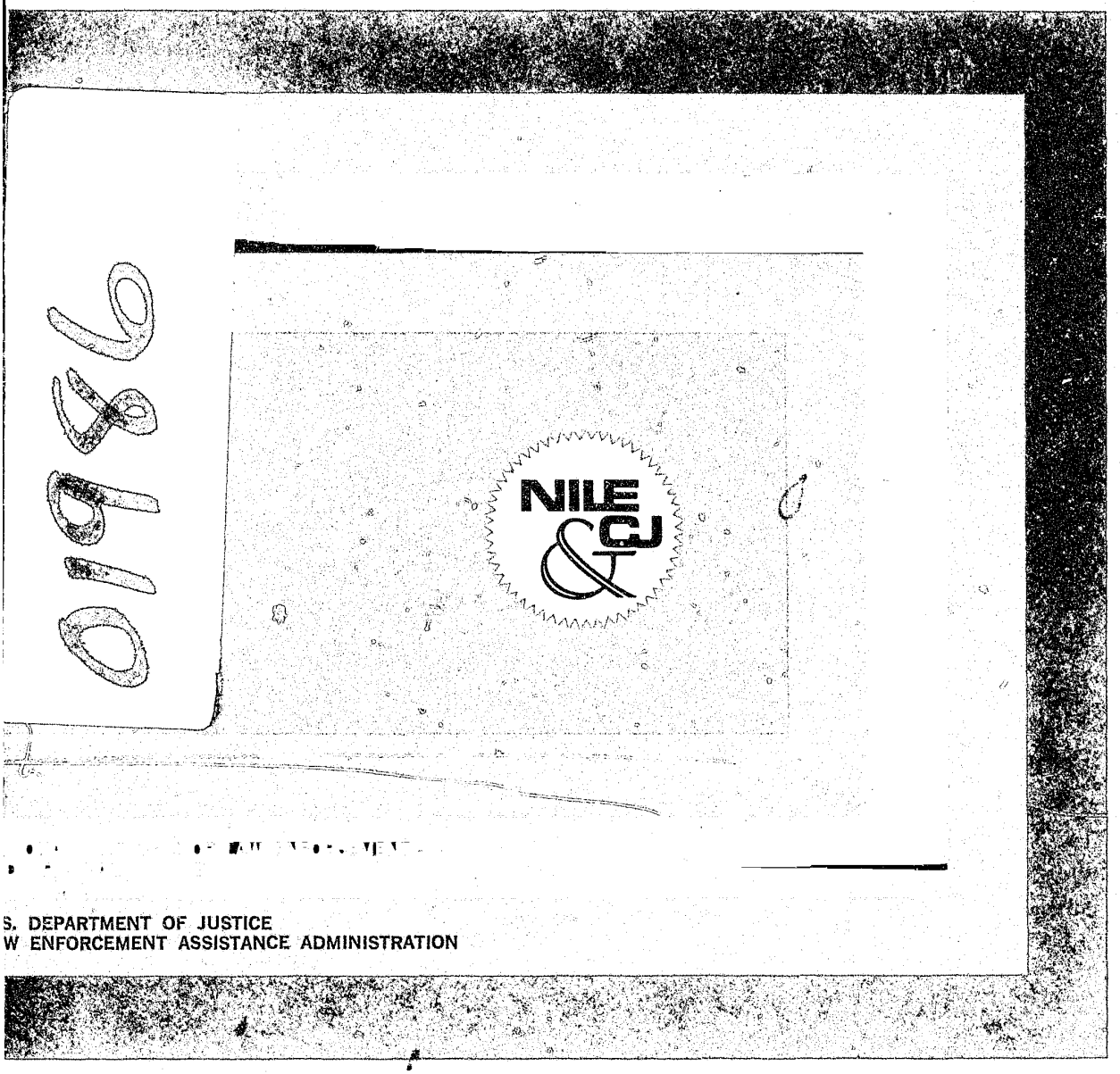
The Examination and Typing of Bloodstains in the Crime Laboratory



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The Examination and Typing of Bloodstains in the Crime Laboratory

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By

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PREFACE

This is not intended as a complete academic treatment of the subjects included, but as a useful and practical basis from which a forensic scientist may make a reasonable start. Work of collecting samples and the evaluations and distribution of blood either at the scene of the crime or on items submitted for laboratory examination have been deliberately excluded on the basis that this volume will cover only scientific techniques used in the laboratory. Sample collection and bloodstain distribution may be dealt with later in a different publication. This document brings together relevant published information and considerable work by the authors that has not been previously published.

The report has been organized into Section 1, preliminary techniques of examination and an introduction to electrophoretic techniques in general; Section 2, serological grouping techniques in the ABO, MN, and Rhesus systems; Section 3, the polymorphic enzyme systems; Section 4, the polymorphic protein systems; and Section 5, other group systems of all types that have been or are being investigated for use in the forensic science field. Appendix A contains information about equipment and materials that could be of value to the scientist performing the type of work discussed in this document.

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PRELIMINARY TECHNIQUES OF EXAMINATION

1.1 INTRODUCTION

Blood is frequently spilt at the scene of a serious crime—a murderer may get his victims's blood on his clothing or a thief may cut himself while entering a building and leave his own blood at the scene. If, therefore, a definitive identification of this blood is possible to connect it with a particular individual, then it can prove very good evidence in court.

It is not yet possible to individualize blood in the same way as one can a fingerprint, but this is because of a lack of knowledge of techniques and not because of the nature of blood. At present it is possible only to work in terms of genetically controlled polymorphisms that occur in the blood, and to calculate from population data the frequency of occurrence of the particular combinations of groups obtained.

For example, using British population data, suppose each of the following groups was found:

Group	Frequency of the Particular Group (%)
O	47
MN	50
R_1r	34
PGM 1	58
AK 1	93
ADA 1	89
PGD^A	94
$G6PD^B$	98
PCE U	96
C_5^-	90
HP 2-1	36
Hb A	98

The combined frequency of this combination of groups is 1.034 percent of the population. In this instance the commonest group within each system has been selected.

A shorter list of groups includes the following:

Group	Frequency (%)
B	8.6
[illegible]	22.0
[illegible]$_O$	2.0
PGM 2	7.0
AK 2-1	7.0
ADA 2-1	11.0

The combined frequency of this combination is 0.00002 percent or 1 in 50,000 of the population. These are not the rarest groups in each system but some that can easily occur. This frequency of occurrence is normally given as evidence to the court in order to help assess the value of the evidence of the blood group type.

Only ten years ago, good reliable techniques were available only for the ABO system grouping of bloodstains. Frequently all those involved in a case were of the same ABO group; therefore, the blood grouping evidence had little value.

The difficulty of having sufficient bloodstain for the macro-methods was also common. Today with micro-methods for most group systems the problem that arises more frequently is which of the group systems available should be used. Ideally perhaps all groups should be done on all stains but for a variety of reasons this is often impossible, not to say uneconomic.

The type of method to be used is a problem that must be decided in each individual case and with reference to the laboratory doing the necessary work on that case. For example there may be only enough bloodstain to attempt grouping in two systems; if the groups of the victim and the suspect are known from their blood samples the choice should be those systems which (1) distinguish one from the other and (2) reduce the population percentage from which the blood could have come.

It is highly desirable, and this cannot be overstressed, that blood samples should be obtained from all those who are or may be involved in the particular case. These can be rapidly processed through all the grouping systems as a routine and the results used in the manner indicated above to make the optimum selection of the work to be done on the bloodstains.

By obtaining blood samples from a large number of possible suspects in a case where the criminal has left his own blood at the scene of the crime, it is possible to save a great deal of the police time by eliminating all those who could not have shed blood. This is an aspect of the work which, in England, is increasing, and the saving in interrogation and checking time can be very great.

Accurate and detailed records of all the work done are absolutely essential, for errors are much more likely to occur in a simple clerical transposition than in the performance or interpretation of the experimental work. These records also build a local knowledge of population frequencies that may be of value in the future.

Methods and some background have been given to each of the blood group systems dealt with in later paragraphs, since it is usually not a matter of following a cookbook and reading answers. Experience and an understanding of the background and the particular material being investigated are essential if the best results are going to be obtained and presented in court.

Little is said here about court presentation because this will vary from place to place according to the local laws of evidence and will have to be worked out by those concerned.

As progress is made in this field, it is possible that convicted criminals will have a complete blood typing on record as well as photographs, fingerprints, and other data. In the future known criminals may be traced by the blood that they leave at the scenes of their activities.

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1.2 ELECTROPHORETIC TECHNIQUES

1.2.1 GENERAL

Since this document is intended to be a practical volume, the theoretical aspects of electrophoresis are largely omitted. Many treatises on this subject have been written and the reader is referred to these for such background.[1, 2, 3, 4] Only those factors that are readily modified in practice will be considered briefly.

1.2.2 BUFFERS

Buffers should always be made from high grade chemicals, and made accurately. Technical grade chemicals may contain impurities that will affect certain proteins, or adversely affect the activity of enzymes which it is desired to detect subsequently. An example from experience was the use of TRIS (tris-hydroxymethyl-methylamine) technical grade which was not earmarked for buffers. This gave extremely poor separation of serum proteins; this fault was immediately rectified by changing to TRIS produced specifically for buffers by BDH Ltd.

Inaccurately made buffers will affect the pH and the mobility of the material under investigation. Having weighed the necessary components they should be made up to the required volume with distilled water and the pH should ALWAYS be checked.

If a buffer is likely to be kept at room temperature for more than a few weeks it is advisable to include a bacteriastat, such as Thiomersal (merthiolate) at 1 part in 10,000.

A buffer, such as the histidine one for adenylate kinase, is particularly prone to bacterial growth which affects the quality of the separation achieved. The bacteriastat used must not alter the pH or the ionic strength of the buffer.

It will seem that innumerable different buffer systems are used for the various investigations, and attempts have been made to achieve a universal buffer system

applicable to all investigations. So far this has failed. The attempts that came nearest to success still did not produce results of such reliably good quality as those using the individual buffer systems.

It has been found that many of the buffers described later deteriorate and the practice is to keep all buffers refrigerated at 0 to 2° C. At the first suspicion of a poor separation the buffer is discarded and a new batch is made.

Buffers are normally made at room temperature (18 to 20 °C), regardless of the temperature they are going to be used at, and all the pH data given for buffers is at that temperature.

The ionic strength of the buffer is the remaining consideration that will be dealt with. If the ionic strength (μ) of the gel buffer is too high, a high current for a given voltage is produced that results in (1) the gel overheating and (2) poor separation of the components. Overheating can disrupt the gel, precipitate protein, cause loss of enzyme activity, and enhance diffusion.

The charges on the molecules under examination attract ions of the reverse charge from the electrolyte forming a double layer that tends to obscure the charge on the molecule. This layer can vary in thickness and this is dependent on the ionic strength (μ) of the electrolyte or buffer.

Thus, with an electrolyte of low μ, the layer is thick but diffuse, but with high μ, the layer is thin but dense and the charge on the molecule is rapidly screened giving no opportunity for an electric field to have its effect on the molecule.

With decreasing values of μ, the thickness of the double layer increases. At low μ, which is often used in electrophoresis, variation in μ can make a considerable difference.

A definition of μ (ionic strength) is shown in the following equation.

$$\mu = \frac{1}{2} \Sigma Mv^2$$

where:

M = Molar concentration
v = valency
Σ = sum

} of each ion or radical

Electrophoretic mobility, as a result of this double layer effect is inversely proportional to $\sqrt{}$ of μ.

$$\text{Mobility} = \frac{1}{\sqrt{\mu}}$$

The order of ionic strengths that are used is between 0.1 and 0.01. The relative mobilities of a protein due to changes in μ can be calculated, for example:

When

$$\mu = 0.01$$

$$\frac{1}{\sqrt{0.01}} = 10$$

When

$$\mu = 0.1$$

$$\frac{1}{\sqrt{0.1}} = 3.162$$

while at $\mu = 1$ Mobility = 1

From this it appears that the lower the μ of the buffer the better. There are, however, other factors involved, such as

a. Precipitation of proteins being run. Some proteins require higher ionic strength in order to remain in a soluble or mobile state.
b. Immuno precipitation requires higher ionic strength for the aggregation of antigen/antibody complexes into a visible precipitate.
c. The lower the ionic strength the thicker the double layer, greater distance of repulsion leading to a greater diffusion of the bands.

Thus it is clear that the choice of the ionic strength of the buffer is a compromise, and in spite of trying to calculate the effects of changing this it is best to try a series of ionic strengths of each buffer to see which, for laboratory conditions, works best.

Later, where detailed methods are given, it is recommended, for example in the 6-phosphogluconate dehydrogenase system, to dilute the tank buffer 1:15 for use in the gel. This is the dilution that works best at Forensic Science Laboratory. Other laboratories use 1:10 or 1:20 dilutions.

It is recommended that if the separations achieved are not as good as they should be, a slight change of the ionic strength of the gel buffer should be tried and the one most appropriate to your own conditions should be adopted.

1.2.3 ELECTRO-ENDOSMOSIS

Electro-endosmosis is a function of the particular substrate or medium being used and it will vary from batch to batch of the same medium. It will also vary with pH and ionic strength of the electrolyte being used.

In a given electrolyte the support medium, such as agar, will itself carry some charge on its molecules. Thus when the electric field is applied the agar will attempt to move towards the anode. This is patently impossible, so a movement of water in the reverse direction occurs. This flow of water is, at the slightly alkaline pH occurring in most gels, in the reverse direction to all the proteins and they have to battle their way against the tide. This slows down their apparent mobility and in fact gives gamma-globulin a mobility towards the cathode instead of its true mobility slightly towards the anode. But for this phenomenon the precipitin method described in paragraph 1.4 would not work.

It has, however, certain dangers in that if the supply of water and its intake into the gel at the anodic end is at all restricted, the gel will shrink at this end. Shrinkage will not only mechanically distort the gel but will increase the ionic strength in that area, increasing the conductivity and so on with all the consequent troubles this brings.

Normally electro-endosmosis is within tolerable limits and work is not only possible with it present but also it can be used as a specific tool. If electro-endosmosis is excessive, then the whole system must be readjusted.

1.2.4 HOMOGENEITY OF THE GEL

The electric field in an electrophoresis plate is determined by the voltage across its ends and the nature of the plate itself. This plate should be so constructed that this field is homogeneous throughout the plate.

In discontinuous buffer systems there will, of course, be a heterogeneity at the leading ion/trailing ion boundary. This sweeps through the gel and improves separation. The heterogeneity to avoid is any minor one that does not affect the whole gel but only part of it.

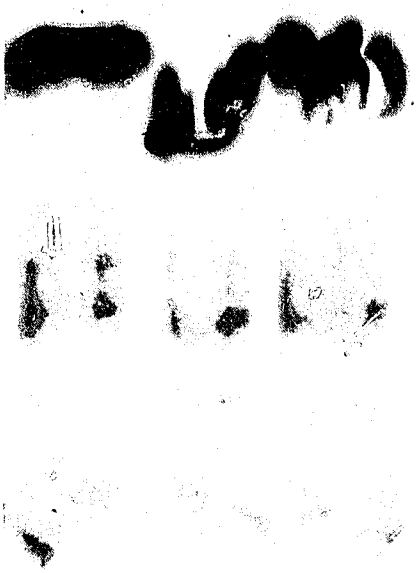
Discontinuities in conductivity seem to be the most common source of error. If a sample is inserted into the gel on filter paper and the sides of the gel slot do not close on the sample properly, then the results will be wrong. Use of a liquid or gel sample insert in a large volume can cause gross change of the ionic strength at this point and cause curvature effects on the sample running. The field appears higher or lower each side of the sample than through its center. If the concept of minimum alteration of the gel by sample insertion is followed, then the heterogeneities introduced will be minimal and will be held within tolerable limits.

Heterogeneity can also be introduced by a badly made gel, for example an incompletely melted agar prior to pouring, or a starch gel allowed to go lumpy. Figure 1-1 shows various faults that can occur.

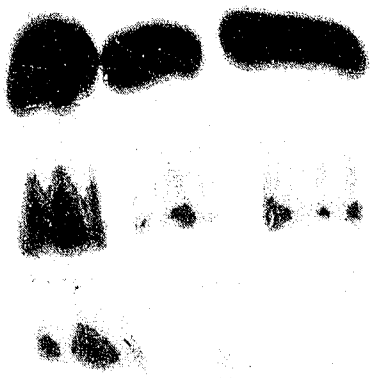
1.2.5 EQUIPMENT

1.2.5.1 General

There is a wide variety of very expensive electrophoresis equipment on the market today. This equipment is largely over-complex and certainly over-priced for the techniques presently used in forensic work and described in later sections.



Badly Made Non-Homogeneous Gel



Discontinuous Bridge Contact

High Ionic Strength of the Right Hand Sample

Discontinuity in the Gel on the Right Side

Figure 1-1. Sample Gel Faults

If accurate quantitation is required of the components of an electrophoretogram, then this type of equipment is necessary. In forensic work this quantitation is not required and simple but adaptable equipment has been found best.

1.2.5.2 Tanks

The most adaptable and useful tank on the market in England many years ago when this work was started was thought to be the Kohn tank made by Shandon Scientific Company priced at about £25. This tank was capable, with the aid of accessories, of dealing with paper, cellulose acetate, thin-layer silica gel or alumina, agar, immuno-electro-phoresis, and starch gel.

Two problems arose, (1) the laboratory's usage of tanks increased to an extent where £25 each meant a very large capital outlay, and (2) with the introduction of cooling plates below the gels, a tank designed for this purpose was needed; therefore, the Shandon tank was unsuitable. (This firm now makes tanks with suitable cooling plates that require only a small modification to the lid.)

The solution at the Forensic Science Laboratory was to design a tank that met the laboratory's requirements. The parts, in 3/16-inch thick sheet Perspex, were bought ready cut to size. All that was needed was to drill a few holes and assemble the parts. A completed tank cost less than £2.50. These tanks proved as good as any commercial tank that was tested.

For those with the same problems the parts required are listed below and the diagrams show the method of assembly.

The following pieces of 3/16-inch Perspex are required:

a. Base:
   - 1 at 10 1/2 inches by 10 inches.
   - 4 at 10 inches by 2 1/2 inches
   - 3 at 10 inches by 1 inch, 2 with 1/4-inch holes for buffer contact.

b. Lid:

- 1 at 10 1/2 inches by 11 inches.
- 4 at 10 1/2 inches by 1 inch.

Other parts also required include a handle for lid, two 1-foot lengths of thin stainless steel wire, two terminals, silicone rubber, two small pieces of Perspex to hold wire, and Perspex glue.

Figure 1-2 shows a completed tank. Note the silicone rubber seals around the ends of the center division to give improved insulation under conditions of high voltage and condensation within the tank.

Various tank accessories are necessary and these can be either made or bought. The tank described will accept the accessories made for the Shandon Kohn tank.

Most of the techniques described involve the use of starch or acrylamide gel.

Experience has shown that it is best to set up at least one tank for each type of examination or group system that is going to be used. Bearing these facts in mind, it is worth buying Shandon tanks with the appropriate accessories (or their equivalent) for precipitins, immuno-electrophoresis and for cellulose acetate membrane (CAM) electrophoresis, using the home-made tanks for everything else. While this is not essential, it saves a lot of fine detail additional work on the home-made tanks.

1.2.5.3 Accessories

1.2.5.3.1 Extended Shoulders

These hold paper or cellulose acetate strips in the tank and with the extended shoulders narrow strips of cellulose acetate membrane (CAM) can be used as described in the technique for detection of haemoglobin variants (paragraph 4.2). They are needed also for the precipitin technique where a 3-inch microscope slide, coated with agar, is inverted onto filter paper bridges supported by these extended shoulders.

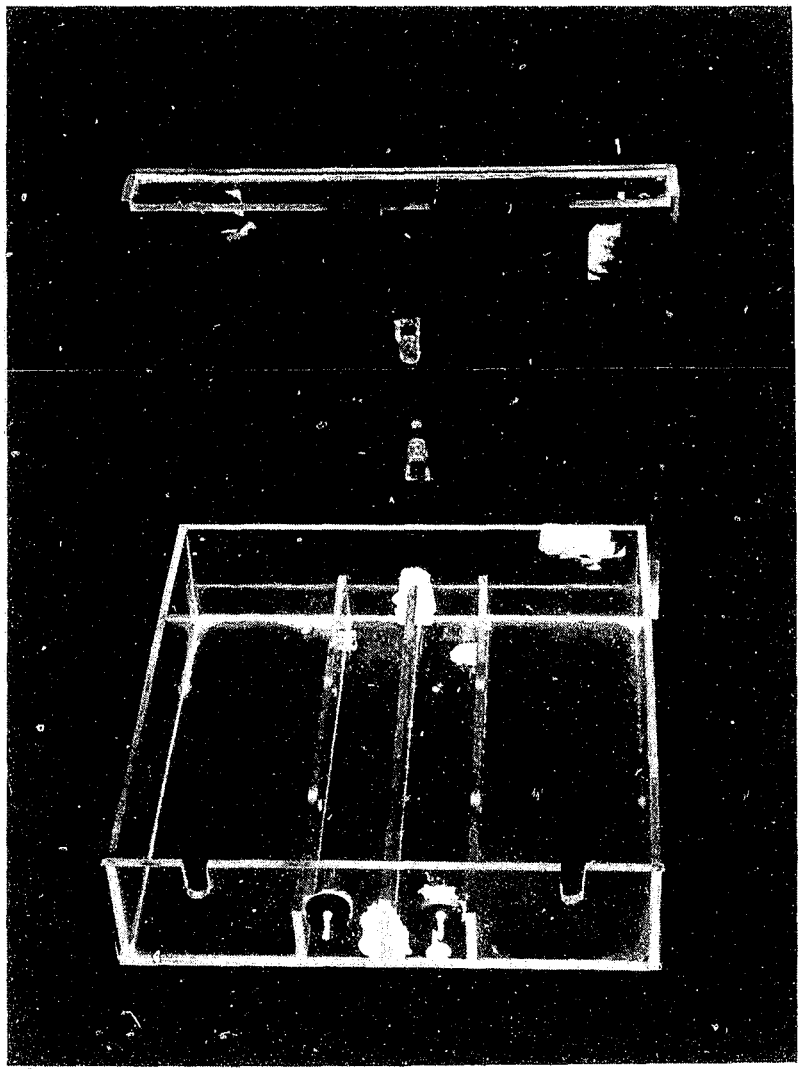


Figure 1-2. Completed Tank

1.2.5.3.2 Immuno-Electrophoresis Accessories

If immuno-electrophoresis is going to be undertaken, such as in Hp groups in bloodstains or Gc groups (see paragraph 4.1 and Appendix A) then a tank or accessories to a tank designed to simplify the procedures is desirable. These accessories include, as a minimum, slide holders for the agar coated microscope slides and a pattern cutter to mark the sample origins and the antiserum trough. Accurate repeatability is more difficult without such a pattern cutter.

If the use of the Laurell technique of two-way electrophoresis is contemplated then suitable tank supports and, if necessary, narrow cooling plates may be needed. (The new Shandon tank is well suited to this purpose. See Figure 1-3.)

1.2.6 TEMPERATURE

Control of temperature during a run is of great importance in many cases. Running at low temperature reduces diffusion and gives a greater clarity of separation than is obtainable at higher temperatures. With some of the enzyme systems, the maintenance of an even low temperature appears to be critical to good quality work.

The removal of heat as it is formed by the current in the plate reduces the effects of heterogeneities mentioned earlier. The most efficient temperature control has been found to be by means of metal surfaced cooling plates that are in the form of flat boxes through which water is pumped at the chosen temperature.

It is difficult to buy ready-made satisfactory cooling plates of the correct size so a detailed description of these is given. (See Figure 1-4.) The sizes given fit the tanks previously described and also fit the size of gel plates that were adopted as a standard.

