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ELECTROPHORETIC AND CHROMATOGRAPHIC TECHNIQUES FOR THE DIFFERENTIAL DIAGNOSIS OF A HAEMOGLOBIN ABNORMALITY: Hb E HETEROZYGOSITY

ADRIANA BIANCHI BOSISIO
S. Carlo Borromeo Hospital, Clinical Chemistry Laboratory, Milan (Italy)
JACQUES ROCHETTE and HENRI WAJCMAN
Institut de Pathologie et Biologie Cellulaires et Moleculaires, INSERM U15, Paris (France)
and
ELISABETTA GIANAZZA and PIER GIORGIO RIGHETTI*
Chair of Biochemistry, Faculty of Pharmaceutical Sciences, University of Milan, Milan (Italy)
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SUMMARY

A method is described for separating haemoglobin (Hb) E (β26 Gly→Lys) from Hb A_2 (a normal minor Hb component in adult blood). The technique allows the distinction between subjects carrying β-thalassaemia trait and patients who are simultaneously α-thalassaemic and heterozygous for Hb E, the standard electrophoretic pattern often being similar in these two circumstances. Complete separation between Hb E and Hb A_2 (2 mm empty space in between) is obtained by isoelectric focusing in immobilized pH gradients in an ultra-narrow pH 7.55–7.65 gradient. The apparent pI values of the two species (at 10°C and at an average ionic strength of 5.6 mequiv. l^{-1}) have been calculated to be 7.603 for Hb E and 7.607 for Hb A_2. Thus, the system reported here affords a resolution of at least 0.004 pH unit.

INTRODUCTION

Hb E, whose molecular structure was first identified in 1961 (β26 Gly→Lys)[1]. is one of the most prevalent Hb variants in man; its occurrence is virtually confined to South East Asia, where it has been estimated to affect about 30 million people[2]. In this part of the world other haemoglobinopathies, mostly α- and β-thalassaemia, are also very frequent[3].

Hb E is, in addition, characterized by a decreased level of biosynthesis, giving rise to a β^+-thalassaemia. The mutation, localized near to the splice junction, leads to abnormal mRNA processing[4]. Individuals homozygous for Hb E present a constant anaemia and the phenotype of a mild homozygous thalassaemia. Heterozygous individuals (Hb E trait) have smaller amounts (*ca.* 35%) of Hb E than Hb A. In addition, when some α-thalassaemia coexists, in relation with preferential subunit association of α-chains with normal β-chains[5], the amount of Hb E may be as low

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as 10% or even less[3]. The electrophoretic and haematological pattern is then very similar to that observed in the case of β-thalassaemia trait in which Hb A_2 is increased. An accurate diagnosis between these two haemoglobinopathies can be achieved either by family studies or by structural studies; an alternative strategy would be an electrophoretic test discriminating Hb E from Hb A_2.

Standard tests currently employed include starch gel electrophoresis in Tris–EDTA–borate (pH 8.6), coupled to citrate agar gel electrophoresis at pH 6.25[6–8]; in the former, Hb E comigrates with Hb A_2, O and C; in the latter, it is separated from Hb C and O, but comigrates with Hb A, A_2, D and G. Hence neither technique is able to separate Hb E from Hb A_2 (in fact, it is the presence of this abnormal protein in the Hb A_2 zone that leads to a suspect presence of an Hb mutant). Not even conventional isoelectric focusing (IEF) in the standard, commercially available pH 6–8 range can achieve such a separation[9]. Therefore, for a proper assessment of these conditions, one has to resort to lengthy chromatographic techniques (DEAE-cellulose separation of native tetramers) followed by preparation of β-chains, trypsin digestion and finally identification of the two abnormal β T3 peptides, usually by high-performance liquid chromatography (HPLC)[10]. Such techniques are not easily amenable to routine clinical analysis.

