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Evaluation of a Nonequilibrium Isoelectric Focusing (IEF) Method for the Simultaneous Typing of Esterase D (EsD), Red Cell Acid Phosphatase (AcP_1), Phosphoglucomutase (PGM_1), Adenylate Kinase (AK), and Adenosine Deaminase (ADA)

REFERENCE: Stockwell, D. C., Gregonis, D. J., and Jones, D. T., "**Evaluation of a Nonequilibrium Isoelectric Focusing (IEF) Method for the Simultaneous Typing of Esterase D (EsD), Red Cell Acid Phosphatase (AcP_1), Phosphoglucomutase (PGM_1), Adenylate Kinase (AK), and Adenosine Deaminase (ADA),**" *Journal of Forensic Sciences*, JFSCA, Vol. 35, No. 1, Jan. 1990, pp. 46–61.

ABSTRACT: A nonequilibrium isoelectric focusing method incorporating the chemical spacers MOPS and HEPES was developed and subsequently evaluated for its ability to reliably discriminate common and rare phenotypes in the esterase D (EsD), red cell acid phosphatase (AcP_1), phosphoglucomutase (PGM_1), adenylate kinase (AK), and adenosine deaminase (ADA) isoenzyme systems. The validation procedures used were blind testing, comparison of results to conventional methods, and evaluation of known rare variant phenotypes. This method proved to be a quick and reliable method for typing all five isoenzyme systems, while providing an excellent probability of discrimination ($PD = 0.96$).

KEYWORDS: forensic science, genetic typing, isoelectric focusing, nonequilibrium isoelectric focusing, chemical spacers, separators, ultrathin polyacrylamide gel, esterase D, red cell acid phosphatase, phosphoglucomutase, adenylate kinase, adenosine deaminase, rare phenotypes

The conception of this isoelectric focusing (IEF) procedure cannot be traced to any particular person or method. Like science itself, it has followed an evolutionary track with theme building upon theme and author adding to author. A complete anthology of this process is neither required nor possible here; still, a brief review of several pivotal papers in the development of this system is in order. Radola [*1*] brought IEF within the means of typical forensic science laboratories with ultrathin-layer polyacrylamide gel isoelectric focusing (ULPAGIEF), lowering the cost of focusing to tolerable levels and at the same time reducing the amount of sample required for analysis and increasing banding resolution. Dykes et al. [*2,3*] used IEF in agarose gels to look at many rare variant phenotypes in the phosphoglucomutase (PGM_1) isoenzyme system. Dorill and

Portions of this paper were presented at the joint California Association of Criminalists/North West Association of Forensic Scientists (CAC/WAFS) 1987 Spring Seminar at Sparks, Nevada. Received for publication 22 Dec. 1988; revised manuscript received 10 Feb. 1989; accepted for publication 28 Feb. 1989.
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Sutton [*4*] used a "split plate" method employing pH 5 to 8 ampholytes to type simultaneously PGM_1 and erythrocyte acid phosphatase (red cell acid phosphatase), (EAP [AcP_1]). Divall [*5,6*] advanced the use of nonequilibrium conditions to discriminate successfully the esterase D (EsD) 1, 2, and 5 alleles. This method, as stated by Divall [*5*], "is essentially one of electrophoresis in a pH gradient," since true equilibrium conditions were not reached. Gill [*7*] described the use of chemical separators to flatten locally the pH gradient curves and effectively increase the separation of isoenzyme banding in the pH range 4 to 6. Whereas Gill and Sutton [*8*] used the chemical separator *N*-(2-hydroxyethyl) piperazine-*N'*-3-propanesulfonic acid (EPPS) for enhanced resolution of PGM_1, Finney, Renshaw, and Werrett [*9*] used an analogous separator MOPS to type simultaneously EAP (AcP_1) and EsD.

S. Kuo [*10*], noting the similarity of these methods, proposed the simultaneous typing of EsD, EAP (AcP_1), and PGM_1 by incorporating the chemical separator MOPS in a pH 4 to 8 interval polyacrylamide gel (PAG) using nonequilibrium conditions. He used a split-plate method similar to Dorrill and Sutton's to type the AcP_1 and PGM_1 isoenzyme systems. The following year, Kuo [*11*] did further research and reported on the reliable typing of the common phenotypes within the EsD, EAP (AcP_1), and PGM_1 systems, as well as expanding the system to include the adenosine deaminase (ADA) and adenylate kinase (AK) isoenzyme systems.

Our study was designed to answer several questions not covered in Kuo's papers. Does this IEF method behave in similar respects to those reported in the literature? Does this multisystem approach compromise any of the isoenzyme systems such that results are ambiguous? Can rare variant phenotypes be confused with common phenotypes, and if so, is there a remedy? To answer these questions a four-step approach was taken:

(1) Review the relevant literature.

(2) Reanalyze samples previously run through conventional electrophoretic methods. This step was run in conjunction with an EsD 2/2-1 study to determine the frequency of the "5" allele.

(3) Analyze available rare variant standards to determine if they act as predicted and to define any limitations of this method.

(4) Perform a blind study.

Methods

The ULPAGIEF gel was prepared using 9 mL of a 5% T 3% C stock acrylamide/bisacrylamide solution incorporating 1 g of sucrose, 0.25 g of MOPS [3-(*N*-morpholino)propanesulfonic acid], 0.20 g of HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 700 μL LKB pH 4–6.5 Ampholines®, 3 mg of ammonium persulfate, and 4 μL of tetra methyl ethylenediamine (TEMED). This solution was cast using the flap technique of Radola [*1*] onto PAG gelBond® in a mold 200 by 200 by 0.2 mm and polymerized in a 50°C oven for 1 h. These gels were then stored refrigerated in a moisture chamber overnight before use. Gels were stored up to three months in this fashion without detrimental effect.

