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# A TECHNIQUE FOR ESTIMATION OF HEMOGLOBIN BIOSYNTHESIS ($\beta/\alpha$ RATIO) BY ISOELECTRIC FOCUSING ON AMPHOLINE-POLYACRYLAMIDE GEL

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## Abstract

**A new method for estimation of the ($\beta/\alpha$) globin chain ratio of hemoglobin biosynthesized in reticulocytes incubated in a culture medium containing radioisotopic leucine is described.**

**Carbonmonoxyhemoglobinized hemolysate prepared from the reticulocytes was treated with PCMB solution, and subjected to isoelectric focusing on an ampholine-polyacrylamide gel plate to separate the PMB-$\alpha$ and the PMB-$\beta$ hemoglobins. They were separately eluted into phosphate buffer solutions, their heme was removed with acid-acetone, and dissolved in Triton X-100 solutions. The radioactivities (dpm) of the solutions of heme-free PMB-$\alpha$ and PMB-$\beta$ were counted. Their specific activities (dpm ÷ absorbance of the eluates at 415 nm), $\alpha$ and $\beta$, were calculated to get the $\beta/\alpha$ ratio of the biosynthesized hemoglobin.**

**The analytical data obtained about the normal subjects and the $\beta$-thalassemia subjects by this method were in good agreement with those obtained by the standard method of Clegg-Naughton-Weatherall.**

## INTRODUCTION

In reticulocytes of normal human blood, the $\alpha$ and the $\beta$ globin chains are biosynthesized at a ratio of approximately 1.0, but in those of $\beta$-thalassemia patients they are not synthesized symmetrically, namely, the $\beta/\alpha$ ratio is significantly low on account of the inhibition of $\beta$-chain production. Therefore, the $\beta/\alpha$ ratio which is obtained by the globin biosynthesis examination is helpful to diagnosis of ambiquous cases of $\beta$-thalassemia.

Clegg-Naughton-Weatherall's column chromatography on urea-carboxymethyl cellulose has been employed traditionally for this purpose as standard method which enables separation of globin chains synthesized in reticulocytes in a culture medium containing radioisotope labelled leucine.

Ueda and Shibata[1], Vettore et al.[2], and Salmon et al.[3] reported new

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simple and rapid procedures for the estimation of $\beta/\alpha$ chain ratio of biosynthesized hemoglobin by means of urea-cellulose acetate membrane electrophoresis[4] ; these would be useful as routine diagnostic test of $\beta$-thalassemia.

Another technique for the measurement of $\beta/\alpha$ biosynthesis ratio was recently invented in our laboratory and reported preliminarily[5]. This employs PCMB treatment of the carbonmonoxyhemoglobinized (COHb) hemolysate and isoelectric focusing on ampholine-polyacrylamide gel plate.

The purpose of this paper is to describe in detail the procedure of our method and the results obtained by it.

## MATERIALS AND METHODS

*Hemoglobin biosynthesis.* Heparinized venous blood (ca. 2 ml) was centrifuged (3,000 rpm for 5 minutes) and the sedimented erythrocytes were washed with physiological saline repeatedly. The upper layer (0.2 ml) of the sediment was mixed with and incubated in a culture medium[6] (1.2 ml) which contained $^{14}$C-leucine (7.5 $\mu$Ci, 0.15 ml) or $^{3}$H-leucine (70 $\mu$Ci, 0.1 ml) at 37°C for 2 hr. At the end of incubation, they were washed 5 times with saline and centrifuged. The packed cells were mixed with distilled water (ca. 0.4 ml) and agitated well. The hemolysate thus prepared was centrifuged to remove stroma and the supernatant was used for the analysis.

*Polyacrylamide gel plate.* A mixture of 30% acrylamide-Bis solution was prepared by dissolving 30 g of acrylamide monomer and 0.8 g of N, N-methylenebisacrylamide in distilled water and made to 100 ml. A 2.5 ml portion of this solution was mixed with distilled water (2.5 ml), 20.8% sucrose solution (9.1 ml), and ampholine (pH range 3.5-10.0, LKB Produkter AB) (0.75 ml). The mixture was degassed under reduced pressure by cooling in ice-water. Then, 0.004% riboflavin solution (50 $\mu$l), TEMED (N, N, N', N'-tetramethylethylenediamine) (50 $\mu$l), and 10% ammonium persulfate solution (50 $\mu$l) were added to it and mixed. The mixed solution was poured into the gel mold and polymerized under ultraviolet light for 3 hr to get a rectangular plate (11 cm x 13 cm x 1 mm).

