Structure and Function of Mammalian Ribosomes

I. Isolation and Characterization of Active Liver Ribosomal Subunits

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We have developed a method for preparation and purification of active ribosomal subunits from rat and mouse liver. Our technique involves incubating purified polysomes with all components for protein synthesis until all competent ribosomes have terminated and released their polypeptide chain. Upon treatment of such an incubation mixture with 0·5 M-KCl, followed by sucrose gradient centrifugation in 0·3 M-KCl and 0·002 or 0·003 M-magnesium acetate, 80 to 90% of all ribosomes are found as 60 s and 40 s subunits. These subunits can be concentrated from the gradient fractions by ethanol precipitation. The purified subunits, when recombined, spontaneously associate to 80 s couples in the absence of transfer RNA, messenger RNA or supernatant factors. When supplied with poly U and the other components required for *in vitro* polypeptide synthesis, the subunits polymerize 15 to 20 phenylalanine residues per subunit couple present in the reaction mixture. Furthermore, at least 50 to 65% of the subunits actually participate in polyphenylalanine synthesis. At least 90% of the RNA of the purified subunits is intact as shown by sedimentation analysis of lithium dodecyl sulfate-treated particles. The purified 60 s subunit contains the enzymic site for the catalysis of peptide bond formation. This reaction is sensitive to anisomycin, an inhibitor of mammalian protein synthesis, but is not affected by the bacterial inhibitor chloramphenicol nor by cyclohexamide, an inhibitor of translocation in mammalian systems. The proteins from 40 s and 60 s subunits and from polysomes were analyzed and compared by acrylamide gel electrophoresis.

1. Introduction

The purpose of our work is to gain insight into the relationship between molecular structure and function in mammalian ribosomes. Our first goal was to isolate structurally and functionally intact ribosomal subunits. Preparation of bacterial subunits with these qualities has been possible for many years (Gilbert, 1963a; Gesteland, 1966). However, until recently the same accomplishment with mammalian ribosomes has posed great difficulties. Most mammalian ribosomes from tissue or cultured cells are isolated in the form of messenger RNA–ribosome complexes carrying peptidyl-transfer RNA. Depending upon the isolation procedure and the tissue source, these complexes may be intact polysomes, highly active in *in vitro* polypeptide chain elongation, or degraded polysomes. This peptidyl-tRNA–ribosome–messenger complex is very stable and requires strong magnesium ion chelating agents such as ethylene diamine

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tetra-acetic acid or pyrophosphate to achieve dissociation of the ribosomes into subunits. These subunits, when recombined and tested with natural or artificial messenger RNA, such as polyuridylic acid, were always inactive in polypeptide synthesis (Lamfrom & Glowacki, 1962: Lazda & Noll, personal communication).

In 1968 Schweet and coworkers reported that reticulocyte ribosomes pelleted after treatment with 0·5 M-KCl were still competent in poly U-directed polyphenylalanine synthesis, and, if supplied with the "KCl extract", these KCl-treated ribosomes were active in hemoglobin synthesis (Hamada, Yang, Heintz & Schweet, 1968; Yang, Hamada & Schweet, 1968). But, in these early studies the subunits were never isolated and the actual state of ribosome association in the KCl-treated samples seemed to change rapidly with time and was, therefore, poorly defined.

In our studies we concentrated our efforts on developing methods for the preparation of high yields of subunits, the majority of which are structurally and functionally intact. This then allows us to correlate function more quantitatively with structure. We wanted to minimize the harshness of the conditions required for subunit dissociation. Therefore, we prepared subunits from free 80 s ribosomes rather than from polysomes. To accomplish this we prepared pure polysomes which demonstrated high efficiency in in *vitro* protein synthesis and converted these polysomes into free 80 s ribosomes by letting them terminate and release their nascent polypeptide chain in an *in vitro* protein synthesis system. This approach also has the advantage that only those ribosomes which are competent in protein synthesis with the endogenous messenger are converted into subunits. Furthermore, by our method for isolation of polysomes, we eliminate practically all 80 s ribosomes present in the tissue homogenate. These *in vivo* 80 s ribosomes might represent a special pool which differs functionally from ribosomes engaged in protein synthesis on polysomes (Girard, Latham, Penman & Darnell, 1965; Hogan & Korner, 1968; Joklik and Becker, 1965a,b). A comparison between 80 s ribosomes divided *in vitro* from polysomes and those present in the tissue homogenate (*in vivo* 80 s ribosomes) will be discussed in the following paper (Falvey & Staehelin, 1970).

The term chain *termination* is used in this and the following articles to describe the events of ribosome disengagement from mRNA coupled with the release of both polypeptide chain and tRNA. Chromatography on DEAE-cellulose of peptidyl-tRNA isolated from polysomes and of protein released during *in vitro* synthesis show that the latter is mainly free and not linked to tRNA. Thus, the important step of peptidyl-tRNA cleavage occurs in our *in vitro* chain termination.

2. Materials and Methods

Buffer solutions used for the various types of homogenization of mouse or rat liver.

Buffer A consists of 0·2 M-sucrose, 0·1 M-NH$_4$Cl, 0·005 M-magnesium acetate, 0·02 M-Tris–HCl pH 7·5, and 0·001 M-dithiothreitol. Buffer A is used for polysome preparation as well as for high speed supernatant enzyme preparation.

Buffer B consists of 0·3 M-sucrose, 0·005 M-magnesium acetate, 0·01 M-Tris–HCl pH 7·5, and 0·001 M-dithiothreitol. This buffer is used for the preparation of pH 5 enzyme.

All solutions containing Mg^{2+} also contain EDTA at one-twentieth the concentration of Mg^{2+}, since our 1 M-magnesium acetate stock solution is actually 1·05 M-magnesium acetate + 0·05 M-EDTA.

(a) *Preparation of mouse liver polysomes*

Male Swiss Albino mice, weighing about 25 g were starved for 16 to 20 hr and exsanguinated in ether anaesthesia by severing the abdominal aorta. The liver was removed

quickly, rinsed in ice-cold Buffer A and placed in 2·5 vol. of buffer A. Eight livers, each weighing about 1 g were homogenized together in 20 ml. of Buffer A in a Potter-Elvehjem type homogenizer by 8 to 10 strokes with a motor-driven loose-fitting Teflon pestle. The homogenate was centrifuged for 10 min at 12,000 rev./min. in a Sorvall centrifuge, and the upper two-thirds of the supernatant fluid (post-mitochondrial supernatant) was removed. Just before further purification, the post-mitochondrial supernatant was made 1·3% in sodium deoxycholate. 20-ml. portions of the sodium deoxycholate-treated post-mitochondrial supernatant were layered over discontinuous sucrose gradients consisting of 7 ml. of 2 M-sucrose and 7 ml. of 0·7 M-sucrose both containing the ionic conditions of buffer A. (Modified method of Wettstein, Staehelin & Noll, 1963). The gradients were centrifuged for 24 hr at 4°C either in the IEC SB110 rotor with an IEC B60 ultracentrifuge or in the SW27 rotor with the Spinco LH65B ultracentrifuge. Following centrifugation the supernatant was removed by aspiration and the pellets quickly rinsed with distilled water and then dissolved in buffer A without sucrose. The polysomes were stored at −80°C in small portions containing 80 to 150 O.D.$_{260}$ units/ml.

(b) *Preparation of pH enzyme*

Our method is a modification of that of Hoagland, Stephenson, Scott, Hecht & Zamecnik (1958). The main difference is the omission of KCl, the use of low Tris–HCl concentration and the threefold dilution of the post-microsomal supernatant with water before acidification. Our method gives high yields of tRNA and of all the factors required for *in vitro* polypeptide synthesis. Mouse livers or rat livers were homogenized in buffer B (2·5 ml./g of liver), and the post-mitochondrial supernatant was prepared by centrifugation at 15,000 rev./min at 2°C for 15 min. The post-mitochondrial supernatant was then centrifuged in an angle rotor at 40,000 rev./min for 3 hr. Approximately two-thirds of the supernatant fluid (post-microsomal supernatant) was then diluted with 2 vol. of cold distilled water containing 0·001 M-dithiothreitol and the pH was adjusted to between 5·1 and 5·2 by dropwise addition of 1 M-acetic acid with constant stirring. The precipitate which formed was immediately centrifuged at 10,000 rev./min for 10 min and then dissolved in buffer A without sucrose. The pH had to be adjusted to 7·5 by addition of small amounts of 1 N-KOH. The final volume was approximately 1 ml./6 ml. of post-microsomal supernatant. After traces of insoluble material were removed by centrifugation at 10,000 rev./min, small portions of the pH 5 enzyme were stored at −80°C until used. Repeated freezing and thawing does not cause loss of activity.

