TECHNICAL NOTE

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The Simultaneous Separation of the Enzymes Glyoxalase I, Esterase D, and Phosphoglucomutase

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ABSTRACT: A procedure for the multisystem analysis of bloodstains using the simultaneous separation of the enzymes glyoxalase I, esterase D, and phosphoglucomutase has been developed. The amount of bloodstain required has therefore been reduced threefold without any loss in resolution and sensitivity. Bloodstains at least seven weeks old have been correctly phenotyped in all three systems.

KEYWORDS: forensic science, blood, genetic typing, enzymes

The enzymes phosphoglucomutase (PGM), E.C.2.7.5.1; esterase D (EsD), E.C.3.1.1.1; and glyoxalase I (GLO), E.C.4.4.1.5 were all shown to be polymorphic, respectively, by Spencer et al. [*1*], Hopkinson et al. [*2*], and Parr et al. [*3*]. All were described as being separated on thick starch gel at neutral pH. Subsequently, the enzymes were adapted to forensic bloodstain analysis [*4-6*] using the same buffer systems as those described by the original authors. The use of thick gels for bloodstain analysis was largely discontinued in 1968 with the development of 1-mm-thick starch gels [*7*] allowing for a more judicious use of limited bloodstain material. Although starch gel has been the method of choice for separating most polymorphic enzymes, Monn in 1968 [*8*] separated the enzyme PGM using agarose gel.

A number of enzymes are routinely separated at similar pHs, strongly suggesting that they can be separated together on one gel. The advantages of a multisystem approach are (1) a

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savings of analyst's time and (2) considerable savings of limited bloodstain material. These considerations become more important now that there are as many as 20 different blood grouping systems available for the typing of bloodstains. Nonetheless, of greater importance, is that poor resolution, loss of activity resulting from nonoptimum pH, or the overlapping of enzyme bands leading to misinterpretation of results should not be introduced to save time and/or bloodstain.

The idea of combining two or more enzymes and separating them at the same time on a single electrophoresis gel is not new. Many authors have shown the possibilities of this approach both in forensic bloodstain analysis and in other scientific disciplines. For example, in 1971, Brinkman and Thoma [9] separated the enzymes 6-phosphogluconate dehydrogenase (6PGD), adenosine deaminase (ADA), and adenylate kinase (AK) on the same gel. In 1982, Conkle et al. [10] used a multisystem approach for starch gel electrophoresis of conifer seeds. For bloodstain analysis, Culliford in 1971 [11] reported the separations of the enzymes pseudocholinesterase/E2 locus (PCE/E2) and AK on one gel, and in 1975, Blake and Sensabaugh [12] reported the separation of esterase D with either erythrocyte acid phosphatase (EAP) or PGM. In 1976, Nielson et al. [13] separated peptidase A (PEPA), PGM, and AK simultaneously using starch gel.

A multisystem analysis can be achieved in several ways. Some workers have used thick starch gels, sliced the gels horizontally, and stained individual slices for separate enzymes. Others have divided the gel vertically after electrophoresis leading in some cases to overlapping of one enzyme with another. Blake and Sensabaugh [12] reported this problem when separating EAP and EsD together. The disadvantages of some of these multisystem approaches are: (1) overlapping of the isozymes from different enzymes, (2) poor separation making interpretation difficult, (3) nondetection of rare variants, and (4) loss of enzyme activity as a result of nonoptimum development.

Obviously then the enzymes to be separated simultaneously have to be very carefully chosen. Similarly the choice of separating pH is equally important. When starting this project it was observed that most of the polymorphic enzymes and proteins that are used in bloodstain analysis were separated at either an acid pH (5 to 6), neutral pH (6.5 to 7.5), or alkaline pH (8.5). These enzymes and proteins were therefore initially placed into three appropriate groups. This paper is concerned with Group I where the three enzymes are separated at neutral pH. The separation of the other enzymes and proteins at acid and alkaline pH (Groups II and III) are the subject of further papers. As was mentioned before, the three enzymes PGM, EsD, and GLO were all separated individually at neutral pH (PGM and EsD at 7.4 and GLO at 6.8). It was apparent that these three enzymes were good candidates for a multisystem analysis and so a study was conducted to ascertain if they could be simultaneously separated. From the results obtained it is now considered that not only can they be separated at the same time, but that there is a substantial improvement of the resolution of the isozyme bands of EsD and GLO compared to their respective single-system methods of conventional electrophoresis.

