POLYRIBOSOME BREAKDOWN AND HEMOGLOBIN SYNTHESIS

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Communicated by David E. Green, September 16, 1963

Recent reports have shown that polyribosomes, structures containing several ribosomes probably held together by messenger RNA, are the major sites of protein synthesis in reticulocytes,¹⁻³ in rat liver,⁴ in HeLa cells,⁵ and in *E. coli*.⁶ Also, these structures may be formed when poly U is added to *E. coli* ribosomes.⁶⁻⁸ We have previously reported briefly on the breakdown of polyribosomes coincident with hemoglobin synthesis. In these earlier studies, 80S ribosomes were also active for hemoglobin synthesis, but joined the aggregates during synthesis and left on completion of a globin chain.⁹ Here we report the orderly breakdown of polyribosomes dependent on hemoglobin synthesis, along with evidence that each ribosome is liberated from the polyribosome structure as it completes and releases a globin chain. Also, evidence for formation of polyribosomes by attachment of 80S ribosomes is presented. A mechanism involving ribosome movement along the messenger RNA coordinated with peptide-chain synthesis was postulated by Gierer,³ Warner *et al.*,¹⁰ and Gilbert.⁶ A similar conclusion was reached by Noll *et al.*¹¹ using liver ribosomes.

Materials and Methods.—Reticulocyte ribosomes were isolated as described by Allen and Schweet.¹² Preparations designated 1X microsomes were centrifuged once and suspended by gentle hand homogenization with a loose-fitting lucite homogenizer in either solution D (10^{-2} M Tris buffer, 10^{-3} M KCl, 10^{-4} M $MgCl_2$), or 0.25 M sucrose in distilled water. Thus, 1X microsomes were pelleted only once before use, whereas 2X microsomes were resuspended in medium B¹² and precipitated by centrifugation a second time before use.

Usually, fresh whole supernatant solution from ribosome preparations was used for the preparation of soluble enzyme fractions. Two types of preparations designated "regular enzyme" and "purified enzyme" were used. Regular enzyme was prepared by making whole supernatant¹² to 0.1 M Tris HCl at pH 7.5. Powdered ammonium sulfate was then slowly added with stirring to give 40% saturation. After 30 min the mixture was centrifuged, the precipitate was discarded, and powdered ammonium sulfate added to the supernatant to give 70% saturation. After 30 min with gentle stirring, the precipitate was collected by centrifugation and then dissolved in 0.1 M Tris-HCl buffer at pH 7.5 and dialyzed for 18 hr against 2 changes of 0.02 M Tris-HCl buffer, pH 7.5 containing 10^{-3} M GSH and 10^{-4} M EDTA. The resulting "regular enzyme fraction" contained sRNA and was relatively rich in hemoglobin even though most of the hemoglobin present in the whole supernatant solution was not precipitated with ammonium sulfate at 70% of saturation.

The preparation of "purified enzyme" differed from that of regular enzyme in that a 1.0% solution of protamine sulfate in 0.1 M Tris HCl at pH 7.5 was slowly added with stirring to the whole supernatant to give a final concentration of protamine sulfate of 0.34 mg/ml. After 30 min, the resulting protamine-RNA precipitate was removed by centrifugation. Ammonium sulfate was then slowly added with stirring to the supernatant as above. After 30 min with stirring, the precipitate was removed by centrifugation, discarded, and ammonium sulfate added to the supernatant as before to give a 70% saturated solution. The precipitate was dissolved in a solution containing 0.1 M Tris chloride at pH 7.5, 5×10^{-4} M GSH, and 10^{-4} M EDTA, and brought to a volume equal to one-half the volume of the whole supernatant originally used. The pH of the solution was adjusted to 7.0 by the cautious addition of 1.0 M acetic acid, and the fraction which precipitated between 40-70% saturation with ammonium sulfate prepared as before. The precipitate was dissolved in the same volume of 0.1 M Tris buffer containing GSH and EDTA as used before. The pH of the resulting solution was cautiously adjusted to 6.4 with 0.1 M acetic acid and ammonium sulfate added to give 70% saturation. The precipitate was collected by cen-