(c) *Preparation of high speed supernatant (S_{100})*

Mouse or rat livers were homogenized in 2·5 ml. buffer A/g of liver. Post-mitochondrial supernatant, prepared as described above, was centrifuged at 40,000 rev./min for 3 hr in an angle rotor at 2 to 4°C. Small samples of this high-speed supernatant were immediately frozen and stored at −80°C until used.

(d) In vitro *polypeptide synthesis*

(i) *Protein synthesis with endogenous messenger leading to polypeptide chain termination and release and to the formation of free 80 s ribosomes*

The reaction mixture contained per ml.: 1 μmole ATP, 0·4 μmole GTP, 10 μmole phosphoenolpyruvate or 10 μmole of phosphocreatine, 10 μg crystalline pyruvate kinase (Sigma) or 50 μg creatine phosphokinase (Sigma), 0·2 ml. pH 5 enzyme, 0·2 ml. S_{100}, between 10 and 20 O.D.$_{260}$ units of polysomes, and 0·5 μc [^{14}C]phenylalanine. The final ionic conditions were 0·15 M-NH$_4$Cl, 0·004 M-magnesium acetate, 0·02 M-Tris–HCl pH 7·5, and 0·001 M-dithiothreitol. Incubation was carried out at the temperature specified for each experiment (25 to 35°C). Portions containing approximately 1 O.D.$_{260}$ unit of ribosomes were then absorbed onto Whatmann 3MM filter papers which were put into 10% trichloroacetic acid and heated to 90°C for 10 min. After a further wash with cold 5% trichloroacetic acid and two washes with ether the filter papers were dried and placed into scintillation liquid and counted in a Beckman LS250 liquid scintillation spectrometer. The counting efficiency for ^{14}C in this system is 62 to 65% and for ^{3}H the efficiency is about 10%.

With the phosphocreatine plus creatine kinase ATP-generating system the rate of

endogenous protein synthesis was approximately 50% faster than with the phospho-enolpyruvate–pyruvate kinase system, and a slightly higher percentage of ribosomes terminated and released their polypeptide chains. The efficiency of the poly U system was almost doubled by substituting the phosphocreatine plus creatine kinase ATP-generating system for the phosphoenolpyruvate–pyruvate kinase system.

(ii) *Polyuridylic acid-directed polyphenylalanine synthesis*

Either 80 s ribosomes liberated during polypeptide chain termination and release in a preincubation (polysome-derived 80 s ribosomes) or purified isolated 60 s and 40 s ribosomal subunits were tested in a system supporting polyphenylalanine synthesis. The incubation mixture contained per ml. the same concentrations of components as used in the system for endogenous protein synthesis except that the magnesium ion concentration was 0·012 M and polyuridylic acid (Sigma) was added at 375 μg/ml.† The radioactive amino acid used was either [^{14}C]phenylalanine or [^{3}H]phenylalanine. 80 s ribosomes were present at a concentration of 8 to 10 o.d.$_{260}$ units/ml., or 60 s subunits and 40 s subunits at 6 to 7 o.d.$_{260}$ units/ml. and 3 to 4 o.d.$_{260}$ units/ml. respectively. Hot trichloroacetic acid-insoluble radioactivity was determined as described above. The cold amino-acid pool in the incubation mixture which was introduced by addition of S$_{100}$‡ was determined both by amino-acid analysis and radioisotope dilution experiments. Various S$_{100}$ preparations contained 80 to 100 mμmole of leucine/ml. and 40 to 45 mμmole of phenyla-lanine/ml. Thus, in our incubation mixtures containing 0·2 ml. of S$_{100}$/ml. the specific radioactivity of phenylalanine ranges from 45 to 55 and that of leucine ranges from 21 to 24 mc/m-mole.

(e) *Isolation and purification of 60 s and 40 s subunits*

In a first step polysomes were preincubated in a protein synthesizing system to terminate and release nascent polypeptide chains in synchrony with the release of free 80 s ribosomes from polysomes. The preincubation was done in a mixture similar to that described in the previous paragraph except that 0·25 ml. of S$_{100}$ and 0·25 ml. of pH 5 enzyme each were included per ml. of reaction mixture. The polysome concentration was 30 o.d.$_{260}$ units/ml. After 50 min of incubation at 35°C, the reaction mixture was chilled in ice and KCl was added to a final concentration of 0·5 M. 5- to 6-ml. samples of this KCl-treated solution were layered onto 30-ml. convex exponential sucrose gradients ranging from 0·35 to 1·1 M-sucrose and containing 0·3 M-KCl, 0·003 M-magnesium acetate, 0·02 M-Tris–HCl pH 7·5, and 0·001 M-dithiothreitol. The gradients were centrifuged at 3 to 4°C for 13 to 14 hr at 27,000 rev./min in an SW27 Spinco rotor. The o.d.$_{260}$ profile of the gradients was recorded continuously by passing the gradient fluid through a flow cell mounted in a model 2400 Gilford spectrophotometer. Fractions corresponding to the 60 s and 40 s peaks were collected according to the o.d.$_{260}$ profile. The subunits in the sucrose gradient fractions were then concentrated by adding 0·7 vol. cold 95% ethanol after raising the Mg^{2+} concentration of the fractions ot 0·008 M. The ethanol leads to instant precipitation of ribosomes. After centrifugation at 10,000 rev./min in a Sorvall centrifuge for 10 min, the subunit pellets were redissolved in 0·1 M-NH$_4$Cl, 0·002 M-magnesium acetate, 0·001 M-dithiothreitol and 0·02 M-Tris–HCl pH 7·5. The ribosomal subunits were stored at concentrations of 60 to 100 o.d.$_{260}$ units/ml. either in ice until use or frozen at -80°C. Unfrozen subunits, kept at 0°C, maintained activity without appreciable loss for at least two weeks.

(f) *Sucrose gradient analysis of ribosomes and their subunits*

Two types of sucrose density gradients were used: (1) low salt gradients and (2) high salt gradients. Low salt gradients contained 0·1 or 0·15 M-NH$_4$Cl, 0·005 M-magnesium acetate, and 0·02 M-Tris–HCl pH 7·5, and high salt gradients contained 0·3 M-KCl, 0·003 M-

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† Due to the extremely high molecular weight of commercial poly U (1 to 2 × 10^6) we found that in the experiments presented in this report the poly U was always slightly limiting. If more poly U is used or the poly U was degraded by 10-min incubation in 0·1 N-KOH at 22°C followed by neutralization and reisolation by ethanol precipitation, we find that over 80% of our subunits are active in polyphenylalanine synthesis.

‡ Abbreviation used: S$_{100}$, high speed supernatant.

magnesium acetate, and 0·02 M-Tris–HCl pH 7·5. The shape of the gradients was convex exponential ranging from 0·4 M-sucrose to approximately 1·0 M-sucrose. Most analyses were done in the Spinco SW50·1 rotor. In these conditions particles move in a nearly isokinetic fashion throughout the gradient (Noll, 1969). In low salt gradient conditions free 80 s couples as well as polysomes are stable. In high salt gradient conditions only polysomes and 80 s ribosomes still attached to mRNA and carrying a peptidyl-tRNA are stable, whereas free 80 s couples dissociate into subunits. Samples to be analyzed in high salt sucrose gradients were made 0·3 M-KCl before being layered onto the gradient.