Aluminum	8 1/2 inches by 6 1/2 inches by 1/4 inch
Perspex	8 1/2 inches by 6 1/2 inches by 3/4 inch
Rubber gasket	

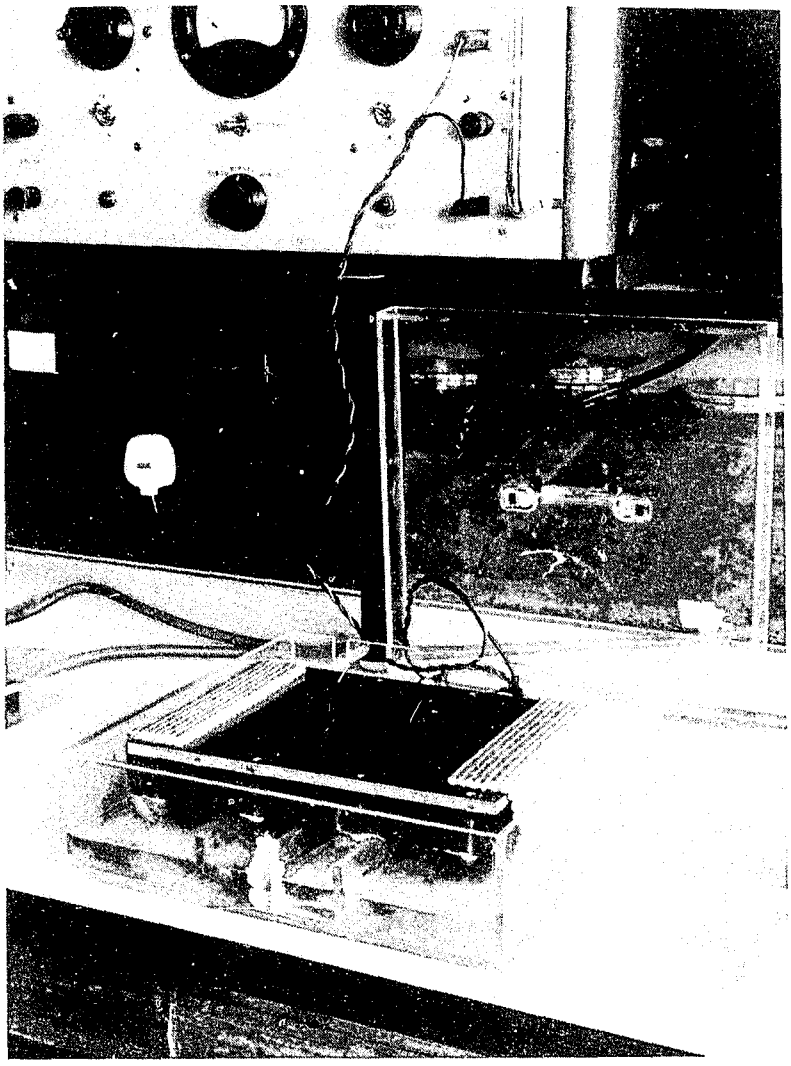


Figure 1-3. Tank with Cooling Plate

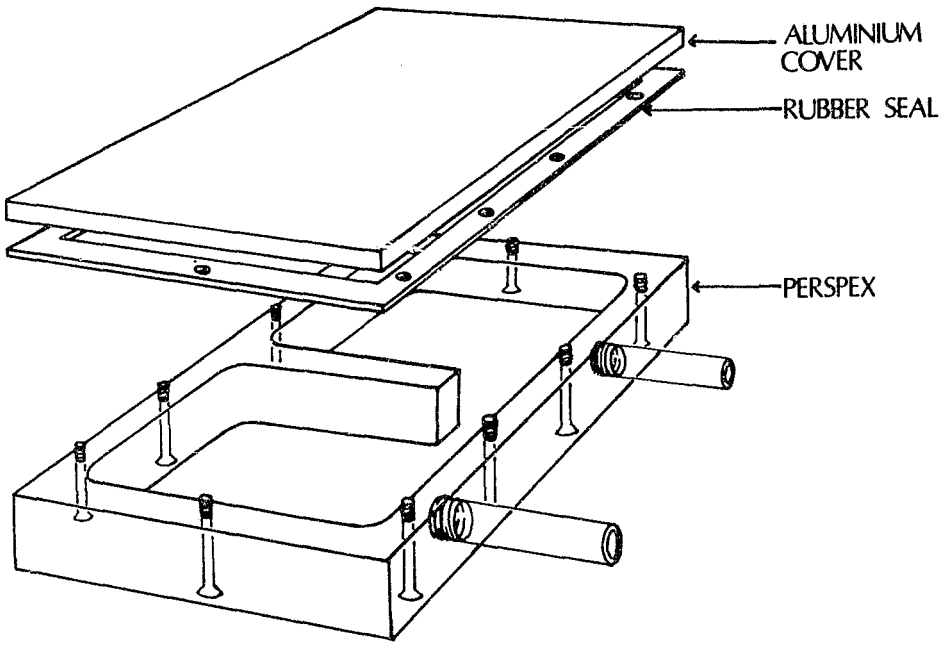


Figure 1-4. Cooling Plate Construction

1.2.7 POWER PACKS

It is difficult to define suitable power packs because this depends on the number of plates being run at a time. All that can be said is that if the voltage and milliamperes output provided is adequate for the user's particular laboratory, then it should be satisfactory. Reliability should be one of the fundamental considerations. The most reliable power packs used have been the Shandon Vokam units (0 to 300 volts, 0 to 80 milliamperes). There are, however, many other makes equally good. Like the supply of tanks in the Forensic Science Laboratory at least one power pack per system is considered to be the ideal situation.

Most power packs are unnecessarily complex and, therefore, unnecessarily highly priced for most of the work described in later sections. A simple power pack of fixed voltage can easily be constructed from spare radio or television parts (See Figures 1-5 and 1-6). The value and type of the components will vary with the power source voltage, the transformer used to achieve the required voltage, and the load on the pack.

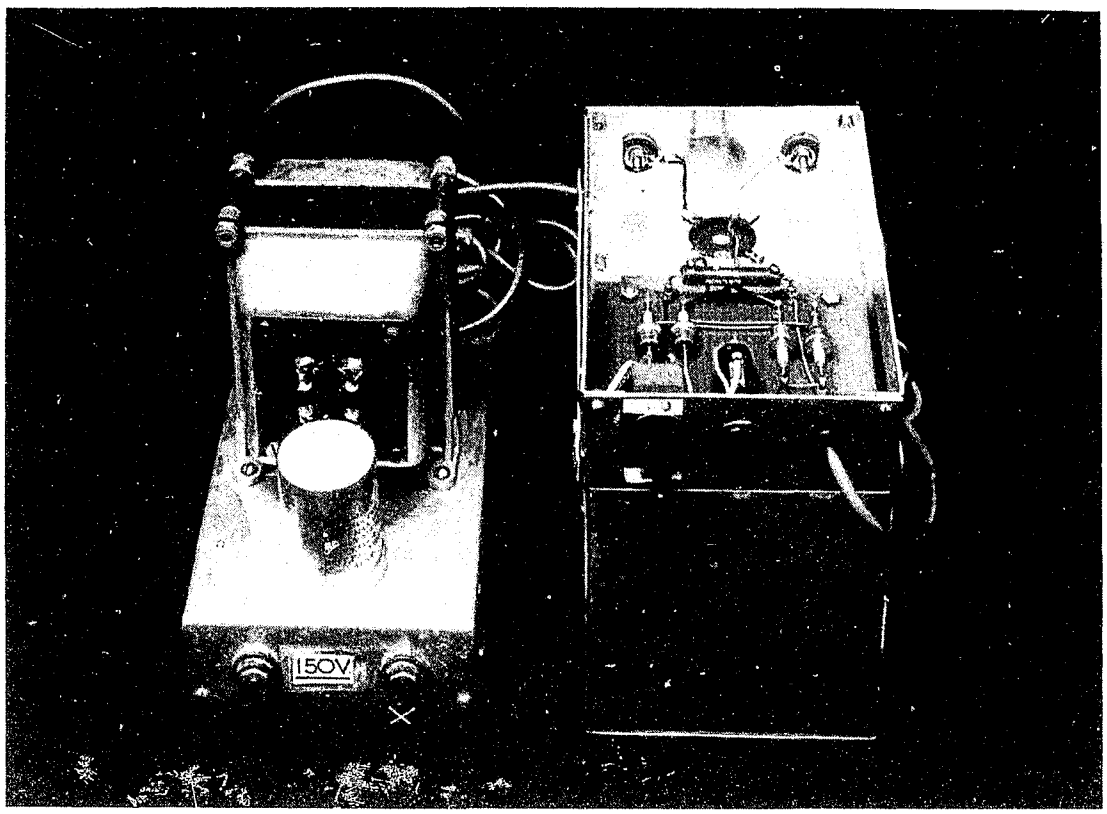


Figure 1-5. Simple Home-Made Power Pack
(Upper and Lower Views)

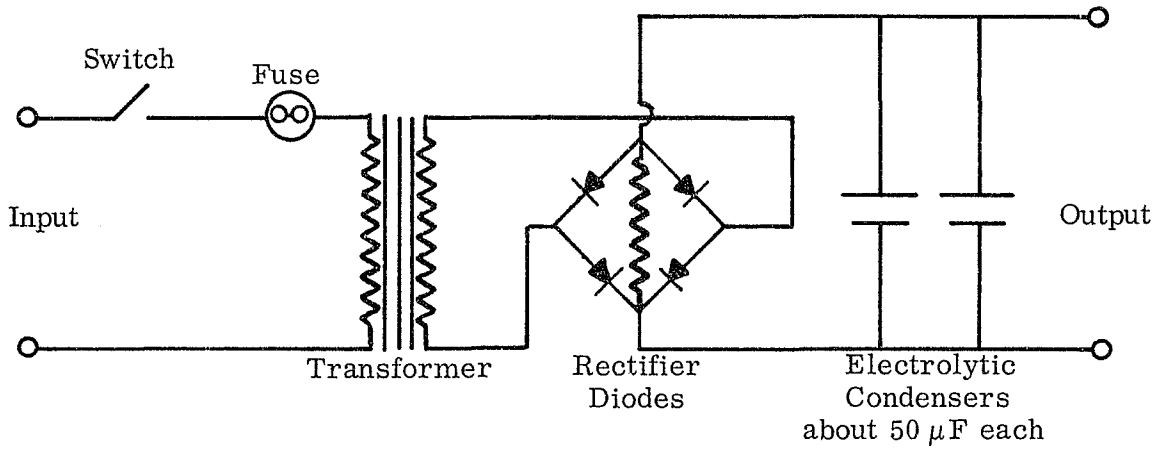


Figure 1-6. Circuit Diagram of Home-Made Power Pack

1.2.8 MEDIA ON WHICH ELECTROPHORESIS IS PERFORMED

1.2.8.1 Paper

This was one of the earliest bases used. The time taken in the run was long and the resolution poor when compared to other quicker running media.

1.2.8.2 CAM (Cellulose Acetate Membrane)

CAM looks like a thin fine paper. When dry it is very brittle and must be handled with care. Wet or dry it fingermarks very easily and is best handled with forceps. The resolution obtainable on this medium is better than that of paper or agar for protein stained material. Kohn[1] has used this medium for immunoelectrophoresis and several others have described its use for enzymograms.[2, 3, 4] It was found that this medium is more difficult to handle for immunoelectrophoresis than agar and not as good on resolution as starch or acrylamide gels. The amount of sample required is smaller than with most other methods. The thin layer starch gel method requires slightly more but is of the same order of sample size. The only variant system using CAM as the base medium in this work is that of haemoglobin.

The dry CAM is floated on the surface of a dish of buffer until wetted through; it is then submerged in the buffer and allowed to soak for a few minutes. If it is immersed in buffer when it is dry, air is trapped in the CAM matrix and the strip cannot be evenly soaked in buffer. After soaking it is removed, blotted lightly with a smooth filter paper to remove excess buffer, and placed in position in the tank. It is at this stage that the strip can dry out unduly and care must be taken to ensure that this does not happen.

Some workers recommend either leaving the strip for one-half to one hour in the tank or one-quarter to one-half hour with the current switched on before application of the sample. This is due to the difficulty of repeatability in the blotting stage of excess buffer removal. Experience, practice, and good technique are the best cure for lack of repeatability and neither of the former processes is necessary.

Care must also be exercised in the application of the sample to the strip of CAM. Many patent applicators have been marketed and many more home-made ones tried but a drawn-out capillary tube still seems to be the easiest and most satisfactory applicator used in conjunction with a ruler to ensure a straight line of sample on the CAM. It is very easy to put too large a sample on the strip. It is adequate to use 1 to 2 μl of serum, for a protein separation, or 0.1 to 0.5 μl of haemolysate for haemoglobin separations, in a line about 1 cm. long.

1.2.8.3 Agar

Agar is a biological product and, therefore, very variable from one manufacturer to another and from one batch to another. The two most important factors in the choice of an agar for electrophoresis are the degree of endosmosis with the buffer system to be used and the clarity of the gel.

A number of agars are now produced specially for electrophoresis and some of these are good quality products. Difco Agar Noble has excellent clarity and is ideally suited to immuno-diffusion techniques but some batches used have all shown a very strong endosmosis if used without a great deal of prior treatment. Difco Bacto Agar has a much lower endosmosis but the clarity is very poor. This latter point of poor clarity applies to almost all the agars produced for bacteriological work. If the agar is as cloudy as most of these, faint precipitate lines cannot be seen properly. Behringwerke agar is excellent but its price is too high for routine use.

It was found that most batches of Oxoid Ionagar No. 2 meet all requirements of clarity, possess a low endosmosis, and are reasonably priced. Some endosmosis is necessary for the precipitin reaction technique (described in paragraph 1.4).

When making agar gels, it is a great deal easier to make a bulk agar in distilled water gel at 2 percent agar and then make each different buffer to be used at double the normal strength. Then it is easy to melt equal quantities of buffer and agar to obtain the final gel quickly, easily, and when it is required. This applies also to the agar gel overlays for the detection of enzymes after starch gel electrophoresis.

1.2.8.4 Starch

There is only one supplier of hydrolysed starch for electrophoresis and that is Connaught Laboratories Toronto. (There are agencies in most countries.) Suitable starch can be obtained, hydrolysed, and then standardized in the laboratory according to the method described by Smithies[63] but this is uneconomic in terms of laboratory staff time.

Quoted on the label of each batch of starch is the quantity of that batch which will give a standard separation. Starch percentages given later assume this concentration quoted on the jar to be 10g per 100 ml. If it is different from this, then the concentration quoted under particular methods should be modified as follows:

$$\text{concentration required} = \frac{\text{conc. quoted on jar} \times \text{conc. quoted in method}}{10}$$

Starch gels at the usual concentrations that are used form a molecular sieve so that proteins and enzymes are separated according to molecular size as well as charge. The gel should be carefully and properly made, it is easy to heat insufficiently and equally easy to burn the starch. Either circumstance will lead to poor quality runs. A description of the method of making starch gels is given later in this Section.

1.2.8.5 Acrylamide

This type of gel gives separations similar to those of starch. It is much easier to control the pore size of the molecular sieve in this gel than in starch. It is an extremely useful medium but experience has shown that it is not as easy to establish as a routine method with this medium within a busy laboratory as it is with starch. Hence, starch methods are used as routine in preference to acrylamide. The exception to this is the cholinesterase C_5 system where the separation is greatly superior using a mixed plate of acrylamide and starch. The method is described in detail under that enzyme paragraph 3.7.

1.2.8.6 Techniques

With the exception of starch gel electrophoresis, all the methods described in the ensuing paragraphs are individual ones restricted to a particular group system or investigation.

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Thick plate (or standard) and thin layer starch gels are used in many of the systems so it is intended to describe each of these here rather than repeat the description under each group system.

The thick starch gel plate is described as it refers to haptoglobin and the thin layer starch gel as it applies to phosphoglucomutase. The necessary buffer and starch concentration changes in order to use these techniques on other group systems are given under the description of each system.

1.2.9 PREPARATION OF STARCH GEL (THICK)

1.2.9.1 Preparation of Plate

An 8 1/2-inch by 6-inch glass plate is made into a mould by sticking two layers of 1/4-inch wide glass strips 3-mm thick around the perimeter of the upper surface with M.S. 4 silicone grease.

1.2.9.2 Buffer for Gel (pH 8.65)

The buffer for gel contains the following:

TRIS	9.196 gm
Citric acid	1.05 gm
Distilled water	1 litre

1.2.9.3 Preparation of Gel

Place 180 ml of gel buffer in a 1 litre conical flask and add 18 gm of hydrolysed starch. This mixture is heated over a bunsen burner with continual shaking and swirling. It will become thick and agitation is essential at this point. Subsequently it will become clear and then thinner when boiling will commence. After very brief boiling, a few seconds, remove from the heat and continue agitation by shaking and swirling.

De-gassing is effected by connecting the conical flask to a water pump until the mixture boils and becomes quite clear and bubble-free. It is then poured into the mould which has been made as described previously. The mould should be placed on a level surface or a leveling table prior to pouring the starch gel into it.

The mixture is allowed to cool to room temperature and a cover of PVC sheet is then put over it.

1.2.9.4 Application of Samples

The PVC cover is removed from the gel and a straight edge, such as a ruler, placed across the gel parallel to one of the short sides, 2 1/2 inches from the cathode end. A 7 to 8 mm wide razor blade is then inserted vertically into the gel towards the top of the plate. A piece of 3MM Whatman filter paper approximately 5 by 8 mm is soaked in the serum sample. This is then inserted vertically down the side of the piece of razor blade so that it enters the gel. The razor blade is withdrawn and a short distance away the next slot is made in the gel and the next sample inserted. Using these sample sizes the gap between samples should be about 1/2 cm or more. When the plate has been filled with samples in this manner, it can be transferred to the appropriate tank.

1.2.9.5 Connection in Tank

The gel is connected to the tanks, which have been filled with tank buffer, by means of either pieces of Spontex sponge dishcloth material or 6 thicknesses of 3MM filter paper running the full width of the gel. These should overlap the gel by approximately 1 cm. The PVC cover is replaced over the gel. The power pack, connected to the tanks, is then switched on and adjusted to a constant voltage of 100 volts. This is the correct voltage when running in a cold room at 4°C. If the run is being made at room temperature, the voltage should be approximately 60 volts. The overnight run is 15 to 17 hours.

1.2.10 THIN LAYER STARCH GEL TECHNIQUE

Materials used for this technique include the following:

a. Two glass plates 22 cm by 15 cm by 3-6 mm thick.
b. Two glass strips 22 cm by 5 mm by 1 mm thick.
c. Two glass strips 14 cm by 5 mm by 1 mm thick.
d. Two glass strips 22 cm by 5 mm by 3 mm thick.
e. Four glass strips 14 cm by 5 mm by 3 mm thick.
f. Two foam plastic (Spontex) buffer bridges 14-cm wide to fit the tank being used.
g. Starch, buffers, etc.

The glass strips b and c are lightly greased with M.S.4 silicone grease and stuck by means of the grease around the perimeter of one of the glass plates a to form a shallow mould 1-mm deep. A permanent mould may be made using Araldite instead of grease. Using the phosphoglucomutase gel buffer 50 ml of 10 percent starch gel is made. (TRIS, 12.11 g; maleic acid, 11.62 g; EDTA, 2.92 g; magnesium chloride, 2.03 g; water to one litre. This is the tank buffer. Dilute 1:15 for the gel.)

The starch gel is boiled and de-gassed as described earlier and poured into the glass mould at one end. A bevelled Perspex starch gel scraper (Shandon Scientific) is drawn across the mould resting on the strips b, starting at the end containing the starch gel. The purpose of this operation is to produce an even layer of starch gel 0.8- to 1-mm thick. There will be an excess of starch gel which flows off the mould at the end of the scraping process.

Most of the failures when using this technique have been caused by either an uneven layer of starch gel or a layer that is too thick.

A check on the thickness of the gel can be made when electrophoresis is started. At 4 volts/cm the current should not exceed 8 ma. If the current is over 10 ma, the plate is probably too thick for the best results.

Origin slots are made 7 to 8 cm from the cathode end of the plate; each slot is 8 to 10 mm long. The inserts used are 8 to 10 mm pieces of cotton thread soaked in lysate or a similar amount of bloodstained thread, when dealing with dried bloodstains. In the case of bloodstained clothing, a thread is carefully removed and allowed to soak in gel buffer for about five minutes prior to insertion into the gel. In the case of weak bloodstains or stains on thin material, several threads should be used.

The Spontex bridges, soaked in tank buffer, are positioned across the ends of the plate overlapping the gel by about 2 cm and are held in contact with the gel by placing the second glass plate over the first thus sandwiching the Spontex bridges at each end.

Electrophoresis is carried out for 17 to 20 hours at 2 to 4 °C with a voltage of 4 volts/cm. On completion of electrophoresis the two glass strips d and two of the strips e are greased and stuck on top of the strips b and c, respectively. The two remaining glass strips are used to block off an area running from the origin to 3 cm from the anode end of the plate.

The reaction mixture is made by dissolving the solid components in the buffer (see paragraph 3.1) and warming to 37 °C in an incubator (in the dark), mixed with the melted agar, which has been cooled to 50 to 55 °C, and immediately poured onto the appropriate area of the starch gel. There is no need to slice the gel as in the thick gel technique. When the agar is set, it is covered with the second glass plate and incubated at 37 °C in the dark for one to two hours, after which the bands of enzyme activity will be seen.

The advantages of the thin gel technique are in the quantities of reagents used, the lack of any need to slice the gel, and in particular the small amount of sample required. This is advantageous when dealing with bloodstains of limited size.

When dealing with blood samples or lysates, the inserts used for most techniques are lengths of cotton thread about 8 to 10 mm long taken from cotton sheeting which has been well-washed. Standard sizes of sewing cotton are rather too thin and two or three thicknesses of this would be required.

With the acrylamide/starch technique used for pseudocholinesterase C_5 system, slightly more material is used, so the insert used is a 1-mm wide strip of Whatman 3MM filter paper, or cotton inserts.

Bloodstained clothing is usually simple to deal with. A thread of bloodstained cloth, approximately equivalent to the standard cotton thread, is removed and used as the insert. In some systems short soaking of this thread in buffer is all that is required; in other systems slightly more preparation is needed.

With bloodstains on carpet or non-spun textile materials, a small bunch of individual fibres is taken and treated similarly to a spun thread of fibres.

Bloodstains often occur on hard porous or non-porous surfaces, such as bricks, wood, knives, glass, etc. In these cases the bloodstain (or a portion of it) is swabbed off using a standard cotton insert soaked in the appropriate gel buffer for the system of grouping for which it is intended to use it.

If, as in adenosine deaminase, a bloodstain sample is normally soaked in buffer containing mercaptoethanol, then the same mixture is used when swabbing a bloodstain from a hard surface.

With some porous substrates it may be better to scrape the blood from the surface onto a glass slide before attempting to swab it onto a standard cotton insert. This is because some very porous materials will absorb liquid from the swab without allowing solution of the bloodstain onto the insert.

The usual practice is to hold the cotton thread inserts in fine watchmaker's forceps (a No. 4 or 5) when swabbing a bloodstain from a hard surface or when putting the inserts into the thin starch gel.

Inserts for haptoglobin typing are larger than those used in any other system and are considered in paragraph 4.1.

1.2.11 TRANSFUSION PROBLEMS

In serious assault cases, the victim often loses a lot of blood, so that on arrival at hospital a blood transfusion is given at an early stage in the treatment. Transfusion services usually type the victims blood only in the ABO and Rhesus systems. The typing in the Rhesus system may not be a complete typing. The remainder of the systems which are of use to the forensic scientist are not determined or matched. Hence a person of type PGM 1 may well receive a transfusion of PGM 2 blood. This will not harm the patient but could make a blood sample taken as a control from the victim unreliable for forensic purposes.

The recognition of transfusion effects is an important factor when typing blood samples. Every effort should be made to ensure that the laboratory knows whether or not a transfusion has occurred, and if possible a pre-transfusion blood sample obtained. If this cannot be done then it may be better to use the

victim's bloodstained clothing as the source of the control blood sample. Otherwise a sample of blood taken 4 to 6 weeks after the transfusion will normally give the victim's correct groups.

Transfusion reactions can be seen when dealing with the enzyme groups since they leave very weak spurious bands. Some examples of these are shown in the Figures 1-7 and 1-8. The sample marked 'T', Figure 1-7, is from a PGM 1 subject who has been transfused with blood type PGM 2; weak additional bands can be seen. This makes it possible to confuse it with a PGM 2-1 by the inexperienced worker. Figure 1-8 is similar to Figure 1-7, but a PGM 2 subject has been transfused with blood of type PGM 1, leading to a similar potential confusion.

