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Note

Characterization of polymerized stroma-free haemoglobin by chromatography, electrophoresis and electrofocusing in gels

T. I. PŘISTOUPIL*, M. KRAMLOVÁ, V. FRIČOVÁ and S. ULRYCH
Institute of Haematology and Blood Transfusion, Prague (Czechoslovakia)
and
J. KRAML and K. JOJKOVÁ
1st Department of Medical Chemistry, Charles University, Prague (Czechoslovakia)
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Polymerized stroma-free haemoglobins (SFHs) are currently being investigated in several laboratories as prospective oxygen transporting blood volume expanders. After infusion, the modified SFHs usually have a slower exclusion rate from the blood stream than native haemoglobin whose tetrameric structure (M_r 64,500) easily dissociates into chain-dimers which are rapidly excluded[1–7]. The analytical control of the quality of such products needs sensitive methods, because aqueous solutions of native haemoglobin contain several haemoglobin subfractions, each exhibiting mutual dissociation–association equilibria[8] dependent on the given conditions. The presence of non-haemoglobin proteins[9] as well as the spontaneous formation of methaemoglobins must also be considered. The addition of cross-linking reagents, *e.g.*, glutaraldehyde, inevitably causes considerable changes in the above heterogeneous system by forming inter- and intramolecular bonds involving all the protein molecules.

In the present work we tested various batches of SFH by means of gel chromatography on Sephadex G-100 in the presence of $MgCl_2$[10] (1 *M*), on Sepharose 6B, by means of electrophoresis in polyacrylamide gels with sodium dodecyl sulphate (SDS) and by means of isoelectric focusing in IEF agarose gels. Attention was focused upon the sensitivity of each method in detecting the presence and mutual proportions of cross-linked derivatives of haemoglobin. Model samples of glutaraldehyde-treated SFHs were selected, which have shown favourably lowered exclusion rates in biological experiments[7,11]. Their oxygen transporting abilities are not reported here.

MATERIALS AND METHODS

Human SFH was a standard sample produced in our laboratory[7]. The reaction of haemoglobin (50–90 g/l) with glutaraldehyde (Serva, Heidelberg, G.F.R.), diluted from a stock solution (250 g/l), took place in a phosphate buffer (0.05 *M*, pH 7.1) at 4°C for 1 h under vigorous stirring in a stream of nitrogen gas. The molar ratios of haemoglobin tetramer: glutaraldehyde were mostly 1:2.5 to 1:8. In a series of experiments the reaction was moderated by the addition of lysine[1]. Lysine was also used to inactivate the unreacted glutaraldehyde at the end of each experiment.

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Gel chromatography of the carbonyl haemoglobin derivatives was performed on columns (27 × 1.5 cm) of Sephadex G-100 Superfine gel in a Tris–HCl (0.0082 *M*) buffer, pH 7.0[10], with $MgCl_2$ (1 *M*) and of Sepharose 6B gel in an isotonic phosphate buffer, pH 7.4. Both eluting buffers were saturated with CO. Chromatography with the buffer containing $MgCl_2$ (1 *M*) was performed at 4°C in the dark to prevent denaturation and precipitation of the dissociated haemoglobin subunits[10]. The flow-rate was 4 ml/cm^2 · h. The elution curves were recorded automatically.

Disc electrophoresis was done in cylinders (10 × 0.6 cm) of polyacrylamide gel (50 g/l) alone or with added sodium dodecyl sulphate (1 g/l)[11,12]. The protein patterns were stained with Coomassie blue G-250. Purified samples of human serum albumin, gamma globulin and haemoglobin, horse myoglobin, bovine fibrinogen and ovalbumin served as standards for the construction of calibration curves for the estimation of relative molecular masses.

Isoelectric focusing was performed in a thin layer of agarose IEF gel and a Pharmalyte pH 5–8 gradient (Pharmacia, Uppsala, Sweden) under conditions described previously[13]. A Vitatron TLD 100 (Eefde, The Netherlands) was used to evaluate the stained patterns from electrophoresis and electrofocusing.