Blood samples were extracted in a 0.05*M* dithiothreitol solution (DTT). Sample size was approximately 4 mm^2 of blood dried on clean cotton sheeting. The samples were moistened with 6 μL of DTT in the cap of a 400-μL Eppendorf centrifuge tube, sealed with tape, and allowed to extract for 20 min at room temperature. The extract was then isolated by piggyback centrifugation. The gel was set up with a 16-cm interelectrode distance, using Serva 3 (*L*-aspartic acid, *L*-glutamic acid, pH 3) as the anolyte soaked onto three thicknesses of Whatman No. 3 paper 1 cm wide, and Serva 10 (ethylenediamine, *L*-arginine, *L*-lysine, pH 10) as the catholyte, likewise soaked onto three thicknesses of Whatman No. 3 paper. The gel was prerun on a cooling platten at 6°C using

8- 10-mA limits until 2000 V was reached (approximately 30 min). Extracts were applied 1 cm anodic of midplate using an overlay mask with 5 by 3-mm wells and allowed to run in for 10 min at 2000-V/10-mA settings. The overlay mask was then blotted and removed. The run was completed at 2500-V/10-mA settings for approximately 60 min. (Using a volt-hour integrator, the total sample run time, including run in, was 2600 V/h.) Development reaction mixtures are those described in Table 1.

The EsD and AcP_1 reaction mixtures were overlayed first. The EsD reaction mixture was soaked onto a cellulose acetate membrane 4.0 cm wide and overlayed from the origin towards the anode. The AcP_1 reaction mixture was soaked onto a cellulose acetate membrane 6.0 cm wide and overlayed from the origin towards the cathode. These overlays were left in place for no more than 3 min before they were removed, inverted, placed on a clean glass plate, and incubated in a moisture chamber at 37°C for 20 min. The ADA, PGM, and AK reaction mixtures were then poured. The ADA mixture was poured from the anode to the origin. The PGM mixture was poured from the origin 6.0 cm toward the cathode. The PGM mixture was allowed to solidify, the cathodal border was removed, and the AK reaction mixture was poured from the PGM overlay to the cathode. This plate was then placed in a moisture chamber and incubated at 37°C. Refer to Fig. 1 for a schematic representation of the overlays with various phenotypes of the five enzyme systems.

Conventional methods used for comparisons in this study included modifications of Wraxall's Group 1 [*12*], Group 2 [*13*], and PGM subtyping [*14*], and an agarose method for ADA and AK.[2]

TABLE 1—*Development reaction mixtures for isoenzyme systems.*

Enzyme	Reaction Buffer	Reaction Mixture
EsD	0.05*M* sodium acetate pH 6.5	4-mg 4-methylumbelliferyl acetate dissolved in 200-μL acetone; diluted with 10-mL reaction buffer
AcP_1	0.05*M* citric acid pH 5.0	6-mg 4-methylumbelliferyl phosphate dissolved in 10-mL reaction buffer
PGM_1	0.29*M* trizma base 0.02*M* $MgCl_2$ 0.01*M*-histidine HCl pH 8.0	35-mg glucose-1-phosphate; 2-mg NADP sodium salt; 25-μL G-6PD; 2.5-mg MTT; 1.0-mg PMS; 10 mL reaction buffer; 10-mL 2% agar
ADA	0.025*M* Na_2HPO_4 0.016*M* NaH_2PO_4 pH 7.0	10-mg adenosine; 25-μL xanthine oxidase; 25-μL nucleoside phosphorylase; 4-mg MTT; 2-mg PMS; 10-mL reaction buffer; 10-mL 2% agar
AK	0.1*M* trizma base 0.02*M* $MgCl_2$ pH 8.0	20-mg glucose; 5-mg ADP; 3-mg NADP sodium salt; 2.5-mg MTT; 2.5-mg PMS; 25-μL G-6PD; 25-μL hexokinase; 10-mL reaction buffer; 10-mL 2% agar

[2]F. Springer, California Department of Justice, Bureau of Forensic Services, Sacramento, CA, February 1986, personal communication.

