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Structure and Function of Mammalian Ribosomes

II.† Exchange of Ribosomal Subunits at Various Stages of *in vitro* Polypeptide Synthesis

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(1) During *in vitro* polypeptide chain termination by rat or mouse liver polysomes, 80 s monosomes briefly dissociate into free subunits, presumably at the moment of their relase from the messenger. The subunits reassociate to form 80 s couples. In this reassociation step labeled 40 s subunits added to the incubation mixture freely compete with unlabeled subunits.

(2) The 80 s ribosomes reformed after termination of the polypeptide chain slowly exchange their subunits with radioactive subunits in the reaction mixture. The equilibrium between free subunits and 80 s couples strongly favors the latter.

(3) In contrast to polypeptide chain termination, at initiation of polyphenylalanine synthesis directed by polyuridylic acid, 80 s ribosomes attach to this messenger without a dissociation step.

1. Introduction

In this report we describe the behavior of mammalian ribosomal subunits during *in vitro* polypeptide synthesis. Specifically, we asked if 80 s couples dissociate and reassociate during protein synthesis, and if they do dissociate and reassociate, at which steps do these reactions occur. Our approach was to add radioactively labeled 40 s subunits to unlabeled ribosomes at various stages of polypeptide synthesis. If at any stage short dissociation followed by reassociation of the subunits occurs, the radioactive subunits added to the incubation mixture should compete in the reassociation reaction and, therefore, enter the reformed 80 s couples.

From these experiments we have found that during termination of protein synthesis and release of the polypeptide chain from the ribosome, the 80 s couple dissociates into 60 s and 40 s subunits which rapidly reassociate to form free 80 s monosomes. These reformed free 80 s couples continue to exchange their subunits with free 40 or 60 s subunits present in the incubation mixture; we call this reaction "post-termination exchange". The post-termination exchange rate is very slow. The dissociation–reassociation equilibrium strongly favors the state of the 80 s species as evidenced from sucrose gradient analysis. The experiments described in this report also demonstrate that at initiation of poly U-directed polyphenylalanine synthesis, preformed

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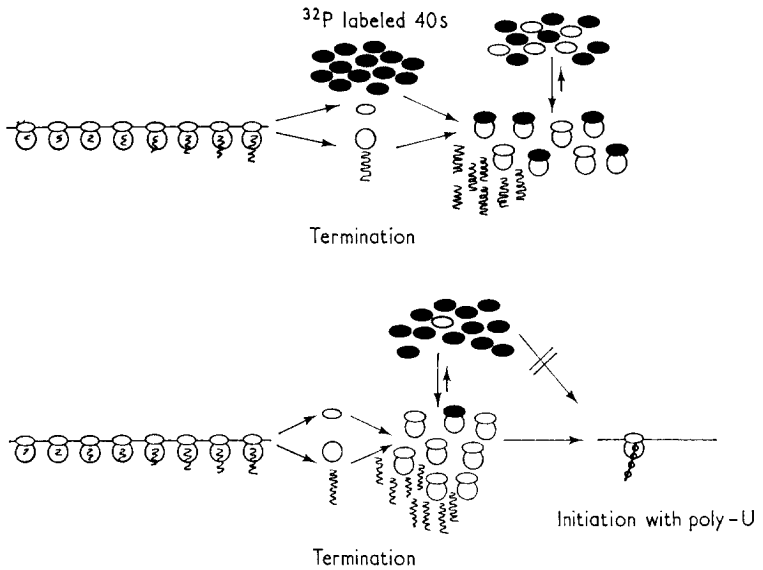

FIG. 1. Illustration of experimental design and main implications of this study. Upper portion: ^{32}P-labeled 40 s subunits were present during the incubation of polysomes in conditions supporting chain elongation and termination. Illustration of subunit exchange during polypeptide chain termination. Lower portion: ^{32}P-labeled 40 s subunits added after preincubation of polysomes for polypeptide chain termination. Illustration of post-termination exchange and of poly U-dependent polypeptide chain initiation.

Single arrows indicate distinct steps. Bidirectional double arrows indicate equilibrium reactions.

80 s couples attach to the messenger without prior dissociation into subunits. These results are schematically summarized in Figure. 1

2. Materials and Methods

(a) *Preparation of radioactive ribosomal subunits*

All methods for preparation and isolation of polysomes and subunits as well as pH 5 enzyme and high speed supernatant are the same as described in the preceding paper (Falvey & Staehelin, 1970). ^{32}P-labeled subunits were prepared from mice which had been injected intraperitoneally with 1 mc of carrier-free ^{32}P-labeled H_3O_4 in 0·15 M-NaCl 18 to 20 hr before exsanguination under ether anaesthesia. The purified ^{32}P-labeled polysomes were exhaustively preincubated in a protein synthesizing system in order to terminate and release the polypeptide chains. The chilled reaction mixture was made 0·5 M-KCl and centrifuged in sucrose gradients containing 0·3 M-KCl, 0·003 M-magnesium acetate, 0·02 M-Tris–HCl pH 7·5 and 0·001 M-dithiothreitol. The gradient fractions containing pure 40 and 60 s subunits were precipitated with ethanol (40% final concentration). Figure 2 shows the analysis in low and high salt of purified ^{32}P-labeled 40 s subunits. The initial, specific activity of various preparations was between 12,000 and 16,000 cts/min per O.D.$_{260}$ unit of ribosomes.

