Recycling during Translation in a Reticulocyte Cell-free System. J. Biol. Chem., 1970. 245, 6237-6239.

91. Henshaw, E. C., Guiney, D. G. and Hirsch, C. A. The Ribosome Cycle in Mammalian Protein Synthesis I. The Place of Monomeric Ribosomes and Ribosomal Subunits in the Cycle. J. Biol. Chem., 1973. 248, 4367-4376.

92. Jacob, F. and Monod, J. Genetic Regulatory Mechanisms in the Synthesis of Protein. J. Mol. Biol., 1961. 3, 318-356.

93. Gros, F. Control of Gene Expression in Procaryotic Systems. Fed. Eur. Biochem. Soc. Lett., 1974. 40, S19-S27.

94. Singer, R. H. and Penman, S. Stability of HeLa Cell mRNA in Actinomycin. Nature, 1972. 240, 100-102.

95. Greenberg, J. R. High Stability of Messenger RNA in Growing Cultured Cells. Nature, 1972. 240, 102-104.

96. Pain, V. M. and Clemens, M. J. The Role of Soluble Protein Factors in the Translational Control of Protein Synthesis in Eukaryotic Cells. Fed. Eur. Biochem. Soc. Lett., 1973. 32, 205-212.

97. Palmiter, R. D. Regulation of Protein Synthesis in Chick Oviduct II. Modulation of Polypeptide Elongation and Initiation Rates by Estrogen and Progesterone. J. Biol. Chem., 1972. 247, 6770-6780.

98. Fan, H. and Penman, S. Regulation of Protein Synthesis in Mammalian Cells II. Inhibition of Protein Synthesis at the Level of Initiation during Mitosis. J. Mol. Biol., 1970. 50, 655-670.

99. McCormick, W. and Penman, S. Regulation of Protein Synthesis in HeLa Cells: Translation at Elevated Temperatures. J. Mol. Biol., 1969. 39, 315-333.

100. Steward, D. L., Schaeffer, J. R. and Humphrey, R. M. Breakdown and Assembly of Polyribosomes in Synchronized Chinese Hamster Cells. Science, 1968. 161, 791-793.

101. Salb, J. M. and Marcus, P. I. Translational Inhibition in Mitotic HeLa Cells. Proc. Nat. Acad. Sci. USA, 1965. 54, 1353-1358.

102. Scharff, M. D. and Robbins, E. Polysome Dissaggregation during Metaphase. Science, 1966. 151, 992-995.

103. Vaughan, M. H. jr., Pawlowski, P. J. and Forchhammer, S. Regulation of Protein Synthesis in HeLa Cells Deprived of Single Amino Acids. Proc. Nat. Acad. Sci. USA, 1971. 68, 2057-2061.

104. Baliga, B. S., Pronczuk, A. W. and Munro, H. N. Regulation of Polysome Aggregation in a Cell-free System through Amino Acid Supply. J. Mol. Biol., 1968. 34, 199-218.

105. Eliason, E., Bauer, G. E. and Hultin, T. Reversible Degradation of Polyribosomes in Chang Cells Cultured in a Glutamine-Deficient Medium. J. Cell Biol., 1967. 33, 287-297.

106. Hodge, L. D., Robbins, E. and Scharff, M. D. Persistence of Messenger RNA through Mitosis in HeLa Cells. J. Cell Biol., 1969. 40, 497-507.

107. Metafora, S., Felicetti, L. and Gambino, R. The Mechanism of Protein Synthesis Activation after Fertilization of Sea Urchin Eggs. Proc. Nat. Acad. Sci. USA, 1971. 68, 600-604.

108. Gambino, R., Metafora, S., Felicetti, L. and Raisman, J. Properties of the Ribosomal Salt Wash from Unfertilized and Fertilized Sea Urchin Eggs and Its Effect on Natural mRNA Translation. Biochim. Biophys. Acta, 1973. 312, 377-391.

109. Hille, M. B. Inhibitor of Protein Synthesis Isolated from Ribosomes of Unfertilised Eggs and Embryos of Sea Urchins. Nature, 1974. 249, 556-558.

110. Palmiter, R. D. and Haines, M. E. Regulation of Protein Synthesis in Chick Oviduct IV. Role of Testosterone. J. Biol. Chem., 1973. 248, 2107-2116.

111. van Venrooij, W. J. W., Henshaw, E. C. and Hirsch, C. A. Nutritional Effects on the Polyribosome Distribution and Rate of Protein Synthesis in Ehrlich Ascites Tumor Cells in Culture. J. Biol. Chem., 1970. 245, 5947-5953.

112. Henshaw, E. C., Hirsch, C. A., Morton, B. E. and Hiatt, H. H. Control of Protein Synthesis in Mammalian Tissues through Changes in Ribosome Activity. J. Biol. Chem., 1971. 246, 436-446.

113. Ames, B. N. and Hartman, P. E. The Histidine Operon. Cold Spring Harbor Symp. Quant. Biol., 1963. 28, 349-356.

114. Strehler, B. L., Hendley, D. D. and Hirsch, G. P. Evidence on a Codon Restriction Hypothesis of Cellular Differentiation: Multiplicity of Mammalian Leucyl-SRNA-Specific Synthetases and Tissue-Specific Deficiency in an Alanyl-SRNA Synthetase. Proc. Nat. Acad. Sci., USA, 1967. 57, 1751-1758.

