Journal of Chromatography, 569 (1991) 297–321
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5955

Review

Electrophoretic phenotyping of erythrocyte enzymes

MASATERU KANE*

*Forensic Science Laboratory, Shiga Prefectural Police Headquarters, Ohtsu 520 (Japan) and *Department of Legal Medicine, Shiga University of Medical Science, Ohtsu 520–21 (Japan)*

and

TATSUSHIGE FUKUNAGA, YOSHIO YAMAMOTO, MITSUKO YAMADA and YOSHITSUGU TATSUNO

Department of Legal Medicine, Shiga University of Medical Science, Ohtsu 520–21 (Japan)

(First received January 29th, 1991; revised manuscript received March 25th, 1991)

ABSTRACT

Erythrocyte acid phosphatase (EAP), esterase D (ESD) and phosphoglucomutase (PGM) phenotypes among the erythrocyte enzyme types of blood groups are surveyed and a modified cellulose acetate membrane isoelectric focusing (CAM-IEF) method for their exploration is described. The phenotyping procedures are usually classified as either equilibrium or non-equilibrium IEF. Equilibrium IEF, which is based on differences in p*I* values, includes three methods: (i) a narrow pH range of carrier ampholytes, (ii) a relatively narrow pH range of carrier ampholytes containing chemical separators and (iii) immobilized pH gradient gels. Among the three methods, immobilized pH gradients provide a better resolution of isozymes. Conversely, the disadvantages of immobilized pH gradients include longer focusing times and complex gel preparations. Moreover, immobilized pH gradients are unsuitable for stain analysis because of the insensitivity of PGM1 detection. A hybrid IEF system and a commercial immobilized pH gradient dry plate have overcome these problems. However, EAP typing is extremely expensive and ESD typing is not well distinguished by hybrid IEF. As each method has both merits and demerits, the most suitable technique should be selected based on the kind of erythrocyte enzyme types and sample conditions. On the other hand, non-equilibrium IEF is a rapid method because isozymes are detected on the basis of their charge differences under non-equilibrium conditions. Moreover, the appropriate addition of chemical separators increases the charge difference and provides a good resolution within a shorter time. Addition of more separators produces a narrow pH range in the gel and takes a substantially longer time to reach the optimum pH range for charge difference. Further, the viscosity due to the excess addition of separators results in poor reproducibility for high-field-strength CAM-IEF. Hence the amount of chemical separators is taken into consideration of obtaining optimum results. If the optimum conditions are established with primary experiments, the combination of chemical separator and non-equilibrium IEF is proposed as a readily available method for routine analysis of erythrocyte enzyme types in forensic science.

0378-4347/91/$03.50

CONTENTS

LIST OF ABBREVIATIONS

ACES	N-(2-Acetamido)-2-aminoethanesulphonic acid
ADA	Adenosine deaminase
AK	Adenylate kinase
BES	N,N-Bis(2-hydroxyethyl)-2-aminoethanesulphonic acid
CAM	Cellulose acetate membrane
DTT	Dithiothreitol
EAP	Erythrocyte acid phosphatase
EPPS	N-(2-Hydroxyethyl)piperazine-N′-3-propanesulphonic acid
ESD	Esterase D
GOT	Glutamate oxaloacetate transaminase
G-6-PD	Glucose-6-phosphate dehydrogenase
GPT	Glutamate pyruvate transaminase
HEPES	N-(2-Hydroxyethyl)piperazine-N′-2-ethanesulphonic acid
HLA	Human leukocyte antigens
IEF	Isoelectric focusing
Ig	Immunoglobulin
IPG	Immobilized pH gradient
MOPS	3-(N-Morpholino)propanesulphonic acid
PAG	Polyacrylamide gel
6-PGD	6-Phosphogluconate dehydrogenase

PGM	Phosphoglucomutase
p*I*	Isoelectric point
Tf	Transferrin

1. INTRODUCTION

Analytical electrophoresis is a technique for protein separation used widely in biochemical research and clinical chemistry. In the medico-legal field, it is a powerful technique for exploring blood groups, especially for identification or paternity testing. More than 150 different kinds of blood groups are known, being divided into four types. The first type is blood group antigens, including ABO typing. These are detected by agglutination, based on an antigen–antibody reaction. The second is serum types, of which polymorphism is derived from serum proteins, immunoglobulins (Igs) and complements. The third is erythrocyte enzyme types, which are considered in this review. The genetically determined systems include erythrocyte acid phosphatase (EAP or ACP1), phosphoglucomutase (PGM1), 6-phosphogluconate dehydrogenase (6-PGD), glutamate pyruvate transaminase (GPT), esterase D (ESD), glucose-6-phosphate dehydrogenase (G-6-PD), adenosine deaminase (ADA), adenylate kinase (AK), glutamate oxaloacetate transaminase (GOT), etc. The fourth type is the human leukocyte antigens (HLA) system, which is a histocompatibility antigen.

Inasmuch as routine analysis of serum and erythrocyte enzyme types are performed with conventional electrophoresis, several methods have been developed to increase the resolution of proteins and the sensitivity of detection. One of these is isoelectric focusing (IEF), which has contributed dramatically to the analysis of transferrin (Tf) in serum types. The allelic frequency of Tf C by conventional electrophoresis is greater than 98% for most populations. However, IEF makes it possible to demonstrate the heterogeneity of Tf C1–C8 [1–6], and its resolving power gives rise to sharper bands. In general, sharper bands result from a significant increase in protein per unit gel volume. With increasing enzyme activity, EAP can be sensitively phenotyped even in ageing blood stains [7]. Further, a new supporting medium has been developed based on the principle that buffering groups are linked to the medium; also, the immobilized dry plate has recently been made commercially available. In routine analysis, polyacrylamide or agarose gels are most commonly used as supporting media for IEF.

The cellulose acetate membrane (CAM) is not used for IEF in spite of its operational simplicity, because drying of CAMs during high-voltage IEF results in poor reproducibility. However, we were able to improve on CAM-IEF and applied it to the analysis of EAP and ESD under non-equilibrium conditions [8]. In this paper, we review EAP, ESD and PGM1 phenotypes with emphasis on the non-equilibrium IEF method.

2. PHENOTYPING

2.1. Erythrocyte acid phosphatase typing

In 1963, Hopkinson *et al.* [9] first described the genetic polymorphism of EAP (or ACP1) (EC 3.1.3.2) using starch gel electrophoresis. EAP is governed by three co-dominant autosomal alleles, namely *EAP*A*, *EAP*B* and *EAP*C*, all located on chromosome 2. The total frequency of the three phenotypes, *i.e.*, CA, CB and C, is about 8% in the Caucasian population, whereas it has been found to be less than 0.1% in the Japanese [10–13].

Fig. 1 shows diagrams of the isozyme patterns of the six common phenotypes of EAP by IEF. In 1977, Burdett and Whitehead [14] demonstrated EAP phenotyping by IEF, but the haemoglobin and EAP A band (combination of A_1 and A_2 bands) overlapped. Randall *et al.* [15] reported that non-equilibrium IEF overcame this problem and showed the separation of A_1 and A_2 bands. As shown in Fig. 1, A_1 and A_2 bands are far from the B_1 (or C_1) band, whereas B_1–B_4 and C_1–C_4 bands are localized in the same position. Although the A phenotype is easily differentiated from the other phenotypes, determination of the B, C, CB, BA and CA phenotypes relies on the intensities of C_1, B_2 and/or both bands. Therefore, the diffusion of bands, especially B_1, B_2, C_1 and C_2 bands, leads to mistyping. In comparison with starch gel electrophoresis, IEF minimizes band diffusion during electrophoresis, thereby allowing more accurate analysis. It has been suggested that an isotachophoretic mechanism might be involved [16,17]. It was not long before methods to preclude band diffusion during isozyme detection were developed. Destro-Bisol and Ranalletta [18] showed sharper isozyme patterns by using hydrophilic cellophane film soaked in prewarmed substrate solution at 50°C to shorten the incubation time. In addition, the isozyme patterns were revealed without removing the film from the gel. Budowle and Gambel [19]

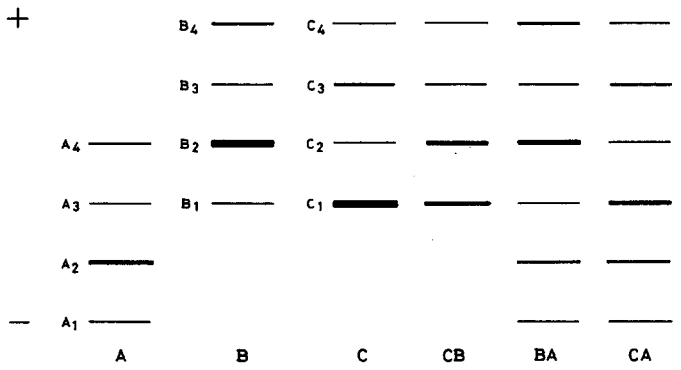

Fig. 1. EAP isozyme patterns obtained by IEF. As bands B1–B4 and C1–C4 are shown at the same positions, determination of the B, C, CB, BA and CA phenotypes relies on the intensities of C1, B2 and/or both bands. Hence band diffusion often causes mistyping.

demonstrated non-diffusing and non-fading fluorogenic zymograms, using the substrate 4-trifluoromethylcoumarin phosphate and cellulose diacetate membranes. Recently, we applied CAM-IEF to an analysis of EAP phenotyping [8]. Direct detection on membranes without transfer provided sharper bands comparable to those of Destro-Bisol and Ranalletta [18].

