Journal of Chromatography, 569 (1991) 197–228
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5947

Review

Isoelectric focusing in immobilized pH gradients: applications in clinical chemistry and forensic analysis

PIER GIORGIO RIGHETTI* and ELISABETTA GIANAZZA

Department of Biomedical Sciences and Technologies, University of Milan, Via Celoria 2, Milan 20133 (Italy)

ADRIANA BIANCHI-BOSISIO

Clinical Chemistry Laboratory, Ospedale di Cernusco sul Naviglio, Milan (Italy)

and

PRANAV SINHA and ECKART KÖTTGEN

Institute of Clinical Chemistry and Biochemistry, Universitätsklinikum Rudolph Virchow, Free University, Berlin (Germany)

(Received October 10th, 1990)

ABSTRACT

The applications of isoelectric focusing in immobilized pH gradients in clinical chemistry and forensic analysis are reviewed. Strong emphasis is given to the separation of serum proteins, in particular α_1-acidic glycoprotein, acid phosphatase, alkaline phosphatase, α_1-antitrypsin, apolipoproteins, complement component, factor B, factor XIIIB, group-specific component, lecithin:cholesterol acyltransferase, phosphoglucomutase, prealbumin, protein C and transferrin. The analysis of human parotid salivary proteins is discussed and an assessment is given of the state of the art in thalassaemia screening.

CONTENTS

0378-4347/91/$03.50

LIST OF ABBREVIATIONS

ACES	N-(2-Acetamido)-2-aminoethanesulphonic acid
ACP	Acid phosphatase
ALP	Alkaline phosphatase
α_1-AT	α_1-Antitrypsin
C3	Complement component 3
CA	Carrier ampholyte
DBP	Vitamin D-binding protein
DMAPMA	Dimethylaminopropylmethacrylamide
FXIIIB	Factor XIIIB
GC	Group-specific component
Hb	Haemoglobin
HDL	High-density lipoprotein
HPLC	High-performance liquid chromatography
IEF	Isoelectric focusing
Ig	Immunoglobulin
IPG	Immobilized pH gradient
K_M	Michaelis constant
LCAT	Lecithin:cholesterol acyltransferase
LDL	Low-density lipoprotein
M_r	Relative molecular mass
NP-40	Nonidet P-40
ORM	Orosomucoid
PAGE	Polyacrylamide gel electrophoresis
PC	Protein C
PGM	Phosphoglucomutase
p*I*	Isoelectric point
PI	Protease inhibitor
R.S.D.	Relative standard deviation
SDS	Sodium dodecylsulphate
Tf	Transferrin
TTR	Transthyretin
VLDL	Very-low-density lipoprotein

1. INTRODUCTION

Isoelectric focusing (IEF) in immobilized pH gradients (IPG) represents the latest development in focusing methods, *i.e.*, electrophoretic techniques able to create and maintain a pH gradient in an electric field throughout the duration of the separation process until the attainment of steady-state conditions. Conventional IEF is based on the use of a multitude of soluble, amphoteric buffers (carrier ampholytes, CAs), able to migrate electrophoretically to their isoelectric point (p*I*) values and, once there, to maintain a constant pH value by exerting a good buffering capacity and providing good conductivity [1]. Conversely, in IPGs the pH gradient is generated prior to the electrophoretic step by casting a gradient gel with the aid of a two-vessel gradient mixer. The buffers (weak acrylamido derivatives containing either carboxyl groups or tertiary amino groups) are covalently affixed to the polyacrylamide gel matrix, and this provides an indefinitely stable pH gradient [2].

The IPG technique was introduced in 1982 [3], but it has had a relatively slow growth owing to some problems connected with the chemicals used to create and maintain the pH gradients (available commercially under the trade name Immobiline from Pharmacia–LKB Biotechnology, Uppsala, Sweden). The second generation of these compounds (Immobiline II), launched in the summer of 1988 [4], now offers a trouble-free technique of unrivalled versatility and resolving power.

The specific advantages of focusing techniques over other electrophoretic procedures are as follows.

(a) To a large extent, the results do not depend on the mode of sample application, the total protein load or the time of operation, as both IEF and IPG are steady-state techniques; an intrinsic physico-chemical parameter of the variant protein (its p*I*) may be measured.

(b) Excellent resolution is possible between variants whose p*I* values differ by only 0.02 pH unit (if analysed in conventional IEF) or by *ca.* 0.001 pH unit (with IPGs); the protein bands are extremely sharp (focusing effect).

The advantages over chromatographic techniques are as follows.

(a) The typical sample load is of the order of 10–50 μg (100 times less when using silver staining), compared with several milligrams required by ion-exchange columns, and 200–300 μg in high-performance liquid chromatography (HPLC).

(b) CA-IEF may be completed in a few hours (considerably less when utilizing miniature gels in automated equipment, such the Fast System from Pharmacia–LKB); with IPGs, results are obtained within one day; several samples are analysed in parallel (>30 on a standard-sized slab and >60 in a gel split into two by a common central electrode).

Notwithstanding these advantages, whereas CA-IEF is enjoying wide popularity in clinical analysis (for a review, see ref. 5), IPG s have found few applications. This is probably due to two main reasons: the relative difficulty in mastering the technique and the labour-intensive and highly manual approach of the IPG method.

However, just as CA-IEF became fairly popular only after the introduction of ready-made gels in pH ranges tailored for specific clinical analyses, IPGs will enjoy greater popularity with the availability of ready-to-use gels. In fact, pre-cast IPG gels have recently been introduced in the following pH ranges: (a) pH 4.2–4.8 (*e.g.*, for α_1-antitrypsin analysis); (b) pH 4.5–5.5 [*e.g.*, for group-specific component (GC) screening]; (c) pH 5.0–6.0 (*e.g.*, for transferrin analysis); (d) pH 5.6–6.6. (*e.g.*, for phosphoglucomutase screening); and (e) pH 4–7 (for general purposes). In addition, especially for use with two-dimensional maps, wide pH 4–10 IPG strips have just been released.

This review is largely devoted to the analysis of serum proteins, as they assume particular importance as biochemical indicators in a host of diseases and are important markers in genetic analysis, owing to their ready availability in a pure form and to the facility for obtaining specific antibodies from commercial sources. From this point of view it should be emphasized that, strictly speaking, examples on the use of IPGs for clinico-chemical analyses are few, wherease there are many more reports on forensic and genetic analysis; however, the results can easily be transferred from one field to the others.

2. SERUM PROTEIN ANALYSIS

Modern clinical tests rely heavily on the qualitative and/or quantitative analysis of a host of serum proteins, as their alteration can often be associated with a particular disease. In addition, serum collection is readily feasible and serum proteins have been extensively mapped in the last decade [6].

2.1. α_1-Acidic glycoprotein

This protein (also known as orosomucoid, ORM) is an acute phase reactant with a molecular mass of *ca.* 40 000 dalton, a high carbohydrate content (up to 55% of neutral sugars) and a large number of sialic acid residues (up to 11%) [7]. After desialylation, the electrophoretic pattern of ORM shows three phenotypes, determined by two co-dominant autosomal alleles, OR^F and OR^S. By CA-IEF, a third allele for OR^F was described by Thymann and Eiberg [8] in a Danish population. ORM levels increase on infection, inflammation or malignancy and also during pregnancy or under pharmacological treatment. In biological terms, ORM shares about 50% sequence homology with immunoglobulins; it affects immuno responses by inhibiting lymphocyte mitogenesis and mixed lymphocyte reaction. ORM has been linked to ABO and adenylate kinase, and it has been assigned to human chromosome 9. By IPG analysis in a pH 2.8–4.5 gradient, ORM is fractionated into six bands, with p*I* values in the pH range 3.4–3.8 [9] (Fig. 1). The sera we have screened had a titre in ORM ranging from 0.17 to 3.02 mg/ml; as the gels were stained with Coomassie Blue, *ca.* 15 μl of the high-titre samples were sufficient for band detection. However, Eap and Baumann [10],

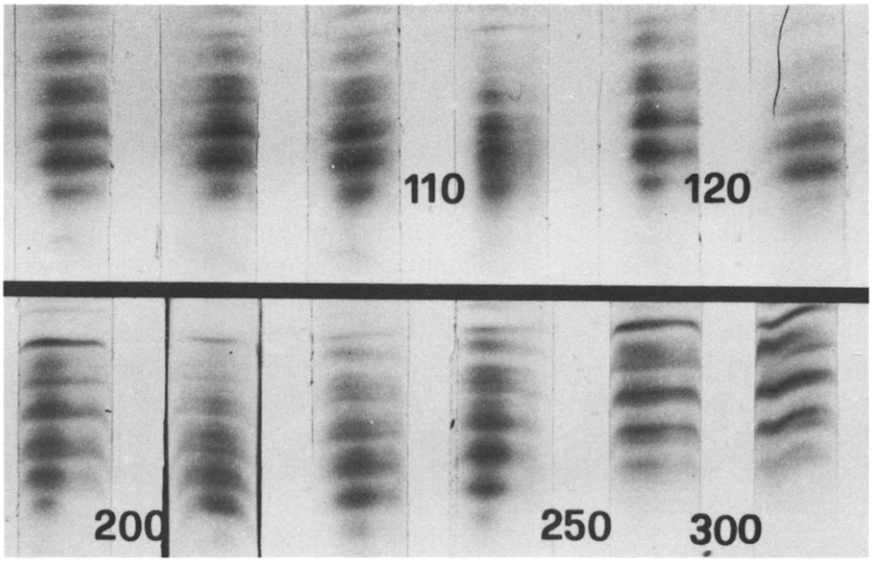

Fig. 1. α_1-Acidic glycoprotein patterns of some serum samples on pH 2.8–4.5 IPG matrices, ordered according to their increasing protein titre. The gel was a 3% C, 4% T polyacrylamide matrix, the average buffering power of grafted Immobilines being 9 mequiv. l^{-1} pH^{-1}. Run: overnight at 200 V, then 90 min at 1500 V, at 6°C. Staining with Coomassie Brilliant Blue. (From Gianazza *et al.* [9], with permission.)

who performed IPG runs with the desialylated forms (which exhibit the same polydisperse spectrum, only shifted by *ca.* 1 pH unit to higher values), could still detect the protein, after blotting and staining with an alkaline phosphatase-linked secondary antibody system, at a dilution of 1:28 672.