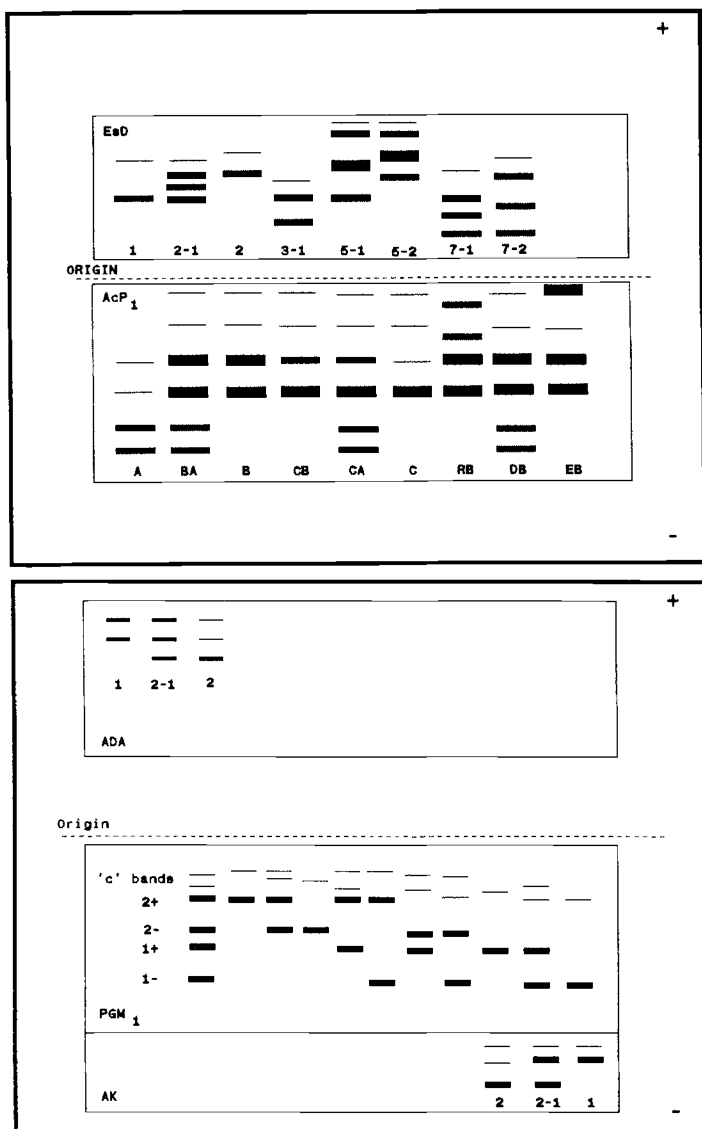

FIG. 1—*Schematic of overlays as they would be placed on the IEF gel as well as a representation of the various phenotypes as they would appear.*

Results and Discussion

EsD

Figure 2 depicts conventional typing of the EsD 1, 2, 3, 5, and 7 alleles. As pointed out by Yuasa [*15*], most conventional systems do not resolve the EsD 1 and 7 alleles or the 2 and 5 alleles, as seen in Fig. 2. Conversely, normal isoelectric focusing with equilibrium conditions differentiate the 1 from the 7 and the 2 from the 5 alleles, but the 1 and 2 alleles are no longer resolved. Different authors took varying approaches to solve

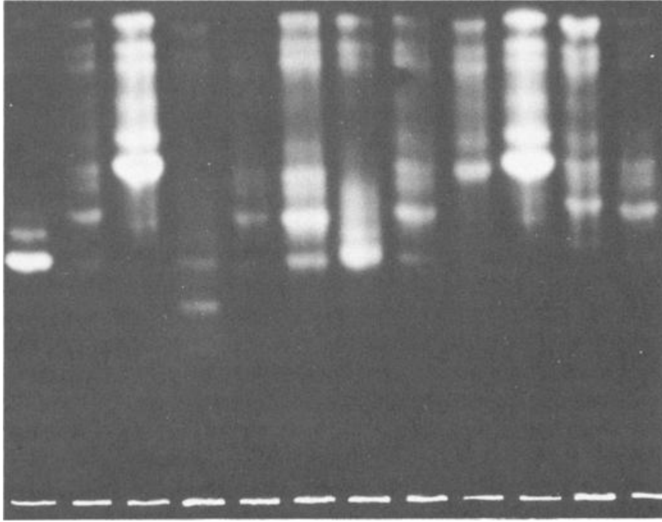

FIG. 2—*Photograph of EsD phenotypes by conventional electrophoresis. Samples from left to right are: 1; 2-1; 2; 3-1 (SERI); 2-1; 5-1; 7-1 (LAPD); 2-1; 5-2; 2; suspected 7-2 (SERI), 2-1. Anode is at top.*

this dilemma, all in essence leading to nonequilibrium IEF conditions. Divall [*5,6*] advanced the use of controlled time and temperature conditions, and Gill [*16*] and Finney [*9*] used chemical spacers which imparted nonequilibrium conditions. Each of these methods proved successful in differentiating the 1, 2, and 5 alleles. Yuasa [*15*], using "low voltage IEF," was able to differentiate the 1, 2, and 7 alleles.

Figure 3 depicts the EsD 1, 2, 3, 5, and 7 alleles as they appear on the IEF method under review. It is apparent that the homozygous 3 band is missing from the known EsD 3-1 variant; therefore, this method cannot be used to confirm the EsD 3 allele. The 1, 2, 5, and 7 alleles are clearly distinguished by this method.

A study was undertaken in our laboratory to reexamine all conventionally diagnosed EsD 2-1 and EsD 2 case samples to determine the frequency of the EsD 5 allele. The results of this study are summarized in Table 2.

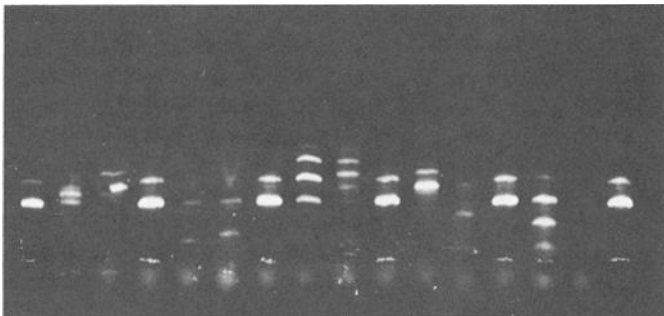

FIG. 3—*Photograph of EsD phenotypes by IEF method reviewed. Samples from left to right are: 1; 2-1; 2; 1; 3-1 (SERI); unconfirmed cathodal variant; 1; 5-1; 5-2; 1; 2; suspected 7-2 (SERI); 1; 7-1 (LAPD); 1; 1. Anode is at top.*

TABLE 2—*Frequency of EsD 1, 2, and 5 allele.*

Phenotypes	Observed	Expected	χ^2	Allele Frequencies
EsD 1	881[a]	877.2	0.016	EsD 1 = 0.883
EsD 2-1	205	207.2	0.023	EsD 2 = 0.104
EsD 2	14	12.4	0.206	EsD 5 = 0.011
EsD 5-1	22	22.5	0.011	
EsD 5-2	3	2.6	0.062	
EsD 5	0	0.1	0.100	
Total	1125	1122	0.418	
	$\Sigma\chi^2 = 0.418$	$df = 2$	$0.90 > P > 0.80$	

[a]EsD results in this table do not reflect the discrimination of EsD 1 from EsD 7-1.