The optical density profile of all sucrose gradients was recorded continuously with a flow cell system mounted in a Gilford model 2400 spectrophotometer. The optical density scale at 260 nm in all Figures is corrected for 1-cm light path. If radioactivity was analyzed, fractions (usually 0·15 to 0·2 ml. per fraction) were collected directly into counting vials. To each of the vials was added 1 ml. of water plus 10 ml. of scintillation liquid consisting of 7 parts toluene–PPO–POPOP (Packard Instrument Co.) solution and 6 parts of Triton X-100 and then the contents were mixed vigorously before counting in a Beckman LS-250 liquid spectrometer. Counting efficiency in this system is 80% for ^{14}C and 16·5% for ^{3}H. Control experiments were performed to show that within such a gradient acid-precipitable radioactivity was identical with respect to distribution and amount to the radioactivity recovered by the direct collection method, except for the radioactivity at the top of gradients which contains the free amino acids. The Triton–toluene scintillation liquid is made according to Noll (1969).

(g) *Assay of peptidyl-transferase activity of mouse liver 60 s subunits and* Escherichia coli *50 s subunits*

We used the fragment assay of Monro & Marker (1967) to test peptidyl-transferase activity of ribosomal subunit preparations. The reaction mixture for the assay contained per 1·0 ml.: 0·33 ml. methanol, 260 μmole KCl, 13 μmole magnesium acetate, 33 μmole Tris–HCl pH 7·5, 0·67 μmole puromycin dihydrochloride, and about 10,000 cts/min of N-acetyl [^{3}H]leucyl-CACCA (the 3′-terminal pentanucleotide of leucyl-tRNA, gift from D. Vazquez and R. Monro). The reaction was carried out at 0°C and was started by the addition of the fragment. At the times indicated 0·1 ml. of the reaction mixture was withdrawn and added to 0·1 ml. of BeCl$_2$–NaAc–MgSO$_4$ salt solution to stop the reaction. The N-acetyl [^{3}H]leucylpuromycin product was extracted with ethyl acetate and counted according to the method of Leder & Bursztyn (1966) as modified by Maden & Monro (1968).

(h) *Analysis of RNA from polysomes and 60 s and 40 s ribosomal subunits*

After disruption of the nucleoprotein structure of polysomes or ribosomal subunits by addition of lithium dodecyl sulfate to a final concentration of 0·5%, the RNA was analyzed in sucrose gradients. 5-ml. convex exponential gradients were used ranging from 0·22 to 0·8 M-sucrose and containing 0·05 M-LiCl, 0·001 M-magnesium acetate and 0·02 M-Tris–HCl pH 7·5. Centrifugation was carried out at 50,000 rev./min for 3 hr at 4°C. The gradients were analyzed as described above.

(i) *Electrophoretic analysis of ribosomal proteins*

Ribosomal proteins were extracted from polysomes and purified 60 s and 40 s subunits by the urea–LiCl method of Spitnik-Elson (1965). After removal of the RNA precipitate, the urea–LiCl extracts were dialyzed against 6 M-urea containing half concentration of spacer gel buffer. We then conducted electrophoresis at 4°C in 6 M-urea at pH 4·5 using 9-cm long gels consisting of 10% acrylamide (this method is basically the procedure of Leboy, Cox & Flaks (1964) and is described by Gesteland & Staehelin (1967)). The protein bands were stained with amido black (1% solution in 7·5% acetic acid) followed by electrophoretic destaining.

3. Results

Figure 1 shows the sucrose gradient analysis of incorporation mixtures incubated at 30°C for various lengths of time. Specifically it illustrates (1) the change of the size
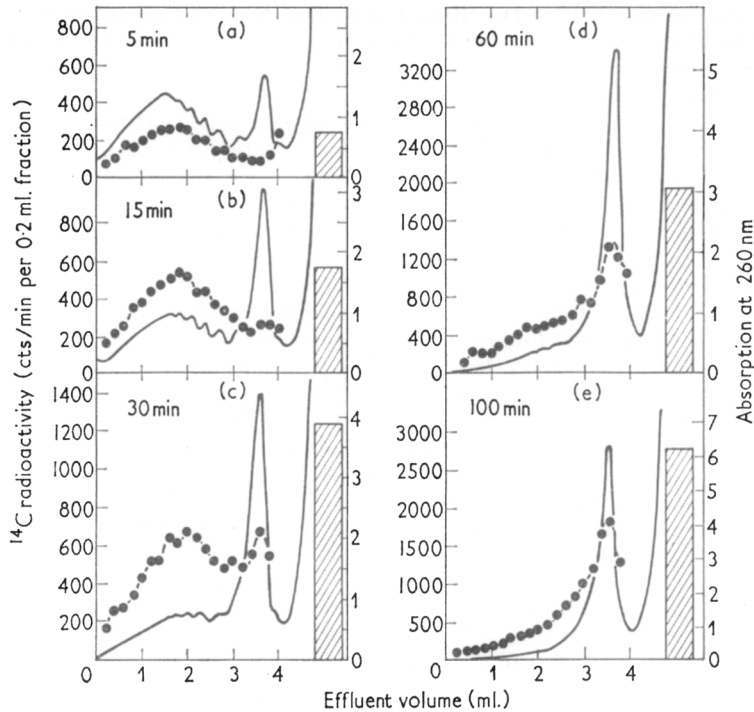
FIG. 1. Kinetics of 80 s ribosome release from polysomes during *in vitro* amino-acid incorpora-tion, analyzed in low salt sucrose gradients. The reaction mixture included per ml: 1 μmole ATP, 0·4 μmole GTP, 10 μg crystalline pyruvate kinase, 10 μmole phosphoenolpyruvate, 0·3 ml. S_{100}, 0·3 ml. pH 5 enzyme, 150 μmole NH_4Cl, 5 μmole magnesium acetate, 20 μmole Tris–HCl pH 7·5, 1 μC [^{14}C]leucine (specific activity 315 mc per m-mole), and 24 o.D.$_{260}$ units of polysomes. The incorporation mixture was incubated at 30°C and samples were removed at 5, 15, 30, 60 and 100 min and chilled in ice. After 30 min of incubation, an additional 5 μmole of phosphoenolpyruvate were added per ml. of reaction mixture. 0.3-ml. samples from each incorporation mixture were then layered onto sucrose gradients ranging from 0·35 to 1·1 M-sucrose and containing the ionic con-ditions of the reaction mixtures. The gradients were centrifuged at 4°C for 45 min at 50,000 rev./min following which they were analyzed as described in Materials and Methods. The top 1·5 ml. of each gradient was collected, and hot trichloroacetic acid-precipitable material was counted to determine the amount of released polypeptides in each gradient (hatched area: corresponding to width of 3 gradient fractions. Therefore, total radioactivity = height (cts/min $\times$ 3)). (————) Ab-sorption at 260 nm; -- ●-- ●--, ^{14}C radioactivity.

distribution of polysomes simultaneous with the release of single 80 s ribosomes during polypeptide synthesis; (2) the radioactivity incorporated into ribosome-bound nascent proteins, and (3) the amount of ^{14}C-labeled protein released from ribosomes during the incubation. The amount of hot trichloroacetic acid-insoluble radioactivity on top of the gradients in Figures 1 and 2 is represented by the hatched area (not just by its height). The width of the area corresponds to three gradient fractions.

