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Control of Hemoglobin Synthesis at the Translation Level. Nascent Polypeptide Chain Distribution on Rabbit Reticulocyte Polyribosomes*

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ABSTRACT: The nascent polypeptide chains present on rabbit reticulocyte polyribosomes were labeled with [^{3}H]valine, and their relative distribution was studied by two different approaches. (a) One approach concerned the determination of the average molecular weight of the nascent chains by sucrose density gradient centrifugation and by Sephadex G-50 gel filtration. The nascent chains were released either by RNase or by alkali treatment at pH 10. (b) The other approach concerned the determination of the specific activity of the tryptic peptides obtained from the growing labeled polypeptide chains, after their completion in a nonradioactive cell-free system. The results obtained are in favor of the uniform distribution of the nascent polypeptide chains, and therefore of the ribosomes, along the α- and β-globin mRNAs, with no detectable rate-limiting steps during the phases of globin assembly and release.

Among the several possible mechanisms controlling the rate of synthesis of a protein molecule, a control at the translation level has been proposed by several authors (Itano, 1966; Ames and Hartman, 1963; Conconi *et al.*, 1966). A nonuniform rate of globin assembly has been proposed by Dintzis (1961) and by Naughton and Dintzis (1962) in rabbit reticulocytes, and by Winslow and Ingram (1966) in human bone marrow. Itano (1966) and Ames and Hartman (1963) have discussed the possibility that a particular "modulating" tRNA might act to slow the synthesis of a given polypeptide chain at some point during the growing process. On the contrary a uniform rate of α- and β-globin synthesis, in rabbit reticulocytes, has been shown by Hunt *et al.* (1968). These authors challenge the assumption of Dintzis (1961) and Winslow and Ingram (1966) that the analysis of the soluble globin, formed shortly after the addition of labeled amino acids to an intact reticulocyte system, can give an accurate picture of the distribution of ribosomes on mRNA and consequently of the rate of translation and elongation of the polypeptide chains (Englander and Page, 1965).

In an effort to establish the existence of a control mechanism for hemoglobin synthesis at the translation level, we have isolated polyribosome complexes from rabbit reticulocytes, previously incubated with labeled amino acids. The experiments have been designed following two different approaches: (1) analysis of the growing polypeptide chains after breaking the aminoacyl binding between tRNAs and polypeptide chains either by alkali treatment or RNAse hydrolysis, and (2) analysis of the nascent chains after their completion in a cell-free system.

The results obtained are in favor of the uniform distribution of the growing chains on the polyribosome complexes and therefore of the uniform rate of assembly of the globin molecules.

Materials

The disodium salt of adenosine triphosphate, sodium phosphoenolpyruvate, pyruvate kinase, and crystalline horse heart cytocrome *c* were obtained from Boehringer und Soehne, Mannheim, Germany. The disodium salt of guanosine triphosphate and crystalline bovine pancreatic ribonuclease (RNase) were purchased from Sigma Chemical Corp., St. Louis, Mo. CM-cellulose (capacity of 0.66 mequiv/g) was a product of Serva, Heidelberg, Germany. L-[3,4-^{3}H]valine (3.25 mCi/μmole) and L-[U-^{14}C]valine (0.2 mCi/μmole) were obtained from New England Nuclear Corp., Boston, Mass.

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Methods

Preparation and Incubation of Reticulocytes. The methods for the preparation and washing of rabbit reticulocytes have been previously described (Conconi *et al.*, 1966). Cells were incubated in two volumes of a modified Krebs–Ringer bicarbonate buffer medium, containing all the amino acids (Burka and Marks, 1964), with the only exception valine. After 10-min incubation at 37° L-[^{3}H]valine was added to give a final concentration of 0.01 μmole/ml, sufficient to give a linear incorporation into soluble proteins for 30 min. During this time, as already shown by Conconi *et al.* (1966), the radioactivity associated with the polyribosomes reached a maximum after 1 min and therefore remained constant. Incubations with labeled valine were continued for 10 min and stopped by the addition of ten volumes of ice-cold 0.01 M Tris-HCl buffer (pH 7.4), 0.01 M KCl, and 0.0015 M $MgCl_2$ (solution A). Cells were then washed twice with saline, and stroma-free lysates were obtained as previously reported (Conconi *et al.*, 1966). Uniformly labeled hemoglobin was prepared by incubation of the cells for 3 hr in the same incubation medium reported above and with the addition of L-[^{14}C]-valine at a final concentration of 0.05 μmole/ml.