(b) *Conditions for incubation*

(i) *Experiments concerning subunit exchange during termination of polypeptide chains as well as "preincubation" of polysomes to obtain 80s monosomes for post-termination and initiation exchange experiments*

The reaction mixtures contained per ml: 1 μmole ATP, 0·4 μmole GTP, 10 μmole phosphocreatine, 40 μg creatine phosphokinase, 0·2 ml, S_{100}†, 0·2 ml. pH 5 enzyme, 150 μmole

† Abbreviation used: S_{100}, high speed supernatant fraction.

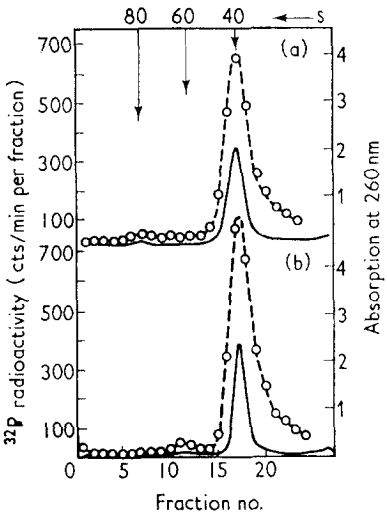

FIG. 2. Purified ^{32}P-labeled 40 s subunits analyzed in low salt (a) and high salt (b) sucrose gradients. The asymmetry of the radioactivity is primarily due to the loss of resolution in the tubing between the ultraviolet absorption flow cell and the counting vial. In later experiments this was partially improved by eliminating a U-turn in the tubing. --○--○--, ^{32}P radioactivity; ———, absorbance at 260 nm.

NH_4Cl, 5 μmole magnesium acetate, 20 μmole Tris–HCl pH 7·5, 1 μmole dithiothreitol, and polysomes (13 to 20 O.D.$_{260}$ units depending on the experiment) and when specified [^3H]phenylalanine with a specific activity of 6·3 c/m-mole (the amount used is specified in each corresponding Figure). The length of time and temperatures used for the incubations are indicated in each Figure.

(ii) *Experiments concerning exchange at initiation with polyuridylic acid template (poly U)*

Following preincubation as described above, we added to the reaction mixtures per ml.: ^{32}P-labeled 40 s subunits (amounts indicated in each Figure), 7 μmole magnesium acetate (final concentration is 12 μmole/ml.), 0·375 mg poly U, [^3H]phenylalanine with a specific activity of 6·3 c/m-mole (amounts indicated in each Figure), 5 μmole phosphocreatine, and 20 μg creatine phosphokinase. The reaction mixtures were incubated at 30°C, for the lengths of time specified in each Figure.

(c) *Sucrose gradient analysis and radioactivity measurements*

All sucrose gradient analyses in this report were carried out using the SW 50·1 Spinco rotor at 50,000 rev./min and 4°C for the times indicated in each Figure. Unless otherwise specified in the Figures, we used 5 ml. convex exponential gradients from 0·4 to 1·0 M-sucrose in which ribosomes sediment almost isokinetically throughout the gradient. After centrifugation, the absorption at 260 nm was monitored continuously and fractions collected directly into counting vials and counted in a Beckman LS250 liquid scintillation spectrometer as described in the preceding paper (Falvey & Staehelin, 1970). In all Figures the optical density scales are corrected to a 1-cm light path. In double label experiments with ^3H and ^{32}P the window discriminators for the two channels were set to give 0% of the ^3H counts in the ^{32}P window and 8% ^{32}P counts in the ^3H window. At these settings the counting efficiency of ^{32}P was 80% and that of ^3H was 16%.

(d) *Analysis of* in vitro *subunit exchange*

(i) *During polypeptide chain termination*

Reaction mixtures containing polysomes and ^{32}P-labeled 40 s subunits plus all the components required for polypeptide synthesis were incubated at 25°C for various lengths of time, then chilled and analyzed by sucrose gradient centrifugation in low salt ionic

conditions (0·1 M-NH_4Cl and 0·005 M-magnesium acetate). The concentration of polysomes was chosen to result in a suitable amount of 80 S monosomes released after a given time of incubation. The amount of radioactive 40 S subunits was chosen to result in a ratio of free 80 S monosomes to 40 S subunits feasible for radioactivity analysis of the gradients. This ratio was varied on purpose in different experiments by a factor of at least 2·5 in order to assure that the degree of subunit exchange was independent of this ratio.

(ii) *After termination*

Reaction mixtures containing unlabeled polysomes were incubated at 30°C to allow complete polypeptide chain termination and release of 80 S couples from mRNA. Then radioactive 40 S subunits were added and the incubation was continued in the same ionic conditions for the specified lengths of time followed by sucrose gradient analysis in low salt conditions.

(iii) *During poly U-directed polypeptide chain initiation*

After preincubation of polysomes to allow complete chain termination, we added to the reaction mixture in the following order at 0°C: ^{32}P-labeled 40 S subunits, [^3H]phenylalanine, poly U, magnesium acetate, phosphocreatine, and creatine phosphokinase in concentrations as specified above. The reaction mixtures were then incubated at 30°C for specified lengths of time, after which they were centrifuged through sucrose gradients containing high salt (0·3 M-KCl and 0·003 M-magnesium acetate). These ionic conditions allow selective analysis of 80 S ribosomes which have initiated polypeptide synthesis with poly U.

