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REVIEW

AN UPDATE ON ELECTROPHORETIC AND CHROMATOGRAPHIC METHODS IN THE DIAGNOSIS OF HEMOGLOBINOPATHIES

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1. INTRODUCTION

The main hemoglobin component in the normal adult is hemoglobin A (Hb-A). This tetrameric protein, made up of two pairs of identical subunits ($\alpha_2{}^A\beta_2{}^A$), is one of the most studied macromolecules.

The interest that has been shown in hemoglobin may be explained by the importance of its physiological function and by the many variants of Hb-A that have been found in man. Since the discovery of Hb-S $\alpha_2{}^A\beta_2{}^6$ (Glu → Val) by Pauling et al. [1] in 1949, three hundred other variants have been described. The study of these mutants has provided explanations of the origin of some inherited diseases, including hemolytic anemias, polycythemias and congenital cyanosis. The hemoglobin variants constitute a valuable tool for the genetic study of any given population. They have also been used to verify and to perfect the atomic model of the hemoglobin molecule [2] established by Perutz and co-workers [3, 4]. As a result of these variants, studies are now in progress to identify the nature of the intermolecular contacts which form during the polymerization of deoxy Hb-S. Such studies are very promising and could eventually lead to medical treatment of sickle-cell anemia.

Despite recent improvement in their management, there is still no specific treatment for abnormal hemoglobins or for thalassemias. Generally, the best thing to do is to diagnose serious hemoglobinopathies as soon as possible to assure a careful follow-up of children, in order to diminish or prevent complications which result from homozygous states of these inherited diseases. Another solution now available is to make a prenatal diagnosis and to interrupt the pregnancy when a homozygous fetus for Hb-S or a β-thalassemia syndrome is detected.

In addition to Hb-A and its variants, minor components may also be present

in man. Thus, it is especially important to measure three of these minor components, since changes in their levels can play a role in the diagnosis of some diseases. For example, the Hb-A_2 ($\alpha_2{}^A\delta_2{}^A$) level is about twice as high in the blood of carriers of the β-thalassemia trait as in the blood of non-carriers [5] and can be modified in other disorders [6, 7]. Hb-F ($\alpha_2{}^A\gamma_2{}^F$), which is the major component in fetal blood and at birth, is normally present at trace levels in the adult but may be elevated in genetically determined or acquired disorders, including thalassemia, hereditary persistence of Hb-F, leukemias, etc. [8]. The third minor component of importance in pathology is Hb-A_{1c}. Hb-A_{1c} is a glycosylated Hb-A [9] whose level is elevated in diabetes mellitus [10]. Precise quantitation of Hb-A_{1c} is of great interest since it may provide the best means of assessing accurately the degree of hyperglycemia on a long-term basis [11—13].

Chromatographic and electrophoretic methods are generally required for the detection, identification and assay of normal and abnormal hemoglobins. Chromatographic procedures are more efficient than electrophoretic ones but are often time-consuming and scarcely compatible with the requirements of clinical biochemistry. However, the recent introduction of powerful electrophoretic techniques (such as isoelectric focusing) and of easier chromatographic techniques (such as microchromatography) may greatly modify the study of hemoglobins in clinical biochemistry.

The first section of this review deals primarily with methods used in separating hemoglobins, while the second presents the main diagnostic problems met within the field of the hemoglobinopathies.

2. METHODS FOR SEPARATION OF HEMOGLOBINS

This section is divided into three parts: electrophoretic methods, isoelectric focusing and chromatographic methods. After each technical description, we will discuss the main advantages or disadvantages of each method. The third section (chromatographic methods) is mainly limited to those procedures that are used in clinical biochemistry. For detailed information on other methods, the reader is referred to the recent work of Huisman and Jonxis [14].

2.1. Electrophoresis

2.1.1. Electrophoresis under non-denaturing conditions

Since the use of paper electrophoresis has been discontinued, cellulose-acetate electrophoresis performed with an alkaline Tris—EDTA—borate buffer (Fig. 1a) has become the most commonly used technique.

The time required for optimal separation is between 20 min and 2 h according to the grade of cellulose-acetate strips. This technique is convenient because ready-to-use strips are produced by several manufacturers. They are easily stained (Ponceau S or Amido Black) and destained and can be scanned when transparent. An alternative procedure for quantitating hemoglobin fractions consists of cutting out the bands of interest and either eluting the hemoglobins in buffer, or dissolving the cellulose-acetate strip in 80% acetic acid. This latter system has been found useful in determining Hb-A_2 levels [15]. Nevertheless,

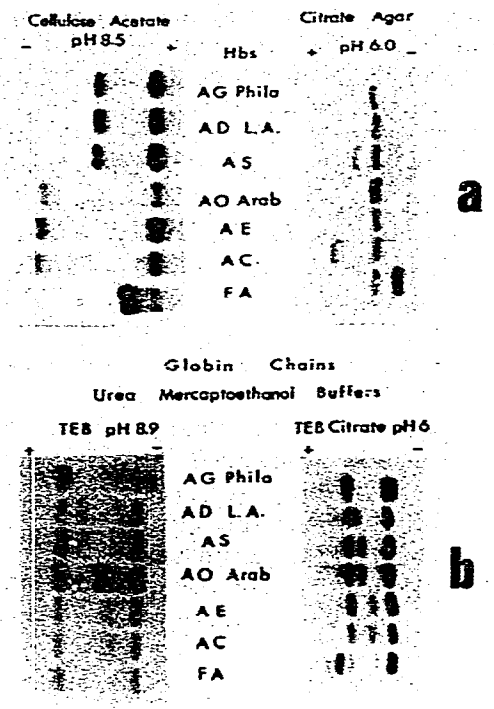

Fig. 1. Electrophoretic patterns of hemolysates containing hemoglobins A, S, G Philadelphia, D Los Angeles, O Arab, E, C and F. (a) Electrophoresis performed on cellulose acetate at pH 8.5 (left) and on citrate agar at pH 6.0 (right). (b) Globin-chain electrophoresis performed on cellulose acetate in urea—mercaptoethanol buffers at pH 8.9 (left) and pH 6.0 (right). (By permission, see ref. 28.)

cellulose-acetate electrophoresis has been largely replaced in many laboratories by the microcolumn technique for the evaluation of Hb-A_2 level (see below).

The main limitation of cellulose-acetate electrophoresis is its low resolving power. For instance, many hemoglobin variants can not be separated from Hb-S. It is therefore necessary to use additional methods such as the solubility test [16], electrophoresis on agar gel, and electrophoresis of globin chains (see below). Furthermore, several abnormal hemoglobins may give a normal pattern on cellulose-acetate electrophoresis. Such a situation is clearly open to criticism since hemoglobinopathies may escape detection in such cases. Nevertheless, the simplicity of this method and its possible application to large-scale screening accounts for its high popularity.

In addition to cellulose-acetate electrophoresis, other techniques are used for specific purposes. Thus, the application of the starch-gel technique to studies of hemoglobins is particularly useful for the resolution of the minor fractions [17] such as free α-chains, Hb Bart's (γ^4) or Hb-H (β^4), which are often difficult to detect by cellulose-acetate electrophoresis. Phosphate buffer systems (pH 6.5—7.0) are necessary for the resolution of the very low amounts of Hb Bart's and Hb-H which are more clearly separated from hemoglobins A and F in this pH range than at alkaline pH (ref. 18, p. 313). Hemoglobin electrophoresis at

alkaline pH under non-denaturing conditions can also be performed on polyacrylamide gels [19], agarose gels [20] and starch blocks [5]; the starch block remains useful for preparative purposes on a relatively small scale.

Another useful system is citrate-agar electrophoresis, which was introduced by Robinson et al. in 1957 [21]. Modifications of the initial procedure have been presented more recently [22, 23]. This method distinguishes hemoglobins S and C from some "S-like" or "C-like" electrophoretic variants (Fig. 1a). Hb-F is clearly resolved from Hb-A, which may be useful in neonatal diagnosis. Unfortunately, many other variants are confused with Hb-A. Moreover, the separation seems to be due in part to adsorption of the hemoglobin on to impurities in the agar [22], which probably explains the variations in electrophoretic patterns from one electrophoretic run to another. Before the introduction of isoelectric focusing, this method, despite its rather complex procedures, was an invaluable tool in the diagnosis of Hb-S.

2.1.2. Electrophoresis of globin chains

2.1.2.1. Cellulose-acetate electrophoresis in urea buffers. This technique was first presented by Cortesi et al. in 1966 [24], and was later modified by Beuzard et al. [25] and by Schneider [26]. It consists of suppressing the tetrameric structure of hemoglobin with urea and mercaptoethanol and then performing electrophoresis of globin chains with buffers at different pH values containing 6 *M* urea and mercaptoethanol. Electrophoresis of globin chains can be successfully carried out at acid and alkaline pH. With this technique it is possible to locate the mutated chain in an abnormal hemoglobin, to distinguish between different variants and occasionally to detect electrophoretically silent variants [27]. It has been used by Schneider et al. [28] on a large scale. The method, however, is unsuitable for screening abnormal hemoglobins, but must be used for the characterization of variants after their detection by, for instance, cellulose-acetate electrophoresis at alkaline pH (Fig. 1b). Using this method, Schneider et al. [28] have been able to differentiate many mutant hemoglobins electrophoretically similar to Hb-S or Hb-C at alkaline pH under non-denaturing conditions. The method is time-consuming, however, since it requires several analyses of each sample. A second limitation arises from the difficulty met in interpreting the electrophoretic patterns in some cases. Electrophoresis of globin chains is nevertheless a very useful method for identifying abnormal hemoglobins, particularly when thin-layer isoelectric focusing is not available (see below) and should also prove to be applicable to the study of in vitro globin chain synthesis [29—31].

2.1.2.2. Chain electrophoresis in the presence of detergent. Recently chain electrophoresis has been performed in acid urea polyacrylamide gels containing Triton X-100 [32]. Non-ionic detergents are known to bind the hydrophobic parts of the polypeptide chains and are thus able to alter electrophoretic mobility as a function of the nature of the apolar groups present on the chain. This method has been successfully applied by Alter et al. [33] to the separation of the two γ-globin chains, γ-Gly and γ-Ala. Such a separation is illustrated in Fig. 2. The two γ-globin chains can be also separated by isoelectric focusing [34] and CM-cellulose chromatography [35] in the presence of Nonidet P-40 or by high-performance liquid chromatography [36, 37].

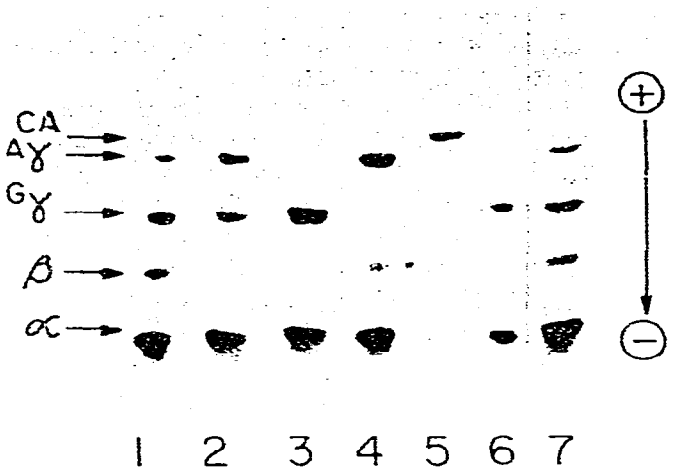

Fig. 3. Electrophoresis of globin chains on Triton-polyacrylamide slab gels; 10 μg were applied to each well. The gel was stained with Coomassie blue. 1, Newborn; 2, HPFH homozygote; 3, G_γ-$\delta^0\beta^0$-thalassemia homozygote; 4, A_γ Hb-F, isolated from A_γ—HPFH heterozygote; 5, carbonic anhydrase; 6, Hb-A_2; 7, Newborn. (With permission, ref. 33.)

2.2. *Isoelectric focusing*

2.2.1. *Background*

The first study of hemoglobins by isoelectric focusing (IEF) was reported by Drysdale et al. [38]. These investigators used polyacrylamide-gel tubes which were inadequate for large-scale screening and not convenient for comparison of the isoelectric positions of several samples. The introduction of IEF in thin-layer gels [39, 40] and the use of high voltage [41] provided the conditions required for the development of a technique applicable to routine analyses.

