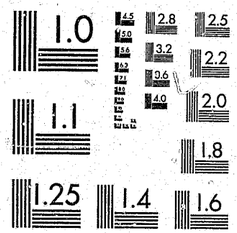

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National Institute of Justice
United States Department of Justice
Washington, D.C. 20531

FINAL REPORT

BLOODSTAIN ANALYSIS SYSTEM

By

Brian Wraxall: Forensic Consultant
Jean Bordeaux: Program Manager
Gary Harmor: Forensic Chemist
Jack Walsh: Manager, Life Sciences

Subcontract 67854

July, 1978

Prepared For:

The Aerospace Corporation
Suite 4040, 955 L'Enfant Plaza, S.W.
Washington, D.C. 20024

BECKMAN®
Advanced Technology Operations
Beckman Instruments, Inc.,
1630 South State College,
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FR-2700-101

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ABSRACT

The Bloodstain Analysis System project was initiated to determine whether existing principles of electrophoretic separation of blood genetic markers could be extended to provide increased capability for discriminating one person from another with improved reliability, ease of analysis, and with limited equipment. Candidate blood constituents were reviewed to determine which had the properties to permit their use to meet program objectives. It was determined that eight constituents would be needed to provide the required 1-in-200 discrimination capability. The selected constituents were separated on various substrates to establish individual optimum methodologies.

After these optimum constituent/substrate combinations were determined, the potential for simultaneous analysis was explored. The eight constituents were grouped according to the pH at which they were normally subjected to electrophoresis. The three groups are (I) GLO I, PGM, EsD; (II) ADA, EAP, AK; and (III) Gc and Hp.

Many iterations using differing analytical conditions were required to develop the procedures that yielded the quality of results necessary for forensic laboratory use. That development objectives had been met was shown in a Feasibility Demonstration Test, using unknown samples of dried bloodstains. A training session was then held with criminalists to demonstrate the capability to transfer the technology to others. Field testing at four participating crime laboratories demonstrated the utility of the Bloodstain Analysis System in the working crime laboratory. Thus, all contract requirements have been satisfied.

CONTENTS

PREFACE

This work was performed under subcontract W-67854 issued by The Aerospace Corporation, under prime contract J-LEAA-025-73. The project was initiated in December, 1976, and completed in June, 1978. This project was part of a program of the National Institute of Law Enforcement and Criminal Justice, Law Enforcement Assistance Administration, U.S. Department of Justice, "...to encourage research and development to improve and strengthen law enforcement." To support this objective, an Equipment System Improvement Program was established within the Institute to promote the development and testing of new or improved equipment and procedures.

The specific goal of this project was the development of improved methods for the electrophoretic identification of blood genetic markers--the bloodstain analysis system. To provide strong technical support for the project, Beckman Instruments, Inc., subcontracted to the University of California at Berkeley to utilize the White Mountain Research Laboratory facilities under the direction of Dr. Benjamin W. Grunbaum. Two Staff Research Associates supported Dr. Grunbaum at the laboratory: Gary C. Harmor, and Benny Del Re. This work was done under subcontract number Z-847905-G. In addition to the technical support of Dr. Grunbaum, Beckman retained two criminalist consultants recognized as expert in the electrophoretic analysis of blood stains: Brian G. D. Wraxall of the Metropolitan Police Laboratory, London, England; and Mark D. Stolorow of the Michigan State Police Laboratory.

After approximately nine months of work, Dr. Grunbaum's support was no longer required, and the University of California subcontract was terminated at his request. A report was submitted by Dr. Grunbaum for this period of performance. A copy of this report has been forwarded to The Aerospace Corporation.

The subsequent work, including final development and feasibility testing of the Bloodstain Analysis System, and training of criminalists in the use of the system, was performed by Wraxall and Harmor using Beckman laboratory facilities in Anaheim, California.

Personnel from several crime laboratories participated in demonstration tests to show that the system, as developed, could be transferred from the development laboratory to the working environment of the crime laboratory.

Their experience with the system in their own laboratories generated suggestions for further methodologic improvements and simplifications which have been incorporated. The individuals and their affiliations are:

Ms. Frances Gdowski, New Jersey State Police Laboratory
Mr. Terry Laber, Minnesota Crime Laboratory
Mr. Stephan Schliebe, Los Angeles County Sheriff's Office
Mr. John Wegel, Georgia Bureau of Investigation
Mr. James Kearney, Federal Bureau of Investigation

Robert Shaler, Ph.D, of The Aerospace Corporation offered valuable suggestions throughout the development phase of the project.

We wish to acknowledge the contributions and support of these individuals and their organizations.

SUMMARY

The purpose of this work was to develop new and improved procedures for rapid, low-cost electrophoretic identification of blood genetic markers. The advanced methodologies and equipment were to be demonstrated, tested, and delivered as a Bloodstain Analysis System. This was to be achieved through the use of existing equipment or modifications thereof.

Program goals were:

- Reduce analyst skill requirements
- Reduce analysis time
- Reduce complexity of interpreting results
- Provide for analysis of old stains (four weeks)
- Achieve a discrimination probability of one person out of two hundred

The plan for achieving the program goals was based on improving existing technology for electrophoretic separation of blood constituents. Candidate constituents were reviewed for inclusion in the system using the discrimination index and chemical stability as primary criteria for selection or rejection. No previously unknown enzymes or proteins were discovered for use with the system. Only one constituent not on the list of constituents in the statement of work--Glyoxalase I--was suitable for use. Our review of the blood constituents showed it would be necessary to use eight to obtain the discrimination capability of 1 in 200. Simultaneous electrophoretic separation would be required as no more than three setups were to be used.

It was not a goal of the program to develop new equipment; it was expected initially that some variation of the Beckman Microzone® cell would be utilized. The major thrust of the program was satisfying method/procedure development. The program requirements depended to a great extent on the substrate used during the electrophoretic separation.

Various substrate materials were investigated. The system required a minimum of eight constituents to provide the 1-in-200 discrimination capability; it was found that no one substrate material was satisfactory for all of them.

The candidate substrates were evaluated using "blind trial" tests. As a result of these tests, and the subsequent development of simultaneous analyses, it was found that cellulose acetate membranes (CAM) did not yield satisfactory separations for a sufficient number of constituents to use them as a basis for our system. Our hardware constraints required separation of more than one component at a time. Therefore, substrates offering greater potential for simultaneous analyses were chosen for further development. The materials selected are: starch, acrylamide, and agarose.

Having decided on the specific constituents and substrates, development of methodologies for the simultaneous separation necessary to provide an integrated system was initiated. The methodologies were optimized through repetitive tests with judicious changes in the controllable parameters such as pH, buffer ionic strength and composition, voltage gradient, and time.

Accuracy of the Bloodstain Analysis System was demonstrated with unknown aged stains. Stains from two to four weeks old were presented for identification by The Aerospace Corporation. The accuracy requirements of the contract were satisfied. The results of these tests are presented in the Feasibility Test Report, Appendix A.

Following the Feasibility Tests, a two-week training program was conducted with forensic scientists. During this time, the new analytical procedures were demonstrated and the scientists obtained "hands-on" experience in the use of the system. Subsequently, the system hardware was shipped to four participating crime laboratories. Under actual field conditions, it has been proved that the system could be used

successfully with case material. It was demonstrated that greater discrimination could be obtained with fewer manhours of effort than was previously possible. This work is reported in the Crime Laboratory Demonstration Test Report, Appendix B.

Simultaneous analysis of multiple constituents suggests that somewhat less than optimal analytical conditions for any single constituent might have to be employed. Thus there was some concern that the conditions necessary for simultaneous separations would result in inferior results. This has not been the case; in fact, some separations are improved over those previously considered optimum. Some problems were observed in the analysis with PGM and GLO I, but these are attributed to the enzyme, not the analytical system.

Operator skill is required. It is necessary to follow the procedures rigorously to obtain satisfactory separations. There are some minimum requirements for a technician if he is to absorb the procedural details in two weeks of training. Sufficient expertise can be gained in a month to six weeks to use the system with confidence for case work.

The program goals were met. Additional refinement is undoubtedly possible, but it appears that the basic system is sound.

Analyst skill requirements are reduced as more detailed procedures which yield clearer and cleaner separations are available. Operator time is substantially reduced as it requires only about 3-1/2 man-hours to identify eight constituents in as many as twelve samples including controls.

Based on the discrimination index for a general population of white caucasians, the probability of discriminating one person out of 200 was achieved.

Four-week-old stains can be identified, but this, of course, can vary depending on the storage conditions of a particular stain.

1.0 INTRODUCTION

The overall objective of this program was to develop methods, procedures, and reagents for the improved electrophoretic analysis of bloodstains and the demonstration of a Bloodstain Analysis System. Technical requirements were listed in the contract Statement of Work. For reference, these requirements are repeated in the following paragraphs.

1.1 Speed of Analysis

The manpower needs for the procedure from receipt of the stain at the laboratory to the reading of the analysis results shall not exceed five (5) manhours. Periods of time during which operations proceed unattended are not counted for the purpose of the manpower requirement. The elapsed time for the entire procedure shall not exceed twenty-four (24) hours.

1.2 Skill Requirements

The method to be developed shall be capable of being learned in two weeks and reliably used by typical crime laboratory technicians with approximately two years of college-level chemistry, including organic or biochemistry, plus one year of applicable serological experience.

1.3 Hazards

Reagents used by the method to be developed shall be nontoxic on skin contact or on vapor inhalation. They shall not introduce radiological hazards under prescribed procedures and currently practiced applicable safety provisions. Any deviations from the requirements established by the Health and Safety Act of 1975 shall be justified and brought to the attention of The Aerospace Corporation for approval.

1.4 Bloodstains

The method to be developed shall be capable of analyzing bloodstains found on a variety of commonly found substrates, such as textiles, glass, plastics, cement, paint, etc.

The method used shall be capable of performing the complete analysis on stain sizes equivalent to 50 microliters of fresh blood without consuming more than half of the sample.

The method used shall be able to verify that genetic markers identified in a dry stain can also be found in a sample of the same whole blood.

1.5 Reagent Characteristics

Reagents required by the method to be developed shall be storable at laboratory freezer temperatures for periods up to six (6) months. They shall be nonhazardous as described above. As a goal, these reagents shall be available from more than one source in the United States.

If required, standards and methods of calibrating these reagents against such standards shall be provided.

1.6 Discrimination Probability

The electrophoretic analysis system developed shall be capable of achieving a degree of discrimination probability of one out of 200 randomly selected individuals, using as a sample a bloodstain aged for four (4) weeks.

1.7 Analysis Ambiguity

The contractor shall reduce the ambiguity resulting from interpreting analysis results so that a serologist trained intensively in this procedure for no more than two (2) weeks can clearly interpret those results.

1.8 Simultaneous Analysis

No more than three (3) electrophoretic setups shall be needed in order to run all of the chosen genetic markers simultaneously.

1.9 Accuracy of Analysis

The requirement for accuracy or reliability of detection of the blood constituents selected by the contractor shall be no less than 99 percent at the 90-percent confidence level. This requirement shall be demonstrated during the Crime Laboratory Demonstration Tests and shall apply only to uncontaminated stains. The actual reliability of analysis of contaminated stains will be determined during future testing.

1.10 Cost

The cost of analysis of a single stain using the method to be developed shall be no more than $25.00, including the cost of reagents, equipment amortization, and maintenance requirements. Any equipment or instrumentation used or modified under this contract must, when produced for crime laboratory use, cost no more than $6,000.

1.11 Scope of Effort

To meet these requirements, the work was broken down into several tasks. The first step was to review known blood constituents to determine those with stability and discriminating power adequate for use in this program. Tests were then conducted with these constituents to verify their persistence. Eight constituents were selected for further work. Next, the selected constituents were separated on various substrates to determine which combinations provided the highest quality separations. The level of performance of these individual separations was evaluated by using a series of blind trials.

After a constituent/substrate combination had been established, system development was initiated. Here, several alternative approaches were available. However, schedule and funding constraints would permit

developing only one "system." It was necessary, then, to decide on which approach we should concentrate our effort. One approach, strongly supported by one investigator, was to separate only one constituent at a time and repeat until all eight constituents had been identified. Such a system would require short electrophoresis times such as provided by celloulose acetate membranes in a Beckman Microzone-type cell. While six of the eight candidate constituents can be separated on CAM, our development work showed only Gc to be satisfactory on CAM, while GLO-I, EAP, and ADA either yield better results or must be separated on starch gel. Based on these results, it was decided not to continue working with CAM. Also, the contract limitation of using only three setups could not be met as there would have to be one setup for each constituent.

The three-setup limitation requires simultaneous separation. This can be done with either a parallel strip or zone-type separation (described and illustrated in Section 4.5). The zone separation permits a much larger number of samples to be processed in each cell than does the parallel separation. Also with parallel separation, the band patterns at the edges of the strips are apt to be distorted.

Based on both test results and program requirements, it was concluded that development effort should be directed toward zone-type separations with substrates of agarose, starch, and acrylamide. Based on buffer pH as the most critical common denominator, it appeared that three constituents could be separated on each of two cells and two on the third cell.

The final phase of development was the optimization of the methodologies for the three groups. This involved approximately 600 trial separations. With at least 15 controllable variables being involved, it is apparent that iterations could continue indefinitely. A practical limit was imposed by schedule and funding constraints, but the accuracy of the system was proven through analysis of "unknown" bloodstains. Following successful completion of feasibility demonstration tests, several forensic scientists were trained in the use of the system.

The system hardware and written procedures were then delivered to participating forensic laboratories for field testing. Through the cooperation of these laboratories, the usefulness of the system was evaluated under actual casework conditions. Based on this evaluation, final adjustments were made in the procedures. Reports were written to present the results of the Feasibility Tests and the Crime Laboratory Demonstration Tests. These reports are included in this report as Appendices A and B, respectively.

2.0 SYSTEM OPTIMIZATION

The first step was to consider all the known polymorphic enzymes and proteins to determine which markers, if any, could be eliminated because of low discriminating probability or unreliable persistence in bloodstains. As shown in Table I, at least fourteen genetic markers were to be considered for use. Many more proteins and enzymes showing polymorphism were also considered, most of which were eliminated because of very low discriminating probability. However, three additional polymorphic enzymes merited serious consideration and were therefore included in the pool. These were Glutathione reductase (GR), Uridine monophosphate kinase (UMPK), and Glyoxalase I (GLO I).

TABLE I. LIST OF BLOOD CONSTITUENTS (GENETIC MARKERS) THAT SHALL BE CONSIDERED (From Statement of Work)

Hemoglobin, Hb
Haptoglobin, Hp
Group specific component, Gc
Glutamate-pyruvate transaminase, GPT
Erythrocyte acid phosphatase, EAP
Phosphoglucomutase, PGM
Adenylate kinase, AK
Adenosine deaminase, ADA
Pseudocholinesterase (E1) and (E2), PCE
6-Phosphogluconate dehydrogenase, 6 PGD
Glucose-6-phosphate dehydrogenase, G6PD
Esterase D, EsD
Peptidase A, PEPA
Carbonic anhydrase, CAII

The seventeen markers were divided into three priority grouping systems (Table II). The first group consisted of six enzymes and proteins with good discriminating probabilities and known to be reasonably stable in bloodstains. The second group consisted of four enzymes arranged in the order in which they would be added to the first group as necessary. The

third group consisted of seven enzymes and proteins which were considered to be completely unreliable or had very low discrimination probabilities in a combined population. These seven markers were therefore rejected from inclusion in the final system.

TABLE II. SELECTION OF GENETIC MARKERS

System	Discriminating Probabilities	Comments
1st Priority Grouping Systems		
EAP	0.35	
Hp	0.39	
PGM	0.25 (0.47)	IEF should be used
GLO I	0.38	
Gc	0.45	
EsD	0.69	
Total	0.0040 (0.0075) = 1 in 250 (1 in 133)	
2nd Priority Grouping Systems		
AK	0.82	Could be completed on one substrate at same time
ADA	0.82	
6 PGD	0.91	
GPT	0.38	Unreliable. Stains are unstable
Systems To Be Rejected		
PCE (E1&2)	0.82	Completely unreliable
UMPK	0.82	Completely unreliable
Hb	0.82 (Blacks)	DP very low in combined population*
GR	0.59 (Blacks)	DP very low in combined population
G6PD	0.41 (Blacks)	DP very low in combined population
Pep A	0.75 (Blacks)	DP very low in combined population
CAII	0.75 (Blacks)	DP very low in combined population

*Discrimination Probability

The discrimination probability values were computed from population data on white Americans, the only significant data available at that time. However, all the values are very similar to values achieved by using population data from British caucasians (Stedman, 1977) and more recently using the data from caucasians in California (Grunbaum, *et al.*, ref 9). These data are shown in Table III.

TABLE III. DISCRIMINATING PROBABILITIES FROM THREE SOURCES

Genetic Marker	SOW	British	California
GLO I	0.38*	0.38	0.38*
EsD	0.69	0.69	0.67
PGM	0.47	0.47	0.47
ADA	0.82	0.83	0.82
EAP	0.35	0.33	0.34
AK	0.82	0.84	0.86
Gc	0.45	0.44	0.43
Hp	0.39	0.38	0.39
Cumulative Discrimination Probability	1 in 196	1 in 210	1 in 208

*No figures available; British figures are used.

The second step was to optimize each individual enzyme and protein in the first priority group (Table II) using already accepted methodologies. On starch gel, PGM was separated by the method of Culliford (1967), EAP by the method Wraxall and Emes (1976), EsD by the method of Parkin and Adams (1975), and GLO I by the method of Parr et al. (1977). Gc was separated on agarose (Wraxall 1975) and Hp on continuous gradient acrylamide gel (Culliford 1971).

On cellulose acetate membrane (CAM) the following methodologies were used: PGM, Grunbaum (1974); EAP, Grunbaum and Zajac (personal communication, published 1978); EsD, Grunbaum, et al. (personal communication, published 1978); and Gc, Grunbaum and Zajac (1977). GLO I would be examined by the method of Kahn and Doppert (1976) using cellogel. Hp could be separated using a step gradient acrylamide gel by the method of Grunbaum (1975).

After optimization of all six genetic markers (except GLO I on acetate), it was decided to follow a program plan which would efficiently compare both substrates and equipment, eliminate unnecessary work, and assure a smooth transition into system development (Figure 1).