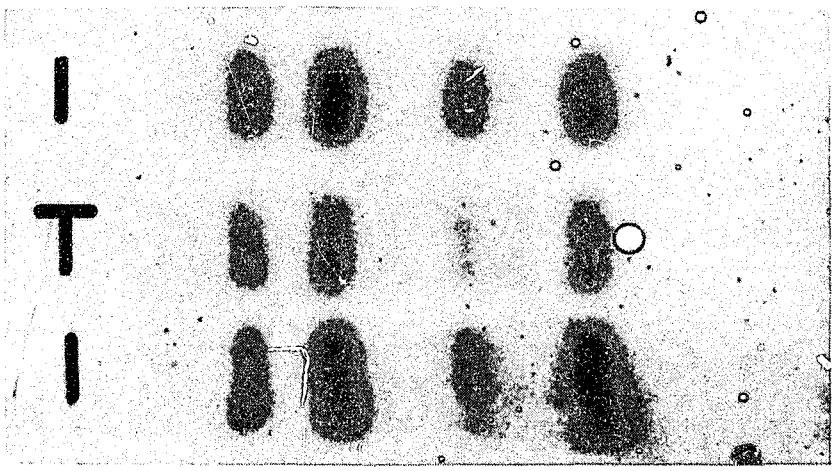

Figure 1-7. PGM 1 Subject Transfused with PGM 2 Blood

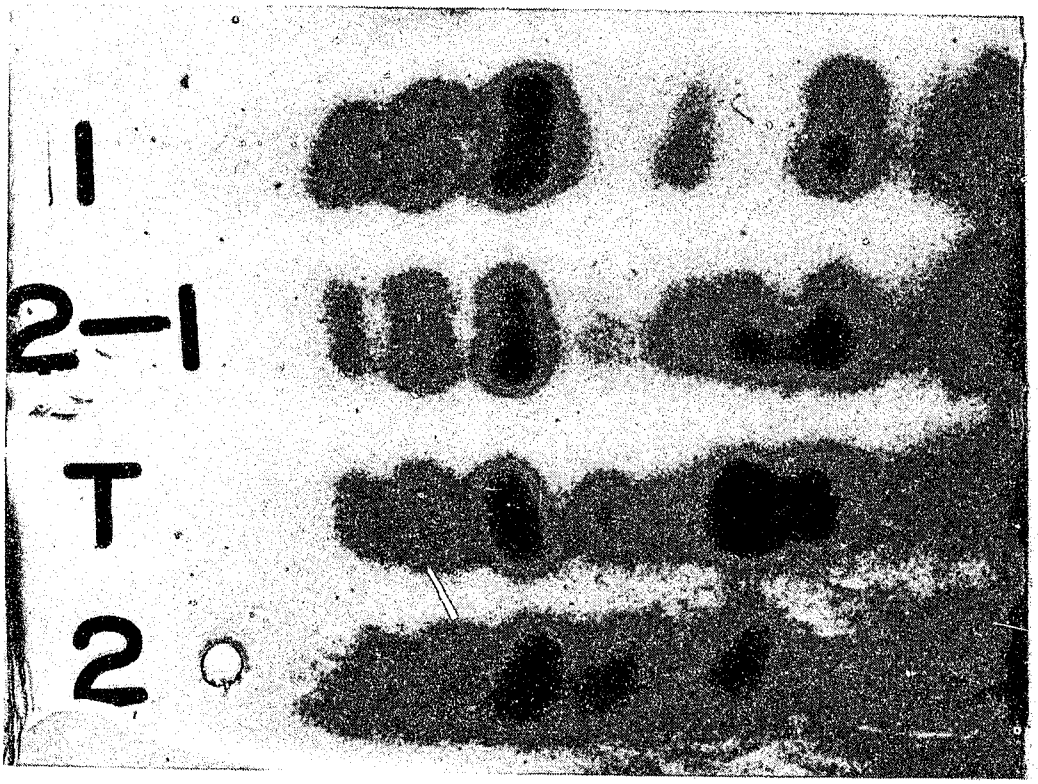

Figure 1-8. PGM 2 Subject Transfused with PGM 1 Blood

1.3 BENZIDINE TEST

1.3.1 HISTORICAL

For many years a search was made for a diagnostic test for blood. This was, of course, of great importance in forensic investigation as well as in clinical pathology.

The first test of any significance was the guaiacum test discovered in 1861[5]. Its inconvenience and erratic nature led to the search for a better method which resulted in the publication of numerous tests including the benzidine test in 1904[6]. This proved to be so delicate and positive in its reaction to blood that it was immediately adopted for clinical work while forensic workers sought to prove its specificity so that its value as evidence in a Court of Law could be determined.

It was, of course, soon shown that the test was not specific, and a number of variants were suggested, such as the leucomalachite green test, the phenolphthaltin test and the luminol test. All these, however, are tests for peroxidase and, therefore, suffer from the same drawbacks as the original test and are, in addition, much less convenient to use and in some cases, less certain in operation. Later in 1968 the use of o-tolidine and o-dianisidine was dropped because they also were reported as carcenogenic. The reagent that was substituted at this time was a modified Kastle-Meyer, an alkaline leuco-phenolphthalein. The modifications improved its sensitivity but it is not considered as good as benzidine and the search for a better replacement to benzidine is continuing.

The results obtained with all these reagents are similar and any reference to benzidine should be regarded as including these other reagents. The benzidine test has been retained as a generic term here, because of its long usage, to describe a particular type of testing in forensic science and because many people know the implications of this test under this name. Throughout this paragraph benzidine test will be used in this broad sense.

The use of the benzidine test in practical forensic science is of great importance and its interpretation and precise value should be clearly understood. Because

of its lack of specificity, the test has gone up and down in popularity over the years. Originally it was accepted as being a specific test for blood. When the lack of specificity of the test in the form in which it was then being used had been conclusively proved, it fell out of favour to such an extent that Glaister in 1931[7] states:

> While some employ this test it has the disadvantage that like the guaiacum test it can only be of value as a negative test. We do not put our trust in this test. We have abandoned completely the guaiac and benzidine tests for the reason chiefly that the reaction obtained in the presence of minute amounts of known blood is uncertain and doubtful and also because a reaction may be produced by substances other than blood.

Because of the methods that were used in performing the benzidine test it would appear that Glaister was misled into considering it useless. Further experience in the Forensic Science Laboratories showed, however, that the test was certainly much more valuable than Glaister stated.

In 1954 Gradwohl[9] took an almost opposite view to that of Glaister.

> Make a solution of the material suspected of being blood. Place a drop of solution of the suspected stain on the new clean glass slide and add a drop of the benzidine/glacial acetic acid/hydrogen peroxide mixture. A green or blue colour indicates the presence of blood.

Experience does not support this view of the simple benzidine test either.

The majority of workers in the medico-legal field writing in the period of 1920 to 1950 decided that the test was only of use when it was negative. This attitude is summed up by Gonzales, Vance, Helpern and Umberger 1950[10]:

> Valuable as a negative test and more sensitive than the guaiac test.

Those who hold this view give considerable weight to the evidence of materials other than blood which are said to give positive results.

The authors' own view of the simple benzidine test has been expressed by Sydney Smith[11] and by Nickolls[12] in a previous publication:

> This is the best preliminary test for blood and by means of it a large number of possible stains may be examined and those that give a positive test marked out for further examination. (Smith)

> The superiority of the benzidine reaction over the phenolphthalein and the leucomalachite green reaction lies in the cleanness of the negative test and the intensity and striking nature of the positive test. The benzidine test gives a colourless blank which only slowly discolours. The blanks from the other two tests are always quite strongly tinted, a fact which obscures the certainty of the test. As a result, it is safe to assume that the benzidine test is the most satisfactory presumptive test for blood notwithstanding the recent strong disparagement given to it by Kirk 1953. (Nickolls)

Nevertheless, as recently as 1960 Hunt et al,[8] in a survey of the testing of bloodstains state that even less reliance should be placed in the benzidine test. Referring to this test:

> Occasions do occur when a garment which is expected to be contaminated with blood gives a positive presumptive test for blood, although the deposit is insufficient or unsuitable for more specific tests. However tempting it may be to use this as evidence, it is scientifically and morally incorrect to do so, for it is clearly recognized that such tests are not specific and their introduction into evidence may well mislead.

The concept implied here that the results of a scientific test should be deliberately withheld from the court or that positive results should be denied is alarming, since it usurps the function and power of the court. Indeed, having obtained a positive benzidine reaction, it would be considered morally wrong to withhold this information.

If a garment had been stained with blood and the quantity was minute (e.g., having been subsequently washed) then one would expect to get a positive benzidine reaction. One would hope to get other reactions to further tests but one would not necessarily get them. In these circumstances, it would be equally misleading to

report that a garment gave no reaction which could indicate the presence of blood as to report emphatically that human blood was present on the garment.

The court should be told the facts of the testing and at the same time it should be told the precise implications of this test.

In the Metropolitan Police Laboratory more than 10,000 articles are examined each year by means of the benzidine test for the presence of bloodstaining and, it has been found that a positive test is very rarely obtained in the absence of blood and in these few cases the reason for the reaction could be easily demonstrated. The Forensic Science Laboratory has, therefore, a very considerable faith in the test as being indicative of blood in the circumstances in which it is performed. It was decided, therefore, that it was necessary to find out exactly what could be said for and against the test and what facts could be stated with certainty. The results of the investigation were surprising.

1.3.2 METHOD AND REAGENTS

There have been innumerable variations of reagents and technique in the benzidine test, since its original description and it is more than possible that these variations account for some of the varying results obtained and opinions formed.

It was originally performed on liquid extracts of bloodstains, a method which few if any workers still use. Scrapings of bloodstains in tubes, wet filter paper blottings (wetted with a variety of solvents), and dry filter paper rubbings have all been used as methods of obtaining a minute sample for testing.

The reagents used have varied also from a weak alcoholic solution of benzidine to a saturated solution of benzidine in glacial acetic acid.

Four samples of the differing peroxide substrates which have been used include three vol. hydrogen peroxide, 100 vol. hydrogen peroxide, 20 percent sodium perborate solution and ozonised turpentine

Usually the benzidine and the peroxide have been together in a single reagent solution. This single solution benzidine reagent has led to the inclusion of chemical oxidants in the list of materials giving a positive benzidine reaction.

The methods used in the Metropolitan Police Laboratory are as follows:

a. Solution of benzidine, o-tolidine or o-dianisidine (0.1 to 0.5 percent) in alcohol/acetic acid 50/50 or in glacial acetic acid.

b. 20 vol. hydrogen peroxide, or Kastle-Meyer reagent

Phenolphthalein	4 g
Sodium hydroxide	40 g
Zinc dust	20 g
Water	1000 ml

This mixture is refluxed until colorless, cooled, decanted and made up to 1200 ml with ethyl alcohol.

Also required in this test are ethyl alcohol and 20 vol. hydrogen peroxide.

The stain to be tested is lightly rubbed with the corner of a small (4 cm) dry filter paper folded into four. It is not necessary to rub to such an extent that the stain is visibly affected or that the filter paper is visibly discolored or abraded.

A single drop of benzidine solution is applied to the corner of the filter paper. After a brief interval to ensure no color develops, hydrogen peroxide is dropped onto the same area of the filter paper. An immediate deep blue coloration indicates a positive reaction (see Figure 1-9).

If Kastle-Meyer reagent is used, then a drop of ethyl alcohol is put onto the corner of the filter paper first, followed by the reagent and finally the hydrogen peroxide. In this instance the color is deep pink for a positive reaction.

The filter paper is used in this manner to avoid undue damage to the stain and to avoid using too much of the available material on a test used solely to sort out potential bloodstains from stains of material other than blood.

Figure 1-9. Use of Small Folded Filter Paper on a Stain Suspected of Being Blood

If specific stains cannot be seen and a general searching for small amounts of weak bloodstaining or washed out (dilute) bloodstaining is required, then a light rubbing over a large area with larger filter papers is used.

Testing these larger papers is similar to that used previously except that more reagent is used.

This general rubbing technique should never be used until scrutiny (if necessary with a low power microscope) and specific spot testing have been exhausted, since other evidence may be lost by its use.

1.3.3 FALSE POSITIVE REACTIONS

In the particular circumstances which are being considered here, a false positive reaction may be defined as any positive reaction given by any substance other than the bloodstaining being sought. These substances may be conveniently divided into four groups.

a. Blood Contamination—The test is so sensitive that quantities of blood far too small to see will give a strong positive reaction. Scrupulous care must be exercised to see that the conditions under which the test is carried out ensure that negative reactions are given in all cases where they should be expected. If unexpected positive reactions are found, a search must be made for the source of the contamination and, for example, the search bench cleansed, blank reagent tests performed, etc., until 100 percent negatives are obtained. A few sporadic false positives can easily produce a feeling of uncertainty in the validity of the test.

b. Chemical Oxidants and Catalysts—The behavior of these chemicals is, in general, different from that of blood. Although they give strong colors, these colors often differ quite obviously from the color given by blood. Nevertheless, it is not desirable that adventitious colors should arise in practice. This, however, can be avoided easily by use of the two-solution test. Chemical oxidants will give a discoloration before the addition of the hydrogen peroxide while blood does not. If a discoloration is obtained the chemical should be sought and its effect eliminated if possible before further tests are performed. If a negative test reaction is obtained, a few drops of 20 volume H_2O_2 are dropped into the benzidine wetted area. In the presence of blood or a vegetable peroxidase an immediate blue color will develop. By the use of this two-solution test, the effect of chemical oxidants can be eliminated.

   Chemical catalysts, however, are not all eliminated by this means and it is only on the addition of peroxide that color develops.

   Those most often quoted are copper and nickel salts. These do, in a strong solution only, give a positive reaction with the benzidine

reagents. The reaction when it does occur is different to that produced by blood. A weak coloration is usually produced before the addition of the hydrogen peroxide. When the peroxide is added there is an immediate removal of the existing coloration and then very slowly a deep blue coloration is produced. This starts as a ring around the wet area on the filter paper and very gradually extends inwards. The reaction is usually still incomplete after 15 to 20 minutes. Thus, the reaction obtained is quite different in its form from that obtained with blood or the chemical oxidants. But when dry stains (or crystals of the chemical) are tested in the manner described, no reaction is obtained.

The color of the stain tested must be considered and the evidence of usual observation added to the intelligent use of this test. A stain on cloth of these salts strong enough to give a positive reaction will (a) discolor the cloth (unless the cloth is of identical color) and (b) show small crystals in the cloth where the salt has dried. Its color will not be that of blood. Invisible traces of blood can be detected. Invisible traces of these salts are not detected using the method described.

c. Other Substances of Animal Origin—The literature states that substances other than those considered in a, b, d will give positive reactions. Such substances mentioned are pus, some nasal secretions and faeces. These need cause no concern. It is impossible to obtain pus completely free from blood. Traces of blood, not obvious to the naked eye, are commonly present in nasal secretion and faeces. The great sensitivity of the benzidine test is such that these invisible traces are ample to give a strong positive reaction. Microscopic examination of such substances will immediately distinguish them.

d. Plant Peroxidases—The most important class of substances that is liable to interfere with the benzidine test and that has always been held to vitiate the specificity of the test is the vegetable peroxidases. There is no doubt that many plant tissues give intense reactions with the benzidine reagents which can be mistaken for blood.

The excellent work of Hunt et al,[8] has confirmed this. Nevertheless, certain facts do not appear to have been noticed or at any rate recorded. The color of the stain must be observed before any tests are done and in most cases this is not the color of bloodstaining. Green and white are the commonest colors to find associated with plant materials. The plant peroxidases appear to reside in the particulate contents of the cells of the plant tissue, the juice of the plant giving a negative or only a faint positive reaction. As was pointed out by Nickolls[12], a strong positive reaction is usually associated with tissue or tissue fragments to be present: such fragments are detectable and identifiable by microscopic examination.

A survey of the literature failed to show an adequate account of the behavior of plant peroxidases. The papers suggested that all the experimental work had been performed by smearing the suspect plant material onto filter paper and testing the smears immediately, or alternatively, testing the fresh plant directly. It would appear profitable to discover whether plant peroxidases differ in their properties from animal peroxidases.

1.3.4 BEHAVIOR OF PLANT PEROXIDASES

1.3.4.1 Thermostability

It has been confirmed that all vegetable peroxidases become inactivated with heat. At a temperature of 100°C the vegetable peroxidases are rapidly inactivated. At the same temperature, the animal peroxidases are relatively stable. A short period (five minutes) of heating to 100°C will, therefore, serve to differentiate between them.

1.3.4.2 Time Factors

Animal haemoglobin peroxidases are very stable substances. Bloodstains many months old give strong benzidine reactions. Stains years old will still give good reactions. Stains received in a forensic science laboratory are usually at least 24 hours old and often older when the examination of the stains is completed.

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This lapse of time in nc way invalidates the benzidine test on the stains. When vegetable stains are treated in this manner, quite different results are obtained. For example, fresh, uncooked vegetables have been pulped and stains have been made from them. These were allowed to dry at room temperature and kept for varying periods of time. The reactions to benzidine were observed at intervals.

The benzidine tests were performed on filter paper rubbings of the stains directly on the stained cloth and on aqueous extracts of the stains. The results are set out in Table 1-1.

Table 1-1

Benzidine Test Results

	Rubbing		Direct On Cloth		Extract	
	After 1 Day	After 3 Days	After 1 Day	After 3 Days	After 1 Day	After 3 Days
Dilute blood 1:100	++++	++++	++++	++++	++++	++++
Onion	±	±	±	±	±	-
Garlic	-	-	±	±	-	-
Beetroot	+	-	±	±	±	-
Cucumber	-	-	±	±	-	-
Horseradish	±	±	±	±	-	±
Horseradish sauce	-	-	±	-	-	-
Lettuce	-	-	±	±	-	-
Tomato	-	-	-	-	-	-
Potato	±	-	+	±	-	-
Cabbage	+	-	+	±	±	-
Bread	-	-	±	±	-	-
Milk	-	-	-	-	-	-
Gluten	-	-	±	±	-	-

After five days it was found that none of the plant material stains gave positive reactions except when benzidine and hydrogen peroxide were applied directly to the stain on cloth, and then weak reactions were obtained only when much cellular material was present. In order to obtain these results undiluted crushed vegetables had to be used and the color of these pulps showed strongly providing the cloth was not of the same color.

If the vegetable extracts were finely sprayed or diluted to an extent where they were almost invisible, no benzidine reaction could be obtained except by flooding the cloth with reagent. In some cases even this did not reveal positive reaction since the residual peroxidase activity of these pulps was so low compared to that of blood.

1.3.4.3 Effect of pH

Plant peroxidases react well in a strongly acid substrate. In general they do not, however, react at a strongly alkaline pH. For this reason the Kastle-Meyer test has been held by many to be much more specific (and even to be specific) for blood. It is, however, an awkward reagent to make, keep, and use. At its most sensitive composition the sensitivity is lower than that of benzidine. At this stage, however, it will rapidly become oxidized and turn pink in air. To make the reagent more reasonable to use one must either remove the air or appreciably reduce the sensitivity to blood. This reagent is also much more fallible as regards chemical agents; more materials in this class produce the pink coloration and they do so at lower concentrations than with benzidine. The standard method of using this reagent is, in our opinion, cumbersome and quite unsuited to a busy laboratory making thousands of tests.

1.3.4.4 Chromatography

The identification of bloodstains by means of paper chromatography was suggested by Fiori[13]. Several methods have been proposed, all of them relying on a specific RF value associated with detection of haemoglobin by means of

benzidine. Fiori, nevertheless, relies on heat to eliminate the plant peroxidases by inactivation and the two-solution technique to eliminate the chemical oxidants.

Some of the chemical catalysts are quoted as having Rf values similar to that of haemoglobin. It is, therefore, difficult to see much advantage in preparing the chromatograms and then using other methods, equally applicable to a rubbing, to distinguish the blood from other peroxidases or oxidizing agents.

1.3.4.5 Electrophoresis

Electrophoresis of small amounts of blood and other peroxidase active materials has been performed and the subsequent detection was by means of the benzidine reaction. The object of this was to try to typify blood in small dilute or dispersed amounts.

It was found that under certain electrophoretic conditions blood from a stain gave a pattern that was drop-shaped, the tail of which had moved towards the anode, the main bulk of the haemoglobin remaining at the origin.

The majority of plant peroxidases moved towards the cathode. An exception which was tomato pulp peroxidase, moved in a single, more or less circular, area towards the anode and was, therefore, just as distinguishable from blood as those which moved towards the cathode.

Chemical oxidants were not considered here since they are so easily detected by the two-solution method. Chemical catalysts were run as a matter of thoroughness and were found to have cathodic mobility. Disruption of the gel used as a substrate also occurred with these when they were present in sufficient concentration to give a reaction. Excessive diffusion also occurred with these chemicals.

This method was found to distinguish bloodstains from all other substances tried which react to the benzidine test in any form, even when using the optimum conditions for these other substances, e.g., freshly pulped vegetables.

1.3.5 ELECTROPHORETIC METHOD

Electrophoresis was carried out in a 1 percent agar gel. The agar used was Ionagar No. 2. The buffer system used was the discontinuous one of Hirschfied[14] for immuno-electrophoresis.

Gel. Buffer

Barbituric acid	1.660 g
Sodium Barbitone	10.510 g
Calcium Lactate	1.536 g
Distilled water to	1500 ml

To make the gel, equal volumes of buffer and a 2 percent ionagar gel in water were heated, mixed and poured onto glass plates to a depth of approximately 2 mm. When the gel had solidified, wells 1 to 1.5 mm in diameter were punched in it using a suitable pasteur pipette. These wells were 1.5 cm apart at right angles to the direction of travel and 7.5 cm apart along the direction of travel. This plate was connected to the electrode tanks by three-layered bridges of Whatman 3 MM filter paper.

The buffer used in the tanks and on the bridges is as follows:

Barbituric acid	1.380 g.
Sodium Barbitone	8.760 g
Calcium Lactate	0.384 g
Distilled water to	1 l

The materials to be tested (bloodstain extracts, vegetable pulps, etc.) were placed one in each of the wells in the gel and electrophoresis was performed at 5 to 6 volts/cm for 30 minutes.

The gel was then stained by pouring a small quantity of benzidine solution in glacial acetic acid onto it and smoothing this out into a film with a glass rod. After 1 to 2 minutes, 10 to 20 volumes hydrogen peroxide is applied in a similar manner.

After a few minutes, when the full color reactions had developed, the excess reagent was washed off with tap water and the gel was placed in a dish of 50 percent alcohol containing a few crystals of phenol. This phenol helps to preserve the staining and to limit fungal growths. After two hours, the gel may be removed and dried at room temperature or in an incubator.

Some examples of this method are illustrated in Figure 1-10. The vegetable extracts were prepared by pounding the fresh material to a pulp in a pestle and mortar and using the pulp undiluted. Gluten and bread were inserted into the wells as moist pellets. Nasal mucus was used fresh.

The bloodstain extract was prepared by taking a stain made 24 hours previously on cloth from a single drop of blood, extracting it with 100 drops of distilled water, and inserting a small portion of this into the well. This was to obtain the equivalent from the stain of 5 ml of a one percent solution of blood. The fresh blood was prepared by diluting a standard haemoglobin A solution until the color approximately matched that of the bloodstain extract.

The remainder of the vegetable pulps, gluten, blood, etc., was used to make stains on cloth. These stains were allowed to dry under normal conditions at room temperature. Extracts were made 24 hours after these stains were made on the cloth. In the case of the bloodstain, the method used was that previously described. In the case of all the other materials, a concentrated pulpy extract was used. The result is shown in Figure 1-11.

In common with most of the vegetable pulps shown in Figure 1-11, none of the remaining substances gave a positive reaction.

After three days those substances that had given a positive result after 24 hours were subjected to electrophoresis again. The only substance other than blood to give a positive reaction was the stain made from raw pulped beetroot (beta vulgaris). Compared to Figure 1-11, Figure 1-12 shows that the intensity of the reaction has dropped markedly between one day and three days.