2.2.2. *Characteristics of an analytical routine technique*

Jeppson's method of thin-layer isoelectric focusing (TLIF) has been adapted to the needs of routine screening [42, 43] by the use of very thin gels. These gels allow the use of higher voltages, thus shortening the duration of the transient state, and at the same time improving resolving power. Reducing the volume of carrier ampholytes makes the cost per sample comparable to that of cellulose-acetate electrophoresis. It is also important to have a uniform pH gradient in the region at which the hemoglobins focus, i.e. between pH 6.5 and pH 7.5 for human hemoglobins. When using Ampholine (LKB, Bromma, Sweden), such a uniform pH gradient can be obtained by mixing equal quantities of Ampholine of pH range 6—8 and Ampholine of pH range 7—9 with a final concentration of 4% [43]. More recently, LKB has commercialized Ampholine PAG plates with narrow pH gradients, including one with a pH range 5.5—8.5 which can be used in the study of human hemoglobins.

Other commercial carrier ampholytes are now available, Servalytes (Serva, Heidelberg, G.F.R.), Bio-Lytes (Bio-Rad Laboratories, Richmond, CA, U.S.A.), Pharmalytes (Pharmacia, Uppsala, Sweden). The equipment necessary to perform TLIF is relatively simple, and requires a cooling system and a constant-

power supply. A cold bath is also convenient but may be replaced, at least for analytical purposes, by tap water. With most commercially available cooling blocks, 48 samples can be analyzed on the same gel. Some cooling blocks (Desaga, Heidelberg, G.F.R.) permit the use of two gels side by side; in this case 96 or more samples can be analyzed at once. The hemolysates are made quickly by using whole blood. The samples are applied to the gel with Whatman paper or application strips, in preference to direct application. A convenient system is to impregnate the papers using a multiple syringe (Desaga) which allows the hemolysates to be transferred twelve at a time [44]. The steady-state is reached in about 1 h. Staining the gel is unnecessary and is replaced by a brief fixation in 12% (w/v) trichloroacetic acid. If the migration has been performed on a gel polymerized on a glass plate, the gel can be stored after drying on a Whatman paper sheet. If the gel was polymerized on a treated Mylar* support, drying can be done directly on the support and the gel can be scanned more easily.

2.2.3. Isoelectric focusing applications

As described above, TLIF appears a convenient method for the screening of abnormal hemoglobins. Fig. 3 represents the result of screening 48 samples on a thin-layer plate, and shows that a clear separation may be obtained between Hb-A, Hb-F and Hb-A_{1c}. Moreover, the same method that is used for screening can be used to identify hemoglobin variants, or for studying minor fractions [43].

It is also suitable in cases in which abnormal fractions are present in small amounts; for example, in cord blood in which hemoglobins A, S and C may be present at low concentration (see below). No other system presently available presents such a possibility.

Purification of hemoglobin variants by IEF is less developed. However, when a clear separation between a variant and Hb-A can not be obtained by either chromatography or by starch-block electrophoresis, preparative IEF is very useful in obtaining relatively small quantities of hemoglobin rapidly (Fig. 4). In this case, the method described by Radola [45], involving the use of layers of granulated gels, is the most convenient method currently available. The main limitation of preparative IEF seems to be the low sample-loading capacity.

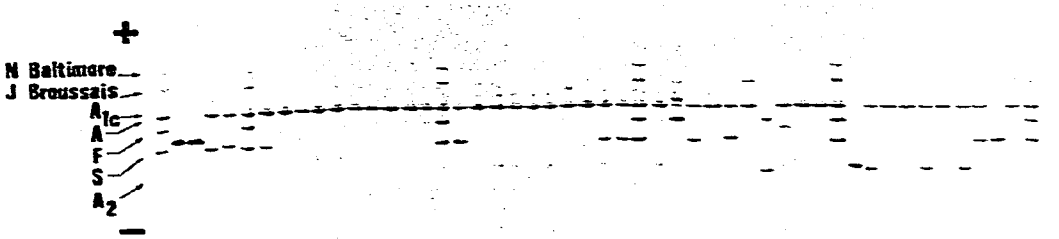

Fig. 3. Mass screening of hemoglobins by isoelectric focusing on polyacrylamide-gel slabs in a pH 6—9 gradient. Dimensions of the gel = 265 mm × 125 mm, 0.5 mm thick; anolite = 1 *M* H_3PO_4; catholyte = 1 *M* NaOH; 44 samples + 4 controls.

*LKB and Gelbond film, marine (Colloids Division, FMC Corporation, Rockland, ME, U.S.A.).

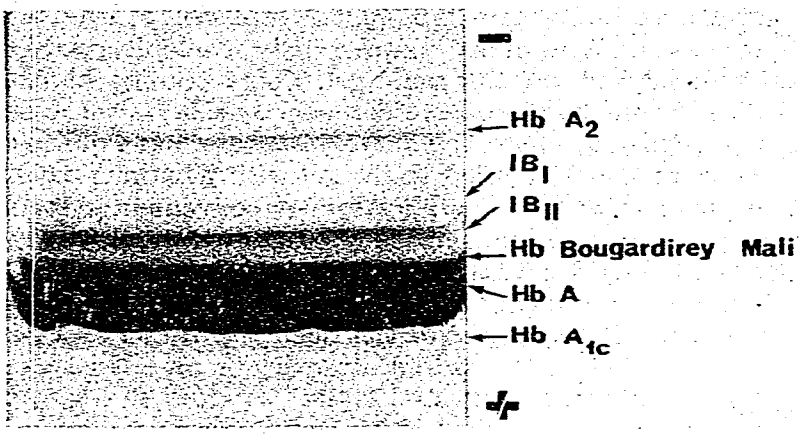

Fig. 4. Preparative isoelectric focusing on Ultrodex (LKB) slab, in a pH gradient range 6—9, of Hb Bougardirey Mali β119 (GH2) (Gly → Val), an "A-like" acetate-electrophoresis hemoglobin variant.

Continuous-flow IEF could be a valuable tool in isolating hemoglobins on a large scale [46].

2.2.4. Resolving power

Rilbe [47] has evaluated the resolving power of IEF in gels as greater than 0.01 pH unit. Allen et al. [48], using the 24 cm long electrode of the LKB Multiphor apparatus, have obtained a resolving power of 0.0025 pH unit. A comparison between the results obtained by TLIF and by a classical electrophoretic system is presented in Fig. 5. This picture compares the migration

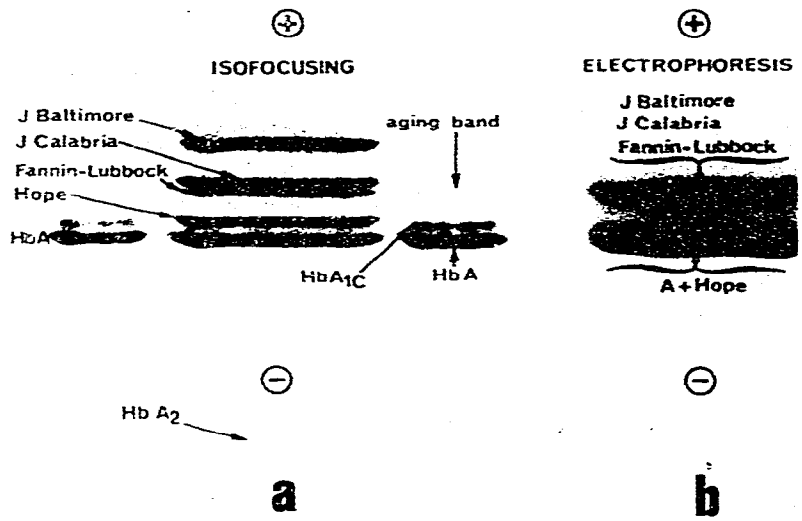

Fig. 5. Migration patterns of a mixture of four variants of Hb-A having the same type of amino-acid substitution: (a) by isoelectric focusing in a pH gradient range 6—9; (b) by electrophoresis on cellulose-acetate strips at alkaline pH.

patterns of four variants of Hb-A having the same type of amino acid substitution, (i.e. glycine replaced by aspartic acid), on TLIF and on cellulose-acetate electrophoresis at alkaline pH. The four variants (Hb-J Baltimore, Hb-J Calabria, Hb Fannin-Lubock and Hb Hope) were mixed together with Hb-A. Electrofocusing distinguishes the five expected bands, only two of which are seen upon electrophoresis.

2.3. Chromatography

Huisman and Jonxis [14] distinguish three types of chromatographic procedure for hemoglobin studies, on the basis of their purpose: (1) microchromatography, for simultaneous identification of hemoglobin variants in a number of samples and for quantitation of certain hemoglobins in a large number of samples; (2) macrochromatography, for quantitation of the various hemoglobin components; and (3) preparative chromatography for isolation of a specific hemoglobin variant for possible structural analysis.

All these chromatographic procedures involve ion-exchange chromatography. In this section we will discuss microchromatographic methods in some detail, these responding most satisfactorily to the demands of clinical biochemistry. Microchromatographic procedures do not call for special equipment, require only small Pasteur pipettes and can be performed at room temperature in 30—200 min. The two other types of chromatography are more briefly treated, and for more detailed information the reader should refer to the recent edition of Huisman and Jonxis's book [14] and Schroeder and Huisman's book [49].

2.3.1. Quantitative determination of Hb-A_2 by microchromatographic procedures

The main application of microchromatography is to quantitate Hb-A_2. The first adaptation of this technique to screening was presented by Efremov et al. [50]. The method is applicable to hemolysates, whole blood or blood collected on filter paper. The anion exchanger is DEAE-cellulose (DE 52; Pharmacia, Uppsala, Sweden) and the developers are Tris—HCl buffer at pH 8.5. Hb-A_2 may be eluted by decreasing the pH to 8.30 in about 1 h. The remaining hemoglobins, Hb-A_0 and Hb-A_{1c}, are then eluted with a second developer (pH 7.00) and the absorbance of the two fractions, collected separately, is measured at 415 nm. Reproducibility of results is good, but depends on the pH and particularly that of the ion exchanger. This method has been improved by Huisman et al. [51] who replaced the Tris—HCl buffer by a glycine—KCN buffer. This improvement allows the quantification of Hb-A_2 without interference from any Hb-S in the sample, and makes the method less sensitive to minor change in the pH of the developer. More recently a further modification of the microchromatographic procedure, involving the addition of sodium chloride to the glycine—KCN developer, has made the method even less sensitive to change in pH of the ion exchanger [52]. Microcolumns for determining the level of Hb-A_2 are now commercially available (Whatman, Helena Labs., Isolab). Recently, Huisman's technique has been adapted to test-tubes [53]. The method uses the same ion exchanger and the same glycine—KCN buffer but no columns. Hemoglobins A and F are bound to the resin while Hb-A_2 remains in the supernatant fraction, allowing its quantitation. More recently Abraham et al. [54] have proposed two

rapid chromatographic procedures for the determination of Hb-A_2 in the presence of Hb-C.

The results obtained by microchromatography in quantitating Hb-A_2 are consistent with the aims of clinical biochemistry. Some 50 samples can be analyzed in a day by one technician using simple equipment. The values obtained easily permit distinction of normal and pathological levels (Fig. 6). The test-tube method seems to give similarly satisfactory results [55].

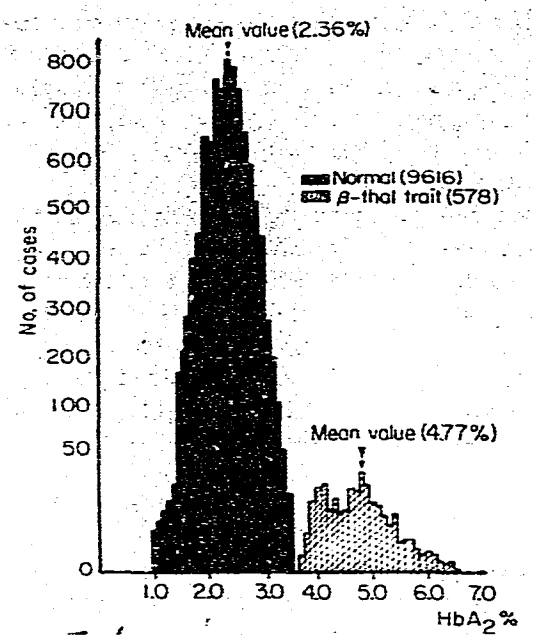

Fig. 6. The level of Hb-A_2 in normal adults and in β-thalassemia heterozygotes determined by microcolumn chromatography. (By permission, see ref. 50.)