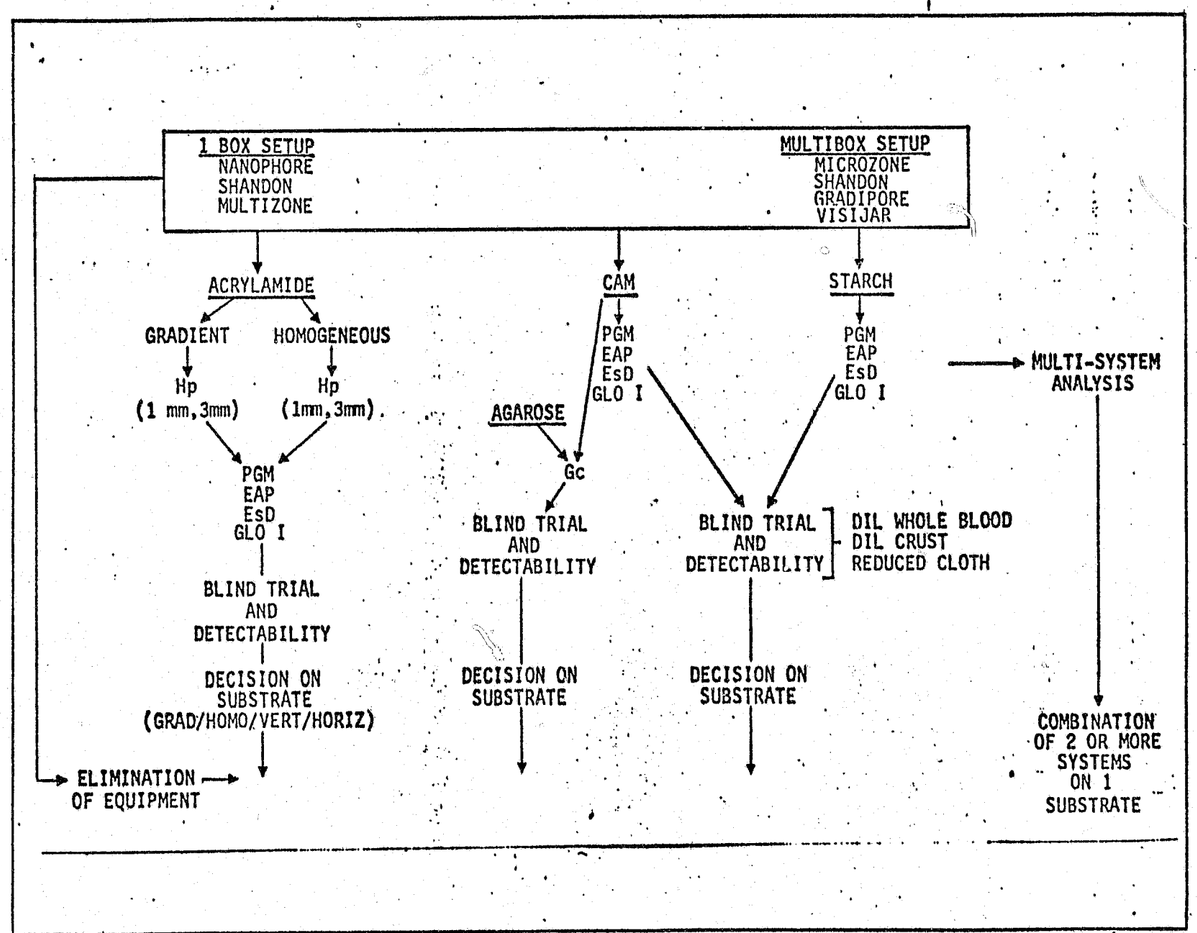

Figure 1. Diagram of Planned Investigation

As required by the Statement of Work, the following six substrates were to be examined and tested:

Acrylamide	Cellulose Acetate
Agar	Cellogel
Agarose	Starch

2.1 Acrylamide

Following the plan (Figure 1), acrylamide was polymerized by various methods and tested for PGM, EAP, EsD, and GLO I. When TEMED was used for polymerizing the gel, inhibition of most of the enzymes occurred. However, when DMAPN was used, inhibition did not occur, but the separation in most cases was not as good as when using a non-molecular sieving media. The only case where molecular sieving was an advantage and even a requirement was when phenotyping Hp. Gc was not tested on acrylamide at this stage as final selection of substrate would be system dependent. Therefore, as far as the four enzymes in this group were concerned, acrylamide was eliminated.

2.2 Agar/Agarose

Several different types and supplies of agar and agarose were examined. The problem with agar, and to some extent agarose, is that it exhibits a property called endosmosis. This gel, being of biological origin, possesses a charge which causes a flow of buffer from the anode to the cathode, thereby retarding electrophoretic migration. It was therefore decided to concentrate on agaroses with low endosmosis values (M_r-) (Wieme, 1965, and Wraxall, 1975). The final selection was the agarose Type I from Sigma having an M_r- value of 0.10. This agarose gave excellent results with PGM, EsD, GLO I, and Gc, but showed inadequate separation with EAP. The agarose provides excellent repeatability even from batch to batch.

2.3 Cellulose Acetate/Cellogel

Several types of cellulose acetate membranes (CAM) had been examined at the U.C. Berkeley laboratory, with the conclusion that the membrane

giving the best results is manufactured by Sartorius. This was also confirmed by the experience of the consultant criminalists. Several different types of acetate membranes were also tested but showed no improvement over the membrane in routine use. As a requirement of the Statement of Work, cellogel was also examined, again showing no improvement over Sartorius. The serum protein, Gc, can be separated on acetate but Hp, which separates by molecular size and not by charge, does not separate on acetate. Three of the four enzymes in the first group separate on CAM, but we were unable to achieve a satisfactory separation of GLO I, despite making more than 150 determinations covering many variables of buffer, voltage, and time.

2.4 Starch

Starch from two different suppliers was examined. All the enzymes plus Hp showed excellent separation when using well-published methods. Gc was not examined on starch gel due to problems with staining the protein.

3.0 BLIND TRIALS

Because starch gel and cellulose acetate both appeared to be the leading candidate substrates for the analysis of PGM, EsD, and EAP, a series of blind trials was initiated to determine quantitative differences in analytical sensitivity as well as subjective differences in interpretability. The trials were designed to test both these parameters, and with this in mind a series of dilutions of different whole bloods was made. Dilutions commenced at 1 in 10 and a total volume of 5 µl was allocated for each substrate. At least 7 samples were subjected to 3 dilutions and the results were read by 4 different readers. The stains consisted of 1, 2, 3, or 4 pieces of 0.5-cm-long bloodstained threads. The same 4 readers were used. The results were tabulated into 4 categories: 1) Correct, where the result agrees with the original phenotype; 2) Incorrect, where a definite reading was given but did not agree with the original phenotype; 3) Questioned, where the sample showed activity but the reader was unwilling to definitely phenotype; and 4) No Activity.

Although the Statement of Work specified that as much as 25 µl of whole blood could be used in making the stains, any misinterpretation of phenotype would most likely occur when using dilute or weak samples. Therefore, the sensitivity studies should show up weaknesses in any of the methodologies.

The first marker to be considered was EAP, and the results on lysates are shown in Table IV. The total number of inconclusives is approximately the same, but although there are more incorrect results on acetate than on starch gel, the results are not significantly different. However, when smaller amounts of bloodstains are used, differences in the results can be seen easily. Table V shows that there are 7 incorrect results given on acetate compared with 0 on starch, and a total of 51 questionable results, 4 on starch. Most of the incorrect results were type BA's being

TABLE IV. BLIND TRIAL — EAP — DETECTABILITY OF LYSATES

Dilution (VWB)*	Substrate	No. of Samples	No. of Readers	Total Readings	Correct	In-correct	Ques-tioned	No Activity	No. of Variants
1 in 10 (0.5 µl)	CAM	7	4	28	26	-	2	-	3
	Starch	7	4	28	24	-	3	1	3
1 in 20 (0.25 µl)	CAM	14	4	56	42	2	12	-	3
	Starch	14	4	49**	32	2	12	3	3
1 in 40 (0.125 µl)	CAM	7	4	28	13	3	8	4	2
	Starch	7	4	21**	12	-	6	3	2

* Volume of whole blood lysate per 5 µl diluted lysate
** Less 1 reader for 7 stains

TABLE V. BLIND TRIAL — EAP — DETECTABILITY OF DRIED STAINS

Size of Stained Thread (cm)	Stain Age (Days)	Substrate	No. of Stains	No. of Readers	Total Readings	Correct	In-correct	Ques-tioned	No Activity	No. of Variants	
4 x 0.5 ≅ 0.7 µl WB	18	CAM	2	4	8	5	1	2	–	2	3
		Starch	2	3	5	5	–	–	–	2	
	25	CAM	2	4	8	5	1	2	–	2	
		Starch	2	3	6	6	–	–	–	2	
	36	CAM	3	4	12	1	3	8	–	2	
		Starch	3	3	9	9	–	–	–	2	
3 x 0.5 ≅ 0.5 µl WB	18	CAM	2	4	8	4	–	4	–	2	3
		Starch	2	4	8	8	–	–	–	2	
	25	CAM	2	4	8	4	–	–	4	2	
		Starch	3	4	12	12	–	–	–	2	
	36	CAM	3	4	12	2	1	9	–	2	
		Starch	3	4	12	12	–	–	–	2	
2 x 0.5 ≅ 0.35 µl WB	18	CAM	2	4	8	6	–	2	–	2	3
		Starch	2	4	8	8	–	–	–	2	
	25	CAM	2	4	8	4	1	3	–	2	
		Starch	2	4	8	8	–	–	–	2	
	36	CAM	3	4	12	4	–	8	–	2	
		Starch	3	4	12	12	–	–	–	2	
1 x 0.5 ≅ 0.18 µl WB	18	CAM	2	4	8	1	–	7	–	2	3
		Starch	2	4	8	5	–	3	–	2	
	25	CAM	2	4	8	2	–	2	4	2	
		Starch	2	4	8	8	–	–	–	2	
	36	CAM	3	4	12	–	–	4	8	2	
		Starch	3	4	12	11	–	1	–	2	

called type B. Further experimentation was conducted where known EAP type BA's were mistyped as B when dilutions were applied to cellulose acetate membranes. The same dilutions were all typed as BA on starch gel.

It can be seen from Table V that as the stain becomes older and/or smaller, the number of questioned and/or no activity results increases. This may be explained by the fact that when applying bloodstains to cellulose acetate, prior extraction is required. It is very difficult to extract all the enzyme or protein from a bloodstain and also to use a volume of extraction liquid small enough so that all can be applied to the membrane. With the gel method, the whole of the bloodstained fiber can be inserted into the substrate. The results obtained indicate that phenotyping EAP on starch gel is more accurate and more sensitive than on cellulose acetate.

The next marker to be examined was EsD. The experimental details are similar to those on EAP except that the lysates are more dilute and only the 1 and 2 bloodstained threads were examined. Also, there are only 3 instead of 4 readers. This is because one reader had great difficulty in interpreting EsD phenotypes on both starch and cellulose acetate so his results have been removed from the table.

It can be seen from Table VI that phenotyping EsD of diluted lysates on starch gel is more accurate and more sensitive than on cellulose acetate.

The large number of questioned results (11) on CAM with the higher concentration samples shows one of the problems in that too much applied sample causes distortion of the pattern. The results obtained from bloodstains, Table VII, shows little difference between the substrates; therefore, no decision on choice of substrate was made at this time.

TABLE VI. BLIND TRIAL — EsD — DETECTABILITY OF LYSATES

Dilution (VWB)*	Substrate	No. of Samples	No. of Readers	Total Readings	Correct	In-correct	Questioned	No Activity	No. of Variants
1 in 20 (0.25 µl)	CAM Starch	7 7	3 3	21 21	7 18	3 –	11 –	– 3	3 3
1 in 40 (0.125 µl)	CAM Starch	7 7	3 3	21 21	12 21	– –	7 –	2 –	3 3
1 in 80 (0.063 µl)	CAM Starch	7 7	3 3	21 21	13 13	2 –	3 8	3 –	3 3
1 in 160 (0.032 µl)	CAM Starch	7 7	3 3	21 21	3 7	– –	1 12	17 2	3 3

*Volume of whole blood lysate for 5 µl diluted lysate

TABLE VII. BLIND TRIAL — EsD — DETECTABILITY OF DRIED STAINS

Size of Stained Threads (cm)	Stain Age (Days)	Substrate	No. of Stains	No. of Readers	Total Readings	Correct	In-correct	Ques-tioned	No Activity	No. of Variants
2 x 0.5 ≅ 0.35 µl WB	11	CAM	2	3	6	4	-	1	1	2
		Starch	2	3	6	6	-	-	-	2
	21	CAM	2	3	6	5	-	-	-	2
		Starch	2	3	6	5	-	1	-	2
	28	CAM	3	3	9	8	-	1	-	2
		Starch	3	3	9	5	1	2	1	2
1 x 0.5 ≅ 0.18 µl WB	11	CAM	2	3	6	3	-	1	2	2
		Starch	2	3	6	2	-	3	1	2
	21	CAM	2	3	6	6	-	-	-	2
		Starch	2	3	6	5	-	1	-	2
	28	CAM	3	3	9	5	-	1	3	2
		Starch	3	3	9	2	-	5	2	2

PGM was only examined on bloodstains of decreasing size, the results of which are tabulated in Table VIII. There were more stains examined on CAM than on starch, but the incorrect answers were a result of incomplete separation of the b and c isozymes. This would cause PGM type 1 to be easily confused for a type 2-1. Further experimentation was carried out which shows that on CAM, misinterpretation of some phenotypes can occur. See Figure 2.

Further blind trials were initiated for EAP, EsD, and PGM to confirm the above conclusions; however, no sensitivity studies were included. The trials consisted of large (approximately 25 µl) bloodstains cut in half and tested on both substrates. The results are summarized in Table IX.

It can be seen from the table that there is substantially no difference in the substrates when phenotyping EAP except that no dilutions were tested. As can be seen from the previous blind trial results (Table V) this is the main problem area for cellulose acetate. EsD phenotyping is inconsistent on CAM whereas starch proved to be reliable. It was determined by experiment that EsD phenotyping of bloodstains on CAM required a different membrane buffer (i.e., a 1:7 dilution of tank buffer) than required on whole blood (1:20). This dilution (1:7) is required to be very accurate or else the final result would be very diffuse (Figure 3). No explanation was found for this problem. PGM phenotyping on CAM produced some interpretation ambiguities even with large amounts of bloodstains and the separation was not improved.

At this point, however, there was still some question as to whether EsD and PGM were equivalent on both substrates. It was therefore decided to run confirmatory tests on all eight genetic markers. The results are summarized in Table X. From this table it can be seen that several questioned calls were made on CAM but not on starch. Also, at this time, after some initial experimentation, EsD, PGM, and GLO I were being phenotyped all on the same starch gel. It was therefore decided to conduct the confirmation tests for starch using this multisystem which

TABLE VIII. BLIND TRIAL — PGM — DETECTABILITY OF DRIED STAINS

Size of Stained Thread (cm)	Stain Age (Days)	Substrate	No. of Stains	No. of Readers	Total Readings	Correct	In-correct	Ques-tioned	No Activity	No. of Variants
4 x 0.5 ≅ 0.7 µl WB	24	CAM	2	4	8	6	-	1	1	1
		Starch	2	3	6	5	-	1	-	1
	31	CAM	2	4	8	8	-	-	-	2
		Starch	2	3	6	5	-	1	-	2
	42	CAM	3	4	12	11	-	1	-	3
		Starch	3	3	9	8	-	1	-	3
3 x 0.5 ≅ 0.5 µl WB	24	CAM	2	4	8	7	-	1	-	1
		Starch	2	3	6	6	-	-	-	1
	31	CAM	2	4	8	8	-	-	-	2
		Starch	2	3	6	6	-	-	-	2
	42	CAM	3	4	12	12	-	-	-	3
		Starch	3	3	9	9	-	-	-	3
2 x 0.5 ≅ 0.35 µl WB	24	CAM	2	4	8	3	2	3	-	1
		Starch	No Test							
	31	CAM	2	4	8	5	-	3	-	2
		Starch	No Test							
	42	CAM	3	4	12	11	-	1	-	3
		Starch	No Test							
1 x 0.5 ≅ 0.18 µl WB	24	CAM	2	3	6	4	2	-	-	1
		Starch	No Test							
	31	CAM	2	3	6	5	-	1	-	2
		Starch	No Test							
	42	CAM	3	4	12	9	-	3	-	3
		Starch	No Test							

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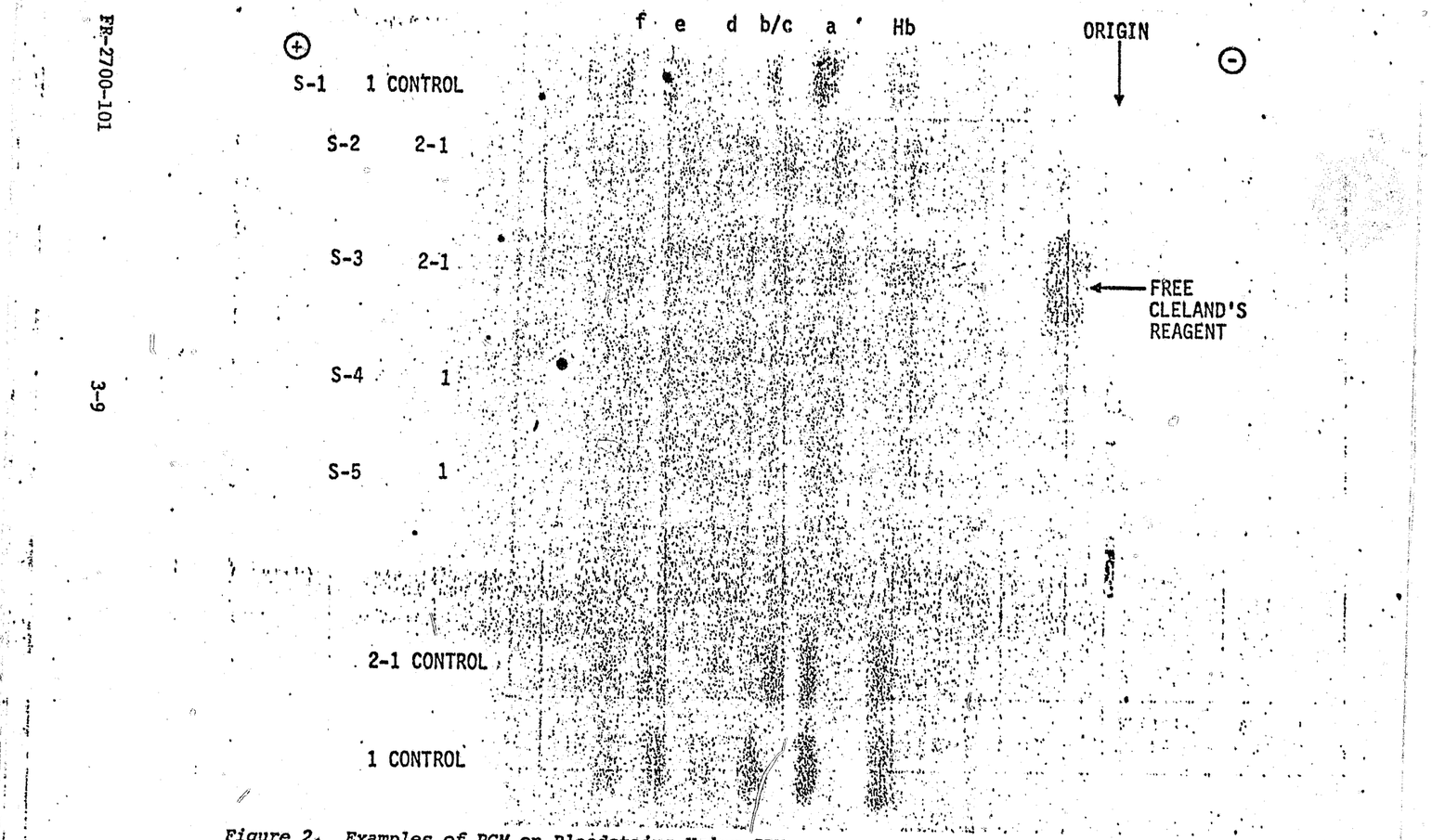