In this paper, we report for the first time a complete separation of Hb E and Hb A_2, achieved by ultra-narrow (0.1 pH unit entire pH span) immobilized pH gradients (IPG)[11]. This investigation was prompted by the hospitalization of a 38-year-old female from Sri Lanka at the S. Carlo Borromeo Hospital; she and her 5-year-old daughter were found to be heterozygous for Hb E.

EXPERIMENTAL

Acrylamide, N,N′-methylenebisacrylamide (Bis) and N,N,N′,N′-tetramethylethylenediamine (TEMED) were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.), Ampholines of pH 6–8 and 7–9, Immobilines of pK 3.6 and 7.0 and Repel Silane from LKB (Bromma, Sweden) and Gel Bond PAG film from Marine Colloids (Rockland, ME, U.S.A.). Red blood cells (RBCs) were washed three or four times in saline (10 ml/ml blood), then lysed in distilled water containing 0.05% of potassium cyanide (5 volumes of lysis solution per volume of packed RBCs). After lysis, 0.5 volume of carbon tetrachloride per volume of packed RBCs was added to precipitate the ghosts. After centrifugation for 10 min at 10 000 *g*, the sample was diluted to about a 1% content, equilibrated with CO and analysed as such by IEF or by IPG. Standard, purified Hb E was a gift from Dr. T. H. J. Huisman (Augusta, GA, U.S.A.) and Hb C from Prof. M. Perrella (Milan, Italy).

Conventional IEF and IPG

IEF analysis was performed in 5%T, 4%C gels, containing 2% of carrier ampholytes (CA) in the pH range 6–9 (a 1:1 blend of Ampholines of pH 6–8 and pH 7–9) and 25 mM histidine (His) for flattening the pH gradient in the region of Hb A_2 and Hb E[12,13]. Focusing was terminated after 3000 Vh^{-1} in an LKB Multiphor II chamber, equipped with an LKB Multitemp thermostat and an LKB Macrodrive (5000 V) constant-power supply.

IPG gels were 0.5 mm thick, with a total acrylamide–Bis concentration of

3.5%T. During the pouring of the solutions into the cassette and the polymerization step, the pH gradient was stabilized by a co-linear density gradient of glycerol (0–16%). The calculations for the narrow (pH 7.4–7.9) and ultra-narrow (pH 7.55–7.65) gradients were performed by linear interpolation of the narrow (pH 7.1–8.1) gradient tabulated in LKB Application Note No. 324 (1984). The amounts of Immobilines used in the two chambers for the pH 7.4–7.9 interval were (for an 8-ml final volume): in the acidic, dense solution, 330 μl of pK 3.6 and 1140 μl of pK 7.0 Immobilines; in the basic, light solution, 170 μl of pK 3.6 and 1610 μl of pK 7.0 Immobilines. For the pH 7.55–7.65 interval the corresponding amounts were: 285 μl of pK 3.6 and 1280 μl of pK 7.0 Immobilines (acidic extreme) and 252 μl of pK 3.6 added with 1375 μl of pK 7.0 Immobilines (basic end) (see ref. 11 for methodological details on casting and manipulating of IPG gels prior to the run).

HPLC analysis

Globin chains from normal and mutant Hbs were prepared by the acid–acetone method and the chains were isolated by CM-cellulose chromatography in 8 M urea. The abnormal β-chain was digested with trypsin and the resulting peptides were separated by reversed-phase HPLC[14]. The HPLC analysis was performed with a Chromatem 800 apparatus (Touzard et Matignon, Ivry, France) using an Aquapore RP-300 column (Brownlee Labs.). A 1-mg amount of tryptic digest was applied to the column and a non-linear gradient of acetonitrile in 0.01 M ammonium acetate (pH 6.0) buffer was developed with a flow-rate of 1.5 ml/min. The column eluate was