2.2. Acid phosphatase

Phenotyping of human erythrocyte acid phosphatase (ACP1) is widely applied in forensic science for, *e.g.*, paternity cases. ACP1 polymorphism was first demonstrated in 1963 by Hopkinson *et al.* [11], using starch gel electrophoresis. Today, however, CA-IEF is routinely used for screening ACP1, either in conventional gels [12] or in ultra-thin matrices [13,14]. Westwood and Sutton [15] applied IPG methodology to the analysis of ACP1, in the pH range 5.5–7.6, as most ACP1 isoforms have p*I* values in the pH range 5.8–7.5. For enzyme detection, the IPG gel, after the run, is overlaid with a foil of cellulose acetate soaked in 0.5 *M* citric acid (pH 5.0) containing 1 mg/ml 4-methylumbelliferyl phosphate. Development occurs in the dark at 37°C for 30 min and the gels are viewed under long-wavelength UV radiation. Successful runs require anodic sample application in slots. It is also possible to type seven-week-old blood stains by extracting the enzyme in the presence of freshly prepared 1% dithioerythritol. The six com-

mon phenotypes in blood lysates and stains (ACP1 A, B, C, CB, BA and CA) were readily identified in IPG gels; in fact, band separation was greater than in CA-IEF.

2.3. Alkaline phosphatase

The role of alkaline phosphatase (ALP) in laboratory and clinical analysis has recently been reviewed [16]. ALP is ubiquitous, being present in nearly every organ, although the highest activities are found in the intestinal epithelium, kidney tubules, osteoblasts, liver and placenta. It is closely associated with the cell membrane [17] and circulates as a dimeric glycoprotein, exhibiting an extensive intra- and inter-tissue charge heterogeneity and variations of the apparent relative molecular mass (M_r) of the purified enzyme. M_r values ranging between 130 and 220 kilodalton have been reported after butanol extraction, 128 kilodalton after detergent solubilization and 122 kilodalton after papain extraction. Three distinct gene loci for the enzyme have already been established, namely the gene for placental ALP, the gene for the adult intestinal ALP and a single gene locus, the socalled tissue-unspecific gene, for the ALP from liver, kidney and bone [18,19]. Extensive polymorphism is demonstrated only by the placental ALP gene, with the presence of three alleles (Pl^1, Pl^2, Pl^3), which give rise to six phenotypes (three heterozygous 2–1, 3–1, 3–2, and 1, 2 and 3 most likely homozygotes) of the heterodimeric enzyme. Further, at least fifteen rare alleles giving rise to 38 phenotypes of placental ALP have been reported [20].

Non-genetic causes for the microheterogeneity of ALP are post-translational modifications of the enzyme, mainly the presence of carbohydrate side-chains: within the same tissue, multiple banding patterns are usually due to terminal N-acetylneuraminic acid residues. Such polydispersity is in general abolished by extensive hydrolysis with neuraminidase [21].

In the routine clinical laboratory, ALP is most commonly used for the diagnosis and therapeutic observation of bone and hepatobiliary diseases. ALP has long been recognized as an osteoblastic marker, as osteoblasts and osteocytes are rich in ALP, but contain little ACP [22]. During osteogenesis ALP is shed into the blood-stream and used as an indicator of bone formation and turnover. In Paget's disease (ostitis deformans) and in familial hyperphosphatasaemia, ALP is the most frequently used biochemical marker [23]. In hepatobiliary diseases, a classical indicator of obstructive jaundice is a strongly elevated level of ALP in serum. During pregnancy, a new form, placental ALP, appears in the blood stream and reaches a maximum at delivery.

Sinha *et al.* [24] and Sorroche *et al.* [25] have applied IPGs to the study of serum ALP in Paget's disease and in hepatobiliary disorders. All known isoforms of ALP are separated in an Immobiline pH 3.5–6.0 gradient, the sample being applied into pockets cast on a pH 8.0 plateau. Normal sera show an array of about ten isobands isoelectric between pH 3.9 and 4.79. In Paget's disease, two

sharp isobands with p*I* values of 4.97 and 5.09 are seen. Placental ALP overlaps with the higher p*I* bands of normal serum; however, on heat destruction of the latter, it shows four sharp isobands with p*I* values of 4.59, 4.62, 4.67 and 4.73. In hepatobiliary disease, the high-M_r ALP isoforms in circulation ($> 10^6$ dalton) are disaggregated in zwitterionic detergents (2% sulphobetaine 3–12 in both sample and gel) and focus in the p*I* range 5–6 (in contrast with ALP in normal sera, which focus in the p*I* range 4–5). However, on treatment with neuraminidase, ALP from normal human sera increase their p*I* values in the pH range 5–6, typical of the hepatobiliary isoforms, sûggesting that the two populations differ in their respective degrees of glycosylation.

2.4. α_1-Antitrypsin (α_1-AT)

Also called PI (protease inhibitor), this is one of the highly polymorphic plasma proteins. Genetic variation was observed first in 1963 by Laurell and Eriksson

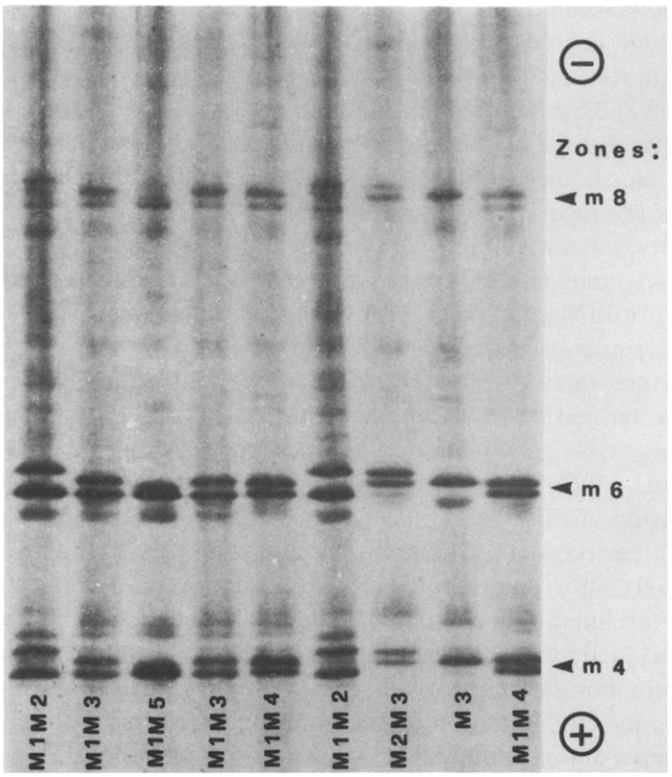

Fig. 2. Demonstration of PIM subtypes as analysed by IEF in IPGs, in the pH range 4.3–4.8, over a 17-cm electrode distance, giving ΔpH = 0.025/cm. Cathodic sample application in 6 × 6 × 0.25 mm slots. Coomassie Brilliant Blue staining. (Modified from Görg *et al.* [31], with permission.)

[26], who reported an inherited deficiency of PI associated with chronic obstructive pulmonary disease. This abnormality is transmitted with an autosomal recessive mode of inheritance. This genotype is now known as PI–ZZ, and is associated with progressive liver disease in infancy and early childhood.

Laurell and Eriksson [26] detected these early variants by agarose gel electrophoresis, while Fagerhol and Braend [27] used an acid–starch gel method. It was only in 1975 that Arnaud *et al.* [28] and Constans and Viau [29] independently suggested the use of CA-IEF in narrow pH ranges for screening of PI polymorphism and reported the first discovery of PI subtypes. Since then, CA-IEF has been the method of choice for the classification of PI variants. The most common allele is PI*M; several subtypes can be distinguished by IEF, which have the notations PIM1, PIM2, PIM3 and PIM4. It has become customary to take the PIM allele as a reference point and thus designate the anodal variants from B to L and the cathodal variants from N to Z.

IPGs were first applied by Görg and co-workers [30,31] and by Weidinger and Cleve [32] to the analysis of the PI system. It was immediately apparent that (a) the classification of the six common PIM1, M1M3, M3, M1M2, M2M3 and M2 could be readily and reliably accomplished, (b) the less frequent PI variants could be demonstrated with a high degree of resolution and (c) the PIZ variant could also be clearly identified in a wider IPG interval.

Görg and co-workers [30,33] proposed two IPG intervals for best resolution: a pH 4.5–4.7 gradient to optimize the pattern of PIM types and subtypes and a pH 4.4–4.8 range to differentiate anodically and cathodically located PI variants. Fig. 2 gives an example of the resolution afforded by the IPG method in PI analysis. At the time of these studies, no new variants were reported, but simply improved and much more reliable screening methods. Soon, however, Weidinger *et al.* [34] reported evidence for a fifth PIM subtype and for a new deficiency allele, called PI*Z Augsburg. Contemporaneously, Yuasa and Okada [35] discovered a new PI allele, called PI*Poki. More recently, Weidinger and Cleve [36] reported two additional PIM subtypes, termed PI Mpassau and PI Mlarisse (observed in an IPG pH range 4.45–4.75), while in Japanese populations a PI Mtoyoura was discovered by Yuasa *et al.* [37] (in an IPG pH range 4.35–4.65).