The chi-square analysis shows an excellent goodness-of-fit for the data presented in Table 2. Additionally, the allele frequencies are close to other published data, such as Dykes et al.'s [*17*].

This study did not attempt to correlate interpretable results to aging. Empirical results support Divall's assertion [*6*] that, of the bands comprising the EsD 2-1 pattern, the heterozygous 2-1 band preferentially loses intensity with age. Caution must therefore be used in attempting to classify aged stains with weak isoenzyme patterns.

AcP_I

Figure 4 depicts the AcP_1 phenotypes used in this study run on a modified Group 2 method. Figure 5 shows these same phenotypes as they appear on the IEF method under study. The common A, B, and C alleles are all clearly identified using either method. The differentiation of the B and C alleles is based upon a subjective assessment of banding intensities as noted by many authors and appears to be independent of the method used.

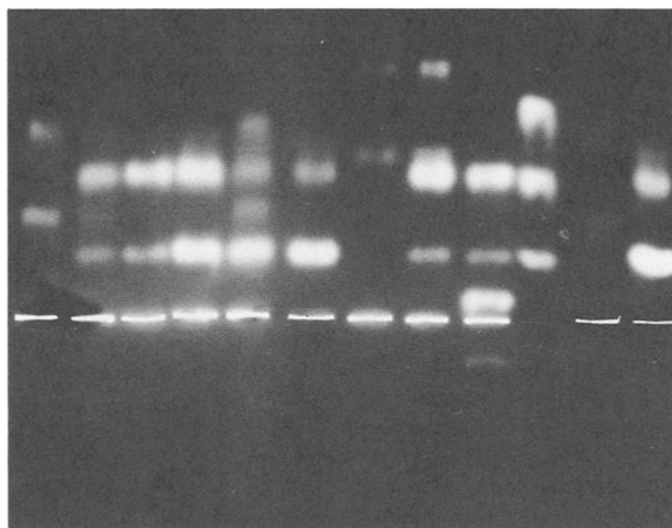

FIG. 4—*Photograph of AcP_I phenotypes by conventional electrophoresis. Samples from left to right are: A; BA; B; CB; CA; C; R (SERI); RB; DB (Dykes); EB (Dykes); A; C. Anode is at top.*

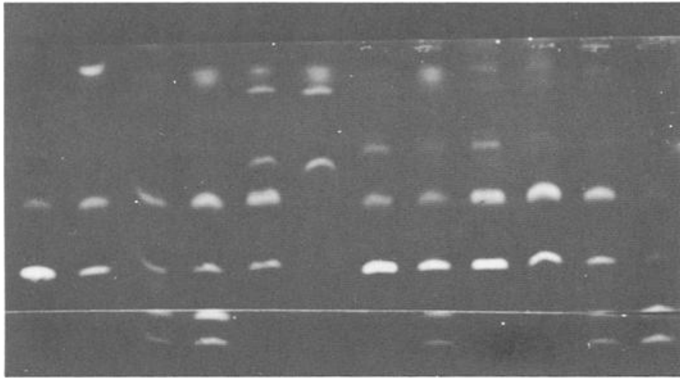

FIG. 5—*Photograph of AcP_1 phenotypes by IEF method reviewed. Samples from left to right are: C; EB (Dykes); BA; DB (Dykes); RB; R (SERI); C; CA; CB; B; BA; A. Anode is at top.*

However, the advantage of the IEF procedure is the increased sensitivity over starch gel electrophoresis. Budowle [*18*] has indicated that improper polymerization of the acrylamide matrix can also result in nearly total C band inhibition. Gelfi [*19–21*] has shown that the conditions used to polymerize polyacrylamide gels can greatly affect the physical structure as well as the amount of unreacted acrylamide, which in turn is free to react at the proper pH with histidine, lysine, tyrosine, cysteine, and the free amino terminus of polypeptides. This may be one possible explanation for the absence of C band activity reported by Budowle. The conditions used for chemical polymerization in our method are stringent, and indeed no AcP_1 C band inhibition has been seen in our laboratory.

The subjectivity of band intensities also presents itself in aging bloodstains. The A bands of AcP_1 lose intensity more quickly upon aging than either the B or the C bands. Again, the fact that the IEF technique is more sensitive than the starch gel helps prevent misclassification of aged and weak stains. In addition, since our method separates the AcP_1 overlay from the original gel, it can be fumed with ammonium hydroxide to enhance weak bands without detriment to the ADA, PGM, and AK enzymes developing on the parent gel.

The rare AcP_1 alleles R, D, and E on this IEF method react in accordance with the observations of Divall [*22*] and Frank [*23*]. The R bands run anodic to the B band and are slightly cathodic and more intense than the storage bands of the B and C alleles. Figure 4 shows that discrimination of the R allele is difficult using the starch gel method noted, whereas the discrimination is easily made using the IEF method, as seen in Fig. 5. The AcP_1 E allele can be distinguished with either method. Using this IEF method it must be stressed that the overlay start at the origin as the E band does not migrate far. The banding of the D allele, while easily distinguished using the starch gel method, is indistinguishable from the A allele banding using this IEF method. Both Divall [*22*] and Frank [*23*] report that the D bands run slightly cathodic of the A bands using their respective methods, although both imply that the D allele is best classified using conventional starch gel electrophoresis. We agree.