115. Sueko, N. and Kano-Sueko, T. Transfer RNA and Cell Differentiation. Prog. Nucleic Acid Res. & Mol. Biol., 1970. 10, 23-55.

116. Heywood, S. M. Specificity of mRNA Binding Factor in Eukaryotes. Proc. Nat. Acad. Sci. USA, 1970. 67, 1782-1788.

117. Heywood, S. M., Kennedy, D. S. and Bester, A. J. Separation of Specific Initiation Factors Involved in the Translation of Myosin and Myoglobin Messenger RNAs and the Isolation of a New RNA Involved in Translation. Proc. Nat. Acad. Sci. USA, 1974. 71, 2428-2431.

118. Wigle, D. T. and Smith, A. E. Specificity in Initiation of Protein Synthesis in a Fractionated Mammalian Cell-free System. Nature New Biol., 1973. 242, 136-140.

119. Nudel, U., Lebleu, B. and Revel, M. Discrimination between Messenger Ribonucleic Acids by a Mammalian Translation Initiation Factor. Proc. Nat. Acad. Sci., USA, 1973. 70, 2139-2144.

120. Rowley, P. T. and Kosciolek, B. Distinction between Two Types of Beta-Thalassaemia by Inducibility of the Cell-Free Synthesis of Beta-Chains by Non-thalassaemic Soluble Fraction. Nature New Biol., 1972. 239, 234-235.

121. Conconi, F., Rowley, P. T., Del Senno, L., Pontremoli, S. and Volpato, S. Induction of β-Globin Synthesis in the β-Thalassaemia of Ferrara. Nature New Biol., 1972. 238, 83-87.

122. Picciano, D. J., Prichard, P. M., Merrick, W. C., Shafritz, D. A., Graf, H., Crystal, R. G. and Anderson, W. F. Isolation of Protein Synthesis Initiation Factors from Rabbit Liver. J. Biol. Chem., 1973. 248, 204-214.

123. von Der Decken, A. Activation of Rat Liver Polyribosomes. J. Cell. Biol., 1969. 43, 138-147.

124. Hoagland, M. B., Scornick, O. A. and Pfefferkorn, L. C. Aspects of Control of Protein Synthesis in Normal and Regenerating Rat Liver II. A Microsomal Inhibitor of Amino Acid Incorporation Whose Action is Antagonized by Guanosine Triphosphate. Proc. Nat. Acad. Sci., USA, 1964. 51, 1184-1191.

125. Content, J., Lebleu, B., Zilberstein, A., Berissi, H. and Revel, M. Mechanism of the Interferon-Induced Block of mRNA Translation in Mouse L Cells: Reversal of the Block by Transfer RNA. Fed. Eur. Biochem. Soc. Lett., 1974. 41, 125-130.

126. Samuel, C. E. and Joklik, W. K. A Protein Synthesizing System from Interferon-Treated Cells that Discriminates between Cellular and Viral Messenger RNAs. Virology, 1974. 58, 476-491.

127. Marcus, P. I. and Salb, J. M. Molecular Basis of Interferon Action: Inhibition of Viral RNA Translation. Virology, 1966. 30, 505-516.

128. Monroy, A. and Tyler, A. Formation of Active Ribosomal Aggregates (Polysomes) upon Fertilization and Development of Sea Urchin Eggs. Arch. Biochem. Biophys., 1963. 103, 431-435.

129. Infante, A. A. and Nemer, M. Accumulation of Newly Synthesized RNA Templates in a Unique Class of Polyribosomes during Embryogenesis. Proc. Nat. Acad. Sci., USA, 1967. 58, 681-688.

130. Rinaldi, A. M. and Monroy, A. Polyribosome Formation and RNA Synthesis in Early Post-Fertilization Stages of the Sea Urchin Egg. Develop. Biol., 1969. 19, 73-86.

131. Lubsen, N. H. and Davis, B. D. Use of Purified Polysomes from Rabbit Reticulocytes in a Specific Test for Initiation Factors. Proc. Nat. Acad. Sci., USA, 1974. 71, 68-72.

132. Lee, S. Y., Krsmanovic, V. and Brawerman, G. Initiation of Polysome Formation in Mouse Sarcoma 180 Ascites Cells. Utilization of Cytoplasmic Messenger Ribonucleic Acid. Biochemistry, 1971. 10, 895-900.

133. Hori, M., Fisher, J. and Rabinovitz, M. Tryptophan Deficiency in Rabbit Reticulocytes: Polyribosomes during Interrupted Growth of Hemoglobin Chains. Science, 1967. 155, 83-84.

134. Staehelin, T., Verney, E. and Sidransky, H. The Influence of Nutritional Change of Polyribosomes of the Liver. Biochim. Biophys. Acta, 1967. 145, 105-119.

135. Bitte, L. F. Phosphorylation of Ribosomal Proteins in Sarcoma-180 Tumor Cells. Ph. D. Dissertation, University of Oregon Medical School, 1973.

136. Muro, A. J., Jackson, R. J. and Korner, A. Studies on the Nature of Polysomes. Biochem. J., 1964. 92, 289-299.

137. Hardesty, B., Hutton, J. J., Arlinghaus, R. and Schweet, R. Polyribosome Formation and Hemoglobin Synthesis. Proc. Nat. Acad. Sci., USA, 1963. 50, 1078-1085.