2.3.2. Other applications of microchromatographic procedures

Microchromatographic procedures have also been used to analyse hemoglobin in cord blood. Detection of the various forms of Hb-S and Hb-C at birth is, as already stated, of considerable importance. On cellulose-acetate strips or starch-gel electrophoresis, such a diagnosis can not be made because Hb-A, present at low levels at this time, can not be separated from the 10—15% of acetylated Hb-F. This situation makes it impossible to distinguish between homozygotes and heterozygotes, and requires a second identification by agar electrophoresis.

Schroeder et al. [56] have developed a microchromatographic method for the study of cord blood and especially for the distinction of AS, AC, SS, SC and CC conditions at birth. The original procedure used columns of CM-Sephadex, but superior results have since been obtained with CM-Cellulose [57]. These authors have been able to analyse more than 10,000 samples. The method is very sensitive since a 2-μl sample is sufficient for detection of Hb-A and

Hb-S at birth. However, this technique seems time-consuming if compared to TLIF which offers the same resolving power and comparable sensitivity (see below). By a modification of the CM-cellulose technique, Henson et al. [58] have used microchromatography for the quantitation of the Hb Bart's and its application in the detection of α-thalassemia at birth. By another modification, Schroeder et al. [59] have developed a microchromatographic procedure for quantitating Hb-F. This method can be used only in cases in which Hb-F is present with Hb-S and/or Hb-C, but in which Hb-A is absent. A faster and more reliable system has been proposed by Abraham et al. [60]. On the other hand, microchromatographic procedures which allow the quantitative determination of Hb-F in the presence of Hb-A are also available [61, 62]; they require more time than alkali-denaturation procedures but can be performed with whole blood.

Finally, the microchromatographic procedures can also be used for detection of hemoglobin variants [54] and for the quantitation of glycosylated hemoglobins (see below).

2.3.3. Chromatographic determination of glycosylated hemoglobins

The glycosylated hemoglobins (Hb-A_{1a1}, Hb-A_{1a2}, Hb-A_{1b} and Hb-A_{1c}) are normal minor fractions deriving from Hb-A. They are particularly interesting because their concentrations are elevated in patients with diabetes mellitus. However, their use in clinical biochemistry has been considerably limited up to now by the lack of a simple rapid assay [63].

All the chromatographic procedures now used to determine glycosylated hemoglobin levels are derived from the method described by Allen et al. [64] on Amberlite IRC 50, and modified later by Trivelli et al. [65]. These original techniques were rather too time-consuming for clinical purposes and many attempts have been made to reduce the duration of the chromatography.

2.3.3.1. Automatic techniques. Automatic techniques can be performed at different pressures and they permit separate quantitation of Hb-A_{1c} and Hb-$A_{1(a+b)}$. High-performance liquid chromatography [66—69] has a performance compatible with the aim of routine measurement of glycosylated hemoglobins but requires rather expensive equipment and still seems too sophisticated for non-specialized laboratories. Medium-pressure liquid chromatography [70] is also limited by cost factors in that it requires an expensive amino-acid analyser. Moreover, this technique does not produce a satisfactory separation of the Hb-A_{1c} and Hb-$A_{1(a+b)}$ peaks. Low-pressure liquid chromatography [71] (Fig. 7a and b) uses simpler equipment and is cheaper to perform than the previous systems. Hb-A_{1c} and Hb-$A_{1(a+b)}$ are clearly resolved and it is easy to analyse twenty or more samples a day.

2.3.3.2. Commercial kits. Microchromatographic methods developed by several authors [72—76] have been adapted to commercial kits (Isolab, Helena Labs., Bio-Rad Labs.). The microchromatographic procedures may quantitate all the glycosylated hemoglobins together. This could be an important limitation since among the glycosylated hemoglobins only one, Hb-A_{1c}, is well-characterized [9, 77] and clearly related to the blood glucose level in patients with diabetes mellitus [65]. Hb-A_{1a1} and Hb-A_{1a2} levels have been found recently to be normal in diabetic patients [78]. However, the quantitative determina-

tion of all forms of Hb-A_1 has been used as a valuable index of long-term blood glucose levels in diabetic patients [79].

The main limitation of the commercial microcolumns is their great temperature-dependence which prevents any reproducible results. This major disadvantage was not found with the Bio-Rad kit which has a satisfactory temperature independence between 20°C and 25°C [80], and which allows the quantitative determination of glycosylated hemoglobins on a large scale.

2.3.3.3. Problems with chromatographic methods. In all chromatographic methods used to assay glycosylated hemoglobins, Hb-F is eluted with Hb-A_{1c}. Thus, when the percentage of Hb-A_{1c} is abnormally elevated, it is important to check the percentage of Hb-F in order to rule out possible contamination of Hb-A_{1c}. A comparable situation has been recently found with Hb-H [81]. Hb Hope [82], which is frequent in black people, is also eluted as a constituent of the Hb-A_{1c} peak. Other hemoglobin variants do not directly interfere with the glycosylated hemoglobin peak, but indirect interference can arise (Fig. 7c and

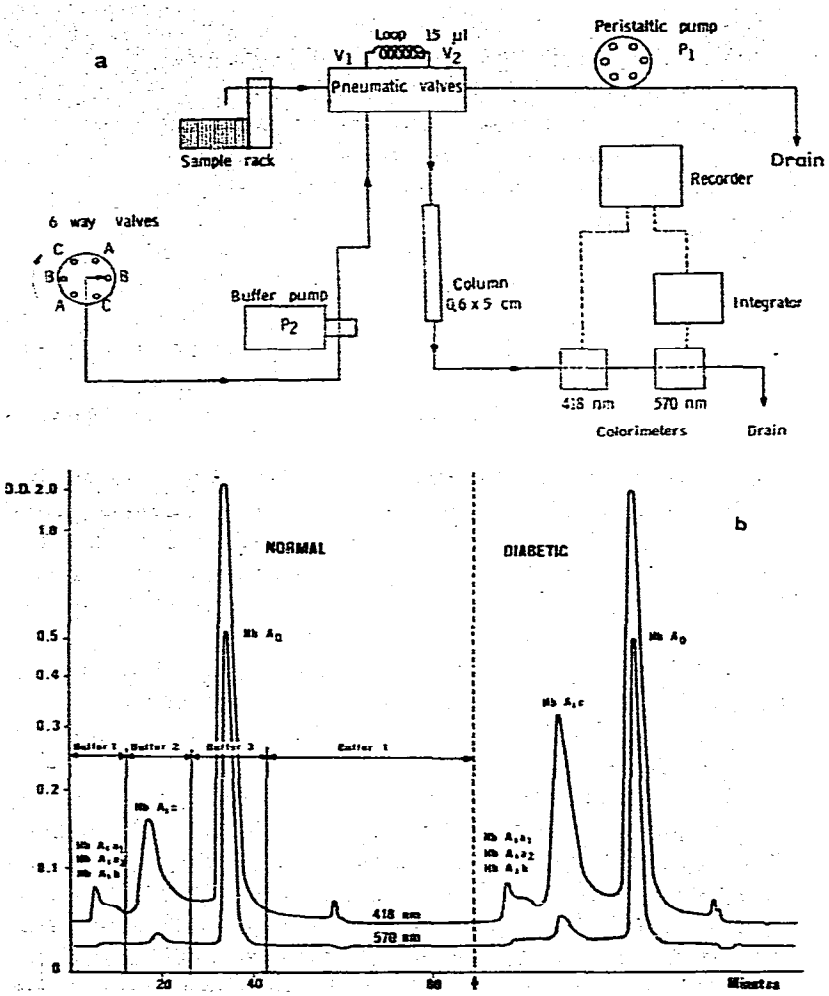

Fig. 7.

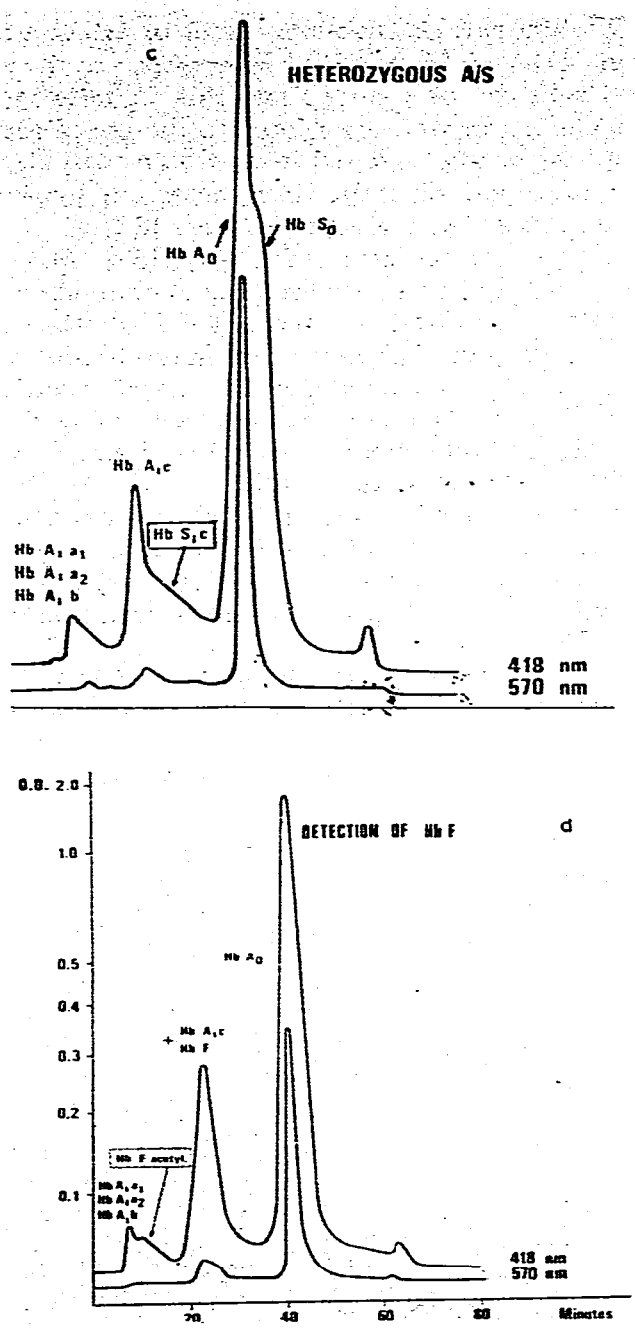

Fig. 7. (a) The overall arrangement of a low-pressure automatic chromatographic system for determination of glycosylated hemoglobins. (b) Typical chromatographic separations of glycosylated hemoglobins. Left: typical profile of a normal hemolysate. Right: profile of a diabetic patient. (c) Typical elution pattern obtained from the hemolysate of patients heterozygous for Hb-S. (d) Chromatographic profile typically seen in hemolysates of patients exhibiting an increased level of Hb-F. In this sample, Hb-F determined by Betke's technique, corresponds to 5% of the total. (By permission, see ref. 71.)

d), since the most frequent hemoglobinopathies involving Hb-S or Hb-C are often associated with an elevated level of Hb-F [83]. Moreover, in these patients the life span of red blood cells can be reduced and this disturbs the significance of the Hb-A_{1c} level, since it is known to be related to the age of red blood cells [84]. Erroneous estimations of glycosylated hemoglobins can also arise from storage of samples over long periods [80], from lactescent hyperlipidemic plasma [80, 85], or when a marked hyperglycemia occurs [86].

Radioimmunoassay has also been used for the assay of Hb-A_{1c} [87]. Moreover, Hb-A_{1c} can be separated from Hb-A by isoelectric focusing in commercial pH gradients [38, 43, 88], but accurate quantitative determination of Hb-A_{1c} under these conditions can be obtained only by high-resolution microdensitometry [89—92]. Improved separations are necessary for the densitometric evaluation of Hb-A_{1c} by conventional scanners. They can be obtained by the use of separators [93, 94] or by the use of home-made short pH gradients (Fig. 8). Radioimmunoassay and isoelectric focusing determinations of Hb-A_{1c} are very promising since they should allow specific evaluation of glycosylated hemoglobins and may resolve problems connected with chromatographic procedures. However, they are not completely adapted to routine assays, and chromatographic methods remain the only techniques that are operational at present.

It has recently been demonstrated that a large amount of Hb-A_0 (8—10%) may be glycosylated at the NH_2-terminus of the α-chains or at lysine amino groups (while Hb-A_{1c} is glycosylated on the NH_2-terminus of the β-chain) [95], but there is still no technique for evaluating the level of this glycosylated Hb-A_0.