The pyridoxalated haemoglobin was the same sample as in our previous paper[13].

RESULTS AND DISCUSSION

Fig. 1 shows that gel chromatography of our samples on Sepharose 6B in the isotonic phosphate buffer gives important information on the gross distribution of the main protein fractions according to their relative molecular mass or size at isotonic salt concentration and physiological pH. However, in all samples treated with glutaraldehyde in the presence of lysine, the main peak corresponded to $M_r \approx 64{,}000$ and there was no difference between the peaks of modified and native haemoglobins. Only those samples modified in the absence of lysine showed a marked asymmetry of the peaks, indicating the presence of a series of haemoglobin polymers up to $M_r \approx 180{,}000$. At elevated glutaraldehyde concentrations (*e.g.*, molar ratio 1:10), marked

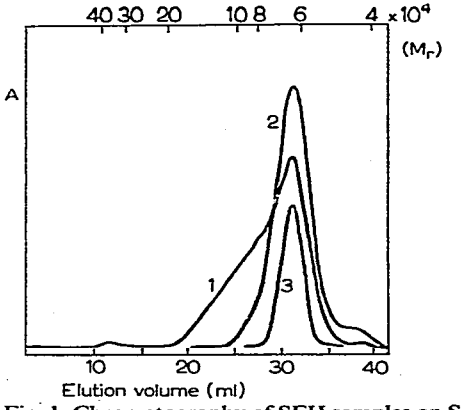

Fig. 1. Chromatography of SFH samples on Sepharose 6B. Curves: 1, SFH treated with glutaraldehyde (1 mole Hb_4 to 8 moles glutaraldehyde); 2, as 1, reaction moderated by lysine; 3, native SFH. Column: 27 × 1.5 cm. Elution rate: 4 ml/cm^2 · h. Eluent: isotonic phosphate buffer pH 7.4, saturated with CO.

amounts of fractions having M_r up to 400,000 were detected. However, those samples have no potential for use in infusions.

Fig. 2 demonstrates that gel chromatography of haemoglobin dissociated by $MgCl_2$ solution (1 mol/l) on Sephadex G-100 Superfine is a very sensitive method of discerning native and modified SFH samples. The main peaks corresponded to mean M_r 70,000 (I), 44,000 (II) and 24,000 (III). The V_e of peak III was similar to that of the subunits of native haemoglobin (evidently chain-monomers and -dimers). The V_e values of the three main peaks were mutually similar in various glutaraldehyde-treated SFH samples. There seems to be a close relationship between the presence of the high-molecular-weight fractions in peaks I and II and the slower exclusion rates from the blood stream of the corresponding modified SFH samples[11].

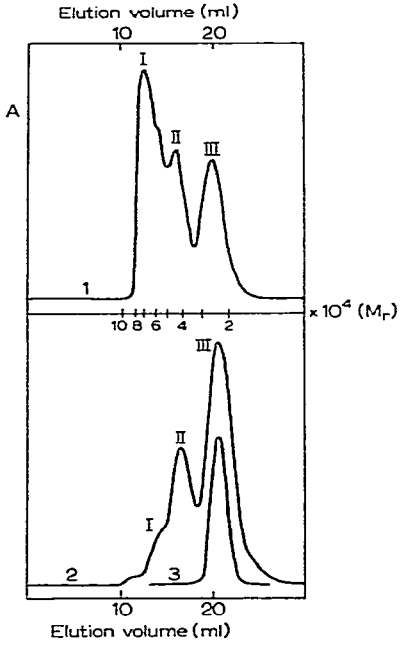

Fig. 2. Chromatography of SFH samples dissociated by $MgCl_2$ (1 *M*) on Sephadex G-100 Superfine. Eluent: $MgCl_2$ (1 *M*) in Tris–HCl buffer (0.0082 *M*) pH 7.0, saturated with CO; temperature 4°C. Other details as in Fig. 1.