138. Lamfrom, H. and Knopf, P. M. Initiation of Hemoglobin Synthesis in Cell-free Systems. J. Mol. Biol., 1964. 9, 558-575.

139. Rodgers, A. Ribosomal Proteins in Rapidly Growing and Non-Proliferating Mouse Cells. Biochim. Biophys. Acta, 1973. 294, 292-296.

140. Craven, G. R., Voynow, P., Hardy, S. J. S. and Kurland, C. G. The Ribosomal Proteins of Escherichia Coli II. Chemical and Physical Characterization of the 30S Ribosomal Proteins. Biochemistry, 1969. 8, 2906-2915.

141. Deusser, E. and Wittman, H. G. Ribosomal Proteins: Variation of the Protein Composition in Escherichia coli Ribosomes as Function of Growth Rate. Nature, 1972. 238, 269-270.

142. Bickle, T. A., Howard, G. A. and Traut, R. R. Ribosome Heterogeneity. The Nonuniform Distribution of Specific Ribosomal Proteins among Different Functional Classes. J. Biol. Chem., 1973. 248, 4862-4864.

143. Van Duim, J. and Van Knippenberg, P. H. Functional Heterogeneity of the 30S Ribosomal Subunits of Escherichia Coli III. Requirement of Protein S1 for Translation. J. Mol. Biol., 1974. 84, 185-195.

144. Barden, N. and Korner, A. A Defect in the 40S Ribosomal Subunit after Hypophysectomy of the Rat. Biochem. J., 1969. 114, 30P (Abstract).

145. Martin, T. E. and Wool, I. G. Formation of Active Hybrids from Subunits of Muscle Ribosomes from Normal and Diabetic Rats. Proc. Nat. Acad. Sci., USA, 1968. 60, 569-574.

146. Rapoport, S. M., Rosenthal, S., Schewe, T., Schultze, M. and Miller, M. The Metabolism of the Reticulocyte. In H. Yoshikawa and S. M. Rapoport (Eds.) Cellular and Molecular Biology of Erythrocytes. Baltimore, London, Tokyo: University Park Press, 1974. pp. 93-141.

147. Lingrel, J. B. and Borsook, H. A Comparison of Amino Acid Incorporation into the Hemoglobin and Ribosomes of Mature Erythroid Cells and Circulating Reticulocytes of Severely Anemic Rabbits. Biochemistry, 1963. 2, 309-314.

148. Borsook, H., Deasy, C. L., Haagen-Smit, Keighley, G. and Lowy, P. H. Incorporation In Vitro of Labelled Amino Acids into Proteins of Rabbit Reticulocytes. J. Biol. Chem., 1952. 196, 669-694.

149. Stohlman, F. jr. and Brecher, G. Regenerative Macrocytosis in Experimental Phenylhydrazine Anemia. J. Clin. Invest., 1961. 40, 1084 (Abstract).

150. Borsook, H. A Picture of Erythropoiesis at the Combined Morphologic and Molecular Levels. Blood, 1964. 24, 202-207.

151. Marks, P. A., Wilson, C., Kruh, J. and Gros, F. Unstable Ribonucleic Acid in Mammalian Red Blood Cells. Biochem. Biophys. Res. Commun., 1964. 8, 9-14.

152. Woodward, W. R., Adamson, S. D., McQueen, H. M., Larson, J. W., Estavik, S. M., Wilairat, P. and Herbert, E. Globin Synthesis on Reticulocyte Membrane-bound Ribosomes. J. Biol. Chem., 1973. 248, 1556-1561.

153. Hunt, R. T., Hunter, A. R. and Munro, A. J. Control of Haemoglobin Synthesis: A Difference in the Size of the Polysomes Making α and β Chains. Nature, 1968. 220, 481-483.

154. Marks, P. A., Burka, E. R., Conconi, F. M., Perl, W. and Rifkind, R. A. Polyribosome Dissociation and Formation in Intact Reticulocytes with Conservation of Messenger Ribonucleic Acid. Proc. Nat. Acad. Sci., USA, 1965. 53, 1437-1443.

155. Bertles, J. F. and Beck, W. S. Biochemical Aspects of Reticulocyte Maturation I. Fate of the Ribonucleic Acid. J. Biol. Chem., 1962. 237, 3770-3777.

156. Miller, A. and Maunsbach, A. B. Electron Microscopic Autoradiography of Rabbit Reticulocytes Active and Inactive in Protein Synthesis. Science, 1966. 151, 1000-1001.

157. Rowley, P. T. and Morris, J. A. Protein Synthesis in the Maturing Reticulocyte. J. Biol. Chem., 1967. 242, 1533-1540.

158. Rowley, P. T., Midthun, R. A. and Adams, M. H. Solubilization of a Reticulocyte Ribosomal Fraction Responsible for the Decline in Ribosomal Activity with Cell Maturation. Arch. Biochem. Biophys., 1971. 145, 6-15.

159. Herzberg, M., Revel, M. and Danon, D. The Influence of Ribosomal Factors during the Maturation of Reticulocytes. Eur. J. Biochem., 1969. 11, 148-153.

160. Kabat, D. Phosphorylation of Ribosomal Proteins in Rabbit Reticulocytes. Characterization and Regulatory Aspects. Biochemistry, 1970. 9, 4160-4175.