trifugation, dissolved in 0.002 *M* Tris HCl, pH 7.5 containing 10^{-3} *M* GSH plus 10^{-4} *M* EDTA to give 1/30 the volume of the whole supernatant used. This solution was then dialyzed against 2 changes of the same solution for 18 hr. The resulting "purified enzyme fraction" contained less than 0.6% nucleic acid by optical density measurements and was only light pink due to contaminating hemoglobin.

Assay procedure: The complete reaction mixture used was that previously described,¹² with minor variations. The complete reaction mixture contained 4.0 mg of ribosomes except for those tubes analyzed for ribosomal composition in the analytical ultracentrifuge or applied to sucrose gradients, in which cases 6.0 mg of ribosomes and 1–2 mg of purified enzyme or 12 mg of regular enzyme plus the other usual components were employed. The mixtures were incubated at 37°C, then either precipitated with 5% TCA in the presence of about 10 mg of casein per tube, or chilled in ice for analysis in the analytical ultracentrifuge or on sucrose gradients. All samples were taken through the complete washing procedure.¹² Radioactivity was determined using either a Nuclear-Chicago gas flow counter or a Packard Tri-Carb scintillation counter. C^{14} -valine incorporation $\times 8.3$ was used to calculate the $m\mu$ moles of total amino acids incorporated into hemoglobin per mg of ribosomes (ribonucleoprotein). For poly-U-induced polyphenylalanine synthesis, the amount of phenylalanine incorporated into hemoglobin was subtracted from the total polyphenylalanine synthesized.

Analysis of ribosome composition: The composition of reticulocyte ribosomes was determined from schlieren images obtained during sedimentation near 0° in a Spinco Model E analytical ultracentrifuge simultaneously using a regular and a wedge window cell. Unless otherwise specified, ribosomes were analyzed at a concentration of 4.25 mg/ml with a bar angle of 40° in the regular complete system incubation mixture. Controls run in 0.01 *M* KCl plus 10^{-4} *M* $MgCl_2$ gave similar patterns. In some cases, reaction mixtures contained relatively large amounts of hemoglobin which made them opaque to light of a wavelength shorter than about 600 $m\mu$, thus necessitating an optical system utilizing red light. This was accomplished by using a Wratten no. 29 red filter with the standard high-pressure mercury arc of the analytical ultracentrifuge and Kodak Type 1-N or Type 103-F spectroscopic plates, either of which gave satisfactory sensitivity in the red region.

Relative concentrations of the ribosomal components were measured by determining the area under the peaks of the schlieren profiles. Appropriate schlieren images were enlarged by projection to about 8×10 in. on single weight Kodak Kodabromide F-5 photographic paper. A base line was fitted to the enlarged profile on the resulting photograph, then each peak was carefully cut out and weighed. After correction for radial dilution within the centrifuge cell, the weights of the photographic paper representing each peak were directly proportional to the concentration of the species of ribosomes present in the sample. This method was found to give greater accuracy than could be obtained with a planimeter. Despite such errors as occur in fitting a base line to the schlieren patterns and determining the outer edges of the peaks, the method gave values that were reproducible within a few per cent, and were in good agreement with percentage compositions of ribosomes determined by sucrose gradient centrifugation.

Sucrose gradients: Linear sucrose gradients were formed for a Spinco SW 25.1 swinging bucket rotor using 7.5–30% w/v recrystallized sucrose (Fisher reagent grade) in solution D. Gradients were formed at room temperature, then the tubes were allowed to stand 30–60 min in ice before 1.0 ml of cold complete reaction mixture containing 4.25 mg of ribosomes was layered on each gradient. These were centrifuged at 24,000 rpm for 4 hr at a refrigeration setting of the centrifuge that maintained the temperature near 0° throughout the run.