Methods for EAP phenotyping are summarized in Table 1. Most of these, with the exception of those of Burdett and Whitehead [14] and Carracedo and Concheiro [20], were performed under non-equilibrium conditions. Although B_1 (or C_1) was far from the B_2 (or C_2) band in all methods, A_1 and A_2 could not be separated in several of the methods [18,21,23,26,27,30]. Among several pH range carrier ampholytes, pH 4–8 and 4–6.5 were useful for separating A_1 and A_2 bands [15,22,24,28,29,31]. Further, Carracedo and Concheiro [20] and Frank and Stolorow [24] described no major difference in isozyme patterns between agarose gel and polyacrylamide gel (PAG). This is taken to suggest that the pH range of carrier ampholytes is one of the factors critically affecting isozyme patterns. We were thus able to modify the isozyme patterns by using a mixture of carrier ampholytes [8]. Minakata and Asano [25] showed the separation of A_1 and A_2 bands using pH 5–7 carrier ampholytes, although Budowle [21] and our group [8] were unable to replicate this. This discrepancy is attributed to the charge difference of isozymes under non-equilibrium conditions.

We shall refer to the effect of the pH range of carrier ampholytes on isozyme patterns under non-equilibrium conditions in Section 4.1.1.

2.2. Esterase D typing

Esterase (EC 3.1.1.1) has several isozyme components (A, B, C and D) in erythrocytes. In 1973, Hopkinson *et al.* [32] demonstrated the genetic polymorphism of ESD, using starch gel electrophoresis and the fluorogenic substrate 4-methylumbelliferyl acetate. ESD is governed by two co-dominant autosomal alleles, namely *ESD*1* and *ESD*2*, both located on chromosome 13. The frequency of ESD2 has been found to be lower in the Caucasian and Negro populations, but higher in the Mongolians [33–35]. Subsequent studies using starch gel or agarose gel electrophoresis revealed the existence of rare alleles, *ESD*3*–*ESD*6*, and a silent allele [36–40]. In medico-legal practice, ESD polymorphism has been detected not only in erythrocytes and blood stains, but also in various organ tissues, hair roots and dental pulps [41–43].

Fig. 2 shows the isozyme patterns of the three common phenotypes (ESD1, ESD2–1 and ESD2), ESD5 and ESD7 variants by IEF. As related here, ESD7 occurs in the Japanese at polymorphic frequencies [44]. In the early stage of IEF adoption, it is much more difficult to discriminate the three common phenotypes because the difference in isoelectric points (p*I*) between ESD1 and ESD2 is too small to allow a distinct separation with a relatively narrow pH range of carrier

TABLE 1

METHODS FOR EAP PHENOTYPING

PAG = polyacrylamide gel; CAM = cellulose acetate membrane; MOPS = 3-(N-morpholino)propanesulphonic acid; HEPES = N-2-(hydroxyethyl)piperazine-N'-2-ethanesulphonic acid; Pre = prefocusing; F = focusing; P = power.

Ref., year	Supporting medium	pH range of carrier ampholytes; chemical separators	Electrode distances (cm)	Focusing condition	Focusing time (min)	Separation of A_1 and A_2 bands
Burdett and Whitehead [14], 1977	PAG	pH 5–9 (pH 5–7–pH 7–9, 1:1)	~10	200 V, 20 mA initial, 1150 V, 14 mA end	150	Combination
Randall *et al.* [15], 1980	PAG	pH 4–8 (pH 4–6–pH 6–8, 1:1)	13	Max. 1600 V, 10 W	75	Separation
Carracedo and Concheiro [20], 1982	Agarose gel and PAG	pH 4–8 (pH 4–6–pH 6–8, 1:1)	Not given	Max. 1200 V, const. 15 W	75	Combination
Budowle [21], 1984	PAG	pH 5–7	5.4	Pre, 250 V; F, 500–2300 V const. P	Pre, 5; F, 17	Combination
Finney *et al.* [22], 1985	PAG	pH 4–8 (pH 4–6–pH 6–8, 1:1); MOPS	12	Pre, 500 V, 150 mA; F, 2500 V, 150 mA, 10 W	Pre, 15; F, 90	Separation
Yuasa *et al.* [23], 1985	PAG	pH 4–8 (pH 4–6–pH 6–8, 1:1)	10	2000 V, 8 mA, 9 W	90	Combination
Frank and Stolorow [24], 1986	Agarose gel and PAG	pH 4–8 (pH 4–6–pH 6–8, 1:1)	~10	1600 V, 5 W initial, 1600 V, 10 W reset	15 75	Separation
Minakata and Asano [25], 1986	PAG	pH 5–7	10	Const. 800 V	120	Separation

Minakata and Asano [26], 1986	CAM	pH 5–8	10	Const. 800 V	50	Combination
Alonso and Gascó [27], 1987	PAG	pH 5–8	4.5	450 V, 1.5 W, 15 mA	35	Combination
Zamir [28], 1988	PAG	pH 4–8 (pH 4–6–pH 6–8, 1:1); MOPS	23	Pre, 700 V, 150 mA, 10 W; F, 4000 V, 10 W	30 90	Separation
Shutler and Tompkins [29], 1988	PhastGel (PAG)	pH 4–6.5	3.2	2000 V, 0.2 mA, 3.5 W 200 V, 0.2 mA, 3.5 W, etc.	10	Separation
Destro-Bisol and Ranalletta [18], 1988	PAG	pH 3.5–10 (pH 3.5–10–pH 5–8, 7:20)	~10	800 V, 2 W	120	Combination
Dimo-Simonin *et al.* [30], 1989	Agarose gel	pH 4.5–7 (pH 4.5–5–pH 5–7, 1:3); MOPS, taurine	9.5	Pre, 1500 V, 150 mA, 3W; F, 1500 V, 150 mA, 3 W 1500 V, 150 mA, 10 W	Pre, 15; F, 15 45–50	Combination
Stockwell *et al.* [31], 1990	PAG	pH 4–6.5; MOPS, HEPES	16	Pre, 2000 V, 8–10 mA; F, 2000 V, 10 mA 2500 V, 10 mA	Pre, 30; F, 10	Separation
Kane *et al.* [8], 1990	CAM	pH 5–8 (pH 5–6.5–pH 5–8, 5:1)	6	Max. 1200 V, const. 4 W	30	Separation
		pH 5–7 or pH 5–8	6	Max. 1200 V, const. 4 W	30	Combination

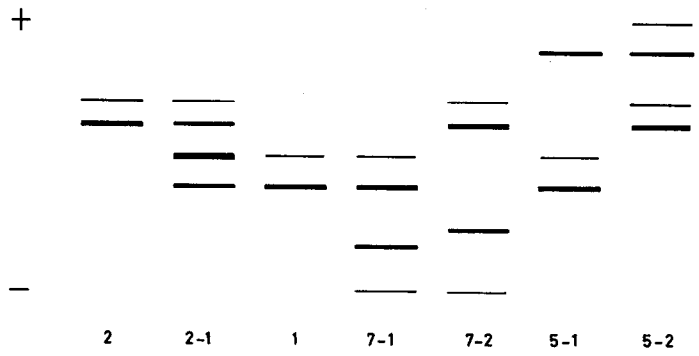

Fig. 2. ESD isozyme patterns obtained by IEF. The three common phenotypes (ESD1, ESD2–1, and ESD2) are usually discriminated by either conventional electrophoresis or IEF. It is usually difficult to differentiate more anodic variants of ESD5 and more cathodic variants of ESD7 from the three common phenotypes by conventional electrophoresis.

ampholytes under equilibrium conditions. However, IEF provided a good separation of ESD5 and ESD7 from ESD1 and ESD2, whereas with conventional electrophoresis it proved difficult [44–46].