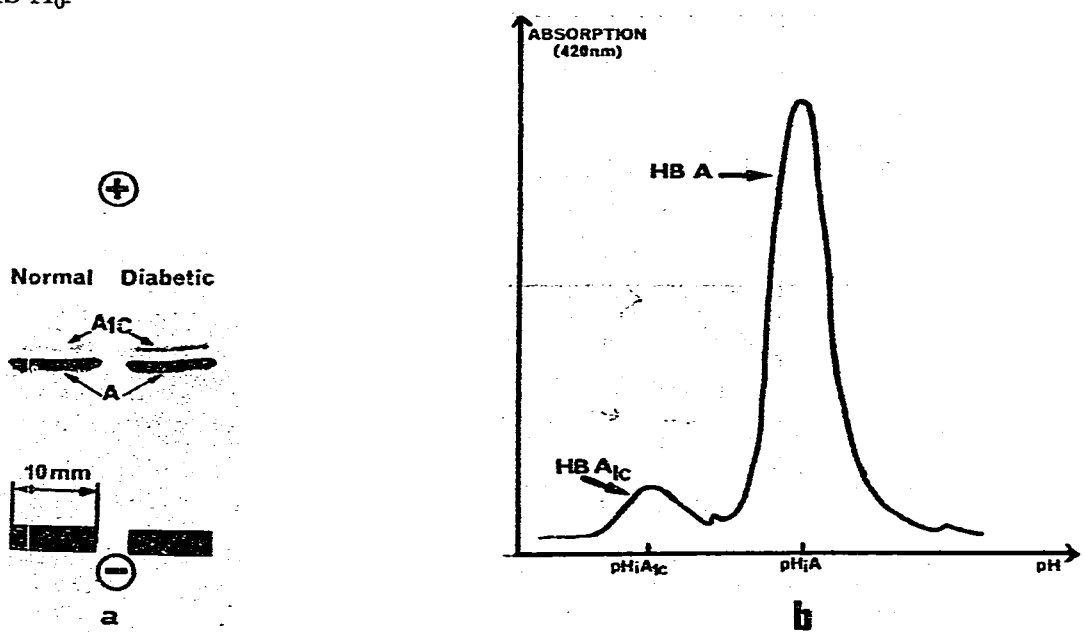

Fig. 8. Separation of Hb-A_{1c} from Hb-A_0 on thin-layer isoelectric focusing in a pH gradient of 1 unit (pH 6.5—7.5). (a) Blood from a normal adult and a diabetic patient are compared. (b) Corresponding densitometric tracing of the diabetic patient.

2.3.4. Chromatographic separation of globin chains

In 1965, Clegg et al. [96, 97] described a chromatographic method permitting separation of the α- and non-α-hemoglobin chains and their quantitative evaluation. Hemoglobin is converted to globin by treatment with HCl—acetone and then dialysed against an 8 *M* urea—0.05 *M* mercaptoethanol—phosphate buffer. Chromatography is performed on a CM-cellulose column.

The procedure is time-consuming but is nevertheless used in many laboratories in view of its numerous applications to the structural study of abnormal hemoglobins. In clinical biochemistry, Clegg's technique, performed in conjunction with studies of globin synthesis in vitro from reticulocytes or bone marrow cells, is currently used in specialized laboratories to diagnose thalassemia syndromes. The original method, presented in 1965 by Weatherall et al. [98], has been recently used to analyse radioactive globin chains for the antenatal diagnosis of hemoglobinopathies. The absorbance profiles of a maternal sample (AS) and from a cord blood sample (AA) are shown in Fig. 9. A reliable alternative to this relatively time-consuming procedure could now be offered by the Triton-gel technique [32, 33] and perhaps by globin-chain electrophoresis on cellulose acetate [29—31]. Other chromatographic techniques have also been presented recently: high-performance liquid chromatography [99—101] which may be about fifteen times faster than the CM-cellulose chromatography but which can not be applied at present for the diagnosis of sickle-cell disease, ion-exchange chromatography on CM-Sepharose CL-6 B [102], and globin-chain affinity chromatography on Sepharose—haptoglobin [103] which is particularly useful in cases where globin chains are synthesized in small amounts.

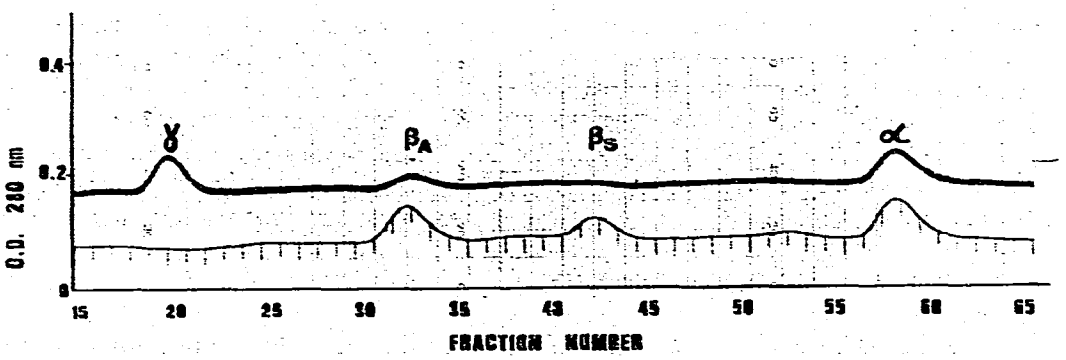

Fig. 9. Elution pattern of the chromatographic separation of globin chains on CM-cellulose in the presence of urea. Upper curve: normal newborn. Lower curve: adult heterozygous for sickle-cell disease.

2.3.5. Chromatographic procedures used in the structural study of abnormal hemoglobins

The identification of many hemoglobin variants, some of which are involved in pathology, requires structural analyses. Chromatographic procedures play a major role in these studies but such techniques are too specialized and time-

consuming for everyday use in numerous clinical laboratories. We will discuss the general principles of these methods; for more detailed information, the reader should refer to Huisman and Jonxis [14] and Schroeder and Huisman [49].

Once detected, an abnormal hemoglobin must be purified in significant quantity, and if possible, separated from Hb-A, since in most cases the patient is heterozygous. Starch-block electrophoresis or preparative IEF can be used for this purpose, but abnormal hemoglobins are more generally prepared by ion-exchange column chromatography. DEAE-Sephadex chromatography using Tris—HCl developers is the most popular of these methods. Several grams of material can be prepared in one experiment but the elution time can reach several days. The recently introduced anionic exchanger DEAE-Sephacel eliminates this disadvantage and in our laboratory has given good results in isolating hemoglobins with p*I* values higher than the p*I* of Hb-A [104]. For other variants, CM-cellulose or CM-Sephadex chromatography are the most advantageous.

Special conditions are sometimes required. Thus, some M hemoglobins have been isolated by chromatography on Amberlite Biorex 70 [105]; this method is also useful for isolating Hb Hope [82]. Variants that are electrophoretically similar to Hb-A may sometimes be isolated by chromatography on an ion exchanger. In general, however, when only small quantities of material are required, preparative IEF is the method of choice.

Once purified, the hemoglobin variant is desheminizèd and the abnormal polypeptide chain prepared by CM-cellulose chromatography as previously described. The abnormal chain is then aminoethylated [106] and submitted to tryptic digestion. The peptides are analysed by fingerprinting on paper, or on silica gel or cellulose thin-layer plates, thin-layer silica-gel plates giving results comparable to those obtained on paper, but more rapidly [107]. The abnormal peptide can also be prepared by automatic peptide chromatography [108, 109] or by high-performance liquid chromatography [110, 111]. The amino-acid analysis of the peptide and its sequence determination involve further chromatographic procedures which include cation-exchange column chromatography, thin-layer chromatography and gas—liquid chromatography. Finally, sequencing is performed manually or, in some cases, with an automatic sequencer.

3. DIAGNOSIS OF HEMOGLOBINOPATHIES

Hemoglobinopathies can be conveniently divided into two main groups: (1) abnormal hemoglobins involving an inherited structural alteration in the hemoglobin molecule; and (2) thalassemia syndromes, which are characterized by an inherited defect in the rate of synthesis of one or more hemoglobin peptide chains, although the structure of the affected chain usually remains normal (ref. 18, p. 43). Methods of diagnosis are therefore very different in the two groups of hemoglobinopathies.

This section deals with the study of diagnoses that are the most frequently encountered in clinical biochemistry in the adult, at birth and in the foetus. The cases open to erroneous interpretations, such as the association of Hb-A (or Hb-S) with a variant which is not separable from Hb-A (or Hb-S) by electro-

phoresis, are discussed in some detail. The end of the section is devoted to the glycosylated hemoglobins and their use in diabetics. For other information on the diagnosis of hemoglobinopathies, the reader should refer to Fairbanks [112].

3.1. Diagnosis of abnormal hemoglobins

The difficulties encountered in the diagnosis differ in the adult and at birth, because the main hemoglobins of adult and cord blood, respectively Hb-A and Hb-F, have distinct electrophoretic and chromatographic properties.

3.1.1. Abnormal hemoglobins in the adult

About 300 variants of hemoglobins A are now recorded. Up-to-date listings are regularly published [113].

The electrophoretic properties of the most important variants, i.e. those with clinical repercussions, are not absolutely specific. For instance, over 50 different variants behave similarly to Hb-S in electrophoresis in alkaline buffers (ref. 14, p. 101). Thus, to identify the abnormal hemoglobin mutants, electrophoretic analysis must be complemented by the study of other properties, such as solubility, stability, affinity, etc. Even then, few known hemoglobin variants exhibit specific properties. Structural studies are therefore necessary, but as we have seen these studies are long and complicated. This explains attempts to develop simplified procedures with sufficient resolving power to identify at least the most important variants. We have presented three procedures of this nature in the preceding section: (1) electrophoresis of globin chains in conjunction with cellulose-acetate electrophoresis at alkaline pH and citrate-agar electrophoresis; (2) microchromatographic procedures; and (3) thin-layer isoelectric focusing (TLIF). The first two systems have been used with success during recent years and remain very popular. More recently, TLIF has been introduced in routine analyses and may provide a reliable alternative. TLIF permits screening and characterization of hemoglobin mutants [42, 43, 114, 115].

From a theoretical point of view it should be possible to characterize a variant according to its isoelectric point [116]. However, TLIF has a resolving power of at least 0.01 pH unit, i.e. far below the performance of the best contact electrodes [117]. Direct p*I* determination cannot now precisely identify an abnormal hemoglobin. Current procedure requires the comparison, on the same plate, of the isoelectric position of an unknown hemoglobin variant with that of control variants of known structure [43]. This method requires a bank of control variants and a precise reference map. Fig. 10 shows a map presenting over 80 variants with over 40 distinct isoelectric positions. The major drawback of this method is that it requires use of control hemoglobins. On the other hand, TLIF requires only a small amount of material; control samples can be stored in liquid nitrogen at −180°C over a long period of time. Inter-laboratory exchanges are increasingly frequent and we envisage that panels of the most important variants encountered in pathology will one day be constituted and become available to interested laboratories.