Ten-drop fractions were collected from each tube. An aliquot of each fraction was removed and diluted for determination of the optical density at 260 $m\mu$; then casein and 5% TCA were added to the remaining portion of each fraction. All fractions were then washed and counted as previously described. Since drop size (and therefore fraction size) was found to vary, data was corrected to and plotted on the basis of 30 fractions per gradient tube. Both optical density and incorporation are expressed as values for 1.0-ml fractions.

Results.—Polyribosome breakdown and protein synthesis: When 1X ribosomes (see *Methods*) were incubated in the complete system and the mixtures cooled to 4° after various incubation times, a progressive breakdown of polyribosomes was

FIG. 1.—Sedimentation patterns of reticulocyte ribosomes after incubation in the cell-free system. Ribosome preparations were incubated in the complete system for hemoglobin synthesis for the indicated number of min. Aliquots were cooled to 0° and analyzed in the analytical ultracentrifuge (see *Methods*). The direction of sedimentation is from left to right. Centrifugation was generally about eight min at 42,000 rpm at 0°.

observed (Fig. 1). The peaks seen in these patterns have sedimentation constants of approximately 190, 170, 150, 130, 110, and 80S, confirming earlier reports.¹⁻³ The percentages of the various sizes of polyribosomes and 80S particles in the 1X ribosomes are similar to those in an un-pelleted extract (unpublished data).

Three points of interest can be seen in Figure 1. The breakdown is essentially a conversion of polyribosomes to 80S ribosomes which is completed in the same time period that amino acid incorporation occurs. The heavier polyribosomes break down first (compare Figs. 1B and 1C). There is a lag period in the breakdown during the earlier time periods even though amino acid incorporation is linear during this time (compare Fig. 1A to 1B, with 1B to 1C). Further documentation of these points is given below.

A quantitative analysis of polyribosome breakdown is shown in Table 1. The percentage of the various sizes of polyribosomes and 80S particles varied to some extent in various preparations. For example, some preparations contained up to 40 per cent of 80S particles. This probably reflects small differences in the handling procedure and in the analysis of the analytical ultracentrifuge patterns (see *Methods*). However, a zero time control of the same preparation was usually run for comparison. The striking dependence of polyribosome breakdown on the presence of energy is evident (Table 1). The polyribosomes remaining after 20 min of synthesis in this experiment (Table 1) reflect the slow incorporation of this experiment, as will be shown below. Puromycin at a concentration which inhibited hemoglobin synthesis by 90 per cent did not inhibit polyribosome breakdown (Table 1). However, this is consistent with evidence that puromycin inhibition does not prevent peptide-bond formation, but results in the premature release of small peptides.^{13, 14}

Polyribosome breakdown was also dependent on, and correlated with, protein synthesis when this was varied by changing the enzyme preparation or omitting the amino acid mixture (Table 2). The proportionality between the amount of protein synthesis and amount of polyribosome breakdown with various enzyme fractions was good in a 20-min assay. The absence of amino acids decreased incorporation more than it did breakdown. In other experiments, the omission of amino

TABLE 1
BREAKDOWN OF 1X POLYRIBOSOMES

Incubation	Per cent Composition					
	80S	110S	130S	150S	170S	190S
Incubated at 4°	31.8	10.4	14.3	20.0	15.2	5.1
Complete system	63.4	8.2	17.2	8.0	...	...
Complete, minus energy	32.6	12.5	15.2	18.6	14.4	5.7
Complete, plus puromycin	70.4	10.3	11.5	7.6	...	...

The samples were incubated in the complete amino acid incorporating system for 20 min at 37° (except for the control). In "No energy," the ATP, GTP, creatine phosphate, and creatine kinase were omitted. Puromycin was added at a concentration of 7×10^{-4} M. The dots indicate no detectable material at this position.

acids had less effect on the rate of protein synthesis, and a more direct correlation with breakdown could be obtained. In addition, there is an early lag in polyribosome breakdown which has not been considered here (see below). All of these results taken together demonstrate that polyribosome breakdown is a function of protein synthesis.