All three buffers used for the individual separation of the enzymes were tried with the multisystem approach. The buffer showing the most promise was the buffer used for PGM by Spencer et al. [1]. Most of the initial work was carried out using a 10% starch gel 1 mm thick. It was subsequently found, however, that agarose gel gives sharper isozyme patterns and was easier to prepare and handle than starch. Two percent of starch gel was initially incorporated into the agarose gel to facilitate development of the GLO (a starch iodine complex formation); however, it was observed that the agarose-starch mixture afforded resolution superior to either starch or agarose alone.

The advantage of separating polymorphic enzymes individually is that the separation distance and development surface area are not restricted and the optimum conditions of both separation and development can be employed. Using the parameters reported above [1-3], it is commonly assumed that all three enzymes, when analyzed individually, are separated un-

der optimum conditions. Therefore, if any two enzymes are separated simultaneously, then at least one will be separated under less than optimum conditions. As we will show, with these three enzymes by this particular method, we believe this is not true.

Materials and Methods

Electrophoresis

Tank Buffer—The tank buffers were:

0.1*M* trizma base, 12.11 g;
0.1*M* maleic acid, 11.62 g;
0.01*M* magnesium chloride, 2.03 g;
0.01*M* ethylenediaminetetraacetic acid (EDTA), 2.92 g; and
0.15*M* sodium hydroxide, 6.00 g.

All dissolved in 1 L of distilled water, giving a final pH 7.4. The gel buffer is made by diluting 1 volume of the tank buffer with 14 volumes of distilled water, final pH 7.4.

The gel consists of 1% agarose (EEO 0.08) and 1% hydrolysed starch. (The starch is required for the development of the GLO. The presence of 1% starch reduces the effects of electroendosmosis in the agarose.) The agarose/starch mixture is dissolved and a 1-mm-thick gel is prepared [*7*]. The separation of the three enzymes requires a minimum distance of 20 cm between anode and cathode.

Sample Application

Blood samples are prepared by washing cells three times in physiological saline and mixing two volumes of packed cells with one volume of freshly prepared 0.05*M* dithiothreitol (Cleland's reagent). Hemolysates are inserted into the gel on 1-cm-long cotton threads (Size 8).

Bloodstains are treated by removing 1-cm-long threads or sections, soaking in 0.05*M* dithiothreitol for 5 to 10 min, and inserting carefully into the gel. Stains on hard surfaces (for example, metal, glass, and so forth) can be removed by swabbing with a minimum amount of Cleland's reagent soaked on No. 8 cotton threads. Dilute or diffuse stains can be extracted into a minimum of Cleland's reagent, concentrated by further extracting more stain in the initial extract, and taking up on cotton threads.

The origin is located 2½ cm from the cathode. The control reference sample consists of a GLO Type 2-1, EsD 2-1, and PGM 2-1.

Electrophoresis is carried out at 20 V/cm for 2½ h on cooling plates at 4°C. The voltage should be carefully measured across the gel with a volt meter. To reduce band distortion, threads may be removed from the gel 20 min after electrophoresis is initiated.

Development

Esterase D

Methylumbelliferyl acetate, 4 mg, is dissolved in a minimum quantity of acetone and mixed with 10 mL of 0.05*M* sodium acetate buffer adjusted to pH 6.5 with 1% acetic acid. The reaction mixture is soaked onto Whatman 3 MM filter paper, excess removed, and the paper overlayed on the gel from the origin to 10 cm toward the anode. The gel is incubated at room temperature for approximately 10 min and the results read under long wave ultraviolet light after the removal of the filter paper overlay. The fast moving EsD 2 isozyme band

should be approximately 8 cm from the origin. After reading the results, a plastic strip is placed on the gel at the EsD 2 position.