3.1.1.1. "C-like" variants. We will call hemoglobins "C-like" those of the ab-

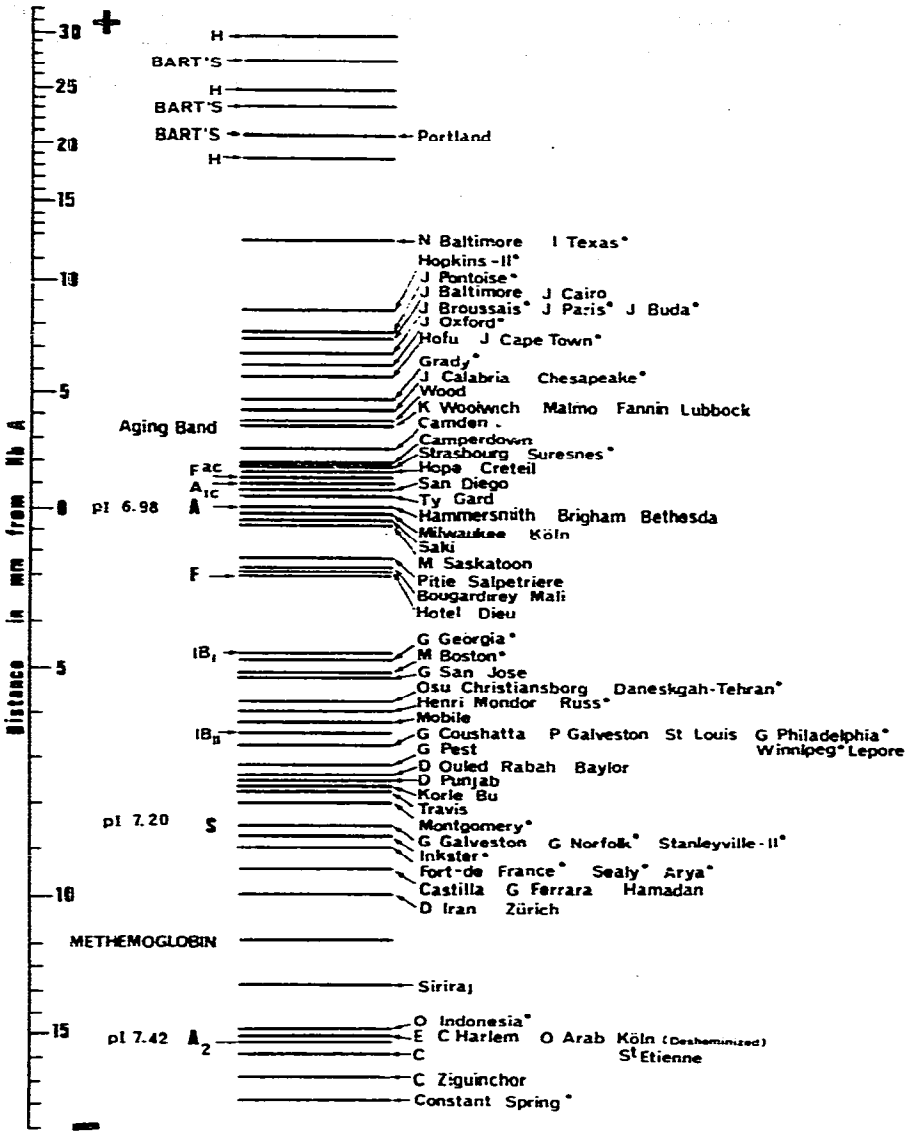

Fig. 10. Isoelectric focusing map of human hemoglobins. Asterisk (*) indicates α-chain mutation. IB_I and IB_{II} are the ferrous—ferric hybrids of Hb-A.

normal hemoglobins which are not separable from Hb-C by electrophoresis on cellulose acetate at alkaline pH, i.e. Hb-E, Hb-C Harlem, Hb-C Ziguinchor, Hb-O Indonesia and Hb-O Arab. A precise diagnosis of these hemoglobins is important since they are all, with the exception of Hb-O Indonesia [118], involved in clinical manifestations, either alone or in association with Hb-S or a thalassemia gene (ref. 18, pp. 43—74) [119]. All these variants can theoretically be separated by citrate-agar electrophoresis [27, 120]. However, certain of the separations obtained are slight and may be difficult to interpret. IEF can be useful in this case.

Hb-E, Hb-O Arab, and Hb-C Harlem all have the same isoelectric position, but TLIF allows identification of the remaining "C-like" variants [43]. Thus, by combining citrate-agar electrophoresis and TLIF, it is possible to identify all the "C-like" variants (Fig. 11).

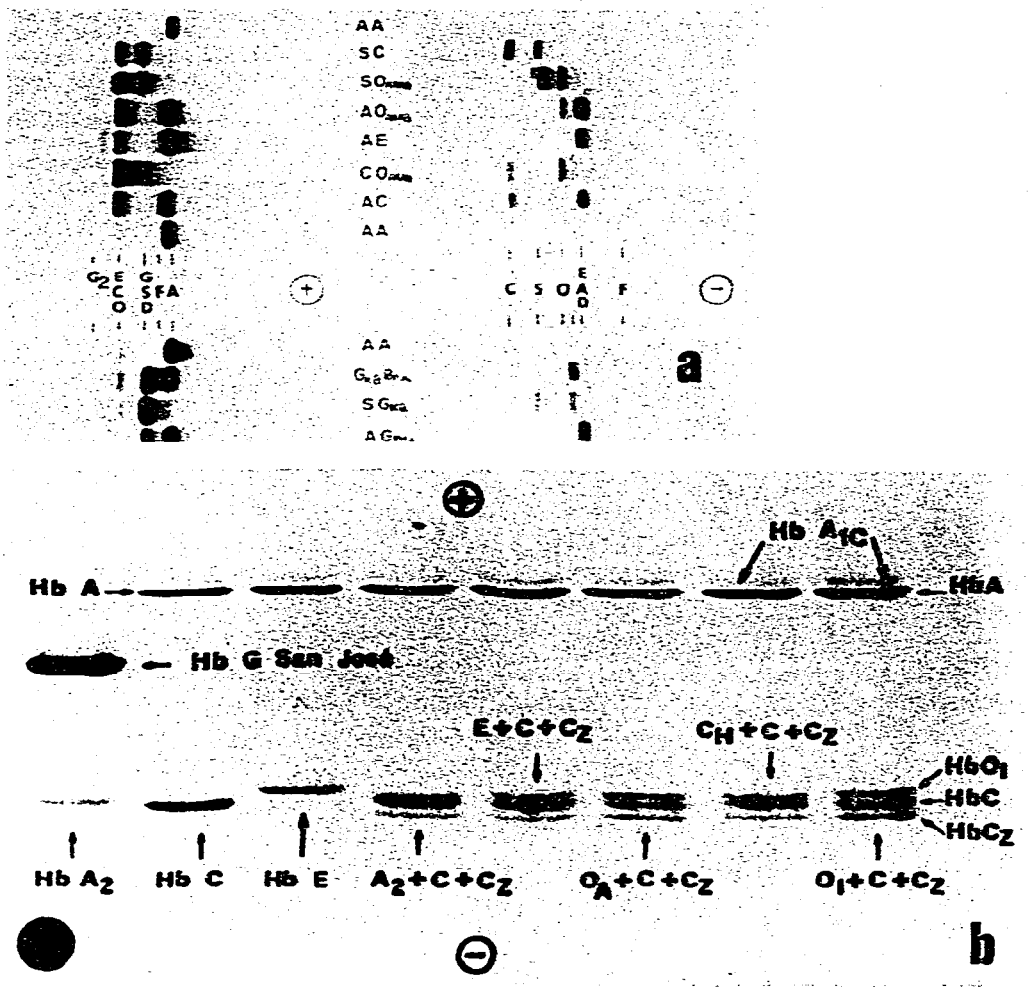

Fig. 11. Identification of abnormal hemoglobins not separable from Hb-C by electrophoresis at alkaline pH. (a) Separations obtained by citrate-agar electrophoresis. (By permission, see ref. 23.) (b) Separations obtained by thin-layer isoelectric focusing with a pH gradient 6—9. C_Z = Hb-C Ziguinchor, O_I = Hb-O Indonesia, O_A = Hb-O Arabia, C_H = Hb-C Harlem.

3.1.1.2. "S-like" variants. Hb-S is the most widely distributed abnormal hemoglobin in the world. Every year, many thousands of children die of sickle-cell anemia. The major diagnostic problem in the case of Hb-S is to avoid confusing Hb-S with one of the 50 other electrophoretically "S-like" variants. Theoretically this should be easily accomplished since Hb-S has the specific property of being insoluble in its desoxy form. However, the homozygous S/S

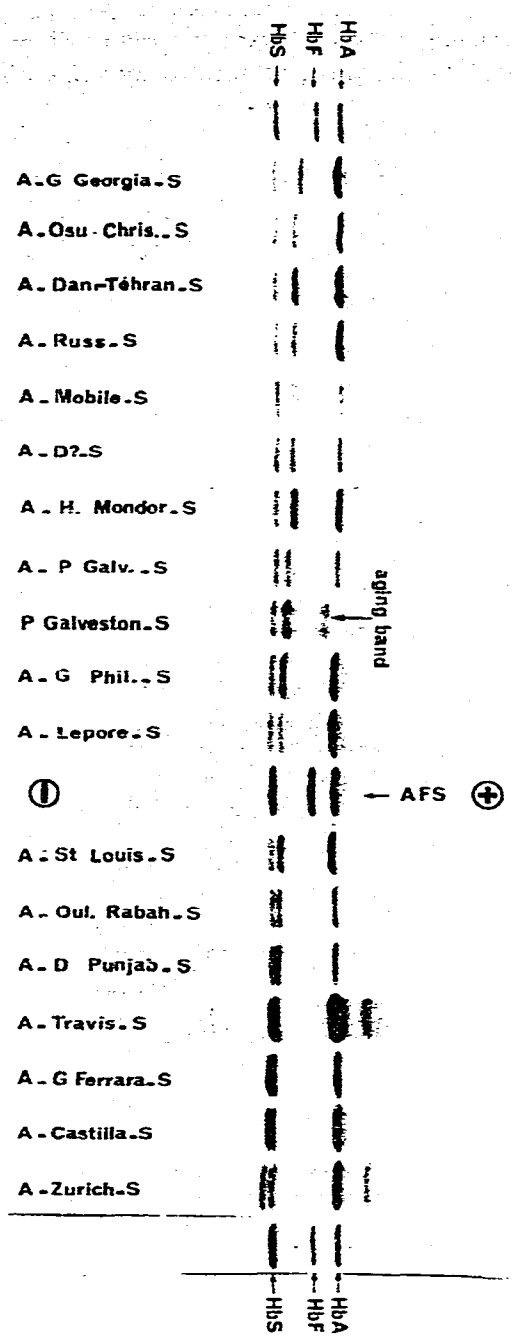

Fig. 12. Thin-layer isoelectric focusing pattern of some abnormal hemoglobins not separable from Hb-S by electrophoresis at alkaline pH. Samples containing both Hb-A and the abnormal hemoglobin were mixed with Hb-S and analyzed in a pH gradient 6—9.

state which is responsible for sickle-cell anemia can be confused with other disorders with less serious consequences. The main source of error is the confusion between the SS phenotype and a double heterozygote for Hb-S and an "S-like" variant, i.e. an Hb-D, -P or -G. In these two cases, the electrophoretic pattern is the same and, moreover, the solubility tests are positive even if the hemoglobin mixture contains only a part of insoluble Hb-S. Several of these associations can be detected by using agar electrophoresis and/or the system of double pH chain electrophoresis described by Schneider and co-workers [26, 28], but such a schedule is time-consuming and has been demonstrated to be inefficient in several cases. We have systematically compared the previous method with TLIF in a large number of variants exhibiting the "S-like" pattern of electrophoresis at alkaline pH. The correct answer may be obtained in most cases of the double heterozygosity Hb-S—Hb-"S-like" [43]. In our hands only one β-variant, Hb-G Galveston [121], exhibited the same isoelectric position as Hb-S (Fig. 10). The analysis of associations such as Hb-S—Hb-"S-like" by TLIF is illustrated in Fig. 12, which represents the separation obtained from artificial mixtures of the patient hemolysate with Hb-S.

Some of these associations of Hb-S with an Hb-"S-like" variant are not uncommon. Diagnosis can be somewhat complicated because some of the electrophoretically frequent "S-like" variants clinically interact with Hb-S. This is the case with Hb-D Punjab (i.e. D Los Angeles), which, when associated with Hb-S, gives a clinical symptomatology very similar to that of sickle-cell anemia [122], and with Hb Korle Bu, which, in contrast to Hb-D Punjab, interacts negatively with Hb-S [123]. The isoelectric positions of hemoglobins D Punjab, Korle Bu*, and of a third variant also involved in sickling, Hb-S Travis [124], are extremely close together (Fig. 10). The procedure used to distinguish these three variants by TLIF is presented in Fig. 13. Its consists of mixing the sample to be analysed with Hb-S. This procedure emphasizes the slight differences existing between the isoelectric position of these variants.

When Hb-S and a β^0-thalassemia gene are present in the same patient, the electrophoretic pattern may also be confused with that of sickle-cell anemia. If the existence of β^0-thalassemia is suspected because of clinical features such as the persistence of splenomegaly in the adult, correct diagnosis must be based on the presence of microcytosis, with an increased level of Hb-A_2, and on a family study [125].