3. Results

(a) *Subunit exchange during polypeptide chain termination*

Since there is negligible reinitiation in our *in vitro* system for protein synthesis using purified liver polysomes, polypeptide chain elongation and termination are the two main processes which occur during incubation. At termination 80 S monosomes are released from the messenger and accumulate. In order to study the fate of the ribosomal subunits during termination, at zero time we added different amounts of radioactively labeled 40 S subunits to the reaction mixtures which we then incubated at 25°C for various lengths of time. Figure 3 shows sucrose gradient analyses of the reaction mixtures. 80 S monosomes and 60 and 40 S subunits are separated and displayed in the gradient while the remaining polysomes have pelleted. It is clear that radioactive 40 S subunits did enter the 80 S ribosomes which accumulated during each incubation period. The pronounced broadening and trailing of the radioactivity behind the 80 S optical density peak, due to some mixing of the more shallow lower part of the gradients in the collecting system, makes a visual estimate of the degree of subunit exchange unfeasible. But from the integrated optical density and radioactivity peak areas we can calculate that during each incubation period the subunits in the accumulated 80 S particles had almost completely equilibrated with free radioactive subunits (for details see Results section (d)). In Figure 4 the sucrose gradients display polysomes, released 80 S ribosomes and free subunits from incubation mixtures in which the polypeptide chains are labeled with [^3H]phenylalanine. This experiment clearly shows that radioactive 40 S subunits exchange with those of released 80 S couples but not with polysomes; thus, dissociation and reassociation of subunits must occur at or after termination. Again, after both five and ten minutes of incubation, the subunits of the 80 S ribosomes have almost completely equilibrated with the free radioactive 40 S subunits. The absence of any significant quantity of ^{32}P-radioactivity in the polysomes, even after ten minutes of incubation, demonstrates that no appreciable reattachment of ribosomes to polysome-associated mRNA occurs. Furthermore, the

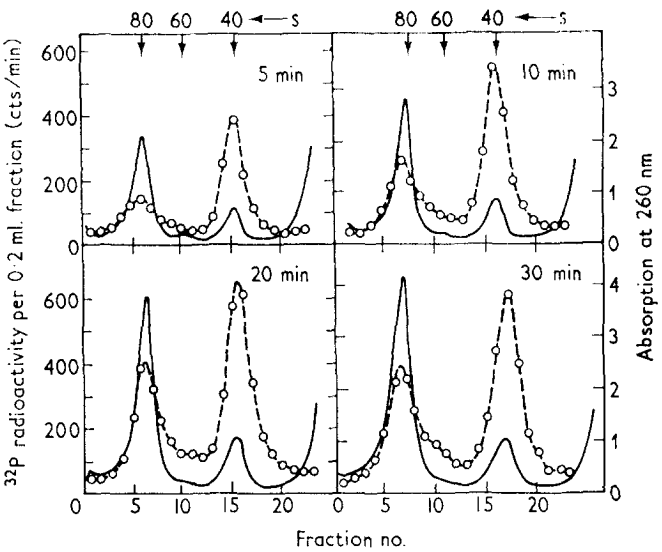

FIG. 3. Exchange of ribosomal subunits during polypeptide chain termination. 0·3-ml. samples of reaction mixture were incubated at 25°C for the indicated lengths of time. Following the incubation the reaction mixtures were chilled and immediately layered onto 5-ml. low salt sucrose gradients which were centrifuged at 4°C for 2 hr at 50,000 rev./min. The reaction mixtures contained the following amounts of polysomes and ^{32}P-labeled 40 s subunits: 5-min incubation, 6 O.D.$_{260}$ units of polysomes and 0·26 O.D.$_{260}$ unit of ^{32}P-labeled 40 s subunits; 10-min incubation, 5 O.D.$_{260}$ units of polysomes and 0·40 O.D.$_{260}$ unit of ^{32}P-labeled 40 s subunits; 20-min incubation, 4 O.D.$_{260}$ units of polysomes and 0·55 O.D.$_{260}$ unit of ^{32}P-labeled 40 s subunits; 30-min incubation, 3 O.D.$_{260}$ units of polysomes and 0·55 O.D.$_{260}$ unit of ^{32}P-labeled 40 s subunits. – –○– –○– –, ^{32}P radioactivity, ———; absorbance at 260 nm.

lack of [^{3}H]phenylalanine radioactivity in the 80 s region shows that most of the polypeptide chains are released from the 80 s particles.

(b) *Post-termination subunit exchange*

From the experiments described above we cannot distinguish whether subunit exchange (that is, dissociation and reassociation) is coupled to, and therefore a direct result of, the termination event or whether subunit exchange occurs very rapidly after termination. In order to distinguish between these two possibilities, we wanted to determine the rate of possible subunit exchange in the absence of polypeptide chain termination. In these experiments we incubated polysomes under conditions for protein synthesis long enough to ensure polypeptide chain termination by all competent ribosomes and then added ^{32}P-labeled 40 s subunits to the reaction mixture which we further incubated for various lengths of time. Sucrose gradient analyses of these reaction mixtures demonstrate that labeled 40 s subunits do exchange with the 40 s subunits of the 80 s couples. Figure 5 shows the kinetics of this post-termination exchange in ionic conditions identical to those used for protein synthesis with endogenous mRNA (0·15 M-NH_4Cl and 0·005 M magnesium acetate). The exchange is, however, very slow and does not reach equilibrium even after 50 minutes of incubation. This is most evident from a comparison between Figure 5 and the 30-minute incubation of Figure 3; all of these sucrose gradients contain about the same ratio of 80 s to free 40 s particles.

