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Note

Electrofocusing of stroma-free hemolyzates of human erythrocytes; comparison of fresh, stored and chemically modified samples

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Infusible stroma-free hemoglobin solution (SFH) as a potential oxygen-transporting blood substitute has attracted increasing attention during the past decade. Generally, SFH samples are prepared from fresh or banked human erythrocytes by various methods of hemolysis, purification and chemical modification, and can be stored under different conditions[1–8]. A detailed analysis of the protein pattern of SFH is important with regard to the non-standard nature of the raw material (pooled blood), to the instability of hemoglobin (Hb) solutions and to the presence of a small amount of non-Hb proteins in purified Hb solutions[9,10].

The present paper shows that electrofocusing on a thin layer of polyacrylamide gel (PAG) may give more detailed information on the protein composition of various SFH samples than previous methods.

MATERIALS AND METHODS

Standard SFH samples were prepared according to ref. 11. A part of each sample was lyophilized with sucrose[7,12]. Hb was modified by glutaraldehyde treatment according to ref. 13. The control samples of crude hemolyzates of human erythrocytes were prepared from fresh human blood (one volume of 3.8% sodium citrate + four volumes of blood). The erythrocytes were separated and washed five times (each for 10 min) by centrifugation in 0.9% NaCl at 2000 *g* at 4 °C. Packed red cells were lysed by adding four volumes of cold distilled water and kept in a refrigerator at 4 °C for 1 h. The hemolyzates were then centrifuged at 6000 *g* for 10 min. Carbon dioxide was bubbled through a part of the supernatant to prevent autoxidation. Prior to electrofocusing, all Hb samples were desalted on a 9 × 0.9 cm column of Sephadex G-25 Superfine in distilled water. Methemoglobin was prepared by addition of $K_3Fe(CN)_6$ to the hemolyzate[14]. The final concentration of Hb in hemolyzates before electrofocusing was 20–24 g/l, and was measured by the standard cyanohemoglobin method[15].

Isoelectric focusing on PAG was performed with the Pharmacia FBE 3000 Flat Bed Apparatus following the procedure recommended by the manufacturer[16]. An ECPS 200/300 power supply (Pharmacia, Uppsala, Sweden) was used. Pharmalyte (Pharmacia) was employed to form a gradient of pH 5–8.

Ammonium persulphate and acrylamide were obtained from Lachema (Brno, Czechoslovakia, N,N-methylenebisacrylamide from Eastman-Kodak (Rochester, NY, U.S.A.). The separated stained components were estimated by densitometric scanning using a Vitatron TLD 100 (Paul Bruning, Eefde, The Netherlands).

Procedure[16]

Solutions of acrylamide (150 g/l) and methylenebisacrylamide (5 g/l) were treated with mixed ion exchanger (H^+ and OH^-) to remove salts. Glass plates (230 × 115 mm) were treated with Silan A-174 before use. To prepare PAG, 10 ml of acrylamide–methylenebisacrylamide solution (ion exchanger removed by decantation), 6 ml of glycerol in distilled water (500 g/l), 2 ml of Pharmalyte (pH 5–8) and 12 ml of distilled water were mixed. This mixture was deaerated in a vacuum bottle for 15 min. Gel polymerization was initiated by addition of 100 μl of sodium sulphite (50 g/l) and 200 μl of sodium persulphate (22.8 g/l) solution in distilled water. This mixture was injected immediately into a cassette constructed from two glass plates, the space between which had previously been filled with nitrogen. The polymerization required 10–15 min.

Porous electrode strips were dipped in the electrode solutions (anode solution, 0.04 mol/l glutamic acid; cathode solution, 1 mol/l ethanolamine) and placed on a filter-paper for 15 sec before setting them along the long sides of the glass plate. Prefocusing lasted 20 min. Small pieces of filter-paper (5 × 5 mm) were soaked in the individual samples and applied to the surface of the gel. The controls of the power supply were set to 30 W per apparatus as follows: range switch, 2000 V, 150 W, 90 mA; meter scale, watts; power limit control, 20%; voltage limit control, 100%; current limit control, 100%; standby-operate switch, operate. Electrofocusing lasted 60 min.