Hb-S can also be associated with an α-"S-like" variant. If the patient is homozygous for Hb-S and heterozygous for the α-"S-like" variant, electrophoresis at alkaline pH demonstrates the presence of two abnormal hemoglobins, one migrating as Hb-S, the second as Hb-C, which is, in fact, the hybrid S—α variant [126]. If the patient is heterozygous for both Hb-S and the α-"S-like" variant, the diagnosis depends on analysis of three major bands on cellulose-acetate electrophoresis at alkaline pH. Fig. 14a presents the association Hb-S—Hb-G Philadelphia which is relatively frequent in the West Indies and in

*In a previous report [43] we presented an erroneous isoelectric position for Hb Korle Bu. Since then, we have found several authentic Hb Korle Bu (diagnosis confirmed by a structural study). The exact nature of the hemoglobin presented as a Korle Bu variant (and as Hb-D? in the present report) is not yet known.

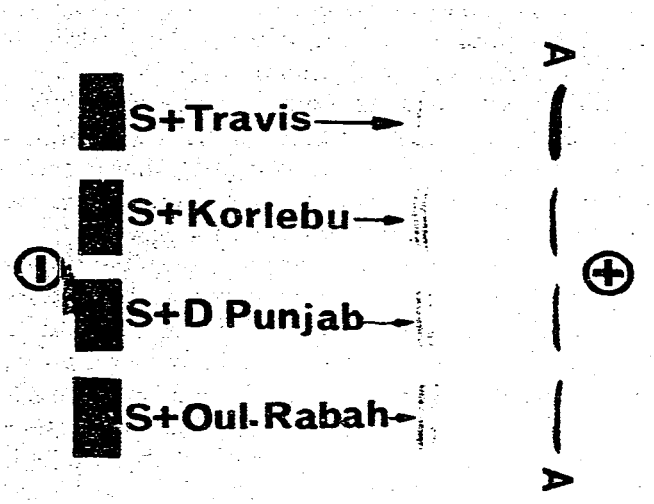

Fig. 13. Identification of Hb Korle Bu and Hb-D Punjab by thin-layer isoelectric focusing (pH gradient 6—9). Interpretation of the isoelectric focusing pattern is made easier by mixing the samples with Hb-S.

North America [126]. The interpretation of these three major electrophoretic bands could not be made definitively but TLIF analysis provided the solution (Fig. 14b). Four major bands were detected and, in addition, a minor fraction resulting from the doubling of Hb-A_2, could be seen close to the cathode. Thus, it was possible to conclude that the patient was a double heterozygote for an

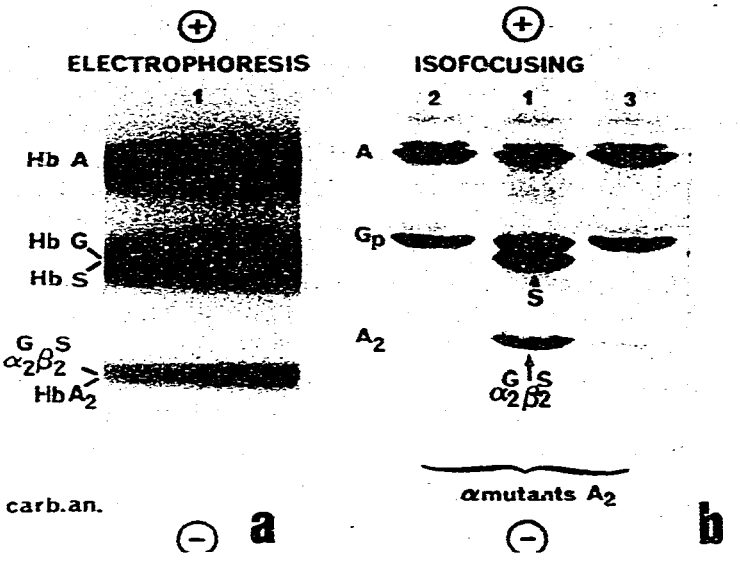

Fig. 14. Electrophoretic and isoelectric focusing patterns of an hemolysate from the same subject, heterozygous for both Hb-S and Hb-G Philadelphia. (a) Electrophoresis on cellulose acetate at alkaline pH. (b) Thin-layer isoelectric focusing pattern (pH gradient 6—9) of the propositus (1) compared with a heterozygous subject for Hb-G Philadelphia (2 and 3).

α- and a β-variant. The β-variant was easily identified as Hb-S (by its isoelectric position and by a positive solubility test) and the α-variant was suspected to be Hb-G Philadelphia (or Hb Winnipeg) (Fig. 10). Structural studies confirmed that the mutant was in fact an Hb-G Philadelphia.

Diagnosis is more difficult in the similar, and quite frequent, association of Hb-S—Hb Stanleyville II [127], because they have the same isoelectric position (Fig. 10). The α-variant is suspected owing to the importance of the intermediate band (which corresponds to Hb-S + Hb Stanleyville II) in comparison with a normal AS sickle-cell trait. In addition, a slight band with a slower migration than normal Hb-A_2 was found. This band corresponds to Hb $\alpha_2\delta_2$ Stanleyville II, but has the same electrophoretic migration as carbonic anhydrase. Diagnosis must then be based on benzidine-specific staining or on migration upon IEF (in this case, since no staining is performed Hb $\alpha_2\delta_2$ Stanleyville II can not be confused with carbonic anhydrase).

Finally, it may be difficult to interpret some other patterns in which Hb-S is found at higher levels than in the classical sickle-cell trait. These patterns can correspond to transfused Hb-S sickle-cell anemia, to Hb-S—β^+-thalassemia, a sickling disorder generally weaker than sickle-cell anemia [122], or to an association of Hb-S with an unstable hemoglobin. Such a situation has been described for Hb Saki, a mild unstable hemoglobin having the same electrophoretic mobility as Hb-A [128]. In this case, the diagnosis was based on the positive results of the stability test, and was confirmed by TLIF, since Hb-A and Hb Saki have slightly different isoelectric positions (Fig. 10).

3.1.1.3. "A-like" variants. Almost all of the known mutants of this type are clinically manifested. This does not represent a mysterious electrophoretic structural relation, but more probably reflects the conditions in which these variants have been detected. In fact, these variants can not be separated from Hb-A by electrophoresis at alkaline pH and have escaped most screenings. However, in cases of patients with hematologic disorders such as hemolytic anemia, cyanosis or polycythemias, even if hemoglobin electrophoresis is normal, complementary investigations should nevertheless be performed. Unstable "A-like" mutants, which may be associated with hemolytic anemia, are the most easily detected among the electrophoretically "A-like" abnormal hemoglobins, since stability tests are easy to perform in all laboratories.

Among these, Hb Köln [129] is one of the most frequent unstable hemoglobins. Its electrophoretic pattern is not specific but is observed in a large number of other unstable hemoglobins such as Hb St. Etienne [130] (Fig. 15a). The slow-migrating bands are constituted by partially desheminized hemoglobin. Heminized Hb Köln is not separable from Hb-A by electrophoretic techniques and must be characterized, for instance, by diagonal printing [131]. A simpler solution is offered by TLIF which allows separation of heminized Hb Köln from Hb-A (Fig. 15b).

The M hemoglobins, which cause congenital cyanosis, exhibit a pattern indistinguishable from that of Hb-A on cellulose-acetate electrophoresis at alkaline pH. Their diagnosis requires spectrum analysis and several electrophoretic runs at various pH values and under various conditions of ligandation. Fig. 16 shows that some Hb-M are easily separated from Hb-A by TLIF. Moreover,

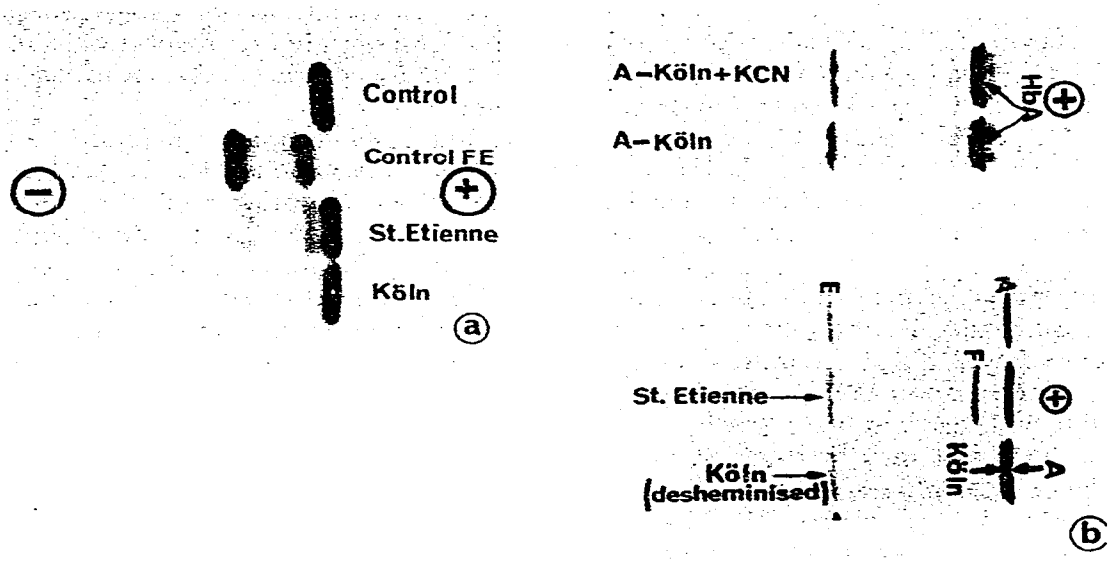

Fig. 15. Electrophoretic and isoelectric focusing patterns of Hb Köln and Hb St. Etienne. (a) Electrophoresis on cellulose acetate at alkaline pH. Unstable fractions migrating slowly are visible. In addition, 20% Hb-F is present in hemolysate St. Etienne. (b) Thin-layer isoelectric focusing (pH gradient 6—9). Isoelectric focusing separated heminized Hb Köln from Hb-A.

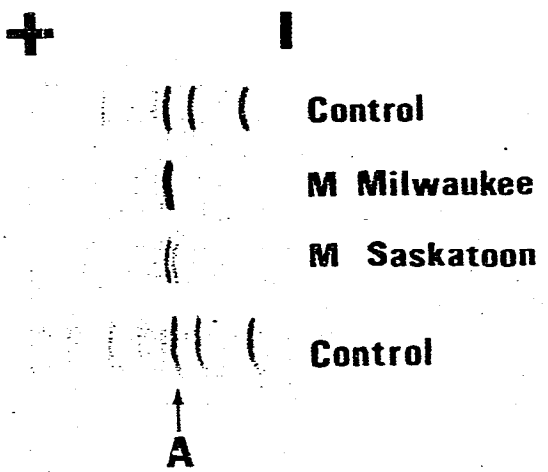

Fig. 16. Separation of Hb-M Milwaukee and Hb-M Saskatoon from Hb-A by thin-layer isoelectric focusing (pH gradient 6—9).

they exhibit specific p*I* values and can be directly identified (Fig. 10). The various states of oxidation of hemoglobins are also separable by IEF as previously decribed by Bunn and Drysdale [132], who identified the ferri form and two intermediary hybrids of valency in addition to oxyHb-A (Fig. 17).

Finally, some of the high-affinity variants are electrophoretically "A-like".

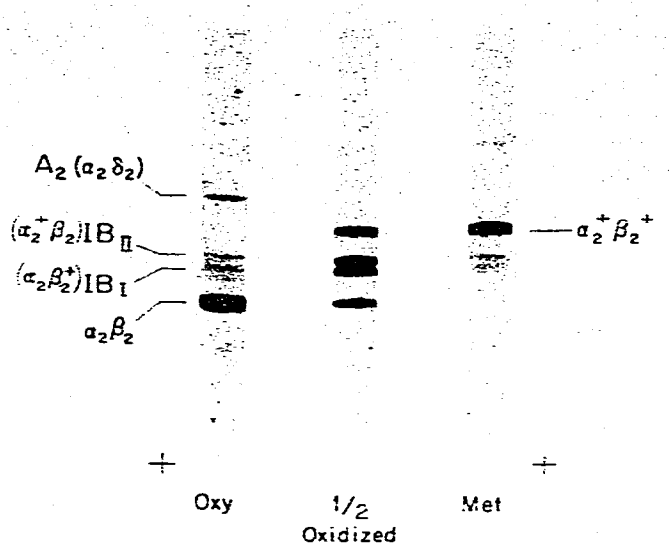

Fig. 17. Separation of partially oxidized hemoglobins IB_I ($\alpha_2\beta_2^+$) and IB_{II} ($\alpha_2^+\beta_2$) from fully oxidized ($\alpha_2^+\beta_2^+$) and unoxidized hemoglobin ($\alpha_2\beta_2$) by isoelectric focusing in a pH gradient 6—8. (By permission, see ref. 134.)