Lysis of Reticulocytes and Isolation of Ribosomes. Reticulocytes were lysed as reported by Conconi *et al.* (1966). Labeled ribosomes were recovered by pelleting 3 ml of the lysate through a 15–30% linear sucrose density gradient (24 ml) in solution A. For each preparation six tubes were used. After centrifugation at 25,000 rpm with a SW 25.1 rotor in a Spinco Model L ultracentrifuge for 6 hr at 4°, the supernatant solution was removed and the hemoglobin-free pelleted ribosomes were resuspended in solution A by standing overnight at 4°.

Release of Nascent Peptide Chains from Polyribosomes. The resuspended polyribosomes were pooled (2 ml) and centrifuged at 10,000*g* for 5 min to remove the insoluble material. The labeled polypeptide chains were released by adding 1.0 ml of triethylamine buffer (0.5 M, pH 10). After standing for 1 hr at 37° (during which time the pH was controlled and kept constant), ribosomes were removed by precipitation at pH 5.1 and centrifuged at 10,000*g* for 5 min. Alternatively the polypeptide chains were released by treatment of the labeled polyribosomes with pancreatic RNase at 37° for 45 min (0.025 mg of RNase/mg of RNA). Prior to RNase treatment the ribosomes were washed twice with Mg^{2+}-free solution A.

Analysis of the Released Polypeptide Chains. Two alternative procedures were used to calculate the average molecular weight of the polypeptide chains released from the polyribosomes. (1) The polypeptide chains (1 ml) were layered on a 2–20% linear sucrose density gradient (26 ml) in solution A and centrifuged at 25,000*g* in an SW 25.1 rotor of a Spinco Model L ultracentrifuge for 72 hr at 4°. Cytochrome *c* and human adult hemoglobin were mixed and used in a separate gradient as external standards. In another experiment sodium acetate buffer (0.002 M, pH 5.9) was used for the gradient solutions. In this case rabbit globin, uniformly labeled with [^{14}C]valine, was used as internal standard. (2) The released nascent polypeptide chains were also analyzed on Sephadex G-50 columns (1.5 cm × 1.5 m), equilibrated with 0.02 M Tris-HCl (pH 7.4). An internal standard of cytochrome *c* was added to the radioactive material. Fractions of 2 ml were collected and analyzed for optical absorbance at 408 mμ and for radioactivity in a Packard Tri-Carb liquid scintillation counter.

Cell-Free Incubation of Prelabeled Ribosomes. Ribosomes, prelabeled with [^{3}H]valine by incubation of intact reticulocytes, were prepared as described above. The labeled polypeptide chains were completed and released in a cell-free system prepared as described by Morris (1964) using a 105,000*g* supernatant obtained from rabbit unlabeled cells. Each incubation mixture contained approximately 5–10 mg of ribosomal material in 5 ml. After incubation at 37° for 60 min, the ribosomes were removed by centrifugation at 39,000 rpm in the SW 39 rotor of a Spinco Model L ultracentrifuge for 2 hr at 4°. The supernatant was used for further analysis.

Preparation of Globin and Aminoethylation. Free globin chains were prepared by acid–acetone precipitation (Anson and Mirsky, 1930) and α and β chains were separated according to Dintzis (1961). Aminoethylated globins were obtained following the procedure described by Jones (1964).