An interesting application of PI screening for forensic hemogenetics is due to Skoda *et al.* [38]. Over a period of three years, they analysed PI types in 347 putative paternity cases; owing to the reproducibility and accuracy of the IPG technique, 54 men were excluded from paternity solely on PI evidence. Even though the number of excluded men in paternity cases was below the theoretical single exclusion chance for non-fathers, for this particular polymorphism (the single exclusion chance in the case of PI should be 27%, compared with a found value of 15.6%, *i.e.*, 54 cases out of a total of 347), this was remarkably superior and more accurate than when using CA-IEF, even in presence of separators. On the basis of these analyses, Skoda *et al.* [38] concluded that the allele frequency from non-related individuals did not deviate from the Hardy–Weinberg equilib-

TABLE 1

DISTRIBUTION OF PI ALLELE FREQUENCIES IN VARIOUS EUROPEAN POPULATIONS

[a] From Alonso [39], with permission.

Population	*n*	M1	M2	M3	M4	S	Z	Others
Spain	164	0.5915	0.1829	0.1067	0.0213	0.0854	0.0122	–
Galicia (Spain)	480	0.6604	0.1146	0.0604	–	0.1490	0.0094	0.0062
Basques (Spain)	166	0.666	0.133	0.066	–	0.117	–	–
Barcelona (Spain)	938	0.6007	0.1908	0.0996	–	0.1044	0.0021	0.0021
Central Spain	103	0.665	0.1699	0.0679	0.0146	0.0825	–	–
Northern Italy	202	0.6757	0.1559	0.0817	0.0371	0.0297	0.0099	0.0074
Southern Italy	260	0.6692	0.1788	0.0788	0.0308	0.0250	0.0116	0.0038
Western Germany	730	0.6774	0.1411	0.1301	–	0.0322	0.0151	0.0041
France	2707	0.613	0.153	0.099	0.014	0.096	0.014	0.011

ria and corresponded well with know frequencies from German and other Causasian populations. They concluded from their experience with PI phenotyping that the method with IPG gels, despite its increased technological demands, represents a major improvement over conventional IEF in the distinction of phenotypes. Similar conclusions were also drawn by Alonso [39] in the screening of a Spanish population. He also studied the effect of reswelling ready-made IPG gels in the presence of different additives: inclusion of dithiothreitol does not improve the protein pattern, but the presence of 50 m*M* N-(2-acetamido)-2-aminoethanesulphonic acid (ACES), a popular additive in CA-IEF, sharpens the PI bands, allowing better distinction of PIM subtypes. Also, the addition of 20% sucrose or sorbitol shifts the relative positions of the M3 and M4 bands, preventing misclassification of these components.

Typical distrubutions of PI allele frequencies in various European populations are shown in Table 1. As a note of caution, however, it should be emphasized that not even IPGs can distinguish any possible subtype: thus, two new subtypes of M1 (M1 Val 213 and M1 Ala 213), discovered by genetic analysis, could not be separated by IEF techniques [40].

2.5. Apolipoproteins

Lipoproteins are macromolecular complexes of lipids and proteins that are synthesized mainly by the liver and intestine and catabolized by hepatic and extrahepatic tissues [41]. Their main physiological function is to transport dietary and/or endogenously synthesized lipids (*e.g.*, cholesterol, triglycerides and phospholipids) from one organ to another. In normal plasma, there are traditionally considered to be four protein classes: (a) chylomicrons, (b) very-low-density li-

TABLE 2

PROPERTIES AND COMPOSITION OF HUMAN PLASMA LIPOPROTEINS

Modified from Zannis [41], with permission.

Properties and composition	Lipoprotein class			
	Chylomicrons	VLDLs	LDLs	HDLs
Size (Å)	750–12 000	300–700	180–300	50–120
Density (g/ml)	0.94	0.94–1.006	1.019–1.063	1.063–1.21
Triglycerides (wt. %)	80–95	45–65	4–8	2–7
Phospholipids (wt. %)	3–6	15–20	18–24	26–32
Free cholesterol (wt. %)	1–3	4–8	6–8	3–5
Esterified cholesterol (wt. %)	2–4	16–22	45–50	15–20
Svedberg unit (S_f^0)	400[a]	20–400[a]	0–12[a]	0–9[a]
Electrophoretic mobility	Origin (cathode)	Pre-β	β	α
Proteins (wt. %)	1–2	6–10	18–22	45–55
Major apoproteins	A-I, A-IV, B, CI, CIII, E	B, E, CI, CII, CIII	B	A-I, A-II, E
Minor apoproteins	A-II, CII	A-I, A-II A-IV	CI, CII, CIII, E	CI, CII, CIII, D, E

[a] Corrected flotation rate at a density of 1.063 g/cm^3, expressed in svedbergs (10^{-13}cm/s. · dyn · g).

poproteins (VLDLs), (c) low-density lipoproteins (LDLs) and (d) high-density lipoproteins (HDLs) (Table 2). The plasma lipoproteins are spherical particles with cores of non-polar neutral lipid consisting of cholesteryl esters and triglycerides and coats or relatively polar materials assembled from phospholipids, cholesterol and proteins. The protein components of lipoproteins are called apolipoproteins and have been designated apoA-I, apoA-II, apoA-IV, apoB, ApoCI, apoC-II, apoC-III, apoD and apoE [42].

For most of the apolipoproteins, at least one physiological function has been identified, but often the metabolic significance is still not completely understood. Variants of most human apolipoproteins have been found, often associated with hyperlipidaemia, and sometimes with a tendency to develop atherosclerosis prematurely. Hence there is an increasing need to identify variants that could warrant therapeutic intervention, particularly before the occurrence of irreversible damage. Table 3 summarizes current knowledge on apolipoproteins and their association with human diseases [43–47].

Polymorphic forms of human apolipoproteins have been described for apoA [48], apoC-III [49], apoC-II [50], apoA-I [51] and apoA-IV [52], simply with the use of CA-IEF. Some of these forms exist owing to differences in sialic acid content, as with apoC-III [49], while apoE also demonstrates genetic polymorphism due to variations in primary structure [53]. Human apoA-I exhibits a few rare mutant forms, such as A-I-Milano [54], A-I-Marburg, A-I-Giessen [52] and

A-I-Munster [51]. In the last instance, Menzel *et al.* [55] decribed three apoA-I-Munster-3 variants, which were designated apoA-I ($Asp_{103} \rightarrow Asn$), apoA-I ($Pro_4 \rightarrow Arg$) and apoA-I($Pro_3 \rightarrow His$), to indicate the position in the chain and the type of mutation occurring. As these mutations involve charged amino acids, they could easily be identified by CA-IEF; however, the sequencing and chemical analysis were made possible by purifying each mutant in a preparative Immobiline gel in the pH range 4.9–5.9. Enough protein (*ca.* 25 mg) could be applied to the IPG gel to allow the recovery of purified zones. Interestingly, as the IPG run was performed in 6 *M* urea, the focused bands could be revealed by simply immersing the gel for a few minutes in distilled water, whereby the isoforms appeared as opaque bands.

Preparative IPG for the purification of apoA-I was also successfully used by Jabs *et al.* [56]. For the identification of apoA-I in analytical IPG gels, Holmquist [57] has proposed that, for blotting purposes, after the IEF run the IPG gel should be covered with a thin (*ca.* 1 mm) layer of 1% agarose (Litex LSL). Thus, after slicing away the plastic backing (the Gel Bond support, both from Marine Colloids, Rockland, ME, USA), an agarose–IPG gel sandwich is obtained with excellent mechanical properties, allowing electroblotting and immunoreactions to be performed without distortion of band patterns. In addition, the agarose layer prevents adhesion of the IPG gel to the nitrocellulose membrane, a common problem when blotting from IPG matrices.

Another class that has been extensively studied is the apolipoprotein C (apoC) family. ApoCs are components of both VLDLs and HDLs, the group being composed of apoC-I, apoC-II, apoC-$III_{0,1,2}$, apoC-IV and apoC-V [58]. The apoCs are polypeptides with different amino acid sequences and molecular masses between 6600 and 9700. ApoC-III is composed of three isoforms, designated C-III_0, C-III_1 and C-III_2, which have a sugar chain at threonine-74 consisting of one galactose, one galactosamine and no, one or two neuraminic acid residues [59]. ApoC-I is an activator of lecithin cholesterol acyltransferase [60], apoC-II activates extrahepatic lipoprotein lipase and apoC-III is an inhibitor thereof [61]. ApoC-II also inhibits hepatic lipase [62]. ApoC-II and apoC-III are therefore important regulators of lipolysis, and an apoC-II deficiency can give rise to severe hypertriglyceridaemia. Most analyses performed so far on apoCs have utilized CA-IEF; in fact, Bugugnani *et al.* [63] gave the following p*I* values for apoCs: apoC-III_0, 5.10; apoC-II, 5.01; apoC-III_1, 4.92; and apoC-III_2, 4.84. Recently, Haase *et al.* [64] applied IPGs, in the pH range 4.8–5.7, to the analysis of apoCs: according to them, the advantages of IPGs lie not only in higher resolution and sharper bands, but also in the better reproducibility from run to run. Weisgraber *et al.* [65] also utilized IPGs for the preparative fractionation of $apoE_3$ (in a pH 4.9–5.9 gradient) and of apoCs (in the pH range 4.0–5.0; the discrepancy with Haase *et al.* [64] is because in the latter instance the IPG gradient is corrected for the presence of 7 *M* urea in the gel, shifting the apparent pH interval from 4.0–5.0 to 4.8–5.7). As described above, in preparative IPGs the apolipoprotein isoforms are simply detected by soaking the gels in distilled water; after excising and mincing the bands, the apoEs and apoCs are extracted at 4°C in 100 m*M* Tris–HCl–4 *M* guanidinium chloride–1 m*M* EDTA (pH 7.4) for *ca.* 24 h.

TABLE 3

APOPROTEINS AND THEIR ASSOCIATION WITH HUMAN DISEASES

[a] From Zannis [41], with permission.