Figure 2. Examples of PGM on Bloodstains Using CAM

TABLE IX. BLIND TRIAL SERIES 2

Test and Substrate	Stain Age	No. of Stains	No. of Readings	Total Readings	Correct	In-correct	Ques-tioned	No Activity	No. of Variants
EAP (Starch)	2-4 wks	10	4	40	40	-	-	-	2
EAP (CAM)	No Test								
EAP (Starch)	4 wks	16	4	64	64	-	-	-	2
EAP (CAM)	4 wks	16	4	64	61	-	3	-	2
EAP (Starch)	No Test								
EAP (CAM)	4 wks	15	3	45	45	-	-	-	2
EsD (Starch)	2-4 wks	10	4	40	37	-	3	-	2
EsD (CAM)	2-4 wks	10 (Test run twice; result unreadable)							
EsD (Starch)	4 wks	16	3	48	48	-	-	-	2
EsD (CAM)	4 wks	16	3	48	18	-	14	16	2
EsD (CAM)	4 wks	15	5	75	73	-	2	-	2
PGM (Starch)	4 wks	16	4	64	63	-	1	-	3
PGM (CAM)	4 wks	15	5	75	70	-	5	-	2

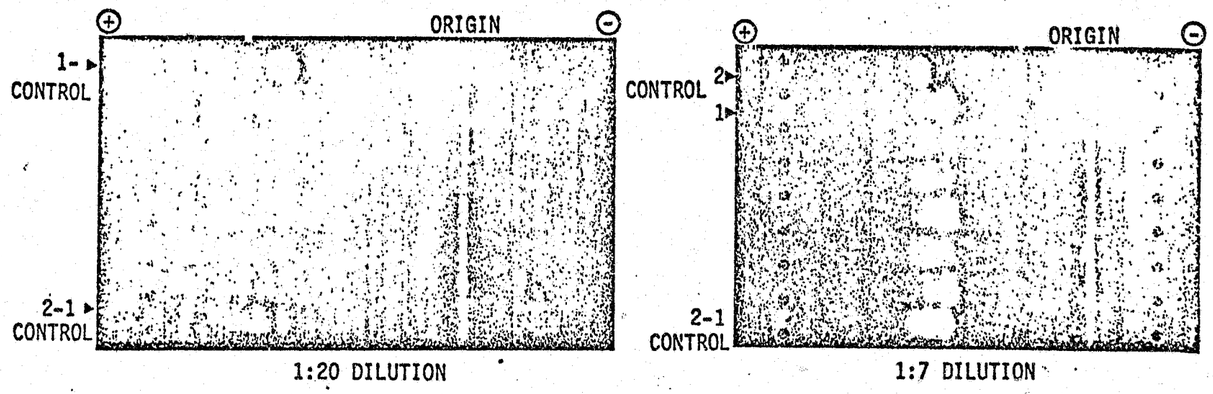

Figure 3. Examples of EsD on Bloodstains Using CAM

TABLE X. BLIND TRIAL SERIES 3

Test and Substrate	Stain Age	No. of Stains	No. of Readings	Total Readings	Correct	In-correct	Ques-tioned	No Activity	No. of Variants
EAP (Starch)	2-3wks	7	4	28	28	-	-	-	5
EAP (CAM)	2-3 wks	7	4	28	26	-	2	-	5
EsD* (Starch)	2-3 wks	7	4	28	27	-	-	1	3
EsD (CAM)	2-3 wks	7	4	28	27	-	1	-	3
PGM* (Starch)	2-3 wks	7	4	28	28	-	-	-	3
PGM (CAM)	2-3 wks	7	4	28	27	-	1	-	3
GLO I*(Starch)	2-3 wks	7	4	28	22	**	3	3	2
GLO I (CAM)	Insufficient separation; No Test								
AK (Starch)	2-3 wks	7	4	28	28	-	-	-	1
AK (CAM)	2-3 wks	7	4	28	28	-	-	-	1
ADA (Starch)	2-3 wks	7	4	28	28	-	-	-	2
ADA (CAM)	2-3 wks	7	4	28	27	-	1	-	2
Gc (Agarose)	2-3 wks	7	4	28	25	-	3	-	3
Gc (CAM)	Results too weak to read; No Repeat								
Gc (Agarose)	4 wks	15	4	60	60	-	-	-	3
Gc (CAM)	4 wks	15	4	60	42	-	7	11	3
Hp (Cont Gradient)	2-3 wks	7	4	28	20	-	2	6	3
Hp (Step Gradient)	2-3 wks	7	4	28	17	-	3	8	3

* EsD, PGM, GLO I phenotyped on one gel using multisystem method on starch.
** Original report of this result (September Monthly Progress Report PR-2700-8) was incorrect.

resulted in no incorrect results for all three markers. Subsequent experiments of these markers on CAM showed that it was only possible to phenotype one marker at a time on this substrate. It was now becoming evident that the use of cellulose acetate membrane would not meet the Statement of Work requirements in terms of reliability, repeatability, and the combining of one or more genetic markers in one setup.

It was, therefore, recommended that CAM should be eliminated from further study for all markers except AK. (It was probable that this marker would be phenotyped on gel, together with another enzyme.) It was further recommended that all effort be directed towards system development with accent on multisystem analysis.

4.0 SYSTEM DEVELOPMENT

Although experiments had been conducted since April 1977 on combining two or more systems on a single substrate, the initial approach to system development was to consider the pH at which each genetic marker was separated on an individual basis. The markers and their separation pHs are listed below:

Marker	pH
AK	5.0
EAP	5.9
ADA	6.8
GLO I	6.8
EsD	7.4
PGM	7.4
Hp	8.3
Gc	8.6

It was decided to examine the enzymes, EAP, GLO I, EsD, and PGM on a variety of buffers. A 1-mm starch gel (20 x 15 cm) was divided into four sections and blood samples representing many of the common phenotypes were applied on cotton threads in each quarter. Electrophoresis was carried out and each quarter stained for a separate enzyme. From these results it was determined whether the enzymes could be separated from each other (by measuring the appropriate migration), and whether each enzyme was separated sufficiently in any given buffer. It was confirmed that EAP phenotyping is improved if citrate ions are present (if not, the "a" isozymes are found in a more cathodic position and are not so easily interpreted, Hopkinson & Harris 1969).

Many buffers were examined, starting with GLO I tank and gel buffers (phosphate at pH 6.8), followed by EAP (citrate/phosphate, at 5.9), EsD (lithium hydroxide/tris at 7.4), and PGM (tris/EDTA/Maleate/$MgCl_2$, at pH 7.4). Small amounts of citrate were added to some of these buffers without resulting in the desired improvement. Different concentrations

of phosphate buffers ranging in pH from 6.8 to 7.4 were tried as well as combinations of different tank and gel buffers (e.g., PGM tank buffer with EsD gel buffer).

It soon became obvious that the best combination of enzymes would be GLO I, EsD, and PGM (pH range 6.8 to 7.4). The buffers giving the best separation were a PGM tank buffer (Culliford, 1967) with an EsD gel (Parkin and Adams, 1975) or a PGM tank buffer with a 1:15 dilution for the gel (Culliford, 1967). It was then decided (before too much work was done on the GLO I, EsD, PGM combination) to examine the other three enzymes--ADA, EAP, and AK--to see if they would separate on the same gel. It soon became apparent that they would; in fact, one of the earlier gels was composed of EAP tank and gel (citrate phosphate pH 5.9 diluted 1:100 for the gel) which gave clear separations of EAP and AK with ADA separated from EAP but with diffuse bands. The conclusion was that all six enzymes could probably be separated on two gels with the two serum proteins, Gc and Hp, possibly phenotyped on a third. It was provisionally decided to call GLO I/EsD/PGM Group I, ADA/EAP/AK Group II, and Gc/Hp Group III.

At this point, it seemed appropriate to consider other substrates, i.e., acrylamide and agarose. It had been reported (Adams & Wraxall, 1972) that the enzyme EAP was inhibited by acrylamide. It was thought that this may apply to other enzymes. The enzymes PGM, EsD, and EAP were separated on 5% acrylamide gel using a cyanogum 41/ammonium persulphate/TEMED mixture in the appropriate buffer. This caused inhibition of each of the three enzymes. Other means of polymerization of the acrylamide gel were tried. These were riboflavin and dimethylaminoproprionitrate (DMAPN) which appeared to remove the inhibition but did not improve the separation. It was concluded that the acrylamide was having a sieving effect on the enzymes which was not obvious in starch or agarose gels. The isozymes of each enzyme are separated by charge alone, and therefore molecular sieving (as provided by acrylamide gel) is not necessary. The separation of the other three enzymes was not attempted on acrylamide

because 1) GLO I requires either the incorporation of starch or the use of the positive stain method (Parr, 1977) using dichloroindophenol (DCIP) which was found to be insensitive, 2) ADA and AK were considered to be enzymes which would be "added on" to one of the other enzymes for phenotyping (e.g., Group II). It was therefore decided that the six enzymes should not be phenotyped on acrylamide but that this substrate would be considered for Group III (Gc/Hp) later.

The other substrate to be considered was agar or agarose. These gels exhibit a property not seen in starch or acrylamide. This property is electroendosmosis and, depending on the degree or M_r-value, results in the retardation of the isoenzymes. Agarose is a purified form of agar and manufacturers are now even listing the M_r-value of their agaroses. Several types of agarose were tested with contrasting results. Beckman manufactures a rehydratable agarose which--if after testing was found to be satisfactory--would have the advantage of limited gel preparation. However, it was found that these gels had a high degree of endomosis resulting in most of the enzymes migrating cathodically. The Sigma Chemical Company produces three agaroses all with different M_r-values and it was found that the lowest endosmosis value agarose gave the best results. Group I enzymes separated better than Group II and even gave better results than on starch gel. It was therefore decided to concentrate our efforts on the separation of GLO I, EsD, and PGM on agarose and consider the Group II separation later.

4.1 Group I

The first important consideration was how to stain for GLO I. The DCIP method was found to be insensitive in that the lysates exhibited more activity when stained by the starch/iodine method. This method, however, relied on starch in the gel. Incorporation of starch, at very low percentage, into the agarose gel was the obvious answer which gave excellent results.

Concentrations of starch ranging from 1 to 3% in agarose gels of 0.8 and 1% were tried, the most successful being 1% agarose with 2% starch. Most polymorphic enzymes are normally separated under optimum conditions of voltage, time, pH, and ionic strength. It was therefore necessary to test all the enzymes in Group I to avoid any loss of sensitivity or reliability. The pH and ionic strength of the tank and gel buffers had already been established in the PGM buffer in terms that it gave the best separation of all three enzymes. It was also better because PGM is routinely separated under those conditions (on starch), and EsD is separated routinely at pH 7.4 even though the ionic strength is different. The next condition to establish was the voltage and time. Sixteen hours of electrophoresis time (at 3 V/cm) was found to be inadequate because of diffusion of the isozymes. Higher voltages and lower electrophoresis times were in order; the choice was between 20 V/cm for 2-1/2 hours, or 15 V/cm for 3-1/2 hours. The remaining conditions to be considered were the pH, ionic strength, and biochemical concentrations of the reaction mixtures. This was to take several months of work before we were satisfied that all enzymes were working under optimum conditions. Some of the problems encountered and solved were: 1) the starch/iodine reaction for GLO I and its relationship with reduced glutathione and methyl glyoxal; 2) Glucose-6-phosphate dehydrogenase enzyme in the PGM reaction (it was found that G6PD from one manufacturer was inferior to that from another); and 3) methyl umbelliferyl acetate (MUA) is very sensitive at alkaline pH but hydrolyzes and gives background fluorescence, while at acid pH it does not hydrolyze but is insensitive.

Obviously, when an enzyme is separated in a different environment than is usual, all the other conditions must be examined for optimum reliability and sensitivity. However, in early September, 1977, even while all these conditions were being examined, a blind trial sent from a serology study group was typed for Group I enzymes. The trial consisted of eight stains between 7 and 10 days old. All eight stains gave clear unambiguous results for all three enzymes indicating that Group I had a high potential for being included in the final system (Table X).

4.2 Group II

In late August, 1977, having established that Group I had good potential, it was decided to examine Group II--ADA, EAP, and AK. In early experiments, it was seen that EAP and AK could easily be separated even though there was a possible overlap of rare variants (i.e., EAP DB, and AK 3-1). This was using standard EAP gel and tank buffers, citrate-phosphate pH 5.9. The results of a blind trial for EAP and AK, shown in Table X, were phenotyped on the same starch gel. The problem was ADA and how it could be resolved on the same gel. The ADA isozymes were well separated and did not overlap the EAP isozymes but the results were weak and diffuse. Further work showed that the liquid enzymes used in the reaction, nucleoside phosphorylase and xanthine oxidase, were partly to blame and when these were purchased from Boehringer, results were dramatically improved. (Further improvement was obtained later when the pH and ionic strength of the reaction buffer was optimized.) The next step was to improve the resolution of the ADA isozymes and this was attempted by altering the pH and ionic strength of the tank and gel buffers. The constituents (i.e., citrate and phosphate) were not altered because of the EAP "a" isozyme's affinity for citrate, Hopkinson & Harris (1969). Buffers between pH 5.0 and 6.3 were examined in order to obtain a good separation of all three enzymes both from each other and for good resolution of the individual markers. Another problem to be taken into account was the heat lability of EAP "a" isozymes in relation to the "b" and "c" isozymes, Wraxall & Emes (1976). Therefore, if a method to keep down the heat generated during electrophoresis could be achieved, this would hopefully resolve this problem. This was accomplished by conducting electrophoresis overnight which not only solved the heat problem, but employed the equipment and personnel time much more efficiently. This will be described in detail later.

At this time, it was decided to examine Group II on agarose with a view to using the same substrate as Group I. A 1% agarose gel gave disastrous results with the electroendosmosis causing EAP and AK to migrate cathodically. The addition of starch helped to counteract this problem but

after many determinations, it was seen that the starch gel was superior to any agarose or agarose/starch gel combination.

The buffer which gives the best separation is the citrate-phosphate buffer at pH 5.5. However, when this is diluted 1:40 for use in the gel, the buffer changes to 6.2 This resulted in the AK 1 isozyme remaining on the origin and causing problems with interpretation. This was overcome by making the gel buffer from dry chemicals to obtain the correct pH (5.5). With the electrophoresis conditions optimized, it was now essential to optimize the reaction conditions similar to Group I.

4.3 Group III

Several ideas had already been tested earlier in the program prior to system development. These concerned the simultaneous separation of Gc and Hp on agarose and also the cleanup of bloodstains before the separation of serum proteins.

Most phenotyping of Hp on bloodstains is accomplished by using gradient acrylamide gel (Culliford, 1971). Although Hp in serum can be separated in starch gel, when bloodstains are applied breakdown products cause severe smearing throughout the gel. A gradient acrylamide gel by virtue of its decreasing pore size allows the small molecular weight breakdown products to move through the gel while retaining the larger haptoglobin proteins according to their molecular weight. However, as these bloodstains become older, the amount of breakdown and aggregation products increases and is not cleared from the gel.

Apart from the time-consuming procedure for preparing the gel, the step gradient acrylamide method (Grunbaum, 1975) consisting of layers of 4, 6, and 8% acrylamide gel presents even more of a problem (see Table X). The gradient, not being continuous, does not allow the breakdown and aggregation products to migrate from the gel while stopping the fast-moving Hp 1 band.

A well-known and widely published biochemical procedure dating back to 1901 (Krüger) uses chloroform to precipitate denatured hemoglobin from certain aqueous solutions. We have utilized this technique to clean up extracts of bloodstains before electrophoresis, thereby eliminating smearing which causes masking of the protein bands. This technique was initially applied to haptoglobin typing on continuous gradient acrylamide gels resulting in the ability to phenotype old (3-4 months) stains. Later, when the homogenous acrylamide technique was developed, the chloroform extraction technique became an integral part of the method.

Another earlier idea was the simultaneous separation of Gc and Hp on agarose. It was already established (Wraxall 1975) that Gc phenotyping could be accomplished by antigen-antibody crossed electrophoresis (AACE). However, although this technique was more sensitive than CAM (Table X), it requires a high degree of technical skill. Haptoglobin is best separated, as previously mentioned, using some form of molecular sieving (e.g., acrylamide) as the polymer bands are separated not by charge but by molecular weight. On agarose, there is a separation of the three main types but the differentiation between the types 2-1 and 2-2 is very small. Even so, it was thought worthwhile to try separating the three Hp types by AACE hoping that some improvement in the differentiation might occur. It was possible to separate the three Hp types and, by using longer electrophoresis times (up to 4 hours), the three Gc types could be separated as well, all at the same time (Figure 4). There was, however, some interaction between the two antigens, resulting in a loss of activity on the Gc. This was attributed to the two antisera being made in two different animals (i.e., goat and rabbit.) This problem could easily be remedied but it was concluded at this time (July, 1977) that the separation was not good and that the skill required would probably make the system impractical.

The simultaneous separation of Gc and Hp was, therefore, left until late September, 1977, when the initial approach was to separate both proteins on acrylamide, staining Hp with o-tolidine and Gc by immunofixation. The

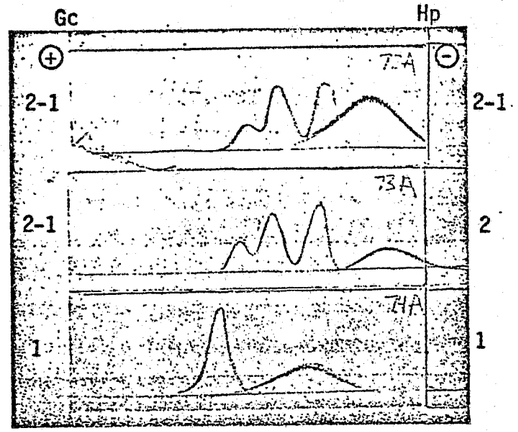

Figure 4. Separation of Gc and Hp

first experiments were very encouraging in that both Hp and Gc separated both individually and from each other on 6% acrylamide. It was then necessary to try different buffers between pH 8.0 and 8.6 and different concentrations of acrylamide, to obtain the best separation. Several buffers were tried, both continuous and discontinuous, the best results being obtained with the simplest buffer--tris-glycine at pH 8.4. Several methods of acrylamide polymerization were tried, the best results being obtained using DMAPN. The concentration of acrylamide selected was 6.5%, thus giving a good separation of Gc and Hp. This was particularly true of the Hp 1 band from free haemoglobin. Preliminary results on Gc showed that the immunofixation procedure would work, and once the separation details had been established it would then only be necessary to work out the immunofixation details to give maximum sensitivity.

When the program was moved to the Beckman facility in mid-November, 1977, system development was well advanced and position of all three groups was as follows:

Group I Separation conditions well established. GLC I, PGM, and EsD all working well on four-week-old stains.

Group II Separation of ADA, EAP, and AK possible on one gel. Reaction conditions of each enzyme need to be established.

Group III Separation of Gc and Hp possible on one gel. Four-week-old stains working for Hp. Gc unknown on stains at this time. Conditions for separation and detection need to be optimized.

After the new laboratory had been established, each group was optimized in preparation for Method Feasibility Testing. In Group I the reaction mixture for GLO I was optimized, the critical positioning of the origin was established, and several types of sponge cloths (used for bridges) from different sources were tested. In Group II the pH was fixed at 5.5, the origin position set at 6 cm, and the reaction mixtures for ADA, EAP, and AK were optimized. In Group III the chloroform extraction procedure was examined using different buffers at varying pH's for the preliminary extraction. The initial selection was the tris glycine buffer at pH 8.4 (this was later changed to water at neutral pH which gave cleaner extracts).