If the cause of the breakdown were the detachment of 80S ribosomes from the messenger as they completed synthesis of a chain, there should be an orderly shift in polyribosome sizes during protein synthesis toward the smaller sizes and a proportional increase in the 80S ribosomes. As shown in Figure 2, this is the case. The heaviest polyribosomes disappear first (90S), followed in order by the other sizes. With longer incubation times, or with more rapid protein synthesis, more complete disappearance of all polyribosomes was observed. There is a small amount of nonspecific breakdown, but the polyribosomes present initially are converted to 80S ribosomes in about 85 per cent yield. Secondly, as shown previously,⁹ the 80S ribosomes are not labeled; that is, the formation of 80S ribosomes was accompanied by the release of the completed chain (Fig. 3). The large increase in labeled hemoglobin occurred between 5 and 20 min, during which period the number of 80S ribosomes increased greatly. During this time, the labeled polysomes maintained a similar steady-state radioactivity or decreased slightly, although further incubation which resulted in complete polyribosome breakdown led to a decrease in radioactivity. In general, the final radioactivity in the polysome peak was 10–15 per cent of that in released hemoglobin.⁹

We have previously noted that 80S ribosomes were active for hemoglobin synthesis and aggregated onto the polyribosome if these were present.⁹ Re-formation of polyribosomes is also indicated by the lag in polyribosome breakdown and formation of 80S particles shown in Figure 2. Polyribosome breakdown did not occur

FIG. 2.—Breakdown pattern of reticulocyte ribosomes. Ribosome preparations were incubated as described for Fig. 1, and the quantitative analysis of several runs were averaged. The points show the percentage of the total of each size of ribosome at various times.

TABLE 2
POLYRIBOSOME BREAKDOWN AND PROTEIN SYNTHESIS

Incubation conditions	Per cent polyribosomes	Per cent breakdown	Incorporation	
			μ moles amino acids	Per cent of total
Zero time	62.5	0	0	0
Purified enzyme	8.0	87.2	9.8	92.5
Regular enzyme	22.0	65.0	7.0	66.0
No enzyme	48.8	21.5	1.6	15.0
Minus amino acids	29.4	53	2.9	27.5

The samples as described were incubated in the complete system for 20 min, then chilled and aliquots taken for ultracentrifuge analysis and determination of radioactivity. The final total incorporation of 10.6 μ moles of total amino acid was reached in 25 min with the purified enzyme, and 60 min with the regular enzyme. The latter was slower than usual. The % polyribosomes was the sum of all sizes greater than 80S.

FIG. 3.—Labeling pattern of reticulocyte ribosomes. 1X ribosomes were incubated in the complete system for the indicated times and then analyzed on a sucrose gradient. The data are given as $m\mu$ moles of total amino acid incorporated per tube. The optical density patterns corresponding to these times would be similar to the results shown in Fig. 1. 80S ribosomes show a peak at tube 20. The bottom of the gradient (heavier polyribosomes) is to the left.

FIG. 4.—Rate of polyribosome breakdown and protein synthesis. The samples were incubated for various periods in the complete system, and aliquots used for determination of radioactivity and for analysis in the analytical ultracentrifuge. The final incorporation at the completion of the reaction was the 100% value (usually 40 min of incubation). The initial amount of all ribosomes larger than 80S was considered the 100% polysome value.

until 30–40 per cent of the total incorporation had taken place and then was proportional to the amount of protein synthesis (Fig. 4). When different enzymes or amounts of enzyme were used, the final amount of synthesis and polyribosome breakdown were similar, but different lengths of time were required. Thus, 80 per cent of the total incorporation gave about 70 per cent polyribosome breakdown, although this required 16 and 30 min, respectively, with different enzyme preparations (Fig. 4). Since incorporation and release of completed chains do not show a lag, re-formation of polyribosomes during the early stages of synthesis could explain the initial delay in polyribosome breakdown (see *Discussion*).