Tryptic Digestion of α and β Chains. In order to measure the specific activity of the tryptic peptides labeled with [^{3}H]-valine, an internal standard of purified α- or β-globin, uniformly labeled with [^{14}C]valine, was added to the [^{3}H]globins before hydrolysis, as suggested by Dintzis (1961). The mixture of the two labeled globins was prepared as to give approximately the same counts per minute in a Tri-Carb liquid scintillation counter set for the double-label determinations. Samples (5 mg of globin/ml) were adjusted to pH 8.5 with 0.2 N NH_4OH and digested with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone–trypsin added in a 50:1 ratio (w/w). Digestion was carried out for 4 hr at 37° with automatic adjustment of the pH in a Radiometer pH-Stat. Samples were taken to dryness in a rotatory evaporator under vacuum. Dried samples were suspended in the same buffer used for the electrophoretic analyses. Slight turbidity was eliminated by low-speed centrifugation.

Separation of Tryptic Peptides. The salt-free digests for the α- and β-globin were applied to Whatman No. 3MM paper (42 × 37 cm), prewet with buffer at pH 4.7 (Dintzis, 1961; Naughton and Dintzis, 1963). Electrophoresis was carried out at 1200 V (28.5 V/cm) and 100 mA for 5 hr. The paper was then dried. Ascending chromatography was conducted at room temperature, using the same conditions described by Dintzis (1961). The dried papers were stained with ninhydrin (0.2% in acetone) and the spots were cut out. Peptides were eluted with 0.01 N HCl directly into scintillation counting vials. The eluates were brought to dryness under vacuum, and redissolved in 0.1 N HCl (Hunt *et al.*, 1968). To each vial 10 ml of Bray's solution (1960) was added. The double-labeled samples were counted in a Tri-Carb liquid scintillation counter with appropriate setting for the ^{3}H and ^{14}C simultaneous determinations. In each preparation the α and β peptides were previously identified on the fingerprintings using specific staining techniques (Easley, 1965; Easley *et al.*, 1969).

Results

Determination of the Molecular Weight of the Nascent Polypeptide Chains Released from the Polyribosomes by Alkali or RNase Treatment. The analysis by sucrose density gradient centrifugation of the labeled nascent chains, released from the polyribosomes either by alkali or RNase treatment, showed in both cases peaks of radioactivity having migration corre-

TABLE I: Specific Activity of the Valine-Containing Tryptic Peptides Obtained from Nascent Ribosomal Chains Prelabeled with [^{3}H]Valine, and Completed in a Cell-Free System with Nonradioactive Amino Acids.[a]

Peptide	α-Globin Peptides			β-Globin Peptides		
	^{3}H cpm	^{14}C cpm	^{3}H cpm/^{14}C cpm	^{3}H cpm	^{14}C cpm	^{3}H cpm/^{14}C cpm
T1	190	143	1.32	565	451	1.25
T4	443	307	1.44	1037	760	1.36
T9	400	452	0.88	590	613	0.96
T10	700	1147	0.61	737	1138	0.64
T12	290	616	0.47	600	1209	0.49
T13	61	184	0.33	58	268	0.21
T14	36	186	0.19	45	274	0.16
T1	1074	1107	1.07	1376	1390	0.99
T2	262	283	0.92	1100	1162	0.94
T3	2041	2195	0.92	4916	5407	0.91
T4	145	178	0.81	1512	1797	0.84
T5	315	407	0.77	282	332	0.85
T6	252	420	0.60	430	660	0.65
T9	438	730	0.60	521	811	0.64
T11	597	1010	0.59	730	1240	0.59
T12	100	850	0.12	21	154	0.14
T13	118	268	0.44	140	310	0.45
T14	60	315	0.19	85	874	0.10

[a] For technical details see the legend of Figure 1 and the section Methods. The table shows the actual values obtained for each peptide for the ^{14}C labeling, while the ^{3}H counts per minutes have been corrected for the overlapping of the ^{14}C into the ^{3}H channel. Blanks prepared from Whatman No. 3MM papers, after electrophoresis and chromatography, gave 15 cpm for both channels.

sponding (Martin and Ames, 1961) to molecular weights ranging from 9200 to 9700. Similar results were also obtained on gel filtration through Sephadex G-50.

Determination of the Specific Activity of the Tryptic Peptides Obtained from Nascent Ribosomal Chains Completed in a Cell-Free System with Nonradioactive Amino Acids. The amount of radioactivity detected as ^{3}H and ^{14}C in the various α and β peptides examined in a typical experiment is reported in Table I. The values of the ratio ^{3}H to ^{14}C are plotted in Figure 1 and indicated by the open and closed circles (the experiment was done in duplicate). The two continuous lines represent the plot of the theoretical ratios expected in the case of uniform nascent chain distribution. As can be seen the experimental values fit satisfactorily the theoretical lines.