They are usually accompanied by erythrocytosis or occasionally by chronic hemolysis, if the variant is unstable [133]. Most of these high-affinity variants can, however, be separated from Hb-A by isoelectric focusing (Fig. 18). Of the four which can not be distinguished by TLIF, Hb Bethesda is separable by citrate-agar electrophoresis [134]. Diagnosis of the remaining three variants (Hb Brigham, Hb Olympia, and Hb Heathrow) may be very difficult and may involve numerous and sometimes useless complementary investigations. It is therefore vital to measure the P_{50} of venous blood in all patients presenting erythrocytosis.

3.1.1.4. A new strategy for diagnosis of abnormal hemoglobins. Usually abnormal hemoglobins have been screened by cellulose-acetate electrophoresis at alkaline pH, and by solubility, stability and alkali-denaturation tests. The detected variants have subsequently been submitted to other electrophoretic procedures (citrate-agar and globin-chain electrophoresis). At this stage, except in the case of common variants, structural studies must be performed.

Our experience has shown that the introduction of TLIF in routine analyses could notably modify this diagnostic procedure. Firstly, cellulose-acetate electrophoresis is no longer absolutely required, since TLIF represents a better approach to screening. As we have seen above, once a mutant has been detected by TLIF, it is compared with a panel of identified variant controls whose p*I* values are close to the p*I* exhibited by the unidentified sample. Comparison of the isoelectric position of the mutant, combined with the results of the biochemical tests (solubility, stability, alkali-denaturation), the hematological data, the clinical studies and the family investigations, generally allows identification of the variant. Otherwise, other electrophoretic procedures (citrate-agar electrophoresis and globin-chain electrophoresis) or chromatographic tech-

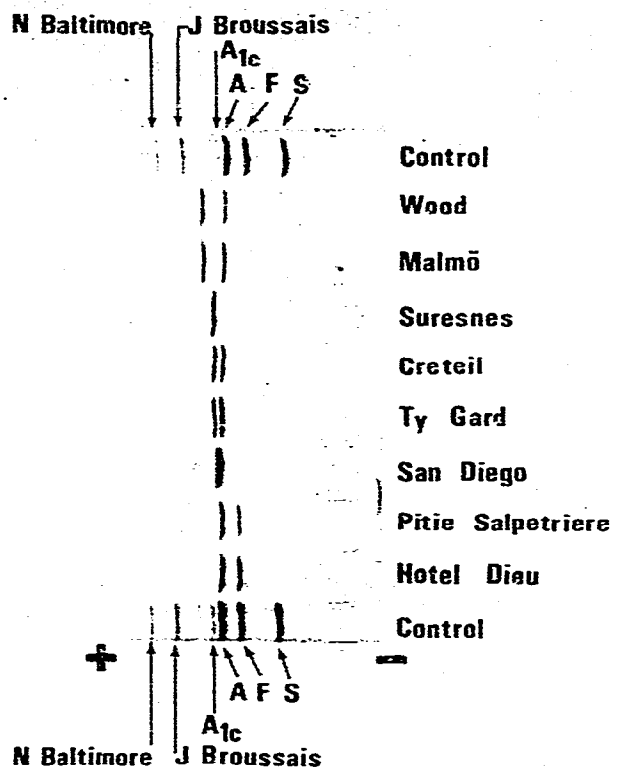

Fig. 18. Isoelectrophoretic pattern of some high oxygen affinity variants in a pH gradient 6—9.

niques could be used. Structural studies should always be limited to difficult cases.

Such a strategy tends to reduce the number of time-consuming structural determinations, but should not be applied too rigidly. In our laboratory, this strategy has produced useful results, but new variants will most probably exhibit the same p*I* as known variants. In the future, this strategy, perhaps complemented by the use of specific antibodies [135], could be the ideal solution for the identification of abnormal hemoglobins without structural studies.

3.1.2. Abnormal hemoglobins at birth

At birth, nearly 70% of the total hemoglobin is constituted by Hb-F and only about 20% by Hb-A.

Since Hb-A, Hb-F and acetylated Hb-F migrate closely in alkaline electrophoresis, most of the cord blood screening programs use, in addition to cellulose-acetate electrophoresis, citrate-agar electrophoresis [136—138] which improves the separation of Hb-A and Hb-F. In the presence of an abnormal hemoglobin at birth, one of the main difficulties is to ascertain whether the newborn is a homozygous or a heterozygous carrier. Such early diagnosis is particularly important when the abnormal hemoglobin is Hb-S, in order to prevent the

disastrous consequences of sickle-cell anemia in the early months and years of life.

TLIF recently provided [139] a reliable alternative in this case, because Hb-A, Hb-F and acetylated Hb-F are clearly resolved by this method, facilitating differentiation between homozygous and heterozygous states (Fig. 19) or allowing the detection of abnormal hemoglobins (Fig. 20).

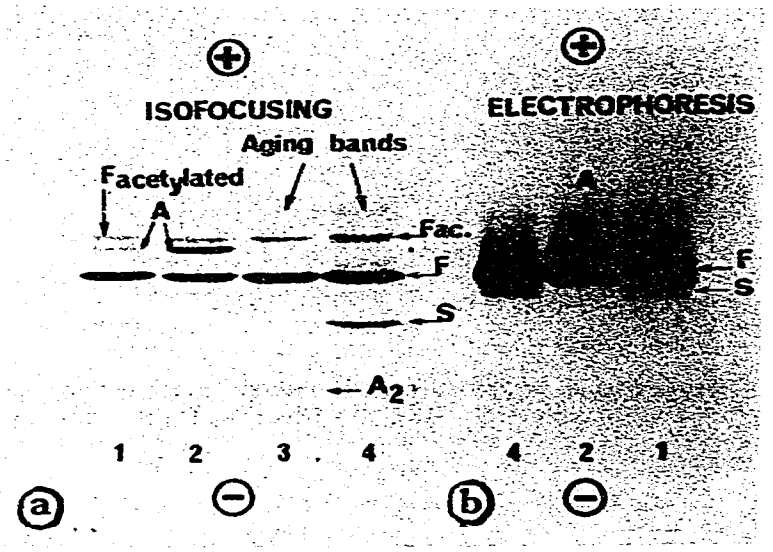

Fig. 19. Electrophoretic and isoelectric focusing patterns of cord blood hemolysates. (a) Thin-layer isoelectric focusing (pH gradient 6—9): 1 = heterozygous for Hb-S; 2 = normal newborn; 3 = sample from an adult with homozygous β^0-thalassemia; 4 = homozygous for Hb-S. (b) Electrophoresis in alkaline buffer of samples 1, 2, and 4.

3.2. Diagnosis of thalassemia syndromes

Thalassemia syndromes are characterized by an inherited defect in the rate of synthesis of at least one of the peptide chains of hemoglobin, although the structure of the affected chains remains normal in most cases. Some thalassemia syndromes are still produced, however, by the presence of an abnormal hemoglobin while others are accompanied by an abnormal level of some hemoglobin component normally found in adults or at birth. In all these cases, diagnostic methods are similar to those described for the abnormal hemoglobins. In contrast, many other thalassemia syndromes are present with a normal or slightly modified hemoglobin pattern. Diagnosis must then be carried out differently.

Thalassemia genes may be associated together or may occur concurrently with abnormal hemoglobins in different ways, giving rise to various phenotypes in the same patient. In this section, we limit our discussion to diagnostic problems connected with the four most frequently encountered situations, i.e. homozygous β-thalassemia, Hb-H disease, the β-thalassemia trait and the α-thalassemia trait.

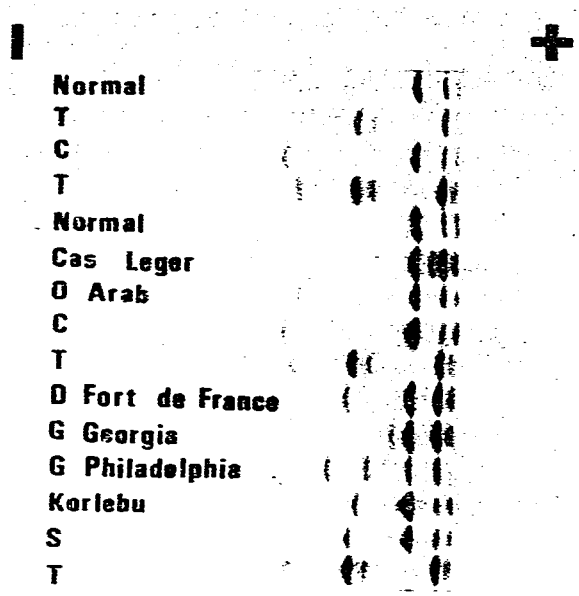

Fig. 20. Isoelectric focusing pattern of some cord blood samples with abnormal hemoglobins from the French West Indies: T = control sample; C = Hb-C; S = Hb-S; Cas Leger = abnormal hemoglobin in current studies.

3.2.1. Homozygous β-thalassemias

β-Thalassemia can be found in most human populations but has a higher incidence in the ethnic groups of the Mediterranean basin, the Middle East and the Far East. The homozygous state is generally accompanied by a chronic hemolytic anemia with a dyserythropoietic component on which the prognostic of the illness mainly depends. In black subjects, the homozygous β^+ state (persistence of some β-chain synthesis) can be almost symptomless [140]. The β^0-thalassemia form (absence of β-chain synthesis) seems, however, to be as severe in black populations as in other ethnic groups [140—142]. Interaction with α-thalassemia seems to be able to modify the usually severe clinical picture to that of mild thalassemia intermedia [143].

Diagnosis is simple since homozygous β-thalassemia is characterized by clinical and hematological features and by an abnormally high Hb-F level. The determination of the in vitro ratio of chain synthesis is exceptionally useful for diagnostic purposes. Moreover, this index does not seem able to distinguish between Cooley's anemia and the thalassemias intermedias [144—146]. At birth, diagnosis is more difficult since a high level of Hb-F is normal in the newborn. However, in the case of homozygous β^0-thalassemia, diagnosis can be made by citrate-agar electrophoresis or by IEF which demonstrate the absence of Hb-A in the cord blood hemolysate. In the presence of a β^+-thalassemia, this approach is less reliable and it is necessary to supervise the babies and to make a follow-up examination some months after birth.

3.2.2. Hemoglobin-H disease

Hemoglobin-H disease is generally found in individuals heterozygous for both the α-thalassemia-1 and the α-thalassemia-2 genes, or for the α-thalassemia-1 and the Hb Constant-Spring genes [147], or in individuals homozygous for the non-deletion determinant α-thalassemia gene [148]. Recently Hb-H disease has been also described in children doubly heterozygous for α-thalassemia-1 gene and Hb Petah Tikva, a new unstable variant [149]. In rare cases, Hb-H disease can be acquired [150]. The clinical picture of Hb-H disease is, in most cases, that of thalassemia intermedia. Diagnosis is based on the presence of Hb-H in the adult. We have seen in the preceding section, that Hb-H is detected by electrophoretic procedures or by IEF. When Hb-H disease is associated with Hb Constant-Spring, the α-variant is also detected by electrophoresis or by IEF. In addition, Hb-H inclusions can be generated in the red cells by incubation in cresyl blue [151].

3.2.3. β-Thalassemia trait

Individuals affected by β-thalassemia trait alone generally have no clinical manifestations. The heterozygous state is accompanied by only mild hematologic and biochemical abnormalities (ref. 18, p. 110). Hematological data are characterized by minor morphological changes of the red cell with a pseudopolycythemia resulting from microcytosis. It is nevertheless important to detect the heterozygous carriers because every year about 100,000 children die from Cooley's anemia in the world [152].

An abnormally high Hb-A_2 level is considered to be the best index for the diagnosis of β-thalassemia trait (ref. 18, p. 124). Nevertheless, the β-thalassemia trait may be present with a normal Hb-A_2 level in about 3% of heterozygotes [153].

