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BRIEF NOTE

ISOELECTRIC FOCUSING OF PCMB-TREATED HEMOLYSATE PREPARED FROM ISOTOPE-LABELED RETICULOCYTES FOR ESTIMATION OF GLOBIN CHAIN BIOSYNTHESIS (β/α RATIO)

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It was demonstrated by Heywood, Karon and Weissman (1964, 1965)[1,2] and Weatherall and Clegg (1972)[3] that the α and the non-α chain were synthesized at the ratio 1 : 1 to compose hemoglobin molecules in erythroid cells of adult human being. They incubated reticulocytes in a culture medium containing radioactive amino acid in order to synthesize hemoglobin in vitro. At the end of incubation they prepared hemolysate of thus treated reticulocytes to subject it to urea CM cellulose column chromatography and isolated the effluents containing the α and the non-α (i. e. β) chain of the synthesized hemoglobin. They measured the radioactivities of the effluents and estimated the β/α biosynthesis ratio.

Recently, a simple method employing urea cellulose acetate membrane electrophoresis in combination with liquid scintillator counting was developed for the test of biosynthesis of globin chains in our laboratories.[4] While we were using it for screening the β/α globin chain synthesis ratio in normal subjects and patients, we obtained a new idea that isoelectric focusing of hemoglobin which has been biosynthesized with isotopic labeling in vitro and then dissociated into its subunits, namely, the α and the non-α chains, by para-chloromercuribenzoate (PCMB) might be useful for the same purpose. The procedure of dissociation of hemoglobin by PCMB was first invented by Bucci and Fronticelli (1965)[5]. Ohba and his coworkers (1966)[6] successfully applied it to the determination of chain anomaly of abnormal hemoglobins by employing starch gel electrophoresis of PCMB-treated hemolysate which contained the normal hemoglobin together with the abnormal. Therefore, we tested our idea. The result of the test showed usefulness of isoelectric focusing of PCMB treated hemoglobin solution which has been isotopically labeled for estimation of β/α synthesis ratio. Our procedure is as described below.

About 10 ml of blood is withdrawn from the antecubital vein of a normal subject or a patient, transferred in a sterilized centrifuge tube containing 2 drops of heparine and mixed. This blood sample is centrifuged (3,000 rpm for 10 minutes) to separate erythrocyte layer from plasma. The plasma is

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removed, and to the erythrocyte layer is added a sufficient amount of sterilized physiological saline and centrifuged. This procedure is repeated three times to obtain washed erythrocytes.

All the washed erythrocytes are introduced into 5 ml of incubation mixture of Lingrel-Borsook (^{14}C-leucine 30 μCi)[7] and allowed to stand at 37°C for 120 minutes. At the end of the times the crythrocytes are washed with physiological saline (centrifugation at 480 G for 10 minutes) 5 times repeatedly. One volume of the washed erythrocyte layer is mixed well with 5 volumes of 2.81 % Dextran T-40 saline solution, and centrifuged at 10,000 rpm (5,200G) at 25°C for 60 minutes to separate reticulocytes in liquid layer lying over sedimented erythrocytes. The layer containing reticulocytes are all collected, washed and centrifuged with sufficient amount of physiological saline. The supernatant layer is discarded. To one volume of reticulocyte sediment are added 1.5 volumes of distilled water and 0.5 volume of carbon tetrachloride, stirred completely to have a hemolyzed solution. The hemolysate in bubbled with carbon monoxide to convert oxyhemoglobin into carbonmonoxy-hemoglobin (COHb).

Aliquot of 20 μl of COHb solution thus obtained is introduced in a test tube, and to this are added 50 μl of phosphate buffer solution (μ=0.2, pH 5.9), 20 μl of 2 M sodium chloride solution, and 20 μl of 1 g/dl PCMB solution, and mixed. The tube is filled with carbon monoxide gas, stoppered tightly, and placed in a cold chamber (4°C) for 4 to 6 hours to dissociate hemoglobin into its α and non-α subunits.