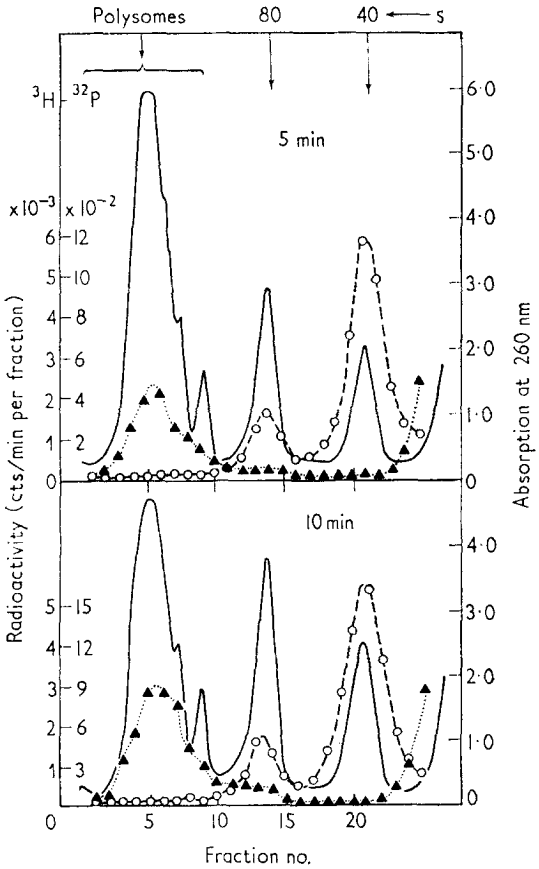

FIG. 4. Exchange of ribosomal subunits during polypeptide chain termination exclusively between released 80 s couples and free subunits. Incubations were carried out as described in Fig. 3 with the following modifications: (1) 0·3 ml. of the reaction mixture contained 1·4 μc [^{3}H]phenylalanine to label the nascent protein chains; (2) the 5-min incubation contained per 0·3 ml. 6 O.D.$_{260}$ units of polysomes and 0·8 O.D.$_{260}$ unit of ^{32}P-labeled 40 s subunits and the 10-min incubation contained per 0·3 ml. 5 O.D.$_{260}$ units of polysomes and 1·2 O.D.$_{260}$ units of ^{32}P-labeled 40 s subunits. These reaction mixtures were analyzed in low salt sucrose gradients which consisted of 0·7 ml. of a 2 M-sucrose cushion, 0·8 ml. of a manually layered gradient from 1·8 to 1·2 M-sucrose, and 3·5 ml. of a continuous convex gradient from 0·4 to 1·0 M-sucrose. (These gradients were prepared 4 to 5 hr before use.) Centrifugation was for 80 min at 50,000 rev./min and 4°C. Polysomes accumulate at the very steep lower part of the gradient whereas 80 s ribosomes and subunits are well separated in the flatter upper part of the gradients.

A possible source of error in the interpretation of the experiments concerning the subunit exchange during or after termination may lie in the fact that as termination proceeds, the amount of free 80 s ribosomes constantly increases from almost zero to the amount present at the end of the incubation period. On the other hand, during the post-termination exchange experiments shown in Figure 5, the ratio of 80 s ribosomes to labeled 40 s subunits was constant throughout the entire incubation period. Therefore, we did post-termination exchange experiments in which we mimicked as closely as possible the kinetics of 80 s accumulation observed in the termination experiments. To an incubation mixture containing labeled 40 s subunits post-termination 80 s ribosomes were added in small increments at short intervals throughout the incubation

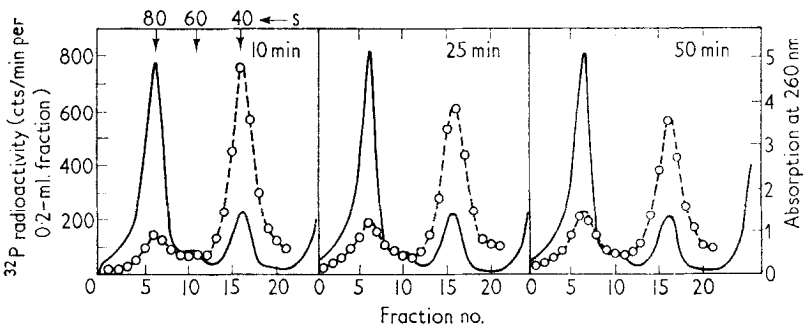

FIG. 5. Kinetics of post-termination exchange. 10 O.D.$_{260}$ units of polysomes per ml. were pre-incubated as described in Materials and Methods to allow complete polypeptide chain termination. 3·7 O.D.$_{260}$ units of ^{32}P-labeled 40 S subunits per ml. were then added and incubation was continued at 25°C for 10, 25 and 50 min. 0·2-ml. samples of the reaction mixtures were immediately centrifuged in 5 ml. low salt sucrose gradients at 4°C for 2 hr at 50,000 rev./min. – – ○ – – ○ – –, ^{32}P radioactivity; ———, absorbance at 260 nm.

period. The results (not shown here) demonstrate that the degree of exchange after 5 and 20 minutes is indistinguishable from that observed when all of the 80 S ribosomes were present from the beginning of the incubation. Thus, the striking difference in the extent of subunit equilibration between termination and post-termination exchange experiments cannot be attributed to the variation in the ratio of 80 S to labeled 40 S particles.

The experiments described so far illustrate that 80 S ribosomes briefly dissociate into free subunits and reassociate to form 80 S couples upon their release from polysomes at termination of the polypeptide chain. In the reassociation step free subunits (such as radioactive 40 S subunits) which are present in the incubation mixture compete with the polysome-derived 40 S subunits. After this distinct dissociation and reassociation, the 80 S couples remain in an equilibrium with free subunits. But the rate of subunit exchange occurring after termination is very slow; and the equilibrium strongly favors the state of 80 S couples.