Apoprotein	Sites of synthesis	Plasma concentration (mg/ml)[a]	Molecular weight	Amino acid sequence of mature protein	Function[b]	Association with clinical disorders
A-I	Liver/intestine, minor sites[c]	1.0–1.2	28 000	243 AA	Activates LCAT	ApoA-I–apoCIII deficiency
A-II	Liver	0.3–0.5	8 500	77 AA		
A-IV	Liver/intestine	0.16	45 000	376 AA	Activates LCAT	
B-100	Liver/intestine	0.7–1.0	513 000	4536 AA	Receptor-mediated catabolism of LDLs	Abetalipoproteinaemia, normotriglyceridaemic abetalipoproteinaemia (B-100 deficiency)
B-48	Liver/intestine		243 000	Similar to amino-terminal portion (residues 1–2152) of B-100		
CI	Liver/adrenal	0.04–0.06	6 500	57 AA	Activates LCAT (moderately)	
CII	Liver/intestine	0.03–0.05	9 000	79 AA	Activates lipoprotein lipase	Familial type I hyperlipoproteinaemia
CIII	Liver/intestine	0.12–0.14	9 000	79 AA	Inhibits catabolism of apoE-containing lipoproteins	ApoA-I–apoCIII deficiency
D	Adrenal gland, kidney, pancreas, placenta, minor sites [d]	0.06–0.07	19 500	169 AA		
E	Liver/peripheral tissues	0.025–0.05	34 200	299 AA	Receptor-mediated catabolism of apoE-containing lipoproteins	Familial type III hyperlipoproteinaemia

[a] The plasma concentrations of apolipoproteins are derived from J. J. Albers, M. C. Cheung, S. L. Ewens and J. H. Tollefson, *Atherosclerosis*, 39 (1981) 395–409.

[b] LCAT = lecithin:cholesterol acyltransferase.

[c] Minor sites of apoA-I mRNA synthesis in fetal human tissues are the adrenal gland, kidney, gonads, ovaries, heart and stomach, whereas ApoE mRNA is present in every tissue analyzed.

[d] Minor sites of ApoD mRNA synthesis are the intestine, liver, spleen and brain.

A last class studied by the IPG technique is the apolipoprotein E (apoE). Although the concentration of apoE in serum is low compared with the other classes (apoA-I and apoB), it is present in all lipoprotein density classes in variable amounts [66]. As it is recognized by the LDL receptor [67] and specific remnant receptors [68], apoE plays an important role in lipoprotein metabolism, namely the catabolism of triglyceride-rich lipoprotein particles [69]. The protein is a single polypeptide of 299 amino acids of known sequence [70]. Three common alleles, E2, E3 and E4, exist at a single gene locus, leading to six different phenotypes that can be distinguished by IEF [71]. A number of less frequently occurring genetic apoE isoforms have been described [72,73]. In addition, each apoE isoform is found in plasma in a number of minor species, differing in their sialic acid content. In general, this produces a train of spots of progressively higher M_r and lower pI values.

Baumstark *et al.* [74] applied the IPG technique to the routine screening of apoEs. The runs are performed in Immobiline Dry Plates (Pharmacia–LKB) pH 4–7 (which allow the simultaneous screening of both apoEs and apoCs), reswollen in 8 *M* urea–30% glycerol–0.5% CAs. Interestingly, addition of CAs to the rehydration solution was found necessary to ensure the entrance of apoE molecules (applied in tabs at the cathodic gel edge). CAs were not necessary to focus apoA-I, apoA-II, apoA-IV, apoC-III and apoC-II isoforms. They also found the IPG technique to be superior to CA-IEF for resolution, reproducibility and (surprisingly) even simplicity (no doubt owing to the availability of precast gel plates).

The use of IPGs avoids several inherent disadvantages of conventional IEF, namely extremely low ionic strength, unknown buffering capacity and instability of the pH gradient. Theoretically, this technique should provide better resolution of apolipoprotein isoforms and especially the degree of sialylation should be readily distinguishable. We summarize here our experience with the IPG fractionation of apolipoproteins. No serious problems are encountered in the sample prepration for the analysis of C-II, C-III, A-I and A-II. In each instance it is sufficient to bring serum or lipoprotein fractions to a final concentration of 9 *M* urea, 5% 2-mercaptoethanol (or 20 m*M* dithiothreitol) and 2% CAs. For the identification of apoE isoforms it is preferable to delipidate serum samples in acetone–chloroform. Delipidation of samples was found to yield sharp bands in the typical apoE positions. It should be noted, however, that it is also possible to analyse apolipoproteins in on-delipidated serum samples. Also, there is no difference in the final steady-state pattern with or without added CAs in the gel. IPG gels in the absence of CAs have, however, the disadvantage of longer focusing times (30 000–40 000 V h, pH 4–7) as opposed to mixed-bed gels, which require much shorter times (*ca.* 15 000 V h).

A number of variations have been proposed for blotting from IPG matrices. In our experience, it is sufficient to wash the gels after electrophoresis in either distilled water or in 0.01% sodium dodecylsulphate (SDS) for 10 min, in order to reduce the urea concentration in the gels. The proteins are then blotted onto

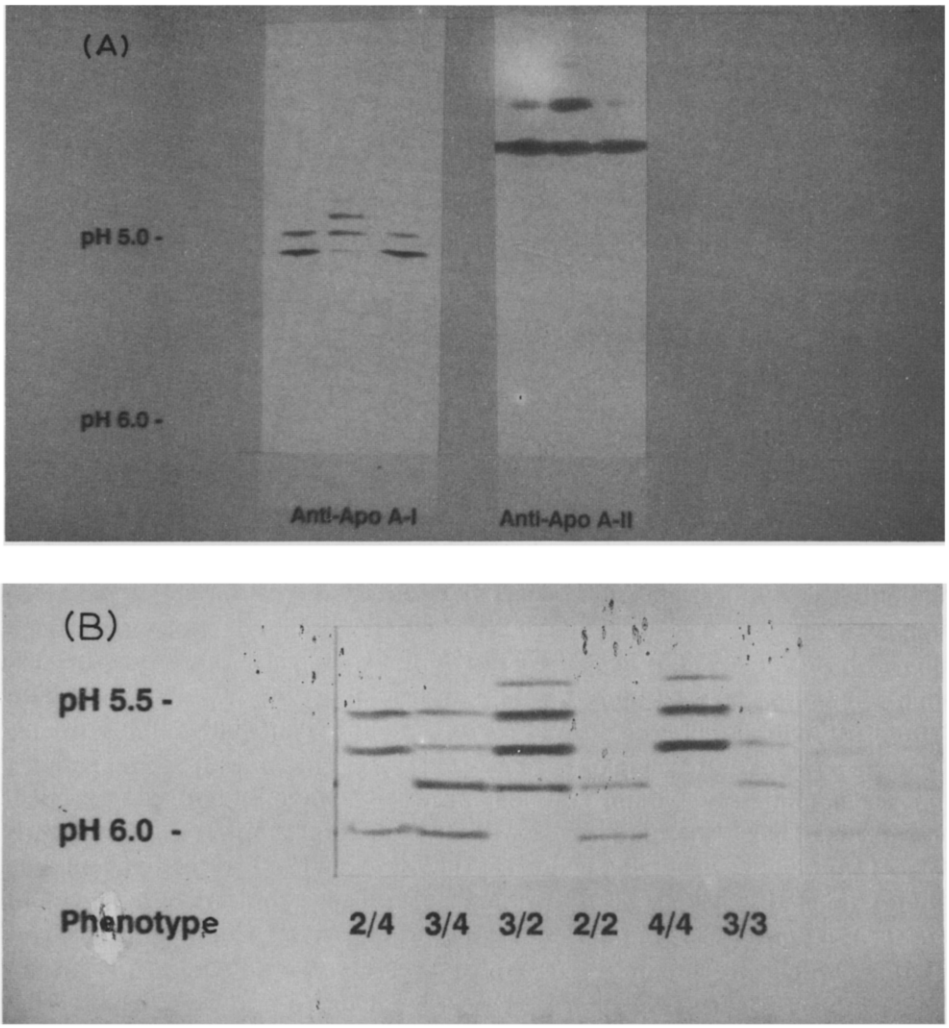

Fig. 3. IEF in IPGs of delipidized serum lipoproteins. (A) Separation of ApoA-I and ApoA-II; (B) separation of ApoE. The gels were 4% T, 4% C and contained a pH 4–7 IPG. A 100-μl volume of serum sample was delipidized using chloroform–acetone. After centrifugation and air-drying, the samples were dissolved in 100 μl of 9 *M* urea–2% pH 4–7 CAs–20 m*M* dithiothreitol. The gels were reswollen in 8 *M* urea–0.5% CAs–10 m*M* DTT. Volumes of 10 μl of serum samples were loaded in each track. Electrophoretic conditions: 300 V, 5 mA, 5 W for 2 h, followed by 2000 V for a total of 20 000 V h. After electrophoresis and a 10-min rinse in water or 0.01% SDS, the gel was capillary-blotted on a PVDF membrane. After blocking the membrane sites with 5% bovine serum albumin in phosphate-buffered saline, the bands were revealed with the peroxidase–antiperoxidase reaction. (P. Sinha, E. Köttgen and M. Kohlmeier, unpublished work.)

either nitrocellulose or preferably PVDF membranes using capillary blotting at elevated temperatures (37–50°C) for up to 1 h. This time is sufficient for optimum transfer of apolipoproteins to the membranes and for immunological identifica-