161. Bitte, L. and Kabat, D. Phosphorylation of Ribosomal Proteins in Sarcoma 180 Tumor Cells. J. Biol. Chem., 1972. 247, 5345-5350.

162. Loeb, J. E. and Blat, C. Phosphorylation of Some Rat Liver Ribosomal Proteins and Its Activation by Cyclic AMP. Fed. Eur. Biochem. Soc. Lett., 1970. 10, 105-108.

163. Blat, C. and Loeb, J. Effect of Glucagon on Phosphorylation of Some Rat Liver Ribosomal Proteins _in Vivo_. Fed. Eur. Biochem. Soc. Lett., 1971. 18, 124-126.

164. Majumder, G. C. and Turkington, R. W. Hormone-dependent Phosphorylation of Ribosomal and Plasma Membrane Proteins in Mouse Mammary Gland _in Vitro_. J. Biol. Chem., 1972. 247, 7207-7217.

165. Correze, C., Pinell, P. and Nunez, J. Effects of Thyroid Hormones on Phosphorylation of Liver Ribosomal Proteins and on Protein Phosphokinase Activity. Fed. Eur. Biochem. Soc. Lett., 1972. 23, 87-91.

166. Krystosek, A., Bitte, L. F. and Kabat, D. Phosphorylation of Ribosomal Proteins in Higher Organisms. In E. H. Fischer, E. G. Krebs, H. Neurath and E. R. Stadtman (Eds.) Metabolic Interconversion of Enzymes 1973. Berlin, Heidelberg, New York: Springer, 1974. pp. 165-173.

167. Krystosek, A., Bitte, L. F., Cawthon, M. L. and Kabat, D. Phosphorylation of Ribosomal Proteins in Eukaryotes. In A. Tissieres, P. Lengyel, and M. Nomura (Eds.) Ribosomes. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory, In Press.

168. Kabat, D. Turnover of Phosphoryl Groups in Reticulocyte Ribosomal Phosphoproteins. J. Biol. Chem., 1972. 247, 5338-5344.

169. Kabat, D. Phosphorylation of Ribosomal Proteins in Rabbit Reticulocytes. A Cell-Free System with Ribosomal Protein Kinase Activity. Biochemistry, 1971. 10, 197-203.

170. Traugh, J. A., Mumby, M. and Traut, R. R. Phosphorylation of Ribosomal Proteins by Substrate-Specific Protein Kinases from Rabbit Reticulocytes. Proc. Nat. Acad. Sci., USA, 1973. 70, 373-376.

171. Traugh, J. A. and Traut, R. R. Characterization of Protein Kinases from Rabbit Reticulocytes. J. Biol. Chem., 1974. 249, 1207-1212.

172. Tao, M., Salas, M. L., and Lipmann, F. Mechanism of Activation by Adenosine 3':5'-Cyclic Monophosphate of a Protein Phosphokinase from Rabbit Reticulocytes. Proc. Nat. Acad. Sci., USA, 1970. 67, 408-414.

173. Traut, R. R., Howard, G. A. and Traugh, J. A. Ribosomal Proteins from Rabbit Reticulocytes: Numbers, Molecular Weights, Relative Amounts and Phosphorylation by Protein Kinases. In E. H. Fischer, E. G. Krebs, H. Neurath and E. R. Stadtman (Eds.) Metabolic Interconversion of Enzymes 1973. Berlin, Heidelberg, New York: Springer, 1974. pp. 155-164.

174. Walton, G. M. and Gill, G. N. Adenosine 3',5'-Monophosphate and Protein Kinase Dependent Phosphorylation of Ribosomal Protein. Biochemistry, 1973. 12, 2604-2611.

175. Eil, C. and Wool, I. G. Phosphorylation of Rat Liver Ribosomal Subunits: Partial Purification of Two Cyclic AMP Activated Protein Kinases. Biochem. Biophys. Res. Commun., 1971. 43, 1001-1009.

176. Eil, C. and Wool, I. G. Phosphorylation of Liver Ribosomal Proteins. Characterization of the Protein Kinase Reaction and Studies of the Structure of Phosphorylated Ribosomes. J. Biol. Chem., 1973. 248, 5122-5129.

177. Cawthon, M. L., Bitte, L. F., Krystosek, A. and Kabat, D. Effect of Cyclic Adenosine 3':5'-Monophosphate on Ribosomal Protein Phosphorylation in Reticulocytes. J. Biol. Chem., 1974. 249, 275-278.

178. Malkin, M. and Lipmann, F. A Stimulation by Cyclic 3',5'-Adenosine Monophosphate of Amino Acid Activation and Polymerization in Reticulocyte Hemolysates. Proc. Nat. Acad. Sci., USA, 1969. 64, 973-980.

179. Jost, J. P. and Rickenberg, H. V. Cyclic AMP. Ann. Rev. Biochem., 1971. 40, 741-774.

180. Eil, C. and Wool, I. G. Function of Phosphorylated Ribosomes. The Activity of Ribosomal Subunits Phosphorylated In Vitro by Protein Kinase. J. Biol. Chem., 1973. 248, 5130-5136.

181. Okamoto, T. and Takanami, M. Interaction of Ribosomes and Natural Polyribonucleotides. Biochim. Biophys. Acta, 1963. 76, 266-274.