*PCMB treatment of hemoglobin.* The procedure of Rosemeyer and Huehns[7] was followed with slight modification. To 20 $\mu$l aliquot of the hemolysate which had been converted to the carbonmonoxy form were added 18 $\mu$l of phosphate buffer solution ($\mu$: 0.2, pH 5.9), 8 $\mu$l of 2 M sodium chloride, and 10 $\mu$l of 1 g/dl PCMB (p-chloromercuribenzoic acid) solution in the order mentioned. The mixture was saturated with CO gas and allowed to stand at 4°C for more than 4 hr so that hemoglobin molecule might be dissociated into its $\alpha$ and $\beta$ hemoglobins (PMB-$\alpha$ and PMB-$\beta$). A small

mount of precipitate appeared at the bottom of the test tube containing the mixed solution.

*Isoelectric focusing of the PCMB-treated hemoglobin.* Wicks of thick rectangular filter paper (1 mm x 6 mm 13 cm) were touched to both ends of the afore-mentioned plate. The anodic wick was moistened with 0.02 M phosphoric acid solution and the cathodic with 20% ethylenediamine solution. A rectangular filter paper strip (5 mm x 30 mm) was soaked with the PCMB-treated COHb hemolysate and placed on the gel plate 1.0 cm distant from the cathodic end and perpendicular to the short (11 cm) axis. Electrophoresis was done at a constant voltage of 300 V/11cm at 8°C overnight (ca. 12 hr).

*Elution of the PMB-$\alpha$ and the PMB-$\beta$ hemoglobin.* The bands (2 mm x 3 cm) of the PMB-$\alpha$ and PMB-$\beta$ hemoglobins were separately removed from the plate with a cutter, put into two test tubes which contained 1 ml aliquots of 0.1 M phosphate buffer solution (pH 7.4) with 0.01% potassium cyanide, and allowed to stand for 3 hours for elution of hemoglobins. The eluates were filtered and collected through funnels set with small cotton plugs.

*Measurement of the absorbance of eluates.* An aliquot of 100 $\mu$l of each eluate was diluted exactly 10 times with distilled water, transferred into a semi-microcuvet, and measured for absorbance ($\alpha$ A and $\beta$ A) in a Gilford spectrophotometer (Model 2400-2) at 415 nm.

*Radioactivity counting (dpm).* A 0.5 ml aliquot of each eluate (PMB-$\beta$ and PMB-$\beta$ hemoglobin) was poured into 10 ml of cold 1% HCl-acetone and mixed well to remove heme from the hemoglobins. The $\alpha$ and the $\beta$ globin chains were sedimented by centrifugation at 3,000 rpm for 10 min, washed two times with cold 1% HCl-acetone, and dried at room temperature. The sediments were dissolved in 1.2 ml of 10% Triton X-100 solution. All these procedures was done in the same test tube. A 1 ml aliquot of each solution was transferred into a liquid scintillation vial which contained 8 ml of Dotite Scintisol 500 (Wake Pure Chemicals Co., Japan), and each vial was shaken until the contents became transparent. After keeping at room temperature for 30 min, their radioactivities ($\alpha$ dpm and $\beta$ dpm) was counted in a Liquid Scintillation Spectrometer (Searle Analytic Inc., Mark III).

*Calculation of the specific radioactivity ratio ($\beta/\alpha$).* This was calculated by the following equation.

$$\beta/\alpha \text{ ratio} = \frac{\beta \text{ dpm}/\beta \text{ A}}{\alpha \text{ dpm}/\alpha \text{ A}}$$

## RESULTS

Treatment of COHb hemolysate with PCMB solution in CO gas atmosphere

for 4 hours was satisfactory for complete dissociation of hemoglobin A into $\alpha$ and $\beta$ hemoglobin, namely PMB-$\alpha$ and PMB-$\beta$ (Fig. 1).