Table 2 shows the methods for ESD phenotyping. In 1984, Divall [47] demonstrated the utility of non-equilibrium IEF using time-chase investigations, indicating that the two important factors for successful and reliable ESD phenotyping were focusing time and gel temperature. Moreover, Yuasa *et al.* [48] performed non-equilibrium IEF under low voltage and observed no major difference in pH gradient in the gel between low- and high-voltage IEF. They suggested that the better resolution resulted from charge differences under non-equilibrium conditions rather than from differences in p*I* values.

On the other hand, Budowle [49,50] demonstrated the separation of the three common phenotypes and ESD5 variants under equilibrium conditions with narrow pH range carrier ampholytes. In addition, Budowle and Gambel [51] modified the isozyme patterns with a mixture of several narrow pH range carrier ampholytes. Weidinger and Henke [52] obtained a good resolution of isozyme patterns with pH 4.5–5.4 carrier ampholytes in agarose gel IEF. Recently, Destro-Bisol and Spinella [53] reported on the combination of chemical separators and non-equilibrium IEF, using pH 4–6.5 carrier ampholytes and N-(2-hydroxyethyl)piperazine-N′-2-ethanesulphonic acid (HEPES). We applied CAM-IEF to analyse ESD phenotypes and demonstrated that pH 5–6.5 carrier ampholytes, combined with N-(2-hydroxyethyl)piperazine-N′-3-propanesulphonic acid (EPPS), allowed sufficient resolution within 25 min [8].

According to the report of Gill and Sutton [54], addition of HEPES resulted in a greatly flattened pH gradient with a linear pH range between 5 and 5.5. The addition of EPPS to the gel produced considerable flattening of the gradient,

resulting in a linear pH range between 5.6 and 5.9. Thus, with pH 5–6.5 carrier ampholytes, the addition of less EPPS produces flattening in the middle of the pH gradient formed in the gel (pH 5.6–5.9 range). With pH 4–6.5 carrier ampholytes, the addition of less HEPES results in a similar effect. This flattening effect increases the charge difference of isozymes under non-equilibrium conditions and provides good resolution.

We shall refer to the effect of concentration of chemical separator on isozyme patterns under non-equilibrium conditions in Section 4.2.2.

2.3. Phosphoglucomutase typing

PGM (EC 2.7.5.1) has several isozyme components (PGM1–PGM4) in erythrocytes and most other tissues. In 1964, Spencer *et al.* [55] reported on the genetic polymorphism of PGM1 using starch gel electrophoresis. PGM1 is governed by two co-dominant autosomal alleles, namely *PGM1*1* and *PGM1*2*, both located on chromosome 1. Subsequent investigations have shown that PGM2 and PGM3 were governed by separate chromosomes [56,57]. In contrast with conventional electrophoresis, IEF is capable of demonstrating four co-dominant autosomal alleles at the PGM1 locus and separating phenotypes into 1+, 1+1−, 1−, 2+, 2+2−, 2−, 1+2+, 1−2+, 1+2− and 1−2− [58]. In addition, PGM1 polymorphism is useful for medico-legal practice not only in erythrocytes and blood stains, but also in semen and hair roots [59,60].

Fig. 3 shows common isozyme patterns of PGM1 obtained by conventional electrophoresis and by IEF. Among the Caucasian, Negro and Mongoloid populations no significant difference in genetic frequencies of PGM1 is shown [61,62].

Table 3 shows the methods for PGM1 phenotyping. In 1983, Divall and Ismail [63] demonstrated the utility of non-equilibrium IEF. Gill and Sutton [64] improved the isozyme patterns by investigating the following parameters: mixtures of several pH range carrier ampholytes, thinness of the gel, focusing time, focusing conditions and application point. Moreover, Gill and Sutton [54] tested the resolution of isozyme patterns by adding various separators to the gel, thus obtaining the optimum pH gradient necessary for the separation of PGM1. They demonstrated that addition of EPPS to the gel produced a gradient range of pH 5.6–5.9 [54]. This narrow gradient provided a good resolution because the p*I* of PGM1 phenotypes ranged from pH 5.7 to 6.0 [65,66]. Budowle *et al.* [67] reported that on addition of EPPS the distance between each PGM1 band was at least twice that in previous methods [56,57,63,68–70]. However, the best separation was obtained by IEF in immobilized pH gradient (IPG) gels [71–73].

Righetti [74] stated that the advantages of IPGs were as follows: (i) increased resolution; (ii) unlimited stability; (iii) insensitivity to salt and buffer disturbances from the sample; (iv) increased load capacity; and (v) higher reproducibility than in carrier ampholyte gels. In medico-legal practice, advantages ii, iii and v are useful for PGM1 phenotyping because wavy or distorted isozyme patterns often

TABLE 2

METHODS FOR ESD PHENOTYPING

PAG = polyacrylamide gel; CAM = cellulose acetate membrane; MOPS = 3-(N-morpholino)propanesulphonic acid; BES = N,N-bis(2-hydroxyethyl)-2-aminoethanesulphonic acid; HEPES = N-2-(hydroxyethyl)piperazine-N′-2-ethanesulphonic acid; EPPS = N-(2-hydroxyethyl)piperazine-N′-3-propanesulphonic acid; Pre = prefocusing; F = focusing; P = power.

Ref., year	Supporting medium	pH range of carrier ampholytes; chemical separators	Electrode distance (cm)	Focusing condition	Focusing time (min)	Equilibrium or non-equilibrium
Olaisen *et al.* [45], 1981	PAG	pH 4–6	20	Const. 1100 V	360	Equilibrium
Dykes *et al.* [46], 1982	Agarose gel	pH 4–6.5	Not given	2000 V, 5W 2000 V, 10 W	10 45	Equilibrium
Nishigaki and Itoh [44], 1984	PAG	pH 4–7 (pH 4–6–pH 5–7, 2:1)	~20	Pre, const. 0.5 mA/cm; F, 0.5 mA/cm, 1800 V, 20 W	30 240	Equilibrium
Divall [47], 1984	PAG	pH 4–6	18	Const. 1900 V	60–180	Non-equilibrium
Budowle [49], 1984	PAG	pH4.5–5.5(pH4.5–5–pH5–5.5, 1:3.4)	8	Pre, 250 V; F, 500–3000 V, const. P	Pre, 15 F, 38	Equilibrium
Yuasa *et al.* [48], 1985	PAG	pH 4–6.5	9.5	1000 V, 4 W	70–80	Non-equilibrium
Finney *et al.* [22], 1985	PAG	pH 4–8 (pH 4–6–pH 6–8, 1:1); MOPS	12	Pre, 500 V, 150 mA, 5 W; F, 2500 V, 150 mA, 10 W	Pre, 15 F, 90	Non-equilibrium
Budowle [50], 1986	PAG	pH4.5–5.4–pH4–6 or pH4–6.5,9:1; BES	9.5	Pre, 250 V; F, 500–3500 V, const. P	Pre, 25 F, 70	Equilibrium

Alonso and Gascó [27], 1987		pH 4–6.5; HEPES	4.5	550 V, 1.8 W, 15 mA	45	Non-equilibrium
Zamir [28], 1988	PAG	pH 4–8 (pH 4–6–pH 6–8, 1:1); MOPS	23	Pre, 700 V, 150 mA, 10 W; F, 4000 V, 10 W	30 90	Non-equilibrium
Budowle and Gambel [51], 1988	PAG	pH 4.5–5–pH 5–5.5–pH 4.5–5.4–pH 4–6, 1.1:3.9:5:1	9.5	Pre, 250 V; F, 500–3000 V, const. P	Pre, 25 F, 60	Equilibrium
Weidinger and Henke [52], 1988	Agarose gel	pH 4.5–5.4	~10	1500 V, 20 mA, 16 W	Pre, 25 F, 120	Equilibrium
Destro-Bisol and Spinella [53], 1989	PAG	pH 4–6.5; HEPES	~10	600 V, 2 W	90	Non-equilibrium
Dimo-Simonin *et al.* [30], 1989	Agarose gel	pH 4.5–7 (pH 4.5–5–pH 5–7, 1:3); MOPS, taurine	9.5	Pre, 1500 V, 150 mA, 3 W; F, 1500 V, 150 mA, 3 W 1500 V, 150 mA, 10 W	Pre, 15 F, 15 45–50	Non-equilibrium
Stockwell *et al.* [31], 1990	PAG	pH 4–6.5; MOPS, HEPES	16	Pre, 2000 V, 8–10 mA; F, 2000 V, 10 mA 2500 V, 10 mA	Pre, 30 F, 10 60	Non-equilibrium
Kane *et al.* [8], 1990	CAM	pH 5–6.5; EPPS	6	Max. 1200 V, const. 4 W	25	Non-equilibrium