It was discovered after optimizing the staining procedure for Gc on acrylamide that the degradation product which occurs in stains interferes with the typing of the protein. In agarose it is known to develop into a fast-moving globulin immunologically identical to Gc protein. In acrylamide it was found to migrate into the slow Gc 1 position and, therefore, cause interpretation problems (Figure 5). It was decided to examine agarose, using the same buffer, to see if stains could be phenotyped for Gc by immunofixation. The results were highly acceptable. With part of the same chloroform extract used for Hp phenotyping, it was possible to phenotype Gc using the same buffer, in the same tank, at the same time as Hp.

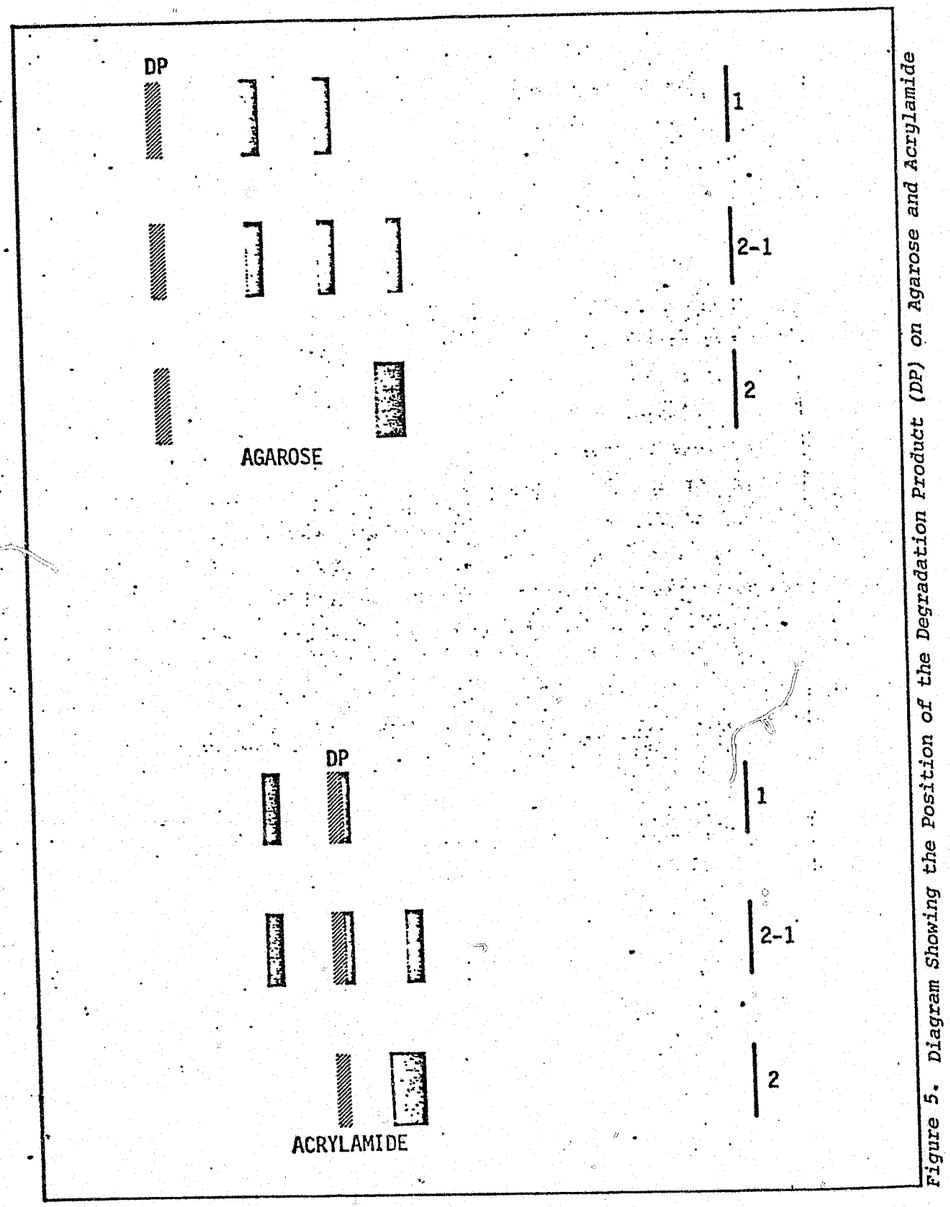

Figure 5. Diagram Showing the Position of the Degradation Product (DP) on Agarose and Acrylamide

All three groups were then subjected to internal blind trials in preparation for method feasibility testing. Some minor flaws were overcome and system development was complete. Methodology is described in Appendix C.

4.4 Hardware Selection

It was not an objective of this program to develop new electrophoresis cells. It was expected that the development work would be done with the Microzone cell which would be modified as required by new methodologies. However, in order to evaluate a variety of substrates in addition to cellulose acetate, it was necessary to work with other types of cells.

Several types of commercially available cells were obtained and evaluated as part of the process of selecting the substrates to use. This work was part of the program plan as summarized in Figure 1. It was decided that for simplicity, only one type of cell would be used for our system.

The electrophoretic cells discussed in the following paragraphs were considered.

4.4.1 Microzone

This cell is designed for use with cellulose acetate membranes. It has been used in clinical laboratories for many years. There is an accessory kit that permits it to be used with acrylamide gel.

The Microzone cell is small, with a separation distance available of about 7.5 cm. This is adequate for the separation of one constituent at a time. However, when it was decided to use simultaneous analyses with a gel substrate, the Microzone cell proved to be much too small. It would also require the addition of a gel cooling plate. These modifications could not be made without a complete redesign of the cell. Therefore, it was eliminated from further consideration.

4.4.2 Gradipore

This apparatus is designed for continuous gradient acrylamide electrophoresis with the plates in a vertical position. While this apparatus can also be used for homogenous acrylamide, it cannot be used for horizontal starch gel plates. There did not appear to be any way to easily adapt this instrument to our needs and it was also excluded.

4.4.3 Visijar

This apparatus is capable of handling all substrates except cellulose acetate on a horizontal basis. However, some modifications were required, both to the cell and the cooling plate, which would prove costly and so this apparatus was eliminated.

4.4.4 Nanophore

Two prototype Nanophore cells were loaned by NASA for this program. The Nanophore unit is similar to the Microzone. It is small and designed primarily for cellulose acetate membranes. A cooling plate can be used for gel substrates. However, the existing cooling capability is not adequate for use with starch gel.

Increased cooling is possible if a metal cooling plate were used in place of the existing plastic one. Another and more significant problem is the small area available for the electrophoretic separation. This is adequate for CAM but not for starch gel when separating more than one component in zones as described in Section 4.5.

In view of the extensive modifications required to optimize the design for this program, it was eliminated from the final system.

4.4.5 Multizone/Shandon

The Multizone and Shandon cells are dimensionally identical and performed equally well in this program. The Shandon cell and cooling plate

is commercially available and can be used for the Bloodstain Analysis System without modification. The Multizone is used primarily in clinical applications and is not sold with a cooling plate. Some modifications must be made to the cell to accommodate a cooling plate. Multizone cells were modified and prototype cooling plates made for support of the development work of this program.

This type of cell is large enough for the multiconstituent simultaneous analysis which must be done to stay within our hardware constraints. The Shandon cell and cooling plate is, therefore, recommended for use with the Bloodstain Analysis System.

4.4.6 System Hardware

The process of selecting the hardware is summarized in Figure 6.

The electrophoresis cell is a major critical part of the hardware. The total system consists of one refrigerated circulating cooling bath, three electrophoresis cells with cooling plates, and two power supplies (Figure 7). The complete list of equipment including accessories is included in Appendix C.

4.5 Parallel and Sequential Analysis

The systems of parallel or sequential analysis which were proposed as alternatives to the multicomponent analysis, require some comment. To help the understanding of these different types of analyses, the following definitions are proposed.

- Sequential Analysis. This is where only one cell is used. The first genetic marker is analyzed and the results recorded. The buffer is replaced, and the second genetic marker is analyzed in the same cell. This procedure is repeated until all the markers have been analyzed. For each marker

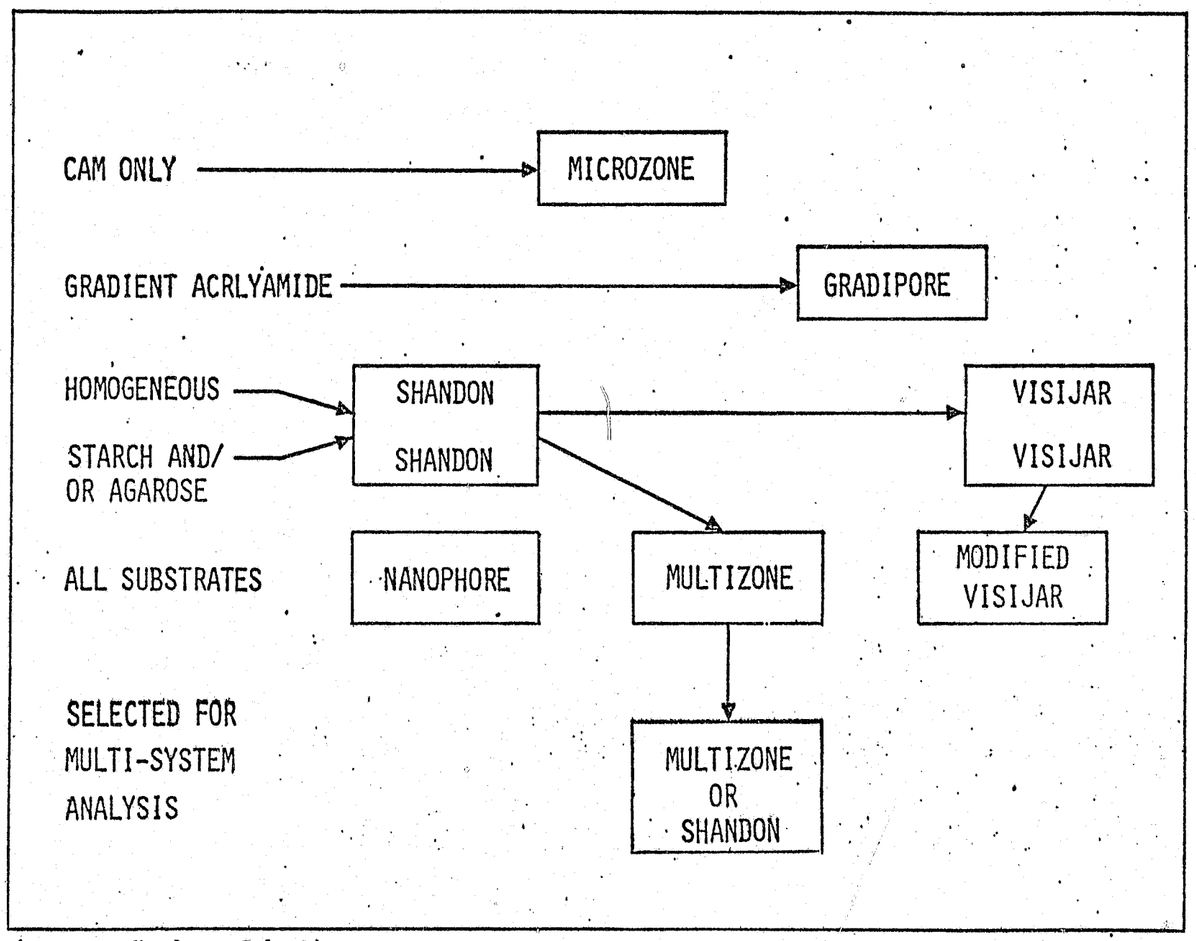

Figure 6. Hardware Selection

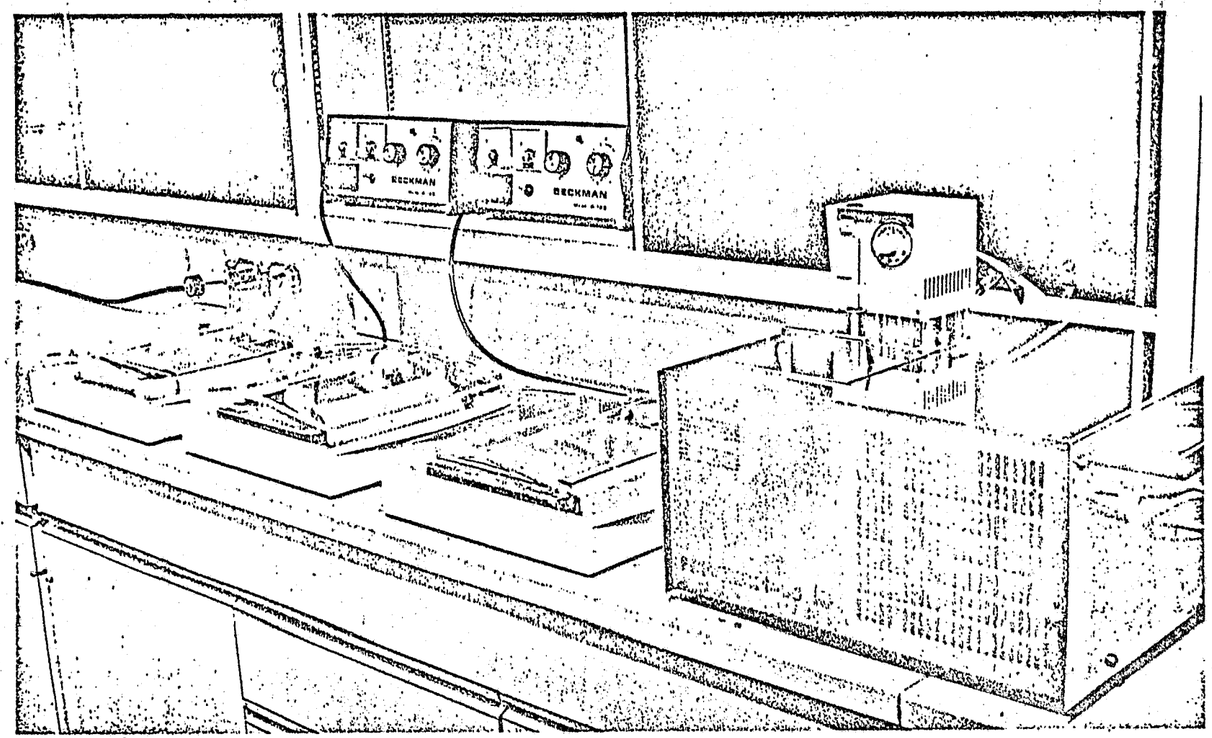

Figure 7. Equipment Setup

at least one control is used and one aliquot of stain is used for each of 8 samples (Figure 8A).

- Parallel Analysis. Here the samples are applied in triplicate to the substrate. After electrophoresis the substrate is divided into three strips, parallel to the direction of electrophoresis (Figure 8B). Each of the three areas is then stained for a separate genetic marker. Allowing for one control per marker, this only allows for two samples to be typed per electrophoretogram, using one aliquot of stain per sample per genetic marker.

- Multicomponent or Simultaneous Analysis. This is the type of system developed in this project where two or more different genetic markers are separated at the same time and are detected in different zones on the same substrate (Figure 8C). Only one control is used and at least 8 unknown samples are phenotyped for two or more genetic markers using one aliquot of stain for each of the samples.

It would be appropriate to compare both the technical and practical problems of these different approaches:

Technical. With the parallel approach and irrespective of substrate, the separating conditions of buffer, time, and voltage have to be researched to ensure that the isozymes of each genetic marker are separated optimally. The same is true for multicomponent analysis. Several tests were made to evaluate the parallel system after these conditions had been determined. On starch gel, hemolysates in triplicate were phenotyped for the Group II systems, i.e., EAP, AK, and ADA. All separations were satisfactory, but when the same three enzymes were examined on cellulose acetate the results were far from adequate. The EAP isozymes were insufficiently separated and the AK and ADA isozymes appeared diffuse. By cutting the membrane into three, parallel to the

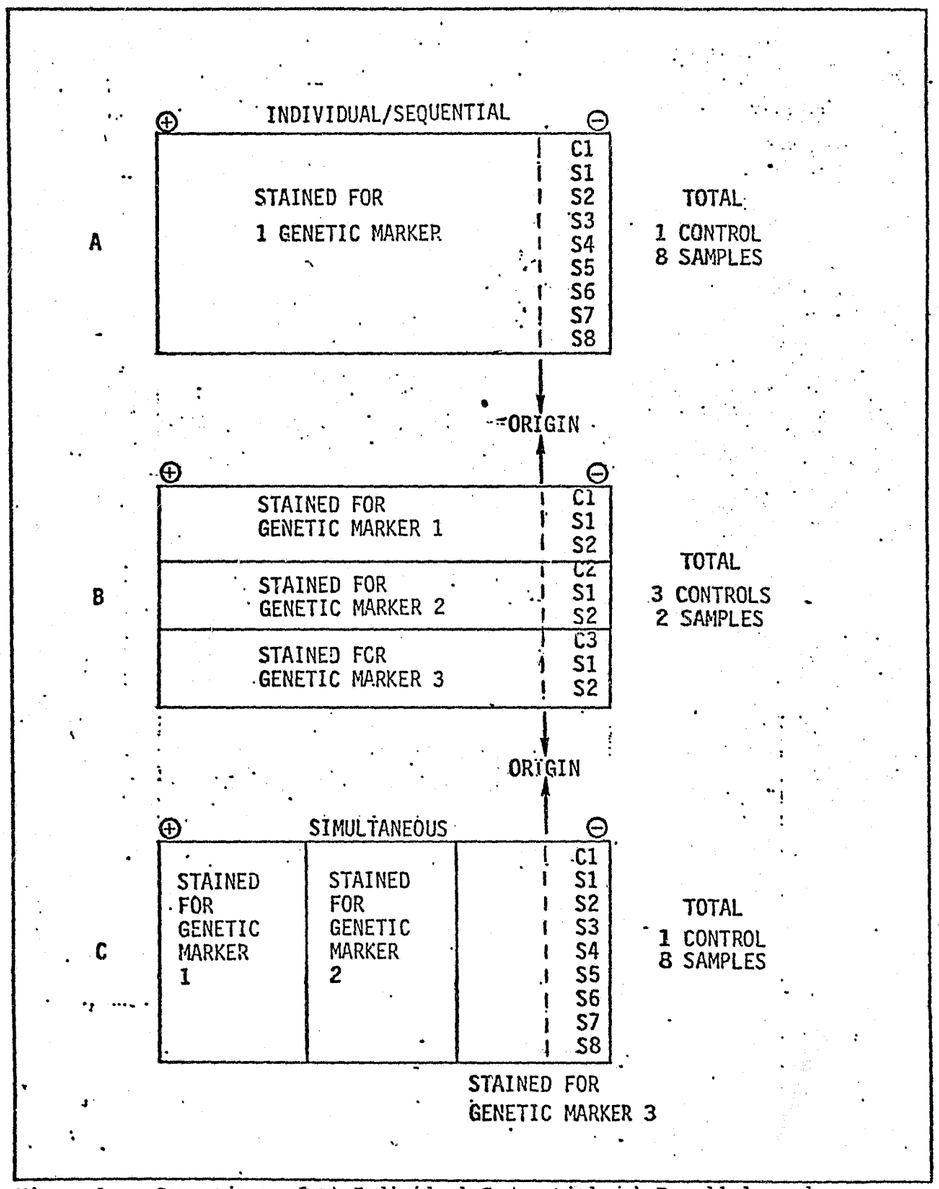

Figure 8. Comparison of A) Individual Sequential, B) Parallel, and C) Simultaneous Separations

direction of electrophoresis, this gave rise to "edge effects" on many samples, resulting in distorted isozyme patterns.