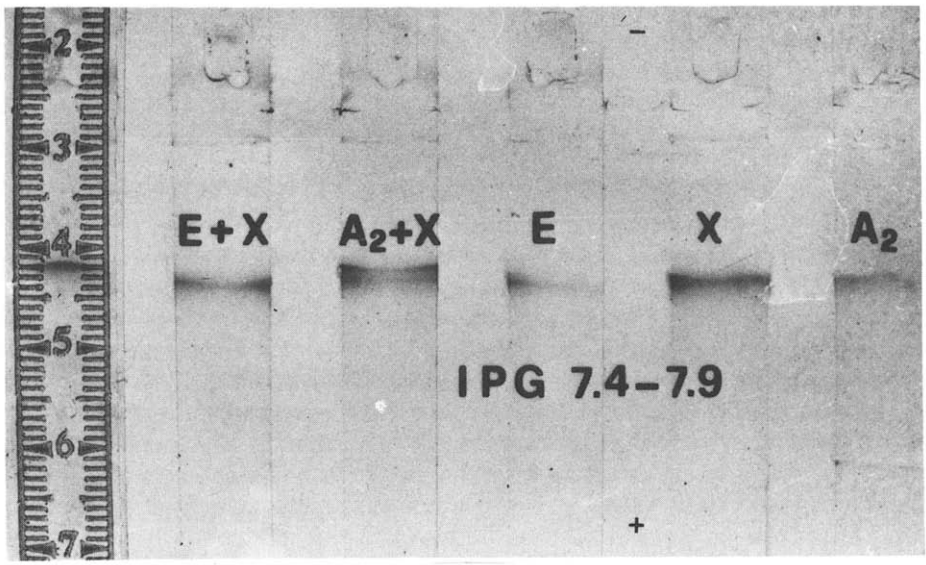

Fig. 1. Separation of Hb E and Hb A_2 in immobilized pH gradients. IPG gel: 3.5%T, 4%C, pH 7.4–7.9. Run: 2500 V, 0.5 mA, 10 h at 10°C. Stain: Coomassie Brilliant Blue R-250. The gel slab (0.5 mm thick) was divided into separate tracks by excising 4 mm wide trenches between adjacent sample lanes. Sample load: 300 μg of each Hb species per track; cathodic sample application in slots, Hb E and Hb A_2 were chromatographically pure samples; Hb X was a lysate from the two prepositi. Electrolytes: 20 mM Glu at the anode and 20 mM Lys at the cathode.

monitored by a flow cell at 220 nm and fractions were collected every 0.7 min. After HCl hydrolysis (standard azeotrope, for 24 h at 110°C), the amino acid composition of the abnormal peptide was determined on a Rank-Hilger J180 analyser.

RESULTS

Fig. 1 shows the separation of Hb A_2, standard Hb E and unknown Hb X from the two prepositi in an IPG of 0.5 pH unit width (pH 7.4–7.9). It can be seen that, whereas Hb E and Hb X, when run in a mixture, focus at the same p*I* in a single zone, a mixture of Hb A_2 and Hb X is split into two bands, Hb X being slightly more anodal than Hb A_2. However, the separation distance, *ca.* 0.5 mm, is not enough for a proper densitometric evaluation of the two bands. Therefore, an ultra-narrow IPG range was run, encompassing barely 0.1 pH unit (pH 7.55–7.65). An artificial 1:1

