Gel Chromatographic Analysis of Nascent Globin Chains

EVIDENCE OF NONUNIFORM SIZE DISTRIBUTION*

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SUMMARY
A gel chromatographic analytical procedure using Bio-Gel A-0.5m in 6 M guanidine HCl-0.1 M 2-mercaptoethanol has been developed and standardized. The procedure permits the molecular weight of peptides in the size range of 2 to 146 amino acids in length to be established from the distribution coefficient of the peptide on the gel column. This procedure has been applied to an analysis of the size distribution of nascent peptides from rabbit reticulocyte ribosomes. These studies indicate that the size distribution of nascent peptides is nonuniform and that translation of hemoglobin messenger RNA molecules does not proceed at a constant rate in the reticulocyte.

The assembly of the polypeptide chains of hemoglobin on rabbit reticulocyte ribosomes takes place by the sequential addition of amino acids, beginning at the NH2-terminal end of the molecule and proceeding toward the COOH-terminal end of the molecule (1). A population of polyribosomes thus contains nascent globin chains of a wide range of sizes. Development of a gel chromatographic procedure for the fractionation of nascent globin chains has permitted a study to be conducted of the size distribution of nascent peptides from rabbit reticulocytes.

While techniques for the measurement of the average rate of polypeptide synthesis during globin synthesis are available (2, 3) determination of the relative rates of translation of specific portions of the globin messenger RNA molecules has not been feasible previously. Since an increased time in residence of a ribosome at a particular position along the messenger RNA will result in an increased amount of the corresponding nascent chain in the population of nascent chains, measurement of the relative amounts of the different sizes of nascent chains in a population of nascent chains provides a means of accessing the relative rates of translation along the messenger RNA molecules.

Data presented here reveal that a nonuniformity of size distribution exists among the nascent globin chains. These results indicate that the rate of translation of the messenger RNA molecules varies during the synthesis of the globin chains in a manner which suggests that slow steps are present during synthesis of specific regions of the globin molecules.

EXPERIMENTAL PROCEDURES
Reagents—Guanidine HCl (grade 1) was purchased from Sigma Chemical Co., St. Louis, Mo., and recrystallized twice according to the procedure of Norzaki and Tanford (4). Cycloheximide and 2-mercaptoethanol were also obtained from Sigma Chemical Co. Bio-Gel A-0.5m (200 to 400 mesh, 10% agarose), Bio-Gel P-10 (50 to 100 mesh), Bio-Gel P-2 (100 to 200 mesh), and Dowex 50-X8 (100 to 200 mesh, H+ form) were purchased from Bio-Rad Laboratories, Richmond, Calif. Blue dextran 2000 (mol wt 2 X 106) and the K 15/90 chromatographic column (1.5 X 90 cm) were purchased from Pharmacia Fine Chemicals, Piscataway, N. J. Labeled amino acids were purchased from Amersham Searle Corp., Arlington Heights, Ill. Aquasol was from the New England Nuclear Corp., Boston, Mass. Cyanogen bromide was bought from Distribution Products Industries, Rochester, N. Y. Trypsin was L-1-toxylamido-2-phenylethyl chloromethyl ketone-treated, obtained from Worthington Biochemicals Corp., Freehold, N. J. Penicillin G was from the Upjohn Co., Kalamazoo, Mich. Streptomycin sulfate (USP) was from General Biochemicals, Chagrin Falls, O. Carboxymethylcellulose (CM32) was from H. Reeve Angel and Co., Clifton, N. J. DNP-alanine was provided by Dr. R. J. Evans of Michigan State University and sparsomycin was donated by Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md.

Column Preparation—Bio-Gel A-0.5m was suspended in water, allowed to settle, and the supernatant solution was decanted. This wash procedure was repeated several times. The agarose gel was then suspended in water and solid guanidine HCl was added to a final concentration of 6 M, 2-mercaptoethanol was added to 0.1 M. The mixture was degassed in vacuum and titrated to pH 6.5 with NaOH. The mixture was then equilibrated overnight at 4° and the gel slurry was added to the chromatography column using 60 cm of solvent hydrostatic pressure. Column beds, ranging in heights from 78 to 83 cm, were prepared and washed for 36 hours with 6 M guanidine HCl-0.1 M 2-mercaptoethanol, pH 6.5. Flow rates from these columns were approximately 3 ml per hour. All gel chromatography analyses were conducted at 4° and 60 cm of solvent hydrostatic pressure.