Discussion

Sucrose density gradient centrifugation and Sephadex gel filtration have shown that the nascent chains released from the polyribosome complexes with alkali or RNase treatment have an average molecular weight lower than 10,000. Assuming the uniform distribution of the nascent globin chains the peak of radioactivity should have a molecular weight of 16,000, corresponding to that of the completed globin. These findings therefore would indicate that the chains of shorter length are more represented than the chains near completion and release and could be therefore interpreted as an acceleration of the globin assembly, close to its COO^--terminal end.

These results are not confirmed by those obtained with the second experimental approach. In fact, the analysis of the ribosomal chains released by the cell-free chain completion technique are very close to the theoretical values expected in the case of uniform chain distribution, thus confirming in a different system and with a different amino acid the results of Hunt *et al.* (1968).

We believe that the results obtained by releasing the nascent chains at pH 10 or by RNase treatment are less reliable than those obtained allowing the growing chains to be completed and released from the polyribosomes as soluble globin in a cell-free system. In fact after pH 10 or RNase treatment the recovery of the ribosomal radioactivity is only about 50% as compared to the 80% of the cell-free system. This low recovery could reflect a selective detachment of chains of shorter length and a retention on the polyribosomes of the chains near completion. Furthermore the pattern obtained on Sephadex filtration and on sucrose density gradient centrifugation could indicate a falsely low molecular weight. It is possible in fact that the released chains, not yet arranged in the final globular shape characteristic of the completed globin, migrate slower than the molecule in its normal conformation. On the contrary the data obtained from the analysis of the ribosomal chains completed in the cell-free system do not present this problem. In fact this system (1) employs physiological means for elongation, completion, and release of nascent growing ribosomal chains; (2) allows the release of most of the radioactivity associated with the polyribosomes (80%); and (3) permits approaching the nascent chain distribution problem through the analysis of the completed globin

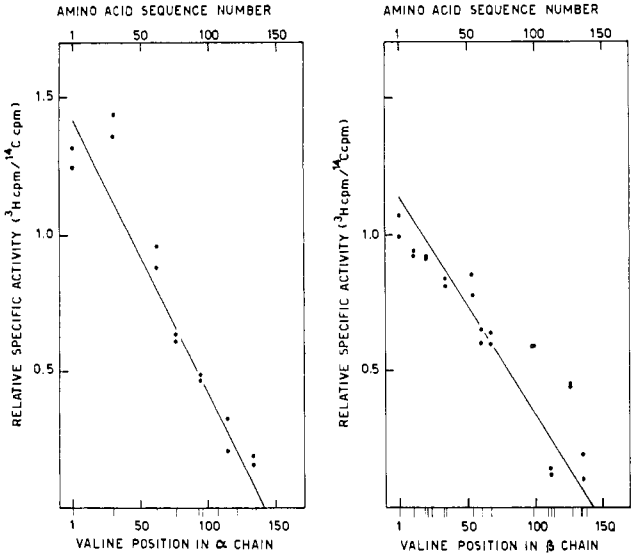

FIGURE 1: Specific activity of the valine containing tryptic peptides obtained from labeled ribosomal globin chains completed in a cell-free system with nonradioactive amino acids. The tryptic digestions were carried out on ^{14}C uniformly labeled α-globin (282,000 cpm) mixed with ^{3}H-labeled α-globin (164,000 cpm) and on ^{14}C uniformly labeled β-globin (430,000 cpm) mixed with ^{3}H-labeled β-globin (248,000 cpm). The tritiated globins used in the tryptic digestions were obtained from prelabeled ribosomal nascent chains, isolated from intact reticulocytes, and released in a nonradioactive cell-free system, as reported under Methods. The two lines represent the theoretical specific activities expected in case of uniform nascent chain distribution; they were calculated taking into consideration the total amount of tritiated material released from the polyribosomes as α-and β-globin, and consequently the relative number of ^{3}H-labeled chains expected for the two chains at the different points of valine insertion. The following are the calculations that were carried out for the two globins.