Figure 2 shows a similar experiment, except that after the incubation the reaction mixtures were made 0·4 M with respect to monovalent cations and then analyzed in sucrose gradients containing 0·2 M-KCl and 0·003 M-magnesium acetate. In these con-ditions polysomes and messenger-bound monosomes remain stable, whereas free 80 s ribosomes dissociate into subunits. In this experiment at least 90% of the monosomes behave like free ribosomes as judged by their lability in high salt. During the early
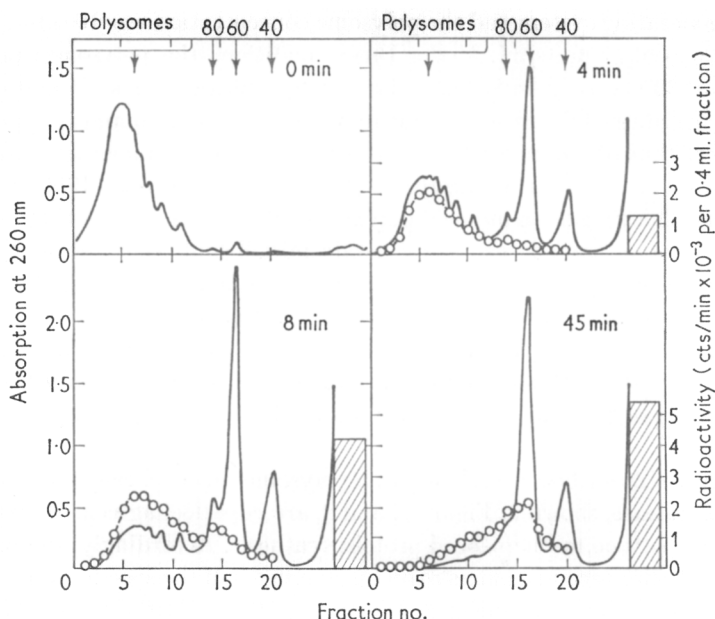
FIG. 2. Kinetics of ribosome release from polysomes during *in vitro* amino-acid incorporation, analyzed in high salt sucrose gradients. Reaction mixtures were similar to those in the experiment shown in Fig. 1 except that polysome concentration was 10 O.D.$_{260}$ units/ml., phosphocreatine and creatine phosphokinase were substituted for phosphoenopyruvate and pyruvate kinase, and the Mg^{2+} ion concentration was 0·004 M. Following incubation at 30°C, the reaction mixtures were made 0·25 M-KCl and centrifuged for 3·5 hr at 41,000 rev./min at 3°C through 12-ml. concave sucrose gradients ranging from 0·48 to 2 M-sucrose and containing 0·2 M-KCl, 0·003 M-magnesium acetate, and 0·02 M-Tris–HCl pH 7·5. The lower two-thirds of the gradients were collected into counting vials after passing through the ultraviolet monitoring system. The upper third of each gradient was collected on to a Whatman 3MM filter paper strip, washed in hot trichloroacetic acid, and prepared for counting as described in Materials and Methods. Hatched areas represent the sum of the radioactivity recovered on the filter paper after correction to give the same counting efficiency as the gradient fractions counted in the aqueous scintillation system. (————) absorption at 260 nm; —○—○—, radioactivity.

periods of incubation only stable 80 s ribosomes carry a labeled polypeptide chain presumably as peptidyl-tRNA. However, during the final stages of incubation some salt-labile 80 s ribosomes seem to still carry peptidyl-tRNA which remains attached to the 60 s subunit. These results suggest that a factor (or factors) necessary for polypeptide chain termination and release become limiting or inactive during the incubation. Experiments supporting this assumption will be reported later.

In contrast to ribosomes released during protein synthesis *in vitro*, 80 s ribosomes created by ribonucleolytic breakdown of polysomes are stable in high salt (results not shown here).

As is evident from Figures 1 and 2, after exhaustive incubation, 80 to 85% of all polysomes have become salt-labile monosomes, while the other 15 to 20% remain as small polysomes and stable monosomes still carrying peptidyl-tRNA. The initial rate of amino-acid polymerization in the experiment shown in Figure 1 is approximately five amino acids/minute/ribosome based on 0·4 leucine residues/minute/ribosome. The experiment shown in Figure 2 differs from that in Figure 1 in that creatine kinase and phosphocreatine were substituted for pyruvate kinase and phosphoenolpyruvate in

the energy generating system and the polysome concentration was 10 O.D.$_{260}$ units/ml.
instead of 20 O.D.$_{260}$ units/ml. Under these conditions the polysomes polymerized
amino acids at 30°C with an initial rate of about 20 to 25 amino acids/minute/ribosome.
This rate is maintained for at least eight minutes of incubation and is approximately
one-twelfth of that of globin synthesis in intact reticulocytes incubated at the same
temperature (Hunt, Hunter & Munro, 1969; Knopf & Lamfrom, 1965: Lamfrom &
Knopf, 1964). This rate of amino-acid polymerization comes close to that of the crude
reticulocyte lysate system described by Lamfrom & Knopf (1964). From Figure 2 we
estimate that after eight minutes of incubation less than 10% of all 60 s subunits
carry a peptidyl-tRNA and after exhaustive incubation at most 15% of all 60 s
subunits still carry a peptidyl-tRNA. This calculation is based on the assumption that
the labeled polypeptide chains on 60 s subunits have a specific activity between that
of the released polypeptide chains and that of the growing polypeptide chains on
polysomes.

The kinetics of 80 s ribosome release from polysomes in synchrony with polypeptide
synthesis and release, shown in Figures 1 and 2, are in perfect agreement with the pre-
dictions from the tape mechanism of protein synthesis. Accordingly, the polypeptide
chains released after termination have on the average half the specific activity of those
still on polysomes (Noll, Staehelin & Wettstein, 1963). Furthermore, the observed
breakdown pattern of polysomes as well as the number of amino acids polymerized
per ribosome during the incubation excludes any significant amount of nucleolytic
breakdown of polysomes into small oligosomes and subsequent translation of only
short stretches of mRNA. Thus, in Figure 2, the pentamers still present after eight
minutes of incubation have incorporated approximately 15 to 16 leucines/ribosome
unit, corresponding to about 180 amino acids; and, the dimers present after 45 minutes
incubation polymerized about 25 leucines or close to 300 amino acids/ribosome.
Accordingly, the mRNA stretches translated are only compatible with no or very little
mRNA breakdown before or during translation (Staehelin, Wettstein, Oura & Noll,
1963; Staehelin, Verney & Sidranski, 1967). This notion is further supported by
control incubations of polysomes in the presence of all enzymes and tRNA but without
ATP, GTP and phosphocreatine. After 15 minutes at 25°C the polysome pattern is
almost indistinguishable from the non-incubated sample (Falvey & Staehelin, un-
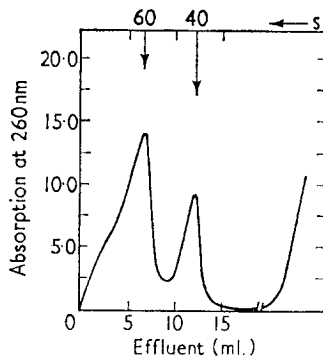published results).

FIG. 3. Preparative sucrose gradient for isolation of subunits. For details see Materials and
Methods.

(a) *Preparative isolation of 40 and 60 S subunits*

Experiments described in the previous paragraph provided the basis for the *in vitro* preparation of large amounts of easily dissociable 80 s ribosomes from active polysomes. Most of these subunits do not carry a peptidyl-tRNA. The incubation of polysomes in a complete system followed by isolation of the subunits in 30 ml. high salt sucrose gradients is described in Materials and Methods. Figure 3 shows the O.D.$_{260}$ profile of a sucrose gradient used for preparation of subunits. Because of the tendency of rat liver 60 s subunits to form 90 s dimers in the cold in low salt ionic conditions (Peterman, 1964; Martin, Rolleston, Low & Wool, 1959), we used mouse liver ribosomes for most of our experiments. Figure 4 shows purified mouse liver subunits analyzed in sucrose gradients containing low salt (0·15 M-NH$_4$Cl and 0·005 M-magnesium acetate) and high salt (0·3 M-KCl and 0·003 M-magnesium acetate).
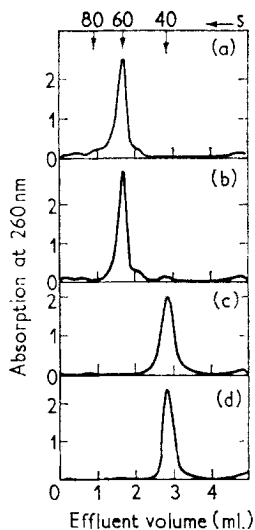

FIG. 4. Sucrose gradient analysis of purified 60 s ((a) and (b)) and 40 s ((c) and (d)) ribosomal subunits in low salt ((a) and (c)) and high salt ((b) and (d)) ionic conditions as described in Materials and Methods. Centrifugation was carried out at 4°C for 2 hr at 50,000 rev./min.