Samples of peptides to be analyzed were combined with blue dextran (0.08 mg) and DNP-alanine (25 µg) in 6 M guanidine HCl-0.1 M 2-mercaptoethanol-7.5% sucrose (total volume 0.21 ml), and titrated to pH 6.5 with NaOH as determined with pH-indicator paper. After standing at room temperature for several hours the solution was adjusted to pH 6.5 with HCl, chilled, and layered onto the top of the Bio-Gel A-0.5m column using a Sage pump (Sage Instruments, Inc., White Plains, N. Y.). Eluate fractions were collected using a Gilson linear fractionator (Gilson Medical Electronics, Middleton, Wis.) equipped with a drop counter attachment, or directly into glass scintillation vials fastened to a round

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1 The abbreviation used is: DNP-alanine, dinitrophenylalanine.
fraction containing (Instrument Specialties Co., Lincoln, Neb.) using the drop counter attachment. Eluate fractions containing the blue dextran, and the DNP-dalanine markers were measured spectrophotometrically at 630 and 360 nm, respectively, in a Gilford spectrophotometer, model 2400S (Gilford Instrument Laboratories, Inc., Oberlin, O.). Counting of radioactivity in the eluate fractions was accomplished using a Unilux 1 Nuclear Chicago liquid scintillation counter (Nuclear Chicago, Des Plaines, III.) for those eluates containing a single isotopic peak. Using a Packard model 3310 liquid scintillation spectrometer for samples containing two isotopes. Aquosol (5 ml) was added to each aliquot or sample counted. The decompositions per min (dpm) in each sample were calculated by the channel ratio method.

**Incubation of Rabbit Erythrocytes**—Rabbit reticulocytes were obtained as described by Slabough and Morris (5). Following an initial 2-min preincubation, to achieve temperature equilibration, to dryness. Removal of guanidine HCl and 2-mercaptoethanol from the fractions containing the smallest peptide (p116) was accomplished by adding the sample to a Dowex 50 column (1.1 X 40 cm) equilibrated with 0.1 M pyridine. The column was washed until the guanidine HCl emerged and the peptide was then eluted with 4 M pyridine and lyophilized to dryness. The eluted peptides were then characterized by paper electrophoresis at pH 4.7 on Whatman No. 3MM paper according to the method of Hunt et al. (2). Labeled tyrosine-containing peptides were located using a Packard model 7301 radiochromatogram scanner equipped with a model 385 recording ratemeter. A trypic digest of "C-labeled β-globin was run in parallel as a reference standard and tyrosine-containing peptides were visualized using a 1-nitroso-2-naphthol stain (14).

**Results**

Nascent rabbit globin chains on a population of reticulocyte polysomes would be expected to contain nascent globin chains in all degrees of completion. Thus, this population of peptides should range in length from a minimum of 2 amino acids to a maximum of 146 amino acid residues. This prediction may be deduced from the NH₂- to COOH-terminal amino acid sequence of rabbit hemoglobin (17). In seeking to characterize the properties of reticulocyte polysomes, it became necessary to construct theoretical elution curves to serve as a reference with which to compare the observed elution profiles for peptides labeled with one amino acid. These elution patterns were calculated using the following assumptions.

1. The size distribution of nascent peptides is uniform.
2. The elution pattern of any single peptide will be a Gaussian curve. The Gaussian curve will be centered at the distribution coefficient (Kd) of its corresponding peptide. The elution profile of each peptide is independent of other peptides.
3. The ratio of nascent β-globin chains to nascent α chains is 1. (Hunt et al. (15) report this ratio to be 1.1 while our own studies yielded a ratio of 1.04 (8).)
4. For a population of nascent peptides labeled with a single amino acid, the area under the elution pattern of any one peptide will be proportional to the number of residues of labeled amino acid present.
5. Molecular weight increases in steps of 110, the average molecular weight of amino acids of β-globin. Nascent α-globin chains have the same number of residues will have the same distribution coefficient. The relationship between the distribution coefficient of each nascent peptide employed in the construction curve was determined from the experimental relationship defined under "Results" (Fig. 2). The construction of the theoretical elution pattern consists of drawing Gaussian curves for each of 145 peptides sizes and adding the ordinates of the resulting curves at each Kd value. The theoretical elution patterns reported here were determined using the CDC 6600 computer of Michigan State University.

