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BRIEF NOTE

A simple radioisotope-labeling method for estimation of globin-chain biosynthesis (α/β ratio) in vitro: use of urea cellulose acetate membrane electrophoresis in combination with liquid scintillator counting.

It was Heywood (1964[1], 1965)[2] and Weatherall (1965)[3] that afforded, for the first time, a substantial evidence for the theory that unbalanced or asymmetrical biosynthesis of globin due to genetic suppression of the α or the non-α chain would be the etiology of thalassemia. They incubated reticulocytes in a culture medium containing radioactive amino acids in order to synthesize hemoglobin in vitro. At the end of incubation they prepared hemoglobin solution from the reticulocytes and subjected to urea CM cellulose chromatography to isolate the effluents containing the α and the non-α (i.e. β) chain. They measured the radioactivities of the effluents, and found that the α/β effluent radioactivity ratio was around 1.0 in the reticulocytes collected from the normal subjects, but it was significantly larger than 1.0 in those obtained from β- thalassemia patients. They interpreted this result as a finding which indicated that radioactive amino acids were incorporated into the α and the β chains in equal amount or symmetrically in normal men, but it was taken less into the β chain than into the α chain in β-thalassemia patients, due to genetic suppression of the β chain synthesis in this disease.

This interpretation has been confirmed by other investigators[4], and, at the present time, test for biosynthesis of globin chains is being demanded as inevitable measures for the diagnosis of thalassemia in dubious cases. However, the demand has not yet been satisfied, because the techniques employed by Heywood and Weatherall are too complicated and time-consuming for use in the ordinary laboratories.

Recently a simple, rapid method for estimation of globin chain biosynthesis (α/β ratio) was invented in our laboratory. This employs urea cellulose acetate membrane electrophoresis instead of the conventional urea CM cellulose chromatography for the purpose of separation of globin polypeptide chains. Our method will be described below.

Ten milliliters of heparinized venous blood were added to 8.8 ml of Lingrel-Borsook's reaction medium[3] containing ^{14}C-leucine (30 μCi), and incubated under constant agitation at 37°C for 120 minutes.

At the end of incubation a sufficient volume of physiological saline solution was added to the mixture, centrifuged (480 G, for 10 min), and the supernatant layer was removed to collect the erythrocyte layer. The erythrocyte layer was washed with 10 -fold volumes of physiological saline 5 times by centrifugation and decantation.

One volume of thus washed erythrocyte layer was mixed with 5 volumes of 28.5 % Dextran T-40 aqueous solution.[6] The mixture was centrifuged at 10,000 rpm (5200 G) and 25°C for 60 minutes. Reticulocytes assembled at the top and upper layers of the Dextran solution, while erythrocytes were sedimented to the bottom.

The Dextran solution layer was transferred to the ordinary conical centrifuge tubes, and physiological saline, equal in amount, was added so that the reticulocytes might be sedimented at the bottom of the tubes by centrifugation (1000 G, 10 min).

To one volume of reticulocyte layer are added 1.5 volumes of H_2O and 0.5 volume of carbon tetrachloride, to hemolyze the reticulocyte by mixing and agitation in a vortex mixer. Clear hemolysate was obtained by centrifugation (1000 G, 20 min) of the mixture.

The hemoglobin in the hemolysate was dissociated into the α and the β chains with mercaptoethanol and 8M urea solution, allowing to stand overnight at room temperature. As to the detail of the procedure Ueda and Schneider (1969)[7] was followed.

The α and the β chains were separated by urea cellulose acetate membrane electrophoresis (pH 8.0) at 200 V/7 cm, 7 mA/20 cm and 4°C for 120 minutes. The buffer solution was prepared by the following prescription: Tris (10.2 g), EDTA (0.6 g) and boric acid (3.2 g) were dissolved in distilled water and made to volume of 1000 ml. Urea (100 g) was dissolved in and made to 300 ml with this solution. Mercaptoethanol (1.8 ml) was added. The pH of the mixture was corrected to 8.0 with 1N HCl,

After electrophoresis the cellulose acetate membrane was stained in Brilliant blue R solution (Brilliant blue R 25 mg, acetic acid 10 ml, isopropanol 25 ml and distilled water 65 ml) for 2 hours, and immersed in 0.3 % acetic acid overnight to destained the background. The bands of the α and the β chain appeared distinctly blue.

The two bands were cut out from the cellulose acetate membrane, regarding that the space circumscribed between the anodal edges of the α band and that of the β band belonged to the α chain area, and the region, the same in size as this area, departing from the anodal edge of

the β band anodewards represented the β chain area. They were cut in small pieces of α and β separately, and put into the scintillation vials, α and β in which 10 ml of scintillation cocktail (PPO 4 g, POPOP 100 mg, protosol 100 ml and toluene 1000 ml) had been introduced, and allowed to stand at room temperature overnight.

Then, the vials were subjected to scintillation spectrometer (Packard, Model 3385) to measure their radioactivities (total counts, cpm). Specific activities (dpm) were read by collation of the total count to the ^{14}C correction curve of the instrument.

Application of this method for the globin chain biosynthesis in normal adult subjects disclosed α/β ratio to be 0.99 ± 0.09. The coefficient of variation was 9.3 %. In the near future its usefulness will be checked by the blood samples of thalassemia patients.

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