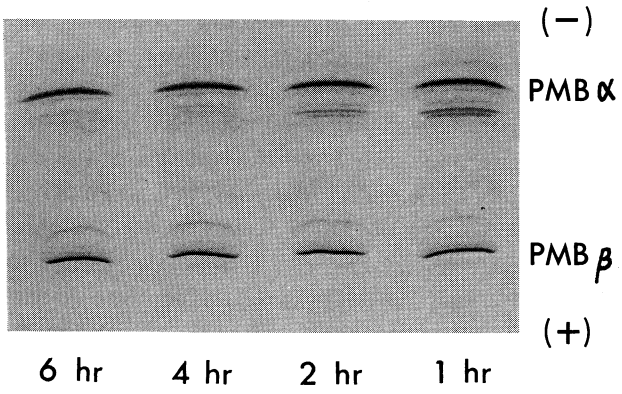


Fig. 1. Isoelectric focusing of PCMB-treated hemolysate at various reaction time. The hemolysate contained 2% of Hb F (by alkaline denaturation method[8]).

However, Hb F ($\alpha_2\gamma_2$) remained undissociated under this condition as had been pointed out by Rosemeyer and Huehns[7] (Fig. 2).

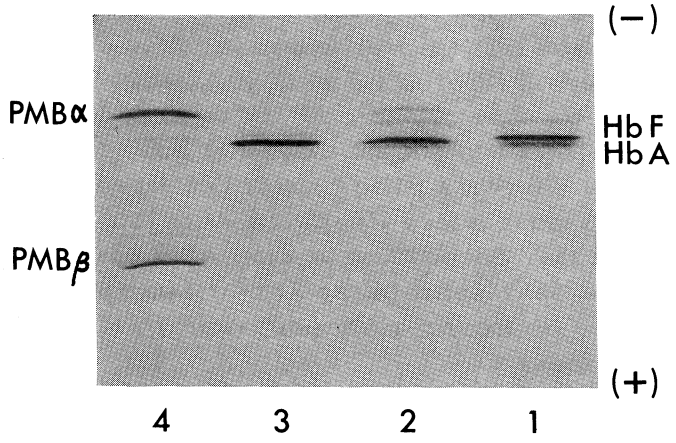


Fig. 2. Isoelectric focusing of PCMB-treated and non-treated hemolysates for 4 hours. 1: Non-treated cord blood hemolysate. 2: PCMB-treated cord blood hemolysate. 3: Non-treated normal adult hemolysate (Hb F contents: 2%). 4: PCMB-treated normal adult hemolysate.

Figures 1 and 2 present the evidences : isoelectric focusing of PCMB-treated hemoglobin on a polyacrylamide gel plate containing ampholine (pH range 3.5–10.0) showed two distinct well-separated main bands of PMB-$\alpha$ and PMB-$\beta$ hemoglobins.

These main bands were able to be identified as the $\alpha$ and the $\beta$ globin chains in the following way. Eluates of PMB-$\alpha$ and PMB-$\beta$ bands were treated with 2-mercaptoethanol and with acid-acetone in order to get their globin chains, and the chains thus obtained subjected to urea-cellulose acetate membrane electrophoresis and their electrophoretic migration was compared with Hb A of the normal subject. The migrations of the globin chains obtatined from PMB-$\alpha$ and PMB-$\beta$ were the same as those of the $\alpha$ chain and the $\beta$ chain of Hb A, respectively (Fig. 3).