In additional control experiments we degraded polysomes by treatment with pancreatic ribonuclease (RNase) into monosomes which are as stable in high salt as undegraded polysomes. As expected these 80 S ribosomes do not exchange subunits with radioactive 40 S subunits in ionic conditions where post-termination subunit exchange occurs.

(c) *Lack of subunit exchange during polyphenylalanine chain initiation*

Like chain termination, when 80 S ribosomes are released from mRNA, polypeptide chain initiation is a distinct and specific mechanism in protein synthesis. We therefore wanted to determine whether the step of ribosome attachment to mRNA and polypeptide chain initiation is preceded by an obligatory dissociation of 80 S ribosomes into subunits, or whether the ribosomes must initiate as an 80 S couple. At this time, for lack of availability of natural mRNA, we are restricted to the use of an artificial template, namely poly U. We are aware of the inherent limitations of this system.

To a reaction mixture containing free 80 S ribosomes formed during exhaustive incubation of polysomes in a complete system, we added at 0°C in the following order: ^{32}P-labeled 40 S subunits, [^{3}H]phenylalanine, and poly U plus other components to

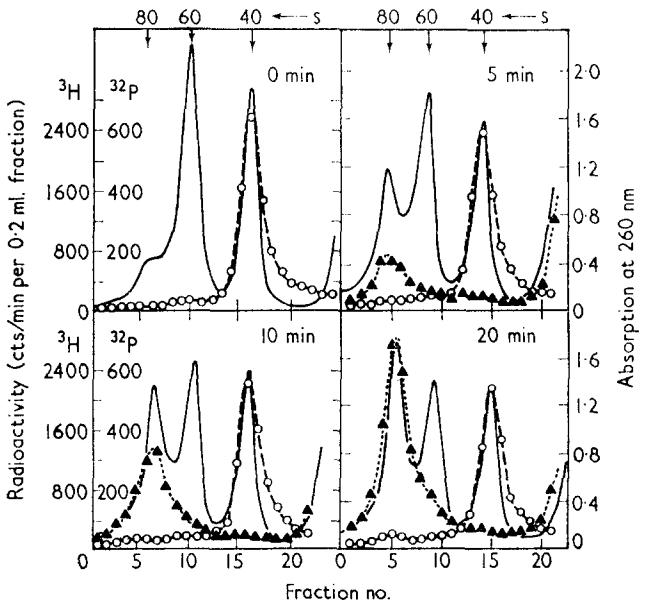

FIG. 6. Absence of subunit exchange during initiation of poly U-directed polyphenylalanine synthesis. After preincubation of polysomes as described in Materials and Methods to allow termination of endogenous protein synthesis, the following components were added in the listed order to each ml. chilled reaction mixture: 2 O.D.$_{260}$ units of ^{32}P-labeled 40 S subunits, 2·3 μC [^{3}H]phenylalanine (6·3 C per m-mole), 0·375 mg poly U, 7 μmoles magnesium acetate, 5 μmoles phosphocreatine and 20 μg creatine phosphokinase. Incubation was then continued at 30°C for 0, 5, 10 and 20 min following which the reaction mixtures were chilled in ice and made 0·5 M-KCl. Immediately 0·2-ml samples were layered onto high salt sucrose gradients and centrifuged at 4°C for 2 hr at 50,000 rev./min and then analyzed as described in Materials and Methods.

adjust the system to conditions needed for polyphenylalanine synthesis. Following incubation at 30°C for 0, 5, 10 and 20 minutes, we made the reaction mixtures 0·5 M-KCl and analyzed them in sucrose density gradients containing 0·3 M-KCl and 0·003 M-magnesium acetate. In these conditions only the active poly U-peptidyl-tRNA–ribosome complexes sediment as stable 80 S particles, while the remaining free ribosomes dissociate into subunits. Such an experiment is shown in Figure 6. Without incubation only the small fraction of ribosomes which did not terminate during the preincubation sediment as "stuck" 80 S particles. After 5, 10 and 20 minutes of incubation, the amounts of stable 80 S ribosomes carrying labeled polyphenylalanine increased, but they contain almost no radioactive 40 S subunits. The small amount of ^{32}P-labeled 40 S subunits which sediment in stable 80 S couples can be accounted for by post-termination exchange occurring before initiation. We measured the rate of this exchange in the absence of poly U but in the same ionic conditions. It is considerably slower than the post-termination exchange at the lower magnesium ion concentrations used for protein synthesis directed by endogenous messenger. (See Fig. 8 which is a summary of all exchange experiments.)

One might still argue that the preferential exclusion of radioactive 40 S subunits in polyphenylalanine synthesis is due to a much lower activity of these purified subunits compared to the polysome-derived 80 S ribosomes. In order to test this possibility, the

80 s ribosomes to be used in polyphenylalanine synthesis were formed by preincubation of polysomes in the presence of purified ^{32}P-labeled 40 s subunits. Then, after addition of [^{3}H]phenylalanine and poly U, we further incubated the reaction mixture in conditions suitable for polyphenylalanine synthesis. Figure 7(a) shows that ^{32}P-labeled 40 s subunits completely equilibrated with unlabeled polysome-derived 40 s subunits