Effects of synthetic polynucleotides: The inhibition of hemoglobin synthesis by poly U has been reported.¹⁵ Inhibition of hemoglobin synthesis by poly U and by poly A is shown in Figure 5. The inhibition of hemoglobin synthesis by poly U was 60 per cent of maximal at levels of poly U where no polyphenylalanine formation took place. Poly A was also inhibitory, although thus far, polylysine synthesis has not been reported in this system. In addition, the maximal inhibition by poly U and poly A was about 65 and 40 per cent, respectively, even at very high levels of these polymers. Despite the inhibition, polyribosome breakdown was accelerated by the addition of poly A, but only when added to the complete system (Fig. 6). The same orderly breakdown was noted (cf. Figs. 2 and 6). No breakdown occurred in the presence of poly A if energy was omitted. Similar results, in general, except for the preservation of 110S and 130S particles, were found with poly U and will be reported in detail.¹⁶ These facts suggested that poly A might inhibit hemoglobin synthesis by preventing attachment of 80S ribosomes to polyribosomes.

This would permit the polyribosomes to complete and release globin chains and 80S ribosomes, but would prevent the starting of new chains and re-formation of polyribosomes. This mechanism for the inhibition caused by poly A is supported by the data in Figures 4 and 6. It can be seen that the more rapid breakdown of polyribosomes in the presence of poly A was due largely to the absence of a lag phase. This was shown most strikingly when polyribosome breakdown was plotted against incorporation (Fig. 4).

Discussion.—The polyribosome structure in reticulocytes breaks down only during protein synthesis in the cell-free system. The breakdown depends on the amount of protein synthesis and on the presence of energy, enzyme, and amino acid mixture. The fact that this can be observed in the analytical ultracentrifuge or on sucrose density gradients implies that a large proportion of the ribosomes in the polyribosome structure are active for protein synthesis. However, the data do not exclude the possibility that some of the ribosomes in these structures are inactive, but remain attached in some way until protein synthesis causes the structure to break down. During protein synthesis the largest polyribosome clusters disappear first, followed by the next largest, and so on. It should be noted that the rate of breakdown depends on the polyribosome size and not on the per cent composition. Such an orderly breakdown as a result of protein synthesis suggests a mechanism involving growth of the polypeptide chain as the 80S ribosomes move along the polyribosome structure, and release of single 80S ribosomes from the end of the cluster upon completion of the polypeptide chain. The association of labeled polypeptide chains with smaller polyribosomes as incubation in the cell-free system continues (Fig. 3) is also consistent with this mechanism. A similar breakdown of liver polyribosomes during protein synthesis has been reported by Noll.¹¹

For this mechanism to be valid, it is important to demonstrate that the detachment of 80S ribosomes from the polyribosome is related to the *completion* of the polypeptide chain. This was not possible in earlier studies where the nascent chains remained attached to the 80S ribosomes.^{6, 11} In the reticulocyte cell-free system, completed globin chains are released, and this was associated with polyribosome breakdown and formation of unlabeled 80S ribosomes (Fig. 3). The synthesis which accompanies the breakdown of polyribosomes should result only in the completion of already started chains. The N-terminal region of such chains therefore would not be labeled during incubation in the cell-free system. Attachment of 80S

FIG 5.—Effect of poly A and poly U on incorporation. The data are given as $m\mu$ moles of total amino acids or polyphenylalanine per mg ribosomes. The synthetic polynucleotides were added in the indicated amounts to the complete system (1.4 ml) at zero time, and standard assay conditions were used.