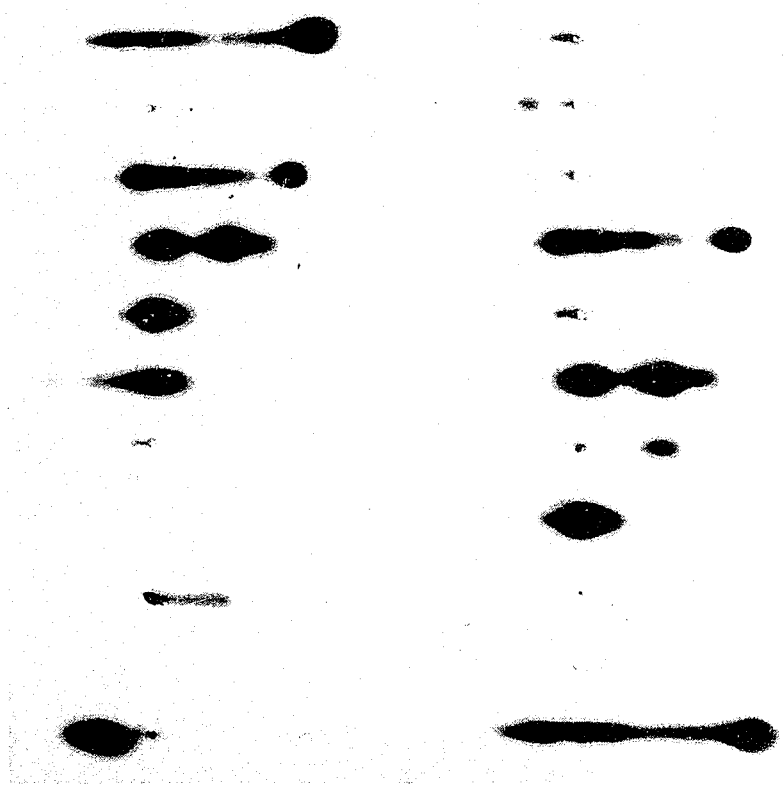

Figure 1-10. The Electrophoresis of Various Peroxidases (Fresh)

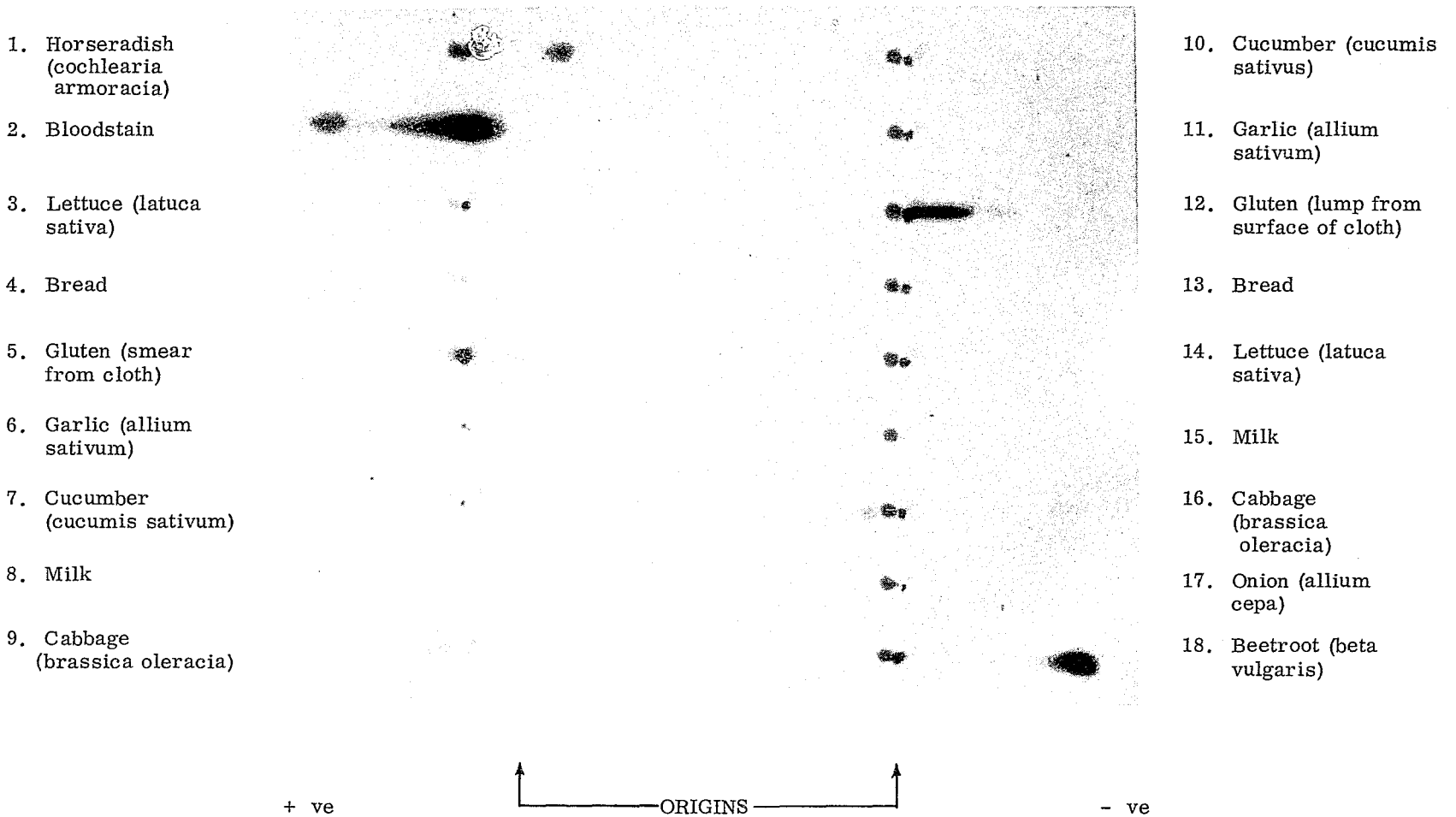

Figure 1-11. Various Peroxidase Reactions From Stains 24 Hours Old

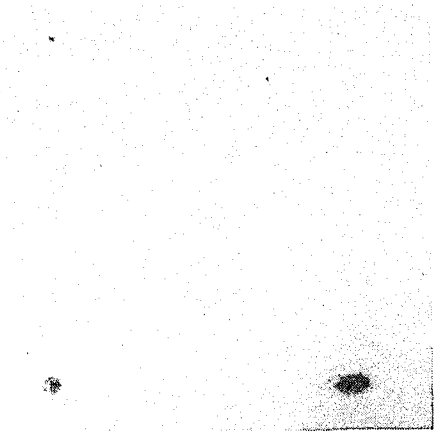

Figure 1-12. Drop in Reaction Intensity

Seven days after making the stains, the only one which gave a positive reaction was blood. No reduction in the pattern or the intensity of the reaction was appreciable up to a month after the stains were made.

1.3.6 CONCLUSIONS

The result of this work has disclosed a number of facts that throw new light on the benzidine test and that explain the conflicting opinions expressed concerning it in literature. The important facts are:

a. The benzidine test is the most positive of the many tests discovered for detecting blood.

b. The benzidine test, in its simplest form, is not specific for blood.

c. This apparent non-specificity has been over-emphasized in the past by workers who have not performed their tests under normal existing conditions prevailing in the practical examination of exhibits in connection with crime. Experiments at the Forensic Science

Laboratory have shown that it is quite useless making desultory spot-tests. The possible confusing substances are:

(1) Chemicals—These can be eliminated by inspection and by the routine use of the two-solution test.

(2) Plant Peroxidases—These are essentially eliminated on standing. Their elimination can be hastened by heating.

(3) Various Other Body Secretions—These cannot always be obtained completely free from blood and, therefore, there is no evidence that the secretions themselves are responsible for the test. (The nasal secretions that were tried at no time gave a positive benzidine).

d. While the benzidine test in the hands of an experienced worker may be regarded as being specific for blood, complete specificity is obtained by electrophoresis. As a result of the work on this subject, much of the information published to date can be completely misleading, particularly if taken out of context.

1.4 PRECIPITIN REACTIONS

1.4.1 INTRODUCTION

Since early this century it has been known that it is possible to distinguish the blood of different animals from one another by means of specific precipitating antisera. Before proceeding to group bloodstains in forensic work it is essential to know if the blood is of human origin. On some occasions blood of other animals may be sought and reliable identification methods for this also are required.

The basic technique of bringing into close reacting contact an extract of a bloodstain and suitable antisera has remained the same since the test was originally described. The means of accomplishing this has, over many years, had many variations and only the major ones will be mentioned.

1.4.2 ANTISERA

Whichever method is being used, the whole technique relies on the quality of the antisera used. An antiserum is a biological product and is, as a result, unpredictable in its manufacture. It may turn out to be univalent or polyvalent. If polyvalent serum is required, the antibodies present in different preparations will vary not only between different species but also from batch to batch. The titre of a specific antibody will also vary from one batch of antiserum to another even from the same animal.

There are two basic forms of precipitating antisera depending on the animal in which they are prepared. Those antisera prepared in horses are known as H type and those prepared in rabbits, sheep, goats, etc., are known as R type. Those prepared in chickens are generally of the R type but have certain peculiarities of their own, requiring higher salt concentrations to give good precipitates.

H type antisera produce a flocculent precipitate which is soluble in excess antigen or antibody and, therefore, requires a narrow range of antigen/antibody concentrations. The R type antiserum produces a much more stable precipitate which is not soluble in excess antibody and only partially soluble in excess antigen.

Horses tend to produce more antibodies than rabbits and the antisera at equivalence produce neater, clearer, cleaner lines than the R type antisera. If the maximum number of antigen/antibody reactions is required, then, the H type is the best. If, however, the concentration and number of antigens is unknown, the R type should be used.

The shape and position of the precipitin band depends on the concentrations of antibody and antigen and also their relative diffusion coefficients. Coefficients of diffusion cannot be changed but antigen and antibody concentrations can be changed to improve the shape of the arc obtained. This is a matter of trial and experience.

The specificity of sera is a matter that must be of considerable concern. The precise specificities of the antisera in the conditions in which they are used must be known. Commercially available precipitin sera can be relied on to be reasonably specific. However, two problems occur here. First, these antisera are not tissue specific and will give a reaction with most tissues from the animal concerned as well as from the blood. Also they will react with blood and tissues from closely related animals. Anticow for example reacts with deer blood. Anti-human serum reacts with certain anthropoid sera.

Secondly, there is the problem of nonspecific precipitation. This occurs when the concentration of the blood extract is too high. For example, the Metropolitan Police Laboratory has found that cow blood at a dilution of 1:400 reacts with certain batches of antisera against human and sheep. Obviously, when dealing with stains, this problem is obviated by working at dilutions greater than 1:400.

1.4.3 SPECIFIC ANTISERA

Recently, antiserum of special interest to the forensic scientist has become available. This is the anti-human semen serum. Unfortunately, several brands of this antiserum that have been tried reacted with high concentrations of blood and saliva and also with vaginal epithelium from some adult donors. It is, therefore, not possible to determine by this method whether there is semen on a vaginal swab.

Most tissue and body fluids can be used to provide tissue specific antisera, such as antisaliva, antiurine, antivaginal secretion, antiamniotic fluid, and antistomach secretion (to identify vomit).

Until these antisera are available commercially, it is possible to tackle the problem in two ways. The first is to use an immuno-electrophoretic technique using the cross-reactivity of a non-specific, anti-blood serum to detect the common proteins between two body fluids. The second method is to absorb the antiserum with the unwanted antigens, for example absorb anti-human semen serum with vaginal secretion, thus removing the cross-reactivity and making it specific for semen. The tests are then made with the suspect material against the absorbed antiserum.

Since the majority of problems are associated with blood rather than any other body fluid or tissue, the routine laboratory procedure for bloodstains will be described and any other problem can be dealt with as a special project. In all normal routine conditions, commercially available antisera are excellent. In some countries, however, specific local problems may well arise where no suitable antiserum is commercially available. In these circumstances some of the possible solutions have been suggested; this does not invalidate the use of the technique but makes all the more important the need to know well and accurately the attributes of all the antisera that are being used.

1.4.4 METHODS

1.4.4.1 Tube Technique

The original technique used in precipitin tests was the tube technique in which a small quantity of the antiserum, centrifuged or filtered clear, is put into a small glass tube about 2 inches by 1/4 inch. The dilute extract of the bloodstain, also centrifuged or filtered clear, is carefully layered over the top of the antiserum without disturbing the interface thus formed. If a reaction occurs, a white precipitate forms at this interface within 20 to 30 minutes.

Several problems arose with this method, the least of these being the quantity of bloodstain extract and antiserum required. A tube of internal diameter of 2 mm was adopted but the liquids still had to be centrifuged and a number of false negative reactions caused by cloudy solutions still occurred. Then, because of the enormous increase in the number of tests being performed, an electrophoretic method was introduced by Culliford (1964).

1.4.4.2 Electrophoretic Method (Crossed over Electrophoresis)

In this method the two reactants are placed in two wells punched in the gel placed close together along the line of electrophoretic movement—the blood extract in the cathodic well and the antiserum in the anodic well. The antigens in the blood extract are serum albumin and alpha and beta globulins, whereas the antibodies in the antiserum are in the gamma globulins. Under electrophoretic conditions, the movement of the gamma globulins is toward the cathode (because of endosmosis) while all other proteins move toward the anode. When appropriate reactants meet in the area between the wells, a precipitate is formed. (See Figure 1-13.)

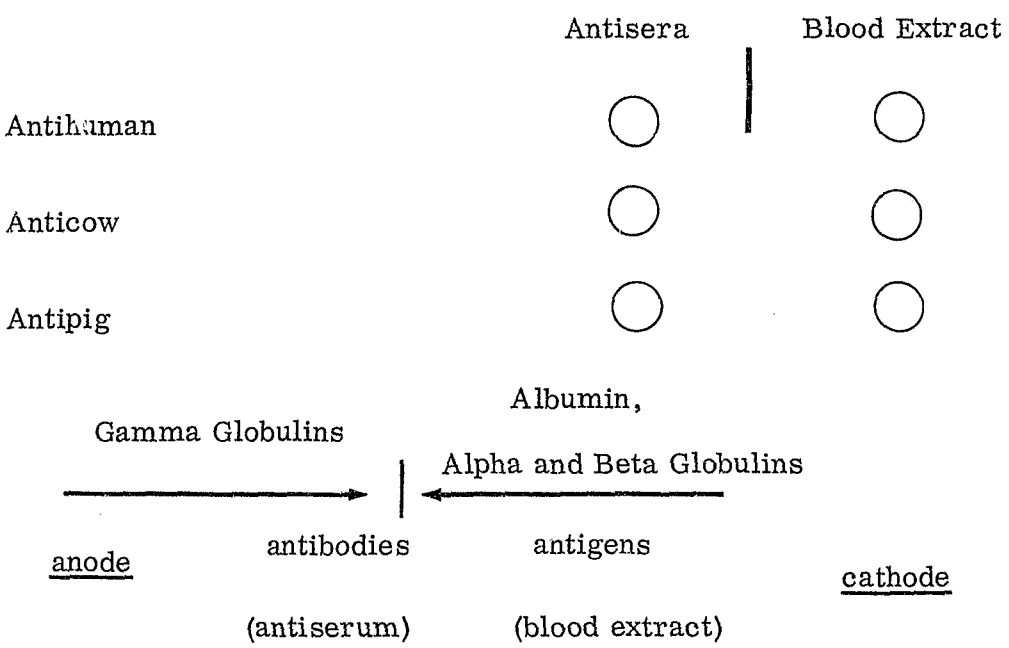

Figure 1-13. The Mechanism of the Electrophoretic Method

When choosing an agar for the electrophoretic technique the prime consideration is the degree of endosmosis that the agar shows. If there is too little, the method will not work; too much, and the gel will shrink badly (see paragraph 1.2). Special Difco Agar Noble has excellent clarity but too much endosmosis and is, therefore, much less suitable than Oxoid Ionagar No. 2. This has the necessary endosmosis to allow the reaction to occur without causing either shrinkage of the gel or formation of the precipitate too close to either of the wells.

Microscope slides 3 inches by 2 inches or 3 inches by 1 inch are degreased using chromic acid or a detergent. They are then rinsed and polished dry. After numbering with a diamond, they are coated with 0.2-percent solution of agar and allowed to dry. The gel is made by mixing equal volumes of 2 percent stock agar in water with gel buffer, heating and pouring onto the plates to a depth of 1-2 mm.

The tank buffer (pH 8.6) is composed of the following:

Sodium barbiturate (Veronal)	8.76 g
Diethyl barbituric acid	1.38 g
Calcium lactate	0.38 g
Distilled water	1 liter

The gel buffer (pH 8.6) is composed of the following:

Sodium barbiturate (Veronal)	7.00 g
Diethyl barbituric acid	1.1 g
Calcium lactate	1.0 g
Distilled water	1 liter

Test tubes containing 7 ml of 1 percent gel in buffer are kept in the refrigerator until needed. This quantity is sufficient to cover a 3-inch by 2-inch slide. After setting, small wells are punched in the gel approximately 1.5 mm apart, as shown in Figure 1-14.

Each bank of six holes represents one test. Care should be taken to ensure that the wells are accurately placed along the line of electrophoretic movement.

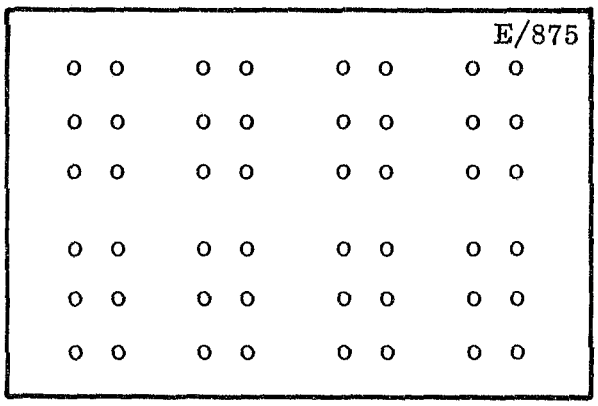

Figure 1-14. Layout of Precipitin Plate (Actual Size)

To produce the correct pattern, a punch obtainable from Shandon Scientific can be used. This cuts the gel and the small plugs of gel are removed using a Pasteur pipette attached to a vacuum. Alternatively, a template can be drawn on a card and placed under the plate and the wells punched out using the pipette and vacuum.

An extract of the bloodstained material is prepared by adding a drop of gel buffer diluted with an equal volume of distilled water to a small quantity of the material and allowing it to soak for 5 to 10 minutes. The ideal extract will appear as a pale straw color. Too strong an extract will cause non-specific precipitation of the control antisera, so it should be diluted with extracting buffer. By means of a very fine Pasteur pipette, the three right hand wells of a bank of six are filled with extract. The three left hand wells are filled with antisera. Normally, two control antisera are used in each test to ensure that the bloodstain is not a mixture of two bloods and that no foreign substance in the material is causing non-specific precipitation. The selection of these two control antisera is largely governed by the circumstances of the case. For example, if a butcher's knife is a suspected murder weapon then the controls would be anticow, antisheep, and possibly antihen and antipig. Similarly in a road accident, the controls should be anticat and antidog. Hence the choice of control antisera must be decided by the worker, taking into account the circumstances of the case and its country of origin.

After the wells have been filled, the slide is inverted onto the tank bridges, consisting of three layers of Whatman 3MM on extended shoulder bridges (Shandon Scientific Company). The stain extracts should be nearest the cathode and the antisera nearest the anode. If the slide is inverted the wrong way, the reactants will be driven apart, not together. Electrophoresis is carried out at 100 to 150 volts for 10 to 20 minutes. A fine line of white precipitate between two holes of a pair represents a positive raction.

Figure 1-15 shows a completed precipitin plate with 30 tests, 10 against each of three antisera—antihuman, antipig, and antisheep.

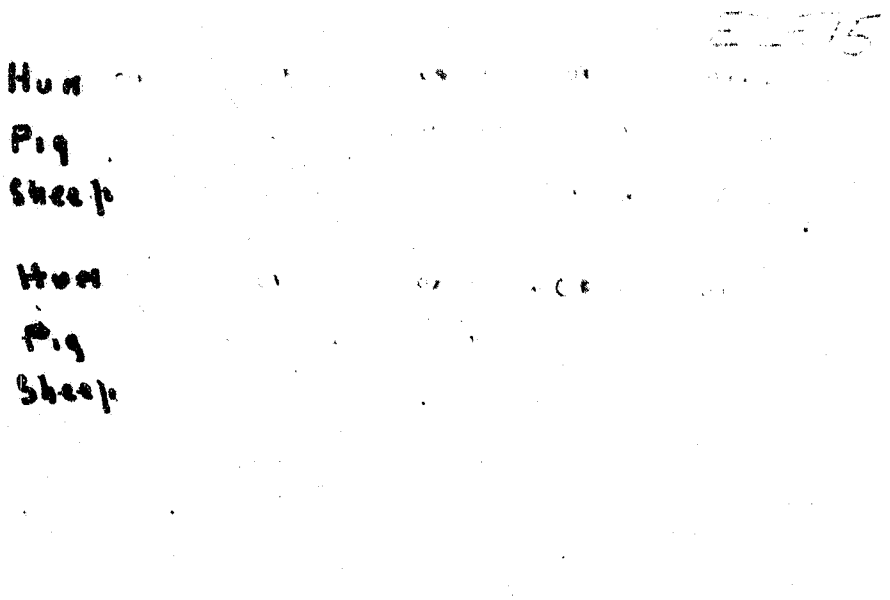

Figure 1-15. Completed Precipitin Plate

After the results have been recorded, the slide is washed in 1 M. saline overnight, rinsed in distilled water, and dried. The slide is then stained in amido black made up as follows:

Amido black 10 B	0.1 g
Methanol	40 ml
Glacial acetic acid	10 ml
Distilled water	50 ml

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After leaving the slide in the stain for 10 minutes, it is differentiated in a wash solution consisting of four parts methanol, five parts distilled water, and one part glacial acetic acid. The precipitates now appear as blue bands. After drying, the slides can be stored as a permanent record.

1.4.5 PROBLEMS

The most frequently encountered problem is the extract being too strong and causing non-specific precipitation. For example, human serum at a dilution of 1:400 will cause precipitation with anticow serum.

Antihuman semen serum can be used when seminal stains are encountered, but it has been found that most of these antisera react with vaginal epithelium, so care should be taken when dealing with this determination.

SECTION 2

RED CELL ANTIGEN SYSTEMS

2.1 INTRODUCTION

Many text books have been written on this subject and there is very ample coverage of both the theoretical aspects and the practical applications in terms of grouping fresh fluid blood samples. It would be superfluous to deal with matters so adequately documented elsewhere. It is therefore proposed to confine this section to the particular problems facing the forensic serologist. For the most part, this is a matter of detection of antigens in dried bloodstains, but it also involves the grouping of lysed blood samples and human tissue (e.g., fragments adhering to vehicles involved in fatal road accidents).

In fluid blood samples the presence or absence of an antigen is ascertained by whether or not the red cells are agglutinated. In dried bloodstains, the cells have ruptured and, therefore, direct agglutination tests are no longer feasible. However, the antigens are not immediately denatured upon drying. Indeed, in the ABO system they survive for many years and retain the capability of combining with specific antibodies. The formation of these antigen-antibody complexes is the basis of all methods employed in the detection of red cell antigens in dried bloodstains. There are several methods of detecting this.

Absorption-inhibition is the classical method of detection of antigens in dried bloodstains. This has been in use for several decades. Aliquots of antisera are added to portions of stains or extracts thereof and, after a suitable period of absorption, the residual sera are tested with red cell suspensions of appropriate groups (e.g., group A cells are added to anti-A serum) and the degree of agglutination is compared with that of controls tested in parallel from the unstained substrate. A substantial reduction in titre of serum added to stained material compared with that of serum in the control indicates the presence of an antigen. For this test, the antisera are usually diluted, but nevertheless, it is necessary for a very high proportion of the antibody content to be absorbed by the red cell antigens in order to obtain a significant reduction in titre. This method is relatively

insensitive when compared with more recent techniques and, in practice, has proved satisfactory only for grouping in the ABO system.

In recent years, methods have been evolved in which, instead of testing residual serum, reactions are obtained directly from the absorbed antibody. Theoretically, it is obvious that such an approach should lead to greater sensitivity and this is born out in practice. To detect a positive reaction, it is only necessary for a very small proportion of the antibody content of the serum to be absorbed, and also it is unnecessary to obtain absorption of all of the antibody components.

One such method is mixed agglutination[15]. A high proportion of bloodstains which are grouped occur on textile fabrics and the corpuscular debris containing the antigenic material adheres tenaciously to the fibres. By this method, finely teased bloodstained fibres are submerged in antiserum and, after a period of absorption, the unbound antibody is washed away leaving only antibody which has combined specifically with the antigens. The fibres are then suspended in indicator cell suspension of appropriate group and rotated. During this period, agglutination occurs between the absorbed antibody and the indicator cells so that, in the presence of a specific antigen, the fibres, when viewed with a microscope, are seen to be coated with a layer of red cells of the same specificity as the stain. This method has proved very satisfactory for the ABO system and can be used on extremely small stains, but, for maximum sensitivity, it is necessary to use specially selected high titre immune anti-A and anti-B sera and the group O indicator cells must be pretreated with enzyme (e.g., papain) in order to detect H antigen. The method has not been found suitable for Rh or MN typing.

Another means of direct testing for absorbed antibody is that of absorption-elution. After absorption and subsequent washing away of unbound serum, the combined antibody is eluted, usually by raising the temperature to about 56°C. The eluate is then tested with indicator cells of the appropriate group; agglutination indicates the presence of an antigen of the same specificity as that of the indicator cells. A variety of adaptations of this basic principle is in use and some of these will be discussed later.

Absorption-elution has proved highly satisfactory for ABO grouping where it is possible to group stains of 1-mm diameter. It is also in routine use for MN and Rh typing in dried bloodstains. Detection of the antigens K, k^{16} and S^{17} have also been reported. This method is both more sensitive and more versatile than mixed agglutination. One possible reason for the greater sensitivity is that in mixed agglutination, reactions are only obtained from the antigenic sites exposed on the surface whereas in absorption-elution the submerged sites are also included. It is also likely that red cells agglutinate more readily with each other than with debris adhering to fibres.