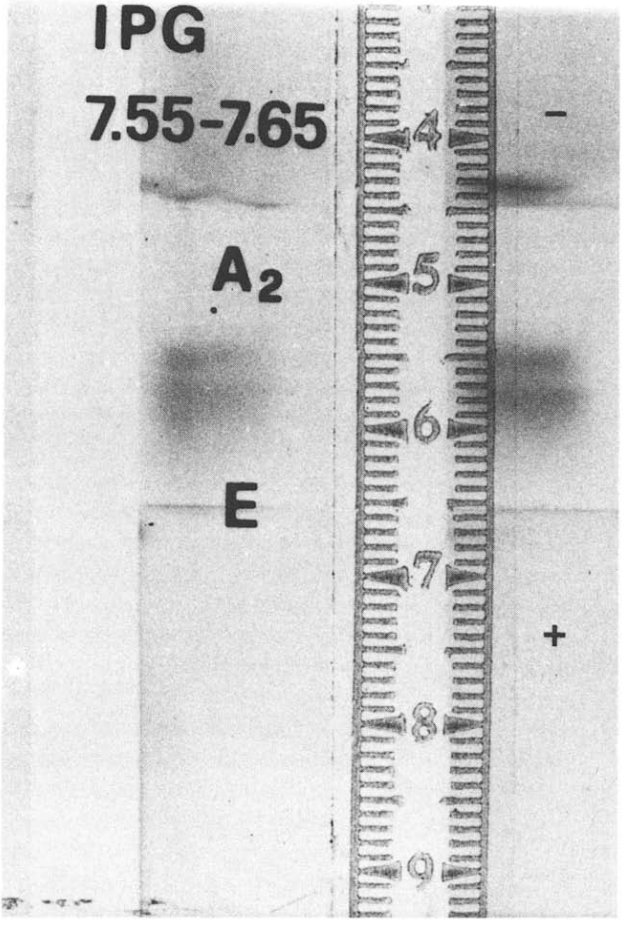

Fig. 2. Separation of Hb E from Hb A_2 in immobilized pH gradients. IPG gel as above, except that the pH interval was 7.55–7.65. Sample: 1:1 mixture of chromatographically pure Hb E and Hb A_2. Run: 2500 V, 0.3 mA, 20 h at 10°C. All other conditions as in Fig. 1.

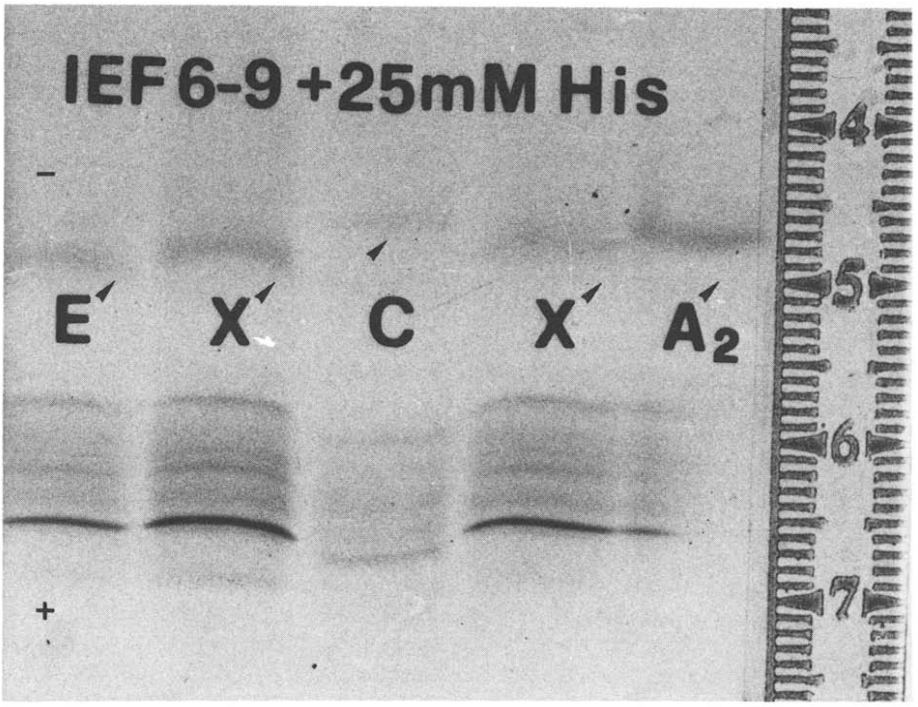

Fig. 3. Separation of Hb E, Hb C, Hb A_2 and Hb X by conventional isoelectric focusing, IEF gel: 4%T, 4%C, 1% Ampholine of pH 6–8, 1% Ampholine of pH 7–9, with 25 m*M* His added. Run: 3000 V h^{-1} at 4°C (V_{max} 1500 V). Stain: Coomassie Brilliant Blue R-250. Sample load: 300 μg Hb per track; cathodic application. Electrolytes: 1 *M* phosphoric acid at the anode and 0.5 *M* sodium hydroxide at the cathode.