Val Position from NH_2-Terminal Amino Acid	^{3}H Chains/ Total Chains		^{3}H cpm Expected	^{14}C cpm Expected	^{3}H/^{14}C
		α-Globin			
1	141/141		40,285	28,200	1.43
29	113/141		32,285	28,200	1.14
62	80/141		22,856	28,200	0.81
70	72/141		20,571	28,200	0.73
93	49/141		14,000	28,200	0.50
96	46/141		13,142	28,200	0.47
107	35/141		10,000	28,200	0.35
121	21/141		6,000	28,200	0.21
132	10/141		2,857	28,200	0.10
135	7/141		2,000	28,200	0.07
		Total cpm	163,396	282,000	
		β-Globin			
1	146/146		26,564	23,889	1.11
11	136/146		24,745	23,889	1.03
18	129/146		23,471	23,889	0.98
20	127/146		23,107	23,889	0.97
23	124/146		22,561	23,889	0.94
33	114/146		20,742	23,889	0.87
34	113/146		20,560	23,889	0.86
54	93/146		16,921	23,889	0.70
60	87/146		15,829	23,889	0.66
67	80/146		14,556	23,889	0.61
98	49/146		8,915	23,889	0.37
109	38/146		6,914	23,889	0.29
111	36/146		6,550	23,889	0.27
114	33/146		6,004	23,889	0.25
126	21/146		3,820	23,889	0.16
133	14/146		2,547	23,889	0.10
134	13/146		2,365	23,889	0.09
137	10/146		1,819	23,889	0.07
		Total cpm	248,030	430,002	

chains, the behavior of which is more understood than that of the growing chains.

The data obtained with the nascent globin chain completion technique fit reasonably well with the theoretical lines of the uniform chain distribution reported in Figure 1. Therefore the globin chains of different length growing on the polyribosomes are equally represented. Furthermore, since each ribosome present on the polyribosome complex and actively engaged in protein synthesis should carry one nascent growing chain (Warner *et al.*, 1962) it can be concluded that the ribosomes are equally distributed among all possible positions of the α- and β-mRNAs.

From the data reported in Figure 1 it is also evident, in agreement with Hunt *et al.* (1968), that no completed globin chains contribute to the results we have obtained in the cell-free experimental conditions. In fact, as graphically shown by Hunt *et al.* (1968), the presence on the polyribosomes of completed chains would give rise to a specific activity pattern parallel to the theoretical lines reported in Figure 1, but with higher intercept values. Our results are in disagreement with those obtained by Colombo and Baglioni (1966). These authors have found in rabbit reticulocytes a ratio of completed *vs.* uncompleted ribosomal α chains of 1 to 5.5, and very few or no completed ribosomal β chains. As previously pointed out by Hunt *et al.* (1968), Colombo and Baglioni (1966) have estimated the number of completed α- and β-globin in separate experiments, using arginine as label for α chain and histidine for the β chains. Hunt *et al.* (1968) have also found varying amounts of completed α- and β-globin chains in different ribosomal preparations, due, according to the authors' interpretation, to uneven hemoglobin contamination; Colombo and Baglioni, therefore, could have observed more completed α than β chains because their observations were obtained in separate experiments. In our experimental conditions, in which the ribosomal material was pelleted through a sucrose density gradient, hemoglobin contamination was completely avoided. Furthermore Colombo and Baglioni (1966) used two different techniques to release the α- and β-peptide chains, namely, a RNase treatment for the α chains and a 4 M urea–6 M LiCl treatment for the β chains. This nonuniform treatment could also give rise to different results for the two chains.

From our data it is possible to draw the following conclusions. (1) Each class of nascent globin chains of different length is equally represented. (2) The ribosomes are therefore uniformly distributed along the α- and β-mRNAs. (3) The speed of peptide-bond formation is approximately the same for every amino acid along the two growing chains. (4) There is no evidence of accumulation of completed α- or β-globin chains on the polyribosome complexes. Taken together our data seem to exclude any rate-limiting step during both the assembly and the release of the rabbit globin chains. The only control mechanism not ruled out by the present results is at chain initiation.