There are no technical problems with either sequential analysis or multicomponent analysis as described. However, one point should be made. Cellulose acetate electrophoresis is normally reported (see references of Sonneborn and Grunbaum on cellulose acetate) requiring only short, 30-60 minutes, electrophoresis times. The same time can be achieved on gels, only it would require a higher voltage power supply. For example, 400 V on an 8-cm-long cellulose acetate membrane is equivalent to 50 V/cm across the membrane. The same voltage applied across a 20-cm-long gel is equivalent to 20 V/cm. Therefore, the gel will take 2.5 times longer to achieve the same separation. The other alternative is to apply 50 V/cm to the gel (i.e., 1000 V) and thereby achieving the same separation in the same time. The differences, however, between sequential and multicomponent analysis become more acute when the practical problems are considered.

Practical. In parallel analysis, unlike simultaneous analysis, any overlap of one genetic marker with another is not important because the whole length of the substrate is stained for each system (see Figure 8B). However, overlap was never a problem in this project because the pH of the buffer chosen for each of the three groups enabled each marker to be well separated from the others. Most of the effort in development was applied to optimizing the separation of the isozymes and the reaction conditions of each genetic marker. The main objection to parallel analysis is that there is no improvement over what is already available to the forensic serologist, i.e., each enzyme being typed individually. There is no saving in terms of time or the amount of bloodstain required. (This becomes more important as the number of genetic markers examined in a bloodstain increases.) As more control samples are required in parallel analysis, fewer bloodstains can be processed per electrophoretogram (Figure 8B).

It was suggested that problems which can occur in multisystem analysis (e.g., two out of the three markers being phenotyped under non-optimum conditions) are overcome by sequential analyses, i.e., analyzing one after another using only one cell. This is assuming that all the methodologies available for individually phenotyping these markers have been optimized. As can be seen under paragraph 4.6, "Interpretation," the new methodologies have shown that the "old" methodologies were not all optimized, particularly those on cellulose acetate (e.g., EAP and EsD) which is the substrate proposed for sequential analysis.

4.6 Interpretation

No matter how simple or fast a methodology on whatever substrate, the ultimate problem is the interpretation of results. If the phenotype is clear to an inexperienced eye, then interpretation is easy. However, the phenotyping of bloodstains has its own problems (e.g., environmental degradation, reaction of the blood with the stain substrate, etc.), and therefore the analyst should not be expected to be fully proficient in interpretation after only two weeks of training. The continuous use of any electrophoretic procedure, with the use of known phenotypes, will always enhance the confidence in interpretation.

Photographs in this section illustrate the separations achieved for all eight genetic markers. The following discussion is intended to help the reader to see the substantial improvements obtained in these separations.

Group I

The separation of the three GLO I phenotypes is shown in Figure 9. When compared with the starch gel procedure (Parr, *et al*, 1977), it can be seen that there is a substantial improvement in the separation. Although a storage band develops anodic to both type 2 and type 1 (see Figure 9), interpretation is very easy. No rare variants have been reported at this time.

The three common EsD phenotypes are shown in Figure 10. Again, there is a substantial improvement in the separation when compared with available methodologies (see Parkin & Adams, 1975; Grunbaum, *et al*, ref. 10). With the secondary gene products (i.e., the weaker band) in a type 1 showing a slower mobility than the primary gene product in a type 2-1 (i.e., the middle strong band), there is no confusion both in hemolysates and bloodstains (Parkin & Adams, 1975). Rare variants should be easily identified.

The three common PGM phenotypes are shown in Figure 11. There is a good separation of the b and c isozymes which is important in differentiating between type 1 and 2-1. The separation of the PGM isozymes is equivalent to the available starch method (Culliford, 1967) and is better than that achieved by Grunbaum (1974) and Zajac and Sprague (1975) (Figure 2). All the rare variants at the PGM_1 locus and the fast-moving variants of the PGM_2 locus are easily observed.

Group II

The three ADA phenotypes are shown in Figure 12. The separation between the isozymes is as good, if not better, than the method of Culliford (1971). Care should be taken when differentiating between a type 2 and a type 2-1. From the figure it can be seen that this differentiation relies on the intensity of the 2 isozyme in relation to the 1 isoenzyme. Variants of ADA should be easily identified.

Four of the six common EAP phenotypes are shown in Figure 13. The separation of the a' isozyme from both the b and c isozymes is particularly important for easy interpretation and it can be seen that this has been more than adequately achieved. When the separation is compared with that reported by Wraxall & Emes (1976), some improvement is noticed. When this separation is compared with that of Grunbaum and Zajac (1978), it will be noticed how much easier it is to interpret this improved isozyme pattern (Figure 14). EAP phenotyping relies on the quantitative

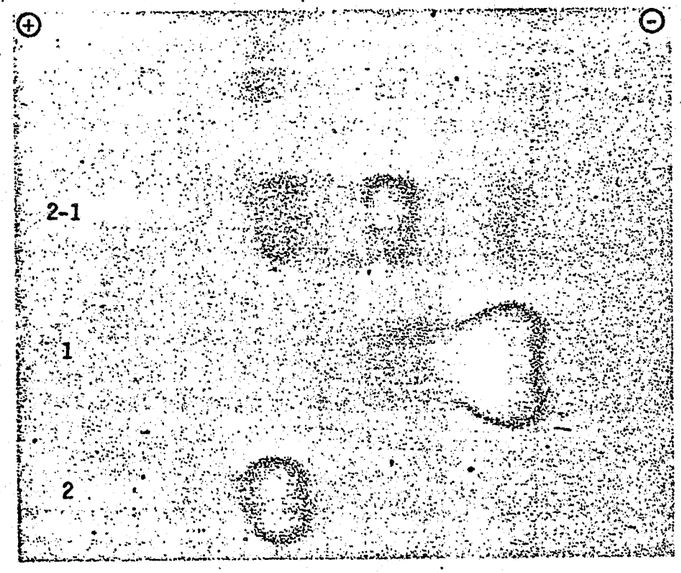

Figure 9. GLO I Phenotypes on Starch/Agarose Gel

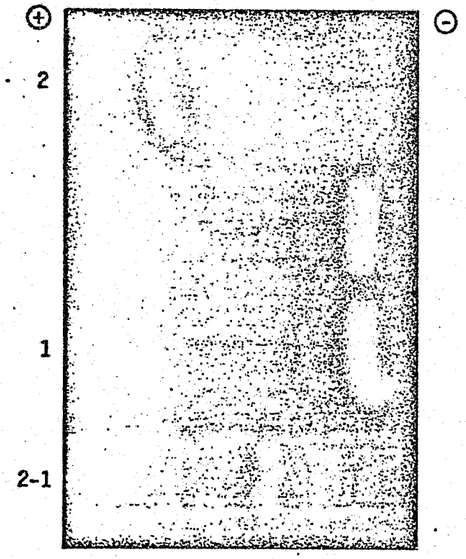

Figure 10. EsD Phenotypes on Starch/Agarose Gel

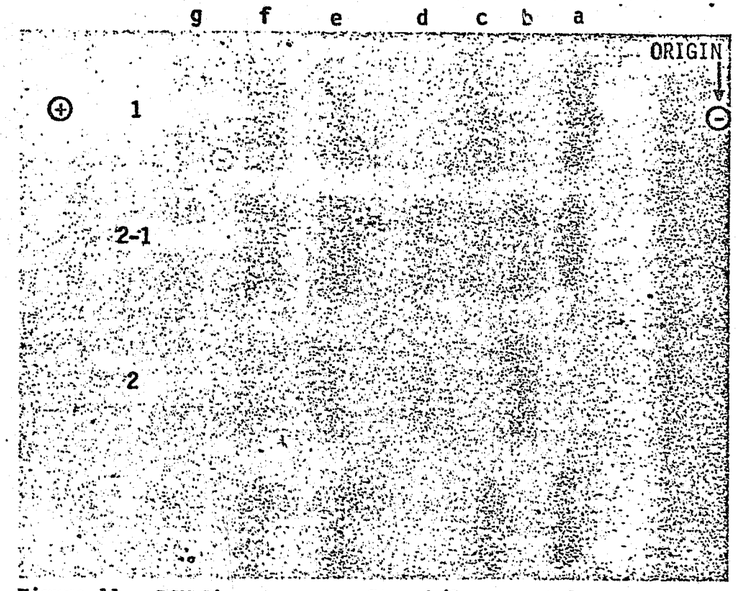

Figure 11. PGM Phenotypes on Starch/Agarose Gel

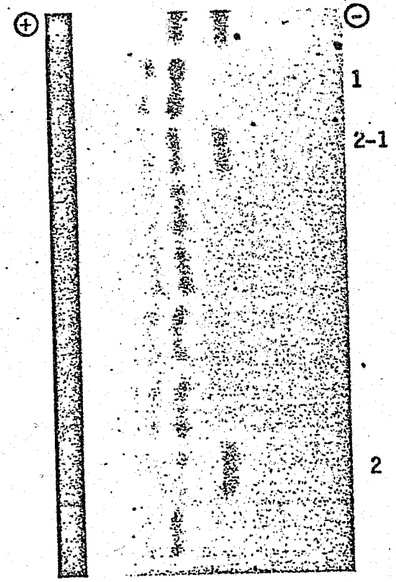

Figure 12. ADA Phenotypes on Starch Gel

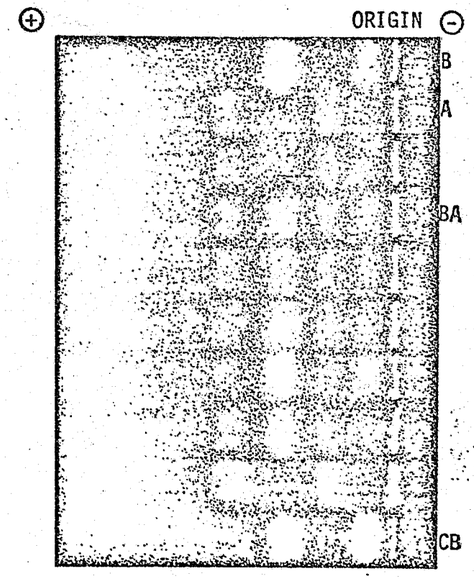

Figure 13. EAP Phenotypes on Starch Gel

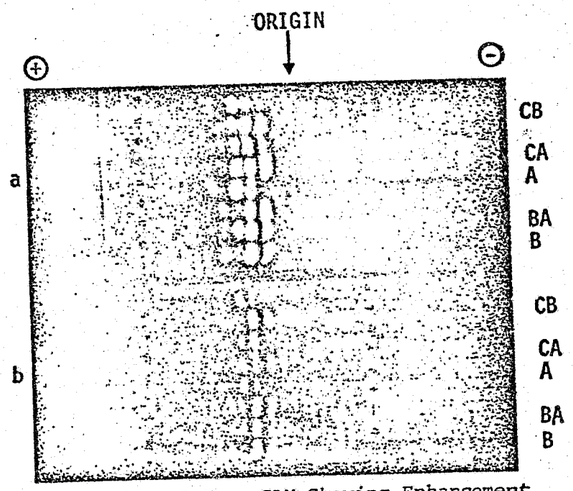

Figure 14. EAP on CAM Showing Enhancement with a) Sodium Carbonate Glycine and b) Glycerol

differentiation as well as the qualitative one, and therefore the intensities of the isozyme bands are important. It should be remembered to compare the relative intensities of the isozymes of each phenotype and not of the different samples. (Compare types CB and B in Figure 13). The rare variants type R and D are separated in this system and are easily identified.

It is reported in the literature (Sensabaugh & Golden 1976, Grunbaum & Zajac 1978) that the use of glycerol is recommended for EAP determinations. Subsequent experiments have shown that the use of glycerol is not desirable in that it is easy to mistype a type CB as a type B due to preferential enhancement of the b isozyme over the c isozyme. If enhancement is required, the use of a sodium carbonate/glycine solution will result in more activity than glycerol without the resulting problems (see Figure 14).

The two common AK phenotypes are shown in Figure 15. Comparisons with the separations of Culliford & Wraxall (1968) and Saenger & Yates (1975) will show improvements. Similar to ADA, differences between the types 2 and 2-1 should be carefully studied.

Group III

The three Gc phenotypes are shown in Figure 16. The interpretation is very easy and when compared with the results of Wraxall (1975), a great improvement can be seen. When compared with Johnson, *et al,* (1975), and Grunbaum and Zajac (1977), the separations are the same. There develops in dried stains a degradation product immunologically identical to the Gc protein. It is the fastest moving of any Gc band and quite often it occurs outside the immunofixation area and therefore does not interfere with the interpretation (see Figure 5).. All known variants of Gc are easily observed.

The three common Hp phenotypes are shown in Figure 17. The differentiation between the types 2 and 2-1 can be difficult but the separation

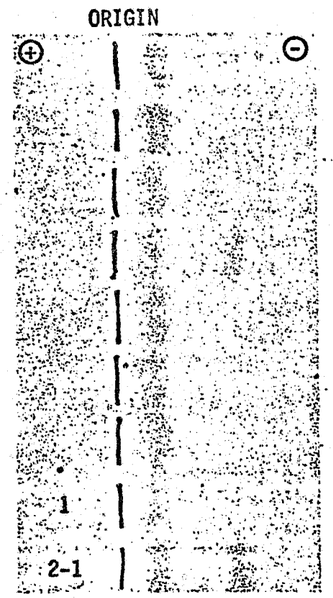

Figure 15. AK Phenotypes on Starch Gel

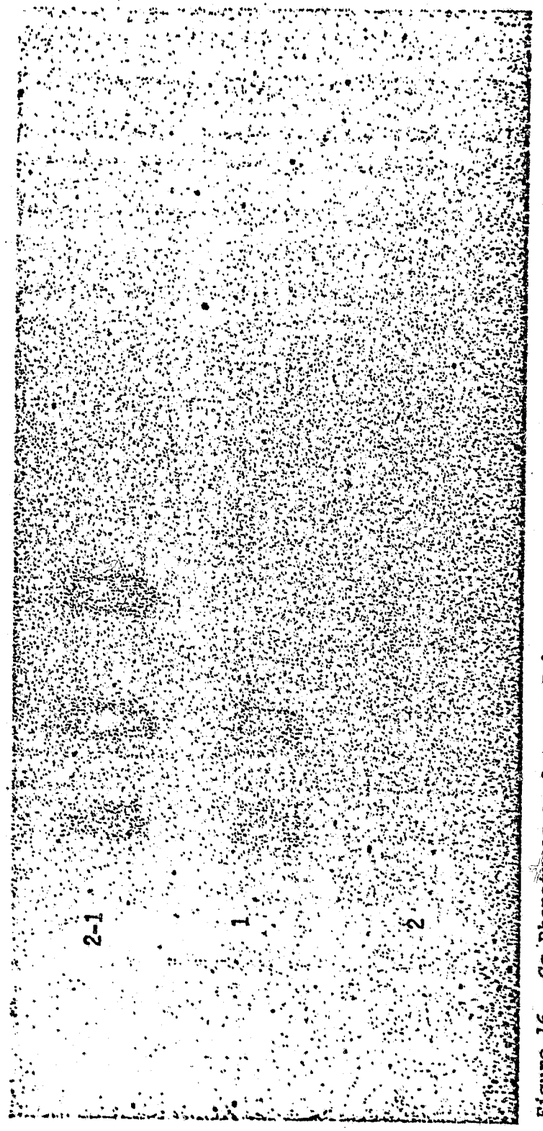

Figure 16. Gc Phenotypes on Agarose Gel

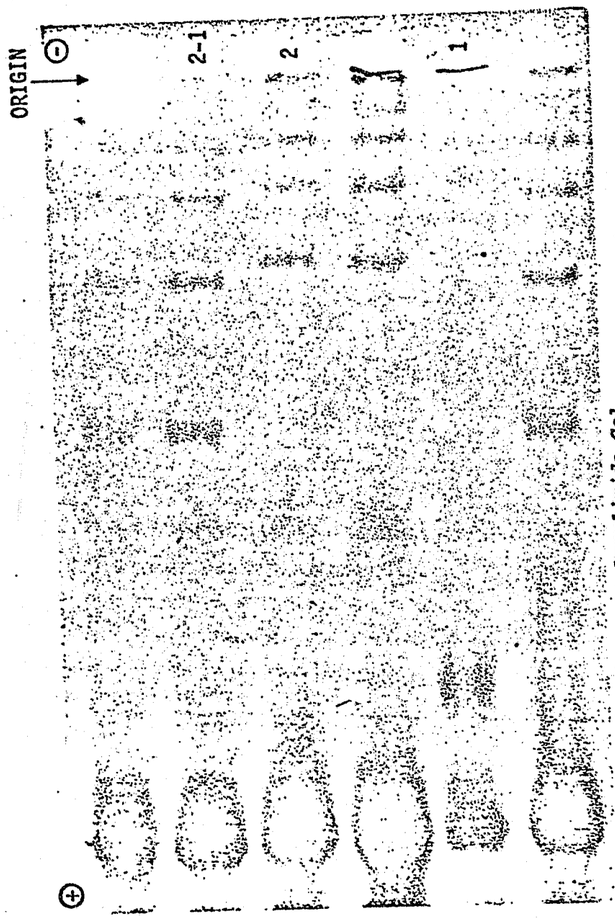

Figure 17. Hp Phenotypes on Acrylimide Gel

is as good as that shown by Culliford (1971) without the need for gradient gel equipment and is better than that of Grunbaum (1975). The following points will help in the differentiation between the types 2 and 2-1. Firstly, it is not always possible to see the haptoglobin 1-band in a haptoglobin type 2-1 Secondly, the first two bands on the type 2-1 are of equal intensity, while the first band of a type 2 is very weak and is found between the positions normally held by the first two bands of a type 2-1. It will also be noticed that the polymer bands on a type 2 are positioned slightly behind those found in the type 2-1. Rare variants can be identified by using the appropriate charts.

In any given system all the known isozymes should be well separated to enable the analyst to easily identify the correct phenotype. Poor or inadequate separations can only lead to misinterpretations. It can be seen from the figures that there is at least equivalent separation, if not a substantial improvement, of all of the eight enzymes and proteins.

5.0 RESULTS

The results obtained during the program are summarized below:

- Discrimination. As seen from Table III, using population data from white caucasians, a discrimination probability of 1 in 200 is possible when using the selected eight genetic markers. The inclusion of minorities in a general population would reduce the discrimination probability depending on the percentage in any given population. For example, the 1974 California population consists of White 73.1%, Black 7.4%, Hispanic 15.5%, and Asian 4.0%. Using the population data of Grunbaum, *et al*, (ref 9), the discrimination probability of a general population in California (26.9% minorities) would be 1 in 170.

- Bloodstain Materials. Bloodstains on both glass and cotton fabric were easily phenotyped and, during the Crime Laboratory Feasibility Testing Phase of the program, it was shown that stains on a wide variety of other materials can be analyzed (Appendix B).

- Volume. Although the volume of blood allowed by the Statement of Work was 25 µℓ, all eight genetic markers could be easily phenotyped using only 5.5 µℓ.

- Age. The age of the stains was required to be four weeks; it was found that all eight enzymes and proteins were stable and therefore able to be typed at this time. Some bloodstains have shown activity for six of the eight systems even at 2-1/2 months, and Hp has been determined (using the chloroform extraction technique) in stains eight months old.