When a β^+-thalassemia gene is associated with a β-variant, β-thalassemia will be suspected because of the abnormally low level of Hb-A. Hemoglobins Lepore, which are β-thalassemia-like genes [147], are also easily identified because of their occurrence at low levels and their isoelectric point. In most cases, however, the main diagnostic problem will be to measure the Hb-A_2 level precisely during routine screening. Cellulose-acetate electrophoresis has until now been the most widely employed method for hemoglobin screening. It was consistently used to assay the Hb-A_2 level, since Hb-A and Hb-A_2 are clearly resolved by this technique. Attempts have been made to develop a densitometric method comparable to that used in clinical biochemistry to perform quantitative determination of the serum proteins. The densitometric methods can lead to many diagnostic errors in β-thalassemia carriers [154]. For some authors, however, electrophoresis with densitometry may be a reliable means for the identification of abnormal Hb-A_2 levels if it is used with carefully controlled techniques and properly calibrated instruments [155, 156]. Nevertheless, the most popular techniques for measuring Hb-A_2 levels are the microchromatographic procedures initially developed by Efremov and Huisman. Their accuracy, use of whole blood and the possibility of analysing up to 50 samples per day, make them very convenient for screening [157]. When this method is not available quantitation of Hb-A_2 must be performed by elution of the electrophoretic fractions.

Although the β-thalassemia trait is symptomless, a false diagnosis may lead to unnecessary difficulties in genetic counselling or to useless explorations in view of a prenatal diagnosis (see the next part of this section). An elevated Hb-A_2 level may also occur in disorders other than β-thalassemias [6, 7], particularly in the case of megaloblastic anemias. The Hb-A_2 level, as already mentioned, can be normal in some β-thalassemia traits. In the presence of β-thalassemia trait and sideropenia, falsely normal Hb-A_2 levels can also be found; these cases must be carefully investigated.

While population screening for β-thalassemia could be envisaged by performing osmotic fragility tests [158], current strategy for detecting β-thalassemia carriers involves study of both Hb-A_2 and hematological data [159—161]. It seems that the value of the function $(M.C.V.)^2 \times M.C.H.$, using the mean corpuscular volume (M.C.V.) and the mean corpuscular hemoglobin (M.C.H.), evaluated with a Coulter Counter could be a good diagnostic index [159].

Lastly, equivocal or borderline cases can be checked by measuring β-chain synthesis, as previously described. However, it must be noted that even β^+-thalassemia is probably a heterogeneous genetic disorder [147]. Thus, although globin-chain synthesis seems to enable diagnosis of the β-thalassemia trait in the Caucasian, this may not be true for all thalassemia traits in black populations [144, 145].

3.2.4. α-Thalassemia trait

The α-thalassemia trait is difficult to detect. However, as in the case of the β-thalassemia trait, it is important to identify the trait carriers. In contrast to β-thalassemia, α-thalassemia trait in the adult is not associated with an easily detected biochemical modification such as a high Hb-A_2 level, except in the case of Hb Constant-Spring and of other similar α-chain variants, which are thalassemia-2-"like" genes [147]. Moreover, α_2-thalassemia trait, which corresponds to the deletion of one of the four α-genes [147], is accompanied by normal hematological data making its diagnosis very difficult. These silent carriers can be detected, however, during family studies undertaken, for instance, when an Hb-H disease is found in a child.

α_1-Thalassemia trait, which corresponds to the deletion of two of the four α-genes [147], is suspected when a decreased M.C.V. and M.C.H. and an abnormal red cell morphology are present, and is sometimes accompanied by a very mild anemia, with a normal or slightly decreased Hb-A_2 level.

Studies of the synthesis of globin chains must be performed in these cases, especially when the serum iron level is normal or elevated. The diagnosis can thus be obtained in most cases. However, a considerable overlap seems to exist between normal people and the heterozygous carriers of thalassemia α-genes without anemia [162].

Diagnosis is easier at birth since the α-thalassemia-1 gene, and eventually, the α-thalassemia-2 gene, are both associated with a slight increase in the level of Hb Bart's [147].

3.3. Prenatal diagnosis of hemoglobinopathies

More than 1500 prenatal diagnoses of hemoglobinopathies have been per-

formed in the last few years. In most cases, fetal blood obtained at 20 weeks' gestation was studied in a three-step procedure: incubation of the fetal red blood cells with [^{3}H] leucine, removal of heme, and, finally, chromatographic separation of globin chains. The diagnosis was based on the ratio of radioactivity incorporated into the globin chains [163–165].

Recently, TLIF has been applied to the study of hemoglobins from fetal blood samples obtained for prenatal diagnosis of hemoglobinopathies [166] (Fig. 21). Comparison of this method with the classical chromatography of globin chains provided identical results for 50 prenatal diagnoses of hemoglobinopathies. TLIF requires 0.1 mg of unlabeled hemoglobin, it is performed in 2 h and several samples can be analysed simultaneously. If present, maternal contamination must be eliminated by selective lysis of maternal cells [166].

A fascinating new approach, using DNA mapping of amniotic fluid cells, is under development for prenatal diagnosis of hemoglobinopathies. It uses the genetic polymorphism of the DNA sequence at the cleavage sites of different

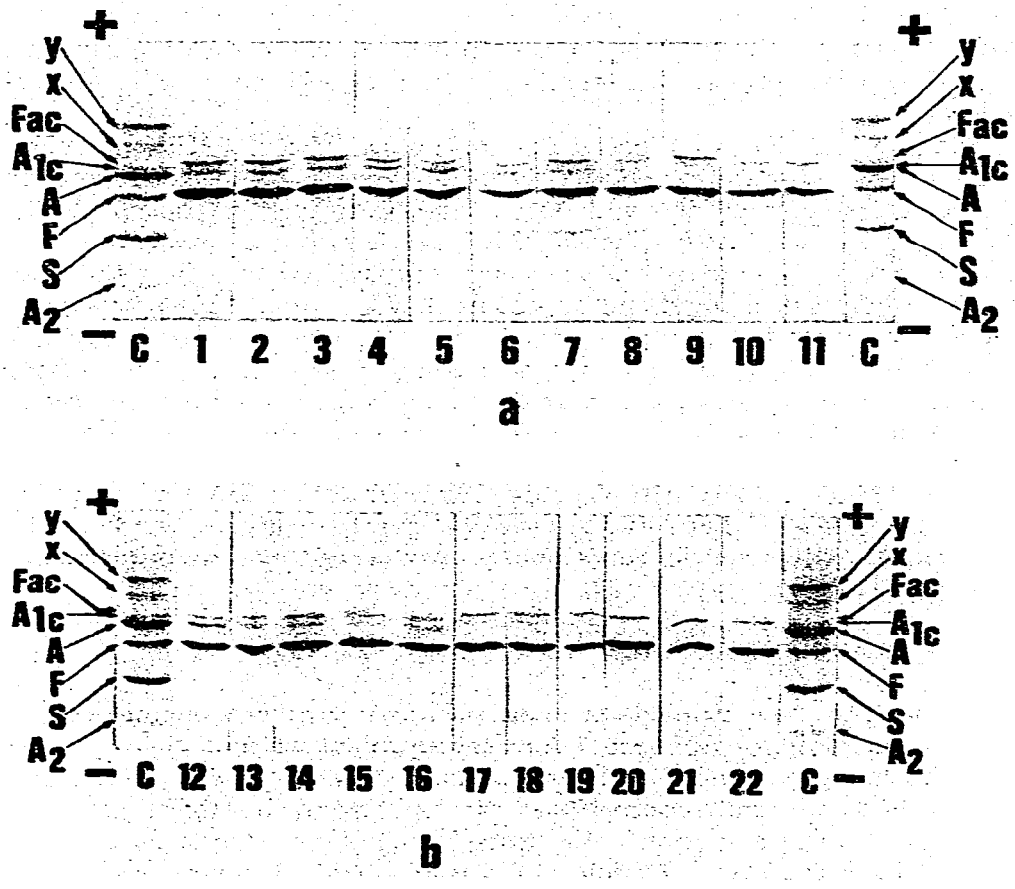

Fig. 21. Prenatal diagnosis of hemoglobinopathies by isoelectric focusing in a pH gradient 6–9. (a) Pattern obtained for 11 fetuses at risk for sickle-cell anemia. C = a control mixture of various hemoglobins including x (Hb-J Calabria) and y (Hb-J Broussais). (b) Pattern obtained for 11 fetuses at risk for β-thalassemia. (By permission, see ref. 166.)

restriction endonucleases [167—169]. The method is not yet applicable in all cases. Its main advantage is to allow diagnosis by amniocentesis instead of foetal blood sampling.

3.4. Glycosylated hemoglobins and diabetes mellitus

The interest in glycosylated hemoglobins is explained by evidence of their increased level in diabetic patients. Until recently the major problem with their clinical use was the lack of a simple rapid assay. As discussed above, the development of automatic chromatographic procedures has significantly modified this situation. More progress may be expected owing to the introduction into clinical routine of radioimmunoassays and of measurement of Hb-A_{1c} after separation by isoelectric focusing. These techniques allow Hb-A_{1c} to be assayed separately, and should make use of this measurement more widespread. As discussed in the preceding section, Hb-A_{1a1} and Hb-A_{1a2} levels have been found to be normal in diabetic patients and the increase in Hb-$A_{1(a+b)}$ seen in diabetics might be due, at least partially, to an increase in the amount of "non-hemoglobin" proteins [78]. Thus, in the future, methods allowing separate measurement of glycosylated hemoglobins will probably be used in clinical biochemistry.

The normal level of Hb-A_{1c} is about 5% and is elevated to 10—15% in diabetic patients. In vivo studies have shown that the glycosylation of Hb-A to form Hb-A_{1c} is a non-enzymatic step and only a slightly reversible reaction [170]. Since red blood cells are freely permeable to glucose [171] and since Hb-A_{1c} is a stable component, this minor fraction can be considered to reflect the average glucose blood level in the diabetic patient. Hb-A_{1c} could thus prove to be the missing index, allowing accurate estimations of the degree of hyperglycemia on a long-term basis [11—13]. Frequent Hb-A_{1c} measurement over a long period could furnish an explanation of the exact relation between the blood glucose level and pathogenesis of diabetic microangiopathy [172]. The measurement of Hb-A_{1c} level could also be a convenient way of detecting latent diabetic states [173]. Finally, Hb-A_{1c} may also provide a model for the pathogenesis of the sequelae of diabetes since the post-synthetic glycosylation of Hb-A to form Hb-A_{1c} alters certain functional properties of this protein such as its oxygen affinity [174].

4. CONCLUSIONS AND FUTURE TRENDS

In this review, we have studied the main diagnostic problems encountered in the field of hemoglobinopathies, and for each problem we have discussed the possible technical approaches. In all cases we have shown that classical chromatographic methods are rarely compatible with the demands of clinical biochemistry, and that classical electrophoretic procedures are rarely sufficiently sensitive. This explains the ever-continuing efforts to adapt chromatographic and electrophoretic techniques to the clinical biochemistry of hemoglobins. Thus microchromatographic procedures have been developed to assay Hb-A_2 and glycosylated hemoglobins. The latter can also be measured more specifically by automatic chromatographic procedures. Thus, IEF has been introduced into

routine analysis for screening and characterization of abnormal hemoglobins in the adult and the newborn. This technique could also soon be applied to the antenatal diagnosis of hemoglobinopathies and to the assay of Hb-A_{1c}. The measurement of Hb-A_{1c} level by such a procedure could provide a useful tool for the detection of latent diabetes by large population screenings. Significant progress could emerge from future production of monospecific antibodies against normal and abnormal hemoglobins. These antibodies could be used to assay minor fractions (Hb-A_2, Hb-F, Hb-A_{1c}) and to identify variants. In the future, the combination of IEF and immuno-identification could provide the ideal system for convenient diagnosis of any abnormal hemoglobin of clinical interest.

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6. SUMMARY

This review primarily deals with methods for separations of hemoglobins.

An introduction considers electrophoretic methods as well as those involving isoelectric focusing and chromatography. The main advantages or disadvantages of each procedure are discussed after each technical description. The chromatographic methods are mainly limited to those used in clinical biochemistry.

The second section treats the main diagnostic problems typically met with in the field of the hemoglobinopathies and deals successively with the diagnosis of hemoglobinopathies in the adult and the newborn. Numerous variants have been described in the adult, and among them Hb-S and Hb-C variants are the most frequent. Unstable or high oxygen affinity variants of hemoglobin are also considered. Finally, a new strategy for diagnosis is proposed. A special section is devoted to the diagnosis of thalassemia syndromes. The prenatal diagnosis of hemoglobinopathies is also discussed in some detail with a view to preventing the birth of homozygous children.

This update ends with a chapter on the interest of the assay of hemoglobins A_{1c} in the pathology of diabetes mellitus.

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