182. Falvey, A. K. and Staehelin, T. Structure and Function of Mammalian Ribosomes II. Exchange of Ribosomal Subunits at Various Stages of in vitro Polypeptide Synthesis. J. Mol. Biol., 1970. 53, 21-34.

183. Williamson, A. R. The Attachment of Polyuridylic Acid to Reticulocyte Ribosomes. Biochem. J., 1969. 111, 515-520.

184. Revel, M. and Gros, F. A Factor from E. coli Required for the Translation of Natural Messenger RNA. Biochem. Biophys. Res. Commun., 1966. 25, 124-132.

185. Stanley, W. M. jr., Salas, M., Wahba, A. J. and Ochoa, S. Translation of the Genetic Message: Factors Involved in the Initiation of Protein Synthesis. Proc. Nat. Acad. Sci., USA, 1966. 56, 290-295.

186. Crystal, R. G. and Anderson, W. F. Initiation of Hemoglobin Synthesis: Comparison of Model Reactions That Use Artificial Templates with Those Using Natural Messenger RNA. Proc. Nat. Acad. Sci., USA, 1972. 69, 706-711.

187. Crystal, R. G., Nienhuis, A. W., Prichard, P. M., Picciano, D., Elson, N. A., Merrick, W. C., Graf, H., Shafritz, D. A., Laycock, D. G., Last, J. A. and Anderson, W. F. Initiation of Globin Synthesis. Fed. Eur. Biochem. Soc. Lett., 1972. 24, 310-314.

188. Smith, K. E., Hirsch, C. A. and Henshaw, E. C. Role of Elongation Factors and the Effect of Aurintricarboxylic Acid on the Synthesis of Polyphenylalanine. J. Biol. Chem., 1973. 248, 122-130.

189. White, A. M., Pederson, S. and Hultin, T. Activation of Single Ribosomes from Ascites Tumor Cells by Polyuridylic Acid. Biochem. J., 1963. 89, 12P (Abstract).

190. Henshaw, E. C., Bojarski, T. B. and Hiatt, H. H. Protein Synthesis by Free and Bound Rat Liver Ribosomes in vivo and in vitro. J. Mol. Biol., 1963. 7, 122-129.

191. Vittorelli, M., Caffarelli-Mormino, I. and Monroy, A. Poly-U Stimulation of Single Ribosomes and of Ribosomes Engaged in Polysomes of Sea Urchin Eggs and Embryos. Biochim. Biophys. Acta, 1969. 186, 408-411.

192. Tissières, A., Watson, J. D., Schlessinger, D. and Hollingworth, B. R. Ribonucleoprotein Particles from Escherichia coli. J. Mol. Biol., 1959. 1, 221-233.

193. Tissières, A., Schlessinger, D. and Gros, F. Amino Acid Incorporation into Proteins by Escherichia coli Ribosomes. Proc. Nat. Acad. Sci., USA, 1960. 46, 1450-1463.

194. Lamfrom, H. and Glowacki, E. R. Controlled Dissociation of Rabbit Reticulocyte Ribosomes and its Effect on Hemoglobin Synthesis. J. Mol. Biol., 1962. 5, 97-108.

195. Tashiro, Y. and Siekevitz, P. Ultracentrifugal Studies on the Dissociation of Hepatic Ribosomes. J. Mol. Biol., 1965. 11, 149-165.

196. Martin, T. E., Rolleston, F. S., Low, R. B. and Wool, I. G. Dissociation and Reassociation of Skeletal Muscle Ribosomes. J. Mol. Biol., 1969. 43, 135-149.

197. Hamilton, M. G., Pvalovec, A. and Petermann, M. L. Molecular Weight, Buoyant Density, and Composition of Active Subunits of Rat Liver Ribosomes. Biochemistry, 1971. 10, 3424-3427.

198. Hamilton, M. G. and Ruth, M. E. The Dissociation of Rat Liver Ribosomes by Ethylenediaminediaminetetracetic Acid; Molecular Weights, Chemical Composition, and Buoyant Densities of the Subunits. Biochemistry, 1969. 8, 851-856.

199. Näslund, P. H. and Hultin, T. Effects of Potassium Deficiency on Mammalian Ribosomes. Biochim. Biophys. Acta, 1970. 204, 237-247.

200. Reboud, A. M., Buisson, M. and Reboud, J. P. Ribosomal Subunits from Rat Liver 2. Effect of Cations on Isolated Subunits. Eur. J. Biochem., 1972. 26, 354-359.

201. Martin, T. E. and Wool, I. G. Active Hybrid 80S Particles Formed from Subunits of Rat, Rabbit and Protozoan (Tetrahymena pyriformis) Ribosomes. J. Mol. Biol., 1969. 43, 151-161.

202. Falvey, A. K. and Staehelin, T. Structure and Function of Mammalian Ribosomes. I. Isolation and Characterization of Active Liver Ribosomal Subunits. J. Mol. Biol., 1970. 53, 1-19.

203. Lawford, G. R. The Effect of Incubation with Puromycin on the Dissociation of Rat Liver Ribosomes into Active Subunits. Biochem. Biophys. Res. Commun., 1969. 37, 143-150.

204. Faust, C. H. jr. and Matthei, H. A Systematic Study of the Isolation of Murine Plasma Cell Ribosomal Subunits, Their Sedimentation Properties and Activity in Polyphenylalanine Synthesis. Biochemistry, 1972. 11, 2682-2691.