Glyoxalase I

Reduced glutathione, 12 mg, and 50 μL of 40% methyl glyoxal are dissolved in 10 mL of 0.2*M* phosphate buffer, pH 6.2. This mixture is soaked onto an 8-cm-wide Whatman No. 1 paper, which, after removing any excess, is laid on the gel, anodic of the plastic strip. The gel is incubated at 37°C for 25 min. After incubation, the paper overlay is removed. Agarose, 0.5 g, is dissolved in 30 mL of distilled water and cooled to 60°C. Iodine solution, 0.2 mL, (1.65 g of potassium iodide, 2.54 g of iodine in 30 mL of distilled water, filtered and stored in a stoppered dark bottle at 4°C) is added and poured onto the anodic portion of the gel. The glyoxalase I isozymes are immediately visible as blue areas on a clear background.

Phosphoglucomutase

Glucose-1-phosphate, 35 mg (containing at least 1% G-1-6-P2), 2 mg of nicotinamide-adenine dinucleotide phosphate (NADP), 2 mg of 3-(4,5-dimethyl thiazolyl-2)-2,5 diphenyl tetrazolium bromide (MTT tetrazolium), 1 mg of phenazine methosulphate, and approximately 2 IU glucose-6-phosphate dehydrogenase are dissolved in 10 mL of 0.1*M* Tris, 0.02*M* magnesium chloride buffer adjusted to pH 8.0 with 1:1 hydrochloric acid (HCl). The reaction buffer is mixed with 10 g of 2% purified agar at 55°C and poured onto the gel between the origin and 8 cm anodic of the origin. The PGM reaction overlay is poured at the same time as the GLO first overlay is applied to the gel. After the iodine/agarose overlay is poured and the GLO results recorded, the gel is reincubated at 37°C for approximately 40 min. Weak stains can be enhanced by a longer incubation time.

Results and Discussion

The results of simultaneous separation of the enzymes GLO, EsD, and PGM (hemolysates and stains) are shown in Figs. 1 through 6. In the EsD system, the isozymes are well separated to the extent that the problem of interpretation of stains, as reported by Parkin and Adams [5], is resolved. The oxidized secondary isozyme bands migrate with a different mobility from the primary isozyme bands, unlike the former methods of EsD electrophoresis. By the Group I method, the center isozyme in an EsD Type 2-1 (the heteromeric band) is more intense, even in older stains, and by this method cannot be easily misinterpreted as a Type 1 as reported by Parkin and Adams. As can be seen from Fig. 4, stains seven weeks old have been successfully typed. Provided a reducing agent is present, it is possible to type bloodstains up to seven months old. The rare EsD Variants 3 and 7 have been easily separated using this system.

Glyoxalase I types are clearly resolved, although like EsD, they do require a reducing agent for optimum activity. An anodic shift of approximately 0.5 cm has been seen on all types if a reducing agent is not present in the sample. The GLO staining reaction is difficult to control at first as it requires a fine balance between the concentration of the reduced glutathione and the concentration of iodine. If the initial overlay is too wet before adding the agarose/iodine, then the iodine is utilized by the excess reduced glutathione before it can react with the exposed starch at the isozyme sites. If the overlay is too dry or the gel is blotted too dry after the first overlay, then insufficient reduced glutathione is present and the iodine reacts with the starch to produce a blue background. If the iodine is applied in excess, a dark blue background is produced.