(b) *Conditions and requirements for reassociation of purified subunits*

In ionic conditions which are suitable for protein synthesis, liver ribosomal subunits spontaneously reform 80 s couples. The association of the subunits occurs in the absence of non-ribosomal cell components such as mRNA, tRNA or supernatant proteins. Figure 5 shows an experiment in which a constant amount of 60 s rat liver subunits was combined with increasing amounts of 40 s subunits (Fig. 5(b) to (d)) as well as 60 s particles and 40 s particles alone (Fig. 5(a) and (e)). It is evident from this experiment that the 60 s particles are titrated almost quantitatively with the 40 s subunits to form 80 s couples. The 80 s ribosomes formed under these conditions are stable only at relatively low salt and high magnesium ion concentrations (that is, where the molar ratio of NH$_4^+$:Mg^{2+} is not more than 35 to 38:1). If centrifuged into a sucrose gradient containing 0·2 M-KCl and 0·003 M-magnesium acetate, these 80 s couples instantly and completely dissociate into subunits again (Fig. 11(a)). We have found potassium ions slightly more effective than ammonium ions in dissociating the free 80 s ribosomes; at 2 to 4°C complete dissociation at 0·004 M-Mg^{2+} concentration
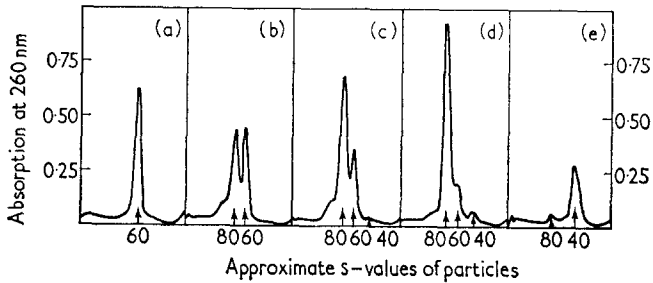
FIG. 5. Spontaneous formation of 80 s ribosomes from purified subunits in 0·15 M-NH$_4$Cl, 0·005 M-magnesium acetate, 0·02 M-Tris–HCl pH 7·5. 0·44 O.D.$_{260}$ units of purified 60 s subunits were incubated 20 min at 30°C: (a) alone; (b) with 0·05 O.D.$_{260}$ units of 40 s subunits; (c) with 0·11 O.D.$_{260}$ units of 40 s subunits; (d) with 0·16 O.D.$_{260}$ units of 40 s subunits; (e) 0·22 O.D.$_{260}$ units of 40 s subunits alone. The sucrose gradients (4 ml. convex exponential from 0·35 to 1·1 M-sucrose, containing 0·1 M-NH$_4$Cl, 0·005 M-magnesium acetate, and 0·02 M-Tris–HCl pH 7·5) were centrifuged for 85 min at 60,000 rev./min at 3°C in the IEC B60 ultracentrifuge.

requires approximately 0·2 M-KCl or 0·25 to 0·3 M-NH$_4$Cl. 80 s couples formed spontaneously from purified subunits are stable in low salt sucrose gradients centrifuged at 28°C indicating that the reassociation is not an artifact caused by low temperature. Furthermore, tRNA, either free or peptidyl-tRNA, is not required for the spontaneous association of free subunits. To show this we incubated polysomes exhaustively in a protein synthesizing system in the presence of ^{32}P-labeled tRNA (33,000 cts/min/ O.D.$_{260}$ unit of tRNA). From this incubation mixture we prepared subunits in two different ways: (1) standard procedure: the reaction mixture, made 0·5 M in KCl, was centrifuged into a high salt sucrose gradient. 60 and 40 s subunits were precipitated with ethanol, and are referred to as "unwashed" subunits: (2) after centrifugation of the reaction mixture through a low salt sucrose gradient the 80 s peak was precipitated with ethanol. These purified 80 s ribosomes were dissociated into subunits by addition of KCl to a final concentration of 0·5 M and the subunits were then separated in high salt sucrose gradients and precipitated with ethanol. They are referred to as "washed" subunits. Both washed and unwashed subunits were allowed to reassociate for ten minutes in 0·15 M-NH$_4$Cl, 0·004 M-magnesium acetate, and 0·02 M-Tris–HCl pH 7·5 at 30°C, and then analyzed in low salt sucrose gradients which are shown in Figure 6.
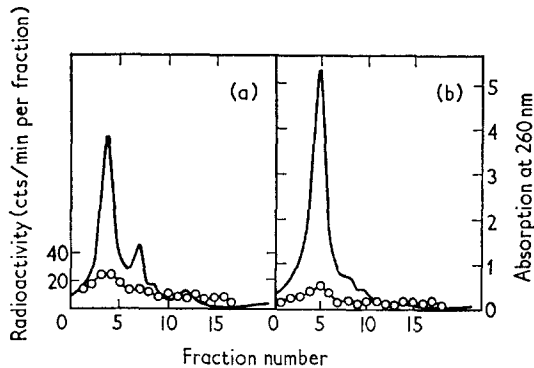

FIG. 6. Determination of the amount of tRNA carried over through two different isolation procedures of subunits. Polysomes were exhaustively incubated in a protein synthesizing system which contained ^{32}P-labeled tRNA. Subunits were then prepared from the resulting 80 s couples as described in the text and referred to as washed and unwashed. The subunits were recombined in buffer A, incubated and then analyzed in low salt sucrose gradients. (a) unwashed subunits; (b) washed subunits. (———) Absorption at 260nm, —○—○—, radioactivity.

Surprisingly, the washed subunits reassociated more completely than the unwashed subunits. The integrated 80 s peak of the unwashed preparation contains approximately 2 O.D.$_{260}$ units and about 100 cts/min of ^{32}P label corresponding to approximately 0·003 O.D.$_{260}$ unit of tRNA indicating that the 80 s peak contains about one-sixth of a tRNA molecule per ribosome. The 80 s peak consisting of washed subunit couples contains approximately 2·7 O.D.$_{260}$ units of ribosomes and 60 cts/min of ^{32}P label corresponding to 0·0018 O.D.$_{260}$ units of tRNA. Thus, there is about one-fifteenth of a tRNA molecule per 80 s couple in this peak. Control analyses showed that during preincubation of the polysomes with ^{32}P-labeled tRNA and ^{3}H-labeled amino acids approximately one tRNA per polysomal ribosome is bound very firmly and cannot be washed off by two consecutive sucrose gradient centrifugations. Close to two tRNA's per ribosome were washed off in the first sucrose gradient centrifugation. These observations are essentially in agreement with the analyses of tRNA binding to liver polysomes by Wettstein & Noll (1955). Furthermore, high salt sucrose gradient analyses of exhaustively incubated reaction mixtures showed that only peptidyl-tRNA remains bound to polysomes and stable 80 s ribosomes and to a small fraction of 60 s subunits. In such a high salt sucrose gradient analysis we find very little loosely bound ^{32}P-labeled tRNA trailing behind the ribosomes. These findings suggest that during our subunit preparation virtually no free tRNA and only a small amount of peptidyl-tRNA is isolated with the subunits.