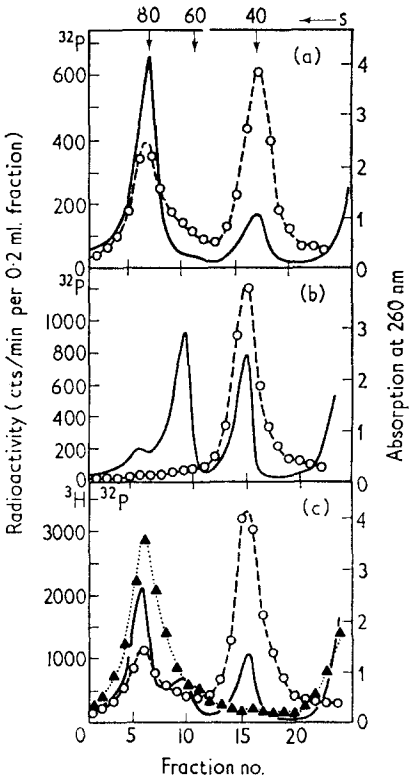

FIG. 7. Exchange of ^{32}P-labeled 40 s subunits into 80 s ribosomes during polypetide chain termination and subsequent participation in poly U-directed polyphenylalanine synthesis. (a) Low salt sucrose gradient analysis after completion of chain termination. (b) High salt sucrose gradient analysis of the same reaction mixture as in (a). (c) High salt sucrose gradient analysis of a similar reaction mixture after further incubation for 30 min in the presence of poly U and [^{3}H]phenylalanine in conditions for polyphenylalanine synthesis. Note the contracted scale of ^{32}P radioactivity in (b). ———, Absorbance at 260 nm.

upon chain termination. Analysis of the same incubation mixture in high salt demonstrates that no measurable 40 s subunits remain in stable 80 s ribosomes (Fig. 7(b)). However, after poly U-directed polyphenylalanine synthesis, a large fraction of ribosomes is now found as high salt stable active 80 s complexes (Fig. 7(c)) which contain about the same proportion of ^{32}P-labeled 40 s subunits as the 80 s ribosomes contained before initiation. This clearly demonstrates that the added ^{32}P-labeled 40 s subunits are about equally competent in polyphenylalanine synthesis as those in the polysome-derived 80 s (with which they exchange during termination). Therefore, the lack of subunit exchange during poly U-directed polypeptide chain initiation is due to the fact that ribosomes initiate as 80 s couples with poly U template.

(d) *Comparison of the rates of subunit exchange during various stages of protein synthesis*

Figure 8 summarizes the results of all subunit exchange experiments. The degree of subunit exchange as "percentage of full equilibration" was calculated in the following way: The optical density (O.D. at 260 nm) of the 80 S peak was integrated and divided by 3·7 in order to compute the amount of 40 S subunits present in the 80 S peak

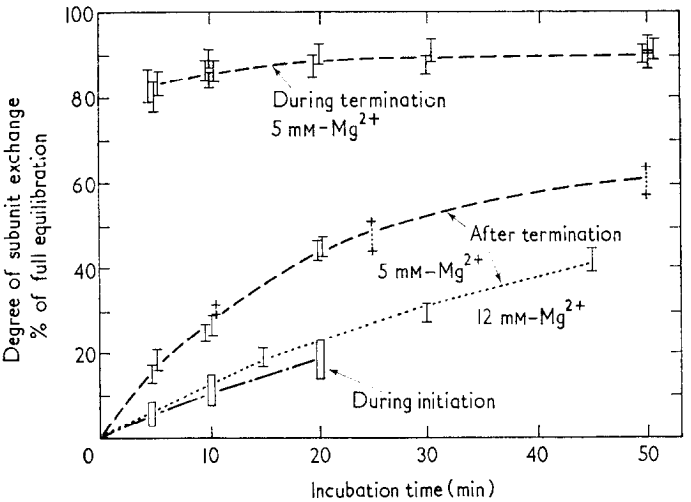

FIG. 8. Summary of data from experiments concerning subunit exchange at polypeptide chain termination, post-termination, and polyphenylalanine chain initiation. The degree of subunit exchange as percentage of full equilibration between radioactive and non-radioactive subunits in the 80 S particles was calculated as decribed in the text. Each solid bar represents the data from one sucrose gradient analysis. Each of the open bars showing the subunit exchange during initiation of polyphenylalanine synthesis represents the data from two independent experiments. The crosses connected by dotted lines represent the experiment shown in Fig. 5.

(designated $[40]_{80}$). The factor 3·7 reflects a 60 to 40 S subunit RNA mass ratio of 2·7:1. The amount of free 40 S subunits are designated $[40]_{40}$. The amounts of radioactivity in the 80 S peak and 40 S peak are designated $[P^{32}]_{80}$ and $[P^{32}]_{40}$ respectively. From these values we calculate the degree of exchange by dividing the fraction of the total radioactivity present in the 80 S peak by the fraction of the total 40 S mass present in the 80 S peak. Thus, the degree of exchange as percentage of full equilibration equals

$$\frac{[^{32}P]_{80}}{[^{32}P]_{80} + [^{32}P]_{40}} \div \frac{[40]_{80}}{[40]_{80} + [40]_{40}} \times 100.$$

Full equilibration (exchange) between labeled and unlabeled subunits has occurred if the mass fraction of 40 S ribosomes found in the 80 S peak equals the fraction of the total radioactivity present in that peak.