205. Infante, A. A. and Graves, P. N. Stability of Free Ribosomes, Derived Ribosomes and Polysomes of the Sea Urchin. Biochim. Biophys. Acta, 1971. 246, 100-110.

206. Blobel, G. and Sabatini, D. Dissociation of Mammalian Polyribosomes into Subunits by Puromycin. Proc. Nat. Acad. Sci. USA, 1971. 68, 390-394.

207. Goldberg, A. Magnesium Binding by Escherichia coli Ribosomes. J. Mol. Biol., 1966. 15, 663-673.

208. Edelman, I. S., Ts'o, P. O. P. and Vinograd, J. The Binding of Magnesium to Microsomal Nucleoprotein and Ribonucleic Acid. Biochim. Biophys. Acta, 1960. 43, 393-403.

209. Rodgers, A. The Exchange Properties of Magnesium in Escherichia coli Ribosomes. Biochem. J., 1964. 90, 548-555.

210. Zitomer, R. S. and Flaks, J. G. Magnesium Dependence and Equilibrium of the Escherichia coli Ribosomal Subunit Association. J. Mol. Biol., 1972. 71, 263-279.

211. Petermann, M. L. and Pavlovec, A. Effects of Magnesium and Formaldehyde on the Sedimentation Behavior of Rat Liver Ribosomes. Biopolymers, 1969. 7, 73-81.

212. Ball, A., Johnson, P. M. and Walker, I. O. The Dissociation of Escherichia coli Ribosomes. Eur. J. Biochem., 1973. 37, 12-20.

213. Morimoto, T. Intermediate Stage in the Association and Dissociation of Escherichia coli Ribosomes and the Combining Properties of their Subunits. Biochim. Biophys. Acta, 1969. 182, 135-146.

214. Petermann, M. L., Pavlovec, A. and Hamilton, M. G. Effects of Agents that Influence Hydrogen Bonding on the Structure of Rat Liver Ribosomes. Biochemistry, 1972. 11, 3925-3933.

215. Morgan, R. S., Greenspan, C. and Cunningham, B. Dissociation of Yeast Ribosomes by Papain. Biochim. Biophys. Acta, 1963. 68, 642-644.

216. Malkin, L. and Rich, A. Partial Resistance of Nascent Polypeptide Chains to Proteolytic Digestion due to Ribosomal Shielding. J. Mol. Biol., 1967. 26, 329-346.

217. Moore, P. B. Studies on the Mechanism of Messenger Ribonucleic Acid Attachment to Ribosomes. J. Mol. Biol., 1966. 22, 145-163.

218. Schlessinger, D. and Gros, F. Structure and Properties of Active Ribosomes of Escherichia coli. J. Mol. Biol., 1963. 7, 350-359.

219. Cammarano, P., Felsani, A., Gentile, M. Gualerzi, C., Romeo, A. and Wolf, G. Formation of Active Hybrid 80S Particles from Subunits of Pea Seedlings and Mammalian Liver Ribosomes. Biochim. Biophys. Acta, 1972. 281, 625-642.

220. Zylber, E. A. and Penman, S. The Effect of High Ionic Strength on Monomers, Polyribosomes, and Puromycin-Treated Ribosomes. Biochim. Biophys. Acta, 1970. 204, 221-229.

221. Beller, R. J. and Lubsen, N. H. Effect of Polypeptide Chain Length on Dissociation of Ribosomal Complexes. Biochemistry, 1972. 11, 3271-3276.

222. Jost, M., Shoemaker, N. and Noll, H. Stepwise Reconstitution of a Ternary Complex in Protein Synthesis. Nature, 1968. 218, 1217-1223.

223. Mechler, B. and Mach, B. Preparation and Properties of Ribosomal Subunits from Mouse Plasmacytoma Tumors. Eur. J. Biochem., 1971. 21, 552-564.

224. Van Holde, K. E. Physical Biochemistry. Englewood Cliffs, N. J.: Prentice-Hall, 1971. (pages 115-118).

225. Warner, J. R. and Rich, A. The Number of Soluble RNA Molecules on Reticulocyte Polyribosomes. Proc. Nat. Acad. Sci., USA, 1964. 51, 1134-1140.

226. Crystal, R. G., Nienhuis, A. W., Elson, N. A. and Anderson, W. F. Initiation of Globin Synthesis. Preparation and Use of Reticulocyte Ribosomes Retaining Initiation Region Messenger Ribonucleic Acid Fragments. J. Biol. Chem., 1972. 247, 5357-5368.

227. Garen, A. and Levinthal, C. A Fine-Structure Genetic and Chemical Study of the Enzyme Alkaline Phosphatase of E. coli I. Purification and Characterization of Alkaline Phosphatase. Biochim. Biophys. Acta, 1960. 38, 470-483.

228. Adamson, S. D., Herbert, E. and Godchaux, W. III. Factors Affecting the Rate of Protein Synthesis in Lysate Systems from Reticulocytes. Arch. Biochem. Biophys., 1968. 125, 671-683.

229. Borsook, H., Fischer, E. H. and Keighley, G. Factors Affecting Protein Synthesis in vitro in Rabbit Reticulocytes. J. Biol. Chem., 1957. 229, 1059-1070.

230. Nienhuis, A. W., Canfield, P. H. and Anderson, W. F. Hemoglobin Messenger RNA from Human Bone Marrow. Isolation and Translation in Homozygous and Heterozygous Beta-Thalassemia. J. Clin. Invest., 1973. 52, 1735-1745.