(c) RNA and proteins of purified subunits

The RNA of subunits was analyzed in sucrose gradients and compared with that of polysomes after disrupting the nucleoprotein structure with lithium dodecyl sulfate. Figure 7 shows that the 40 s subunits contain intact 18 s RNA and that more than 90% of the 60 s particles contain intact 28 s RNA. A small fraction contains fragmented
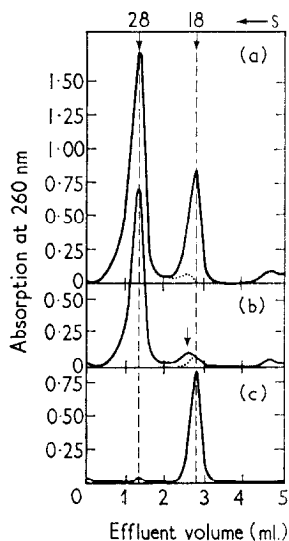

FIG. 7. Analysis of the RNA derived from polysomes and ribosomal subunits. RNA was extracted from the ribosomal particles and analyzed by sucrose gradient centrifugation as described in Material and Methods. (a) 1·25 O.D.$_{260}$ units of RNA from polysomes. The dotted line represents an estimation of 28 s RNA breakdown products. (b) 0·58 O.D.$_{260}$ units of RNA from 60 s subunits. The dotted line represents 40 s subunit-derived 18 s RNA. (c) 0·33 O.D.$_{260}$ units of RNA from 40 s subunits.

28 s RNA which sediments at about 20 s and is poorly resolved from the 18 s RNA derived from contaminating 40 s subunits. This analysis would not reveal hidden breaks in double-stranded regions of the RNA. In Figure 7(a) the optical density ratio of the 28 s: 18 s peak of polysomal RNA is 2·45:1. To obtain the theoretical ratio of 2·7:1 we have to assume that about 3% of the 28 s RNA is fragmented and sediments in the 20 to 18 s region. The predominant 28 s breakdown product observed in 60 s preparation sedimenting at 20 s (arrow Fig. 7(b)) seems to consist of $\frac{1}{2}$ 28 s molecules.

Ribosomal proteins analyzed by acrylamide gel electrophoresis at pH 4·5 in 6 M-urea and stained with amido black are shown in Plate I. The patterns of the 60 and 40 s subunits are quite distinct from each other, although in this type of gel many of the 40 s proteins seem to have identical or very similar electrophoretic mobilities to 60 s proteins. Furthermore, the 60 s proteins show a quite similar band pattern to the polysome proteins. But this does not necessarily mean that many of the 40 s proteins are chemically identical with 60 s proteins. It is possible that the 2 to $2·2 \times 10^6$ daltons of mammalian 80 s ribosomal protein consist of 80 to 100 components of different primary structure although many of these components have the same mobility in acrylamide gel electrophoresis. For *E. coli* ribosomes several groups already have shown that 30 s and/or 50 s ribosomal proteins which are electrophoretically super-imposed have different primary structures (Fogel & Sypherd, 1968; Hardy, Kurland, Voynow & Mora, 1969; Traut, Moore, Delius, Noller & Tissières, 1967).

(d) *Poly U-directed polyphenylalanine synthesis with 80 s ribosomes and purified subunits*

We used two approaches to analyse quantitatively the activity in poly U-directed polyphenylalanine synthesis of either purified subunits or polysome-derived 80 s ribosomes. First, we measured the average number of phenylalanine residues poly-merized per ribosome in the reaction mixture, and second, we determined the fraction of ribosomes actually participating in polypeptide synthesis. The first type of analysis requires knowledge of the specific radioactivity of phenylalanine in the reaction mix-ture which we determined both by radioisotope dilution experiments and by quanti-tative amino-acid analysis of the high speed supernatant (S_{100}); the two values agree very closely. The second approach requires the physical distinction between active and inactive ribosomes. In our previous experiments we have shown that the mRNA-ribosome–peptidyl-tRNA complex is stable in 0·2 M-KCl and 0·003 M-magnesium acetate in which conditions free 80 s couples dissociate into subunits. For analysis of the poly U system we used more rigorous conditions of 0·3 M-KCl and 0·002 or 0·003 M-magnesium acetate to detect in sucrose gradients the active ribosomes, i.e. poly U–80 s–polyphenylalanyl–tRNA complexes. In these conditions natural polysomes become slightly unstable as indicated by their poor resolution in sucrose gradients.

Table I shows the over-all phenylalanine polymerization in several experiments. "Run-off" 80 s ribosomes derived *in vitro* from polysomes usually polymerize between 25 and 30 phenylalanine residues per ribosome. After dissociation and purification of the subunits, the over-all activity is somewhat less and ranges from 15 to 20 phenyl-alanine residues per limiting subunit present. This compares favorably with most published data from bacterial systems (Gilbert, 1963*a,b*; Gesteland, 1966). We usually use a molar ratio of 40 to 60 s subunits of about 1·2. The significant activity of 60 s preparations alone is due to the presence of contaminating 40 s subunits. The number of phenylalanine residues polymerized per 40 s subunit present in a 60 s preparation
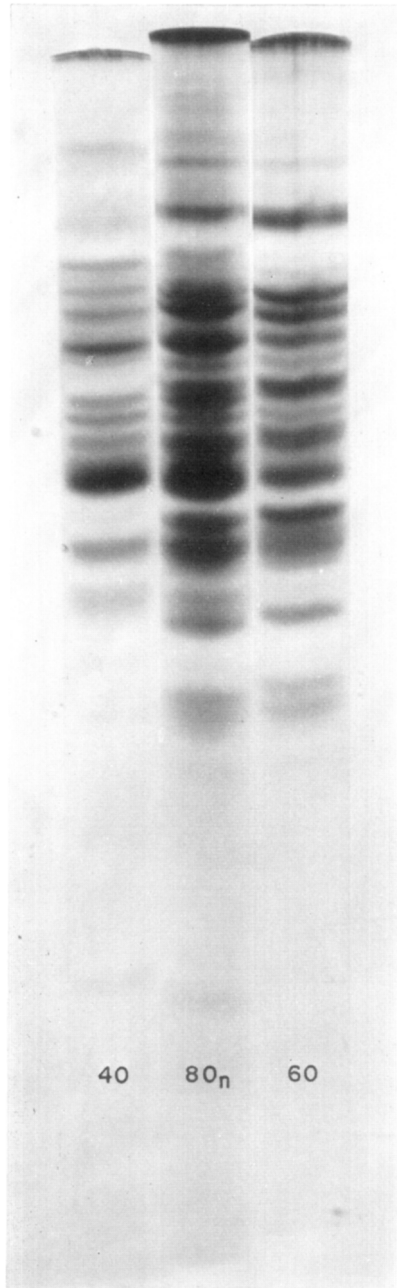
PLATE I. Electrophoretic analysis of proteins extracted from polysomes and 60 and 40 s ribosomal subunits. The proteins of polysomes (80_n) and ribosomal subunits (60 and 40 s) were extracted and analyzed by acrylamide gel electrophoresis at pH 4·5 as described in Materials and Methods.

TABLE 1

Activity of polysome-derived (run-off) 80 S ribosomes and of purified subunits in poly U-directed polyphenylalanine synthesis

Ribosome species tested	Ribosome input ($\mu\mu$mole)	Counts/min /assay	No. of phe polymerized /ribosome unit
80 s	7·7	17225 [14]C	28
80 s	7·7	19420 [14]C	33
80 s	7·7	16983 [14]C	27
60 s + 40 s	17 + 20	22065 [14]C	19
60 s	17	3470 [14]C	2·9
40 s	20	730 [14]C	0·5
60 s + 40 s	17 + 21	63812 [3]H	22
60 s	17	7620 [3]H	2·6
40 s	21	1851 [3]H	0·5

Incubations and preparation of samples for radioactivity analysis were carried out as described in the text. Control reaction mixtures either without poly U or without ribosomes incorporated 80 to 150 cts/min.

is approximately the same as that per 60 s subunit in the presence of excess 40 s (15 to 20 amino acids per limiting subunit).

