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Note

Comparative chromatography of fresh, freeze-dried and chemically modified human haemoglobin on the cation exchanger Mono S

T. I. PŘISTOUPIL*, M. KRAMLOVÁ and V. FRIČOVÁ
Institute of Haematology and Blood Transfusion, U nemocnice 1, CS-128 20 Prague (Czechoslovakia)
and
J. KRAML
1st Department of Medical Chemistry, Charles University, Prague (Czechoslovakia)
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Monodisperse microbeads of the Mono S™ cation exchanger have recently been introduced by Pharmacia as a new material for fast protein liquid chromatography (FPLC). In the present note we report results achieved with this technique during the characterization of stroma-free haemolyzates (SFHs) of human erythrocytes, of chemically modified haemoglobin variants and of reconstituted oxyhaemoglobin which had been freeze-dried with sucrose and then stored dry for 3 years. An hypothetical mechanism for oxyhaemoglobin stabilization by sucrose against methaemoglobin formation is discussed.

MATERIALS AND METHODS

The preparation of SFH, its freeze-drying with sucrose and its modification with pyridoxal-5-phosphate and glutaraldehyde have been described[1,2]. The dry oxyhaemoglobin sample was batch number 01-190483. Chromatography was performed on the Pharmacia FPLC system using a prepacked Mono S HR 5/5 column in 0.01 *M* malonic acid, pH 5.7 (eluent A). Samples of 50 μl were applied and eluted with 0.01 *M* malonic acid containing 0.3 *M* lithium chloride, pH 5.7 (eluent B)[3]. The gradient was 0–100% B in 17 min. The flow-rate was 2 ml/min.

RESULTS AND DISCUSSION

Typical elution patterns of haemoglobin samples (Fig. 1) show marked peaks due to the main haemoglobin subfractions in native samples and less distinctly separated peaks due to the polydisperse moiety of pyridoxalated and polycondensed samples. Use of the cation exchanger Mono S and the FPLC technique in combination[3] led to standard and reproducible results within a short time.

The rather diffuse elution curves of modified haemoglobins reflect the high polydispersity of these preparations due to the different electrokinetic charges of the present protein molecules. The polydispersity remained practically the same in fresh samples as well as in redissolved freeze-dried ones, stabilized with sucrose as usual[2].

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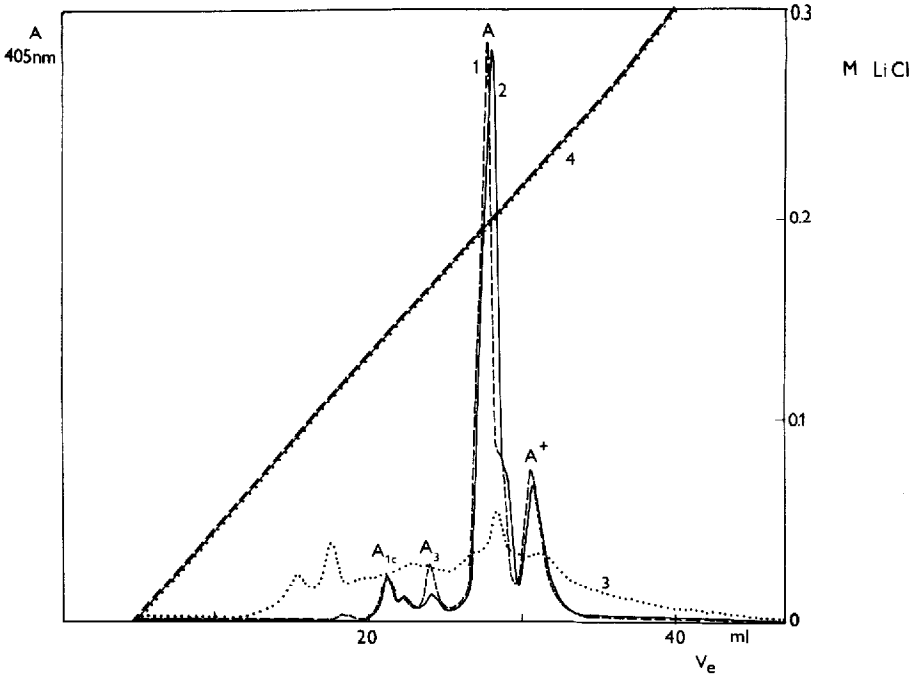

Fig. 1. Chromatography of haemoglobin samples on Mono S HR 5/5. 1 = Fresh native oxyhaemoglobin; 2 = native oxyhaemoglobin freeze-dried with sucrose and reconstituted in water after 3 years of storage; 3 = polycondensed pyridoxalated haemoglobin; 4 = gradient of lithium chloride; A = absorbance; V_e = elution volume. Symbols "A" indicate the peaks due to the main haemoglobin subfractions.

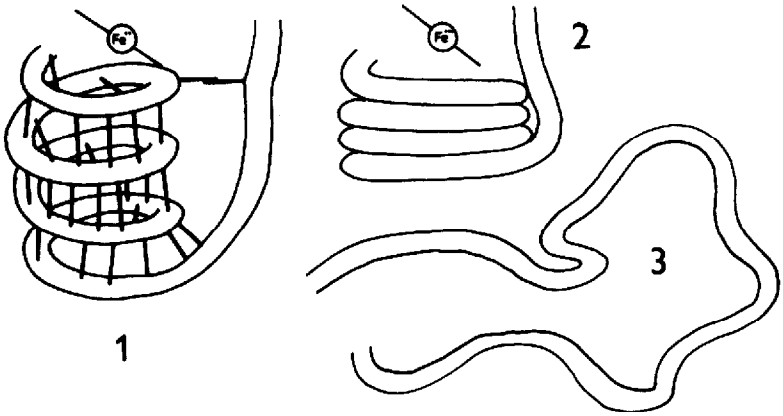

Fig. 2. Scheme of the assumed mechanism of oxyhaemoglobin stabilization during freeze-drying with sucrose. 1 = Native architecture of a part of oxyhaemoglobin supported by a dry rigid sucrose net after sublimation of the bulk of the water molecules[2]; during rehydration the native conformation and ferro form remain unchanged. 2 = Collapsed conformation and oxidation to the ferri form during freeze-drying without sucrose. 3 = Irregular "denatured" form after rehydration of the unstabilized freeze-dried haemoglobin.

The present results correspond well with those derived from earlier experiments by isoelectric focusing, chromatofocusing and gel permeation techniques where a high polydispersity of modified haemoglobins was also found[1,4,5].

The close mutual similarity between the elution peaks of fresh and reconstituted freeze-dried native oxyhaemoglobin after long storage of the latter again demonstrates that sucrose exhibits a very marked stabilizing effect upon the native molecular structure of oxyhaemoglobin during the freeze-drying and redissolution processes. We assume that (*cf.*, ref. 2) the basic principle of stabilization is predominantly mechanical at the molecular level. A very schematic representation of this idea is shown in Fig. 2. Sucrose, and in shorter periods of storage also glucose and certain salts of amino acids, are the most active stabilizers among many other substances tested. This seems to be due to the fact that the spacial features and sizes of the stabilizing molecules fit better those sites of the oxyhaemoglobin molecules which are crucial for the formation of a rigid, dry supporting network[2]. Other effects, *e.g.*, scavenging of superoxide radicals, stabilization by the thin layer of rightly bound water molecules remaining in the lyophilizate, are probably also involved[2,6], however, we assume that they play a secondary rôle.

In conclusion, FPLC on Mono S HR 5/5 columns proved a most useful and reliable method for the characterization of haemoglobin samples investigated as potential infusable oxygen carriers.

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