- Analysis Time. Simultaneous analysis of the eight genetic markers has been accomplished using only three electrophoretic cells as described in the methodologies (Appendix C). The analysis of at least 9 samples phenotyped for all eight markers can be completed in 24 hours, using a maximum of 3.5 manhours of labor. The electrophoresis of Group II enzymes for 16 hours is carried out for the convenience of the operator, allowing him to utilize the equipment and his time much more efficiently.

- Reagent Selection. All reagents required in the methodologies are normally obtainable from more than one source in the United States. However, it has been recommended that certain reagents should be obtained from specific suppliers in preference to others (e.g., the liquid enzymes for the reactions of PGM, AK, and ADA supplied by Boehringer).

- Health and Safety. There are no deviations from requirements established by the Health and Safety Act of 1975 when using the reagents. Normal safety provisions should apply.

- Accuracy and Reliability. As can be seen from the Method Feasibility Test Report (Appendix A), the accuracy and reliability of detection of the eight markers is more than 99% at the 90% Confidence Level.

- Training. Four analysts were trained in the methodologies for two weeks. It can be seen from the Crime Laboratory Feasibility Report that this was successful. All four analysts successfully phenotyped 30 blind trial samples and are now routinely using most, if not all, of the genetic markers in casework in their own laboratories.

Where an analyst is familiar before training with all the phenotypes of a particular enzyme or protein system, then the phenotypes obtained using these methodologies give clearer, more unambiguous results than previously encountered. However,

some of these markers are new to nearly all forensic serologists (e.g., GLO I and Gc) and therefore a longer exposure to the phenotypes than two weeks is required. It should be noted that there was an improvement in the results after the first and second blind trial.

- System Cost. The total cost of the equipment required, consisting of a refrigerated cooling bath, three electrophoretic cells, three aluminum cooling plates, two power supplies, one centrifuge, and all the small accessories, is approximately $3,500. The cost per analysis is much less than $25 per stain (see Feasibility Test Report, Appendix A).

6.0 CONCLUSIONS

After a review of the completed program, we have drawn the following conclusions:

1. The technical requirements have been satisfied.
2. A four-week-old bloodstain of only 5.5 µℓ of whole blood can be phenotyped for eight genetic markers using only three electrophoretic cells.
3. At least nine stains can be phenotyped for all eight markers in only 24 hours, using only 3.5 manhours of labor.
4. There has been a vast improvement in the clarity of many phenotypes using less bloodstained material.
5. Analysts competent to perform single component electrophoresis can learn the methodologies for multisystem analyses in a two-week period of instruction.
6. Analysts without prior experience with electrophoresis will probably require more than two weeks of instruction. Academic background and specific work experience would be important for the rapid understanding of all eight genetic markers.
7. The probability of discrimination of one person out of 200 appears to be close to the maximum achievable with only three setups as allowed by the contract. Additional setups would provide increased discriminating capability, as other blood genetic markers can be identified.
8. The Bloodstain Analysis system represents a significant advance in forensic science in reducing time and equipment requirements and expanding capabilities for processing evidence.

7.0 RECOMMENDATIONS

- The methodologies developed under the Bloodstain Analysis System program should be transferred to crime laboratory personnel through a specific training program. Such a program should be instituted as soon as possible.

- Continuing effort should be applied to incorporate other blood constituents into the BAS System to provide the greatest possible discrimination.

- Recent developments in separation techniques, such as isoelectric focusing, indicate that much greater resolution can be achieved, which will increase discrimination. A considerable increase in discrimination has already been achieved in this program. If still greater discrimination is of value, there are two ways to obtain it. One is to simply add additional constituents for analysis with the existing recommended hardware. This would not yield a greatly enhanced capability, but would require relatively little effort in development. An alternate approach is to develop isoelectric focusing for use with the constituents that have been proven to retain prolonged enzymatic activity.

With the few constituents that have been explored, it is reported that discrimination might be improved several-fold.

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APPENDIX A

FEASIBILITY TEST REPORT

FEASIBILITY TEST REPORT

BLOODSTAIN ANALYSIS SYSTEM

Subcontract 67854

January 30, 1978

Prepared for:

The Aerospace Corporation
Suite 4040, 955 L'Enfant Plaza, S.W.
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CONTENTS

1.0 TEST OBJECTIVE

Prior to presenting the Bloodstain Analysis system to selected crime laboratories for field trials, it was required that tests be conducted to verify that the system met the requirements of the Statement of Work. Those requirements are listed below. The performance of the system for each of the requirements is discussed in Section 3 of this report.

The tests were performed on January 16 and 17, 1978, in accordance with an approved plan. For reference, this plan is enclosed as Appendix A. This report is submitted in accordance with paragraph 5.4.3 of the subcontract Statement of Work.

2.0 REQUIREMENTS

2.1 Accuracy and Reliability

Accurately identify the constituents in a minimum of 90% of the unknown stains.

2.2 Sample Volume

Perform the complete analysis on a stain equivalent to 50 µl of fresh blood using no more than half the sample.

2.3 Degree of Individualization

The system shall provide a probability of discriminating one person in 200.

2.4 Rapidity

It shall not require more than five manhours in a 24-hour period to identify the constituents of one stain.

2.5 Clarity of Reading

The reading of the stains shall be unambiguous.

2.6 Costs

It shall not cost more than $25.00 to analyze one stain.

3.0 RESULTS

3.1 Summary

The test results show the Bloodstain Analysis system met all the performance requirements. Using much less than 25 µl of sample allotted, more than 90% of the unknown stains were accurately identified. All eight of the selected blood constituents were identifiable which provides a probability of discriminating one person in 200.

Out of 144 readings, only one was not correct. A minor change in the development buffer was made to eliminate an ambiguity in that constituent. The system now provides clear and unambiguous phenotypes.

With less than four manhours of work, as many as 12 samples can be processed in a 24-hour period. In practice, it may be more convenient to use about 26 hours total time to allow an overnight run. Capital costs of the system are substantially less than the $6,000 allotted. Amortized costs per analysis are of course dependent on usage and time. However, even with only a few samples a month the cost would be less than $25.00 per sample using a five-year depreciation base. Although four substrates are required, they can be handled in three set-ups. Overall, we conclude that the feasibility of the proposed system has been proven and it is ready for presentation to selected crime laboratories.

3.2 Accuracy and Reliability

There were a total of 18 stains presented by Aerospace Corporation for identification. A summary showing the code number of each sample, the referee analysis and our result is presented in Table 1.

Table 1. Feasibility Test Readings

Stain Code	Stain Date	Group I GLO	Group I ESD	Group I PGM	Group II ADA	Group II EAP	Group II AK	Group III Gc	Group III Hp	Notes
T1	12/20	2	2-1	2	1	BA	1	2-1	2	1
T2	12/20	2-1	1	2-1	1	CA	1	1	2-1	1
T3	12/20	2	2-1	2-1	1	A	1	1	1	1
T4	12/20	2	2-1	1	1	BA	1	2-1	2-1	1
T5	12/20	2-1	1	2-1	2-1	B	1	1	2	1
T6	12/20	2	1	2-1	1	B	1	2-1	2-1	1
T7	12/27	2-1	1	1	1	BA	1	1	1	1
T8	12/27	2	2-1	1	1	B	1	1	1	1
T9	12/27	2	2-1	2-1	1	B	1	2	2	1
T10	12/27	2-1	1	2-1	1	B	1	2-1	2	1
T11	12/27	2	1	1	1	B	2-1	2-1	2	1
T12	1/3	2	1	1	1	B	1	1	2-1	1
T13	1/3	2-1	2-1	1	1	A	1	2	2-1	1
T14	1/3	2	1	1	1	BA	1	2-1	2-1 2	2
T15	1/3	2	2-1	2-1	1	BA	2-1	2-1	2-1	1
T16	1/3	2	1	1	1	B	1	1	2-1	1
T17	1/3	2	1	1	1	BA	2-1	2-1	2-1 2	3
T19	12/22	2-1 2	2-1	2-1 2-1	2-1 2	BA	1	1	2-1	4

Notes:
1. All constituents correctly identified without question.
2. Stain T14: Hp questioned. Retest identified as 2.
3. Stain T17: Hp questioned. Retest identified as 2.
4. Stain T19: GLO questioned. Retest identified as 2.
   PGM questioned. Retest verified as 2-1.
   ADA miscalled. Test sample confirmed as 2.

CONTINUED

1 OF 2

The age of the stain was not to exceed four weeks. The date at which each of the unknown stains was deposited is also shown in Table 1. The age ranged from two to four weeks with seven of the stains being four weeks old.

There was a total of 144 readings. Of this total, there was only one that was miscalled. It was an unusual variant of ADA in a stain approximately one month old. Four other variants were questioned and on retesting were typed correctly. The ADA variant was called a 2-1 whereas it was a 2. During the development testing this variant had not been encountered. The problem was restricted to the development of the ADA reaction and therefore did not affect the electrophoretic separation of the constituents of the group. The problem was easily corrected by reducing the pH of the reaction buffer to 7.0 thereby allowing the reaction to proceed at its optimum.

The tests have demonstrated very conclusively that the system is capable of meeting the accuracy and reliability requirements.

3.3 Sample Volume

The stains were prepared by pipetting 50 µl of blood onto clean cotton cloth. By comparing the area of stain used with the total area, an estimate of the volume of sample used can be made. This was done and an average of 5-1/2 µl of each stain was used. In the cases where a retest was made, approximately 10 µl were used. This is well below the allowed volume. No particular effort was made to minimize the volume used. Our development work indicates the limit of sensitivity is significantly less than our average use for these tests.

3.4 Degree of Individualization

Based on the discrimination factors available for our selected constituents, we have achieved the required 1 in 200 probability of discrimination. Within the constraints of the Statement of Work which allows only three set-ups, there is a practical limit to the number of constituents which can be analyzed for. We have selected the constituents carefully and believe we have achieved the greatest discrimination practical for the general population.

3.5 Rapidity

The analyses of the eight constituents are handled in three set-ups. A time log was made for each of the set-ups. For group one, 65 minutes were recorded. For group two, forth-three minutes, and group three, 98 minutes. A total of three and a half hours were required during which a total of 12 stains were identified. It is obvious that the average time per stain is greatly less than the allowed five manhours.

The total elapsed time was slightly less than 24 hours. It is possible to complete the analyses in 16 hours but in practice it may be more convenient to take 26 hours. This would permit an overnight run that allows efficient use of hardware and technician time.

3.6 Clarity of Reading

The test samples represented a broad coverage of the variants of the system constituents. In fact, one sample contained a rare variant of ADA. As discussed previously in the section on Accuracy, this led to an erroneous call. It was concurred by the Aerospace Corporation observer that the clarity of the phenototypes was very acceptable. This is substantiated by the fact that of the 144 readings only four were not easily readable and a retest was required.

With the correction of ADA, it can be concluded that the system meets the requirement for clarity of reading in providing unambiguous phenotypes.

3.7 Cost Per Analysis

Detailed hardware costs have been presented to the Aerospace Corporation. The total capital costs for the recommended system is approximately $2,000. This is only a fraction of the $6,000 limit. Costs for chemicals and other expendables would probably not exceed $500 for about 100 analyses. Some chemicals have a limited shelf life. The total cost per analysis will vary with the number of samples processed over the life of the equipment. Assuming a five year life, only one sample a week need be processed to keep the average cost under $25.00.

FEASIBILITY TEST PLAN

Subcontract 67854

November 2, 1977

Prepared for:

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CONTENTS

1.0 OBJECTIVE

Prior to presenting the Bloodstain Analysis system to selected crime laboratories for field trials, it must be demonstrated that the system satisfies the criteria of the Statement of Work. It is the objective of the Feasibility Tests to verify that these criteria have been met. For convenience, the criteria from the S.O.W. are listed here. The plan provides details as to how each criterium is addressed and how specific tests will be performed.

- Accuracy and Reliability

  Accurately identify the constituents in a minimum of 90% of the unknown stains.

- Sample Volume

  Perform the complete analysis on a stain equivalent to 50 µl of fresh blood using no more than half the sample.

- Degree of Individualization

  The system shall provide a probability of discriminating one person in 200.

- Rapidity

  It shall not require more than five manhours in a 24-hour period to identify the constituents of one stain.

- Clarity of Reading

  The reading of the stains shall be unambiguous.

- Costs

  It shall not cost more than $25.00 to analyze one stain.

2.0 TEST SAMPLES

Beckman Instruments will supply clean cotton cloth to the Aerospace Corporation for staining. A single stained spot containing 50 µl of whole blood will constitute one sample. The stain will be deposited and allowed to air dry at ambient temperature in a clean laboratory environment. The stained cloth shall be protected from contamination.

Aerospace shall retain in a frozen form portions of the test samples as serum and hemolysate. These samples may be analyzed in the event of a difference between the feasibility test results and the referee coded results. Approximately 18 samples shall be presented for test after aging a maximum of four weeks.

Prior to the Feasibility Tests, a comparative analysis will be made on samples by the referee laboratory and Beckman. There should be approximately 14 such samples tested.

3.0 ASSESSING PERFORMANCE

3.1 Accuracy and Reliability

The variants present in each constituent which is a part of the analysis system will be identified and logged under the code number for each stain analyzed. A comparison of the test results will then be made against the referee results of record as provided by Aerospace personnel at completion of the test. The stain shall be judged to be identified correctly if the test results agree with the referee results.

The percent of the stains accurately identified will then be calculated. A mistrial shall not be part of the percent accuracy calculation.

3.2 Sample Volume

The complete analysis shall be made by cutting out one-half of the stained area of cloth which should contain the equivalent of 25 µl of whole blood.

3.3 Degree of Individualization

The index of discrimination for each of the constituents of the Bloodstain Analysis system shall be obtained from published data from a source acceptable to Beckman and Aerospace. Based on the demonstrated capability to identify a constituent, the cumulative degree of individualization shall be calculated.

3.4 Rapidity

It is planned to have one individual perform the analysis and identification of all of the test stains. A time log will be made so that the total "hands-on" time to analyze one stain can be measured. The time shall accumulate when the coded stains are available to the laboratory technician so that work directly associated with the analysis can begin. The time required by the technician to perform each task will be noted. Intervals of time for electrophoresis, incubation, or other steps which do not require technician participation shall not be included. The total time from beginning the analyses until the last of the system constituents have been identified shall be noted.

3.5 Costs

The costs of reagents and supplies will be determined from prices of recent procurements. The cost of analysis per stain shall be calculated on the proportionate cost of the amounts of materials used. The cost of the hardware involved in making the electrophoretic separation shall be provided to Aerospace Corporation and shall be based on the latest published sales price of the equipment involved.

3.6 Clarity of Readings

No unusual or exotic lighting or optical aids will be used in the phenotyping. Proof of the clarity of readings will be in meeting the accuracy requirements.

4.0 METHODOLOGY

4.1 Test Set-Up

The procedure for the analysis of each constituent is included in an attachment to this plan. The procedures shall be followed, as written, in identifying each of the test samples.

5.0 PHENOTYPING

Phenotyping shall be done by the laboratorian performing the analyses. The phenotyping readings shall be recorded by an observer from Beckman Instruments and witnessed by an Aerospace representative.

In the event a phenotype cannot be interpreted, the validity of the test shall be questioned. This will be done in accordance with the following section titled Mistrial.

6.0 MISTRIAL

A sample whose constituents are known shall be used as a standard. One or more standards may be used with each gel plate. If the standard sample does not separate and react in the normal manner to produce a readable phenotype, a mistrial shall be declared. The cause of the abnormal result shall be determined and corrected before continuing the feasibility test.

7.0 STATISTICAL SIGNIFICANCE OF TEST RESULTS

It is planned to analyze 18 stains. Eight constituents in each will be identified. There will then be a total of 144 readings. As this is a qualitative measurement, there are no averages or standard deviations calculable. The requirement to accurately identify 90% of the stains means that one would be correct 90 times out of 100. It does not necessarily follow that one is wrong the other 10 times. It may be that no answer is given in those instances. For the feasibility tests, we will calculate the percentage based on the total number of readings correct to the total number made (144).

ATTACHMENT

METHODOLOGIES

(These methodologies have been superceded by those in Appendix C)

3.0 METHODOLOGIES

3.1 General

The following notes apply to all three groups.

- Buffers. All chemicals used in buffer making should be of very pure grade. The weights given in the methodology sheets should always be checked against the molarities which are also given. The pH of any buffer should be carefully checked before use. All buffers are best stored in the refrigerator. Approximately 800 ml of buffer are required to fill a tank to the upper line. The tank buffer should only be used for five electrophoretic separations and then discarded. The reaction buffers are best made only in small quantities and care should be taken to clean the containers before re-use.

- Bridges. Sponge bridges are used although some manufacturers do employ softeners and fungicides which can cause problems during electrophoresis. Therefore all bridges should be carefully washed in distilled water before use. If, however, problems continue then three or four thicknesses of Whatman 3MM can be used. All bridges should be cut to the exact width of the electrophoretic plate and held in contact with the gel by means of a second glass plate which is placed on top.

- Reducing Agents. All reducing agents should be prepared fresh weekly and stored while not in use in a refrigerator. Haemolysates are prepared by washing whole red cells three times with saline and lysed. They are best stored in the presence of reducing agents.

- Electrophoresis. The meters on some power supplies are not very accurate so the voltage between the bridges are best checked by using a voltmeter. The times given for electrophoresis should be strictly adhered to or else there will be overlap of one enzyme with another.

- Staining. When using paper overlays care should be taken here to avoid excess reaction mixture which will cause diffusion of the enzyme bands. When using agar overlays, the 2% agar is first boiled and the constituents are mixed together with the reaction buffer. The agar is cooled in running water to approximately 55°C, mixed with the reaction mixture and immediately poured over the surface of the gel.

3.2 Group I

The gel is prepared by weighing the agarose and hydrolyzed starch and boiling until all the agarose has dissolved. The gel is poured into the gel mold, which should be on a level surface, and immediately scraped using the plastic scraper so as to produce an even 1-mm-thick gel.

One-mm-thick strips of stain, 1 cm long, are removed and soaked in a minimum of reducing agent. Slots are cut in the gel using the metal slot makers. After electrophoresis, Esterase D is developed first. Care should be taken not to use too much acetone to dissolve the Methyl Umbelliferyl Acetate; this will produce a large amount of background fluorescence. After leaving the plate at room temperature for five minutes, the Esterase D banding can be read under UV light. The position of the Esterase D 2 band should be marked as this will discern the dividing line between the GLO I and the PGM reaction mixtures.

The 2.5% Methyl Glyoxal is prepared by adding 1.25 ml of 40% Methyl Glyoxal to 20 ml of distilled water and adjusting to pH 7 with a 1% solution of sodium hydroxide. The iodine solution is prepared by dissolving the potassium iodide and the iodine in 5 ml of distilled water. The solution is allowed to stand for 24 hours and then added to the remaining 25 ml of distilled water filtered and stored in a dark amber bottle. To visualize the Glyoxalase I bands, the iodine solution should be added to the agarose solution when it is hot and immediately poured onto the surface of the gel.

3.3 Group II

The gel is prepared by adding hydrolized starch to the gel buffer, bringing to the boil by heating in a 250 ml Erlymeyer flask. (Note: the flask should be kept moving in the flame to avoid burning of the gel!) When boiling, the gel is degassed using a vacuum pump drawing approximately 15 psi pressure and then poured into the gel mold. The starch solution is immediately leveled to a 1 mm gel by using the gel scraper, care being taken to insure that the mold is on a level surface before pouring.