PGM_1

Literature sources dictated that a slightly different approach be undertaken for the PGM_1 study. Various authors have cited the relative positioning of the c± bands in relation to the b± bands. Bark [*24*] originally postulated that "it is probable that the

1+ and 1− bands and the 2+ and 2− bands by isoelectric focusing result from splitting the a and b bands respectively. Some of the bands focusing at lower pH would then result from the c and d bands of starch-gel electrophoresis." Bark made no mention of the c bands causing problems with interpretation. Burdett [25], using Pharmalyte® pH 4 to 6.5 ampholytes, referred to "storage bands intermediate between the b− and b+ isoenzymes," which White [26] in an editorial response postulated was actually the c− band. White further added that in his experience, Pharmalyte pH 4 to 6.5 ampholytes focused the c− band between the 2+ and 2− bands, whereas Ampholine pH 5 to 7 ampholytes focused both c+ and c− anodal to the 2+ band. Burdett's reply [27] essentially said that no matter what the band was called, "it in no way compromises the identification of the PGM phenotype." Divall [28], using Ampholine pH 5 to 7 ampholytes, apparently observed the c− band near the 2+ position. In his opinion, "the juxtaposition of the b+ and c− bands means that the isozyme patterns of PGM (1−) and (2+1−) could easily be confused if band intensities are not considered." Divall adds, "this is, in our opinion, the most likely explanation for anomalies between results obtained by starch gel electrophoresis and IEF."

Sutton and Westwood [29] acknowledged the problem of the b+ with *c*± confusion and offered possible solutions: (1) admixture of pH 5 to 7 with pH 6 to 8 ampholytes (unpublished) and (2) use of an immobilized pH gradient presented in their paper. "The electrofocusing pattern of the a± and b± isoenzymes conformed to the separation normally achieved on a pH 5 to 7 Ampholine gradient except that the c−/c+ isoenzymes were excluded. This system therefore eliminates the possibility of mistyping errors occurring especially with the homozygotes PGM_1 1− or 1+." Budowle [30], using the separator EPPS with pH 5 to 7 ampholytes, also reported c− with 2^+ coincidence. His conclusion: "Band intensities play a part in the interpretation of the PGM_1 subtypes. In particular, the c− band lay approximately in the same position of the gel as the 2+ band. However, the c− band was far less intense than the 1− band. Thus in a 1−2+ phenotype, the 2+ band was equal to or slightly greater in intensity than the 1− band." However, Budowle adds, "Currently, we are investigating methods for separating the c− and 2+ bands to eliminate any possible confusion with typing."

Obviously, these authors were concerned enough about possible c± and 2+ banding confusion to cite their respective opinions. Only Sutton, using an immobiline method, felt the problem was entirely resolved even though no blind proficiency was mentioned. If there is consensus among the other authors it would most likely follow the opinions of Divall and Budowle that an assessment of band intensities will suffice to distinguish true phenotypes. This assessment of PGM_1 band intensities, akin to the AcP_1 b and c bands, is subjective, dependent on both the method used and the experience of the analyst. Subjectivity is not a deadly sin. However, subjectivity does require more stringent precautions on the part of the analyst.

The PGM_1 blind study was conducted in two parts because of the preceding discussion. The first blind test was designed to test the ability of the analyst to identify correctly PGM_1 subtypes without benefit of conventional PGM_1 typing results. Subsequently, the samples were reviewed with consideration given to conventional PGM_1 typing. This tandem method approach is not novel. Dykes [2] proposed this method to resolve rare variants which could not be distinguished by IEF or conventional methods alone. It is of interest that, of the authors reviewed, only Dykes professed to using conventional PGM_1 typing to supplement IEF.

Figure 6 depicts common phenotypes of the PGM_1, ADA, and AK isoenzyme systems as they appear on the method under review. Focusing on the PGM_1 system for the moment, it can be seen that the common phenotypes are differentiated. Two samples deserve special attention. Sample 5 in Fig. 6 is conventionally typed as a 2-1. It can be seen that the 2+ isoenzyme is slightly less intense than the 1+ isoenzyme. If we were to

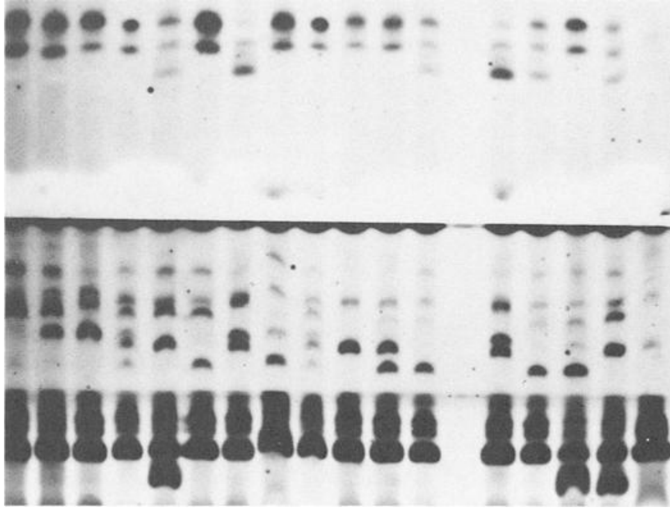

FIG. 6—*Photograph of PGM_1 sub, ADA, and AK by IEF method reviewed. PGM_1 subtypes* (center) *starting from left are: 2^+; 2^+2^-; 2^-; $2^+2^-1^+1^-$; 2^+1^+; 2^+1^-; 2^-1^+; 2^-1^-; $2^+2^-1^+1^-$; 1^+; 1^+; 1^+1^-; 1^-. ADA types* (top) *starting from the right are Knotts (SERI, weak); 2-1; 1; 2-1; 2 (LASO). AK types* (bottom) *starting from the right are: 1; 2-1; 2 (LASO); 1; 1. Note that the AK isozymes are overdeveloped. Anode is at top.*