Another obvious approach in the grouping of dried bloodstains is the use of labelled antibody. This can be achieved either by conjugation with a fluorescent dye or with radioisotopes. Theoretically, these methods should be infinitely more sensitive than those previously described. In practice it is difficult to obtain clear-cut negative reactions. For this reason neither method is in routine use for red cell antigen detection. However, it is likely that these techniques may be improved.

2.2 ABO SYSTEM

2.2.1 METHODS

In 1960, S. S. Kind introduced absorption-elution for the detection of ABH antigens[19,20]. The original method was crude but modifications of this principle have been described by a number of workers including Kind[18,21,23,24,25,29]. In the Metropolitan Police Laboratory, the method of Nickolls and Pereira[24] was in use for many years. This facilitated the grouping of very small stains using short pieces of bloodstained threads, the entire test being carried out on cavity slides. To achieve satisfactory removal of unbound antibody, the threads were teased into individual fibres and each tuft of fibre was rinsed separately using a wash bottle containing chilled saline and a pipette attached to a suction pump for removal of the saline. It was a matter of some skill to ensure that the fibres were adequately washed without being lost! When dealing with large numbers of stains it was also very time consuming.

A modification by Howard and Martin[23] has provided a very satisfactory solution to these problems. The tests are carried out on cellulose acetate sheet with the pieces of thread being firmly secured to it with an adhesive of cellulose acetate in acetone. The washing then can be achieved by soaking the entire sheet in chilled saline without fear of losing the threads. Furthermore, because it is possible to allow prolonged soaking in chilled saline, it has been found unnecessary to tease the fibres. Bulk handling of a fairly large number of stains is possible by this method.

Another method which facilitates a batch procedure is that of Outteridge[25]. He recommends working on water extracts of stains that are fixed by immersing in a buffer solution at 99°C. The use of an extract has the advantage of ensuring that precisely the same quantity of material is tested against the various antisera. There is a serious disadvantage, however. A high proportion of the corpuscular debris remains firmly attached to the fabric and cannot be utilized.

The use of 5 percent ammonia solution for extracting stains[21] is far more efficient for the purpose. It is argued by Kind that, with such an extraction method,

it is possible to expose a large surface area of antigenic material to antiserum and because of this, greater sensitivity can be achieved. It is highly probable that a more rapid antigen-antibody raction will occur, but Kind's argument that antigen-antibody reaction takes place only at the solid/liquid interface is probably unfounded when dealing with undenatured stains. Unfixed protein will absorb water and it is likely, therefore, that serum antibodies can penetrate to unexposed sites given adequate time. Comparative studies on several series of stains produced by double-diluting whole blood have shown that the Howard and Martin method is more sensitive than the ammonia extraction method[26]. There is a very marked decrease in sensitivity in reactions against anti-H, although those for anti-A and anti-B are less seriously affected.

Fiori[18] recommended fixation of bloodstained fibres with methyl alcohol prior to absorption-elution. He claims that weaker reactions are obtained from unfixed material. Experience at the Forensic Science Laboratory is a complete contradiction of this. All forms of fixation cause a reduction in antigenic sensitivity. This may be because only surface antigens are involved in the reaction, but the possibility of an adverse effect on the antigens cannot be excluded. The disadvantages of fixation are even more apparent with the MN and Rh systems.

2.2.2 HOWARD AND MARTIN TECHNIQUE

2.2.2.1 Materials

The materials used for this technique include the following:

a. Humid Chambers—Polystyrene sandwich boxes (obtained from Woolworth's) used upside down are ideal for this. The tops are lined with damp blotting paper.

b. Cellulose Acetate Sheets—The optimum thickness is 0.4 mm. They are cut to fit the humid chambers and should be ruled out into a grid of approximately 1.5-cm squares using a wax pencil. Alternatively, the sheets can be moulded into a series of cavities using a suitably constructed press.

c. Cellulose Acetate Adhesive—The proportion of powdered cellulose acetate to acetone is to some extent a matter of personal choice.

Respective proportions by volume of 1:2 are satisfactory. It is essential that the adhesive should be sufficiently viscous to avoid absorption by the threads, since this results in a coating of cellulose acetate on the threads and negative reactions at the conclusion of the test. On the other hand, if the adhesive is too viscous, handling becomes very difficult. During use, there is evaporation of acetone which can be replaced from time to time.

d. Serological Reagents

Anti-A (α)

Anti-B (β)

Anti-A + B ($\alpha\,\beta$)

Anti-A and Anti-B sera which are normally used for grouping whole blood are satisfactory for this purpose. Selection of anti-A + B serum is desirable. This is discussed later.

2.2.2.2 Anti-H Lectin

The anti-H lectin is an important ingredient in the Howard and Martin technique. This is an extract of gorse seeds (ulex europaeus) in normal saline. The seeds are ground in a pepper mill and 10 gm of crushed seed are added to 100 ml of saline and left at 4° C for a few days. The extract is then passed through a muslin bag to remove gross particles and centrifuged. Following this, the supernatant is flocculated at 60° C for 30 minutes and re-centrifuged.

The supernatant is tested against A_1B and O cells. A negative reaction should be obtained from A_1B cells and strong agglutination from the O cells. A preparation having a titre of less than 1/32 is unlikely to be suitable for dried stain work. By this method of extraction, providing the seeds are in good condition, it is usually possible to obtain titres of 1/128 or 1/64. In the experience of the Metropolitan Police Laboratory, anti-H prepared by this simple method is superior to the purified commercial products.

Suitable seeds can be obtained from Thompson & Morgan Ltd., Ipswich, England. It is advisable to check the preparation against some known stains before finally accepting it as suitable. It should then be bottled and stored at -20° C.

2.2.2.3 Indicator Cells

A_1, B and O cells are thrice washed in normal saline and very weak suspensions, pale pink in color, are prepared in 0.3 percent bovine albumin in saline (i.e., 1 percent of a 30 percent commercial preparation). This diluent helps to avoid lysis during the elution phase and also increases the sensitivity to a small degree. It is advisable to select suitable donors of O cells since some show greater sensitivity to the lectin than others. This can be done by comparative titration against anti-H, those producing the highest titres being most satisfactory. It is ideal to take about 10 donors from which to make the selection. The A, B, and O cell suspensions should be freshly prepared each day.

2.2.2.4 Method

The following method is used for the Howard and Martin technique:

1. Cellulose acetate sheet is ruled out and labelled as shown in Figure 2-1.
2. Five pieces of bloodstained thread and five unstained threads are secured to the sheet in the appropriate squares by inserting one end into a spot of cellulose acetate adhesive. The optimum length of thread protruding from the adhesive is about 2 mm, except for very fine fabrics when the length should be increased a little.
3. After the adhesive has hardened, which is complete in 5 to 10 minutes, a drop of the appropriate antiserum is added to each square, making certain that each thread is submerged in serum.
4. The sheets are placed in humid chambers and left at 4° C for a minimum period of 2 hours. For maximum sensitivity, overnight absorption is preferable.
5. The serum is rinsed off the sheets by spraying from a wash bottle of chilled saline. Each sheet is then blotted dry and immersed in a tank of saline at 4° C and left 20 minutes. Preferably, there should be constant agitation of the saline during this period but it is adequate to agitate the sheets occasionally.
6. The sheets should then be removed from the saline and blotted dry. A drop of appropriate indicator cells added to each square (i.e., A_1 cells to the threads treated with anti-A and one of each pair

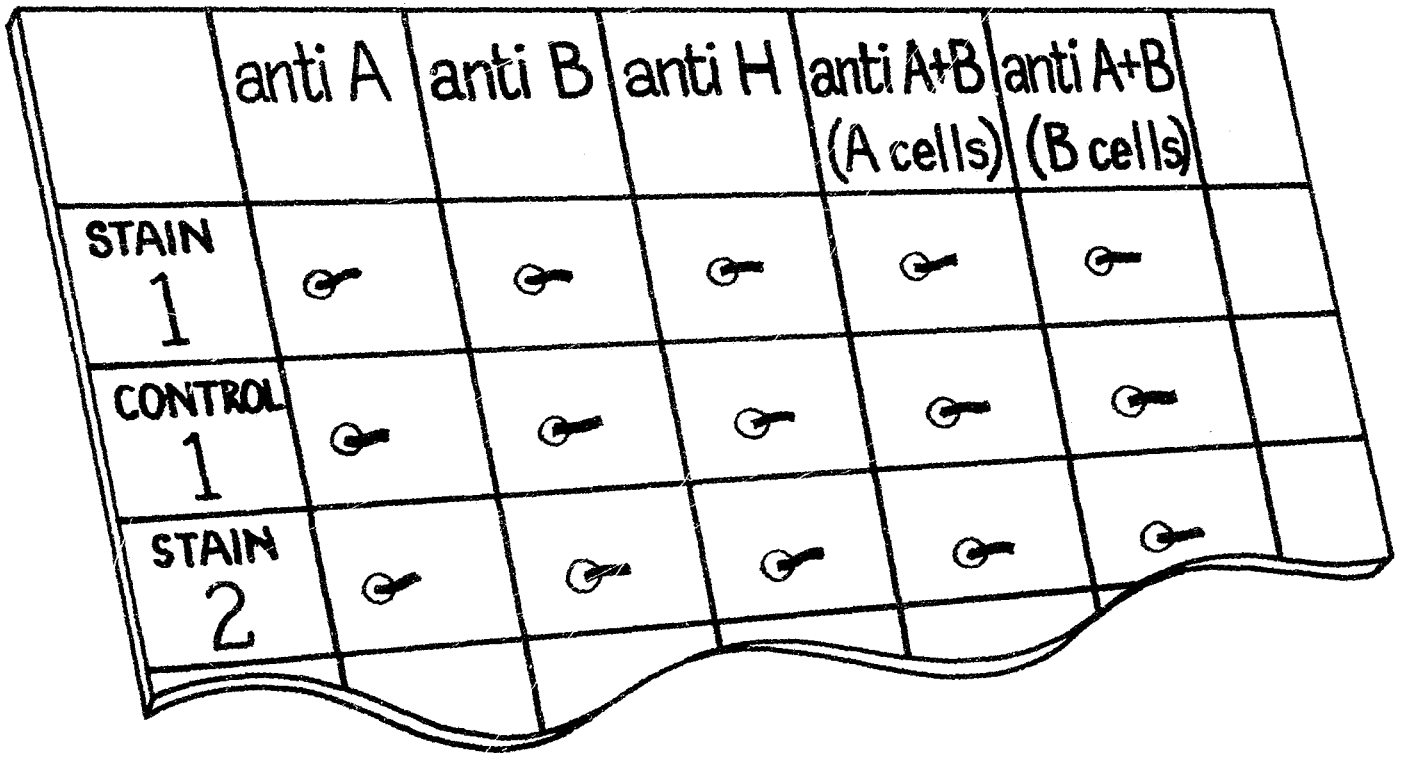

Figure 2-1. Lay-out of Cellulose Acetate Sheet for Howard and Martin Technique

treated with anti-A + B, B to those treated with anti-B and the second of each pair treated with anti-A + B, and O cells to those treated with anti-H).

7. The sheets are replaced in humid chambers and left in an incubator at 50° C for 15 minutes. During this period absorbed antibody is eluted.
8. The humid chambers should then be put on a low speed rotator and left for 30 minutes at room temperature for the development of agglutination.
9. The degree of agglutination is read using a microscope. With heavy stains it is not uncommon to find large agglutinates and no free cells. With weaker stains the agglutination is correspondingly less.

2.2.2.5 Grouping of Stains

In experienced hands, the grouping of dried bloodstains in this system presents relatively few problems. The antigens are very stable and the concentration of antigenic sites is high. With some stains it is also possible to confirm the antigen results by detection of serum antibodies. However, because these antigens also occur in a water soluble form in other body fluids, there can be a problem caused by reactions from the unstained substrate. For example, this may arise when dealing with garments where sweat from the wearer gives specific reactions. Because of this, the necessity for adequate controls from an unstained portion of the item cannot be over-emphasized. These should be taken from as near as possible to the bloodstain. Despite the greater sensitivity of absorption-elution over absorption-inhibition, difficulties caused by control reactions are more common with the latter. This may be because of the water soluble nature of the antigens in sweat.

The possibility of obtaining false positive reactions caused by bacterial contamination cannot be ignored but, in practice, this is not a problem. However, in the case of large stains that have dried slowly there can be a loss of antigenic activity. It is advisable, therefore, to take the material for grouping from the edge of such stains or, in some circumstances, it may be possible to group from a discreet stain on the same item.

When bloodstains occur on non-absorbant surfaces, it is necessary to absorb the dried blood onto a piece of cotton thread moistened with distilled water. It is preferable to use threads of woven cloth from white cotton items which have been laundered many times because the presence of dressings can give rise to weak control reactions. It is obviously necessary to obtain an even distribution of blood along the thread. A control should be taken from an unstained part of the surface in the same manner. The threads should be dried as soon as possible and moderate heat (i.e., up to 50° C) is an advantage. It is also necessary to adopt the same approach with waterproofed fabrics because the proofing material prevents the normal degree of adhesion of corpuscular debris to the fabric.

2.2.2.6 Detection of A_x in Stains

The use of anti-A + B serum is a refinement that is not absolutely necessary and was not included in the original method. It has been incorporated for two reasons. First, it is possible to detect A_x; whereas, without its use, such stains would be grouped as O because, as with whole blood samples, A_x is not detected by normal anti-A. Since A_x is very rare this is not a serious problem. Second, it provides confirmation of reactions obtained by the anti-A and anti-B sera. In this context it is not entirely satisfactory because a high proportion of group O sera cross react when used in an absorption test (i.e., a group A stain will absorb not only the anti-A fraction of the serum but the anti-B fraction also). With a serum of a cross reacting type, in addition to detection of A_x, its use is limited to a confirmation that anti-A and/or anti-B sera have not failed to react. It may therefore be argued that testing stains with a cross-reacting serum and eluting into B cell suspension is of little value. However, by careful selection, it is possible to obtain anti-A + B sera which show little or no cross reaction. Some commercial anti-A sera give weak positive reactions with A_x stains. This may be because of the presence of immune anti-A.

Obviously, if bloodstains are very small it is possible to test using only anti-A, anti-B, and anti-H sera. In these circumstances the bloodstained threads should be secured by inserting an unstained portion into the adhesive.

2.2.2.7 Diluted Bloodstains

Dilute bloodstains can present a problem. If they are diluted to a considerable degree with water there is usually no difficulty in detecting the antigens present. However, if they are diluted with saliva or semen there is a risk of wrong interpretation. This is only too obvious if the blood and saliva or semen originate from different individuals but, there is also a danger of erroneous interpretation if they originate from the same individual, due to the possible presence of excess water soluble antigens. This problem will be discussed further when dealing with saliva and semen grouping.

2.2.2.8 Grouping Fragments of Muscle, Skin, Tissue

Fragments of muscle, skin, etc., usually can be grouped using the Howard and Martin method. These are commonly contaminated with grease or fat. A rapid wash in methyl alcohol followed by a wash in ether is usually adequate to remove this. The sample is then dried, cut into thin strips about 3 mm in length, and secured to cellulose acetate sheet in the normal manner. After absorption, the wash period is increased to one hour. Otherwise the procedure is exactly as previously described.

2.2.2.9 Grouping of Lysed Blood Samples

Lysed blood samples can be tested by immersing pieces of cotton thread into them and, after drying, the usual procedure can be followed. Results obtained from samples showing obvious signs of putrefaction must be viewed with circumspection.

2.2.3 SUBGROUPS OF A

Various methods have been described for distinction between A_1 and A_2 stains. Poon and Dodd[28] reported the use of mixed agglutination in conjunction with Dolichos biflorus lectin. Hayward[22] has given an absorption-elution method also using Dolichos biflorus. Experience at the Forensic Science Laboratory shows that neither of these methods gives completely unequivocal results. The use of anti-H lectin has also been advocated on the basis of the strong positive reactions obtained from A_2 stains compared with negative or weak reactions usually obtained from A_1 stains[26]. Without doubt, anti-H reactions give a strong indication

of the sub-group, but it is unwise to form a definite opinion on this basis alone. A potent human anti-A_1 is probably the most likely answer to this problem, but such a serum is, to say the least, difficult to find. A completely reliable method for A_1/A_2 distinction in dried stains has yet to be developed.

2.3 MN SYSTEM

The use of absorption elution for the detection of MN antigens in dried bloodstains was reported by Pereira[32] and also by Fiori[18] in 1963. The techniques involved are similar to those used for ABO grouping and the Howard and Martin modification[23] provides the same advantage. Immune anti-M and anti-N produced in rabbits are used together with MM and NN indicator cells.

In the detection of MN antigens in dried bloodstains, there are serious problems that are not encountered in ABO grouping. These are inherent in the system and a simple solution is unlikely. Without delving too deeply into theoretical concepts, it is well established that some N is normally present on cells of MM individuals[30,31,33], and it is reasonable to assume that N is the basic substance and the precursor of M. This is the fundamental cause of the difficulties encountered in dried stain grouping in this system. It is also undoubtedly the cause of the great difference in quality between the anti-M and anti-N sera produced in rabbits.

In practice, it is found absolutely essential to carefully select suitable antisera for work on dried stains. The only satisfactory means of selection is to make tests using a range of stains of known group and of various ages. The titre of the serum is no guide as to suitability. Some of the best sera have been of relatively low titre. Of those tested, approximately 50 percent of anti-M sera have been found to be ideal, giving no indication of cross reaction, yet being highly sensitive. Of anti-N sera, less than 10 percent have been satisfactory. Reactions obtained from some are very weak with a serious risk of failure to detect N, particularly in MN stains. Others show cross reactions with MM stains to varying degrees. In some this is only slight but with others, there is little distinction between stains of MM and MN. It is also found that some MM individuals show a higher level of cross reaction than the majority. Stains from a donor of this type should always be included in tests for selection of antisera. Another hazard involved in dried stain grouping in this system is that, because of the greater potency of anti-M compared with that of anti-N, in an old MN stain it might be possible to detect M but not N.

In selecting sera it is also necessary to determine the optimum period for absorption and also the optimum temperature. Some sera require overnight absorption, while others are specific only when absorbed for a shorter period (i.e., 3 hours). Some sera are more specific when absorption is at room temperature and others are lacking in sensitivity unless absorbed at 4°C.

With each batch of stains under test it is necessary to include controls of stains of the three groups, including a stain from a cross-reacting type of M. Results obtained from the unknown stains can only be regarded as significant if the reactions from the control stains are completely satisfactory. For the grouping of fresh fluid blood samples, anti-N lectin obtained from the seeds of vicia graminae is highly potent and frequently used as a substitute for anti-N serum. It has proved unsatisfactory for absorption-elution techniques because of strong cross reaction.

The problems outlined above are formidable but nevertheless, providing antisera are carefully selected and rigid control tests are made, accurate grouping is possible from recent stains (up to 4 to 6 weeks old) in good condition. On some occasions, even though the group of a stain cannot be definitely established, one can exclude the possibility that it has originated from a certain individual. For example, if a stain gives strong positive reactions for M and weak positive reactions for N, the stain may have originated from a cross reacting MM individual or it may be an MN stain in which, perhaps because of age, the N is only reacting weakly. It is absolutely certain, however, that the person who produced that stain was not of group NN. In Britain this means that more than one-fifth of the population could be excluded.

In view of the problems in this system, interpretation of results should be made with great caution. When weak reactions are obtained, no positive identification of the group can be made. Dilute stains are likely to be unsatisfactory, particularly stains contaminated with saliva, semen, or other body fluids. Stains that have dried slowly usually lose all detectable trace of M and N antigens. The advantage of MN grouping of dried bloodstains compared with many other systems is that first, only a small bloodstain is necessary and second, there is a favourable distribution of the three major groups as compared with the Kell system, for instance, where over 90 percent of individuals are of the same type.

2.4 RHESUS SYSTEM

2.4.1 ADAPTION OF ABSORPTION-ELUTION

Because of the complexity of the Rh system, it is potentially capable of providing results of greater significance than any other single red cell antigen system or indeed any other form of blood typing with the possible exception of Gm. It has therefore been the subject of research in forensic serology over many years. With the advent of absorption-elution, the prospect of full Rh geno typing in dried bloodstains became more feasible. In 1967, Bargagna and Pereira[34] published a report on the use of absorption-elution for the detection of Rh antigens in dried bloodstains. In the intervening period, improvements in technique have been made and Rh geno typing of adequate stains is now a routine procedure in the Metropolitan Police Laboratory.

The simple absorption-elution technique used for ABO and MN grouping is, for a variety of reasons, unsuited to the Rh system although the general principle involved is precisely the same. The quantity of bloodstain required is very much greater than with the ABO and MN systems. This is largely because of the lower concentration of Rh antigenic sites on the cell membrane. The use of larger portions of bloodstain in turn necessitates a more prolonged washing procedure.

In grouping fluid blood samples in the Rh system, slide techniques are less satisfactory than grouping in tubes. For working on dried stains the use of tubes is essential. Apart from the risk of loss of sensitivity because of the use of slides, the evaporation problems during the absorption period would be enormous in view of the optimum temperature of 37° C. Elution directly into indicator cell suspensions is impossible because, after such exposure to heat, cells are no longer capable of being agglutinated by anti-Rh sera. It is therefore necessary to elute into saline. The absence of cells during elution gives greater latitude regarding duration and temperature as there is no limitation imposed by red cell fragility. An increase of both duration and temperature produces more efficient elution.

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2.4.2 SELECTION OF ANTISERA

As with MN grouping of dried stains, selection of antisera is important, but there is less difficulty in obtaining suitable sera. While it is possible to use complete antisera, incomplete sera are more satisfactory. Sensitivity is generally greater and specificity is more certain. Unlike anti-M and anti-N sera, those of highest titre are usually most sensitive under absorption-elution conditions. Incomplete sera (slide test) marketed by Hyland, Dade and Ortho have been found very satisfactory. Ortho sera tend to be more viscous than others and therefore, additional care is necessary in washing away unbound antibody. Because of the sensitivity of absorption-elution and the fact anti-Rh sera may contain more than one antibody, it is necessary to test each new batch of serum with stains from a wide range of Rh groups before putting into routine use.

For full genotyping, the following sera are necessary: anti-C, -$\bar{c}$, -D, -E, and anti-e. Anti-C^W is also commercially available and capable of detecting the C^W antigen in dried bloodstains. Anti-$\bar{c}$ and anti-D are usually more potent than anti-C, anti-E, and anti-e.

The quantity of bloodstain necessary is dependent upon the quality of the antisera in use. This means that it is usually possible to detect $\bar{c}$ and D antigens from a smaller quantity of stain compared with antigens C, E and e. For reasons of economy of bloodstain and, more important, to avoid the risk of detecting some of the antigens in a stain and missing others, it is necessary to attempt to restore the balance between the degree of reaction of the five antigens by suitably adjusting the relative proportion of stain tested with each antiserum. In practice, portions of stain 2 to 3 mm^2 have been found suitable for testing against anti-$\bar{c}$ and anti-D. The quantity required for $\bar{c}$ is usually a little less than for D. Stain portions of 4 to 5 mm^2 are usually adequate for C, E and e. Obviously, this is to some extent dependent on the nature of the bloodstain and the thickness of the fabric. As the quality of antisera varies so much, proportions must be readjusted to the serum in use and no hard and fast rules can be made. If certain antisera are of low titre, the balance can also be adjusted by increasing the quantity of serum used. Because the quantity of bloodstained material is large, there is difficulty in washing out unabsorbed serum. It is therefore preferable to work

with all sera diluted. With anti-C, -E and -e, dilutions of 1/2 are usually adequate but for some anti-$\bar{c}$ and anti-D sera it may be necessary to dilute as much as 1/20.

When incomplete antisera are used, any of the established techniques recommended for such sera can be employed for testing the eluates. The albumin addition method is simple but less sensitive than enzyme techniques. The indirect Coomb's test can also be applied but this is time consuming and not well suited for testing large numbers of stains. A highly satisfactory alternative to conventional methods is the use of the Technicon Antibody Screening Auto-analyzer.

2.4.3 MANUAL TECHNIQUES[27]

There are two manual techniques in the rhesus system, (1) using bovine albumin, and (2) using papain. The materials necessary include the following:

a. Tubes—Glass or plastic tubes approximately 8 mm in diameter are suitable.
b. Anti-Rh Sera—High titre incomplete anti-C, -$\bar{c}$, -D, -E, and anti-e are used. These should be tested for suitability using stains of a wide range of Rh genotypes.
c. Indicator Cells—Freshly prepared R_1 R_1 and R_2 R_2 are used.
d. Bovine Albumin—30 percent bovine albumin is recommended.
e. Papain[35]—A stock solution of papain is prepared as a 1-percent suspension in saline and is stored at -20°C. For use, the stock solution is diluted 1/10 with M/15 Sorensen buffer of pH 7.3.
f. Racks—These should accommodate the tubes in rows of five so that each row can be labelled for one of the antisera and rows at right angles can be numbered.