Between 80 and 90% of full equilibration is attained at all times during the termination experiments. After very brief incubations (5 and 10 min), the percentage of exchange was slightly lower, most likely resulting from the presence of some 80 S ribosomes in the original polysome preparation. These 80 S monosomes are high salt labile and therefore messenger-free couples which participate in the much slower post-termination exchange. In contrast to the instant subunit exchange occurring at polypeptide chain termination, the subunits of free 80 S ribosomes equilibrate very slowly

with subunits present in the reaction mixture. Increasing the magnesium ion concentration of the medium results in even slower rates of post-termination (subunit) exchange in spite of the fact that the temperature for incubation was increased 5 C degrees (to 30°C). This experiment was done to determine the rate of post-termination exchange in conditions of poly U-directed polypeptide chain initiation. During initiation of polyphenylalanine synthesis, very little subunit exchange occurs. The small amount of labeled 40 s subunits found in the stable 80 s ribosomes can be accounted for by the slow exchange occurring before 80 s couples initiate and become stabilized in the active complex of polypeptide synthesis.

4. Discussion

It has been well established for bacteria (Kaempfer, Meselson & Raskas, 1968; Kaempfer, 1968) and at least one eucaryotic organism, yeast (Kaempfer, 1969), that the two ribosomal subunits making up the particle active in protein synthesis are not permanently associated. For *Escherichia coli*, Mangiarotti & Schlessinger (1966) first suggested that ribosomes not engaged in protein synthesis exist as a pool of 30 and 50 s subunits which only combine to form stable 70 s couples associated with mRNA at the time of polypeptide chain initiation. At polypeptide chain termination the subunits are thought to dissociate and enter the pool of free subunits. Kaempfer *et al.* (1968) presented evidence for this model of ribosome cycling. They demonstrated with the use of heavy isotope labeling experiments that in growing bacteria the 30 and 50 s subunits found in polysome-derived 70 s particles exchange partners. And, extending this work to protein synthesis *in vitro* Kaempfer (1968) showed that when crude extracts from unlabeled and heavy isotope labeled bacteria were mixed under conditions supporting protein synthesis with endogenous mRNA, heavy and light subunits rapidly exchanged resulting in the formation of hybrid 70 s couples. The kinetics of this exchange were compatible with an exchange of subunits for each round of protein synthesis; thus, the subunits are at least temporarily, if not permanently, dissociated between chain termination and initiation. In an *in vitro* system with bacteriophage RNA as template Grubmann & Nakada (1969) also found that 70 s ribosomes dissociate into subunits before they reinitiate a new polypeptide chain. Furthermore, *in vitro* experiments on polypeptide chain initiation with viral mRNA or polyribonucleotides containing the initiation codon AUG, suggest that natural chain initiation with formylmethionyl-tRNA involves first the formation of a 30 s–mRNA complex. Fmet-tRNA is then bound to this complex followed by the joining of the 50 s subunit which results in the formation of the 70 s complex required for polypeptide synthesis. (Ghosh & Khorana, 1967; Guthrie & Nomura, 1968; Nomura, Lowry & Guthrie, 1967; Nomura & Lowry, 1967; Pestka & Nirenberg, 1966). At the present time the most important features of the model of the ribosome cycle in bacteria are: (1) after termination of a polypeptide chain the 70 s ribosome couple dissociates into its subunits, and (2) the mechanism of polypeptide chain initiation involves the formation of the mRNA–ribosome complex from free 30 and 50 s subunits joining in a sequence rather than the joining of a preformed 70 s couple to the mRNA in a single step.

In ionic conditions used for *in vitro* protein synthesis, the high affinity between 40 and 60 s subunits of mammalian ribosomes makes the free 80 s couple the predominant and almost exclusive state of mRNA-free ribosomes. Analysis of incubation mixtures in sucrose gradients at 28 to 29°C demonstrates that even at this temperature

free 80 s ribosomes are stable. If anything, we actually observed a smaller fraction of subunits in these gradients when compared with those analyzed at 2 to 4°C. Therefore, dissociation of free 80 s ribosomes into subunits could be either a rare fortuitous event or a quickly reversible specific step in the mechanism of protein synthesis.

In our present study we demonstrate clearly the following events in the cycle of protein synthesis. (a) During polypeptide chain termination there is a specific dissociation step of the 80 s ribosomes into subunits followed immediately by reassociation of the free subunits to couples. We visualize the dissociation as the result of an obligatory conformational change of the ribosomes during the mechanism of polypeptide chain termination and release, probably coupled to ribosome disengagement from mRNA. (b) After polypeptide chain termination the dissociation and reassociaton of a free 80 s couple is a very rare event. (c) Poly U-directed polypeptide chain initiation occurs through the attachment of an 80 s couple and not of free subunits to the template.