mixture of chromatographically pure Hb A_2 and Hb E was well separated (2 mm empty space between the two bands), thus allowing for unambiguous quantitation of the two zones by densitometric analysis (Fig. 2). By linear interpolation, the p*I* values of the two zones were calculated to be 7.603 for Hb E and 7.607 for Hb A_2 (at 10°C and at a theoretical ionic strength of 6.6 mequiv. l^{-1}, or 5.6 mequiv. l^{-1} when corrected for 85% incorporation efficiency of the Immobiline chemicals)[15]. Thus, the resolution of this system is at least of the order of 0.004 pH unit. For comparison, a pH 6–9, conventional Ampholine gradient was run, with 25 m*M* His added as a spacer. This molecule, with a p*I* of *ca.* 7.6, should flatten the pH gradient in the region where Hb A_2 and Hb E are isoelectric, and presumably completely separate these species. However, as shown in Fig. 3, the system is ineffective and barely any separation is obtained between Hb E and A_2. In fact, only Hb C (which has the highest p*I* among the three Hbs) is resolved from Hb A_2.

For a complete identification of Hb X, structural analysis was performed. Pure Hb X was obtained by preparative IPG, as described elsewhere[16]. The purified βX chains were digested with trypsin and analysed by reversed-phase HPLC. As shown in Fig. 4, two abnormal βT3 peaks were present (the position of the normal, missing T3 peak run in parallel is shown by the arrow), whose amino acid sequence revealed

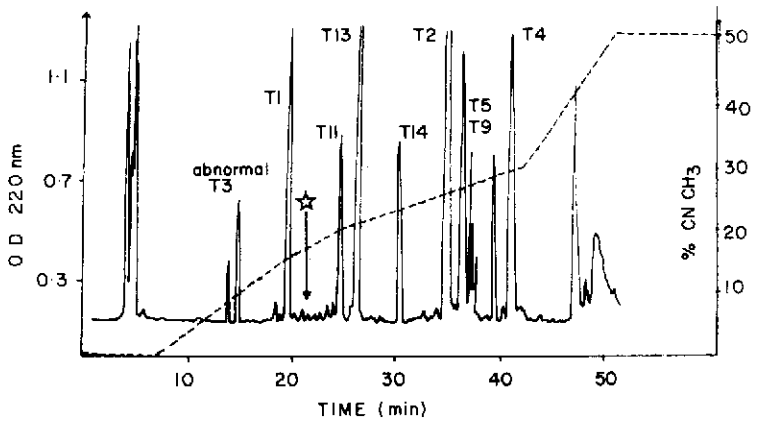

Fig. 4. Reversed-phase HPLC elution pattern of the tryptic digest of the β-chain of Hb X; 1 mg of a tryptic digest of purified βX chain was loaded on an Aquapore RP-300 column and eluted with a non-linear gradient of acetonitrile in 10 m*M* ammonium acetate buffer (pH 6.0) (broken line). Two abnormal T3 peptides are eluted before peptide T1: by amino acid analysis, they were shown to correspond to β 18–26 (Lys) and β 27–30. The missing peak in a tryptic digest of a normal β-chain in indicated by the arrow.

the mutation β26 Gly→Lys, characterizing Hb E. Thus, the identity of the Hb X from the two prepositi with standard Hb E was proved.

DISCUSSION

General considerations on the use of spacers

With CA-IEF, Hb E is barely resolved from Hb C but comigrates with Hb A_2 in a regular 2 pH unit gradient[17]. The use of His as a spacer does not allow the complete separation of Hb E from Hb A_2; in addition, the protein bands are diffuse and the degree of resolution may vary, depending on the experimental conditions.

