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Note

Analytical isoelectric focusing of native and modified haemoglobins after treatment with 4-hydroxymercuribenzoate

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Analytical isoelectric focusing (IEF) has been very useful for the characterization of stroma-free haemoglobin solutions (SFHs) investigated as oxygen-transporting cell-free blood substitutes[1–3]. Some of the most interesting pyridoxalated and glutaraldehyde-treated SFH variants represent a highly polydisperse system of modified haemoglobin molecules. However, the p*I* values of the individual subfractions are very similar mainly between about 6.6–7.2, so that the IEF patterns are very diffuse and provide little information to enable an effective analytical monitoring of SFH batches. For this purpose a more discrete fractionation of modified SFH or of its dissociated subunits would be of great assistance.

The sodium salt of 4-hydroxymercuribenzoic acid (4-HMB) has been widely used for determinations of SH groups[4] and for dissociation of haemoglobin tetramers into their subunits[5]. An erroneous designation of 4-HMB as *p*-chloromercuribenzoate (PCMB) was generally used in earlier literature.

The present paper describes our experiments on the fractionation of haemoglobin subunits after treatment of SFH with 4-HMB. The possible application of 4-HMB-treated dry human oxyhaemoglobin as a coloured multicomponent p*I* marker for the range p*I* 5–8 was also tested.

EXPERIMENTAL

Native human and bovine haemolyzates of washed erythrocytes were prepared and freed from stroma by centrifugation and membrane filtration as is usual[6]. Samples were stored dry below 0°C after lyophilization with sucrose[1,7]. The modification of haemoglobins with pyridoxal-5-phosphate, glutaraldehyde, borohydride and in some experiments also with serum albumin (PHIR-PGA) was performed according to refs. 8–10. The reaction with 4-HMB was carried out according to ref. 5. An IEF flat-bed apparatus Multiphor (LKB, Bromma, Sweden) equipped with 0.5-mm thin layers of IEF agarose and Pharmalyte 5–8 (Pharmacia, Uppsala, Sweden)[11] was used. To avoid the formation of air bubbles during filling the 0.5-mm wide space with hot agarose, the following procedure was adopted: the glass plates were positioned horizontally from the beginning, instead of at an angle of 30° as is usually recommended; the warm agarose solution was poured slowly from a beaker into one corner of the glass plates, instead of using a syringe to apply the solution at the centre. IEF patterns were stained with Coomassie Blue 250.

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RESULTS AND DISCUSSION

Fig. 1 shows the marked differences between the IEF patterns of native human SFH, its modified variant PHIR-PGA[8] and of both 4-HMB-treated samples. During destaining in diluted acetic acid plus ethanol[11], two zones at p*I* about 6.5 and 6.8 showed a yellowish fluorescence when observed under direct sunshine against a dark background. A third fluorescent zone at p*I* 5.8 appeared after treatment of PHIR-PGA with 4-HMB. The very diffuse pattern of undissociated PHIR-PGA changed after treatment with 4-HMB into a series of 15–20 relatively distinct zones along a gradient of pH 5.5–7.7. Nevertheless, the diffuse background of the IEF pattern indicates a certain fine polydispersity even after dissociation of modified haemoglobin. Some subunits derived from PHIR-PGA showed lower p*I* values than those prepared from native haemoglobin. This is evidently due to the modification of basic, mostly amino, groups of the haemoglobin molecules by glutaraldehyde and pyridoxal-5-phosphate and to the presence of small amounts of albumin.

Glutaraldehyde and pyridoxal-5-phosphate are known to react also with the reactive SH group of β-93 cysteine during modification of SFH. This reaction, as

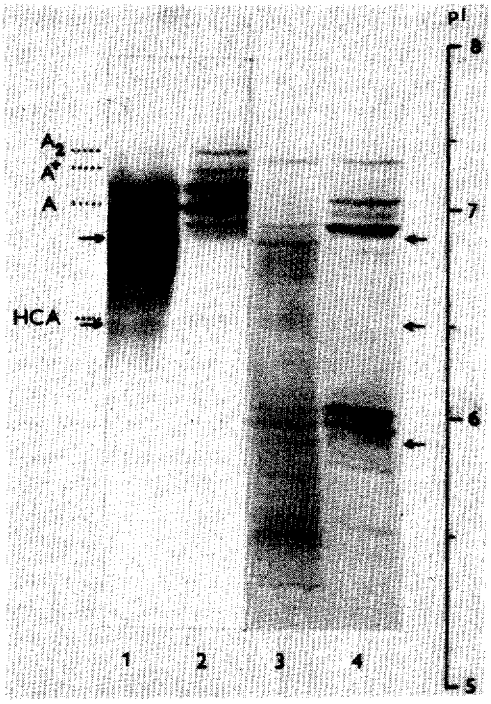

Fig. 1. Isoelectric focusing of human haemoglobin samples and their subunits after treatment with 4-hydroxymercuribenzoate. Samples: 1 = human stroma-free haemoglobin (SFH) modified with pyridoxal-5-phosphate, glutaraldehyde, borohydride and serum albumin[6]; 2 = native human SFH; 3 = sample 1 treated with 4-hydroxymercuribenzoate (4-HMB); 4 = sample 2 treated with 4-HMB. A, A^+, A_2 = Positions of typical haemoglobin subfractions; HCA = human carbonanhydrase. The arrows indicate fluorescent zones. A 0.5-mm thin layer of IEF agarose, Pharmalyte 5–8, was stained with Coomassie Blue 250.