**Discussion**

Nascent peptides of rabbit globin contained on a population of reticulocyte polysomes would be expected to contain nascent globin chains in all degrees of completion. Thus, this population of peptides should range in length from a minimum of 2 amino acids to a maximum of 146 amino acid residues. This prediction may be deduced from the NH₂- to COOH-terminal direction of synthesis of globin chains during the translation of the hemoglobin messenger RNA molecules (1) and from the amino acid sequence of rabbit hemoglobin (17). In seeking to develop an analytical method which would allow accurate measurement of the full range of size distribution of the nascent peptides, a study was conducted of the properties of Bio-Gel A-0.5m column eluted with 0 M guanidine HCl-0.1 M 2-mercaptoethanol.
globin (17). Position of the peptides indicated (see Table I). Were calculated from the amino acid sequence of rabbit hemoglobin samples (C, D, E, and F) compared to the known composition of tyrosine-labeled P-globin (PCB-1). Particularly characteristic is the absence of phenylalanine and the low leucine content. The presence of the homoserine produced by the action of cyanogen bromide is suggested by the observed value of 4 lysine residues since homoserine and lysine are not resolved in these analyses. Peptide E matches the composition of the 55 amino acid-containing cyanogen bromide fragment of β-globin (βCB-1). Particularly noticeable is the absence of isoleucine. Peptides D and C are resolved incompletely from one another and therefore the amino acid composition of the peptides analyzed should show only a degree of enrichment of one peptide over another. The results obtained are consistent with the designation of Peptide D as βCB-2 and Peptide C as αCB-2. This conclusion is further substantiated by the elution profile obtained from a

**Table I**

<table>
<thead>
<tr>
<th>Designation</th>
<th>Compound</th>
<th>No. of amino acid residues</th>
<th>Mol wt</th>
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</thead>
<tbody>
<tr>
<td>A</td>
<td>Blue dextran</td>
<td>2 × 10^4</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>β-Globin</td>
<td>146</td>
<td>16,003</td>
</tr>
<tr>
<td>C</td>
<td>αCB-2</td>
<td>109</td>
<td>11,996</td>
</tr>
<tr>
<td>D</td>
<td>βCB-2</td>
<td>91</td>
<td>10,008</td>
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<tr>
<td>E</td>
<td>βCB-1</td>
<td>55</td>
<td>5,980</td>
</tr>
<tr>
<td>F</td>
<td>αCB-1</td>
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<td>8,413</td>
</tr>
<tr>
<td>G</td>
<td>βT14</td>
<td>12</td>
<td>1,520</td>
</tr>
<tr>
<td>H</td>
<td>βT4</td>
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</tr>
<tr>
<td>I</td>
<td>βT16</td>
<td>2</td>
<td>316</td>
</tr>
<tr>
<td>J</td>
<td>DNP-alanine</td>
<td>255</td>
<td></td>
</tr>
</tbody>
</table>

* Peptides from cyanogen bromide and trypsin treatment have been numbered according to their position of occurrence relative to the NH2-terminal end of the globin chains of rabbit hemoglobin (18). The size and composition of each compound indicated were calculated from the amino acid sequence of rabbit hemoglobin (17).
mixture containing [3H]tyrosine-labeled αCB-2 and [14C]tyrosine-labeled βCB-2 (see inset, Fig. 1).

The smaller peptides were characterized on the basis of their electrophoretic mobilities at pH 4.7 using the known mobilities of the tryptic peptides of β-globin as the reference standard (2). These results (not shown) indicate Peptide G is the 12 amino acid-containing Peptide βT14, Peptide H is the 10 amino acid-containing Peptide βT4, and Peptide I is the dipeptide βT16.

Gel chromatographic analysis of [3H]labeled tryptic peptides of β-globin had previously shown that no radioactivity was eluted in the fractions near the blue dextran marker. Since high molecular weight materials were removed from the tryptic digestion mixture with P-10 chromatography (see “Experimental Procedures”), the peak of [3H]labeled material eluting in Fractions 90 to 99 was deduced to be the added [3H]labeled β-globin.