231. Schafritz, D. A., Drysdale, J. W. and Isselbacher, K. J. Translation of Liver Messenger Ribonucleic Acid in a Messenger-dependent Reticulocyte Cell-free System. Properties of the System and Identification of Ferritin in the Product. J. Biol. Chem., 1973. 248, 3220-3227.

232. Gilbert, J. M. and Anderson, W. F. Cell-free Hemoglobin Synthesis. II. Characteristics of the Transfer Ribonucleic Acid-Dependent Assay System. J. Biol. Chem., 1970. 245, 2343-2349.

233. Aviv, H. and Leder, P. Purification of Biologically Active Globin Messenger RNA by Chromatography on Oligo-Thymidylic Acid-Cellulose. Proc. Nat. Acad. Sci., USA, 1972. 69, 1408-1412.

234. Gilham, P. T. Synthesis of Polynucleotide-Celluloses and Their Use in Fractionation of Polynucleotides. J. Amer. Chem. Soc. 1964. 86, 4982-4985.

235. Penman, S. RNA Metabolism in the HeLa Cell Nucleus. J. Mol. Biol., 1966. 17, 117-130.

236. Loening, U. E. The Fractionation of High-Molecular-Weight Ribonucleic Acid by Polyacrylamide-Gel Electrophoresis. Biochem. J., 1967. 102, 251-257.

237. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem., 1951. 193, 265-275.

238. Zucker, W. V. and Schulman, H. M. Stimulation of Globin Chain Initiation by Hemin in the Reticulocyte Cell-Free System. Proc. Nat. Acad. Sci., USA, 1968. 59, 582-589.

239. Adamson, S. D., Herbert, E. and Kemp, S. F. Effects of Hemin and Other Prophoryrins on Protein Synthesis in a Reticulocyte Lysate Cell-free System. J. Mol. Biol., 1969. 42, 247-258.

240. Waxman, H. S. and Rabinovitz, M. Iron Supplementation _in vitro_ and the State of Aggregation and Function of Reticulocyte Ribosomes in Hemoglobin Synthesis. Biochem. Biophys. Res. Commun., 1965. 9, 538-545.

241. Waxman, H. S. and Rabinovitz, M. Control of Reticulocyte Polyribosome Content and Hemoglobin Synthesis by Heme. Biochim. Biophys. Acta, 1966. 129, 369-379.

242. Gross, M. and Rabinovitz, M. Control of Globin Synthesis in Cell-Free Preparations of Reticulocytes by Formation of a Translational Repressor that is Inactivated by Hemin. Proc. Nat. Acad. Sci., USA, 1972. 69, 1565-1568.

243. Gross, M. and Rabinovitz, M. Control of Globin Synthesis by Hemin: Factors Influencing Formation of an Inhibitor of Globin Chain Initiation in Reticulocyte Lysates. Biochim. Biophys. Acta, 1972. 287, 340-352.

244. Adamson, S. D., Yau, P. M.-P., Herbert, E. and Zucker, W. V. Involvement of Hemin, a Stimulatory Fraction from Ribosomes and a Protein Synthesis Inhibitor in the Regulation of Hemoglobin Synthesis. J. Mol. Biol., 1972. 63, 247-264.

245. Hunt, T., Vanderhoff, G. and London, I. M. Control of Globin Synthesis: The Role of Heme. J. Mol. Biol., 1972. 66, 471-481.

246. Mizuno, S., Fisher, J. M. and Rabinovitz, M. Hemin Control of Globin Synthesis: Action of an Inhibitor Formed in the Absence of Hemin on the Reticulocyte Cell-Free System and its Reversal by a Ribosomal Factor. Biochim. Biophys. Acta, 1972. 272, 638-650.

247. Beuzard, Y. and London, I. M. The Effects of Hemin and Double-Stranded RNA on α and β Globin Synthesis in Reticulocytes and Krebs II Ascites Cell-Free Systems and the Relationship of these Effects to an Initiation Factor Preparation. Proc. Nat. Acad. Sci., USA, 1974. 71, 2863-2866.

248. Balkow, K., Mizuno, S., Fisher, J. M. and Rabinovitz, M. Hemin Control of Globin Synthesis: Effect of a Translational Repressor on Met-tRNA$_f$ Binding to the Small Ribosomal Subunit and Its Relation to the Activity and Availability of an Initiation Factor. Biochim. Biophys. Acta, 1973. 324, 397-409.

249. Balkow, K., Mizuno, S. and Rabinovitz, M. Inhibition of an Initiation Codon Function by Hemin Deficiency and the Hemin-Controlled Translational Repressor in the Reticulocyte Cell-Free System. Biochem. Biophys. Res. Commun., 1973. 54, 315-323.

250. Kruh, J. and Borsook, H. Hemoglobin Synthesis in Rabbit Reticulocytes in Vitro. J. Biol. Chem., 1956. 220, 905-915.

251. Beuzard, Y., Rodvien, R. and London, I. M. Effect of Hemin on the Synthesis of Hemoglobin and Other Proteins in Mammalian Cells. Proc. Nat. Acad. Sci., USA, 1973. 70, 1022-1026.

252. Lodish, H. F. Biosynthesis of Reticulocyte Membrane Proteins by Membrane-Free Polyribosomes. Proc. Nat. Acad. Sci., USA, 1973. 70, 1526-1530.