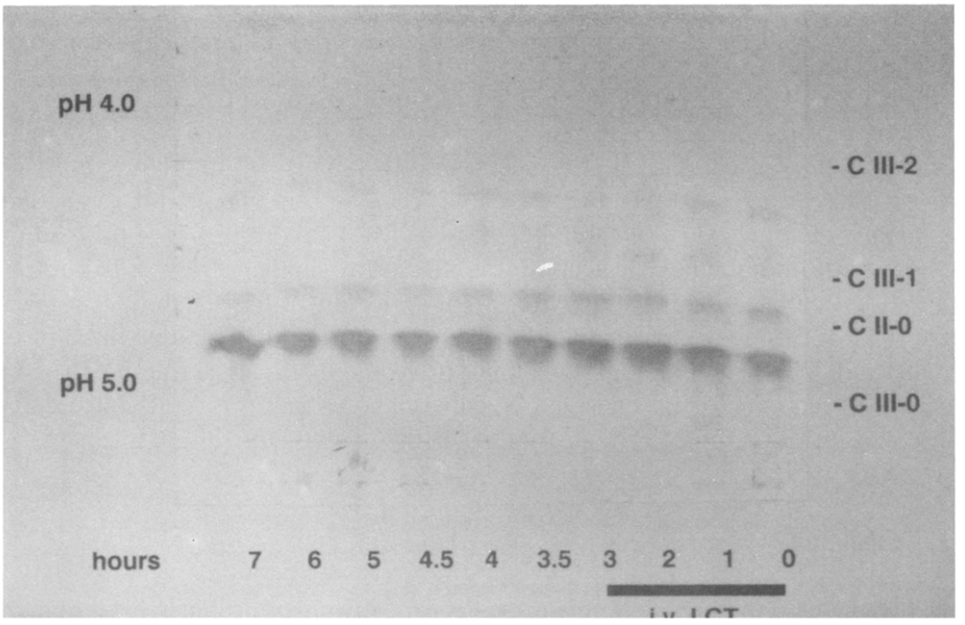

Fig. 4. IEF in IPGs of delipidized serum lipoproteins. Conditions as in Fig. 4, except that IPG pH 3.6–6.0 was used. Screening of ApoC-II and apoC-III species, followed by immunoblotting. (P. Sinha, E. Köttgen and M. Kohlmeier, unpublished work.)

tion using either the indirect peroxidase or for further amplification of the immuno signal by the peroxidase–antiperoxidase technique. Figs. 3 and 4 summarize our experience with focusing and immunoblotting of apoAs, apoEs and apoCs.

2.6. Complement component 3 (C3)

C3 is the most abundant complement component in human serum. It is a 185-kilodalton protein consisting of two non-identical polypeptide chains, called α (110 kilodalton) and β (75 kilodalton). The native C3 molecule is the specific substrate for the classical and the alternative C3/C5 convertases, the proteolytic cleavage of C3 being the critical step in the activation of the complement system. A genetic polymorphism of human C3 was demonstrated by Wieme and Demeulenaere [75] in 1967 and later confirmed by Alper and Propp [76] and Azen and Smithies [77]. They used electrophoresis in either starch or agarose gels. They described a system with two co-dominant alleles, C3*S (slow) and C3*F (fast). Additional studies revealed 22 less common variants [78].

The C3 locus has recently been located on human chromosome 19 [79] and shown to segregate independently from the major histocompatibility complex in man. A linkage has also been discribed between the C3 locus and the loci for apoE, ABH secretion, Lewis secretion and Lutheran blood group [80]. Like other

genetic markers in human blood, C3 polymorphism has been mainly used in forensic medicine and in anthropological studies [81].

Charlionet *et al.* [82] applied the IPG technique for a potential subtyping of the C3 system. Analysis was performed in very narrow IPG gels (pH 5.5–5.7) equilibrated in 10% saccharose, as this sugar seems to stabilize C3 in the proximity of its p*I*. These gels are unique in that itaconic acid was used as buffering group and dimethylaminopropylmethacrylamide (DMAPMA) as a titrant base (instead of commercial Immobilines). Detection was effected by capillary transfer to a nitrocellulose membrane, incubation with rabbit antiserum anti-C3, subsequent reaction with a secondary, peroxidase-labelled goat anti-rabbit immunoglobulin G (IgG) and final zymogramming in *o*-dianisidine and hydrogen peroxide. The aim of this subtyping is to find a correlation between potential C3 variants and certain diseases corresponding to the disfuntion of the components of complement, as suggested by Porter [83].

2.7. Factor B

The factor B of human complement is genetically polymorphic in most human populations, as first reported by Alper *et al.* [84]. They concluded that the factor B variants are coded by a series of codominant alleles at an autosomal locus BF. Two common alleles, BF*S and BF*F, predominate whereas two others, BF*SO7 and BF*F1, appear with a lower incidence in most populations. About then other alleles were described later and appear to be restricted to particular cases [85]. The method commonly used to detect factor B variants is high-voltage agarose gel electrophoresis at pH 8.6, followed by immunofixation [86]. More recently, factor B polymorphism has been studied by CA-IEF in polyacrylamide [87] or agarose gels [88], both followed by immunofixation. Davrinche *et al.* [89] applied the IPG technique to the screening of factor B polymorphism. They used two types of IPG ranges, either pH 5.2–6.1 or pH 5.4–5.9. They also, instead of using the Immobiline chemicals, produced the gradients by utilizing itaconic and methacrylic acids as buffering ions and DMAPMA as a titrant base. After focusing, the bands are transferred to nitrocellulose by quick capillary blotting (10 min), followed by a primary and a secondary immunofixation step, the latter utilizing horseradish peroxidase, which is then revealed by 3,3′-diaminobenzidine and hydrogen peroxide. No new subtypes have been found, at present, even with the IPG technique.

2.8. Factor XIIIB

In plasma, most of the coagulation factor XIIIB (FXIIIB), also known as fibrin-stabilizing factor, exists as a proenzyme with two A- and two B-polypeptide chains (A_2B_2). After activation with thrombin the B-subunits are cleaved off. Only the A-subunits have enzymatic activity and take part in the coagulation

process. The B-subunit, also designated as FXIIIB, F13B, B-protein or S-subunit, acts as a carrier protein for the A-chain and is also found in serum. FXIIIB is a glycoprotein with a molecular mass of 87 800 [90]; the carbohydrate moiety contains neuraminic acid. The p*I* is in the pH range 5.1–5.6 and after desialylation is increased to pH 5.6–8.8 [91]. The genetic polymorphism of FXIIIB was first described by Board [92] using agarose gel electrophoresis. Subsequently, by IEF, a series of new rare variants was discovered [93]. Leifheit and Cleve [94] have recently applied IPGs (in the pH range 5.6–6.6) to the analysis of FXIIB. After focusing, the zones are transferred to nitrocellulose by capillary blotting, precipitated with a primary antibody and then detected with a peroxidase-conjugated anti-rabbit IgG antiserum. The bands are revealed with 4-chloro-1-napthol in ethanol and hydrogen peroxide at pH 7.3. By this method, all common FXIIIB phenotypes (1, 2–1, 2, 3–1, 3–2 and 3) could be revealed with good precision and reproducibility. As this phenotyping is used in forensic haemogenetics, its was found that the exclusion rate in cases of disputed paternities, for non-fathers, with this system alone is 20.35%.

2.9. Group-specific component

The GC of human serum was discovered in 1959 by Hirschfeld [95]. Simultaneously and independently, a vitamin D-binding protein (DBP) was found in human serum [96]. The identity of the two proteins was established only in 1975 by Daiger *et al.* [97]. Since then, the system has been referred to as GC/DBP.

The genetic variants of this protein were originally classified by immunoelectrophoresis on agarose gels [86]. A major advance was the application of IEF in polyacrylamide gels, with subsequent immunoprint, allowing the separation of GC subtypes [98]. The currently employed methods for the analysis of the GC/DBP are CA-IEF, polyacrylamide gel electrophoresis (PAGE) and IEF in the presence of 3 *M* urea [99]. Routine classification of the different genetic types of the GC/DBP system for studies in population genetics, for investigation of disputed paternities and for twin diagnosis is usually carried out by CA-IEF. Six common GC phenotypes are distinguished, corresponding to six genotypes, determined by three alleles: GC*1F, GC*1S and GC*2. The notations for the six common GC types are thus 1F, 1S, 2, 1F-1S, 2-1F and 2-1S. Their mode of inheritance is autosomal co-dominant and the gene locus is mapped on the long arm of chromosome 4 at 4q12. In addition to the six common GS types, a total of more than 120 uncommon GC variants has been identified [100].

A number of worker have proposed IPGs for the analysis of GC/DBP. The first report was by Cleve *et al.* [101]. Subsequently, Westwood [102], Pflug [103,104], Pflug and Laczko [105] and Constans and Cleve [106] investigated various aspects of GC screening by the IPG technique. In particular, Pflug [103] proposed an IPG method based on pouring 250-μm-thick gels (compared with a customary 0.5 mm thickness) and suggested re-using the same gel, after washing

and drying, up to six times, for improved reproducibility. Pflug [103] also investigated the detection of the focused GC zones by direct immunofixation on cellulose acetate membranes overlaid on the IPG gel, as opposed to capillary blotting on nitrocellulose. The sensitivity of the former method was stated to be much better than that of the latter. By detection with an ALP-linked secondary antibody, Pflug [103] demonstrated a sensitivity of about 150 pg of GC in a 1:12 800-fold diluted serum samples. Even in six-year-old badly soluble blood stains kept at room temperature, GC could be phenotyped.

One point is noteworthy: according to Constans and Cleve [106], some rare variants, such as 1A23, 1A28, 1A30 and 1C53, which co-focus in CA-IEF, could only be separated in IPG gels. An interesting point (which applies also to focusing of α_1-AT) is that the order of p*I* values in CA-IEF *versus* IPGs varies for some of the mutants. The phenomenon is unexplained but a likely cause is that, as the two techniques act on the same principle, in CA-IEF some variants might not focus as "stripped" forms, but as complexes with carrier ampholytes: this outer CA coating would then be responsible for the observed p*I* in CA-IEF [107].

Pflug *et al.* [108] and Pötsch-Schneider and Klein [109] also applied IPGs to the typing in GC in human semen and vaginal fluid. This GC screening in semen stains seems to be of practical value in criminal investigations of sexual delinquencies. GC is present in normospermia and azoospermia seminal fluids and found in about 20% of the vaginal secretions. The GC patterns in these fluids were similar and in accordance with the bands of the individual GC type in plasma (serum).

Klein and Stiefel [110] reported that the use of Repel-Silane (dimethyldichlorosilane) as a surface coating on the cover of the gel reswelling cassette can produce blurred and split GC bands.