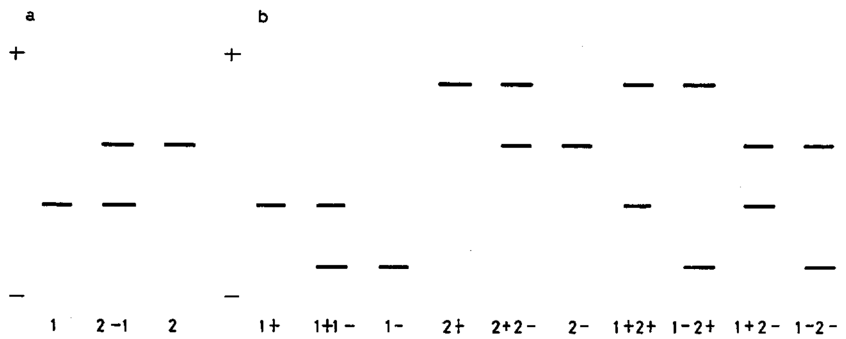

Fig. 3. PGM1 isozyme patterns obtained by conventional electrophoresis and by IEF. (a) PGM1 phenotypes by conventional electrophoresis are classified into three common phenotypes. (b) IEF is capable of resolving PGM1 locus into four alleles and ten subtypes are revealed.

cause mistyping. Conversely, the disadvantages of IPGs include a longer focusing time and complex gel preparations [74]. Moreover, IPGs are unsuitable for stain analysis because of the insensitivity of PGM1 detection. Therefore, a hybrid IEF system has been developed [75,76] in which IPG gels are rehydrated in a low-concentration solution of carrier ampholytes. This method allowed shortening of the separation time and prevented contamination of the sample lane [77]. In addition, Burgess *et al.* [72] reported that the sensitivity of PGM1 detection by the hybrid IEF system was twice that of the carrier ampholyte IEF system or ampholyte–separator IEF system.

Recently, the availability of a commercial IPG dry plate has reduced the complexity of gel preparation [78]. On the other hand, Pflug [79] introduced wedge gels which combined the features of ultra-thin gels, such as good resolution, high sensitivity and small sample size, with the benefits of thicker gels, such as high loading capacity and improved pH gradient stability. Destro-Bisol and Spinella [53] reported on the combination of chemical separators and non-equilibrium IEF, using pH 5–7 carrier ampholytes and EPPS. Although carrier ampholytes IEF and ampholyte–separator IEF are sufficiently capable of phenotyping PGM1 from blood or relatively fresh stains, it may be difficult to analyse ageing stains with low enzyme activity. Hybrid IEF overcomes this problem because of the advantages cited earlier, and also its high sensitivity of detection [72,74].

However, Muñoz-Barús *et al.* [73] described that EAP typing by hybrid IEF was extremely expensive because a wider pH range for EAP entails five different immobilines. Further, the EAP A band was not perfectly separated from haemoglobin. In addition, ESD1 and ESD2 alleles were not well distinguished by hybrid IEF [73]. Therefore, these methods must be applied based on the kind of erythrocyte enzyme types and sample conditions.

3. CELLULOSE ACETATE MEMBRANE ISOELECTRIC FOCUSING

As the use of PAG or agarose gel as a medium for IEF is now well established, we describe here a modified CAM-IEF version.

3.1. Sample preparation

Erythrocyte lysates are prepared from saline-washed, centrifuged erythrocytes by freezing and thawing. The lysates are diluted eight-fold with 0.05 dithiothreitol (DTT) for EAP typing and four-fold for ESD typing. The pretreated samples are kept for 15 min at room temperature before typing.

Divall [47] observed that numerous bands appeared on the cathodic side of ESD isozyme patterns if samples were not pretreated with DTT. Dimo-Simonin *et al.* [30] reported that the intensity of the main bands decreased whereas that of the minor bands increased without this pretreatment. In most previous studies lysates were diluted with 0.05 *M* DTT, although various dilution ratios for EAP and ESD phenotypings were applied. According to the methods of Randall *et al.* [15] and Divall [7], lysates for EAP typing were diluted 1:1 with DTT and further diluted 1:4 or 1:6 with distilled water prior to application. Samples for ESD typing were prepared by diluting two- or four-fold with DTT. It is assumed that the different dilution ratio is derived from the isozyme patterns. EAP phenotyping relies on the intensities of bands, whereas ESD is phenotyped based on the positions of the bands. EAP phenotyping requires sharper isozyme patterns because band diffusion decreases the intensity of isozyme profiles. Recently however, the lysates for EAP typing were diluted two- or four-fold with DTT [18,20,22,23,27,30]. Although this dilution is capable of phenotyping EAP, broader bands still appear occasionally. We were able to obtain sharper bands with unambiguous discrimination by diluting lysates with DTT [8]. Therefore, we recommend the eight-fold dilution for EAP typing.

3.2. Membrane preparation

Carrier ampholytes, pH 5–6.5 (LKB, Bromma, Sweden) or pH 5–8 (Pharmacia, Uppsala, Sweden) were diluted ten-fold with 10% (w/v) sucrose solution. The different concentrations of EPPS were tested for ESD separation under non-equilibrium conditions. EPPS was added to pH 5–6.5 carrier ampholyte solution at a final concentration of 2.5 or 5% (w/v).

CAMs (7 cm × 10 cm × 0.14 mm; Separax EF, Fuji Film, Tokyo, Japan) were floated on the carrier ampholyte solution for 30 s and placed on a glass plate cooled on a flat bed (Pharmacia FEB 3000) at 4°C. Excess of solution was removed by blotting with filter-paper.

In 1975, Harada [80] described IEF on Separax EF by analysing 6-PGD in erythrocyte enzyme types and α_1-antitrypsin in serum types. He listed the follow-

TABLE 3

METHODS FOR PGM1 PHENOTYPING

PAG = Polyacrylamide gel; IPG = immobilized pH gradient; HEPES = N-2-(hydroxyethyl)piperazine-N′-2-ethanesulphonic acid; EPPS = N-(2-hydroxyethyl)-piperazine-N′-3-propanesulphonic acid; MOPS = 3-(N-morpholino)propanesulphonic acid; MES = 2-(N-morpholino)ethanesulphonic acid; Pre = prefocusing; F = focusing; P = power.

Ref., year	Supporting medium	pH range of carrier ampholytes; chemical separators	Electrode distances (cm)	Focusing conditions	Focusing time (min)
Dykes *et al.* [66], 1982	Agarose gel	pH 4–8 (pH 4–6.5–pH 6–8, 1:1)	9	2000 V, 6 W	15
				2000 V, 8–12 W	45
Divall and Ismail [63], 1983	PAG	pH 5–7	12	Const. 1 W, max. 1600 V	180
Gill and Sutton [64], 1984	PAG, 1 mm	pH 5–7	9.5	Const. 9 mA, 600 V, 6 W	210
				Const. 22 mA, 800 V, 18 W	90
		pH 5–7–pH 4–6, 3:1		Const. 25 mA, 750 V, 20 W	90
		pH 5–7–pH 6–8, 2:1		Const. 18 mA, 850 V, 15 W	120
		pH 5–7–pH 7–9, 3:1		Const. 22 mA, 750 V, 17 W	90
		pH 5–7–pH 4–6–pH 6–8, 3:1:1		Const. 25 mA, 700 V, 17 W	120
		pH 4–6.5		Const. 22 mA, 700 V, 15 W	Pre, 30; F, 120
	PAG, 0.15 mm	pH 5–7–pH 6–8, 3:1		Const. 2000 V, 10 mA,	180
		pH 5–7–pH 4–6–pH 6–8, 3:1:1		Const. 2000 V, 9 mA	180
Sutton and Westwood [71], 1984	IPG gel	pH 5.8–6.8	Not given	1000 V, 5 mA, 5 W	10
				2000–3500 V	120