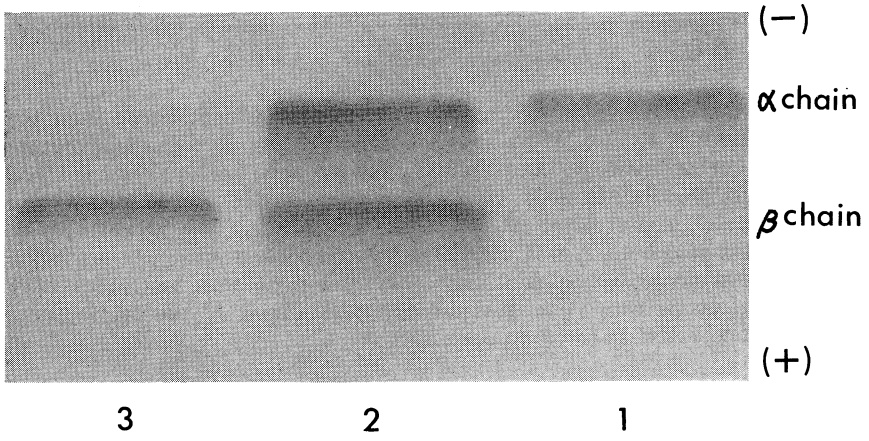


Fig. 3. Identification of globin chains ($\alpha$ and $\beta$) obtained from PMB-$\alpha$ and PMB-$\beta$ hemoglobins (isoelectrofocussed on ampholine-polyacrylamide gel plate) by urea-dissociation cellulose acetate membrane electrophoresis (5.5 M urea-Tris-EDTA-Borate buffer (pH 8.3)). 1: From PMB-$\alpha$ hemoglobin. 2: Normal control hemoglobin A. 3: From PMB-$\beta$ hemoglobin.

Figure 4 shows hemoglobin A recovery from the gel after isoelectric focusing of Hb A, which was prepared from the hemolysate containing isotope ($^3$H)-labelled hemoglobin by DEAE-cellulose (DE 52, Whatman Ltd.) column chromatography in 0.2M glycine-0.01% KCM-NaCl buffer solution (pH 7.8, $Na^+$ ion gradient : 5 mM→40 mM)[9]. The elution of hemoglobin A from the gel was completed at room temperature (25°C) at the end of 3 hours and the vicissitude of the radioactive specific activities of the eluate became stationary at the same time.

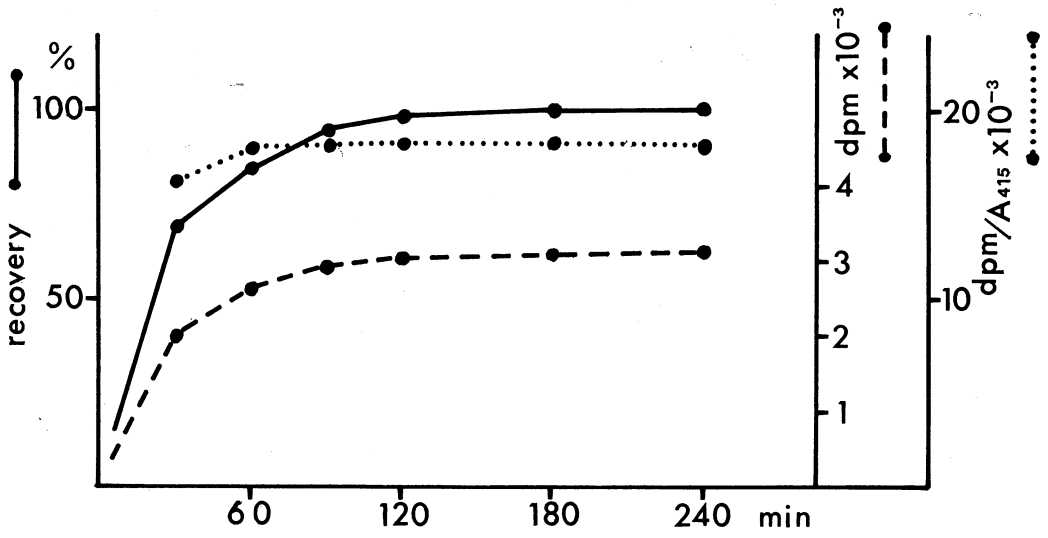


Fig. 4. Elution of biosynthesized radioisotope-labelled hemoglobin A from the gel with 0.01% KCN-0.1M phosphate buffer (pH 7.4) at room temperature (25°C). Purified hemoglobin A solution (40 $\mu$l) was isoelectrofocussed and treated as described in text. Hemoglobin contents (Hb) of the eluate were analysed by measurement of absorbance at 415 nm. The percentages of released hemoglobin A was estimated by comparison with non-isoelectrofocussed standard hemoglobin A solution, and these were regard as 100% recovery. At the same time the radioactivities (dpm) of the eluates were recorded to calculate the ratio dpm/Hb.

The radioactivities of the eluate ran parallel with its hemoglobin concentration.

It will be noticed from Table 1 that a small portion of PCMB-treated isotope-labelled hemoglobin A remains undissociated at the reaction time of 2 hours, but the specific activities of the separated PMB-$\alpha$ and PMB-$\beta$ attain the plateau and their $\beta/\alpha$ ratio became settled constant at that time.