The absence of a distinct dissociation step before initiation with poly U is an interesting observation since it is in contrast to the mechanism of natural initiation in bacterial systems. Yet, this does not allow us to make any predictions concerning natural chain initiation in mammalian protein synthesis. Quite likely mammalian protein synthesis also requires several initiation factors, one of which might induce and maintain subunit dissociation similar to the mechanism suggested by Subramanian, Ron & Davis (1968) in *E. coli*. If mammalian polypeptide chain initiation is similar to that in bacteria, requiring free subunits for the formation of the active mRNA–ribosome complex, we would predict that one of the initiation factors causes ribosome dissociation by binding to one of the subunits (probably the 40 s subunit). Two types of observations in the literature suggest that free subunits rather than 80 s couples are competent in polypeptide chain initiation with natural messenger. Bishop (1966) observed that reticulocyte ribosome fractions rich in free 60 and 40 s subunits are most active in hemoglobin chain initiation *in vitro*. In intact reticulocytes Colombo, Vesco & Baglioni (1968) observed that NaF seemed to inhibit protein synthesis by interfering with ribosome dissociation. Thus, free subunits disappear during NaF treatment and reappear only during the recovery of the cells from NaF. And, Girard, Latham, Penman & Darnell (1965) and Hogan & Korner (1968*b*) showed that in tissue-culture cells free 40 and 60 s subunits become rapidly labeled. These newly synthesized labeled subunits first enter polysomes and appear in the free 80 s pool at a much slower rate. It seems then that at least part of the large amount of 80 s particles often found in animal cells represents a special pool of ribosomes which do not participate in protein synthesis. Yet, it is has been shown that these 80 s ribosomes *in vivo* can be activated to participate in protein synthesis as polysomes. For instance, force feeding starved animals a complete amino-acid mixture (Baliga, Pronczuk & Munro, 1968; Staehelin, Verney & Sidransky, 1967) or transferring ascites tumor cells from an amino-acid-poor into an amino-acid-rich medium (Hogan & Korner, 1968*a*) causes a rapid decrease of 80 s monosomes concomitant with an increase of polysome mass and size, even if mRNA synthesis has been blocked by actinomycin D. It appears therefore that in animal cells a regulatory mechanism may reversibly inactivate the 80 s ribosomes thereby enabling the cell to adjust its over-all rate of protein synthesis to changing physiological conditions.

In order to test the possibility that *in vivo* 80 s ribosomes differ functionally from polysome-derived 80 s particles, we compared the competence in poly U-directed

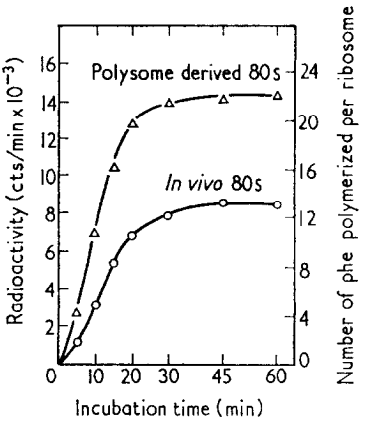

FIG. 9. Comparison of the kinetics of phenylalanine incorporation by polysome-derived and *in vivo* 80 s ribosomes. *In vivo* 80 s ribosomes were purified by sucrose gradient centrifugation of the postmitochondrial supernatant from mouse livers. Polysome-derived 80 s ribosomes prepared by incubation of purified polysomes in a protein synthesizing system were also isolated from sucrose gradients. The 80 s ribosomes in the sucrose gradient fractions were concentrated by alcohol precipitation and dissolved in low salt buffer. Reaction mixtures containing 7 O.D.$_{260}$ units of ribosomes per ml. were incubated at 33°C in a complete poly U system as described in Materials and Methods. At various times 0·1-ml. portions were withdrawn and prepared for radioactivity counting as described in Materials and Methods.

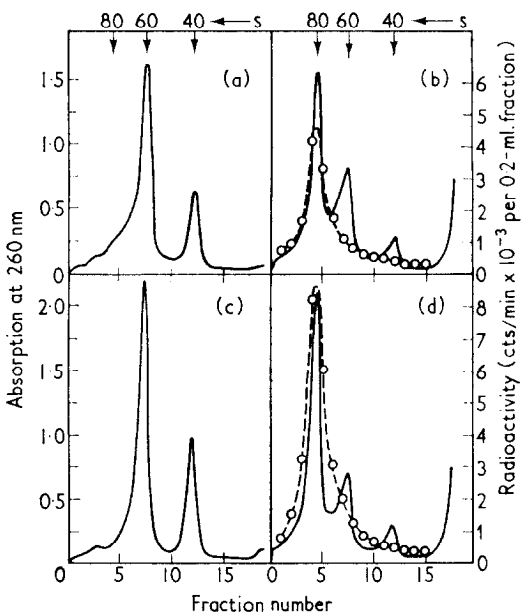

FIG. 10. High salt sucrose gradient analyses (0·3 M-KCl, 0·003 M-magnesium acetate. 0·02 M-Tris–HCl pH 7·5) of *in vivo* 80 s ribosomes ((a) and (b)) and polysome-derived 80 s ribosomes ((c) and (d)) before ((a) and (b)) and after ((b) and (d)) incubation in a complete polyphenylalanine synthesizing system. Reaction mixtures similar to those described in Fig. 9 were incubated at 25°C for 60 min.

polypeptide synthesis of 80 s ribosomes isolated from fresh tissue homogenates with those derived from polysomes after *in vitro* chain termination. The experiment shown in Figure 9 compares the time dependence at 33°C of polyphenylalanine synthesis by *in vivo* and polysome-derived 80 s ribosomes. The kinetics are very similar, but polysome-derived 80 s ribosomes incorporate 1·6 to 1·7 times more phenylalanine than *in vivo* 80 s particles. Figure 10 shows the sucrose gradient analysis in high salt of a similar experiment, but using different preparations of *in vivo* and polysome-derived 80 s ribosomes. Only about 40% of the 60 s and 40 s subunits present in the *in vivo* 80 s preparation participate in polyphenylalanine synthesis compared to about 65% of those from polysome-derived 80 s couples. These results confirm and extend observations reported by Bishop (1965).

Further investigations are needed to understand fully and expand some of the implications of our data. More detailed physical, chemical and functional studies of the *in vivo* 80 s particles should clarify the nature and cause of their low activity in polypeptide synthesis. And, investigation of initiation with natural messenger is required in order to determine whether subunits of 80 s couples participate in the natural ribosome–mRNA binding reaction.

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