2.10. Lecithin:cholesterol acyltransferase

The esterification of the free cholesterol of human plasma lipoproteins is catalysed by lecithin:cholesterol acyltransferase (LCAT; EC 2.3.1.43). In this reaction a fatty acid acyl moiety of lecithin is transferred to the 3β-hydroxyl group of cholesterol, with the ultimate formation of cholesterol ester [111]. LCAT is a key component in the cholesterol transport process between plasma and tissue.

Purified LCAT shows charge polymorphism in CA-IEF [112]. This microheterogeneity reflects the presence of molecular species of the enzyme with a different number of sialic acid residues [113], even though there are discrepancies in the literature on the number of isoforms and their respective p*I* values.

Holmquist and Bjellqvist [114] applied the IPG technique to the analysis of LCAT polymorphism. Owing to the strong tendency of purified LCAT to aggregate in the absence of dissociating agents, the IPG run (in the pH range 4.2–4.9) had to be performed in the presence of 8 *M* urea, 0.5% Triton X-100 and 2 mmol/l 2-mercaptoethanol. Seven isoforms were characterized, having p*I* values

(at 15°C) of 4.37, 4.42, 4.48, 4.53, 4.60, 4.67 and 4.74 (S.D. = 0.03 in all instances); of these, the p*I* 4.48 isoform was the most abundant species.

2.11. Phosphoglucomutase

Phosphoglucomutase 1 (PGM1) polymorphism was first demonstrated by Spencer *et al.* [115] using starch gel electrophoresis. They discovered three different phenotypes: PGM1 1, PGM1 2-1 and PGM1 2. These phenotypes are coded for by two autosomal codominant alleles PGM1*1 and PGM1*2. By IEF, Bark *et al.* [116] demonstrated that the PGM1 polymorphism is determined by four alleles, thus resulting in ten different PGM1 phenotypes. Sutton and Burgess [117], Kühnl *et al.* [118] and Kühnl and Spielmann [119] confirmed the existence of the common PGM1 phenotypes.

Up to recent times, for routine forensic analysis of blood stains, PGM1 subtyping was performed essentially by CA-IEF in ultra-thin polyacrylamide or agarose gels. It was in 1984 that Sutton and Westwood [120] proposed the use of IPGs in the pH range 5.8–6.8, where the best separation of the four main PGM1 bands, 1A, 1B, 2A and 2B*, is achieved. At the end of the run, the enzyme is revealed by a zymogramming procedure, consisting on a 2% agarose overlay, containing 8 mg of NADP, 45 mg of glucose-1-phosphate (the latter in the presence of 1% glucose-1,6-diphosphate), 1.5 mg of phenazine methosulphate, 1.5 mg of MTT and 8 U of glucose-6-phosphate dehydrogenase in 10 ml of buffer (consisting of 0.3 *M* Tris–2.5 m*M* EDTA–4.9 m*M* $MgCl_2$–5 m*M* histidine · HCl, adjusted to pH 8.0). According to Pflug [121], by this method it is also possible to determine the PGM1 subtypes of semen stains, vaginal secretions and mixtures of both. In addition, it is possible to obtain PGM1 patterns directly from hair root cells. The hair roots are cut from pulled hairs and deposited directly on the IPG gel surface, whereby the enzyme forms are electroeluted during the run.

2.12. Prealbumin

As for prealbumin (also calles transthyretin, TTR) Altland and Banzhoff [122] demonstrated inherited variants by IPG in a pH 4–7 gradient rehydrated in the presence of 8 *M* urea, 50 m*M* DTT, 0.5% CA and 6% dextran 8. In order to clarify the region where TTR focuses, they used a technique called double one-dimensional electrophoresis, consisting in first subjecting the sample to a CA-IEF step, locating the band of interest, cutting it out of the gel with a blade and re-submitting the gel block to a second IPG run. In this way, the protein of interest is already desalted and free from neighbouring contaminating macroions. By this method they were able to detect a mutant (called TTR-Met^{30}) differing from the normal (TRR-Val^{30}) for a Met substituting a normal Val residue at position 30 from the amino end. This variant is consistently found in individuals with familial amyloidotic polyneuropathy of Portuguese type I.

2.13. Protein C

Protein C (PC) is a vitamin K-dependent plasma component that is a precursor of a serine protease [123]. It is a glycoprotein containing 23% carbohydrate, with an M_r of 62 000 dalton, composed of a heavy chain (M_r 41 000 dalton) and a light chain (M_r 21 000 dalton) held together by disulphide bonds. Incubation of PC with human α-thrombin results in the cleavage of a dodecapeptide (M_r 1400 dalton) from the amino terminal region of the heavy chain and in the formation of activated PC, an enzyme with serine amidase activity that has strong anticoagulant properties owing to selective inactivation of the active forms of factor V and VIII in plasma. There is evidence [124] that PC contains ten γ-carboxyglutamic acid residues per mole of protein, the two carboxyls in the γ-position being necessary for Ca^{2+} binding and for acting on the surface of biological membranes.

The importance of PC as a major regulator of blood coagulation is now established by the finding that patients with hereditary deficiencies of this protein often develop venous thromboembolic disorders [125], and by the discovery of low PC levels in acquired conditions associated with thrombosis, such as disseminated intravascular coagulation syndrome and the postoperative period [126]. Owing to

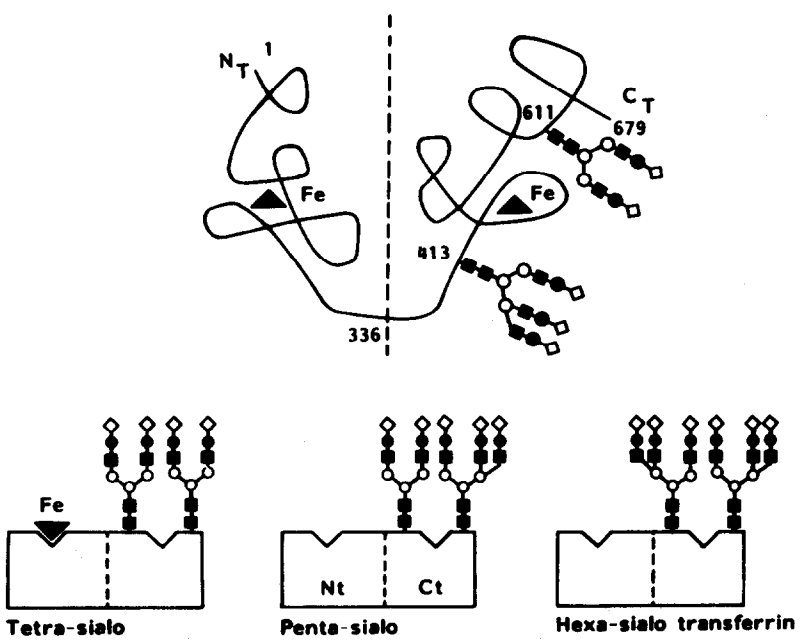

Fig. 5. Schematic representation of the polypeptide chain and two N-linked glycans in the C-terminal domain of three microheterogeneous forms of human transferrin. The dashed line indicates the division between the two globular domains, N_t and C_t, each of which can bind on Fe^{3+} iron. (From de Jong and Van Eijk [146], with permission.)

the lack of a properly purified preparation of PC and of specific antibodies, no studies had been performed on the surface charge properties of PC and on the possible existence of isoforms. Gelfi *et al.* [127] applied directly the IPG technique to the study of PC. The protein has been analysed in IPG gels in the pH range

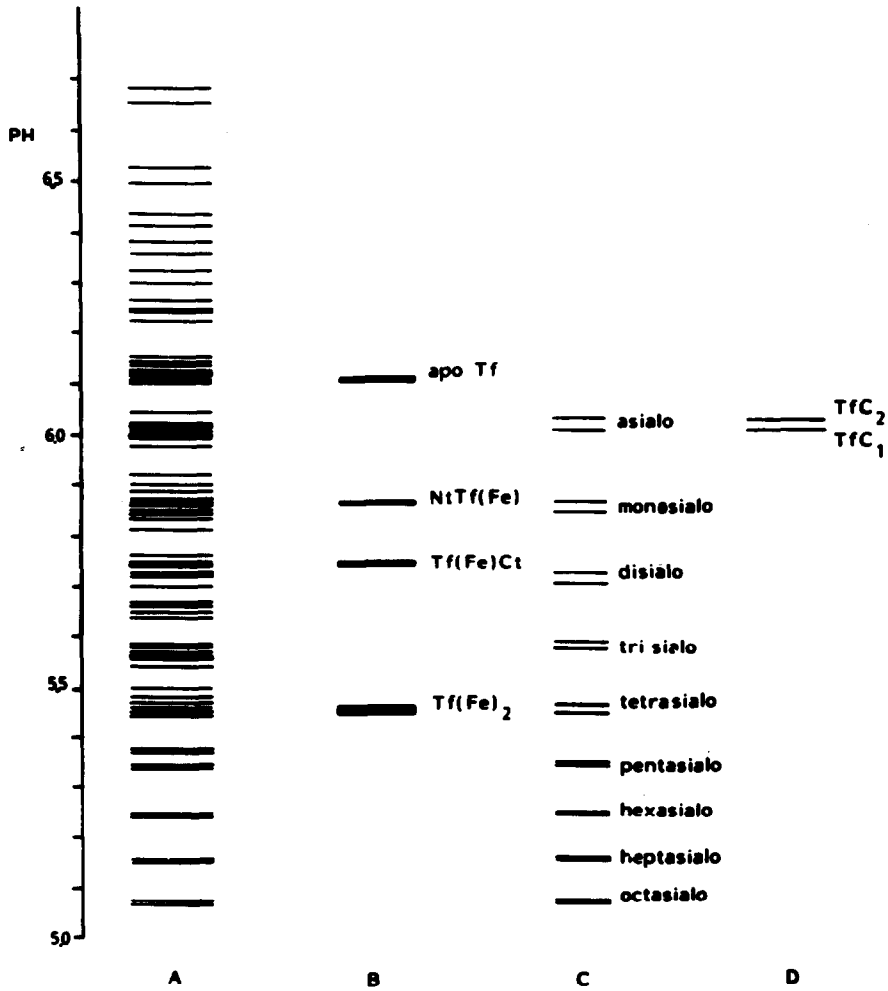

Fig. 6. Diagramatic representation of transferrin microheterogeneity of an individual with the C_1C_2 genotype detectable on IEF. (A) Total number of bands that can theoretically be distinguished on an Immobiline gel (as many as 72!). (B) Major bands as revealed by CA-IEF. Before the introduction of Immobilines, these bands, together with their genetically determined variations, were believed to comprise the total spectrum of transferrin heterogeneity. They correspond to the tetrasialotransferrins that can be separated because of differences in iron content. (C) Microheterogeneous forms of transferrins that can be separated and isolated from an iron-saturated serum sample. (D) Reduction of the number of bands from 72 (in A) to 2 by iron saturation and neuraminidase treatment of the serum sample, a procedure that can be useful in the assessment of genotypic variations. (From De Jong and van Eijk [146], with permission.)