Similarly, if the agarose is too hot when the iodine solution is added, then the iodine sub-

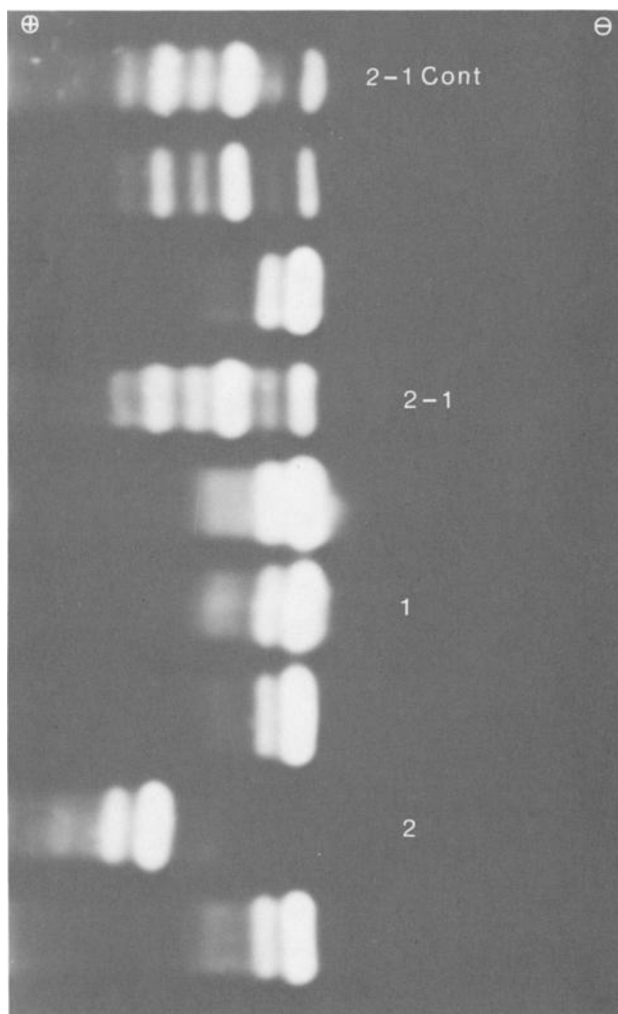

FIG. 1—*EsD phenotypes on hemolysates.*

limes before being poured on the gel causing the failure of blue bands to develop with sufficient intensity. However, once this balance has been achieved on a regular basis, reproducible results are easily obtained (Fig. 2). If the incubation of the first overlay exceeds 25 min, then smears and blurring may occur around the isozymes. It should be remembered that if no GLO isozyme develops, the possibility exists that the enzyme has migrated into the bridge. Bloodstains seven weeks old have been successfully phenotyped in GLO using this system (Fig. 5).

PGM types on hemolysates are shown in Fig. 3. The important isozymes b and c are clearly resolved, thus enabling the analyst to distinguish easily between the PGM 1 and 2-1. Both the anodic second locus isozymes PGM_2 and the slow cathodic variant isozymes PGM_1 6 and 8 are easily seen using this system. Stains dried at room temperature as old as ten months have been successfully phenotyped in the PGM system. For results on dried stains