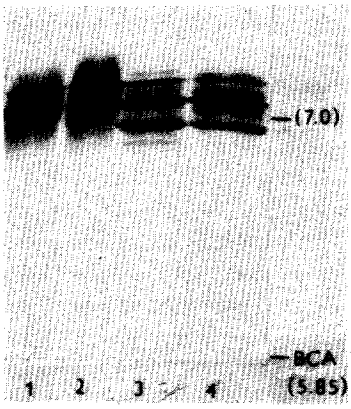

Fig. 2. Isoelectric focusing of bovine haemoglobin samples: 1 = bovine haemoglobin modified with pyridoxal-5-phosphate and glutaraldehyde followed by treatment with 4-HMB; 2 = sample 1 before 4-HMB treatment; 3 = bovine haemoglobin treated with 4-HMB; 4 = native bovine haemoglobin. BCA = Bovine carbonanhydrase. IEF conditions as in Fig. 1.

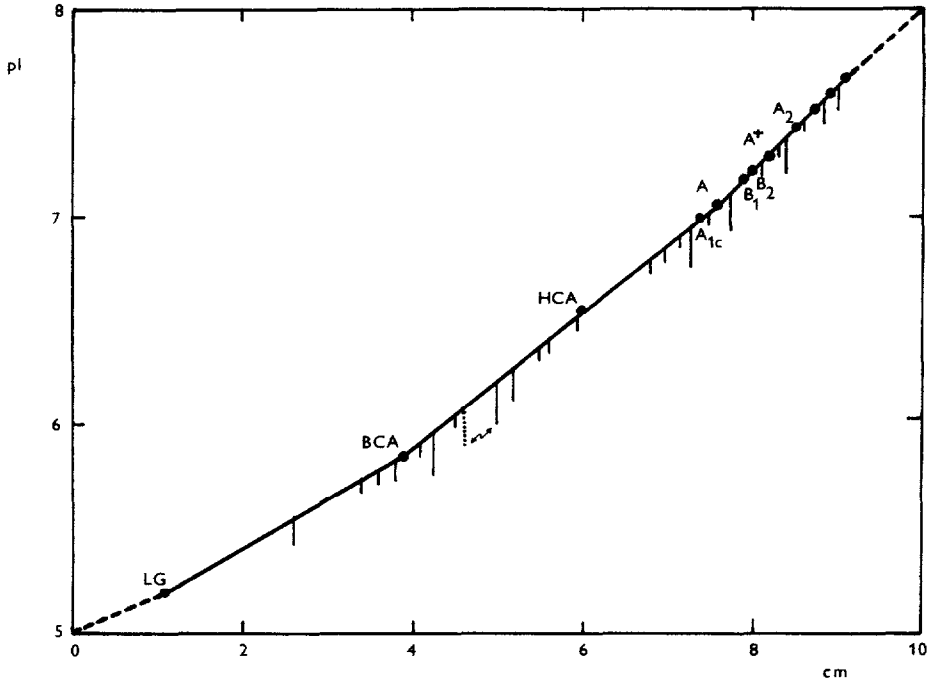

Fig. 3. Calibration curve for estimation of p*I* values from isoelectric focusing patterns. Full circles: LG = β-lactoglobulin; BCA = bovine carbonanhydrase; HCA = human carbonanhydrase; A_{1c}, A, B_1, B_2, A^+, A_2 and three unidentified dots are characteristic human haemoglobin subfractions. Perpendicular lines indicate the positions of zones of 4-HMB-treated native human haemoglobin. The length of the lines is roughly proportional to the intensity of the zones. For details see text.

well as that with amino groups, is not complete under the given conditions, since about 10–20% of the haemoglobin molecules in PHIR-PGA still behave as native ones. The unreacted part of β-93 cysteine and the less reactive α-104 cysteine and β-112 cysteine[12] react after longer treatment with 4-HMB. This leads to the dissociation of haemoglobin (Hb) subfractions, *e.g.*, HbA, HbA_2, etc., into their corresponding subunits and modified subunits, respectively. These subunits can be fractionated into more distinct zones by IEF. A given pattern may then serve as a characteristic fingerprint for a given variant of modified human SFH and as an indicator of the reproducibility of batches. A detailed identification of the separated subunits was not the aim of the present study.

In contrast to human SFH variants, only small changes were observed in IEF patterns of bovine SFH after treatment with 4-HMB (Fig. 2). This is due to the fact that SH groups are absent in the α-chains of bovine haemoglobin and only one (β-92 cysteine) is present in each β-chain[12]. No differences were found between 4-HMB-treated and untreated samples of bovine SFH modified with glutaraldehyde and pyridoxal-5-phosphate.

Fig. 3 shows a typical pH gradient along the IEF pattern based on the relative positions of zones of native proteins with known p*I* values. The positions of human haemoglobin subunits after treatment of SFH with 4-HMB are indicated by perpendicular lines. This multicomponent system usually contains 20–24 distinct subfractions which can serve as p*I* markers covering the range p*I* 5.5–7.7. This colour kit is relatively easy to prepare from a stock of dry oxyhaemoglobin[1,7] whenever needed. The positions of some zones around p*I* 6 are sensitive to the amount of sample applied. This is possibly due to some interaction of the subfraction with the components of the ampholyte. Further investigation of this effect is in progress. A combination of human haemoglobins and of their dissociation products after treatment with 4-HMB seems to be useful means for calibration of IEF in the range p*I* 5–8.

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