4–6, in the presence of 8 *M* urea and 2 m*M* EDTA (the latter present initially in the gel and sample zone). After IEF, the IPG gel is separated from the plastic foil and electroblotted against a nitrocellulose sheet. After fixation with a primary antibody, the membrane is confronted with a peroxidase-labelled secondary antibody, which is then revealed with 4-chloro-1-naphthol and hydrogen peroxide. PC is thus resolved into six isoforms, having p*I* values (corrected for the presence of 8 *M* urea) of 4.80, 4.86, 4.92, 4.96, 5.02 and 5.1. If PC is activated (thus losing the amino-terminal dodecapeptide), it still shows the same group of six bands, with p*I* values *ca.* 0.5 unit higher. At present, no studies are available on the genetic background of these bands or on the possible linkage of some isoforms with coagulation disorders.

2.14. Transferrin

Transferrin (Tf, also called siderophilin) is an iron-binding, monomeric glycoprotein found in the biological fluids of invertebrates [128] and vertebrates [129]. The encoding gene for human Tf is on chromosome 3 [130]; also its complete amino acid sequence has been established [131].

Human Tf consists of a single polypeptide chain containing 679 amino acid residues and two N-linked complex-type oligosaccharide chains, which results in a calculated molecular mass of 79 570. The Tf molecule can be divided into two homologous domains, the N-terminal (residues 1–336) and the C-terminal domain (residues 337–679), with both sites of glycosylation in the carboxyl-terminal domain at positions 413 and 611 (Fig. 5). Each domain contains a metal-binding site, binding an FE^{3+} ion with a Michaelis constant (K_M) of *ca.* 10^{22} l/mol. The concomitant binding of an anion (carbonate or hydrogencarbonate) is essential for metal binding at each site. The bilobal structure together with the existence of a high degree of internal homology between the two domains [132] has led to the hypothesis [133] that mammalian two-lobed transferrins arose during the course of evolution by duplication and fusion of a gene specifying a simpler single-domain protein.

The first factor determining the electrophoretic behaviour of transferrin is its iron content. Under physiological conditions serum Tf is *ca.* 30% saturated with iron; consequently, in fresh serum four different forms of Tf with respect to iron content can be distinguished and isolated electrophoretically: apo-Tf, (Fe)NtTf, Tf(Fe)Ct and diferric transferrin (Fig. 6, track B). The second determinant of electrophoretic behaviour, genetic polymorphism, is best revealed by CA-IEF of serum samples that have been saturated with iron and then desialylated by neuraminidase treatment. In fact, genetic polymorphism of Tf was already reported in 1957 by Smithies [134], simply by zone electrophoresis in starch gels.

The most common phenotype in all human populations has been designed TfC, the more anodal variant (lower p*I*) TfB and the more cathodal form (higher p*I*) TfD. Up to 1978, TfC was considered to be a single variant. By IEF, however,

TfC was shown to consist of two subtypes, Tf^{C1} and Tf^{C2} [135,136]. Subsequently, a third, relatively common, allele (Tf^{C3}) was reported by Kühnl and Spielmann [137]; additional C variants were then detected by IEF, bringing the total to thirteen [138]. The third determinant of Tf electrophoretic behaviour is the carbohydrate moiety.

The two N-linked oligosaccharide chains of transferrin have been shown to be structurally variable [139]. The glycans can differ in their degree of branching as bi-, tri- and tetra-antennary structures have been shown to exist (Fig. 5). As a result, nine different Tf variants, with p*I* values ranging from 5 to 6, can be distinguished electrophoretically owing to differences in sialic acid content [140] (Fig. 6, track C).

The first to utilize IPGs for the analysis of Tf variants were Görg *et al.* [33]. In a subsequent study, Weidinger *et al.* [141], by screening 1125 unrelated individuals in Southern Germany, determined the following allele frequency for TfC: Tf*C1 = 0.787; Tf*C2 = 0.136 and Tf*C3 = 0.067.

A new subtype, called C10, was observed and identified for the first time by the IPG technique. Evidence for a Tfnull allele was obtained in a child and the putative father. By IPG analysis, Weidinger *et al.* [141] calculated a theoretical exclusion rate for paternity examinations, in the case of the Tf system, of 17.95%. The above data agree fairly well with the frequencies proposed by Scherz *et al.* [142], who also gave essentially the same value for the average chance of exclusion in a case of disputed paternity (18.19%). In addition, they found three new variants located in the cathodal region of Tf bands, and thus considered to be D variants. These three new Tf species were given the names of the towns of origin of the families of the carriers: $D_{Mortsel}$, D_{Sorens} and $D_{Bellegarde}$.

Scheffrahan [143] additionally reported the analysis of Tf subtypes among Brazilian indians. Pascali *et al.* [144] also analysed Tf in human sera, and gave guidelines for proper sample handling in IPGs. According to D'Alessandro *et al.* [145], CA-IEF has a distinct disadvantage over IPGs, in that the former technique always produces a multiplicity of bands, possibly due to a whole series of partially saturated Tf molecules, in which the iron is partially depleted during the run, owing to the strong chelating power of CAs. In fact, if we sum all the potential sources of microheterogeneity (genetic variability, partial iron saturation and differences in the carbohydrate moiety), they could potentially produce, in an individual, as many as 72 discrete zones focusing in the pH range 5.0–6.8, fully resolvable by the IPG technique (Fig. 6) [146]. Thus, analysis of Tf under uncontrolled conditions could lead to disaster. However, it is comforting to note that, if all the sources of this microheterogeneity are eliminated (by complete desialylation and full iron saturation), an individual with a C_1C_2 genotype should produce by IEF analysis only two (as opposed to 72!) zones (Fig. 6, track D).

3. HUMAN PAROTID SALIVARY PROTEINS

The analysis of human salivary proteins by IEF has been invaluable for providing information regarding genetic variants in salivary proteins, such as variant α-amylase isozymes [147], or for studying salivary protein abnormalities in cystic fibrosis [148], Sjögren's syndrome [149] and rheumatoid arthritis [150]. The problem with CA-IEF of human saliva is that it has a high level of electrolytes, whereas its protein content is low and variable (0.5–4.2 g/l), so that a desalting and concentration step is essential. Khoo and Beeley [151] applied IPGs to the analysis of saliva: broad (pH 4–9) and narrow (pH 3.5–5.0) IPGs have been used, followed by either Coomassie Brilliant Blue or silver staining. In the wide pH range, they were able to resolve 25–30 bands, with a total protein load of only 20 μg. The narrow pH range (3.5–5.0) was found to be particularly useful for studying acidic proline-rich proteins, whose variation has been attributed to genetic polymorphism [152] and has been associated with rheumatoid arthritis and Sjögren's syndrome. It is noteworthy that Khoo and Beeley [151] found that, over the same acidic pH range (3.5–5.0), carrier ampholyte focusing produced virtually no separations.

4. THALASSAEMIA SCREENING

Prenatal diagnosis of inherited haemoglobin (Hb) disorders has been adopted in laboratory practice over the last decade [153]. The usual method for the diagnosis of β-thalassaemia is the analysis of β-chain synthesis in foetal blood obtained by either foetoscopy or placental aspiration. The foetal reticulocytes are labelled *in vitro* and the radioactive globin chain analysed by different chromatographic or electrophoretic techniques. The widespread carboxymethylcellulose procedure of Clegg *et al.* [154] has been replaced by faster methods, such as HPLC [155] and electrophoresis in polyacrylamide gels [156] or on cellulose acetate membranes [157,158]. Already in 1979 Righetti *et al.* [159] had devised a method for the separation of globin chains by CA-IEF in the presence of urea and detergents. The detergent (Nonidet P-40, NP-40), probably by binding to denatured globins, greatly increases the separation between γ- and β-chains, thus allowing for a proper densitometric or fluorographic determination of their relative ratios. In addition, the detergent splits the γ-zone into two bands, which have been demonstrated to be the two phenotypes of the human foetal γ-globin chains, called A_γ and G_γ. According to Righetti *et al.* [160], this NP-40 effect might be due to preferential binding of the detergent micelle to the hydrophobic stretch ^{133}Met to ^{141}Leu in A_γ chains. On binding, the detergent could sorb the ^{132}Lys in this stretch into its Stern layer or bury it within the micelle, thus inducing a total loss of one proton from otherwise identically-charged phenotypes. This unique separation between A_γ- and G_γ-chains has made possible studies on the switch from G_γ- to A_γ-chain synthesis during the development and maturation of erythroblasts [161].