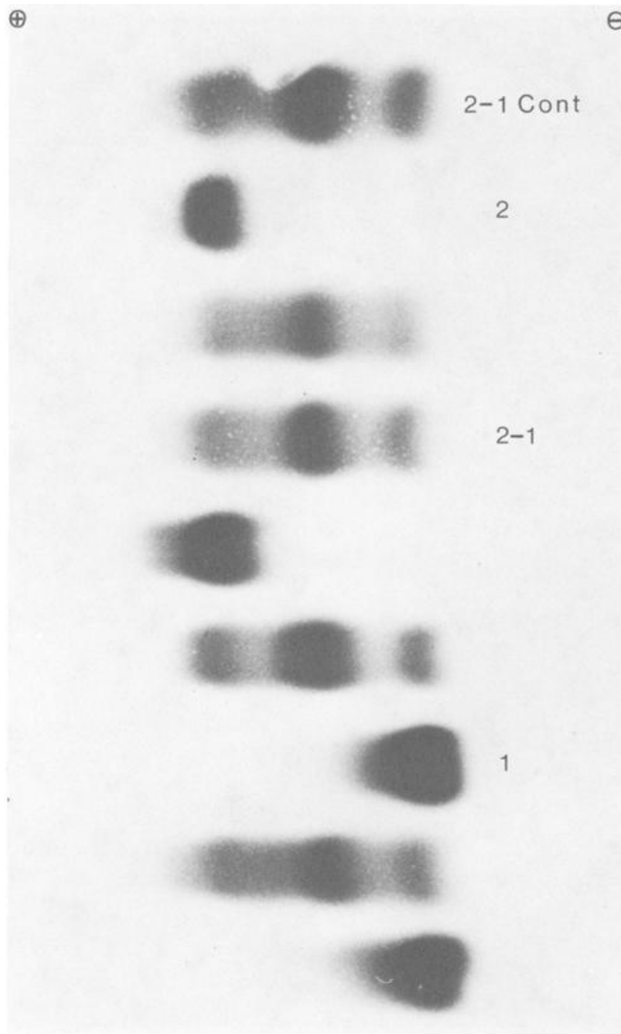

FIG. 2—*GLO phenotypes on hemolysates.*

see Fig. 6. Three common problems associated with this enzyme are (1) distortions of the first locus isozymes caused by too much sample, (2) the use of an agarose with too high an endosmosis, and (3) Locus 1 isozyme activity weak in comparison with Locus 2 caused by reduced 6PGD enzyme activity.

All of the reaction buffers and mixtures have been optimized to give the greatest sensitivity. It is important to remember that regular reproducible results can only be achieved if a fine balance between temperature, voltage, and time is controlled.

After the system had been developed, two series of blind trials were initiated. The first consisted of eighteen bloodstains aged from two to four weeks supplied by an external agency. All stains were subjected to analysis by one of us (BW) using the Group I procedure. Fifty-four readings were made resulting in all stains being correctly phenotyped.

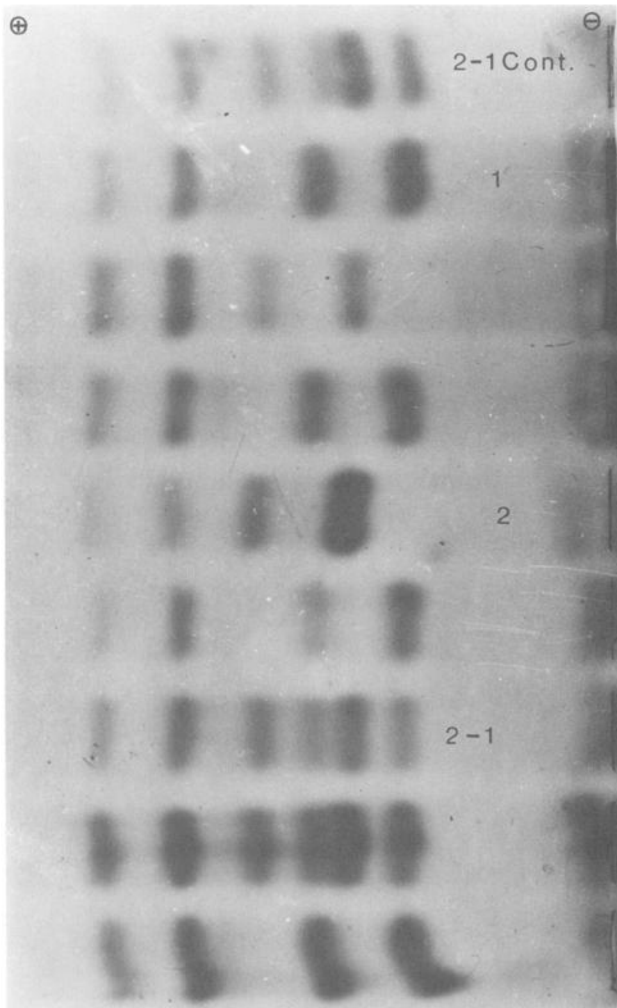

FIG. 3—*PGM phenotypes on hemolysates.*

The second blind trial consisted of 30 bloodstains ranging in age from 1 to 7 weeks which were sent to a representative serologist from each of 4 evaluating crime laboratories who had been trained in the new methods. Using the Group I system for analysis of the stains, 360 readings were made. All stains were correctly phenotyped in the GLO, EsD, and PGM systems.