assign a phenotype based only on band intensities, would this constitute a homozygous 1+ with an intense c+ band, or a true heterozygous 2+1+? For an even more prominent difference, draw your attention to Fig. 6, Sample 8: this sample types conventionally as a PGM_1 2-1, and yet here the 2− isoenzyme band is much less intense than the 1− isoenzyme. Although no confusion would result from c± bands in this case, there is every reason to assume the same intensity difference could also occur at the problematic 2+ position in other samples. Analysts in this laboratory have seen these disparaging intensity differences too many times to feel confident in assessing PGM_1 phenotypes based on banding intensity alone. Indeed, our study showed, at least for our method and experience, that some phenotypes were misclassified if IEF results were interpreted alone (see Table 2).

Another aspect to bear in mind is that if these were evidential stains with an unknown history, the possibility of mixed blood or other biological fluid could not be ignored. In such cases, a band appearing in the 2+ region which might otherwise be dismissed as a c band might appropriately be classified as a 2+ if a conventional PGM_1 type is obtained. To prevent misclassifications as a result of these problems, our laboratory uses the tandem method approach.

The rare PGM_1 variants used in this study were by no means a comprehensive list. The reader is referred to Dykes [*2,3,31*] for a more comprehensive coverage of PGM_1 rare types. Our study attempted to include those rare alleles which would cover the range of cathodal, midrange, and anodal variants. Figure 7 depicts the ability of the IEF method under study to differentiate the 3, 4, 6−, 6+, 7, and 8 alleles. Of these, the 3 and 7 alleles focused anodic to the 2+ isoenzyme band, the 4 focused just anodic of the 2− isoenzyme band, the 6+ allele focused just cathodic to the 1− isoenzyme band, and both the 6− and 8 alleles focused substantially cathodic to the 1− isoenzyme band. Referring to Dykes's papers, we would say we had about equal success in discriminating

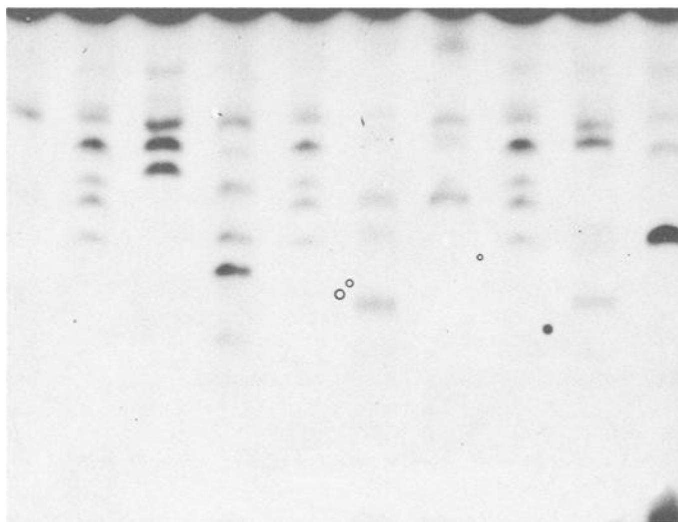

FIG. 7—*Close-up of PGM_1 overlay of IEF plate showing rare PGM_1 phenotypes. Samples from left to right are: (3)1^+; $2^+2^-1^+1^-$; (4)2^+; 6^+1^-; $2^+2^-1^+1^-$; 6^-1^+; (7)1^+; $2^+2^-1^+1^-$; (8)2^+; 1^-. [The (3)1^+, and (8)2^+ were obtained from Dykes. The (4)2^+, 6^+1^-, 6^-1^+, and (7)1^+ were obtained from SERI.] Anode is at top.*

among these rare alleles and would definitely concur with his assertion that to be able to distinguish rare PGM_1 alleles, especially those anodic to the 2+ allele, a conventional PGM_1 method must be used (Fig. 8).

ADA

Figure 9 depicts various ADA phenotypes as they appear on conventional electrophoresis. These same ADA phenotypes can be seen as they appear on the IEF method in the right anodic section of Fig. 6. Both systems distinguish the phenotypes equally well. Kuo [*11*] reported that ADA bands in his original method were distorted by wavy iso-pH lines near the anode. Our method does not suffer from this problem, perhaps as a result of the use of the pH 4 to 6.5 interval instead of pH 4 to 8, as Kuo used. No rare ADA variants other than a weak ADA Knotts were available for this study.

AK

Figure 10 represents the common AK phenotypes as they appear on conventional electrophoresis. Note that the band intensities are very light because the photograph was taken after only 10 min of incubation. This is necessary to distinguish the homozygous AK 2 phenotype from the heterozygous 2-1 phenotype. If incubation proceeds too long, then the homozygous AK 2 develops to look like a heterozygous 2-1. This fact is readily apparent in the AK phenotypes represented in the right cathodic corner of Fig. 6. This idiosyncrasy of AK typing is independent of the method used. Both conventional electrophoresis and this IEF method are equally capable of distinguishing the common phenotypes of AK. No rare variants of the AK system were available to test on the IEF method under review.