The following is a description of the method.

1. Divide stains into five suitable portions and place in tubes in the rack.
2. Prepare controls from an unstained portion of the fabric.
3. For positive controls, take five portions of an R_1 R_2 stain and for negative controls take portions of stains lacking the antigens corresponding to each antiserum.

4. Record the sequence of stains and controls in the racks.
5. Add one drop of appropriate antiserum to each cup and dilute with a suitable quantity of saline.
6. Ensure that all of the pieces of fabric are immersed in diluted antiserum, cork the tubes, and leave overnight in a 37° C incubator.
7. After removal from the incubator, fill the tubes with saline, remove the saline/serum mixture, and replenish with fresh saline. For cotton, linen, woolen, and all heavy fabrics, exchange saline six times during two hours. For delicate fabrics and lightweight synthetics, the duration of washing should be reduced. For very lightweight nylon fabric, half an hour is adequate.
8. Remove saline from all tubes and add two drops of fresh saline to each.
9. Immerse tubes in a water bath at 60° C for 40 minutes and agitate occasionally.
10. Take racks from the water bath, agitate vigorously and remove the pieces of cloth from the eluate as quickly as possible using a fresh piece of applicator (swab) stick for each tube.

Albumin Method

10a. Prepare indicator cells as 3 to 5 percent suspensions. Add one drop of $R_1 R_i$ cell suspension to each of the tubes originally treated with anti-C and anti-e. Add one drop of $R_2 R_2$ cell suspension to each of the remaining tubes. Place the tubes in a 37° C incubator for 1-1/2 hours.

11a. Remove the tubes from the incubator and run a little 30 percent bovine albumin down the side of each tube taking care to avoid disturbing the button of cells at the bottom. The albumin should then form a layer between the cells and the supernatant. Replace the tubes in the 37° C incubator and leave for 1/2 hour.

12a. Remove the tubes from the incubator. Taking up the contents from each tube in turn, streak out on a microscope slide and, in the absence of strong positive macroscopic agglutination, examine under a microscope. Record the results.

Papain Method

10b. During the washing or elution stages, treat the indicator cells with papain as follows: after washing the cells three times, add one volume of packed cells to two volumes of buffered papain and incubate for 10 minutes at 37°C. Wash the cells three times and resuspend in normal saline as a 3 to 5 percent suspension.

11b. Add one drop of appropriate indicator cells to each eluate and incubate for two hours.

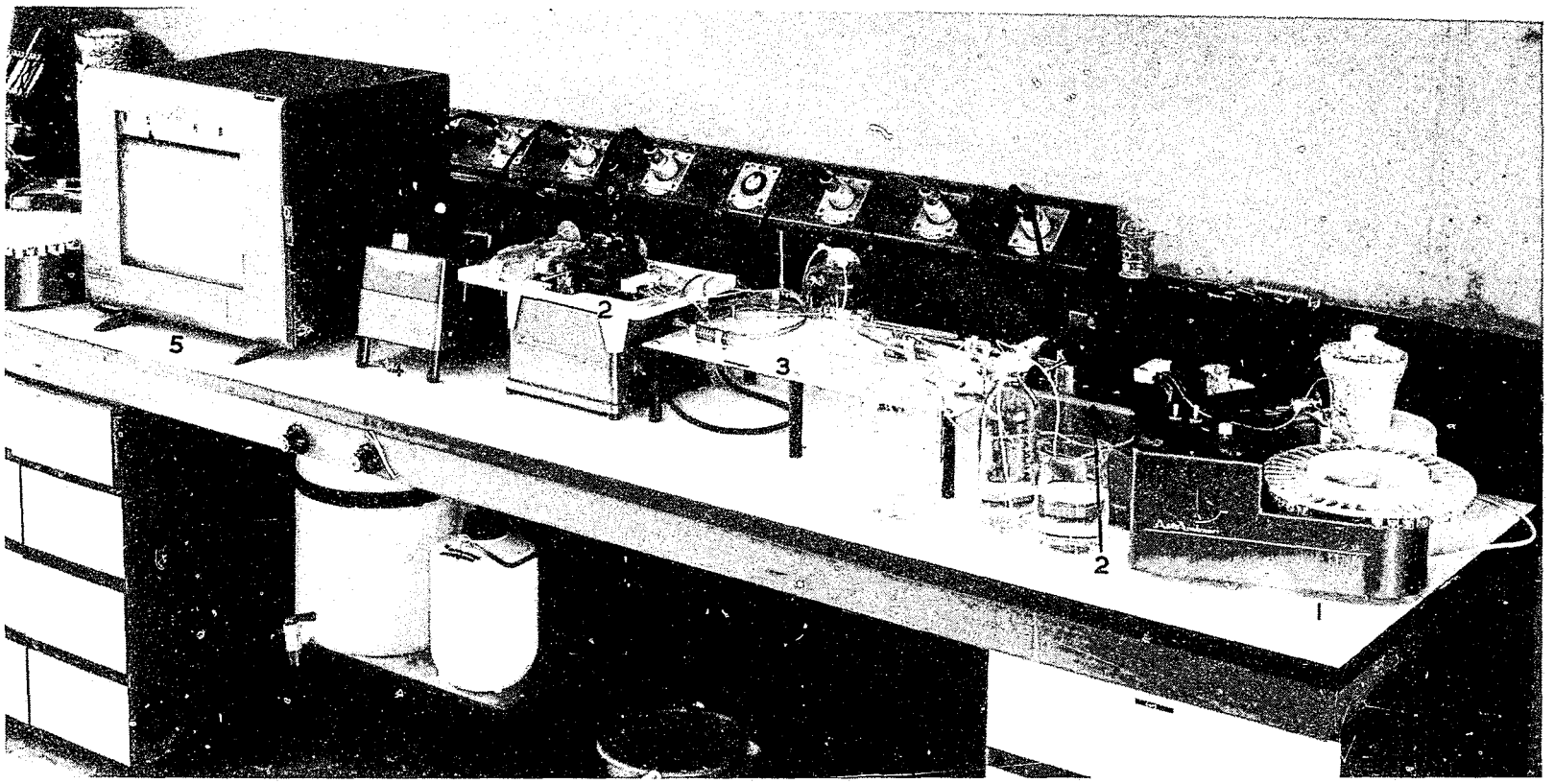
12b. Remove the tubes from the incubator and examine for agglutination as with the albumin method.

2.4.4 AUTO-ANALYZER[36]

In the Rh system, the auto-analyzer (see Figure 2-2) designed for antibody screening is capable of detecting very low concentrations of antibody and its sensitivity is considerably greater than that of normal methods. It is therefore well suited to testing the eluates produced in the typing of dried bloodstains.

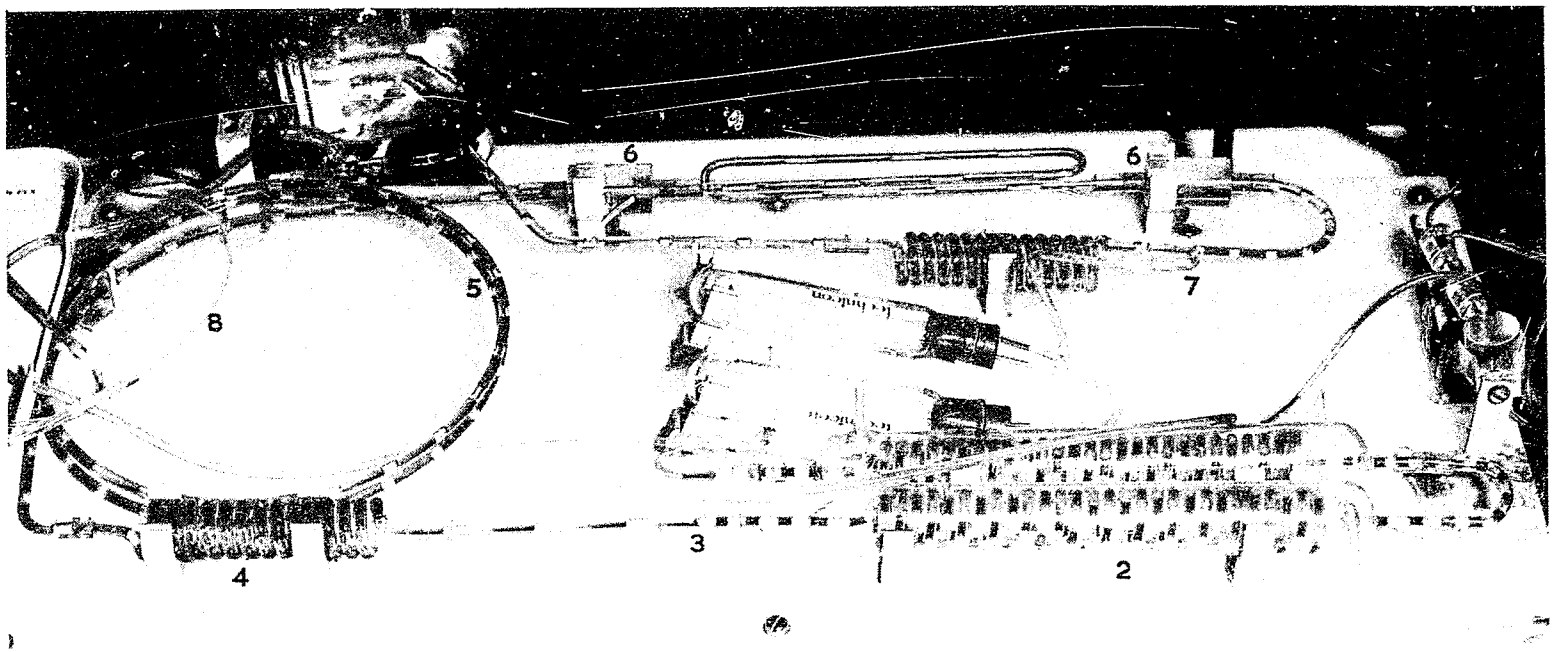
The auto-analyzer is a continuous flow system driven by peristaltic pumps with the flow divided into segments by air to combat carry over between samples. The flow rate of the various liquids in the system is controlled by the diameter of the pump tubes. Indicator cells of group R_1 R_2 are supplied continuously together with solutions of bromelin and methyl cellulose.

The test samples are introduced in rotation from cups in a revolving tray. As the various reagents enter the system, they pass into a mixing chamber and subsequently into a pair of reaction coils (see Figure 2-3). During the passage through these coils, rouleaux formation develops rapidly due to the methyl cellulose. In this condition, the cells are in close physical contact and, in the presence of antibody, the degree of agglutination developed is much greater than in the absence of rouleaux. As the flow emerges from the second reaction coil, saline is introduced in sufficient quantity to break down rouleaux so that only true agglutination remains. After passage through a delay coil to ensure complete elimination of rouleaux, the flow enters a wide settling coil and the agglutinates fall gradually to the bottom of the coil. In the absence of agglutination,



1—Sampler, 2—Proportioning pumps, 3—Glass manifold, 4—Colorimeter, 5—Recorder

Figure 2-2. Technicon Antibody Screening Auto-Analyzer



1—Mixing chamber, 2—Reaction coils, 3—Saline entry point, 4—Delay coil for dispersal of rouleaux, 5—Settling coil, 6—T-junction decanters, 7—Triton entry point, 8—Tube connected to colorimeter via second proportioning pump

Figure 2-3. Glass Manifold

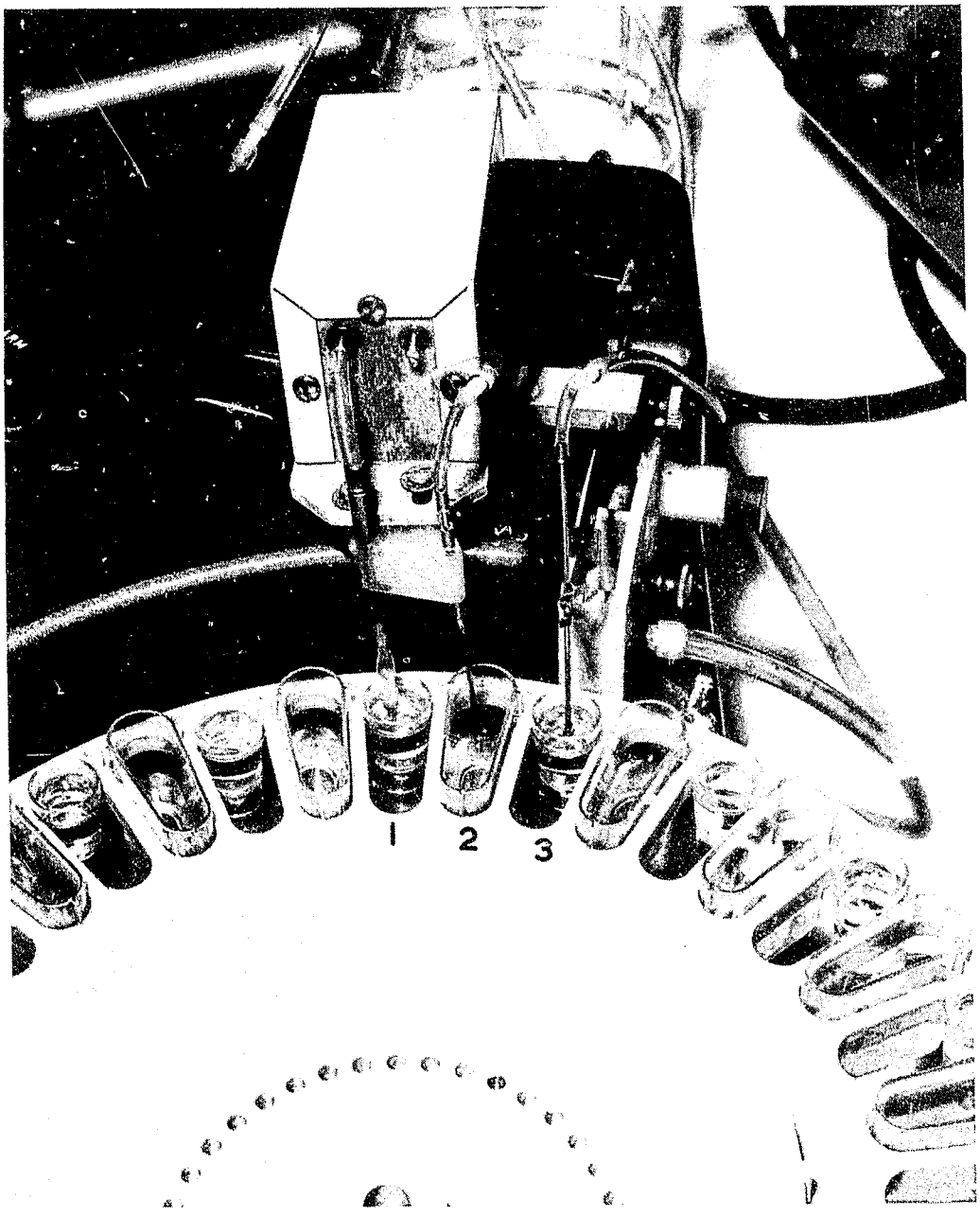
the stream remains homogeneous. On leaving the settling coil, the flow passes over a pair of T-junction decanters through which a constant volume of the flow is removed. All agglutinates are thus removed so that the concentration of cells present in the flow at this stage is inversely proportional to the degree of agglutination, and hence to the concentration of antibody present in the respective samples. A haemolytic reagent is then introduced and, after completion of lysis, a sample of the flow passes through a colorimeter and the relative concentrations of haemoglobin are charted by a recorder. In the absence of agglutination, the haemoglobin concentration is constant and a relatively high optical density is obtained. This forms the baseline on the recorder chart. Optical density is reduced because of the loss of haemoglobin and thus, where reactions have occurred, peaks are formed in proportion to the degree of agglutination.

In addition to increased sensitivity, other advantages in the use of the auto-analyzer are the economy of labour and the fact that the assessment of the degree of agglutination is truly objective. The apparatus is, however, very costly and some experience is necessary before it can be run with efficiency.

2.4.5 AUTO-ANALYZER USE FOR ABSORPTION-ELUTION[27]

2.4.5.1 General

One of the most important and potentially time consuming operations in absorption-elution is the washing away of unbound antibody. By using auto-analyzer equipment, it is p ,sible to construct a washing machine for this purpose (Figure 2-4). It consists of a peristaltic pump and a sampler fitted with a 120/hour adjustable cam in which the wash phase spaces are reduced to a minimum. The 2-ml cups containing pieces of fabric to be washed are placed in the circular sample tray in sequence but alternating with 8-ml cups. All of the cups are filled with saline. The sampler is equipped with a stirrer and two probes set up so that after each cup is stirred, saline is drawn off through the first probe and fresh saline is added by the second. The suction probe is coupled to a pump tube through which the flow is 3.9 ml per minute and for the second probe, the flow rate is 2.9 ml per minute. Each piece of cloth remains soaking in saline during the rotation of the sample tray. The 8-ml cups are included to avoid the pieces of cloth being



1—Stirrer, 2—Suction probe, 3—Probe dispensing saline

Figure 2-4. Washing Machine

carried over into the next sample. In the event of the suction probe carrying over a piece of cloth, the latter is trapped by sinking to the bottom of the 8-ml cup and can easily be restored to its correct position. The sample trays accommodate 40 cups and, with a 120/hour cam, it is possible to carry out three changes of saline per hour. If required, two or three samplers can be operated by one pump. This washing method is unsuitable for loosely woven fabrics or cotton wool. In such cases, a manual method must be employed.

With an automated technique, it is obviously essential that each sample can be accurately related to a particular part of the recorder chart. For this reason, a regular routine should be developed and the sequence of samples recorded and rigidly maintained. The absorption is set up in 2-ml auto-analyzer cups and each piece of cloth remains in the same cup until after elution when the cloth is removed but the corresponding eluate remains. Metal racks are available which accommodate 2-ml auto-analyzer cups in rows of five and are ideal for use with the five anti-Rh sera. Each row therefore can be used for either a complete test on a bloodstain or its control and be labelled accordingly.

If washing is carried out by the automated method, it is necessary to transfer the cups into sampler trays for this process and later to transfer them back into the metal racks for elution. Finally, the cups are transferred to sampler trays prior to testing the eluates. It is essential to maintain an exact sequence of the cups at all stages. When using the auto-analyzer it is necessary to use markers at regular intervals in order to precisely locate the reaction from each sample on the recorder chart.

Anti-D diluted 1: 100 is usually suitable. At such a dilution very strong agglutination is obtained but carry-over problems are avoided. The optimum dilution for the marker is obviously dependent upon the quality of the serum. It is convenient to put a marker between groups of five eluates corresponding to individual stains or unstained cloth controls.

2.4.5.2 Reagents for Auto-Analyzer

2.4.5.2.1 Cell Suspension

R_1 R_2 cells are freshly prepared and 20 ml of packed cells are suspended in 20 mm of 30 percent bovine albumin, 15 ml of AB serum, and 45 ml of normal saline. During use, the container should stand in melting ice and the cells should be mildly agitated by a magnetic stirrer.

2.4.5.2.2 Rouleaux Inducing Agent

Methyl cellulose is prepared as a 0.3 percent aqueous solution. Since this is difficult to dissolve, it is best prepared on the previous day. The use of a magnetic stirrer can expedite preparation. Before use, the preparation should be centrifuged.

2.4.5.2.3 Enzyme

One gm of bromelin is added to 100-ml of saline. This is best prepared using a homogeniser. If this is not possible, the preparation should be left to extract overnight at 4° C. Before use, the preparation should be centrifuged and, during use, the container should stand in melting ice.

2.4.5.2.4 Wash Solution

This is supplied to the wash trough of the sampler and is used for flushing through the probe between samples. It consists of 15 ml of 30 percent bovine albumin in 885 ml of saline.

2.4.5.2.5 Haemolytic Reagent

This is a 0.5-percent aqueous solution of Triton X100. It is best to prepare this on a daily basis. For rapid dissolution a magnetic stirrer is useful.

2.4.5.2.6 Antisera

The following antisera should be used: anti-C, anti-$\bar{c}$, anti-D, anti-E, and anti-e. All should be of the incomplete type having an auto-analyzer titre of at

least 1/2000. They should be tested for specificity with stains of a wide range of Rh groups before putting into routine use with unknown stains.

2.4.5.3 Method

The following method is used with the auto-analyzer.

1. Divide bloodstains into five suitable portions as discussed in paragraph 2.5.4.1 and place in 2 ml auto-analyzer cups arranged in labelled metal racks.
2. Prepare similar controls of unstained fabric.
3. For positive controls, take five portions of an R_1 R_2 stain and for negative controls take portions of stains of different groups that lack antigens corresponding to each antiserum.
4. Record the sequence of stains and controls as they are arranged in the racks.
5. Add one drop of appropriate antiserum to each cup and dilute with a suitable quantity of saline.
6. Ensure that all of the pieces of fabric are immersed in diluted antiserum, cap the cups and leave overnight in a 37° C incubator. It is necessary to place another rack or suitable object on top of the cups to prevent the caps from blowing off during incubation.
7. After removing from the incubator, either follow the washing procedure recommended for the manual method or remove unabsorbed serum using a washing machine. Load the cups in sequence into sampler trays and wash to 1/2 to 2 hours according to the type of fabric.
8. Replace the cups in the same sequence in the labelled racks. Withdraw the saline from all of the cups and replenish with 0.3 ml of fresh saline. Ascertain that every piece of fabric is immersed in saline.
9. Immerse cups in a water bath at 60° C for 40 minutes and agitate occasionally.
10. Take the racks from the water bath, agitate them vigorously and remove the pieces of cloth from the eluate as quickly as possible using a fresh piece of applicator stick for each cup.

11. During the washing process, the auto-analyzer should be set in motion so that, by the time the eluates are prepared, a steady baseline has been established and positive controls using the marker anti-D have been made.
12. Cups containing saline are placed in the even numbered spaces in the sampler tray. This is a further precaution against carry over. The marker is placed in the first space and eluate from anti-C in the third with the eluates from the remaining four antisera in the uneven spaces up to No. 11. A second marker is placed in space No. 13. This sequence is continued until all of the eluates are accommodated. The final cup should contain marker.
13. The sampler trays are run in sequence on the auto-analyzer. Most satisfactory results are obtained when the sampler is fitted with a 40/hour cam, 2:1 wash ratio.
14. After the final marker peak has appeared on the chart, the latter should be removed from the recorder. The marker peaks should be located and labelled and, from the record prepared of sequence of tests in the racks, the results from each stain and control can be identified and labelled.

2.4.5.4 Interpretation of Results

Before an interpretation can be made regarding the results from any particular stain, it is necessary that there should be large, well-defined peaks from the R_1 R_2 positive control stain and no peaks from the negative control stains and also from the cloth control from the individual item (see Figures 2-5 and 2-6). With very strong positive reactions differences in optical density of 0.5 may be obtained, but with small or poor quality stains differences of as little as 0.2 or even 0.15 are acceptable.

