45. Improved Method for Quantitation of Biosynthesized Human Globin Chains in Reticulocytes by Use of Urea Cellulose Acetate Membrane Electrophoresis

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The α and the non-α (represented by β) globin chains have been said to be biosynthesized in normal human blood reticulocyte at a β/α ratio of approximately 1.0. However, in β thalassemia as well as α thalassemia the globin chain biosynthesis is not symmetrical and the β/α ratio is significantly or remarkably deviated from 1.0 on account of the suppression of either β or α globin chain production.\(^1\)\(^-\)\(^3\). Accordingly, measurements of relative amounts of the α and the non-α globin chain biosynthesized in reticulocytes are often necessary for the diagnosis of thalassemia. Clegg-Naughton-Weatherall's urea-CMC column chromatographic separation of α and non-α globin chains which are synthesized by reticulocytes in a culture medium containing radioisotope-labelled leucine has hitherto been employed as standard method.\(^4\) Regretfully, this procedure is complicated and time-consuming for the routine use in the ordinary clinical laboratory. Simpler method has therefore been desired. Recently, a new convenient method for the estimation of β/α biosynthesis ratio by use of urea-cellulose acetate membrane electrophoresis\(^5\) for separation of the α and the non-α chain was developed in our laboratory and reported elsewhere.\(^6\) However, it was soon noticed that the β/α ratio tended to be somewhat larger than the one obtained by the standard method due to contamination of the β chain by the γ and the δ chains which were the dissociation products of Hb F and Hb A\(_2\). This contamination was successfully eliminated lately and the method was improved to a system which gave the β/α ratio quite well comparable with that obtained by the standard urea-CMC column chromatographic method.

Procedure. Heparinized venous blood (about 2 ml) is collected. The red cells are washed with saline to remove the buffy coat and the plasma. Packed red cells are obtained by centrifugation of the washed red cells at 15,600 \(\times\) G (max.) at 4°C (Eppendorf centrifuge Model 5412). The top layer of the packed cells (about 0.2 ml) to which
reticulocytes are abundantly gathered is taken, mixed with the reaction mixture (1.2 ml) of Lingrel and Borsook\(^7\) containing \(^3\)H-leucine (New England Nuclear, 0.1 ml: 75 \(\mu\)Ci), and the mixture is incubated at 37°C for 2 hrs. After incubation, the red cells are washed five times with saline, lysed with 0.1% saponin solution (ca. 0.4 ml) and the lysate is filtered through a milipore filter (Milipore Co., pore size: 1.2 \(\mu\)m) to prepare clear hemolysate. The hemoglobin of the hemolysate is converted to carbonmonoxy-form and stored in a refrigerator at 4°C. To 10 \(\mu\)l of the hemolysate are added 50 \(\mu\)l of 8M urea-Tris-EDTA-Borate buffer solution\(^8\) and 10 \(\mu\)l of 2-mercaptoethanol, and allowed to stand at room temperature for about 1 hr so that the globin moiety of hemoglobin molecule may be split into its \(\alpha\) and \(\beta\) chains. This is called globin mixture. A sheet of cellulose acetate membrane (Sartorius, 7 cm \(\times\) 20 cm) is soaked in the buffer solution containing urea\(^9\) and set up in an electrophoretic assembly. Then the globin mixture (10–15 \(\mu\)l) is streaked (in a line of 4 cm) on the membrane at the site 2 cm distant from its anodal margin. An electric current of 150 volts/7 cm and 2 mA is run at 8°C for 2 hrs to separate the stripes of the \(\alpha\) and the \(\beta\) chains by electrophoresis. At the end of electrophoresis, the cellulose acetate membrane is stained in a dye solution (0.1% Ponceau 3 R in 6% TCA) for 1 min and soaked in 0.1% acetic acid five times repeatedly, for 30 min every time by exchange of washing to destain the background. The \(\alpha\) and the \(\beta\) chains are separated clearly in this way, forming distinctly pink stripes. The membrane is dried at room temperature for 2 hrs. It is scissored along the cathodal top margins of the \(\alpha\) and the \(\beta\) stripes, and each stripe area is cut in rectangular pieces of the size of 4 mm \(\times\) 4 cm, which are put separately into test-tubes A and B, each containing 2 ml of 0.2 N NaOH solution. Concurrently the same manipulation and treatment are done in test-tube b with a 4 mm \(\times\) 4 cm piece which is taken from the same membrane at the portion away from the \(\alpha\) and the \(\beta\) stripes. This serves as blank contrast of the background of membrane. After the \(\alpha\) chain stripe pieces, the \(\beta\) chain stripe pieces and the blank background pieces are eluted in 0.2 N NaOH solution for 1 hr, the membrane pieces are removed and the eluates in A, B and b are allowed to stand at room temperature for another 2 hrs. Then, their absorbances, \(E_\alpha\), \(E_\beta\) and \(E_b\), are measured at 510 nm in a Gilford spectrophotometer model 2400-2. Aliquots of one ml of the eluates