FIG. 6.—Breakdown patterns of reticulocyte ribosomes in the presence of poly A. Conditions are the same as in Fig. 2, except for the addition of 100 μ g of poly A.

ribosomes to polyribosomes in the cell-free system would be required for complete *de novo* synthesis of globin chains. Earlier, we reported the *de novo* synthesis of globin chains in the cell-free system.⁹ However, 80S ribosomes were active, and it was indicated that they could attach to polyribosomes. Attachment of 80S ribosomes in the present studies is suggested by the lag in polyribosome breakdown at early time periods. The peculiar inhibition of hemoglobin synthesis by poly A, which does not exceed 40 per cent inhibition and which

accelerates polyribosome breakdown, can be explained if poly A acts to prevent attachment of 80S ribosomes to polyribosomes.

These conclusions have been tested directly¹⁷ by following hemoglobin formation with ribosomes labeled in the intact cell with H^3 -leucine and incubated in the cell-free system with C^{14} -leucine, by measuring the appearance of N-terminal C^{14} -valine (a measure of *de novo* synthesis), and by studying reattachment of P^{32} -labeled 80S ribosomes to polyribosomes. The results of these experiments support the mechanism of hemoglobin synthesis proposed here.

Since polyribosomes do not break down during protein synthesis in the intact cell for at least 60 min (unpublished data), it appears that the limiting factor in the cell-free system is the reattachment of 80S ribosomes to the polyribosome structure.

Summary.—The breakdown of polyribosomes in the reticulocyte cell-free system occurred only during protein synthesis and was accompanied by the release of completed chains. The largest sizes of polyribosomes disappeared first, followed in order by the other sizes. Inhibition of hemoglobin synthesis by poly A resulted in an increased rate of orderly breakdown. Indirect evidence for reattachment of 80S ribosomes to polyribosomes was presented. These results support a mechanism of protein synthesis, involving attachment of 80S ribosomes to the polyribosome which initiates the formation of the peptide chain, followed by chain growth and finally detachment of the ribosome and release of the completed polypeptide chain.

Note added in proof: After this manuscript was completed, evidence for this type of mechanism of protein synthesis in HeLa cells was reported by Goodman, H. M., and A. Rich, *Nature*, **199**, 318 (1963).

We wish to thank Mrs. Margaret McMillan and Mrs. Frances Chrostowski for expert technical assistance.

* Postdoctoral fellow (BH) and career research awardee (RS) of the National Institutes of Health. These studies were supported by research grants from the National Science Foundation (G-21026) and the National Institutes of Health (H-5293).

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ROLE OF SERINE IN CHYMOTRYPSIN ACTION. CONVERSION OF THE ACTIVE SERINE TO DEHYDROALANINE*

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Communicated by D. D. Van Slyke, September 12, 1963

Serine is apparently *at* or *near* the active center of many enzymes. It is the residue that is phosphorylated or acylated by inhibitors and substrates in such enzymes as chymotrypsin,¹ trypsin,¹ thrombin,² elastase,³ subtilisin,⁴ cholinesterase,⁵ phosphoglucomutase,⁶ and alkaline phosphatase.⁷ Although powerful arguments can be presented^{8, 9} that it is indeed the key nucleophilic residue in the catalytic action of these enzymes, its role remains a source of controversy.

The reasons for this controversy are threefold. The first stems from the fact that essentially all of the "serine enzymes" have hydroxylic substrates, e.g., glucose and water. These acceptors have essentially the same reactivity as serine, and hence it is difficult to conceive of a catalytic role for a residue having the same properties as the final acceptor. The second objection stems from kinetic evidence¹⁰⁻¹² which is difficult to reconcile with an intermediate step involving serine; instead it suggests, or apparently requires, a direct attack by the water or glucose acceptor. Finally, all the reported modifications of serine which render an enzyme inactive involve replacing the H of the hydroxyl with a bulkier group. Hence, it can be argued that such serine modification prevents enzyme action because access to the true catalytic group, e.g., histidine, is blocked rather than because any intrinsic property of serine is changed.

To evaluate these arguments, a chymotrypsin in which the "active serine" residue was converted into a dehydroalanine residue (cf. equation 1) seemed highly desirable.