Gill and Sutton [54], 1985	PAG	pH 5–7; HEPES or EPPS or MES	9.5	Const. 1 W, max. 1500 V Const. 4 W,	30 150
Pflug [79], 1985	PAG (wedge gel)	pH 5–7–pH 4–5, 3:1	~ 12	2000 V, limit. 7–9 mA	100–120
Budowle *et al.* [67], 1986	PAG	pH 5–7; EPPS	8	Pre, 250 V F, 500–2300 V, const. P	Pre, 25 F, 55
Burgess *et al.* [72], 1987	IPG gel and PAG	pH 5.6–6.6 (IPGs)	Not given	1000 V, max. 5 W 2000 V	30 150
		pH 5–7; EPPS	9.5	Max. 1500 V, const. 1 W Const. 4 W	30 150
		pH 5–7	9.5	Max. 1200 V, 9 W	210
Zamir [28], 1988	PAG	pH 4–8 (pH 4–6–pH 6–8, 1:1); MOPS	23	Pre, 700 V, 150 mA, 10 W; F, 4000 V, 10 W	30 90
Destro-Bisol and Spinelli [53], 1989	PAG	pH 5–7; EPPS	~10	700 V, 2 W 1200 V, 3.5 W 2000 V, 4.5 W	35 45 45
Mañoz-Barús *et al.* [73], 1989	IPG gel	pH 5.6–6.6	~10	3000 V, 4 mA, 7 W	180
Stockwell *et al.* [31], 1990	PAG	pH 4–6.5; MOPS, HEPES	16	Pre, 2000 V, 8–10 mA; F, 2000 V, 10 mA 2500 V, 10 mA	Pre, 30 F, 10 60

ing advantages of Separax EF: (i) no gel preparation needed; (ii) ready for use soon after soaking Separax EF in the optimum carrier ampholyte solution; (iii) separation of higher-molecular-mass proteins such as IgM in the membrane, etc. Farrell *et al.* [81] also reported on the three lysosomal acid hydrolytic enzymes (arylsulphatase, hexosaminidase and β-galactosidase) using Separax EF. On the other hand, Ambler [82] described IEF on Cellogel (Chemetron, Milan, Italy) which was pretreated with boron trifluoride in methanol. Although Dobosz and Koziol [83] phenotyped PGM1 by Cellogel-IEF, a seven-day period for membrane preparation was required. Cellogel strips were washed overnight in methanol and methylated in 5% boron trifluoride for 2 h at 45°C. Later, methylated Cellogel strips were washed for 48 h in methanol and five days in distilled water. Separax EF is stored dry and is readily made available for use by simply soaking it in the carrier ampholyte solution.

Toda *et al.* [84] developed a two-dimensional electrophoresis in which IEF was carried out on Separax EF. However, CAMs are not widely used in IEF analysis because their drying during high-voltage IEF results in poor reproducibility. Recently, Toda *et al.* [85] improved the electrophoretic chamber for high-voltage CAM-IEF and applied it to the analysis of serum proteins with favourable results. More recently, Shiba [86] overlapped six CAMs at once by the method of Toda *et al.* [85]. The six CAMs were separately stained, *i.e.*, the first was stained for protein and the second to sixth were stained for IgD, IgE, IgM, IgA and IgG, respectively. We were able to improve on CAM-IEF after modifying the focusing conditions [8], which will be described in detail in the next section.

3.3. Focusing conditions

IEF was performed on a Pharmacia system (ECPS 3000/150 constant-power supply and VH-1 volt hour integrator). An initial voltage of 900 V (150 V/cm) was applied under a constant power (*ca.* 4 W) and unlimited current, with the maximum voltage set at 1200 V (200 V/cm). Samples were applied to CAM at distances of 1 cm from the anode for EAP typing and 0.5 cm from the cathode for ESD typing. The electrode solutions were 0.2 *M* sodium hydroxide (cathodic) and 1 *M* phosphoric acid containing 30% sucrose (anodic). Sample tabs (7 × 5 mm, Whatman No. 1) were removed after 5 min. The focusing time was controlled by volts × hours (V h).

In the early stage of CAF-IEF adoption, a constant voltage of 800 or 1000 V with a 10-cm electrode distance was usually applied [26,80,81]. Toda *et al.* [85] developed an electrophoretic chamber for high-voltage CAM-IEF, whereby CAM-IEF was carried out at 300 V for 10 min, 500 V for 10 min and 1500 V for 45 min after prefocusing at 500 V for 30 min. We shortened the electrode distance from the normal 10 cm to 6 cm [8]. On applying the same low voltage, the shorter electrode distance provided a higher field strength and likewise prevented the CAMs from drying. A higher field strength increases not only the sharpness, but

also the migration rate of bands [87]. Further, introduction of the non-equilibrium IEF method provides good reproducibility for CAM-IEF because of its shorter focusing time.

3.4. Detection

EAP and ESD activities are detected by a modification of the method used by Divall [7,47]. A filter-paper (Whatman No. 2) is soaked in EAP staining solution (3 mg of 4-methylumbelliferyl phosphate dissolved in 5 ml of 0.05 *M* citrate buffer, pH 5.0), and another in ESD staining solution (2 mg of 4-methylumbelliferyl acetate dissolved in 0.3 ml of acetone and 5 ml of 0.05 *M* acetate buffer, pH 5.2). A piece of filter-paper soaked in the staining solution is placed on the focused CAMs and any excess solution is removed by blotting. After incubation of the membrane for 5–10 min at 37°C, the isozymes are revealed under 365-nm UV light after removing the filter-paper.

4. ISOZYME ANALYSIS

4.1. Carrier ampholytes

Of the several methods listed in Tables 1 and 2, in this section we describe the IEF method by carrier ampholytes without chemical separators.

4.1.1. Erythrocyte acid phosphatase typing

Under the equilibrium conditions applied by Burdett and Whitehead [14], the EAP A band (combination of A_1 and A_2 bands) could not be separated from haemoglobin. In the method of Carracedo and Concheiro [20], the EAP A band was found in close proximity to haemoglobin. However, other methods under non-equilibrium conditions [8,15,18,21–31] provided a good separation of the A band and haemoglobin. Unfortunately, several other methods have failed to separate the A_1 and A_2 bands [18,21,23,26,27,30]. With pH 4–8 carrier ampholytes, Randall *et al.* [15] and Frank and Stolorow [24] were nonetheless able to separate the A_1 and A_2 bands. Using the same pH range carrier ampholytes, however, Yuasa *et al.* [23] were unable to separate these bands. Using different pH range carrier ampholytes, a similar result was observed. With pH 5–7 carrier ampholytes, Minakata and Asano [25] separated the A_1 and A_2 bands under low-voltage IEF. With a high field strength, A_1 and A_2 were not separated with pH 5–7 or 5–8 carrier ampholytes [8,21,27].

This discrepancy in isozyme patterns is attributed to the charge difference of isozymes under non-equilibrium conditions. It is difficult to distinguish A_1 and A_2 bands with relatively narrow pH range IEF, because the charge difference between them is reduced on approaching their p*I* values. An alternative method based on the difference in p*I* between A_1 and A_2 bands is to adopt a narrow pH

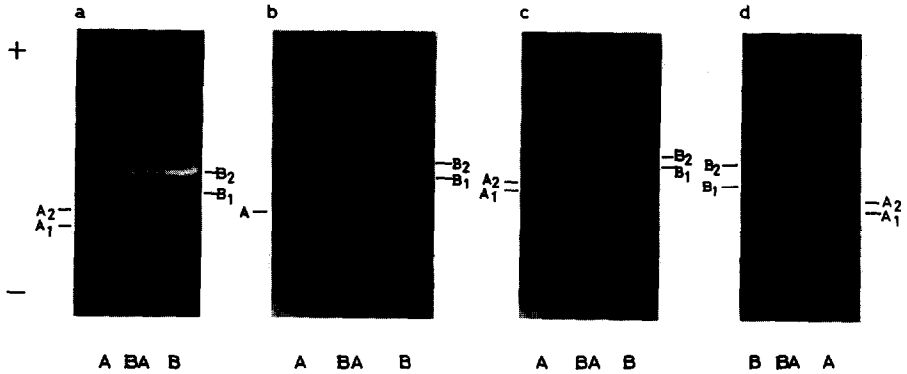

Fig. 4. EAP isozyme patterns obtained with various carrier ampholytes in CAM-IEF: (a) pH 5–6.5; (b) pH 5–8; (c) pH 5–8–pH 5–6.5, 1:3; (d) pH 5–8–pH 5–6.5, 1:5. Electrode distance, 6 cm; focusing time, 30 min; 550 V h.

range IEF. However, narrow pH range carrier ampholytes are incapable of covering the p*I* range of both B_1 (or C_1) and B_2 (or C_2) bands. In addition, EAP A_1 and A_2 bands overlap with haemoglobin under equilibrium conditions.

On the other hand, we demonstrated that the isozyme patterns obtained with pH 5–6.5 and 5–8 carrier ampholytes under the same IEF conditions were different, as shown in Fig. 4a and b. With pH 5–6.5 carrier ampholytes, the A_1 and A_2 bands were separated, whereas the distance between the A_2 and B_1 bands became smaller. With pH 5–8 carrier ampholytes, the opposite result was obtained. Hence the higher pH range formed in the gel is suitable for separating A_2 and B_1 (or C_1) bands, whereas a lower pH range provides a good separation of A_1 and A_2 bands. As the p*I* difference between the A_2 and B_1 (or C_1) bands is large, the charge difference between the A_2 and B_1 (or C_1) bands increases on approaching their p*I* values. In the case of A_1 and A_2 bands having a small difference in their p*I* values, the opposite result is obtained. The focusing conditions with pH 5–6.5 carrier ampholytes are incapable of improving the isozyme patterns, because a maximum pH of 6.5 is formed in the gel even if A_1 band focuses at the cathode side. A mixture of carrier ampholytes modifies the isozyme patterns.