The instruction note of the LKB production Co.[8] is followed in the preparation of polyacrylamide gel containing ampholine (pH ranging from 5 to 8.5). With 2.5 ml of acrylamide-Bis (N, N′-methylenebisacrylamide) solution (30 g of acrylamide monomer are dissolved in 100 ml of distilled water containing 0.8 g of Bis) are mixed 9.1 ml of sucrose solution (20.8 %), 0.375 ml of ampholine (pH range: 5∼7), 0.375 ml of ampholine (pH range: 7∼9) and 2.5 ml of distilled water. Then the mixture is deaerated. To this mixture are added 50 μl of 0.004 % riboflavin solution, 50 μl of TEMED (N,N,N′,N′-tetramethylenediamine) and 50 μl of 10 % ammomium persulfate solution, and mixed. This mixture is polymerized to form a gel layer (1 mm in thickness) between two parallel glass plates (11×13 cm) by illumination with ultraviolet light for about 5 hours. In this way a gel plate with rectilinear pH gradient ranging from 5 to 8.5 which is suitable for isoelectric focusing is obtained.

Wicks of thick rectangular filter papers (1mm×6mm×13cm) are touched to both ends of the gel plate. The anodic wick is moistened with 0.02 M

phoshoric acid, and the cathodic with 20 % ethylenediamine solution. A rectangular filter paper strip of 5 mm × 50 mm is soaked appropriately with the PCMB-treated COHb solution and placed on the gel plate 1.0 cm distant from the cathodic end and perpendicularly to the short (11 cm) axis. An electric current is run at a constant electric pressure of 200 V/11cm in a cool chamber (8 °C) overnight.

The electrophotogram (Figure 1) shows the α chain band and the β chain band which splits into a stripe of PMB-β chain and that of β chain dimer. The former is situated around the position of pH 7.3 and the latter at the area of pH 6.2 to 6.3, respectively.

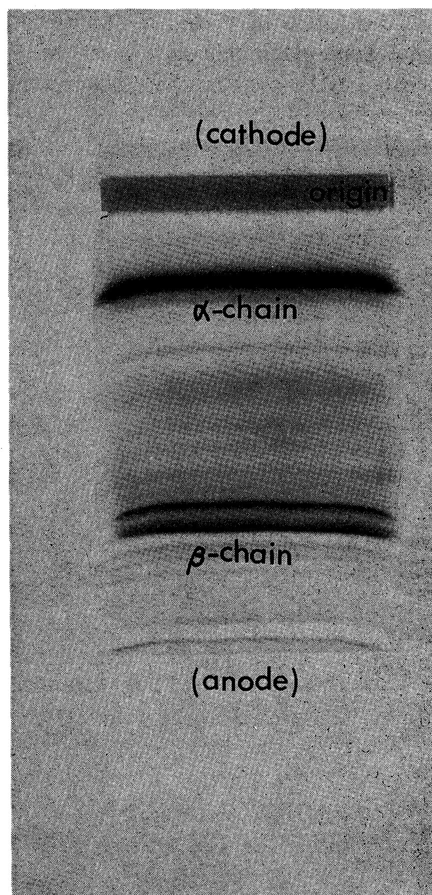

Fig. 1. Isoelectric focusing pattern of hemoglobin treated with p-chloromercuribenzoate.

The acrylamide gel bands containing the α and the β chain are separately enucleated from the gel plate with a cutter, and put into two vials (α and β) containing 10 ml aliquots of liquid scintillation cocktail (PPO 4g, POPOP 100 mg, and protosol 100ml are dissolved in toluene and made to volume of 1,000 ml) individually to be eluted completely until the gel bands become transparent and colorless. They are subjected to liquid scintillation spectrometry. The β/α biosynthesis ratio is calculated from the counts of the eluates (α and β).

The β/α ratio is around 1.0 in normal subject (Table 1), and it was 0.67 in a human heterozygous for β thalassemia.

TABLE 1.
The radioactivities (dpm) of α-and β-chains after isoelectric focusing of hemoglobin biosynthesized in vitro and treated with p-chloromercuribenzoate.

Case	dpm		
	β-chain	α-chain	β/α
Normal subjects			
I	794	810	0.98
II	155	164	0.95
III	67	68	0.99
IV	177	182	0.97
β-thalassemia minor			
V	970	1467	0.66

Fetal hemoglobin ($\alpha_2\gamma_2$) migrates as an undissociated fraction between the bands of the α and the β chains by isoelectric focusing, because Hb F scarcely undergoes dissociation by PCMB treatment of the hemolysate. Accordingly, the value of β/α biosynthesis ratio is little affected by Hb F which is coexistent with Hb A in the hemolysate.

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