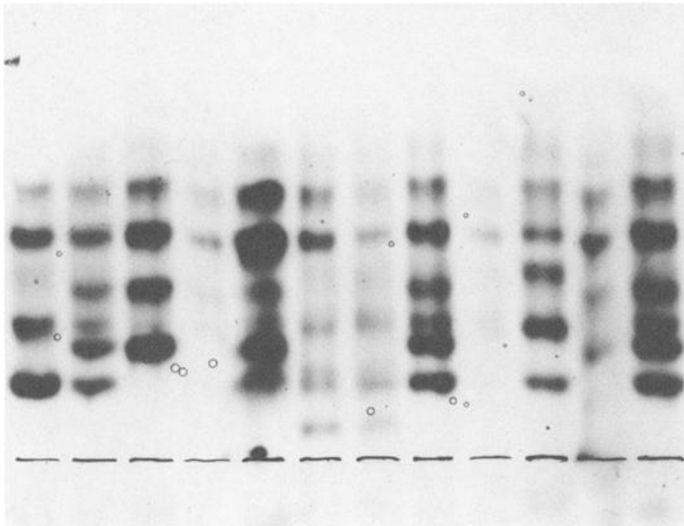

FIG. 8—*Photograph of PGM_1 phenotypes by conventional electrophoresis. Samples from left to right are: 1; 2-1; 2, 3-1 (weak); 4-2; 6-1; 6-1; 2-1; 7-1 (weak); 7-1; 8-2; 2-1. (The 3-1, first 7-1, and 8-2 obtained from Dykes. The 4-2, both 6-1's, and second 7-1 obtained from SERI). Anode is at top.*

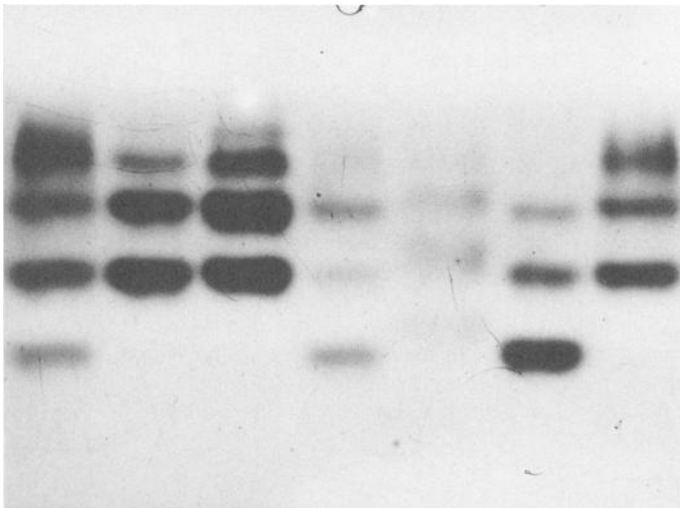

FIG. 9—*Photograph of ADA phenotypes by conventional electrophoresis. Samples from left to right are: 2-1; 1; 1; 2-1; Knotts (SERI, weak); 2 (LASO); 1. Anode is at top.*

General Discussion

The development of multiple isoenzymes with this method requires a discussion of advantages as well as pitfalls of using multiple overlays. The technique of removing the cellulose acetate membrane (CAM) overlays does allow the five enzyme systems to be incubated at the same time. The inversion of the CAM strips on glass plates provided more intense and less diffuse banding when compared to viewing isoenzyme patterns

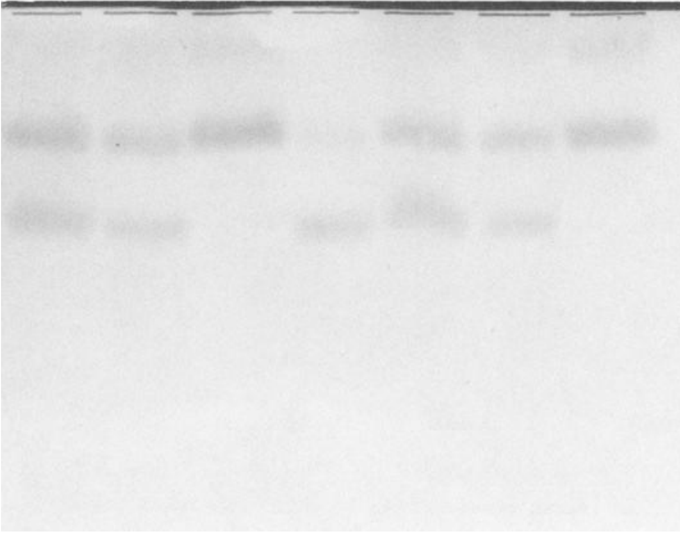

FIG. 10—*Photograph of AK phenotypes by conventional electrophoresis. Samples have incubated for only 10 min at 37°C. Samples from left to right are: 2-1; 2-1; 1; 2(LASO); 2-1; 2-1; 1. Anode is at top.*

through the CAM material. Note in particular that the split plate method of Dorill [*4*] and Kuo [*10,11*] for AcP_1 and PGM_1 was not used in this study.

Concerning the use of a multiple overlay technique to develop AcP_1 and then PGM_1, we designed a simple experiment to determine what detrimental effect, if any, the AcP_1 overlay would have on the PGM_1 isoenzymes. Figure 11 depicts an IEF plate where two equal sets of serial dilutions were run on the gel. The set of dilutions on the right was overlayed in the usual manner with the AcP_1 and EsD overlays, while the left set was not. The gel was then overlayed for PGM, AK, and ADA isoenzymes. After incubation, the gel was examined to determine the last dilution where all PGM_1 isoenzymes were clearly visible. Comparison between the two sets of dilutions reflects a loss of approximately one doubling dilution as a result of the AcP_1 overlay. No detectable difference was seen in band resolution as a result of the AcP_1 overlay. Although some serologists question the use of "multisystems," and in particular multiple overlays, it is our considered opinion that this method does not seriously compromise the viability of the PGM isoenzymes, it does not result in misclassification of phenotypes, and it substantially reduces the sample amount required to perform these analyses as compared with conventional methods.