The limitation to the effectiveness of amphoteric spacers for the resolution of protein species with minute p*I* differences is in fact connected with the (relatively) low voltage gradients allowed in CA-IEF before the onset of gradient instability (cathodic drift) and of local overheating. Unless the field strength (V/cm, over a given pH interval) is adequately increased, however, shallow gradient slopes merely increase the spacing between band centers while leaving unmodified the pH region over which the protein is allowed to diffuse before the electric field is able to drive it back towards its equilibrium p*I* position.

Among the reasons calling for especially accurate standardization of the experimental conditions when using spacer IEF are the following: (i) the p*K* values of the (usually) primary amino groups of the spacers depend on temperature to a different extent than the (mostly secondary and tertiary) amino groups in CAs; (ii) owing to the shallow slope of the gradient and the low voltage gradient, the approach to the equilibrium (p*I*) position is slow, so the experiment is often terminated when sufficient separation has been achieved, but proteins, and possibly the high-concentration spacers, are still in the transient state; (iii) for the reason above and because, owing to the lack of suitable compounds, spacers are generally found among molecules with p*I* values close to, but rarely intermediate between, those of the two sample

components whose resolution is to be enhanced, the time required to reach the equilibrium position, and the overall resolution and band width, are markedly different whether the p*I* is approached from the one or the other side of the pH plateau (*i.e.*, from the anode or from the cathode).

General considerations on the use of IPGs

With IPGs the limitations typical of spacer IEF either are not encountered or can be easily circumvented. Any pH slope down to 0.01 pH unit/cm may be obtained in any pH region between 3.5 and 10. The negligible conductivity contributed by the immobilized ionic charges in IPGs allows voltage gradients even as high as 1000 V/cm to be applied without appreciable Joule heating. The grafted pH gradient is indefinitely stable to the passasge of the current and no drift occurs, so the run can be prolonged until true equilibrium is reached and p*I* values, or at least p*I* differences, may be evaluated for the proteins under analysis. The linear course of the gradient throughout the width of the separation medium leaves the velocity of the approach to the p*I* position by the protein dependent only on the shape of its titration curve.

In this work, Hb E may be resolved from Hb A_2 with a gradient slope of 0.05 pH unit/cm at a field strength of 250 V/cm. The separation is, of course, increased at 0.01 pH unit/cm, but in this instance, to sharpen the bands, higher field strengths should be applied, either during the whole duration of the run or, more conveniently if only power supplies limited to 2–2500 V are available, by moving the electrode wires across the Hb bands *ca.* 2 cm apart during the last hour or so of the run. On decreasing the gradient slope to its lower limit, *i.e.*, 0.01 pH unit/cm, the p*I* difference between Hb A_2 and Hb E can be evaluated as not more than 0.004 pH unit.

In the past, we have published extensive tables on protein p*I*s as determined by conventional IEF[18,19]. Values to the second decimal digit were reported, although we feel that barely the first decimal might have any significance, owing to uncertainties in the ionic strength of the focusing medium, to temperature differences during the IEF run (in general 4°C) and the pH measurements (20–25°C) and to the non-linearity of the pH gradient. With IPGs, considerably greater precision of p*I* measurements can be obtained because (a) both the p*K* values of Immobilines are measured and the IPG run is performed at 10°C, (b) the ionic strength is accurately known and reproducible from run to run and (c) the pH gradient is linear along the separation track (deviation from linearity less than 1% of the pH span). For these reasons, we are able to give p*I* values to the third decimal digit. Although we agree that there might be some uncertainty in the third, the second decimal digit should be taken as accurate; in fact, variations at this level, given such a shallow pH gradient, should move the protein zone by a few centimeters along the separation track. Hence we might be witnessing the dawning of a new era, in which p*I* values might be used to characterize proteins with the same physico-chemical significance as a molecular mass determination.

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