Stains are prepared by cutting 1-mm-thick strips, 1 cm long from the stain and soaking with the minimum amount of reducing agent. Application slots are cut int the gel using the metal slot makers. The application of stains or lysates to the gel should be kept as narrow as possible so as to produce sharp isozyme banding.

After electrophoresis it will be noted that the cathodic portion of the gel is covered with condensation droplets. These should be removed by blotting with Whatman 3MM paper. EAP is developed in an area 1 cm cathodic of the origin to 7 cm anodic of the origin. ADA is developed anodic to the EAP area. Care should be taken not to expose the ADA reaction mixture to strong light. After the EAP results have been recorded the cathodic half of the gel should again be blotted before covering the area with AK reaction mixture.

3.4 Group III

The important point to note here is that the acrylamide and agarose gels can be run in the same tank at the same time. For screening of serum both proteins can be phenotyped using the acrylamide gel. However, the degradation product that is found in varying amounts in stains unfortunately migrates amongst the Gc bands and can lead to misinterpretation. Therefore, all stains must be phenotyped for Gc using the agarose gel.

- Acrylamide. The acrylamide gel is prepared by dissolving all the constituents in the buffer, degassing and pouring into the gel mold. A clean glass plate is carefully overlaid, excluding

all the air and the gel allowed to polymerize for 30 to 45 minutes. Several gels may be made at the same time and then stored in the refrigerator. When the gel is ready for use the top plate is removed by carefully inserting a razor blade between the two plates and gently levering the upper plate from the gel.

- Agarose. This is prepared by dissolving the agarose in gel buffer by boiling and pouring the hot solution onto a plain glass plate on a level surface and allowing to solidify.

- Extraction. The amount of stain required to obtain results will be decided by experience but the size given (i.e., 3 x 3 mm) is recommended when using cotton cloth as the substrate. The amount of buffer required again will be decided by experience but each gel uses approximately 10 mm of extract for each application. It is important, however, to obtain very clear extracts or background smearing on the gels will result. Longer centrifugation can be used if necessary.

- Application. The two-sided metal slot makers are used for making the application slots. For serum samples the anodic slit only is used. One serum sample should contain bromophenol blue as the marker. For stains a 1.5 mm wide plug of gel is removed and the stain extract carefully pipetted into the well. It is preferable to start both gels at the same time removing the agarose after two hours and allowing the acrylamide to continue for a further two hours.

- Immunofixation-Agarose. After two hours the albumen as noted by the position of the tagged bromophenol blue has traveled approximately 10 cm and the hemoglobin about 4.0 cm. The cellulose acetate membrane overlay containing the diluted anti-serum should not be too dry and is placed on the gel approximately 5.5 cm from the origin and covering the whole

width of the agarose. It is allowed to incubate for two hours at room temperature in a moist atmosphere.

- Immunofixation-Acrylamide. After electrophoresis the gel is divided just ahead of the hemoglobin band (which has moved approximately 6 cm) and the cathodic portion of the gel removed into a staining dish. If required a cellulose acetate membrane soaked in diluted anti-serum is overlaid on the remaining portion of the gel at the dividing line. This is allowed to incubate at room temperature for two hours (or overnight if this is more convenient).

- Staining. The haptoglobin is stained immediately after electrophoresis using the orthotolidine mixture. If the results are too weak the percentage of peroxide should be increased. If, however, there is bubbling within the gel the peroxide content should be reduced.

The agarose is reduced to a very thin film by overlaying the gel with wet Whatman 1 paper and several thicknesses of blotting paper and applying approximately 5 kilograms weight for 30 minutes. The gel is then washed overnight in a 1 molar saline solution, repressed for 30 minutes and dried in an oven at approximately 75°C for 15 minutes. The Gc bands can then be visualized by staining in Coomassie blue for five minutes and destained in the higher methanol destain solution.

If immunofixation has been carried out on the acrylamide no pressing and very little washing is required. The bands are visualized by immersing in Coomassie blue stain for 30 seconds. (Any longer and the acrylamide gel will take up the stain which is very difficult to remove.) Destaining is carried out using the lower methanol destain solution.

APPENDIX B

CRIME LABORATORY DEMONSTRATION TEST REPORT

1.0 INTRODUCTION

The development of the Bloodstain Analysis System was based on tests with aged bloodstains from glass slides and cotton cloth. These stains were made under controlled conditions to provide reproducible test conditions. The crime laboratory demonstration tests were conducted to obtain an independent appraisal of the system's capabilities to perform in the actual environment of the criminalistic laboratory. The ability of the system to cope with various types of bloodstains deposited on, or absorbed by, various materials was determined by these tests. The participation of the crime laboratories also served to show how effectively this technology could be transferred to trained serologists.

The demonstration test period lasted approximately three months. In the beginning, personnel from four participating laboratories completed a two-week training program conducted in Beckman laboratories.

The laboratories represented were selected by The Aerospace Corporation. Participating personnel were from the Georgia Bureau of Investigation, the Los Angeles County Sheriff's Office, the Minnesota State Police, and the New Jersey State Police.

Subsequent to the training program, the system hardware was shipped to the various participating crime laboratories. Each individual received the same hardware on which he had been trained. After the system hardware had been received and put into operation at the crime laboratories, blind trial samples were given out to permit checking the accuracy of analysis at each laboratory.

Results of these tests, and reports from the serologists, permitted an evaluation of the overall utility of the Bloodstain Analysis System under

actual field conditions. The detailed results of this effort are presented in this report.

2.0 TRAINING PROGRAM

A representative serologist from each of the four evaluating crime laboratories, plus observers from both the FBI laboratory and The Aerospace Corporation, began a training course at the Beckman laboratories on January 30, 1978. The course was conducted by Brian Wraxall with the assistance of Gary Harmor, and followed the schedule outlined in Table I. The two-week course followed the schedule exactly with each serologist completing each group five times. For the last three attempts at each group, blind samples consisting of bloodstains of varying ages were analyzed. Although some serologists had some problems in interpretation of phenotypes with which they were unfamiliar, by the end of the training course very few mistakes were being made.

At the completion of the course, the equipment--including the method manual and all the biochemicals--was packed and shipped to the four crime laboratories. Each serologist assembled the equipment in his own laboratory and established a routine system very quickly. Some serologists were subjected to the pressure of casework and therefore were not able to devote all their time to the new methodologies. The effects of this can be seen in the initial blind trial results. However, close contact was maintained between the instructors and the students to ensure that any problems could be resolved. The problems that were encountered were very minor and included broken electrode wires, reducing agent being too strong, and GLO I reaction being weak. These difficulties were easily resolved, and within a few weeks all the serologists had their complete systems working routinely.

TABLE I. TRAINING SCHEDULE (January 30 to February 10)

1st Week		
Monday:	Welcome and Introduction.	Jean Bordeaux
	Introduction to Multisystem Analysis and Aims of the Course	Brian Wraxall
	Laboratory Inspection and Equipment Setup	
	Group I Demonstration	
Tuesday:	Group I (1)	
	Group II Demonstration	
Wednesday:	Group I (2) + Stains	
	Group II (1)	
	Group III Demonstration	
Thursday:	Group III (1)	
	Group II (2) + Stains	
Friday:	Group III (2) + Stains	
	Lab Cleanup	
	Buffer Replacement	
	Summary and Question Session	
2nd Week		
Monday:	Group I (3)	
	Group II (3)	
	Group I Interpretation	
Tuesday:	Group III (3)	
	Group II (4)	
	Group II Interpretation	
Wednesday:	Group I (4)	
	Group III (4)	
	Group III Interpretation	
Thursday:	Group I (5)	
	Group III (5)	
	Group II (5)	
Friday:	Options on Groups I and III	
	Dismantle Equipment	
	Summary and Conclusion	

3.0 BLIND TRIAL

Four weeks after the completion of the training course, the blind trial program was started. This consisted of sending a batch of six blood-stains of varying ages to each serologist for phenotyping in all eight genetic markers. Five such batches of stains were sent out over a two-month period. When the analysis was completed, the results were returned to Beckman for checking. Twenty-four out of a possible twenty-seven common phenotypes of the eight genetic markers were used in the trials.

4.0 RESULTS

The results obtained from the blind trials are summarized in Table II. Out of a total of 912 readings, only one reading was made incorrectly. This result was a Gc type 2-1 being called a type 1. The analyst was unfamiliar with phenotyping Gc, and from the photograph of the result, it was obvious that the phenotype of that particular sample should not have been called. It can be seen from the results that there is an increase in the number of positive readings given with each successive trial, and as each analyst acquires more confidence from completing more tests, so the quality of the results increases. It should also be noted that every blind trial was sent to each crime laboratory at the same time, but due to excessive casework in some laboratories the samples were not always analyzed at the same time--hence the difference in age of the stains from laboratory-to-laboratory. This is most noticeable in laboratory D where some stains are very old, which would account for the high number of questioned and no-activity results. It should also be noted that due to excessive casework, laboratory A is routinely testing only for Groups I and II.

5.0 CASEWORK

Any technique, however well it behaves in the research laboratory, is useless unless it can be used successfully in the crime laboratory. It has been demonstrated that these methodologies can be used successfully on case material in the crime laboratory. Three of the four evaluating laboratories have routinely been using all three groups (i.e., eight genetic markers) on case material and reporting the results. The fourth laboratory is using routinely and reporting the results of Groups I and II (i.e., six genetic markers). In the process of evaluation, many different materials containing bloodstains were encountered. These included the following fabrics: wool, cotton, silk, vinyl, nylon, polyester, denim, corduroy, carpeting, and other fabrics representing synthetic fibers. Also examined were paper, wood, metal, glass, leather, concrete, and fingernails. No problems were encountered that could specifically be attributed to the material containing the bloodstains. All four laboratories have replaced their old methodologies with the new because they are achieving not only better results from the markers that they normally examine, but more information from the same amount of bloodstain in the same amount of time.

The following examples are from actual cases examined using the new methods. The laboratories are not identified for obvious reasons, but there is one example from each laboratory.

5.1 Laboratory 1

A homicide occurred earlier in the year. Victim and suspect blood samples were taken and were submitted to the laboratory together with blood from the scene (swabbed by a piece of wetted cotton cloth) and a bloodstained knife. The blood samples were refrigerated and the stains stored at room

TABLE II. CRIME LABORATORY DEMONSTRATION — BLIND TRIALS

Crime Laboratory	Blind Trial	No. of Stains	No. of Readings	Correct	In-correct	Quest-tioned	No Activity	Age of Stains
A	1st	6	48	32	-	13	3	3-5 wks
	2nd	6	36	31	-	1	4	1-4 wks
	3rd	6	36	36	-	-	-	4-6 wks
	4th	6	36	36	-	-	-	4-5 wks
	5th	6	36	36	-	-	-	4-7 wks
	Total	30	192*	171	-	14	7	
B	1st	6	48	37	-	2	9	3-5 wks
	2nd	6	48	48	-	-	-	1-4 wks
	3rd	6	48	46	-	2	-	2-4 wks
	4th	6	48	48	-	-	-	3-4 wks
	5th	6	48	48	-	-	-	3-6 wks
	Total	30	240	227	-	4	9	
C	1st	6	48	48	-	-	-	3-5 wks
	2nd	6	48	48	-	-	-	1-4 wks
	3rd	6	48	48	-	-	-	3-5 wks
	4th	6	48	48	-	-	-	3-4 wks
	5th	6	48	48	-	-	-	4-7 wks
	Total	30	240	240	-	-	-	
D	1st	6	48	36	-	4	8	6-8 wks
	2nd	6	48	37	1	7	3	2-5 wks
	3rd	6	48	33	-	4	11	4-6 wks
	4th	6	48	45	-	3	-	4-5 wks
	5th	6	48	47	-	1	-	3-6 wks
	Total	30	240	198	1	19	22	

*Group III not run on BT 2-5 because of excessive casework

temperature for 2-1/2 months. After this time, the case was examined and the following results obtained:

Sample	Age of Sample	GLO I	EsD	PGM	ADA	EAP	AK	Gc	Hp
Stain from floor	12 wks	-	-	2	1	B	1	1	2-1
Stain on knife	12 wks	-	-	2	1	B	1	1	2-1
Victim's blood	12 wks	incon-clusive	1	2	1	B	1	1	2-1
Suspect's blood	12 wks	1	1	1	1	BA	1	2-1	2-1

These results confirm what was observed during the optimization phase of the project in that GLO I and EsD are the least persistent in stains and that it is possible to type bloodstains much older than 4 weeks.

5.2 Laboratory 2

A case of auto theft occurred where the perpetrator bled in the automobile. A suspect was apprehended and a sample of his blood, his bloodstained clothing, and the bloodstain from the truck submitted to the laboratory. The following results were obtained.

Sample	Age of Sample	GLO I	EsD	PGM	ADA	EAP	AK	Gc	Hp
Suspect's blood	Unknown	2	1	2-1	2-1	B	1	2	2
Blue Jeans	Unknown	2	1	2-1	2-1	B	1	2	2
Stain from truck	Unknown	2	1	2-1	2-1	B	1	2	2

It can be seen that the bloodstains match the suspect's blood. The frequency of occurrence in a general population of this combination of blood groups is 1 person in 14,000 (0.0069%). This laboratory, prior to the training course, was phenotyping bloodstains for EsD, PGM, and EAP. If only these genetic markers had been examined in this case, the frequency of occurrence would

have been 1 person in 10 (10.36%). Obviously the new methodologies are able to provide a substantial increase in the evidential value of the serological results.

5.3 Laboratory 3

A man was disturbed burglarizing a trailer home by the owner and his son. A fight ensued whereby all three people were injured. A suspect was arrested, and his clothing plus bloodstains from the scene were submitted for analysis. The following results were obtained.

Sample	Age of Sample	GLO I	EsD	PGM	ADA	EAP	AK
Owner's blood	Unknown	2-1	1	2-1	1	B	1
Son's blood	Unknown	1	1	2-1	1	B	1
Suspect's blood	Unknown	2-1	1	2	2-1	B	1
Stain from porch	Unknown	2-1	1	2-1	1	B	1
Stain from inside trailer #1	Unknown	1	1	2-1	1	B	1
Stain from inside trailer #2	Unknown	2-1	1	2	2-1	B	1
Stain from back steps	Unknown	2-1	1	2	2-1	B	1
Stain from suspect's clothing	Unknown	2-1	1	2	2-1	B	1

This laboratory, prior to the training course, was grouping bloodstains only for the ABO and the PGM system. In this case, all three persons were the same ABO group (type A) and the owner and his son could not be differentiated in the PGM system. It can be seen that bloodstains from three different people are present at the scene and that they match the three people involved. The blood grouping results helped to substantiate the victims' stories and disprove the story of the suspect.

5.4 Laboratory 4

This case concerns the assault, battery, and rape of a woman. Two suspects were arrested, both of whom had a very small bloodstain on their own pants. The blood samples from the victim and the two suspects were analyzed for Groups I, II, and III. There was only sufficient bloodstain for one test, so only one group could be used. The best differentiation was in Group II as follows:

Sample	Sample	ADA	EAP	AK
Victim's blood	6 weeks	1	B	2-1
#1 Suspect's blood	6 weeks	1	B	1
#2 Suspect's blood	6 weeks	1	BA	2-1
Pants from suspect #1	6 weeks	1	B	2-1
Pants from suspect #2	6 weeks	1	BA	2-1

It can be seen that the bloodstain on the pants from suspect #1 matches the victim, while the stain from the pants of suspect #2 is similar to his own blood. Prior to the training course, this laboratory was not phenotyping for AK or ADA, and therefore it would not have been possible to differentiate in all three genetic markers on such a small bloodstain.

6.0 Conclusions

The results obtained in these case examples show conclusively that these new methodologies, having phenotyped old and very small bloodstains, have the capability of differentiation, individualization, and optimization of serological evidence.

APPENDIX C

DESCRIPTION OF METHODOLOGY

METHODOLOGY (Sheet 1A)

SYSTEM: Group I--GLO I/EsD/PGM

TANK BUFFER:	12.11 g Trizma Base (0.1 M) 11.62 g Maleic acid (0.1 M) 2.92 g EDTA free acid (0.01 M) 2.03 g $MgCl_2 \cdot 6H_2O$ (0.01 M) 6.0 g NaOH (0.15 M)	1 liter distilled H_2O
pH 7.4		
GEL BUFFER:	1:15 dilution of tank buffer	30-ml tank buffer plus 420 ml distilled H_2O
pH 7.4		
TYPE STRENGTH AND VOLUME:	1 mm thin gel, 1% Agarose, Sigma Type I, 2% Hydrolyzed Starch	
	20 cm x 20 cm 50 ml buffer 0.5 g agarose 1.0 g starch	
	20 cm x 15 cm 40 ml buffer 0.4 g agarose 0.8 g starch	
	20 cm x 10 cm 20 ml buffer 0.2 g agarose 0.4 g starch	

APPLICATION:

Lysate	Stain
2 drops neat lysate 1 drop 0.05 M Cleland's Reagent (Dithiothretol) Allow to mix. One cotton thread insert, size 8	Soak with Cleland's Reagent (0.05 M) (7.7 mg/ml) for 10 min
	Origin
	3 cm from cathode

CONTROLS:	GLO I 2-1
	EsD 2-1
	PGM 2-1
TEMPERATURE AND CONDITIONS:	On cooling plate at 5°C with tank buffer at room temperature
VOLTAGE PLUS TIME:	400 V (20 V/cm) 2.5 hours
	300 V (15 V/cm) 3.5 hours

METHODOLOGY (Sheet 1B)

REACTION BUFFERS:

EsD	0.41 g Sodium acetate anhyd (0.05 M) Adjust to pH 6.5 with 1% acetic acid	100 ml H_2O pH 6.5
PGM	1.2 g Trizma Base (0.1 M) 0.40 g $MgCl_2 \cdot 6H_2O$ Adjust to pH 8.0 with 1:1 HCl	100 ml H_2O pH 8.0
GLO I	2.42 g NaH_2PO_4, anhyd (0.2 M) 1.31 g Na_2HPO_4, anhyd (0.11 M)	100 ml H_2O pH 6.2

Iodine solution: 1.65 g KI, 2.54 I_2, 30 ml H_2O — Dissolve in warm H_2O. Stir for 5 min and filter. Store in stoppered dark bottle at 4°C

REACTION MIXTURES:

EsD: 4 mg MU acetate dissolved in a small amount of acetone (approximately 300 µl) and made up to 10 ml with reaction buffer. Soak onto Whatman 3MM paper 10 cm wide

(for 20 x 15 cm plate)

PGM:
- 10 g 2% agar at 55°C
- 35 mg Glucose-1-phosphate + 1% G-1-6 P_2
- 2.0 mg NADP, sodium salt
- 5 µl G6PD (1.7 iu/plate)
- 2.5 mg MTT
- 2.5 mg PMS
- 10 ml PGM reaction buffer

GLO I
a.
- 12 mg reduced glutathione
- 50 µl 40% Methyl glyoxal
- 10 ml GLO I reaction buffer
- Soak onto Whatman 3MM paper

b.
- 0.5 g Agarose Sigma Type I
- 30 ml H_2O
- 0.2 ml Iodine solution

NOTES:

1. Place Whatman paper from origin forward. Leave at room temperature for 5 minutes. Read EsD by using UV light. Mark position of EsD 2 band. Remove Whatman paper, blot dry. Divide gel at EsD 2 band position with plastic strip.
2. Cover anodic portion of gel with GLO I mixture (a).
3. Cover remaining portion of gel to the origin with PGM agar overlay. Incubate at 37°C for 30-40 min. Remove GLO I overlay, blot gel, add mixture (b). Incubate at 37°C for further 1/2 hour if necessary.
4. For 20 x 20 cm plate, multiply the amounts in PGM reaction mixture by 1.5.