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\(^8\) The buffer solution containing urea is prepared in the following way: Tris (12 g), EDTA (0.6 g) and boric acid (3.0 g) are dissolved in distilled water and made to volume of 1000 ml. Urea (100 g) is dissolved in a portion of Tris-EDTA-borate buffer solution thus prepared and made to 300 ml with the same buffer solution. Then, 2-mercaptoethanol (1.8 ml) is added to it and the pH of the mixture is adjusted to 8.3 with HCl.
(α chain in A, β chain in B and blank background in b) are transferred individually into the scintillation vials (A), (B) and (b), each of which contains 8 ml of Dotite Scintisol-500 (Wako Pure Chemicals Co.). Vials (A), (B) and (b) are subjected to a liquid scintillation spectrometer for 10 min (Searle Analytic Inc., Model Mark III) to measure their radioactivities [α], [β] and [b] dpm respectively. After confirming that E_b and (b) are virtually 0, the β/α biosynthetic ratio is calculated by the following equation:

$$\frac{\beta/\alpha \text{ ratio}}{E_b} = \frac{[\beta] \text{ dpm}}{[\alpha] \text{ dpm}}$$

In order to appraise the accuracy of the present method, Clegg-Naughton-Weatherall's standard procedure was carried out simultaneously with the same blood samples, and the estimation by these two methods were compared with each other.

Results and discussion. The globin chains migrate toward the cathode in the urea-cellulose acetate membrane electrophoresis at pH 8.3. The α chain goes farthest from the origin, followed by the δ, the β and the γ chains in the order mentioned. Chains α and β are separated discretely with good reproducibility. Although chains δ and γ migrate closely around the β chain stripe, the β chain is isolated discretely by the present method, without any contamination by δ and γ chains. The relative rates of globin chain biosynthesis are able to be determined by the measurement of radioactivity (³H-leucine) incorporated in the relevant chain, namely by the radioactivity count of the stripes of the α and the β chains.

Estimations of β/α ratio obtained by the present improved method were made with the blood samples collected from patients with β thalassemia and α thalassemia and those with iron deficiency anemia as well as from the normal subjects. The comparison of these estimations with those by the standard method disclosed a satisfactory parallelism and agreement as shown in Fig. 1.

The normal range of β/α ratio values ranged from 0.9 to 1.2 in both methods. In iron deficiency anemia the β/α ratios were above 1, being distributed between 1.1 and 1.5.

Urea-cellulose acetate membrane electrophoresis is simple and rapid in manipulation, and it has good reproducibility. The variation of the heptaplicate estimation of β/α ratio is within ±0.05 of the mean value.

In our experience, the reticulocytes were concentrated by centrifuging so efficiently that the radioactivity count of ³H-leucine incorporated in the globin chains was satisfactorily large for exact calculation of the β/α biosynthesis ratio.

The volume of the hemolysate for electrophoresis is very small,
being less than 10 μl, and it is possible to analyse a large number of samples concurrently and within a short period of time. As for instance, four samples could be dealt with on a sheet of cellulose acetate membrane within 9 hrs, whereas the standard chromatographic procedure requires 30 hrs for one sample.

This method is particularly recommended for use in routine clinical laboratories. It is expected that the exact diagnosis of β thalassemia will be much simplified and facilitated by its application.

This system was first invented by us in 1977. Similar procedures were published a year afterwards by Vettore et al. and Salmon et al.

Fig. 1. The biosynthetic ratio (β/α) analyzed by urea cellulose acetate membrane electrophoresis in comparison with that obtained by carboxymethyl cellulose chromatography (standard method).

References