Since the development of this technique many serologists have been taught this technique both at the Serological Research Institute (SERI) and the Training and Applications Laboratory in Joliet. All trainees have been subjected to blind trial testing at the training facility and occasionally at their home laboratory. Over 200 serologists have received this training, many of whom are now using the procedure in their case work. The documentation of the blind trial results is by no means complete, but a review of available data allows us to present Table 1.

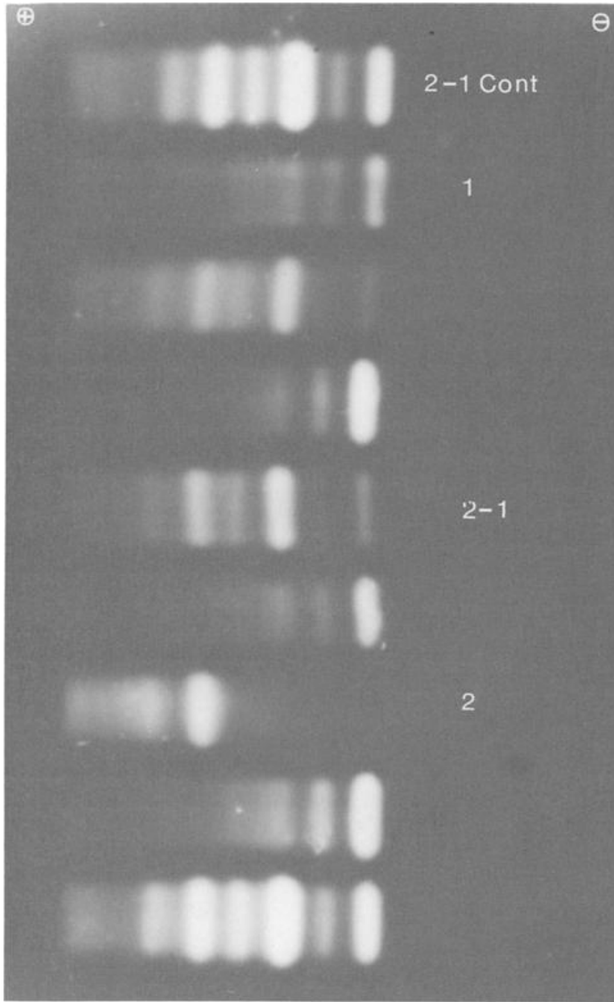

FIG. 4—*EsD phenotypes on dried bloodstains. EsD 2-1 control at top. Remaining eight slots consist of bloodstains from one to seven weeks old.*

Note that the error rate at the Training Facility is approximately 1%, whereas at the home laboratory the error rate is 0.6%. The majority of errors tended to be confined to the GLO system and also to those students who were inexperienced in electrophoretic techniques at the time of training.

Conclusion

As can be seen from the photographs, substantial improvement in the separation of the EsD and GLO isozymes has been achieved. There has been no loss of resolution in the PGM system. We believe that this multisystem approach substantially improves the grouping of bloodstains, allowing savings of time and bloodstain with band resolution superior to that seen with many other published procedures.