2.4.6 LESS COMMON Rh ANTIGENS

2.4.6.1 D^u

This is fairly rare among caucasians but common in negroes. With whole blood samples the cells usually fail to agglutinate with anti-D sera unless the indirect

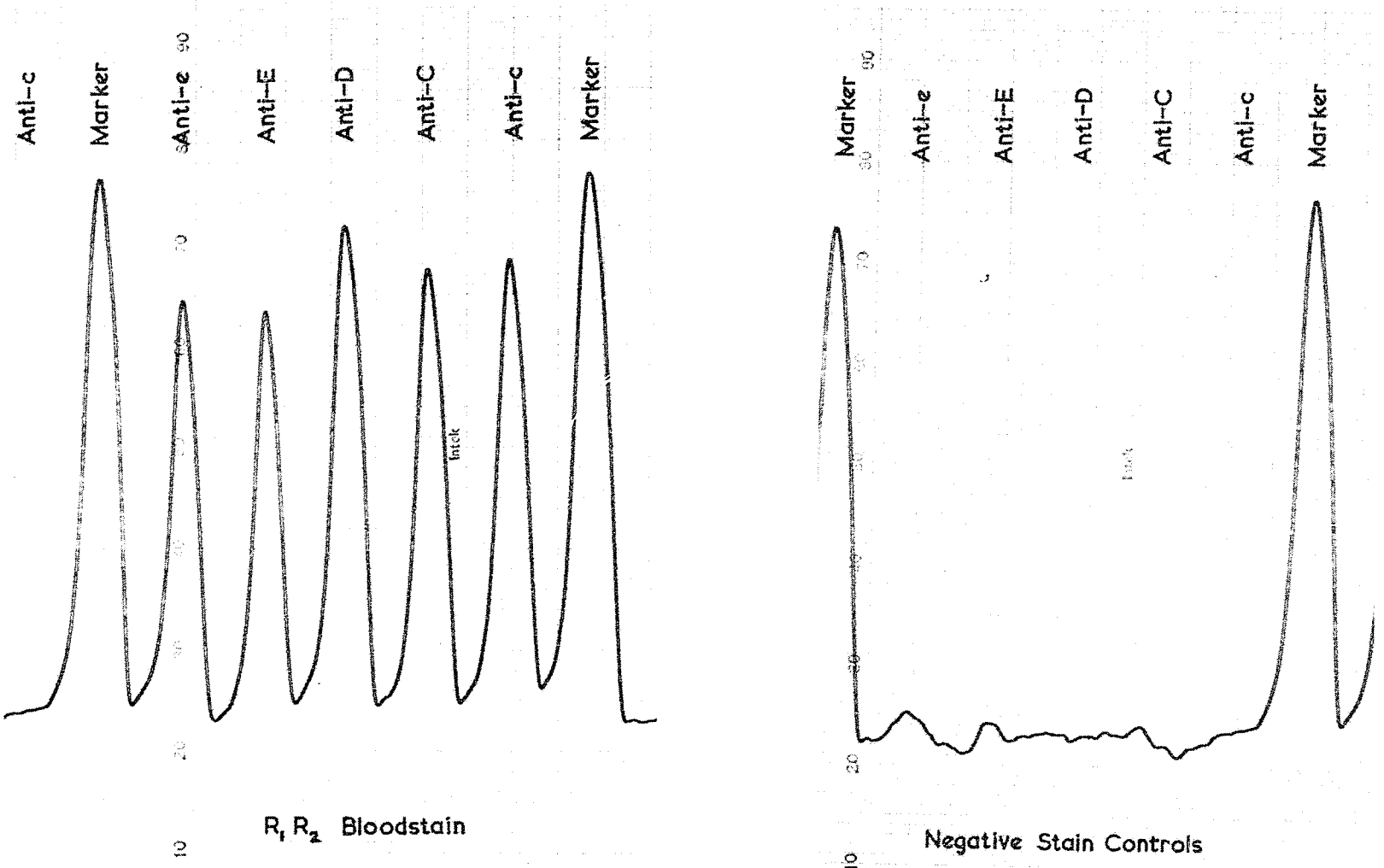


Figure 2-5. Auto-Analyzer Results from Positive and Negative Control Bloodstains

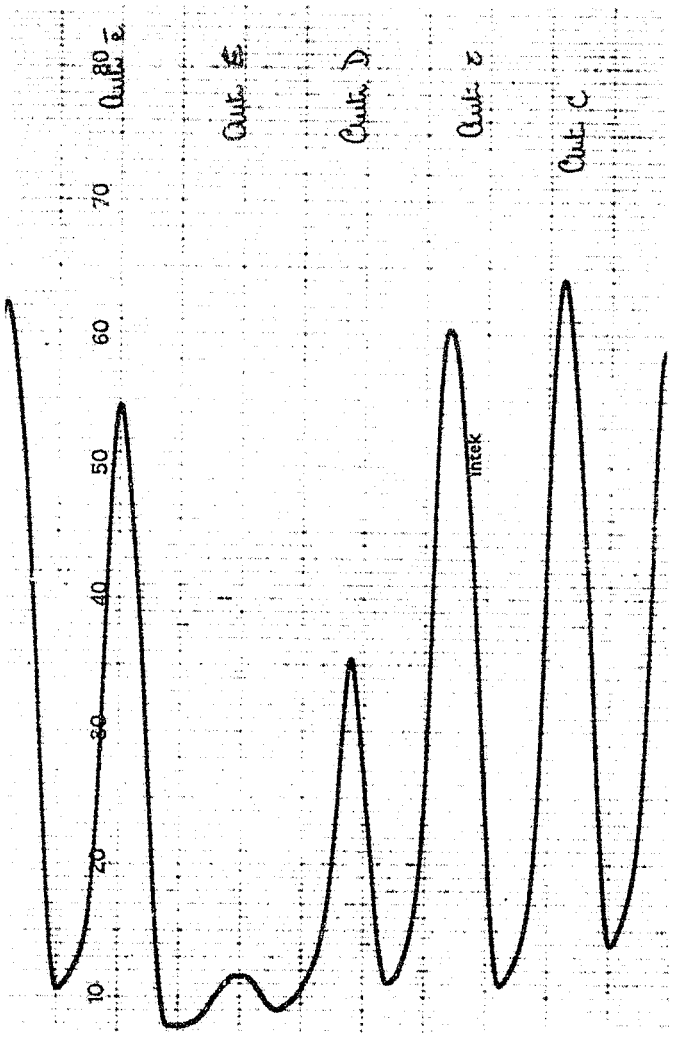


Figure 2-6. Auto-Analyzer Result from a Stain of $R^{u}{}_{1}r$

Coombs test is used. When using the manual techniques, one would normally obtain no agglutination from eluates of D^u stains. It is possible to detect the presence of D^u in dried stains by incubating a second anti-D eluate with $R_2 R_2$ cells for two hours, washing the cells twice, and testing with antihuman globulin serum. If the stain contained the D^u antigen, the cells should be agglutinated. It is possible, however, that some forms of D^u may not be detected and, therefore, with manual techniques there is always a small risk that a stain giving negative reactions for D is in fact a D^u. With the auto-analyzer, the situation is much more satisfactory as eluates from stains of D^u individuals produce well defined peaks but of a reduced size compared with normal D. The peaks are usually between 1/4 and 1/2 of the normal size.

2.4.6.2 C^w

Good quality anti-C^w sera are available and can be used in exactly the same way as other anti-Rh sera. For reasons of economy it is usually best to test for C^w only on stains that have previously given positive reactions for C. If anti-C^w is only available as a complete antibody, it must be checked for the absence of incomplete antibodies of other specificity before it is used for any of the techniques described on the foregoing pages. In the presence of other antibodies, the eluates can only be tested by a saline method. It is necessary to employ a manual technique and, after elution, the indicator cells are left in the eluate at 37° C for two hours and then examined for agglutination. Albumin and enzyme methods must not be used. Obviously, the indicator cells must contain the antigen C^w.

2.4.7 PROBLEMS ASSOCIATED WITH Rh GENOTYPING OF DRIED BLOODSTAINS

With adequate recent bloodstains that have dried quickly, Rh genotyping is highly reliable. With old stains or those of inadequate quantity, it is only safe to draw conclusions from positive reactions. In these circumstances, one cannot be certain of the absence of an antigen when a negative reaction is obtained. Stains that have dried slowly present the greatest danger of erroneous typing and it is never safe to rely on results from those smelling of putrified blood. The appearance of the bloodstain to an experienced eye is a good guide to its suitability for

grouping. The behaviour of the stain when tested for other components is also a useful guide to its quality for Rh typing. For example, those which have lost PGM activity are likely to give negative reactions in the Rh system.

Problems arising from the D^u antigen have been mentioned previously. In a caucasian society the incidence of R_o is low and R_o^u is extremely rare and, therefore, when reactions for rr are obtained, the chance that the stain is actually R_o^u is remote. In a negro population or one with a high proportion of negroes, the possibility of the presence of D^u is considerably greater.

In some cases a bloodstain might be too small for full Rh genotyping but it can be profitable to test for certain antigens. For example, if a victim possessed the antigen E and the suspect did not, useful information could be obtained by testing for E and e. If the presence of C^w was suspected in a stain of inadequate size for full genotyping, it would obviously be of great value if C^w could be detected.

When stains occur on non-absorbant surfaces, they can be absorbed onto small pieces of white cotton fabric moistened with water and, after drying, the cloth can be tested in the normal way. When stains occur on carpets it is best to scrape the blood from the carpet and absorb this on cotton cloth. A serious problem is encountered in grouping in the Rh system on denim material. It is so closely woven that it is impossible to completely remove the unabsorbed serum. If the bloodstains are in a crusty condition it is better to scrape off the blood and treat as for carpets.

The detection of Rh antigens in dried bloodstains is exacting and time consuming but, because of the variety of genotypes within the system, it can be highly rewarding. Despite the heavy financial outlay involved in the automated method it has many advantages over manual techniques.

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2.5 ABO IN SALIVA, SEMEN AND OTHER BODY FLUIDS

2.5.1 GENERAL

Approximately 80 percent of individuals are secretors and produce ABH group specific substances in high concentration in saliva, semen, vaginal secretion, and gastric juice. In other body fluids, such as sweat, tears and urine, the concentration is fairly low. These substances differ from those of blood in that they are mucoids instead of glycolipids and are readily soluble in water. In fluids, such as saliva and semen, the relative concentration of ABH antigens is much greater than in blood. Because of this, absorption-inhibition is more reliable for testing saliva etc., than it is for blood. In non-secretors there are low concentrations of group substances in body fluids and these can be detected by absorption-elution.

Classification into secretor and non-secretor is usually a very simple matter, but occasionally it is difficult to assess. There is also evidence that some individuals secrete in one body fluid and not in another. In forensic serology, the grouping of seminal stains is a routine occurrence but, for the purpose of eliciting whether or not a person is a secretor, it is usual to test saliva and, in these circumstances, a suspect can only be eliminated with certainty on the basis of his ABO group and not by the secretor status indicated by his saliva.

Despite the high concentration of group specific substances in the saliva and semen of secretors, there are serious problems involved in group determination. A small proportion of individuals of groups A and O exhibit B activity in body fluids and some people of groups B and O show A activity. The latter is more common. Most of the work on this subject has been carried out on saliva but there is evidence of similar difficulties in semen and vaginal secretion. Fortunately, the levels of spurious A and B are much lower than those of genuine group specific substances and, by adopting semi-quantitative methods and using absorption-inhibition and absorption-elution in parallel, it is usually possible to determine the group. However, in some cases the results are contradictory and the group cannot be determined with certainty.

The cause of this spurious A and B activity has not been ascertained, but it is likely to be caused by bacteria. Although cultures of bacteria from saliva samples that gave spurious reactions were tested and failed to show the corresponding A or B reactions, it is possible that the problem arises because of the activity of bacterial enzymes that may convert a small proportion of the terminal sugars of the group substances.

As previously stated, the levels of spurious A and B activity are much lower than those of true group specific substances. With both absorption-inhibition and absorption-elution techniques, it is normally possible to dilute saliva or other body fluids to an extent where false activity is not detected but specific reactions are obtained. Because there is considerable variation in the reactions of samples and stains from different individuals, it is preferable to carry out tests on a series of dilutions rather than attempt to select an optimum working dilution. When testing fluid saliva by absorption-inhibition, it is common to find strong inhibition with saliva diluted 1/1000, whereas false positive A and B reactions are rarely detected in dilutions of 1/10. Because of the soluble nature of blood group substances in body fluids, it might be expected that their detection by absorption-elution without prior fixation would be impossible because of the washing process involved, but this is not the case. Tests are carried out by immersing pieces of cotton in the successive dilutions and, after the cotton has dried, a simple technique such as that of Howard and Martin[23] is followed. A and B substances from secretors can be shown at dilutions of 1/4000 or more. It is rare to detect spurious A and B activity at dilutions of more than 1/40. With neat saliva from secretors, it is common to obtain negative or very weak reactions for the appropriate A or B antigen but, with successive dilutions, the strength of agglutination increases progressively up to a maximum which is maintained over many dilutions[37]. This prozone effect is undoubtedly caused by the presence of an excess of water soluble antigens that dissolve in the grouping serum and absorb a high proportion of the antibody content so that insufficient remains to combine with the antigens absorbed onto the cotton fibres. In practice, this prozone reaction is advantageous, since it provides very convincing evidence of the presence of true group specific substance compared with the reaction obtained from false A and B where there is a progressive diminution in agglutination.

On account of difficulties arising from spurious reactions, the use of absorption-inhibition and absorption-elution in parallel is highly desirable for grouping both fluid samples and dried stains. Of the two methods, because of the lower sensitivity, inhibition is less prone to the detection of false A and B and is therefore more reliable. However, with some AB secretors, the level of A activity is very low and can remain undetected by absorption-inhibition although clearly demonstrated by absorption-elution. In grouping dried stains, the highly sensitive elution method can serve as a confirmation that negative reactions obtained by inhibition are caused by absence of antigen and not by the presence of insufficient material.

2.5.2 GROUPING TESTS ON FLUID SALIVA SAMPLES

2.5.2.1 Preliminary Treatment

On receipt in the laboratory, samples should be centrifuged to remove as much cellular debris as possible. The supernatants are transferred to suitable tubes and these are placed in a boiling water bath for ten minutes. After further centrifugation, the supernatants are ready for serological testing but they may be stored at -20° C and tested later if this is more convenient.

2.5.2.2 Absorption-Inhibition

2.5.2.2.1 Materials and Reagents

The tests may be carried out in tubes or welled tiles, etc., according to personal preference. For absorption, tubes of 5 to 8 mm in diameter are suitable.

Anti-A, anti-B and anti-H are used in conjunction with A_2, B and O indicator cells in 2 percent suspensions.

2.5.2.2.2 Method

The following method is used for absorption-inhibition testing:

1. By double dilution, select the optimum dilution of each antiserum. This is the penultimate dilution capable of giving large agglutinates without free cells.

2. Prepare saline dilutions of the saliva samples as follows: 1/10, 1/100, and 1/1000.
3. To aliquots of neat saliva and the three dilutions add equal quantities of diluted anti-A, anti-B, and anti-H.
4. To aliquots of saline add equal quantities of diluted anti-A, anti-B, and anti-H to provide unabsorbed serum controls.
5. Cork the tubes and leave for 2 hours at 4° C.
6. To one drop of the contents of each tube add one drop of appropriate indicator cells in well tiles or tubes.
7. Leave at room temperature to allow development of agglutination—with welled tiles, 30 minutes is appropriate. The method of testing should be precisely the same as that employed to select the optimum dilutions of antisera.
8. Examine for agglutination and record the results.

2.5.2.3 Absorption-Elution

2.5.2.3.1 Technique

The Howard and Martin technique is employed using undiluted anti-A, anti-B, and anti-H. Anti-A + B serum is not used.

2.5.2.3.2 Method

The following method is used for the absorption-elution tests:

1. Prepare the following dilutions of saliva in water: 1/20, 1/40, 1/80, 1/160.
2. Immerse short cotton threads in neat saliva and in the four dilutions. Leave for a few minutes.
3. Thoroughly dry the threads. Secure short pieces to cellulose acetate sheet with adhesive leaving 2 to 3 mm of cotton exposed for subsequent treatment with antisera.
4. Proceed as for blood giving 2 hours absorption at 4° C and using A_1, B and O indicator cells.

2.5.2.4 Interpretation of Results

The results obtained by the two methods must be assessed together. With the majority of samples, little evidence of spurious activity is found, but occasionally it is more pronounced. The results given in Table 2-1 are fairly typical of a B secretor showing spurious A activity. The quantity of detectable H substance in A and B secretor saliva various greatly. Typically, it can easily be detected by absorption-inhibition but, in some cases, it is only demonstrated by absorption-elution. In such individuals, the relative concentration of A or B group specific substance is very high and this would suggest that a high proportion of H substance has been converted to A or B.

2.5.3 GROUPING TESTS ON DRIED STAINS

2.5.3.1 General

The difficulties involved in grouping dried stains are greater than with fluid saliva samples. Once the fluid has dried on an absorbent surface, the extent to which the substances can be re-dissolved varies considerably. In some cases it is easy to prepare an extract and results similar to those obtained with fluid saliva are obtained. In others, the solubility is low and it is then impossible to distinguish between reactions because of spurious A and B and true group specific substances. In sexual crimes, semen is frequently mixed with vaginal secretion. A critical microscopic examination is therefore necessary to form an opinion as to whether or not a particular stain is neat semen. Grouping tests may also be made on vaginal swabs, but these can only be of use if the group of the woman concerned is known. If she is of group AB, such tests would be useless, but if she is group O, then there is a high probability of obtaining useful information.

2.5.3.2 Absorption-Inhibition

2.5.3.2.1 Materials and Reagents

These are exactly the same as for testing fluid saliva.

Table 2-1

Test Results for a B Secretor

Absorption-Inhibition				Absorption-Elution			
Saliva	Anti-A	Anti-B	Anti-H	Saliva	Anti-A	Anti-B	Anti-H
Neat	2	-	-	Neat	2	-	4
1/10	3	-	2	1/20	1	4	4
1/100	4	-	3	1/40	-	2	1
1/1000	4	1	4	1/80	-	3	-
Unabsorbed Serum	4	4	4	1/160	-	4	-
				Cotton Control	-	-	-

4 = large agglutinates with non-free

3, 2 and 1 = successively weaker degrees of agglutination

2.5.3.2.2 Method

The method for absorption-inhibition testing of dried stains is as follows:

1. To 3 pieces of stained material 2 to 3 mm^2, add one drop of saline and one drop of anti-A, anti-B, and anti-H respectively.
2. Take controls of unstained material and treat in a similar manner.
3. Prepare saline extracts of the stain using 6 to 9 mm^2 of stain and adding sufficient saline for the later recovery of six drops of extract. Leave to soak at approximately 50° C for one hour in corked tubes.
4. Prepare dilutions of the extract in saline of 1/5, 1/10, and 1/20.
5. To aliquots of the neat extract and the three dilutions add equal quantities of diluted anti-A, anti-B and anti-H.
6. Proceed thereafter as with fluid saliva testing.

Providing dilution tests are made in the elution technique, stages 3 to 5 are not essential and may be omitted for routine work. However, if there is evidence of spurious A or B activity, the inclusion of these stages can sometimes clarify the situation. In practise, there is frequently insufficient material for the inclusion of stages 3 to 5.

2.5.3.3 Absorption-Elution

2.5.3.3.1 Materials and Reagents

These are the same as for testing fluid saliva samples.

2.5.3.3.2 Method

The method for absorption-elution of dried stains is as follows:

1. Take three short pieces of stained thread and secure to cellulose acetate sheet in the same way as for testing dried bloodstains. For cigarette ends, small strips of paper should be used.
2. Take unstained controls in a similar manner.

3. Prepare an extract of the stain by adding two drops of water to a piece of stained material approximately 3 mm^2 in size and leaving to soak for 1 hour at approximately 50°C.
4. Prepare a control extract in a similar manner.
5. Make dilutions of the extract in water of 1/5, 1/10, and 1/20.
6. Proceed as for fluid saliva samples testing the extracts together with the neat stain.

2.5.3.4 Interpretation of Results

Obviously, this is exactly the same as for fluid saliva samples. Because of difficulties encountered because of lack of solubility of stains and reactions from unstained fabric, some results are equivocal and no positive interpretation can be made.

2.6 OTHER GENOTYPING SYSTEMS

2.6.1 Gm AND Inv SERUM GROUPS

The complexity of the Gm system is such that, if it was possible to carry out full genotyping on dried bloodstains, it would be more useful than any single system. In practice, the quality of many of the antisera is inadequate for use in stain grouping. Gm(1) and Gm(2) can be easily detected by absorption-inhibition and this is routine in some European countries. Preliminary work in the Metropolitan Police Laboratory has shown that it is also possible to detect Inv types in dried bloodstains.

2.6.2 OTHER RED CELL ANTIGEN SYSTEMS

It is highly likely that, in time, given adequate quantities of bloodstain and good quality antisera, it will be possible to detect most, if not all of these in dried bloodstains.

SECTION 3

POLYMORPHIC ENZYME SYSTEMS

3.1 PHOSPHOGLUCOMUTASE (PGM)

3.1.1 PHYSICAL DATA

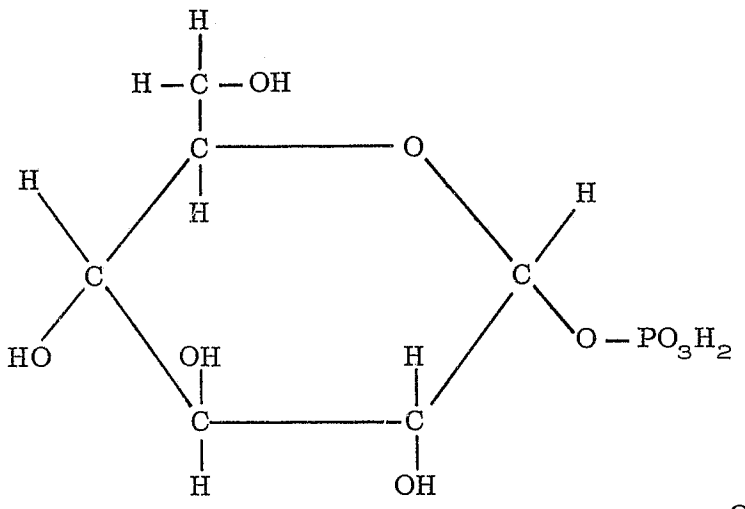
Phosphoglucomutase transfers the position of the phosphate radical from the 1-position to the 6-position on the glucose molecule (see Figure 3-1). This is one of the necessary stages early in glycolisis just prior to the dehydrogenation steps of the pentose phosphate pathway or prior to the isomerase step of the conversion of glucose-6-phosphate to fructose-6-phosphate in the Emden-Meyerhof pathway.

The glucose-1-phosphate substrate is formed by the action of phosphorylase on glycogen (Figure 3-2).

The equilibrium of the phosphoglucomutase reaction is strongly towards the production of glucose-6-phosphate. At equilibrium 5.5 percent of the glucose-1-phosphate remains unchanged, 94.5 percent having been converted to glucose-6-phosphate[39]. This equilibrium is only reached under laboratory conditions with a purified enzyme, since within the red cell the product, glucose-6-phosphate, will be utilized as a substrate by both glucose-6-phosphate dehydrogenase and phosphoglucose isomerase.

The molecular weight of the enzyme (purified from rabbit muscle) is quoted as 74,000 by Bocchini et al. [38] and as 77,000 by Najjar et al.[56]

Najjar gives an electrophoretic mobility for the rabbit muscle enzyme of 2.01 cm^2/volt/sec. at pH 5 in an acetate buffer of ionic strength 0.1[54]. Phosphoglucomutase is active over a pH range from 4.5 to 8.5. Above and below these limits it is rapidly inactivated[54].

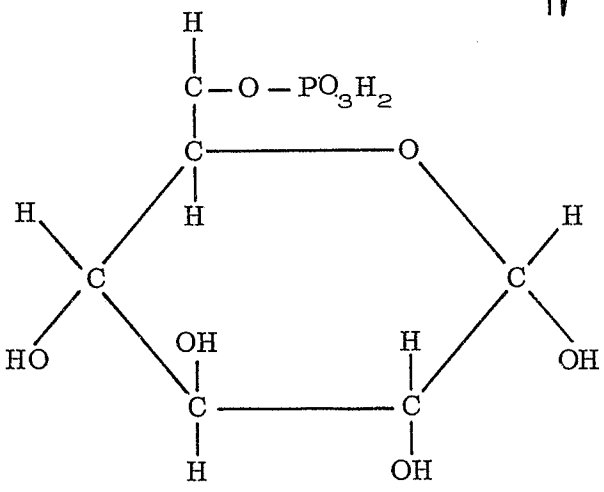


Glucose-1-phosphate

PHOSPHOGLUCOMUTASE

Glucose-1:6-diphosphate

Mg



Glucose-6-phosphate

Figure 3-1. Phosphoglucomutase Reactions

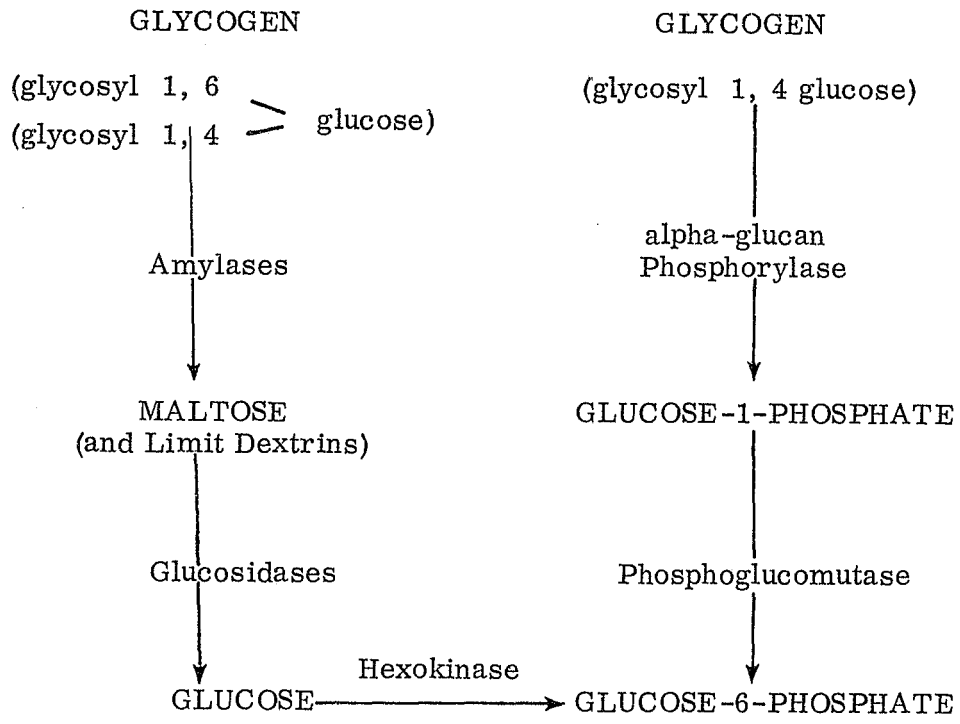


Figure 3-2. Action of Phosphorylase on Glycogen

3.1.2 ACTIVATORS AND INHIBITORS

Activation appears to occur occasionally in samples. These are usually post-mortem samples that have been taken a considerable time after death. There is often a high bacterial content to these samples. The basic pattern of isozymes does not appear altered except as described later under effects of age.