Figure 8 shows the kinetics of polyphenylalanine synthesis by polysome-derived 80 s ribosomes at 25 and 33°C. The over-all rate at 33°C is slightly more than twice that at 25°C. But the final incorporation is almost the same at both temperatures. Figure 9 shows high salt sucrose gradient analyses of incubation mixtures from a similar experiment incubated for various amounts of time at 25°C. The formation of messenger–peptidyl-tRNA–ribosome complexes, i.e. high salt stable 80 s ribosomes, continues for more than 30 minutes. After 60 minutes of incubation, approximately 60% of all
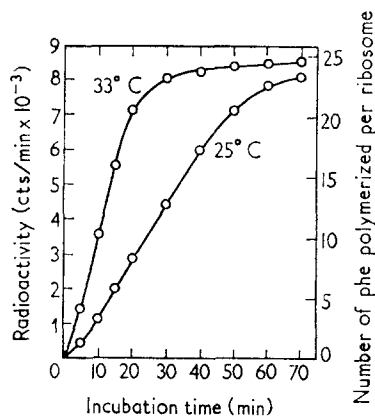

Fig. 8. Kinetics at 25 and 33°C of poly U-directed polyphenylalanine synthesis by polysome-derived 80 s ribosomes. Polysomes (10 o.d.$_{260}$ units per ml.) were incubated in the conditions for protein synthesis which lead to polypeptide chain termination and release of 80 s ribosomes from endogenous mRNA. To such a reaction mixture we added per ml.: 375 μg poly U, 0·5 μc [14C]phenylalanine, 5 μmole phosphocreatine, 25 μg creatine phosphokinase, and 8 μmole magnesium acetate (final ionic conditions are 0·15 M-NH$_4$Cl, 0·012 M-magnesium acetate, 0·02 M-Tris–HCl pH 7·5, and 0·001 M-dithiothreitol). The reaction mixtures were incubated at 25 and 33°C. At various time intervals (from 0 min of incubation to 60 min of incubation) samples containing 0·43 o.d.$_{260}$ unit were removed and absorbed onto Whatmann 3MM filter paper disks which were placed into 10% trichloroacetic acid. The hot trichloroacetic acid-insoluble material was prepared and counted as described in Materials and Methods.
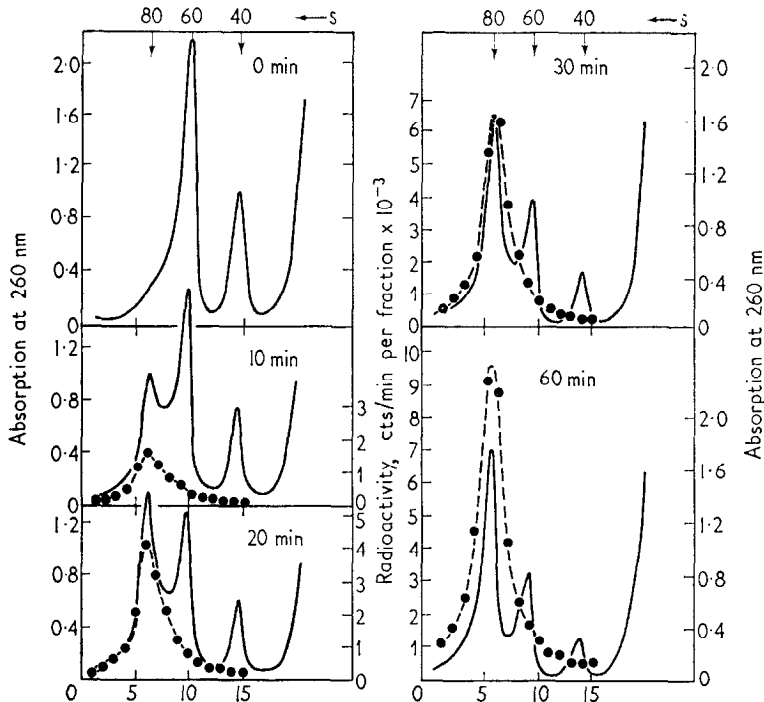
FIG. 9. Sucrose gradient analysis of the kinetics of formation of stable 80 s–poly U–polyphenyl-alanyl–tRNA complexes. Portions from a reaction mixture identical to the reaction mixture incubated at 25°C and described in Fig. 8 were analyzed in high salt sucrose gradients. The type of gradients used, centrifugation and analysis of the gradients are described in Materials and Methods.

ribosomes are found in the stable active complex carrying about 35 phenylalanine residues per 80 s couple. The pronounced trailing of radioactivity behind the 80 s peak, particularly after short periods of incubation, is probably due to the relative instability in high salt of 80 s complexes containing short peptide chains. This is also evident from the large amount of optical density between the 80 and 60 s peaks which results in poorer resolution of these two peaks in analyses of reaction mixtures incubated for 10 and 20 minutes.

Figure 10 shows sucrose gradient analyses of an experiment in which purified 60 and 40 s subunits were recombined and tested for activity in poly U-directed polyphenyl-alanine synthesis. The molar ratio of 40 to 60 s subunits is 1·2. After 60 minutes of incubation at 30°C approximately 60% of the 60 s subunits have formed high salt stable 80 s couples with 40 s subunits. In this particular experiment even in low salt there is a substantial amount of free 60 s subunits plus a component sedimenting between the active 80 s complex and the 60 s subunits. This component probably represents free couples. The number of phenylalanine residues polymerized per high salt stable 80 s ribosome is about 25 in this experiment.

(e) *Catalysis of peptide bond formation by purified 60 S ribosomal subunits*

Purified mouse liver 60 s subunits are able to catalyze the formation of peptide bonds using as substrates puromycin and *N*-formyl- or *N*-acetylaminoacyl-oligonucle-otides from tRNA as described by Monro & Marker (1967). In the experiments shown
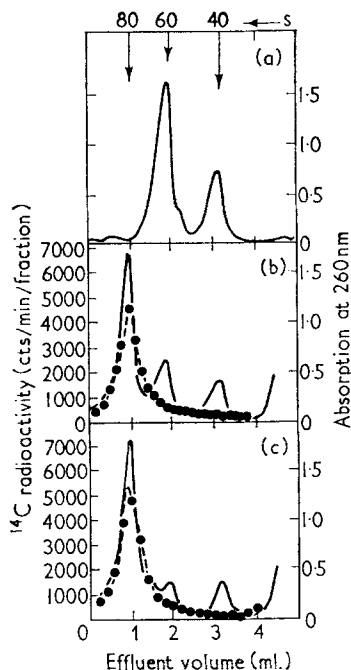
FIG. 10. Sucrose gradient analysis of reaction mixtures containing purified 60 and 40 s subunits recombined and incubated at 30°C in conditions for polyphenylalanine synthesis. 60 and 40 s subunits were combined in a molar ratio of 1:1·2. (a) Non-incubated mixture of ribosomal subunits analyzed in high salt; (b) reaction mixture incubated for 40 min and analyzed in high salt; (c) reaction mixture incubated for 40 min and analyzed in low salt.

in Figure 11 we compare the rates of N-acetyl-leucyl-puromycin formation catalyzed by $E.$ $coli$ 50 s subunits and mouse liver 60 s subunits. The reaction rate with $E.$ $coli$ subunits is approximately twice that with liver subunits. Anisomycin, an inhibitor of mammalian protein synthesis, specifically inhibits the 60 s subunit-catalyzed reaction and has no effect on $E.$ $coli$ ribosomes, whereas chloramphenicol, a strong inhibitor of bacterial peptidyl-transferase, has no effect on the mammalian system. Cyclohexamide,
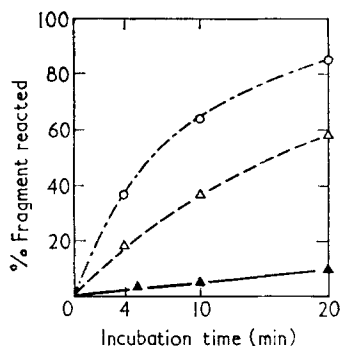

FIG. 11. Comparison of the peptidyl-transferase activity of mouse liver 60 s and $E.$ $coli$ 50 s ribosomal subunits as measured by the fragment assay. See Materials and Methods for details of the assay. —○·—·○—, $E.$ $coli$ 50 s + or − anisomycin; --△--△--, mouse liver 60 s − anisomycin (or + chloramphenicol); — ▲— ▲—, mouse liver 60 s + 10^{-5} M-anisomycin.

an inhibitor of mammalian protein synthesis, presumably affecting translocation, does not inhibit the peptidyl-transferase reaction as tested by the fragment assay. Our results are in agreement with and confirm the work with human tonsil ribosomes by Vazquez, Battaner, Neth, Heller & Monro (1959).