The glass plates were then treated as follows: (1) 30 min in fixing solution (50 g/l trichloroacetic acid + 50 g/l sulphosalicylic acid in distilled water); (2) 30 min in rinsing solution (450 g/l methanol + 100 g/l acetic acid in distilled water); (3) 45 min in staining solution (2 g/l Coomassie blue G-250 + 450 g/l methanol + 100 g/l acetic acid in distilled water); (4) 15 min in rinsing solution; (5) final destaining solution (225 g/l methanol + 75 g/l acetic acid + 50 g/l glycerol in distilled water) until the background was clear.

RESULTS AND DISCUSSION

Fig. 1 compares the Hb fractions of standard SFH and of SFH samples treated in different ways prior to electrofocusing. Seven to eight red, red-brown and brown zones were observed for most samples. Application of higher sample concentrations as well as staining with Coomassie blue enabled visualization of even the very faint zones in the region of coloured zones, usually up to fifteen zones. Sometimes, however, densitometric estimation of the A band could not be carried out due to its high relative concentration in the gel. The main bands of the hemoglobins were

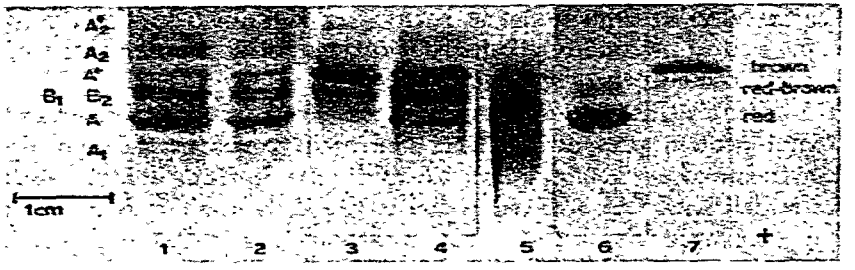

Fig. 1. Isoelectric fractionation of SFH samples in PAG thin layer. 1 = SFH stored in frozen state at −10 °C for 7 months; 2 = SFH stored in lyophilized state at +20 °C for 10 months; 3 = SFH treated with potassium ferricyanide; 4 = SFH stored at +4 °C for 2 months; 5 = SFH treated with glutaraldehyde; 6 = fresh crude hemolyzate through which carbon monoxide has been bubbled; 7 = fresh crude hemolyzate treated with potassium ferricyanide. Electrolyte: 0.04 mol/l glutamic acid (anode), 1 mol/l ethanolamine (cathode). Pharmalyte pH range 5–8. Samples 1–5 were stained with Coomassie blue; samples 6 and 7 are unstained.

better discernible after staining. There were no marked differences between the patterns of different batches of SFH prepared by the standard procedure, either fresh, stored 7 months at −10 °C or lyophilized with sucrose and stored 10 months at 20 °C. However, samples treated in a non-standard way during preparation or stored at $\geqslant$ 4 °C for longer periods showed a marked formation of partly oxidized forms (red-brown) and of methemoglobin (brown bands)[14]. SFH modified with glutaraldehyde formed only one broad diffuse zone of Hb proteins. Fig. 2 shows schematically the whole pattern of both hemoglobin and non-Hb proteins of standard SFH samples,