253. Palmiter, R. D. Ovalbumin Messenger Ribonucleic Acid Translation. Comparable Rates of Polypeptide Initiation and Elongation on Ovalbumin and Globin Messenger Ribonucleic Acid in a Rabbit Reticulocyte Lysate. J. Biol. Chem., 1973. 248, 1095-1106.

254. Lodish, H. F. and Desalu, O. Regulation of Synthesis of Non-Globin Proteins in Cell-free Extracts of Rabbit Reticulocytes. J. Biol. Chem., 1973. 248, 3520-3527.

255. Mathews, M. B., Hunt, T. and Brayley, A. Specificity of the Control of Protein Synthesis by Haemin. Nature New Biol., 1973. 243, 230-233.

256. London, I. M. and Beuzard, Y. The Role of Hemin in the Control of the Synthesis of Hemoglobin and of Other Proteins. In H. Yoshikawa and S. M. Rapoport (Eds.) Cellular and Molecular Biology of Erythrocytes. Baltimore, London, Tokyo: University Park Press, 1974. pp. 307-320.

257. Ottolenghi, S., Comi, P., Giglioni, B., Gianni, A. M. and Guidotti, G. G. Dissociation of Single Ribosomes as a Preliminary Step for their Participation in Protein Synthesis. Eur. J. Biochem., 1973. 33, 227-232.

258. Infante, A. A. and Kraus, M. Dissociation of Ribosomes Induced by Centrifugation: Evidence for Doubting Conformational Changes in Ribosomes. Biochim. Biophys. Acta, 1971. 246, 81-99.

259. Baglioni, C., Jacobs-Lorena, M. and Meade, H. The Site of Action of Inhibitors of Initiation of Protein Synthesis in Reticulocytes. Biochim. Biophys. Acta, 1972. 277, 188-197.

260. Baglioni, C. Heterogeneity of the Small Ribosomal Subunit and Mechanism of Chain Initiation in Eukaryotes. Biochim. Biophys. Acta, 1972. 287, 189-193.

261. Bonanou-Tzedaki, S. A. and Arnstein, H. R. V. Incorporation of Rat Liver 40-S Ribosomal Subparticles into Rabbit-Reticulocyte Polysomes. Eur. J. Biochem., 1971. 24, 156-162.

262. Colombo, B., Felicetti, L., and Baglioni, C. Inhibition of Protein Synthesis in Reticulocytes by Antibiotics. I. Effects on Polysomes. Biochim. Biophys. Acta, 1966. 119, 109-119.

263. Colombo, B., Felicetti, L. and Baglioni, C. Inhibition of Protein Synthesis by Cycloheximide in Rabbit Reticulocytes. Biochem. Biophys. Res. Commun., 1965. 18, 389-395.

264. Stewart, M. L., Grollman, A. P. and Huang, M.-T. Aurintricarboxylic Acid: Inhibitor of Initiation of Protein Synthesis. Proc. Nat. Acad. Sci., USA, 1971. 68, 97-101.

265. Schrier, M. H. and Staehelin, T. Initiation of Mammalian Protein Synthesis: The Importance of Ribosome and Initiation Factor Quality for the Efficiency of _in vitro_ Systems. J. Mol. Biol., 1973. 73, 329-349.

266. Schrier, M. H. and Staehelin, T. Translation of Rabbit Hemoglobin Messenger RNA In Vitro with Purified and Partially Purified Components from Brain or Liver of Different Species. Proc. Nat. Acad. Sci., USA, 1973. 70, 462-465.

267. Vournakis, J. and Rich, A. Size Changes in Eukaryotic Ribosomes. Proc. Nat. Acad. Sci., USA, 1971. 68, 3021-3025.

268. Leader, D. P., Klein-Bremhaar, H. and Wool, I. G. Distribution of Initiation Factors in Cell Fractions from Mammalian Tissues. Biochem. Biophys. Res. Commun., 1972. 46, 215-224.

269. Clegg, J. C. S. and Arnstein, H. R. V. The Controlled Dissociation of Proteins from Rat Liver Ribosomes by Potassium Chloride. Eur. J. Biochem., 1970. 149-157.

270. Reboud, A.-M., Arpin, M. and Reboud, J.-P. Ribosomal Subunits from Rat Liver. 1. Isolation and Properties of 40-S and 60-S Subunits. Eur. J. Biochem., 1972. 26, 347-353.

271. Dice, J. F. and Schimke, R. T. Turnover and Exchange of Ribosomal Proteins from Rat Liver. J. Biol. Chem., 1972. 247, 98-111.

272. Garrison, N. E., Bosselman, R. A. and Kaulenas, M. S. The Effect of Protein Exchange on the Activity of Xenopus Laevis Ribosomes. Biochem. Biophys. Res. Commun., 1972. 49, 171-178.

273. Delauney, J. and Schapira, G. Questioning the In Vitro Protein Exchange between Ribosomes and Post-Microsomal Supernatant. Biochim. Biophys. Acta, 1974. 349, 262-268.

274. Sundkvist, I. C. and Howard, G. A. Comparison of Activity and Protein Content of Ribosomal Subunits Prepared by Four Different Methods from Rabbit Reticulocytes. Fed. Eur. Biochem. Soc. Lett., 1974. 41, 287-291.