Treatment of Data—Fish et al. (16), using Bio-Gel A-5m, studied the relationship between the molecular weight of peptides in the range of 76,000 to 1,540 and the relative positions of those peptides in the elution diagram. Using blue dextran and DNP-alanine as reference markers, a plot of the cube root of the distribution coefficient (Kd) of the polypeptide versus the molecular weight of that peptide raised to the 0.555 power yielded a straight line relationship. A similar treatment of data reported here using Bio-Gel A-0.5m and peptides in the molecular weight range 16,003 to 316 is shown in Fig. 2. It may be seen that the relationship between Kd and (molecular weight)0.555 remains linear throughout the molecular weight range of the materials obtained from labeled rabbit hemoglobin.

The studies which follow report the application of this gel filtration technique, using Bio-Gel A-0.5m, to the investigation of the size distribution of nascent globin chains of rabbit reticulocyte ribosomes.

Analysis of Size Distribution of [3H]Tyrosine-labeled Nascent Globin Chains—Tyrosine residues occur in the amino acid sequence of α-globin chain of rabbit hemoglobin at positions 24, 42, and 140 (17). The β-globin chain contains tyrosine at residues 35, 130, and 145. On the premise that a uniform size distribution of α- and β-globin chains exist in the nascent globin fraction one can construct a theoretical elution pattern for tyrosine-labeled nascent globin peptides, which are uniformly labeled, on the basis of the relationships between molecular weight and Kd described in Fig. 2. The elution profile shown in Fig. 3 is that expected for a population of tyrosine-labeled nascent globin peptides which possess a uniform distribution of peptide sizes. The pattern reflects the positions of incorporation of tyrosine during hemoglobin synthesis. The properties of the Bio-Gel A-0.5m column in fractionating a mixture of peptides of different sizes results in a smaller increase in Kd value between any one peptide and the peptide which is one amino acid longer as the size of the peptides increase. Consequently an eluate fraction collected at Kd = 0.3 would be expected to contain more members of the nascent peptide population than an eluate fraction of the same volume collected, for example, at Kd = 0.6. This latter feature results from a theoretical elution profile having an increasing slope with decreasing Kd.

The elution profile observed when a sample of [3H]tyrosine-labeled nascent globin peptides was analyzed by gel chromatography on Bio-Gel A-0.5m is shown in Fig. 4. While the observed elution profile possesses the general dimensions predicted in Fig. 3, several unexpected features are present. Most notable of these is the presence of "peaks" and "troughs" which are apparent in the profile. In a uniformly labeled population of nascent peptides these "peaks" and "troughs" indicate that certain members of the peptide population are present in in-
creased numbers while other members are present to a lesser extent.

The radioactive materials which elute from the column near the blue dextran marker have been found in all analyses conducted to date. Since digestion of the peptidyl-tRNA preparation with pancreatic ribonuclease, prior to alkaline hydrolysis of the peptide to tRNA ester bond, does not change this component one may conclude that the material is not unhydrolyzed peptidyl-tRNA. Conditions under which samples are prepared for gel chromatography (6 M guanidine-0.1 M 2-mercaptoethanol at room temperature for several hours) tend to rule out aggregation due to disulfide bond formation. While the exact nature of this component has not been established Lodish (19) has recently shown that soluble reticulocyte ribosomes synthesize small amounts of high molecular weight membrane proteins. The nascent peptides of these proteins may be present in this peak of radioactivity.

Analysis of Size Distribution of [3H]Tryptophan-labeled Nascent Globin Chains—Tryptophan residues are incorporated early during globin biosynthesis since they occur at position 14 of the α-globin chain and 15 and 37 of the β-globin chain. Consequently, the theoretical elution pattern (Fig. 5) is less complex for tryptophan-labeled peptides. Data obtained from Bio-Gel A-0.5m chromatographic analysis of [3H]tryptophan-labeled nascent peptides is shown in Fig. 6. The elution profile contains the peaks and troughs noted earlier with [3H]tyrosine-labeled peptides (Fig. 4).

Size Distribution of Nascent Peptides Prepared from Fresh Whole Blood—Tepper and Wierenga (20) have reported that rabbit reticulocytes maintained in the cold rapidly lose their polyribosomal structures. These polyribosomes re-form following incubation at 37° for 2 min. These authors noted an oscillatory rate of hemoglobin biosynthesis in rabbit reticulocytes following chilling, presumably due to synchronous initiation of globin chains during that period. Reticulocytes kept at 37° prior to incubation in the presence of labeled amino acids synthesize hemoglobin at a constant rate.