TABLE 1.

The $\beta/\alpha$ ratio and the specific activities of PMB-$\alpha$ and PMB-$\beta$ hemoglobins in the PCMB-treated hemoglobin at various reaction time.

| reaction time (hours) | specific activities ($dpm/A_{415}$) | | $\beta/\alpha$ ratio |
|---|---|---|---|
| | $\alpha$ | $\beta$ | |
| 2 | 12587 | 14460 | 1.15 |
| 4 | 11206 | 13065 | 1.17 |
| 6 | 12583 | 14394 | 1.14 |

A typical profile of radioactivities (PMB-$\alpha$ and PMB-$\beta$ dpm) and absorbances at 415 nm of the eluates from 0.5 cm (3 cm width) parallel section of the gel is presented in figure 5.

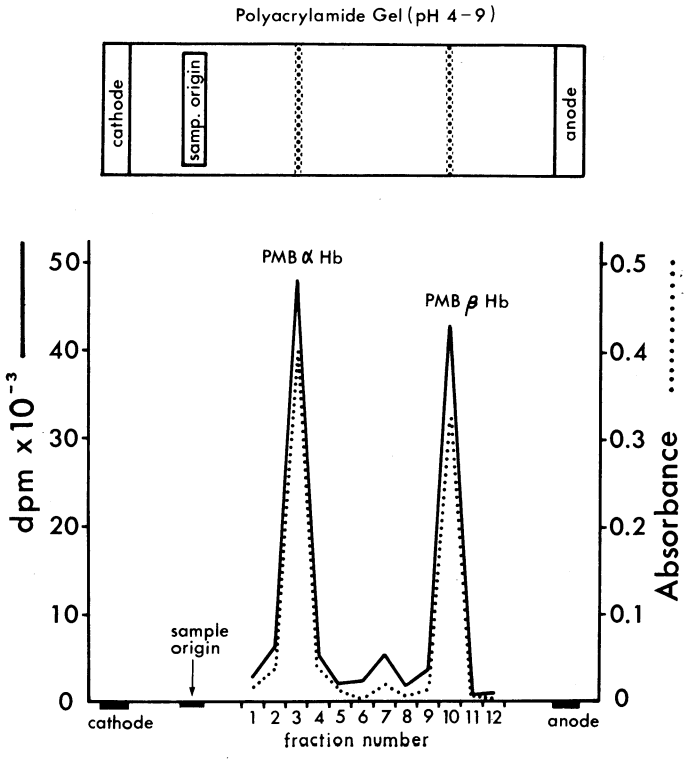


Fig. 5. Separation of the PMB-$\alpha$ and the PMB-$\beta$ hemoglobins on ampholine-polyacrylamide gel after treatment of the biosynthesized radioisotope-labelled hemoglobin with PCMB (upper part), together with the profiles of radioactivity (dpm : solid line) and absorbance of each PMB-hemoglobin bands (dotted line in the lower part). After isoelectric focusing, the gel was cut in 0.5 cm (3 cm width) sections, eluted, measured for their absorbances at 415 nm and counted for their radioactivities. The Hb F and the Hb $A_2$ contents of the hemolysate containing biosynthesized hemoglobin were 0.5% and 2.5%, respectively.

Maximum peaks of the radioactivity coincided with those of the absorbances quite well. Accordingly, specific activity of the $\alpha$- and the $\beta$-hemoglobin can be calculated by dividing the radioactivity (dpm) by the absorbance at 415 nm.

As shown in the Table 2, the $\beta/\alpha$ ratio of the biosynthesized hemoglobin

analyzed by this isoelectric focusing in 35 normal adult subjects ranged from 0.9 to 1.2 with a mean value of 1.04. These were entirely the same as normal range and average values obtained by the column chromatographic standard method of Clegg-Naughton-Weatherall. In $\beta$-thalassemia, the $\beta/\alpha$ ratio was significantly lower than 1.0, ranging over from 0.25 to 0.75. It is apparently that there was a satisfactorily good agreement in the values of $\beta/\alpha$ ratio between the present (PCMB-isoelectric focusing) method and the standard method.