As shown in Fig. 4c, a 1:3 mixture of pH 5–8 and 5–6.5 range carrier ampholytes allowed each band to be approximately equidistant from each other. An even better resolution was obtained with a 1:5 mixture of pH 5–8 and 5–6.5 range carrier ampholytes (Fig. 4d). This was also observed by Destro-Bisol and Ranalletta [18] with a 7:20 mixture of pH 3.5–10 and 5–8 range carrier ampholytes.

However, it takes more than 75 min to achieve sufficient separation with wider pH range carrier ampholytes, such as pH 4–8 [15,20,23,24] and pH 3.5–10 [18]. Using a Phast gel system (pH 4–6.5), Shutler and Tompkins [29] were able to separate each band with only 10 min of focusing. With pH 5–7 carrier ampho-

lytes, Budowle [21] phenotyped EAP after 23 min of focusing. With a 1:5 mixture of pH 5–8 and 5–6.5 range carrier ampholytes, we were able to separate each band after 30 min of focusing [8].

Hence, the relatively narrow pH range carrier ampholytes are suitable for EAP analysis when the analytical time is taken into consideration. A mixture of carrier ampholytes is also useful for obtaining better separations.

4.1.2. Esterase D typing

In the early stages of IEF, three common phenotypes (ESD1, ESD2–1 and ESD2) could not be discriminated under equilibrium conditions with the relatively narrow pH range carrier ampholytes [44–46]. With pH 4–6 range carrier ampholytes, Divall [47] was able to discriminate the three common phenotypes and ESD5 variants using non-equilibrium IEF. Yuasa *et al.* [48] carried out non-equilibrium IEF at low voltage and showed the three common phenotypes and ESD7 variants with pH 4–6.5 carrier ampholytes. Based on photographs, however, the method of Divall [47] allowed a better separation of the three common phenotypes and ESD5 variants than that of Yuasa *et al.* [88]. However, the three common phenotypes by Yuasa *et al.* [88] were more easily distinguished than that of Divall [47].

Komatsu [89] investigated the three common phenotypes and ESD7 variants by using two different methods [47,48]. She reported that the method of Divall [47], with slight modification, yielded a better separation of the ESD7 variants from the three common phenotypes, but discriminating the three common phenotypes was difficult [89]. At a lower pH range, ESD5 and ESD7 variants are easily discriminated from the three common phenotypes, because these variants have a different p*I* to that of the three common phenotypes. The nearer the three common phenotypes are focused to their p*I* values (at the lower pH range formed in the gel), the more difficult it is to distinguish them with the relatively narrow pH range carrier ampholytes. When the three common phenotypes were focused at a higher pH range by the method of Yuasa and co-workers [48,88], the charge difference was increased and the three common phenotypes were well distinguished.

The narrow pH range carrier ampholytes are often used as an alternative method for ESD typing. Budowle and Gambel [51] modified the isozyme patterns with a mixture of several narrow pH range carrier ampholytes. Weidinger and Henke [52] reported the use of an agarose gel with pH 4.5–5.4 carrier ampholytes. However, more than 95 min were needed to attain their p*I* values in this procedure [51,52]. With the relatively narrow pH range carrier ampholytes under non-equilibrium conditions, more than 60 min were also required [47,48,88]. Recently, we demonstrated the separation of ESD typing in only 25 min, using chemical separators and relatively narrow pH range carrier ampholytes [8], as described in the next section.

4.2. Chemical separators

In this section, we describe an IEF method using a combination of carrier ampholytes and chemical separators. We shall deal with the effect of the concentration of chemical separators on isozyme patterns under non-equilibrium conditions in Section 4.2.2.

4.2.1. Erythrocyte acid phosphatase typing

Finney *et al.* [22] described a method for the combined phenotyping of EAP and ESD, using pH 4–8 carrier ampholytes and 3-(N-morpholino)propanesulphonic acid (MOPS). They showed that addition of MOPS tended to draw and space the ESD phenotypes. Further, Finney *et al.* [22] and Zamir [28] reported that MOPS was essential for maintaining the A_1 and A_2 bands in the gel. As the sample for simultaneous phenotyping is applied in the middle of the gel or on the cathode side, the effect of flattening around pH 5 by the addition of MOPS [90] is useful for EAP typing. Dimo-Simonin *et al.* [30] phenotyped EAP and ESD with pH 4.5–7 carrier ampholytes combined with MOPS and taurine. They tested MOPS in combination with other separators, such as HEPES, taurine, N,N-bis (2-hydroxyethyl)-2-aminoethanesulphonic acid (BES) and N-(2-acetamido)-2-aminoethanesulphonic acid (ACES), and concluded that MOPS and taurine gave the best results as taurine increased the ESD resolution without interfering with the EAP separation [30]. However, the A_1 and A_2 bands were still not separated. This isozyme pattern is attributed to the charge difference of isozymes under non-equilibrium conditions. Possibly the addition of taurine flattens the pH gradient at a higher pH range in the gel. As mentioned in Section 4.1, the three common phenotypes of ESD are readily distinguishable at a higher pH range, while a higher pH range also produces the EAP A band (combination of A_1 and A_2 bands).

Recently, Stockwell *et al.* [31] presented a method for simultaneous typing of EAP, ESD, PGM1, AK and ADA by using pH 4–6.5 carrier ampholytes, MOPS and HEPES. As the addition of HEPES amplifies the pH range between pH 5 and 5.5 [54], the distance between the A_1 and A_2 bands increases, in comparison with those of Finney *et al.* [22] and Zamir [28] with the addition of MOPS alone.

Although simultaneous phenotyping is useful for medico-legal practice, it is difficult to obtain the pH range suitable for various erythrocyte enzyme types in one gel. In the analysis of single phenotyping for EAP, application on the anodic side requires no addition of MOPS. As the optimum pH range for EAP typing is relatively wide, it is not necessary to flatten the pH range by the addition of chemical separators.

4.2.2. Esterase D typing

We have demonstrated the satisfactory separation of ESD isozymes in 25 min using 10% carrier ampholyte solution of pH 5–6.5 containing a final concentra-

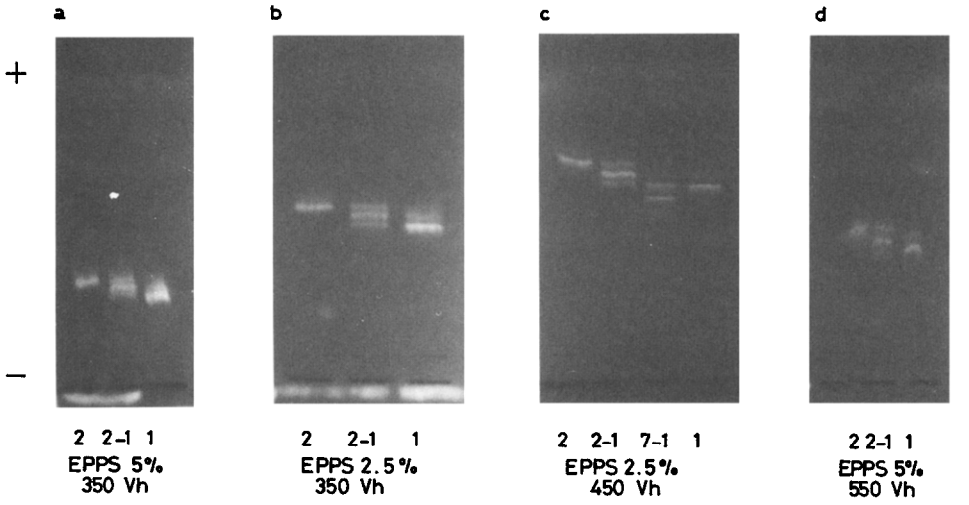

Fig. 5. ESD isozyme patterns containing chemical separators obtained by CAM-IEF: (a) 10% pH 5–6.5 solution containing 5% EPPS, 350 V h; (b) 10% pH 5–6.5 solution containing 2.5% EPPS, 350 V h; (c) 10% pH 5–6.5 solution containing 2.5% EPPS, 450 V h; (d) 10% pH 5–6.5 solution containing 5% EPPS, 550 V h. Electrode distance, 6 cm.

tion of 2.5% (w/v) EPPS [8]. In this section, the effects of the concentration of chemical separators on isozyme patterns under non-equilibrium conditions using modified CAM-IEF are reviewed. As shown in Fig. 5a and b, the isozyme patterns containing 2.5 and 5% EPPS, respectively, are different under the same V h conditions. The isozyme patterns containing 5% EPPS are ambiguous at 350 V h (Fig. 5a), showing no improvement after continuous focusing at 550 V h (Fig. 5d). At 350 V h (20 min of focusing), a good separation is obtained by the addition of 2.5% EPPS. Continuous focusing until 450 V h (25 min) provided a better separation (Fig. 5c).