4. Discussion

While our work was in progress several laboratories reported the isolation of mammalian ribosomal subunits (Martin *et al.*, 1969; Martin & Wool, 1968, 1969; Dickmann, 1969; Petermann, Pavlovec & Weinstein, 1969; Terao & Ogata, 1970: Lazda & Noll, personal communication). Some of our results confirm observations by these workers, particularly the spontaneous reassociation of subunits in ionic conditions used for polypeptide synthesis. We also found that hybrid 80 s ribosomes formed from rat and mouse subunits are as active in polyphenylalanine synthesis as homologous 80 s couples. The most complete data on the function of subunits are those of Martin & Wool. Their phenylalanine incorporations range from about two to seven amino-acid residues polymerized per ribosome couple. As far as quantitative analyses from published data are possible, it seems that only a minor fraction of the ribosomes in the purified subunit preparations were able to function in poly U-directed polypeptide synthesis.

The aim of our study was first to develop and optimize a method for the preparation of mammalian ribosomal subunits, the majority of which function in polypeptide synthesis if recombined and supplied with an artificial messenger. We considered it important physically and functionally to characterize the starting material and intermediate steps of our isolation procedure in detail. The starting material consists of purified polysomes, which only through active polypeptide synthesis *in vitro* are converted into free 80 s couples used for subunit preparation.

Since there still exist some opposing opinions on the state of bacterial ribosomal subunits between polypeptide chain termination and reinitiation, we wanted to describe and define the physicochemical conditions and requirements for dissociation and association of the mammalian subunits. Of particular interest was the question of whether or not tRNA, either free or as peptidyl-tRNA, was responsible for the stabilization of messenger-free 80 s couples in ionic conditions used for *in vitro* protein synthesis. Our results show quite conclusively that tRNA is not needed for maintaining the association of 40 and 60 s subunits in 80 s couples. Free or aminoacyl-tRNA binds only loosely, if at all, to such 80 s couples. And, it washes off very rapidly in sucrose gradients containing the ionic conditions used for *in vitro* protein synthesis.

As we shall show in more detail in the following report, the free 80 s ribosome is a loosely associated subunit couple. However, its predominance in sucrose gradients, even at 28°C, implies that the 80 s couple may be a natural state of mammalian ribosomal subunits not engaged in protein synthesis. We want to emphasize that in this study we intentionally eliminated from our preparation the 80 s ribosomes which are found in crude tissue extracts, because it seems likely that at least a major fraction of the monosomes found *in vivo* represent a special pool (Bishop, 1965; Joklik & Becker, 1965a,b) which can be activated reversibly for protein synthesis (Bagliga, Pronczuk & Munro, 1968; Fleck, Shepherd & Munro, 1965; Hogan & Korner, 1968; Staehelin, Verney & Sidransky, 1967). This problem is discussed in more detail in the following paper.

For the present study we would like to emphasize the following points:

(1) Subunits derived from active polysomes after polypeptide chain termination have a strong tendency to form couples in the same conditions in which they function on polysomes, even in the absence of tRNA and mRNA. These free couples or monosomes are quite distinct from mRNA-bound monomers carrying peptidyl-tRNA. In 0·004 M-Mg^{2+}, 0·15 M-NH_4Cl and 0·02 M-Tris pH 7·5 they sediment as a distinct peak at about 70 s, between the 60 s subunit and the 80 s mRNA–peptidyl-tRNA–monomer complex (Falvey & Staehelin, results to be published).

(2) During our preparation procedure the highest concentration of KCl to which the ribosomes or their subunits are exposed is 0·5 M in the presence of 0·004 M-magnesium acetate (KCl treatment following preincubation of polysomes) and 0·3 M in the presence of 0·002 or 0·003 M-magnesium acetate (conditions used for the preparative subunit sucrose gradients). All preparative procedures were carried out at 2 to 4°C. The methods of Martin et al. (1969) for preparation of subunits involves centrifugation of mixtures of polysomes and free 80 s couples into gradients containing up to 0·880 M-KCl (with 0·012 M-magnesium acetate) for three-and-a-half hours at 28°C, or eight hours at 4°C. The subunit fractions from these preparative gradients are then dialyzed overnight. In our procedure the subunit fractions are concentrated by ethanol precipitation, dissolved in lower ionic strength buffer and immediately frozen.

(3) We chose to convert polysomes into free 80 s couples by the natural process of polypeptide chain elongation and termination, rather than by premature chain termination using puromycin. We compared the two methods and obtained consistently more active subunits, particularly 60 s subunits, without the use of puromycin. We tried two approaches with puromycin.

(a) *Exhaustive preincubation of polysomes in a complete system with puromycin until all polysomes were converted into 80 S monosomes.* These 80 s ribosomes were then dissociated in KCl and the subunits separated in sucrose gradients as described in Materials and Methods. 60 s subunits obtained by this procedure had consistently poor activity.

(b) *A short two- to ten-minute incubation of polysomes with puromycin and all the components for protein synthesis.* This rendered polysomes salt-labile similar to free 80 s couples. However, in high salt many of the 40 s subunits were not found in the 40 s peak, but rather sedimented presumably still as messenger-bound poly–40 s complexes between 60 and 200 s. The result of such an experiment is shown in Figure 12. After a five-minute incubation with puromycin, a large fraction of the total 60 s subunits present in polysomes and monosomes of the low salt gradient analysis sediment as free 60 s particles in the high salt gradient. However, the optical density ratio of the 60 s to the 40 s peak is approximately 5. A ratio of 2·7 would reflect an equimolar ratio of 60 to 40 s subunits. After ten minutes incubation the 60 to 40 s optical density ratio has improved to about 4·5. This result predicts poor recovery of 40 s subunits and heavily contaminated 60 s subunits unless harsher conditions for dissociation are used. We therefore did not pursue this approach any further.

We believe that this study has provided a useful and better defined system to study mammalian protein synthesis further, particularly the relationship between ribosome structure and function. We showed that our ribosomal subunit preparations are functionally and physically intact. They should lend themselves to studies of further ribosome dissociation and reconstitution similar to those done with bacterial
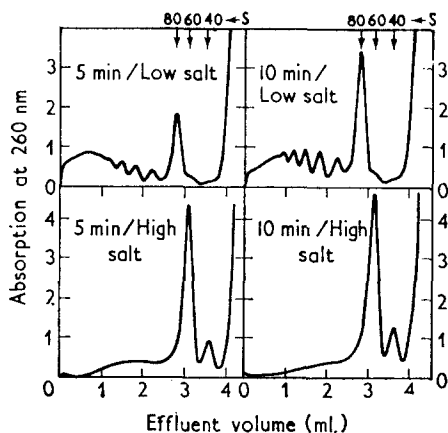
FIG. 12. Sucrose gradient analysis of puromycin-treated polysomes. Polysomes (10 O.D.$_{260}$ units/ml.) in a protein synthesizing system were incubated at 25°C. The reaction mixture contained 0·002 M-puromycin and the other components for protein synthesis as described in Materials and Methods. After 5 and 10 min of incubation, samples were analyzed in low salt (0·15 M-NH$_4$Cl, 0·005 M-magnesium acetate, and 0·02 M-Tris–HCl pH 7·5) and high salt (0·2 M-KCl, 0·003 M-magnesium acetate, 0·02 M-Tris–HCl pH 7·5). The gradients (ranging from 0·35 to 1·1 M-sucrose) were centrifuged at 3°C for 50 min at 50,000 rev./min and then analyzed as described in Materials and Methods.

ribosomes (Hosokawa, Fujimura & Nomura, 1966; Staehelin & Meselson, 1966). Furthermore, the mechanism of chain initiation and its detailed requirements with natural mRNA might be approached with a system like ours. In a first application of this system we have analyzed dissociation and association of ribosomal subunits during various stages and specific steps of protein synthesis *in vitro*. These results are presented in the following paper.

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