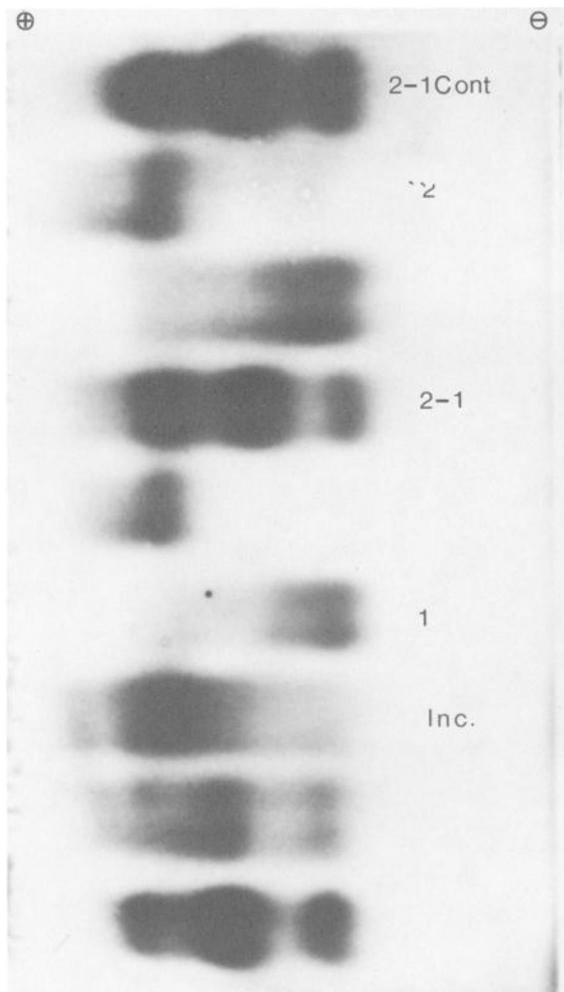

FIG. 5—*GLO phenotypes on dried bloodstains. GLO Type 2-1 control at top. Remaining slots consist of bloodstains from one to seven weeks old.* Inc. = *inconclusive.*

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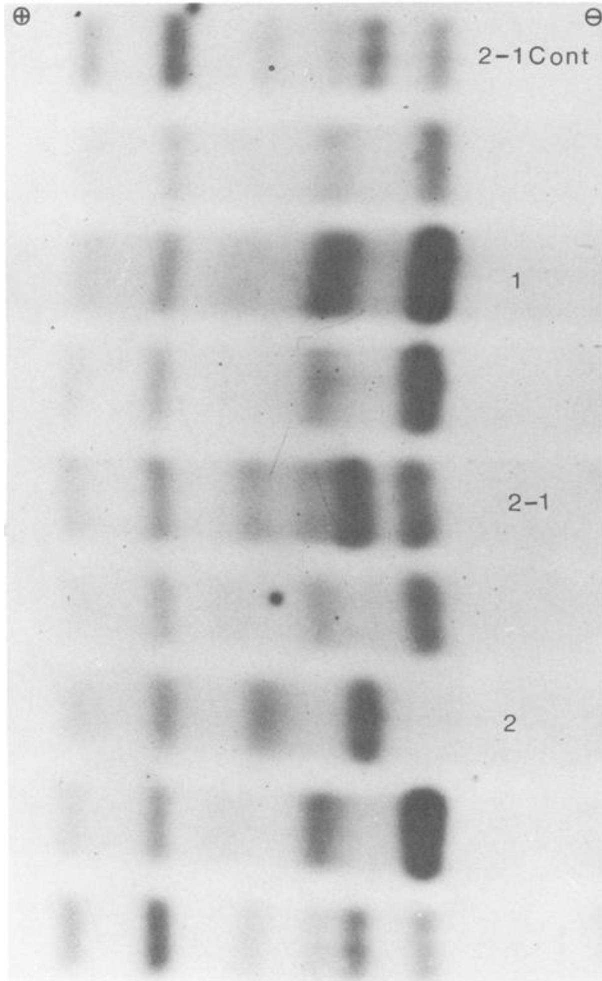

FIG. 6—PGM phenotypes on dried bloodstains. PGM Type 2-1 control at top. Remaining slots consist of bloodstains from one to seven weeks old.

TABLE 1—*Blind trial results (SERI) with five stains three days to four weeks old.*

Total No. of Students	Total Readings	Correct	Incorrect	Questioned	No Activity
		TRAINING FACILITY			
93	1395	1258	14	88	35
		HOME LABORATORY			
54	789	656	5	78	50

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