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Interactions of the Endopeptidase Subunit of Bovine Procarboxypeptidase A-S6*

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ABSTRACT: Activated procarboxypeptidase A-S6 (PCP A-S6) and fraction II, the endopeptidase subunit of PCP A-S6, are both irreversibly inactivated by diisopropyl phosphorofluoridate (DFP), *N-trans*-cinnamoylimidazole (at pH 5.0), and L-(1-carbobenzoxyamido-2-phenyl) ethylchloromethyl ketone (ZPCK). Inhibition by ZPCK occurs considerably more slowly than inhibition of α-chymotrypsin. Quantitative measurements of the incorporation of these inhibitors indicate that the two activated zymogens possess only a fractional value for the number of active sites per molecule, *i.e.*, 0.60 and 0.14, respectively. However, the catalytic activity per mole of "active sites" is the same for both. Isolation of the corresponding [^{32}P]DIP and [^{14}C]ZPCK peptides shows that these reactions are specific for seryl and histidyl residues, respectively, the isolated peptides displaying close similarity to analogous peptides derived from trypsin or chymotrypsin.

Isolation of [^{14}C]ZPCK-labeled fraction II (containing approximately 0.25 active site/molecule) and from [^{14}C]ZPCK–procarboxypeptidase A-S6 (containing 0.59 active site/molecule) suggests that the zymogen contains two endopeptidase subunits of similar if not identical nature.

Bovine pancreatic procarboxypeptidase A-S6 is the zymogen of two enzymatically and physically distinct enzymes: an endopeptidase which resembles chymotrypsin in some of its enzymatic and chemical properties, and the exopeptidase, carboxypeptidase A (Keller *et al.*, 1956, 1958; Brown *et al.*, 1961, 1963). Yamasaki *et al.* (1963) have shown that procarboxypeptidase A is an aggregate of three subunits and that each of the two enzymatic activities, endopeptidase and exopeptidase, is associated with distinct subunits. Brown *et al.* (1963) were able to dissociate procarboxypeptidase A-S6 into three different fractions by incubation in aqueous solution at pH 10.5 and, more recently, Freisheim *et al.* (1967) described the disaggregation of the procarboxypeptidase A complex by succinylation with succinic anhydride and the isolation of viable succinylated fraction I which could be converted by the action of trypsin to succinyl carboxypeptidase A.

Fraction II, the zymogen of the endopeptidase, exhibits both esterase and endopeptidase activity after activation with trypsin. These activities can be generated either in the trimeric precursor (PCP A-S6)[1] or after isolation of the individual subunit(s). A third chromatographically distinguishable protein component, designated fraction III, is of unknown function; no enzymatic activity could be associated with this protein before or after incubation with trypsin (Brown

* From the Department of Biochemistry, University of Washington, Seattle, Washington 98105. *Received July 28, 1969.* This work has been supported in part by research grants from the Public Health Service, National Institutes of Health (GM 13401 and GM 04617), the American Cancer Society (P-79), the National Science Foundation (GB 4990x), and the Office of Naval Research, Department of the Navy (NONR 477-35).

† Taken in part from a dissertation submitted by W. D. B. to the Graduate School of the University of Washington in partial fulfillment of the requirements for the degree of Doctor of Philosophy. Present address: Biophysics Laboratory of the Department of Biological Chemistry, Peter Bent Brigham Hospital, Harvard Medical School, Boston, Mass. 02115.

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[1] The following abbreviations are used: PCP A-S6, bovine procarboxypeptidase A with a sedimentation velocity of approximately 6 S; TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone; ZPCK, (1-carbobenzoxyamido-2-phenyl)ethyl chloromethyl ketone; DNS-Cl, dimethylaminonaphthalenesulfonyl chloride; DNS-OH, dimethylaminonaphthalenesulfonic acid; DNS-NH_2, dimethylaminonaphthalenesulfonamide; Ser·PO_3^{2-}, *o*-phosphoserine; HPLA, hippuryl-DL-β-phenyllactate.