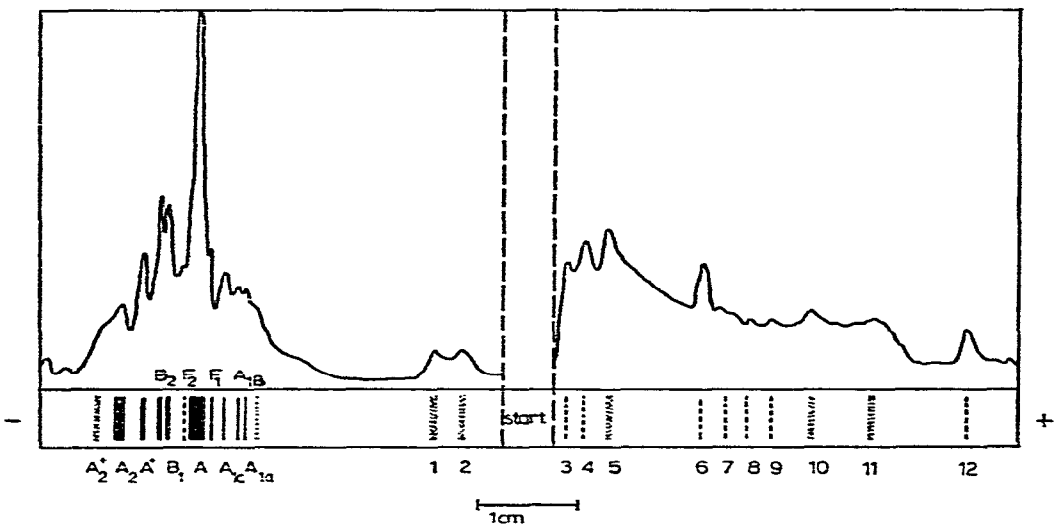

Fig. 2. Densitometric scan of SFH after isoelectric focusing in PAG thin layer. The lower part of the figure shows a schematic drawing of the electrofocused SFH with the identification of the Hb fractions (the ferric forms are indicated by +). Non-Hb proteins are indicated by arabic numerals (1 and 2 = carbonic anhydrase). The sensitivity of the scan to the right-hand (anodic) side from the start is 8.3 times higher than that to the left-hand (cathodic) side. Electrolyte: 0.04 mol/l glutamic acid (anode), 1 mol/l ethanolamine (cathode). Pharmalyte pH range 5–8. Stained with Coomassie blue.

together with the corresponding densitometric scan after staining. Besides up to fifteen Hb zones in the neutral or slightly alkaline region, twelve or thirteen zones of non-Hb proteins are detectable in the acid region.

According to Vitatron scans of several samples, the non-Hb proteins represent *ca.* 4–12% of the total protein present in SFH. The main zones of the non-Hb proteins (*ca.* 90%) correspond to carbonic anhydrase[9,17]. Crude fresh hemolyzates, both treated and untreated with CO, showed patterns generally similar to those of SFH. However, they contained significantly greater amounts of non-Hb proteins but smaller amounts of methemoglobin than standard SFH.

The above results show that electrofocusing on a thin layer of PAG using Pharmalyte (pH 5–8) is very suitable for the investigation and control of SFH samples. The zones are sharp and the protein patterns distinct and reproducible. A simultaneous run of about twenty-two comparative samples on one plate make it possible to quickly evaluate each pattern as a fingerprint and thus to recognize easily any marked deviations from the standard. The main Hb fractions were identified from data in the literature[14,18,19]. Although some of the minor unidentified zones might be artefacts, their position is reproducible and is part of the fingerprint of each SFH sample. Most typical changes of the protein pattern were connected with the different degrees of oxidation of the hemoglobins, the different contents of non-Hb proteins and with the chemical modification. The pattern of the modified SFH and its slight shift towards the acid pH region indicates that the Hb subfractions have lost the distinct net-molecular charges due to the reaction of basic groups with glutaraldehyde.

Our finding that hemoglobins lyophilized with sucrose (or glucose) and stored at 20 °C have identical patterns to those of the fresh samples corroborates earlier results achieved by other methods, and shows that lyophilization is a very suitable way of storing Hb[2,7,12].

The complex pattern of the non-Hb proteins shows that this group of proteins is more heterogeneous than found in earlier papers dealing with the analysis of SFH by other methods[9,10]. These proteins have their origin in the traces of contaminating blood plasma and in the cytoplasm of red blood cells, and perhaps also of leucocytes accompanying the packed erythrocytes. However, the identification of the individual components of non-Hb proteins was not the aim of this paper.

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