In order to examine whether the results observed during Bio-Gel A-0.5m chromatography were in some way related to the chilling of reticulocytes during isolation and washing of the cells prior to incubation, an analysis of [3H]tryptophan-labeled nascent peptides from reticulocytes labeled in unchilled whole blood was conducted. Fig. 7 shows that the size distribution of the nascent globin peptides is unrelated to the temperature during reticulocyte preparation.

Size Distribution of [35S]Methionine-labeled Nascent Peptides—While each globin chain contains only 1 methionine in its final amino acid sequence (at position 32 of α-globin and position 55 of β-globin) the initiation of globin biosynthesis has been reported to involve insertion, and subsequent removal, of an NH2-terminal methionine residue (21-24). Fig. 8 presents the results of an analysis of [35S]methionine labeled nascent peptides.

From Fig. 2 one may calculate that the internal methionine
residues should first appear in α-globin peptides which elute at \( K_d = 0.65 \) and in β-globin peptides which elute at \( K_d = 0.52 \). Radioactivity associated with peptides eluting after \( K_d = 0.65 \) must arise from those peptides containing NH₂-terminal [3H]-methionine. One would also expect that some of the labeled material at \( K_d = 1.0 \) is free methionine liberated from methionyl-tRNA during alkaline hydrolysis of the peptidyl-tRNA fraction since some amino acyl-tRNA would be expected to be present. No theoretical elution profile for methionine-labeled peptides was constructed due to the uncertain contribution of the NH₂-terminal to the expected profile. The peaks and troughs observed in the elution profile of [3H]methionine-labeled nascent peptides may be seen to resemble closely those obtained with [3H]tyrosine (Fig. 4) and [3H]tryptophan (Figs. 6 and 7). Thus these features of the elution profile are independent of the labeled amino acid employed.

**DISCUSSION**

The agarose gel chromatography described here may have general applicability for the fractionation of low molecular weight substances where knowledge of the relationship between molecular weight and the distribution coefficient is desired. Swank and Munkres (25) have used polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate to resolve polypeptides in the 1,200 to 10,000 molecular weight range. Fish et al. (16) obtained a useful range of resolution of polypeptides in the molecular weight range of 1,540 to 76,600 using Bio-Gel A-5m gel chromatography. Our modification of the latter procedure using the more concentrated agarose gel Bio-Gel A-0.5m provided an orderly resolution of peptides in the molecular weight range from 316 (dipeptide) through 16,000 (146 amino acids) and hence provides a useful method for the analysis of the size distribution of nascent globin chains which would be expected to span that particular range of molecular weight values.

Several workers have attempted to assess the size distribution of the nascent globin fraction using the specific activity of tryptic peptides of nascent chains as a measure of uniformity or nonuniformity (15, 20–28). While earlier studies indicated nonuniformity to exist among the nascent globin population more recent work has indicated quite the opposite, suggesting that the size distribution was, indeed, uniform.

The analysis of tryptic peptide specific activities has been shown to reveal nonuniform size distribution in instances of severe amino acid starvation or inhibition of initiation of protein synthesis by NaF (15). The ability of this procedure to detect more subtle deviations from uniformity may be questioned, however. Since each tryptic peptide analysis is obtained from a sample of the nascent peptides more directly. While the elution profiles reported here are complex, the accumulation of nascent peptides of approximately 40, 57, and 89 amino acids in length may be recognized as consistent features of the nascent peptide population. In addition, these data suggest that an accumulation of peptides in the 120 to 145 amino acid size range may be present. The accumulation of the completed α-globin chain, still attached to tRNA (α-globin-tRNA), on rabbit reticulocyte ribosomes has been reported earlier (6).

Since the length of a nascent peptide is indicative of the relative position of the ribosome along the messenger RNA during the course of translation one may conclude that at any one instant ribosomes are more apt to reside at certain regions of the messenger RNA strand than at others.

We interpret these data to indicate that the rate of translation of the globin messenger is not constant but proceeds more rapidly in some regions (hence producing the observed troughs) and more slowly in other regions (producing the observed peaks). These data also indicate that more than one slow region is encountered during translation. The etiology of variations in the translation rate of the hemoglobin messenger RNA molecules presents an intriguing subject for future investigations.

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