METHODOLOGY (Sheet 2A)

SYSTEM: Group II ADA/EAP/AK

TANK BUFFER: pH 5.5	41.2 g Na_2HPO_4 anhy (0.29 M) 19.21 g Citric acid anhy (0.1 M)	1 liter distilled H_2O
GEL BUFFER: pH 5.5	0.405 g Na_2HPO_4 (0.0057 M) 0.24 g Citric acid (0.0025 M)	500 ml H_2O

TYPE STRENGTH AND VOLUME OF GEL: 1 mm thin gel, 10% (W/V) Hydrolyzed Starch

20 cm x 20 cm	50 ml buffer	5 g starch
20 cm x 15 cm	40 ml buffer	4 g starch
20 cm x 10 cm	20 ml buffer	2 g starch

APPLICATION:

Lysate
- 2 drops neat lysate
- 1 drop mercaptoethanol diluted 1:250 with H_2O
- Allow to mix. One cotton thread insert, size 8

Stain
- Soak with 1:500 dilution of mercaptoethanol for 10 minutes

Origin
- 6 cm from cathode

CONTROLS: ADA 2-1 EAP BA & CB AK 2-1

TEMPERATURE AND CONDITIONS: On cooling plate at 5°C with tank buffer at room temperature

VOLTAGE PLUS TIME: 150 V (7.5 V/cm) overnight 16 hours

REACTION BUFFER:

EAP	0.96 g Citric acid anhyd (0.05 M) 0.4 g NaOH (0.1 M)	100 ml H_2O	pH 5.0
AK	1.20 g Trizma Base (0.1 M) 0.4 g $MgCl_2 \cdot 6H_2O$ (0.02 M) Adjust to pH 8.0 with 1:1 HCl	100 ml H_2O	pH 8.0
ADA	0.355 g Na_2HPO_4 anhyd (0.025 M) 0.19 g NaH_2PO_4 anhyd (0.016 M)	100 ml H_2O	pH 7.0

REACTION MIXTURES:

(for 20x15 cm plate)

EAP
4 mg MUP in 10 ml reaction buffer
(MUP - 4 methylumbelliferyl phosphate)
Soak on Whatman 3MM paper, 14 cm wide

ADA
10 g 2% agar at 55°C
10 mg Adenosine
25 µl Xanthine oxidase (0.10 iu/plate)
25 µl Nucleoside phosphorylase (0.5 iu/plate)
2 mg PMS
4 mg MTT
10 ml ADA reaction buffer

AK
10 g 2% agar at 55°C
20 mg Glucose
5 mg Adenosine diphosphate
3 mg NADP sodium salt
2.5 mg PMS
2.5 mg MTT
10 µl G6PD (3.4 iu/plate)
10 µl Hexokinase (2.8 iu/plate)
10 ml AK reaction buffer

NOTES:

1. Place Whatman paper between cathode and 13 cm towards anode
2. Cover remaining anodic portion of gel with ADA reaction mixture
3. Incubate at 37°C for 1/2 to 3/4 hour. Cover ADA reaction mixture with black paper. Read EAP and photograph under UV light
4. Cover area 1 cm anodic of origin to cathode with AK reaction mixture
5. Incubate at 37°C for 1/2 hour
6. For 20 x 20 cm plate, multiply the amounts in reaction mixtures by 1.5

SYSTEM: Group III Gc/Hp

TANK BUFFER: 21.8 g Glycine (0.29 M), 4.5 g Trizma Base (0.037 M), 1 liter distilled H_2O
pH 8.4

GEL BUFFER: As for tank buffer:
pH 8.4

TYPE STRENGTH AND VOLUME OF GEL:

Hp — 1 mm thin gel 6.5% Acrylamide — 20 cm x 10 cm
1.62 g Cyanogum 41
2 mg Potassium ferricyanide
25 mg Ammonium persulphate
100 µl Dimethyl aminopropionitrile
25 ml Gel buffer

Gc — 1% Agarose Sigma Type I — 20 cm x 10 cm
0.4 g Agarose
40 ml Gel buffer

APPLICATION:

Serum

Diluted 1:2 with H_2O. Add hemoglobin if necessary. Whatman 1 paper insert.

Stain

Cloth approximately 3 x 3 mm + 30 µl distilled water, extract 1/2 hour. Add approximately 0.4 ml chloroform, vortex, and spin for 5 minutes at approximately 9000 g

Pipet into slot 0.5 x 0.15 cm

Origin

3 cm from cathode

CONTROLS: On cooling plate at 5°C with tank buffer at room temperature

VOLTAGE PLUS TIME:

Hp 400 V, 15 V/cm, 4 hours Final Hb position 6 cm

Gc 300 V, 15 V/cm, 2 hours Final Hb position 4 cm

REACTION MIXTURE:

Hp 0.20 g O-tolidine
4 ml Ethanol
6 ml Glacial acetic acid
10 ml 0.6% H_2O_2

Gc 400 µl of diluted anti-Gc antiserum (diluted 1:4 w/H_2O). Soaked on 9 x 4.5 cm. CAM

0.1% Coomassie Blue R250 in methanol/acetic acid/H_2O (50/10/50)

NOTES: Hp

1. Stain Hp with reaction mixture from origin to Hb band
2. Gc can be stained on the acrylamide
   a. CAM soaked in 400 µl diluted antiserum is overlaid from 1 cm ahead of Hb band and incubated overnight at room temperature in a moisture box
   b. The gel is washed for 5 minutes in distilled H_2O, stained for 5 seconds in Coomassie Blue, and de-stained in methanol/acetic acid/H_2O (50/75/875)

Gc

1. Overlay agarose in an area from 1.5 cm in front of Hb band anodicalloy with Gc antiserum on CAM. Incubate in moisture cabinet at room temperature for 2 hours
2. Press for 30 minutes
3. Wash plate overnight in 1 M saline solution
4. Rinse in distilled H_2O for 5 minutes
5. Press for 30 minutes
6. Dry at 65°C
7. Stain Gc in Coosmassie Blue R250
8. Destain in methanol/acetic/H_2O (50/10/50)

General

The following notes apply to all three groups:

- Buffers. All chemicals used in buffer making should be of very pure grade. The weights given in the methodology sheets should always be checked against the molarities which are also given. The pH of any buffer should be carefully checked before use. All buffers are best stored in the refrigerator. Approximately 800 ml of buffer are required to fill a tank to the upper line. The tank buffer should only be used for five electrophoretic separations in one week and then discarded. The reaction buffers are best made only in small quantities and care should be taken to clean the containers before re-use.
- Bridges. Sponge bridges are used although some manufacturers do employ softeners and fungicides which can cause problems during electrophoresis. Therefore all bridges should be carefully washed in distilled water before use. If, however, problems continue, then three or four thicknesses of Whatman 3MM can be used. All bridges should be cut to the exact width of the electrophoretic plate and held in contact with the gel by means of a second glass plate which is placed on top.
- Reducing Agents. All reducing agents should be prepared fresh weekly and stored while not in use in a refrigerator. Hemolysates are prepared by washing whole red cells three times with saline and lysed. They are best stored in the presence of reducing agents.
- Electrophoresis. The meters on some power supplies are not very accurate so the voltage between the bridges are best checked by using a voltmeter. The times given for electrophoresis should be strictly adhered to or else there will be

overlap of one enzyme with another or loss of the enzyme into the anodic tank.

- Staining. When using paper overlays, care should be taken to avoid excess reaction mixture which will cause diffusion of the enzyme bands. When using agar overlays, the 2% agar is first boiled and the constituents are mixed together with the reaction buffer. The agar is cooled in running water to approximately 55°C, mixed with the reaction mixture, and immediately poured over the surface of the gel.

- Sample Application. For hemolysates, a cotton thread is soaked in the lysate and carefully laid in the gel slot. If the lysate has been over-diluted, then a second thread should be applied on top of the first.

For bloodstains on solid surfaces, either swab the area with a buffer-moistened cotton thread containing the appropriate reducing agent, or if possible take a small flake and add the minimum amount of reducing agent and when dissolved, soak onto a cotton thread. For stains on cloth, either pull 1-cm-long threads or cut thin 1-mm-wide slivers no longer than 1 cm. Always endeavor to keep the application line narrow, and do not allow the stain to protrude above the surface of the gel. Do not make the applications smaller than 1 cm--this will lead to diffuse bands instead of sharp ones. If the stains are less than 1-cm long, build up the stain at the application point. Do not use excessive amounts of buffer for moistening the stain.

Group I

The gel is prepared by weighing the agarose and hydrolyzed starch and boiling until all the agarose has dissolved. The gel is poured into

the gel mold, which should be on a level surface, and immediately leveled using the plastic scraper so as to produce an even 1-mm-thick gel.

One-mm-thick strips of stain, 1 cm long, are removed and soaked in a minimum of reducing agent for ten minutes. Slots are cut in the gel using the metal slot makers. After electrophoresis, Esterase D is developed first. Care should be taken not to use too much acetone to dissolve the methyl umbelliferyl acetate; this will produce a large amount of background fluorescence. After leaving the plate at room temperature for five minutes, the Esterase D banding can be read under UV light. The position of the Esterase D 2 band should be marked as this will discern the dividing line between the GLO I and the PGM reaction mixtures.

The iodine solution is prepared by dissolving the potassium iodide and the iodine into 30 ml of warm distilled water (65°C). The solution is allowed to stir for 5 minutes and then filtered and stored at 4°C in a dark amber stoppered bottle. To visualize the Glyoxalase I bands, the iodine solution should be added to the agarose solution when it is hot and immediately poured onto the surface of the gel. (The gel should be placed on a level cooling plate to ensure fast gelling of the agarose.)

Group II

The gel is prepared by adding hydrolized starch to the gel buffer, bringing to the boil by heating in a 250-ml Erlynmeyer flask. (Note: the flask should be kept moving in the flame to avoid burning of the gel.) When boiling, the gel is degassed using a vacuum pump drawing approximately 15 psi pressure and then poured into the gel mold. The starch solution is immediately leveled to a 1-mm gel by using the gel scraper. Care should be taken to ensure that the mold is on a level surface before pouring.

Stains are prepared by cutting 1-mm-thick strips, 1 cm long from the stain and soaking with the minimum amount of reducing agent. Application slots are cut into the gel using the metal slot makers. The application of stains or lysates to the gel should be kept as narrow as possible so as to produce sharp isozyme banding.

After electrophoresis it will be noted that the cathodic portion of the gel is covered with condensation droplets. These should be removed by blotting with Whatman 3MM paper. EAP is developed in an area from the cathodic bridge to 7-cm anodic of the origin. ADA is developed anodic to the EAP area. Care should be taken not to expose the ADA reaction mixture to strong visable or UV light. After the EAP results have been recorded, the cathodic half of the gel should again be blotted before covering the area with AK reaction mixture.

Group III

The important point to note here is that the acrylamide and agarose gels can be run in the same tank at the same time. For screening of serum, both proteins can be phenotyped using the acrylamide gel. However, the degradation product that is found in varying amounts in stains unfortunately migrates amongst the Gc bands and can lead to misinterpretation. Therefore, all stains must be phenotyped for Gc using the agarose gel.

- Acrylamide. The acrylamide gel is prepared by dissolving all the constituents in the buffer, degassing and pouring into the gel mold. A clean glass plate is carefully overlaid, excluding all the air and the gel allowed to polymerize for 30 to 45 minutes. Several gels may be made at the same time and then stored in the refrigerator. (This will facilitate top plate removal.) When the gel is ready for use, the top plate is removed by carefully inserting a razor blade between the two plates and gently levering the upper plate from the gel. Occasionally the gel will stick to the top plate; this is still usable as long as the gel is in one piece.

- Agarose. This is prepared by dissolving the agarose in gel buffer by boiling and pouring the hot solution onto a plain glass plate on a level surface and allowing to solidify.

- Extraction. The amount of stain required to obtain results will be decided by experience, but the size given (i.e., 3 x 3 mm) is recommended when using cotton cloth as the substrate. The amount of buffer required again will be decided by experience, but each gel uses approximately 10 µl of extract for each application. It is important, however, to obtain very clear extracts or background smearing on the gels will result. Longer centrifugation can be used if necessary.

- Application. The two-sided metal slot makers are used for making the application slots. For serum samples the anodic slit only is used. For stains, a 1.5-mm-wide plug of gel is removed and the stain extract carefully pipetted into the well. It is preferable to start both gels at the same time, removing the agarose after the hemoglobin has migrated 4 cm (~2 hours), and allowing the acrylamide to continue for a further two hours (Hb 6 cm).

- Immunofixation-Agarose. The cellulose acetate membrane overlay containing the diluted antiserum should not be too dry and is placed on the gel approximately 5.5 cm from the origin and covering the whole width of the agarose. It is allowed to incubate for two hours at room temperature in a moist atmosphere.

- Immunofixation-Acrylamide. After electrophoresis, the gel is divided just ahead of the hemoglobin band (which has moved approximately 6 cm) and the cathodic portion of the gel removed into a staining dish. If required, a cellulose acetate membrane soaked in diluted antiserum is overlaid on the remaining portion of the gel at the dividing line. This

is allowed to incubate at room temperature for two hours (or overnight if this is more convenient).

Staining. The haptoglobin is stained immediately after electrophoresis using the orthotolidine mixture. If the results are too weak, the percentage of peroxide should be increased. If, however, there is bubbling within the gel, the peroxide content should be reduced.

The agarose is reduced to a very thin film by overlaying the gel with wet Whatman 1 paper and several thicknesses of blotting paper and applying approximately 5 kilograms weight for 30 minutes. The gel is then washed overnight in a 1-molar saline solution, rinsed in distilled H_2O, repressed for 30 minutes, and dried in an oven at approximately 65°C for 15 minutes. The Gc bands can then be visualized by staining in Coomassie Blue for five to ten minutes and destained in the higher methanol destain solution.

If immunofixation has been carried out on the acrylamide, no pressing and very little washing is required. The bands are visualized by immersing in Coomassie Blue stain for 5 seconds. (Any longer and the acrylamide gel will take up the stain which is very difficult to remove.) Destaining is carried out using the lower methanol destain solution.

EQUIPMENT, SUPPLIES, AND CHEMICALS RECOMMENDED FOR MULTISYSTEM ANALYSIS

EQUIPMENT

Item Description	Model Type	Manufacturer
Cooling Bath and Circulator with Heater		BI
Fridgmix	1495	
Thermomix	1441	
Neslab	RTE 4	N
Power Supply, ΔC, 0-500 V Variable, 0-100 mA	R120	B
Cooling Plates, aluminum, 20 x 25 cm		SH
Multizone Electrophoresis Cells		SH/B
Volt/Ohm Meter (0-500 volts)	22-201	RS
Centrifuge (capable of 9000 g)	Microfuge-B	B
Variable Pipetter (disposable tips)	10 µl 20 µl 25 µl	E
Pipetter	5 µl	E

MATERIALS

Item Description	Model Type	Supplier
Sponge Bridges		AS
Glass Plates, 20 x 20 cm with 1 mm x 5 mm glass or plastic strips		
Glass Plates, 20 x 15 cm with above		
Glass Plates, 20 x 10 cm with above		
Glass Plates without strips		LS
20 x 20 cm 20 x 15 cm 20 x 10 cm		
Slot Makers		B/SH
Development Strips, 19 x 0.5 x 0.3 cm		
Development Strips, 14 x 0.5 x 0.3 cm		LS
Development Strips, 9 x 0.5 x 0.3 cm		
Tygon Tubing, 3/8 ID x 1/8 Wall	R3603	N
Mylar Plastic Sheets, 25 x 60 x 0.1 cm		C
Cellulose Acetate Strips, SM 40 x 360 mm	Sartorius	
Centrifuge Tube, 400 µl	19926	BA

CHEMICALS

Item Description	Order Code	Supplier
Magnesium Chloride, 6 H_2O	M0250	S
Maleic Acid	M0375	S
Trizma Base	T1503	S
Glycine, Ammonia Free	G7126	S
Citric Acid, Anhydrous	C0759	S
Sodium Phosphate, Monobasic, Anhydrous	S0751	S
Sodium Phosphate, Dibasic, Anhydrous	S0876	S
Starch, Hydrolyzed, Powder (Connaught)	S676	F
Agarose, Type 1	A6013	S
Agar, Special, Noble	0142-01	V
O-Tolidine, practical grade	T3501	S
Cyanogum 41, Gelling Agent	C588	F
Dextrose (α-D(+) Glucose) Grade III	G5000	S
Potassium Ferricyanide, Grade I	P8131	S
Ammonium Persulfate (ACS Standard)		LS
Brilliant Blue R (Coomassie Blue R250)	B0630	S
2-Mercaptoethanol, Type 1	M6250	S
3-Dimethylaminopropionitrile	D1008	S
DL - Dithiothreitol (Cleland's Rgt)	D0632	S
4 - Methylumbelliferyl Acetate	M2881	S
Glucose-1-phosphate (with 1% Gl, $6P_2$)	G1259	S
MTT Tetrazolium	M2128	S
Phenazine Methosulfate	P9625	S

CHEMICALS (Cont'd)

Item Description	Order Code	Supplier
NADP, Sodium Salt	N0505	S
Glutathione, Reduced	G4251	S
Methyl Glyoxal, 40%	M0252	S
4-Methylumbelliferyl Phosphate	M8883	S
Adenosine-5-diphosphate, Grade I	A0127	S
Adenosine	102-075 A9251	B-M S
Nucleoside Phosphorylase	107-956	B-M
Xanthine Oxidase	110-434	B-M
Glucose-6-Phosphate Dehydrogenas, Grade I	127-035	B-M
Hexokinase	127-175	B-M
Anti-Human Gc Globulin	Gc-P003-S	AA

SUPPLIER CODES

AA	Atlantic Antibodies 52 Anderson Road Westbrook, Maine 04092	(207) 856-6392
AS	American Sponge & Chamois Company Los Angeles, California	(213) 639-9900
B	Beckman Instruments, Inc. Advanced Technology Operations 1630 S. State College Boulevard Anaheim, California 92806	(714) 634-4343
BA	Bel Art Products Pequannock, New Jersey 07440	
BI	B. Braun Instruments 805 Grandview Drive South San Francisco, California 94080	(415) 589-9217
BM	Boehringer-Mannheim Biochemicals 7724 East 89th Street P.O. Box 50816 Indianapolis, Indiana 46250	(800) 428-5433
E	Eppendorf Dist by Brinkmann Instruments, Inc. Westbury, New York 11590	(516) 334-7500
F	Fisher Scientific 2761 Walnut Avenue Tustin, California 92680	(714) 832-9800
LS	Local Supplier	
N	Neslab Instruments, Inc. 871 Islington Street Portsmouth, New Hampshire 03801	(603) 436-9444
RS	Radio Shack Nationwide Catalogs	
S	Sigma Chemical Company P.O. Box 14508 St. Louis, Missouri 63178	(800) 325-3010
SH	Shandon Southern Instruments, Inc. Sewickley, Pennsylvania 15143	(412) 741-8400
V	Van Waters Rodgers Scientific P.O. Box 3551, Rincon Annex San Francisco, California 94119	(800) 792-8030

END