Electrophoresis of modified SFH in polyacrylamide gel in the presence of SDS has shown that the differences between the patterns of the smallest dissociable fragments are quantitative rather than qualitative at M_r lower than. The marked difference between native and glutaraldehyde-treated SFH (Fig. 3) suggests the formation of covalent pentyl bridges. However, it is difficult to predict the biological effect (exclusion rate) of a given SFH because SDS splits both electrostatic and hydrophobic bonds and the resulting fragments are too small. Electrophoresis in polyacrylamide gel without SDS was not sensitive enough to reflect the differences between samples prepared under markedly different conditions.

Fig. 4 shows that isoelectric focusing was very sensitive to the differences be-

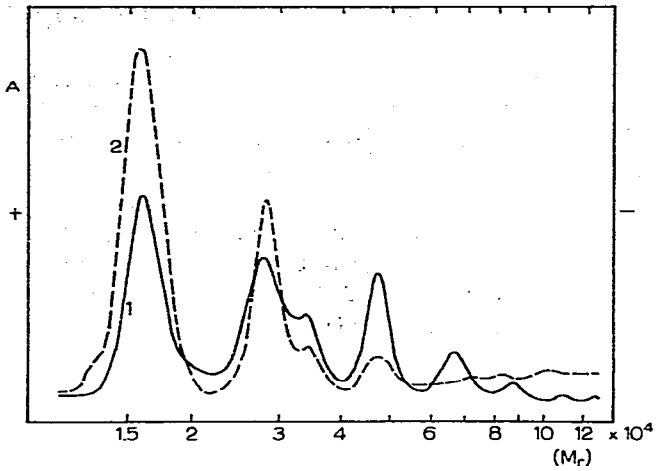

Fig. 3. Electrophoresis of SFH samples dissociated by SDS in polyacrylamide gel (50 g/l) columns. Curves: 1, SFH treated with glutaraldehyde as in Fig. 1 in the presence of lysine; 2, native SFH. Electrolyte: phosphate buffer (0.1 *M*), pH 7.2; SDS concentration 10 g/l. Stained with Coomassie blue G-250, scanned with Vitatron TLD 100.

tween native and modified haemoglobins. The distortion of the native pattern increased with elevation of the glutaraldehyde concentration is evidently due to the blocking of positively charged amino groups. The same explanation is valid for the slight shift of the diffuse patterns towards a more acidic pH region. The patterns of

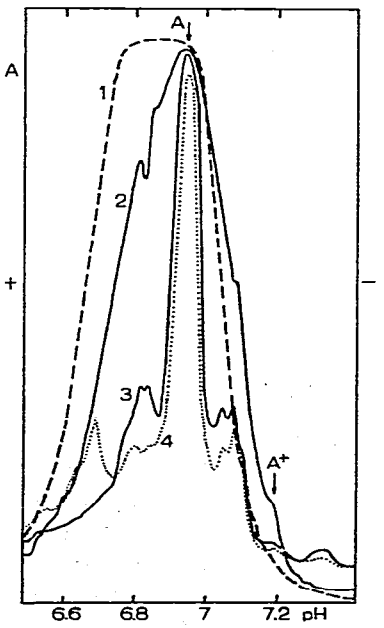

Fig. 4. Thin-layer electrofocusing of SFH samples in IEF agarose gel. Curves: 1, SFH treated with glutaraldehyde; 2, SFH as in 1, but in the presence of lysine; 3, native SFH; 4, pyridoxalated SFH. Pharmalyte pH range 8–5. Stained and scanned as in Fig. 3. The arrows indicate haemoglobin A and methaemoglobin A^+, respectively.

SFH modified in the presence of lysine still retain some features of the native patterns. The orientation experiment with pyridoxalated SFH showed a charcteristic peak in the region of pH 6.7. The results indicate the usefulness of IEF agarose gel as a carrier medium for electrofocusing in a thin layer.

The results of the above model experiments confirmed that each of the four techniques contributes in different ways to the characterization of modified SFH. Both gel chromatographic methods offer a prediction of the persistence of SFH in the blood stream, while gel electrophoresis with SDS and isoelectric focusing are more useful from the analytical point of view. This combination of methods is a useful way of checking the quality of polymerized and modified SFH, offering a high information output.

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