One precaution should be noted with respect to the EsD and ADA overlays. In our method we attempt to limit the EsD overlay to just anodic of the EsD homozygous 5 band, thereby preventing the overlay of any ADA isozyme bands. If the EsD overlay extends too far towards the anode, one or more of the ADA isoenzyme bands could be overlayed. This might lead to selectively diminished banding intensities. For example, it might be possible to overlay just the homozygous ADA 2 band, causing a selective lessening of band intensity at that position. This might cause an ADA 2 phenotype to appear as a 2-1, or an ADA 2-1 phenotype to appear as a 1. Note that if this condition does arise, the ADA 2-1 standard will be affected in the same way.

Samples in a blind testing study were analyzed using both the IEF method and con-

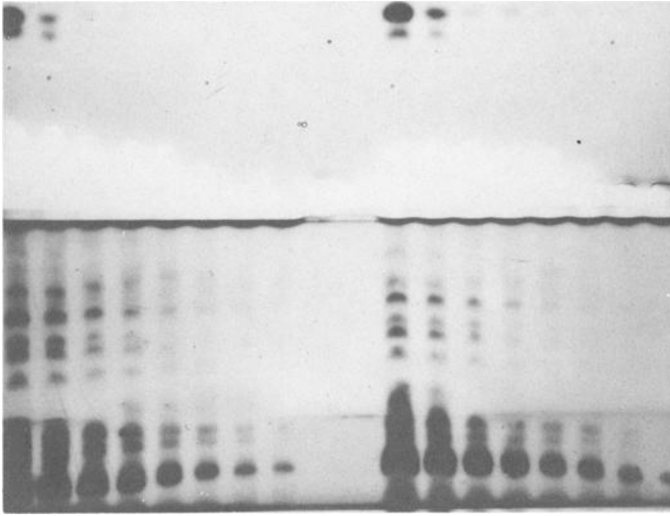

FIG. 11—*Photograph of IEF plate showing two sets of doubling dilutions of a PGM_1 four-band standard. Right half of gel was overlayed for AcP_1 and EsD as our method describes; left half of gel was not overlayed for AcP_1 or EsD. Plate was then developed normally for ADA, PGM_1, and AK. Anode is at top.*

ventional electrophoresis. Table 3 summarizes a comparison of the phenotyping results between these methods.

The blind study contained only two discrepancies, both of which are suspected rare variants. These results simply reiterate that all testing results must be viewed with a watchful eye for rare types. We know of no electrophoretic method which is immune to these subtleties. Still, with proper precautions and by applying known limitations, qualified analysts will have no problems using this method. However, we do have one caveat. Because this method employs nonequilibrium conditions, optimization of run conditions will likely be required on a laboratory-to-laboratory basis. Generally this will require varying time, voltage, or temperature conditions, or some combination of these until the right combination is achieved.

Another very real concern to a serologist contemplating using this system is the judicious choice of standards. Having to represent so many alleles can easily result in more standards than questioned samples on a plate. Our system routinely includes the standards shown in Table 4, with case samples always juxtaposed to a PGM_1 four-band standard.

Summary

We have found that the time spent in researching this method has easily repaid itself, if not several times over. This is due primarily to our ability to screen rapidly many bloodstains through a single method which offers a high degree of discrimination while at the same time conserving sample for subsequent analyses. In a recent case, one analyst examined 30 bloodstains from a homicide case through the 5 isoenzyme systems in under 12 h of analytical time. One of the stains was inconsistent with the victim's blood. This single stain, consistent with the suspect and 0.03% of the general population, was enough to hold the suspect through the arraignment process while further evidence was developed.

Assuming, as with any electrophoretic method, that proper standards are used with

TABLE 3—*Comparison of IEF method and conventional electrophoresis in a blind testing study.*

Isoenzyme System	EsD	AcP_1	AK	ADA	PGM_1 Part 1	PGM_1 Part 2
Total samples examined	246	170	162	157	129	129
Total IEF samples confirmed	246	169	162	157	125	128
Total number of discrepancies	0	1	0	0	4	1
Comments	[a]	[b]			[c]	[d]

[a]EsD 5-1 and 5-2 results recorded as consistent with conventional 2-1 and 2 results, respectively.

[b]Lone discrepancy is classified conventionally as an AcP_1 DB, which by IEF is indistinguishable from an AcP_1 BA.

[c]Part 1 results were collected without benefit of PGM conventional results, three of which were misclassified because of the 2^+ versus c $\pm$ relationship discussed in the body of the text.

[d]Part 2 results rectified the three misclassifications in Part 1 by simple comparison to PGM_1 conventional results; remaining discrepancy is a suspected rare type diagnosed by IEF but not distinguishable on conventional typing.

TABLE 4—*Standard routinely used by this system.*

Standard	EsD	AcP_1	PGM_1sub	AK	ADA
1	2-1	B		2-1	2-1
2	5-1	CB		1	2-1
PGM 4 Band	1	BA	2+2-1+1-	1	1

the proper precautions, this method provides an excellent discriminating tool for forensic serologists. It behaves similar to the observations of other authors who explored the abilities of IEF to type reliably common and rare alleles of the EsD, AcP_1, and PGM_1 isoenzyme systems. Although no comparable IEF system was found for the AK or ADA systems, our testing shows common phenotypes of these isoenzymes are reliably typed as well.

Acknowledgments

The authors are greatly indebted to Dale Dykes of the Minneapolis Memorial Blood Bank, Gary Harmor of the Serological Research Institute, Barbara Johnson of Los Angeles County Sheriff's Criminalistics Laboratory, and Sze Ern Kuo, formerly of the Los Angeles Police Department Criminalistics Laboratory, for their contributions of rare variant standards without which this research could not have been conducted.

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