According to the report of Gill and Sutton [54], the addition of EPPS resulted in a greatly flattened pH gradient with a linear pH range between pH 5.6 and 5.9. They added 2.5% (w/v) of EPPS (final concentration) to a 5% solution of carrier ampholytes. We added 2.5 or 5% (w/v) of EPPS (final concentration) to a 10% solution of carrier ampholytes. Thus, from the point of view of the separator/carrier ampholyte solution ratio, the addition of 5% EPPS serves as well as the addition of 2.5% EPPS in the method of Gill and Sutton [54]. With pH 5–6.5 carrier ampholytes, the addition of 2.5% EPPS creates a flattening in the middle of the pH gradient formed in the CAM. This flattening effect increases the charge difference of isozymes under non-equilibrium conditions and provides good resolution. On the other hand, the addition of 5% EPPS produces a narrow pH range in the CAM and requires a long focusing time to achieve sufficient resolution. Further, the viscosity of 5% EPPS results in drying of CAMs during high-field-strength IEF, the drying being a factor causing poor reproducibility.

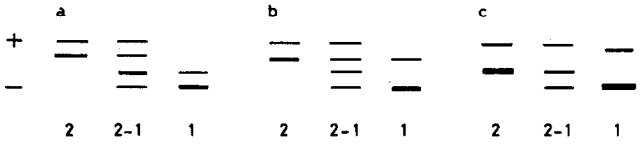

Fig. 6. ESD isozyme patterns obtained by several methods: (a) by our method [8]; (b) by the methods of Yuasa *et al.* [48] and Destro-Bisol and Spinella [53]; (c) by the methods of Budowle [50] and Weidinger and Henke [52].

Destro-Bisol and Spinella [53] and Alonso and Gascó [27] added HEPES to pH 4–6.5 carrier ampholytes. Destro-Bisol and Spinella [53] added 1.3% (w/v) of HEPES (final concentration) to a 2.7% solution of carrier ampholytes and reported that a low concentration of carrier ampholytes and separators provided the best results. In their method [53], however, the ratio of separator/carrier ampholyte solution is relatively high. Therefore, it is possible that the addition of a large amount of HEPES extends to the middle of the pH gradient (pH 5–5.5 range). Moreover, even after performing non-equilibrium IEF at low voltage, their procedure still required 90 min of focusing time. On the other hand, Alonso and Gascó [27] added 3% (w/v) of HEPES (final concentration) to a 4% solution of carrier ampholytes, phenotyping on a miniature gel with an electrode distance of 4.5 cm. However, sufficient separation of the three common phenotypes was not obtained owing to the smaller electrode distance.

Under equilibrium conditions, Budowle [50] demonstrated the separation of the three common phenotypes and ESD5 variants, using pH 4.5–5.4 carrier ampholytes (containing 10% of either pH 4–6 or pH 4–6.5 ampholytes) and BES. It was found that the addition of BES increased the distance between ESD1 and ESD2 by at least 30%. However, Weidinger and Henke [52] showed that pH 4.5–5.4 carrier ampholytes alone was sufficient to yield a good separation. Judging from photographs, no major difference in the three common phenotypes is evident using the two methods [50,52]. Thus, the addition of BES under equilibrium conditions is useful for increasing the distance between the three common phenotypes rather than improving the isozyme patterns.

As shown in Fig. 6, the ESD isozyme patterns among the three common phenotypes are classified into three types. Fig. 6a shows that based on our method [8], the three common phenotypes are focused far from their p*I* values. In Fig. 6c, the three common phenotypes are focused at their p*I* values, based on the methods of Budowle [50] and Weidinger and Henke [52]. The middle of the isozyme patterns is shown in Fig. 6b, using the methods of Yuasa *et al.* [48] and Destro-Bisol and Spinella [53]. Although each method is useful for ESD phenotyping, our method of rapid focusing and good separation under more non-equilibrium conditions is suitable for routine analysis. It is assumed that the non-restrictive properties of CAMs are also favourable for such a short focusing time.

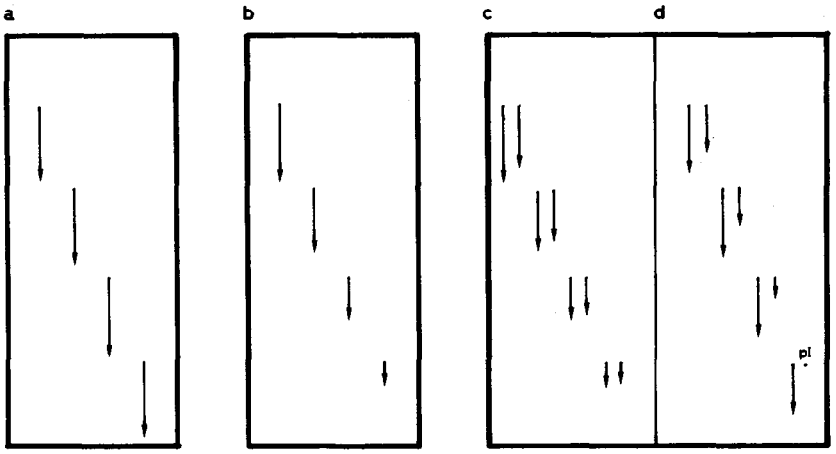

Fig. 7. Diagrams of different migration rates of isozyme by charge: (a) constant migration in conventional electrophoresis; (b) variable migration in IEF; (c) migration by charge difference of isozymes with similar p*I* values; (d) migration by charge difference of isozymes with different p*I* values.

5. CONCLUSION

In conventional electrophoresis, isozymes are separated according to the rates of migration of isozymes based on factors of charge and size. Fig. 7a shows that the rate of migration by charge is constant in conventional electrophoresis. In IEF, however, the rate of migration is reduced on approaching the p*I* value, because a pH gradient is produced along the gel (Fig. 7b). In equilibrium IEF, isozymes are detected based on their p*I* values, whereas charge difference provides the separation of isozymes in non-equilibrium IEF. Thus, a variable charge difference along the gel plays an important role for good separation. If the difference in p*I* values between isozymes is smaller, *e.g.*, A_1 and A_2 bands in EAP typing or the three common phenotypes in ESD typing, the charge difference is reduced towards their p*I* values (Fig. 7c). In a contrasting situation, *e.g.*, A_2 and B_1 (or C_1) bands in EAP typing or ESD5 (or ESD7) variants and the three common phenotypes in ESD typing, the charge difference increases on approaching the respective p*I* values (Fig. 7d). Moreover, the addition of chemical separators increases the charge difference if part of the pH gradient is flattened appropriately. Addition of more separators produces a narrow pH range in the gel and takes a substantially longer time to obtain a good separation. If the optimum pH range for charge difference is obtained on adding less separators, the increasing charge difference provides a good resolution in a short focusing time. Hence conditions such as the optimum pH gradient, chemical separator and focusing time are factors that need to be taken into consideration for obtaining optimum results.

6. ACKNOWLEDGEMENT

We thank Dr. P. Novales-Li for critical comments on the manuscript.