The reproducibility of this method was satisfactory. Figute 6 shows the day-by-day variation of the $\beta/\alpha$ ratio in the same normal subject for the period of 8 days.

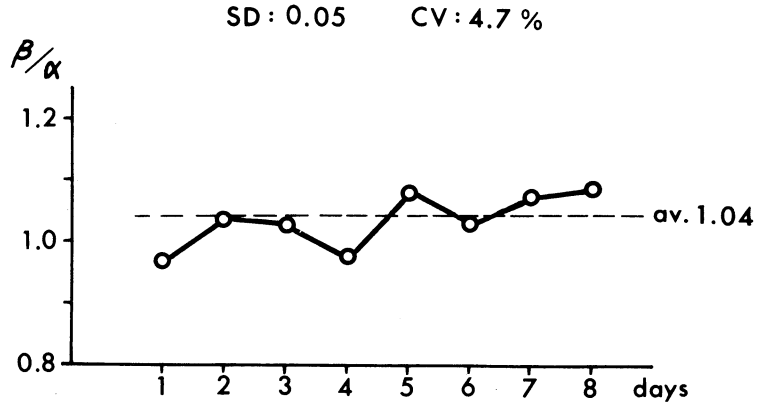


Fig. 6. Day-to-day variation of the $\beta/\alpha$ ratio of hemoglobin biosynthesized in reticulocytes of a normal subject.

TABLE 2.
Comparison of the analytical data ($\beta/\alpha$ ratio) obtained by isoelectric focusing (I. E. F.) and standard column chromatographic (C. Chr.) methods.

| subjects | I. E. F. | C. Chr. | expected genotype |
|---|---|---|---|
| Normal (35) | 0.9-1.2 | 0.9-1.2 | |
| $\beta$-Thalassemia | | | |
| T. K. | 0.35 | 0.28 | $\beta^0\beta^+$ |
| Y. M. | 0.51 | 0.59 | $\beta^0\beta$ or $\beta^+\beta^+$ |
| T. M. | 0.68 | 0.76 | $\beta^+\beta$ |
| T. B. | 0.52 | 0.53 | $\beta^0\beta$ or $\beta^+\beta^+$ |
| S. T. | 0.52 | 0.60 | $\beta^0\beta$ or $\beta^+\beta^+$ |
| N. Y. | 0.47 | 0.55 | $\beta^0\beta$ or $\beta^+\beta^+$ |

## DISCUSSION

Clegg-Naughton-Weatherall's urea-carboxymethyl cellulose column chromatography has hitherto been employed as the standard method for the analysis of the globin chain biosynthesis. However, this is complicated in manipulation, time-consuming and requires a large quantity of blood sample. Recently, Ueda and Shibata[1], Vettore et al.[2], and Salmon et al.[3] have reported simpler and rapid methods that employ urea dissociation and cellulose acetate membrane electrophoresis of the biosynthesized hemoglobin (ca. 10 $\mu$l of hemolysate). However, it was noted that there was slight deviation of $\beta/\alpha$ ratio toward unduly larger value on account of the contamination of the $\beta$ chain with the $\gamma$ and the $\delta$ chain which were the dissociation products of Hb F and Hb $A_2$. The present method by isoelectric focusing which has been discribed in this paper is exempted from this kind of shortcoming, because isoelectric focusing yields excellent and complete separation of the PMB-$\alpha$ and PMB-$\beta$ produced from the biosynthesized hemoglobin on ampholine-polyacrylamide gel plate without any contamination of the PMB-$\alpha$ and the PMB-$\beta$ by the PMB-$\gamma$ and the PMB-$\delta$.

It is true that isoelectric focusing is not so simple as cellulose acetate membrane electrophoresis, but it is not so complicated and time-consuming as urea-carboxymethyl cellulose column chromatography. In our experience, Clegg-Naughton-Weatherall's standard procedure took 25 hours, Ueda and Shibata's method 9 hours, and the present procedure 17 hours, for the complete performance of the analysis of biosynthesized $\beta/\alpha$ ratio.

The present method employing isoelectric focusing gives the results of analysis well comparable with those obtained by the standard procedure of Clegg-Naughton-Weatherall. It is recommended as a useful tool for the analysis of biosynthesized $\beta/\alpha$ globin chain ratio to the personnels who are familiar with the technique of isoelectric focusing.

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