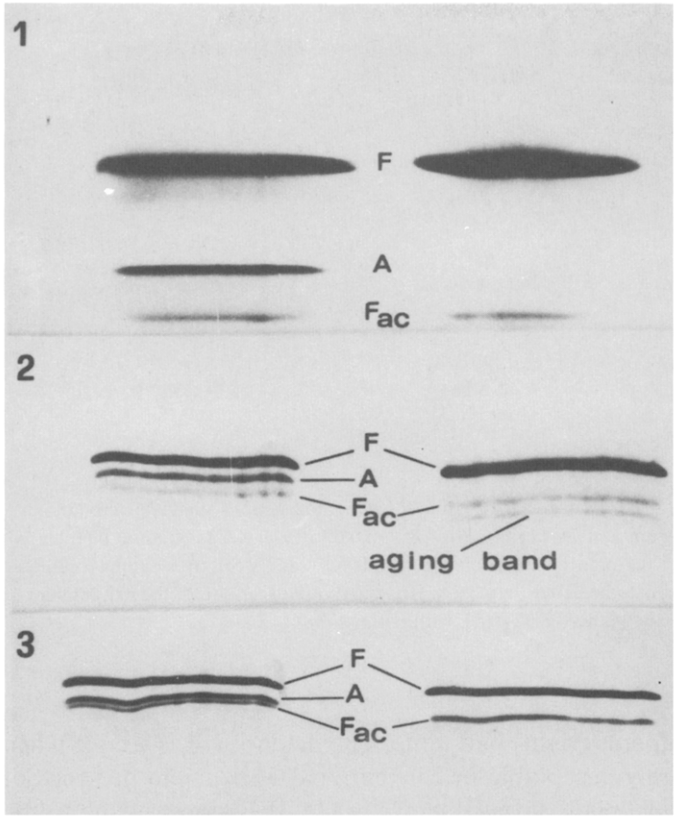

Fig. 7. Separation of Hb F, Hb A and Hb F_{ac} from foetal cord blood (week 18 of pregnancy). (1) IPG gel, pH 6.8–7.8; (2) pH 6–8 Ampholine added with 0.2 *M* β-alanine and 0.2 *M* 6-aminocaproic acid; (3) conventional pH 6–8 Ampholine. Left, normal foetus; right, homozygous β^0-thalassaemic foetus. Staining: Coomassie Brilliant Blue R-250 in Cu^{2+}. (From Manca *et al.* [163], with permission.)

With the advent of IPGs, owing to the high resolution intrinsic to the technique, it has been possible to perform diagnosis for thalassaemias directly on umbilical cord lysates in newborns and in foetuses. In reality, diagnosis of β-thalassaemia in newborns could also be performed well with the CA-IEF technique in the presence of separators, able to flatten the pH gradient in the region where Hb F, A and F_{ac} focus [162]. For antenatal diagnosis, however, because of the ethical problems connected with genetic counselling of couples at risk, and the possibility of abortion, a foolproof screening technique coupling a high resolving power with a high sensitivity for the detection of even minute amounts of Hb A in foetal blood is necessary. IPGs have proved to fulfil these requirements. Fig. 7 shows the separation of the three main components of cord blood (foetal, adult and acetylated foetal) in foetuses at the eighteenth week of pregnacy [163]. Normal foetuses produce substantial amounts of Hb A (left track), whereas a homo-

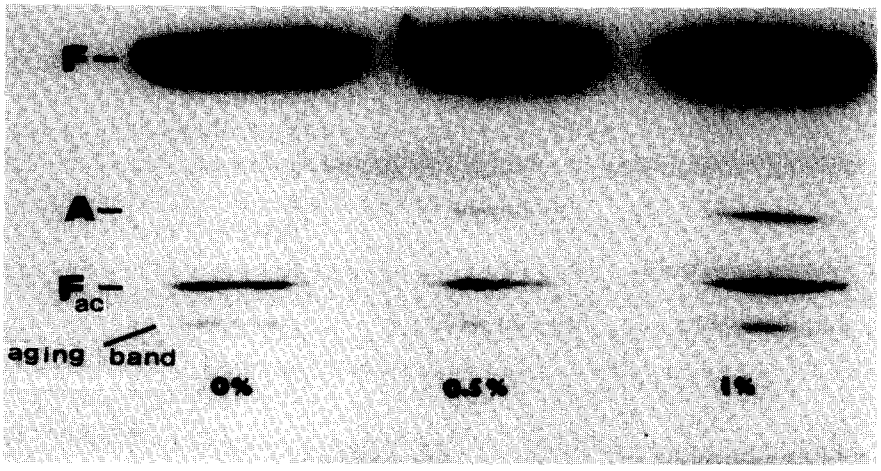

Fig. 8. Minimum detectability of Hb A in foetal cord blood. A homozygous β^0-thalassaemic umbilical lysate was added with increasing amounts of purified Hb A (from 0.5 to 1%). A total amount of 1.2 mg of protein per track was applied to a 0.5-mm-thick Immobiline gel, pH 6.8–7.6. By this overloading technique, the minimum detectability appears to be better than 0.5% Hb A without disruption of the pH gradient or distortion of protein bands. (From Manca *et al.* [163], with permission.)

zygous β^0-thalassaemic foetus (right) had none. The diagnosis was simple when utilizing IPGs (top), barely acceptable in conventional IEF in the presence of separators (central) and definitely unsatisfactory in CA-IEF alone, owing to the poor resolution afforded (bottom). In this last case, a correct diagnosis of the homozygous condition would be extremely difficult, especially at earlier weeks of pregnancy, when the amount of adult Hb could be below 1%.

Just to check how accurate the assessment would be when using IPGs, a homozygous β^0-thalassaemic umbilical lysate was added with increasing amounts of purified Hb A. As shown in Fig. 8, with the IPG method as little as 0.5% of Hb A could be clearly detected in the focusing pattern. Such a high sensitivity was made possible by grossly overloading this analytical gel. In the case of Fig. 8, as much as 1.2 mg of protein was applied per track, in an analytical gel only 0.5 mm thick. In CA-IEF such a high sample load would have produced pH gradient distortion, sample smearing and precipitation all along the separation track. These data are summarized in a review that gives practical hints on how to perform all the IPG steps [164].

IPGs have also proved extremely powerful in separating a number of "silent" variants *i.e.*, mutant Hbs bearing no charge changes in the replaced amino acids. Several examples can be found in the literature [165–168]. Recently, Paleari *et al.* [169] compared chromatofocusing and IPGs for the characterization of a number of Hb variants of clinical and geographical importance, including four silent variants (Hb Brockton, Hb Cheverly, Hb Köln and Hb Waco). Both techniques

TABLE 4

COMPARISON BETWEEN CHROMATOFOCUSING (CRF) AND ISOELECTRIC FOCUSING IN IMMOBILIZED pH GRADIENT (IPG): ANALYSIS OF HAEMOLYSATES FOR HAEMOGLOBIN VARIANTS

From Paleari *et al.* [169], with permission.

Hb variants examined (Hb X)	CRF				IPG				ΔpH[d]
	n	t_R (min)[a]	pH_{el}[b]	Hb X (%)	*n*	*d* (mm)[c]	p*I*	Hb X (%)	
J-Paris	3	+34.0	7.09(0.01)[e]	13.2	4	+19.6	7.08(0.02)[e]	12.2	0.01
Malmö	4	+11.0	7.22(0.04)	50.5	7	+10.1	7.16(0.02)	45.0	0.06
A_{1c}	5	8.0	7.36(0.05)	9.6	6	+3.9	7.22(0.01)	8.4	0.14
A	28	0.0	7.42(0.08)	0	17	0.0	7.26(0.04)	0	0.16
Brockton[f]					2	−4.0	7.27	ND[h]	ND
Köln[f]					2	−4.0	7.27	ND	ND
Cheverly[f,g]	1	+4.0	7.36	48.5	4	−5.0	7.27(0.01)	42.1	0.09
M-Saskatoon[g]	1	−4.0	7.50	37.9	2	−2.5	7.28	47.7	0.22
Waco[g]	4	+10.7	7.47(0.18)	38.8	4	−4.0	7.29(0.01)	47.8	0.18
F	3	+19.3	7.14(0.01)	69.4	4	−9.1	7.34(0.01)	71.9	−0.20
D-Los Angeles	3	−23.3	7.54(0.03)	43.7	5	−20.8	7.45(0.03)	47.4	0.09
Lepore	4	−29.0	7.52(0.09)	14.4	3	−22.5	7.46(0.02)	15.0	0.06
Hasharon	3	−22.0	7.53(0.04)	15.6	3	−23.2	7.49(0.03)	16.3	0.04
S	6	−14.9	7.49(0.12)	38.4	9	−25.5	7.50(0.02)	46.0	−0.01

[a] Retention time (difference with respect to that of Hb A).
[b] pH of elution.
[c] Separation from the Hb A band. The positive sign indicates that the band has an anodic mobility with respect to Hb A.
[d] $pH_{el} - pI$.
[e] Mean (and S.D.).
[f] IPGF range: 0.2 pH unit.
[g] Haemolysate analysed with a flow-rate of 12 ml/h.
[h] ND, not determined, because of sample instability (see text).

showed a good intra-run precision (relative standard deviation, R.S.D. = 0.87% for chromatofocusing and 0.27% for IPG) and a high and similar resolving power (0.01 pH unit in a 1 pH interval). However, only IPGs, when used in ultra-narrow pH intervals, were able to separate the four "silent" Hb variants mentioned above. Table 4 summarizes the chromatofocusing and IPG data pertaining to the separation of fourteen different Hbs.

5. CONCLUSIONS

Clearly the IPG technique has much to offer to the clinical chemist, especially when confronted with very difficult separation problems. However, the technique has so far been scarcely adopted in clinical analyses. In contrast, it is already

widely used in forensic medicine. We believe that the reason is that the IPG technique is still relatively labour-intensive and the casting of gradient gels is not routine for use in clinical laboratories. The availability of ready-made gels would greatly alleviate the problem and encourage more clinicians to enter the field. However, this step requires extra effort.

6. ACKNOWLEDGEMENTS

This work was supported in part by contracts form the Consiglio Nazionale delle Ricerche (CNR, Rome, Italy), Progetti Finalizzati Biotecnologie e Biostrumentazione and Chimica Fine II, and by the Ministero della Pubblica Istruzione.

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