REFERENCES

1 P. Kühnl and W. Spielmann, *Hum. Genet.*, 43 (1978) 91.
2 P. Kühnl and W. Spielmann, *Hum. Genet.*, 50 (1979) 193.
3 J. Constans, P. Kühnl, M. Viau and W. Spielmann, *Hum Genet.*, 55 (1980) 111.
4 M. I. Kamboh and R. L. Kirk, *Hum. Hered.*, 33 (1983) 237.
5 V. L. Pascali and P. Auconi, *Hum. Genet.*, 64 (1983) 232.
6 D. D. Dykes, C. M. DeFurio and H. F. Polesky, *Electrophoresis*, 3 (1982) 162.
7 G. B. Divall, *Forensic Sci. Int.*, 18 (1981) 67.
8 M. Kane, Y. Yamamoto, M. Yamada, T. Fukunaga and Y. Tatsuno, *Electrophoresis*, 11 (1990) 318.
9 D. A. Hopkinson, N. Spencer and H. Harris, *Nature (London)*, 199 (1963) 969.
10 D. A. Hopkinson, N. Spencer and H. Harris, *Am. J. Hum. Genet.*, 16 (1964) 141.
11 K. Omoto and S. Harada, *Jpn. J. Hum. Genet.*, 14 (1970) 298.
12 S. Harada, S. Misawa and K. Omoto, *Jpn. J. Hum. Genet.*, 16 (1971) 22.
13 G. Ishimoto, M. Kuwata and S. Kubota, *Jpn. J. Legal Med.*, 27 (1973) 23 (abstract in English).
14 P. E. Burdett and P. H. Whitehead, *Anal. Biochem.*, 77 (1977) 419.
15 T. Randall, W. A. Harland and J. W. Thorpe, *Med. Sci. Law*, 20 (1980) 43.
16 H. Haglund, *Sci. Tools*, 17 (1970) 2.
17 P. J. Svendsen and C. Rose, *Sci. Tools*, 17 (1970) 13.
18 G. Destro-Bisol and D. Ranalletta, *Electrophoresis*, 9 (1988) 106.
19 B. Budowle and A. M. Gambel, *J. Forensic Sci.*, 33 (1988) 915.
20 A. Carracedo and L. Concheiro, *Z. Rechtsmed.*, 88 (1982) 143.
21 B. Budowle, *Electrophoresis*, 5 (1984) 254.
22 S. T. Finney, N. A. Renshaw and D. J. Werrett, *Forensic Sci. Int.*, 27 (1985) 237.
23 I. Yuasa, N. Tamaki and R. Inuyama, *Acta Crim. Jpn.*, 51 (1985) 145.
24 W. E. Frank and M. D. Stolorow, *J. Forensic Sci.*, 31 (1986) 1089.
25 K. Minakata and M. Asano, *Jpn. J. Legal Med.*, 40 (1986) 146.
26 K. Minakata and M. Asano, *Jpn. J. Legal Med.*, 40 (1986) 335.
27 A. Alonso and P. Gascó, *J. Forensic Sci.*, 32 (1987) 1558.
28 A. Zamir, *J. Forensic Sci. Soc.*, 28 (1988) 219.
29 G. G. Shutler and D. C. Tompkins, *Forensic Sci. Int.*, 39 (1988) 97.
30 N. Dimo-Simonin, C. Brandt-Casadevall and H. Gujer, *Electrophoresis*, 10 (1989) 718.
31 D. C. Stockwell, D. J. Gregonis and D. T. Jones, *J. Forensic Sci.*, 35 (1990) 46.
32 D. A. Hopkinson, M. A. Mestriner, J. Cortner and H. Harris, *Ann. Hum. Genet.*, 37 (1973) 119.
33 A. C. Ebeli-Struijk, E. M. Wurzer-Figurelli, F. Ajmar and P. M. Khan, *Hum. Genet.*, 34 (1976) 299.
34 N. M. Blake, *Hum. Genet.*, 35 (1976) 91.
35 S. S. Papiha and A. Nahar, *Hum. Hered.*, 27 (1977) 424.
36 K. Bender and R. Frank, *Humangenetik*, 23 (1974) 315.
37 K. Berg, F. Schwarzfisher and H. Wischerath, *Hum. Genet.*, 32 (1976) 81.
38 W. Martin, *Ärztl. Lab.*, 25 (1979) 65.
39 G. Radam, H. Strauch and W. Martin, *Blut*, 40 (1980) 337.
40 M. P. Marks, T. Jenkins and G. T. Nurse, *Hum. Genet.*, 37 (1977) 49.
41 B. Hoste, J. Brocteur and A. Andre (Editors), *Internationale Tagung der Gesellshaft für Forensische Blutgruppenkunde, Hamburg, 1977*, Vol. 7, p. 349.
42 J. Twibell and P. H. Whitehead, *J. Forensic Sci.*, 23 (1978) 356.
43 J. Henke, L. Bauer and H. Schweitzer, *Z. Rechtsmed.*, 88 (1982) 271.

44 I. Nishigaki and T. Itoh, *Hum. Genet.*, 66 (1984) 92.
45 B. Olaisen, A. Siverts, R. Jonassen, B. Mevåg and T. Gedde-Dahl, *Hum. Genet.*, 57 (1981) 351.
46 D. D. Dykes, H. F. Polesky and S. Miller, *Hum. Genet.*, 62 (1982) 162.
47 G. B. Divall, *Forensic Sci. Int.*, 26 (1984) 255.
48 I. Yuasa, N. Tamaki, K. Suenaga, K. Itoh, T. Inoue and K. Okada, *Electrophoresis*, 6 (1985) 588.
49 B. Budowle, *Electrophoresis*, 5 (1984) 314.
50 B. Budowle, *Electrophoresis*, 7 (1986) 141.
51 B. Budowle and A. M. Gambel, *J. Forensic Sci.*, 33 (1988) 738.
52 S. Weidinger and J. Henke, *Electrophoresis*, 9 (1988) 429.
53 G. Destro-Bisol and A. Spinella, *Forensic Sci. Int.*, 42 (1989) 43.
54 P. Gill and J. G. Sutton, *Electrophoresis*, 6 (1985) 23.
55 N. Spencer, D. A. Hopkinson and H. Harris, *Ann. Hum. Genet.*, 32 (1964) 9.
56 A. Jongsma, H. V. Someren, A. Westerveld, A. Hagemijer and P. Pearson, *Humangenetik*, 20 (1973) 195.
57 P. J. McAlpine, in *Proceedings of the Third International Isozyme Conference*, Academic Press, New York, 1974.
58 J. E. Bark, M. J. Harris and M. Firth, *J. Forensic Sci. Soc.*, 16 (1976) 115.
59 J. G. Sutton, *J. Forensic Sci.*, 24 (1979) 189.
60 M. E. Lawton and J. G. sutton, *J. Forensic Sci. Soc.*, 22 (1982) 203.
61 S. G. Welch, C. A. Swindlehust, I. A. McGregor and K. Williams, *Hum. Genet.*, 43 (1978) 307.
62 B. Budowle, S. Sundaram and R. Wenk, *Forensic Sci. Int.*, 28 (1985) 77.
63 G. B. Divall and M. Ismail *Forensic Sci. Int.*, 22 (1983) 253.
64 P. Gill and J. G. Sutton, *Electrophoresis*, 5 (1984) 274.
65 R. Scherz, R. Pflugshaupt and R. Bütler, *Hum. Hered.*, 31 (1981) 187.
66 D. D. Dykes, B. A. Copouls and H. F. Polesky, *Electrophoresis*, 3 (1982) 165.
67 B. Budowle, R. S. Murch, L. C. Davidson, A. M. Gambel and J. J. Kearney, *J. Forensic Sci.*, 31 (1986) 1341.
68 P. Kühnl, U. Schmidtmann and W. Spielmann, *Hum. Genet.*, 35 (1977) 219.
69 J. G. Sutton and R. Burgess, *Vox Sang.*, 34 (1978) 97.
70 W. Pflug, V. Vigne and W. Bruder, *Electrophoresis*, 2 (1981) 327.
71 J. G. Sutton and S. A. Westwood, *Electrophoresis*, 5 (1984) 252.
72 R. M. Burgess, J. G. Sutton and D. J. Werrett, *Forensic Sci. Int.*, 35 (1987) 213.
73 I. Mũnoz-Barús, M. V. Lareu, I. López-Rodriguez, M. S. Rodriguez-Calvo and A. Carracedo, *Z. Rechtsmed.*, 102 (1989) 271.
74 P. G. Righetti, *J. Chromatogr.*, 300 (1984) 165.
75 J. S. Fawcett and A. Chrambach, *Protides Biol. Fluids Proc. Colloq.*, 33 (1985) 439.
76 K. Altland and U. Rossmann, *Electrophoresis*, 6 (1985) 314.
77 K. Altland and U. Rossmann, *Application Note 346*, LKB, Bromma, 1987.
78 *Application Note 473*, LKB, Bromma, 1986.
79 W. Pflug, *Electrophoresis*, 6 (1985) 19.
80 S. Harada, *Clin. Chim. Acta*, 63 (1975) 275.
81 D. F. Farrell, M. P. Macmartin and A. F. Clark, *Clin. Chim. Acta*, 89 (1978) 145.
82 J. Ambler, *Clin. Chim. Acta*, 85 (1978) 183.
83 T. Dobosz and P. Koziol, *Hum. Genet.*, 56 (1980) 119.
84 T. Toda, T. Fujita and M. Ohashi, *Anal.Biochem.*, 119 (1982) 167.
85 T. Toda, K. Shiba, H. Cho, P. L. Soon, M. Nakao and M. Ohashi, *Electrophoresis*, 9 (1988) 149.
86 K. Shiba, *Physico-Chem. Biol. (Seibutsu Butsuri Kagaku)*, 34 (1990) 245 (in Japanese).
87 H. Svensson, *Acta Chem. Scand.*, 15 (1961) 325.
88 I. Yuasa, N. Tamaki, T. Inoue and K. Okada, *Forensic Sci. Int.*, 28 (1985) 63.
89 N. Komatsu, *Jpn. J. Legal Med.*, 39 (1985) 339.
90 *Isolectric Focusing: Principles and Methods*, Pharmacia, Uppsala, 1982, p. 114.