The purified enzyme is almost inactive when diluted with water. Cysteine, hydroxyquinoline, histidine and other metal-binding agents activate the enzyme[54, 61]. Mg^{++} produces an increase in activity. Some other ions also increase the enzyme's activity[39]. Many metals are inhibitory—copper, zinc, mercury, lead, and silver, particularly[61].

Fluoride is also an inhibitor of PGM but the degree of inhibition produced is dependent on the concentration of substrate and other ions present. Sodium fluoride is often used as a preservative in blood samples but it has never been found to have adverse effects on the PGM determination. This may well be because of

because of the thorough washing of the red cells prior to lysis and use. The effect of fluoride on lysed blood samples is described in paragraph 3.1.8.

The presence of some metals (found in dyes) on the cloth substrate of a bloodstain may sometimes be the cause of loss of activity, but there is no direct evidence for this.

3.1.3 COENZYMES

Glucose-1:6-diphosphate is normally required as a co-factor. The rate of activity of the enzyme in the presence of this is very much higher than in its absence.

Passonneau et al.[57] report that the enzyme can synthesize its own co-factor G-1:6-diP from fructose-1:6-diP, glucose-1-phosphate, or glucose-6-phosphate. The rate of synthesis is described as low and this may be the answer to low enzyme activities in the absence of the co-factor, glucose-1:6-diphosphate, as a component of the reaction mixture. A very low level of co-factor is possibly synthesized slowly from the substrate glucose-1-phosphate.

3.1.4 POLYMORPHISMS

Polymorphic forms of human red cell phosphoglucomutase (PGM) were first described in 1964 by Spencer et al.[59] They discovered two alleles at one locus, the heterozygote and the two homozygotes giving three commonly occurring variants, denominated PGM 1, PGM 2-1, and PGM 2.

These three types were found to occur in a British population at 58 percent PGM 1, 36 percent PGM 2-1, and 5 percent PGM 2. More detailed population data is given later in this paragraph.

Later Hopkinson and Harris[47] found variation at a second locus. Rare variants have been described in various papers[47, 51, 52] and summarized by Hopkinson and Harris[48], and Giblett[44]. In 1968 Harris et al.[46] introduced evidence of variation at a third structural locus for PGM in placental material.

The first locus, PGM_1, bands have the lowest mobility with the second locus, PGM_2, bands somewhat more anodic, while the third locus, PGM_3, bands have the highest mobility. These are illustrated in Figure 3-3.

Of the three loci governing the production of PGM isozymes, only one, PGM_1, is of any real consistent value to the forensic scientist. The PGM_1 locus determines the bands a, b, c, and d of the three common phenotypes, the bands cathodic to the 'a' band of the rare forms PGM_1^6 and PGM_1^8, and those overlapping the PGM_2 locus up to the PGM_1^3 bands. These are illustrated in Figures 3-3 and 3-4.

Each allele at the PGM_1 locus appears to determine two of the bands seen on the electrophoretic plate. Thus, PGM_1^1 has bands 'a' and 'c', PGM_1^2 has bands 'b' and 'd'. The heterozygote PGM_1^{2-1} will thus have the four bands a, b, c, and d. These are illustrated in Figure 3-5.

The mechanism suggested by Najjar[55] and Najjar and Pulman[56] and Gounaris et al.[45] was that the phospho-enzyme was the active form and the dephospho-enzyme was non-active. Joshi et al.[50] intimated the possibility that the 'a' and 'c' bands of a PGM 1 or the 'b' and 'd' bands of PGM 2 may represent respectively the phospho- and dephospho-forms of the enzyme. This does not seem reasonable in view of the activity of the 'c' and 'd' bands; also in view of experimental evidence that phosphorylation with glucose-1:6-diphosphate or dephosphorylation with glucose-1-phosphate, prior to electrophoresis, did not change the pattern of isozymes either in mobility or in their relative intensity of reaction.

Dawson and Mitchell[43] also were unable to detect any dephospho-enzyme after electrophoresis and found that prior treatment with both glucose-1-phosphate, to produce the dephospho-form, and glucose-1:6-diphosphate, to produce the phospho-enzyme, did not alter the electrophoretic pattern. These authors[43] suggest that there may be a conformational difference between the isozymes of the two bands produced by each gene, without a change in molecular composition There is, however, no evidence at present to support this theory and they also say that there are other possibilities which have, as yet, not been ruled out.

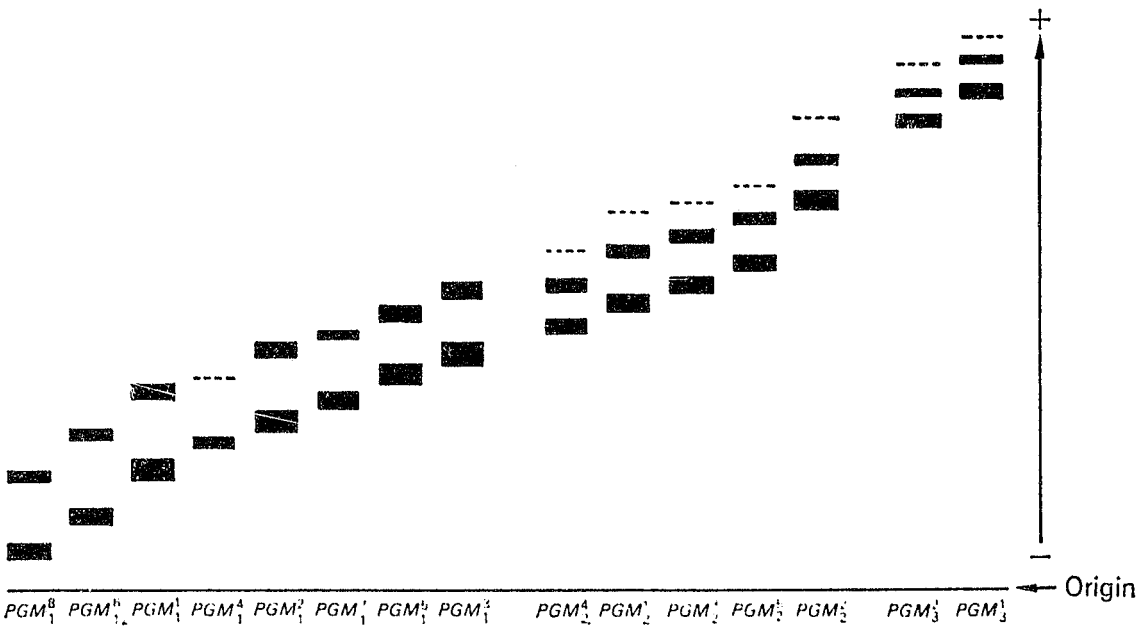


Figure 3-3. Bands of Isozymes After Electrophoresis by the Various PGM Alleles. (Reprinted from Hopkinson and Harris, Ann. Hum. Genet. 1968, 31: 359)

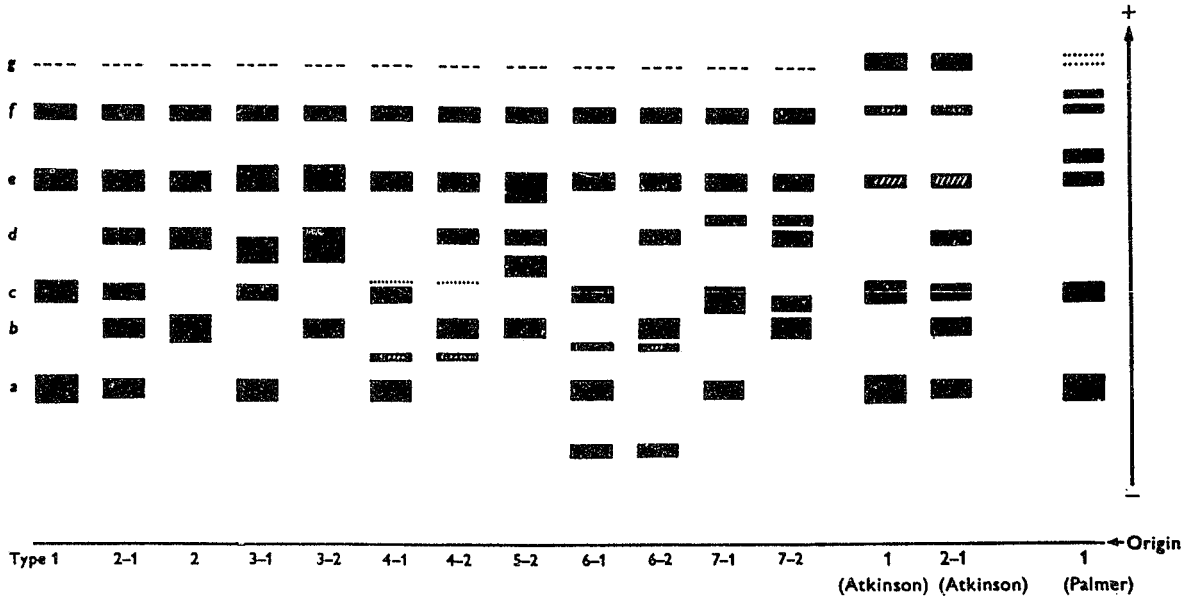


Figure 3-4. Patterns of Various PGM Types. (Reprinted from Hopkinson and Harris, Ann. Hum. Genet. 1966, 30: 167)

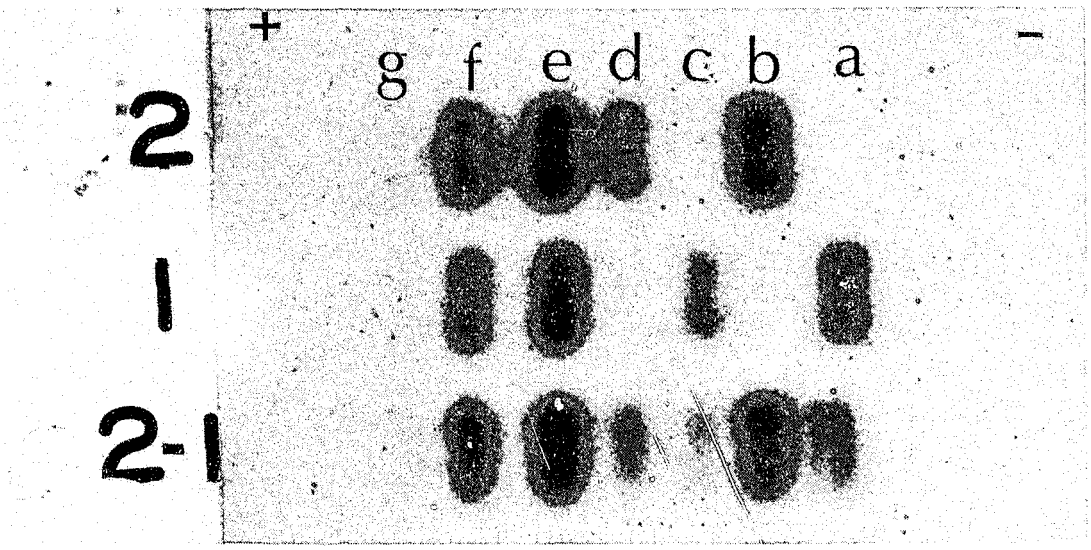


Figure 3-5. Three Common Variants at Locus PGM_1 with Nomenclature Bands Marked.

There is a certain amount of indirect evidence[49] for a storage effect, defined in this instance as the production of a secondary or modified enzyme from the original gene-determined enzyme. This secondary enzyme, in this case, has an altered electrophoretic mobility, one gene thus apparently producing two different isozymes.

This type of modification can occur in vivo or in vitro storage and may be by addition, deamination, degradation, or any similar mechanism that does not affect the active site of the enzyme molecule.

If one considers the quantitative difference of the 'a' band compared with the 'c' band in a PGM_1^1 type from various tissues, changes in proportion of 'a' and 'c' can be seen. In young red cells 'a' dominates over 'c' while in old red cells the reverse may be seen. In white cells and in other nucleated tissues, such as muscle, the dominance of the 'a' band activity over that of the 'c' band will be seen.

This seems to be the most satisfactory of possible explanation so far of multiple products from a single gene.

In practice there are few problems in dealing with PGM_1 locus variants in bloodstains. Most of the time these are detectable and there has never been any indication of one type being converted to another among the common variants.

Locus PGM_2 determines the isozymes in the faster running (more anodic) a - h bands. There is no reason why in theory these variants should not be of use in the examination of bloodstains that are in good condition except that the variants from $PGM_2{}^1$ are very rare. The bands here also appear as pairs as in PGM_1 presumably for the same reasons.

In practice, however, it is not usually possible to use PGM_2 variation, since the activity of the e - h bands diminishes on the drying of a blood into a stain; and these bands then disappear as visible entities on the electrophoresis plate more rapidly than the a - d PGM_1 locus bands. This is a distinct contrast to PGM_1 variants where the enzyme is reasonably stable by comparison.

In one case, for example, a $PGM_1{}^1$ $PGM_2{}^{2-1}$ (PGM 1-Atkinson) was examined as bloodstained exhibits and as test stains made in the laboratory from the blood sample. The $PGM_2{}^{2-1}$ pattern could be detected only for a relatively short time after the stain dried (7 days). A second $PGM_2{}^{2-1}$ sample reacted better and in this case stains two weeks old could be satisfactorily typed. These latter remarks also apply to a $PGM_1{}^1$ $PGM_2{}^{3-1}$ (PGM 1- Palmer). See Figure 3-6.

The PGM_3 locus determines the bands anodic to 'h'. A similar form of polymorphism occurs here which is not apparently linked to PGM_1 or PGM_2.

Three phenotypes have been described, two homozygotes and a heterozygote, with a similar nomenclature to the variants at the other loci. These occur in a British population at 54 percent $PGM_3{}^1$, 39 percent $PGM_3{}^{2-1}$ and seven percent $PGM_3{}^2$.[46]

Most of the work on these PGM_3 types has been done using placental tissues which is richer in its proportion of the PGM_3 enzyme than other tissues. PGM_3 produces only 1 to 2 percent of the total PGM activity in red cells and it

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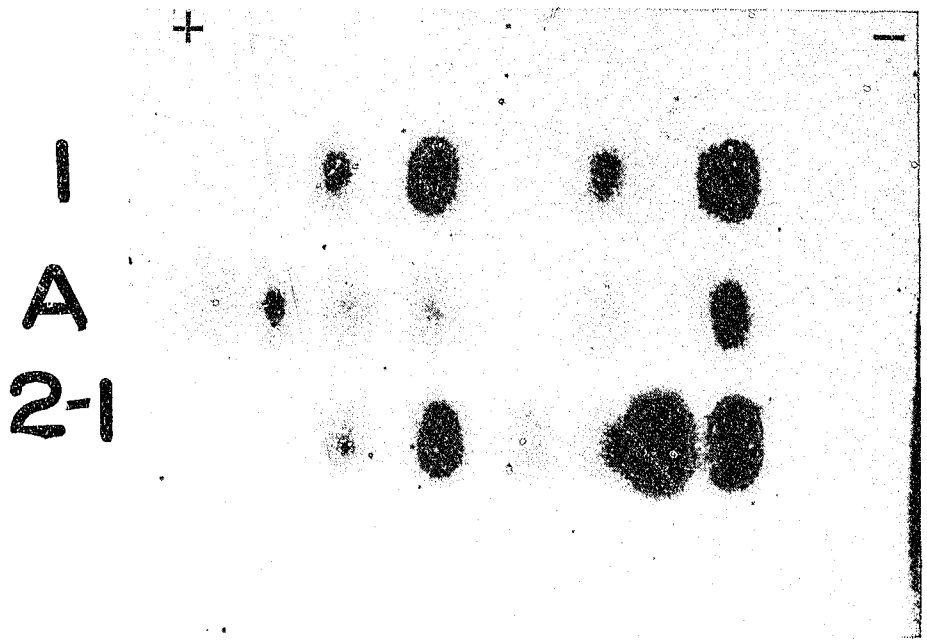

Figure 3-6. A PGM_2 Locus Variant PGM_1^{1}, PGM_2^{2-1} (PGM 1-Atkinson) Between (1) a PGM_1^{1}, PGM_2^{1}, and (2-1) a PGM_1^{2-1}, PGM_2^{1}.

is not possible to determine these from lysates or stains using the usual micro-methods. The activity is too low to obtain any results without partial purification and concentration.

3.1.5 PGM IN BLOOD AND OTHER TISSUES

PGM is present in many tissues of the body, in fact in red cells the level is lower than in many other tissues. Consequently PGM becomes useful at those times when after murder the body is not found for a considerable time. The blood often starts by having an increased activity, but this can soon reduce to nil. Nil activity in a post-mortem blood sample is fortunately rare, but where the blood is not in a satisfactory condition, suitable material for typing can be obtained from any tissue surviving in good condition, such as deep-seated muscle. The same conditions apply if some attempt has been made to destroy the body. Bones only may remain but the PGM type can be determined from the bone marrow, if this has not been damaged in the processes of body destruction or the destruction of the soft tissues.

The extent of the expression of the genes at each of the three loci PGM_1, PGM_2, and PGM_3 vary from one tissue to another. In placental tissue, for example, there is a high proportion of the locus PGM_3 enzyme, while in some other tissues the PGM_1 locus enzyme may form 80 to 90 percent of the total activity.

The correlation of any group systems in semen with those in blood is an advantage in cases of sexual offences. Until recently, only the secretion of ABO groups has been used on stains of semen. PGM is also present in semen and PGM_1 types can be determined from semen[41] and are now being used in selected cases. The isozymes determined by PGM_2 and PGM_3 are virtually absent, but those of PGM_1 are present and are identical in electrophoretic mobility with those in blood.

Numerous homologous blood samples and semen samples have been examined and no instances of discrepancy have been observed (Figure 3-7).

There is no reason why, if stain material is available, this system should not be used in typing of semen stains. More material is required than with a blood-stain because of the lower level of PGM present.

Of the numerous semen samples examined, none have shown a detectable amount of the PGM_2 locus enzymes (e-h bands).

Vaginal secretion sometimes contains PGM in detectable amounts so that the use of this in semen typing on vaginal swabs must be considered with some circumspection. If the woman is a type 1, then semen of a type (2 - 1) or 2 can be identified. If the woman is a type 2 then semen of types 1 or types 2 - 1 may be identified (see Figure 3-8). If the woman is a type 2 - 1 then no identification of the semen can be made unless a clear-cut type 1 or type 2 only is seen, showing that in this case the vaginal secretion does not contain PGM in detectable quantity. The PGM_2 locus e - h bands occur in this vaginal secretion and the presence of these in seminal stains might be used to suggest admixture with vaginal secretion.

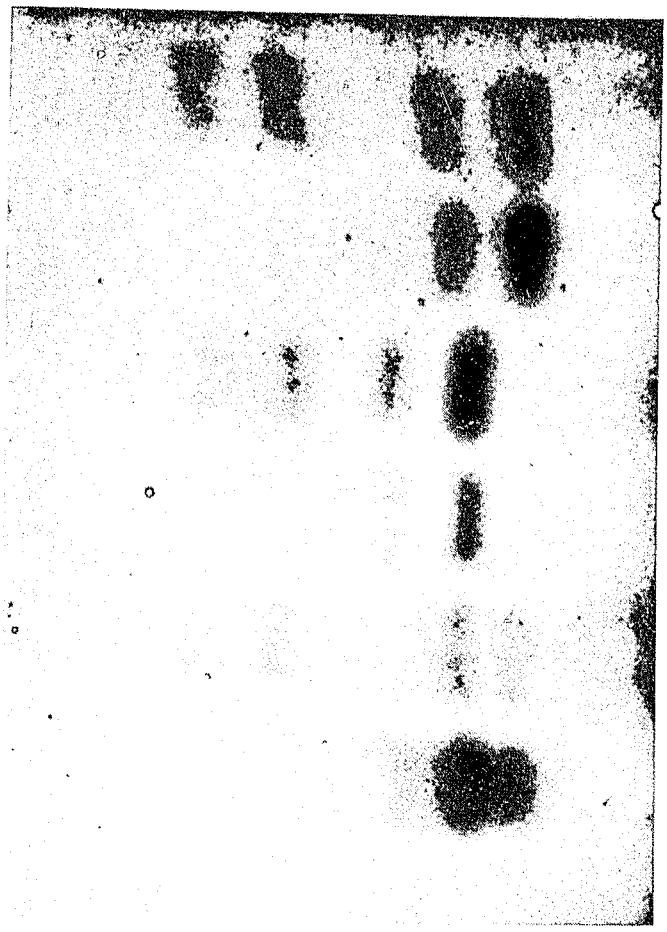

Figure 3-7. Numerous Homologous Blood and Semen Samples

Figure 3-8. PGM in vaginal secretion. (S—bloodstain, PGM 1; VS—vaginal secretion, PGM 1; L—red cell lysate, PGM 2-1.)

3.1.6 EFFECTS OF AGE

The effects of age on stored blood samples vary according to the conditions of storage. These effects fall into three categories:

a. Enhancement of activity—usually accompanied by considerable bacterial growth.

b. Loss of activity, dropping to no activity at all.

c. The development of a diffuse band in the 'd' region.

Figure 3-9 shows the development of effect (a) from normal samples in positions 1 and 2, through a gradual increase shown in positions 3, 4, 5, 6, and finally 7. All the samples 1 through 7 are PGM 1. Position 8 shows a weak PGM 2-1, 9 shows a weak PGM 1, and 10 shows slight enhancement of a PGM 2. All the samples started with approximately the same amount of blood on the insert.

Figure 3-10 illustrates effect (c), shown weakly in positions 3 and 4 and strongly in position 6. Effect (b) is shown by the almost complete inactivity in positions 5 and 7.

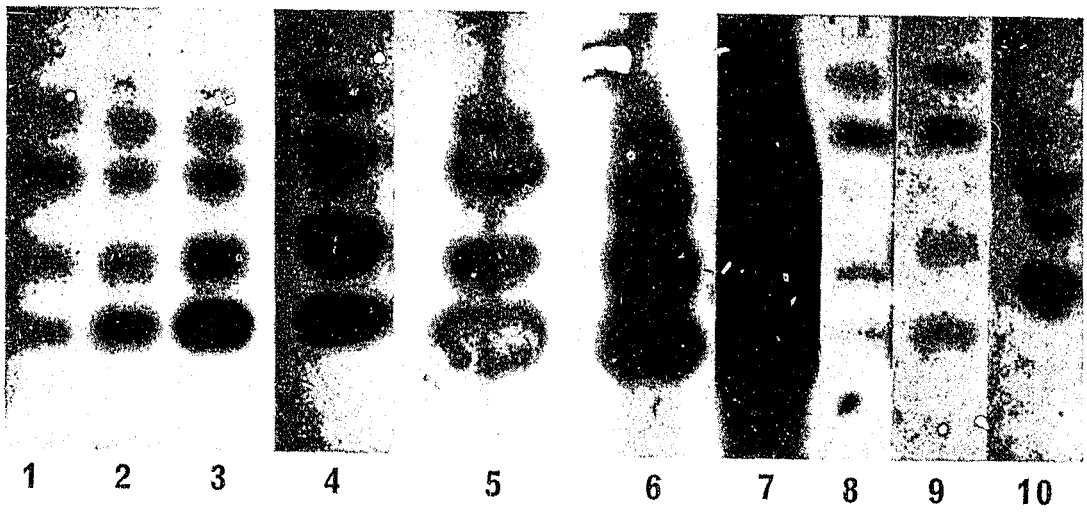

Figure 3-9. Enhancement of Activity in Stored Blood Samples

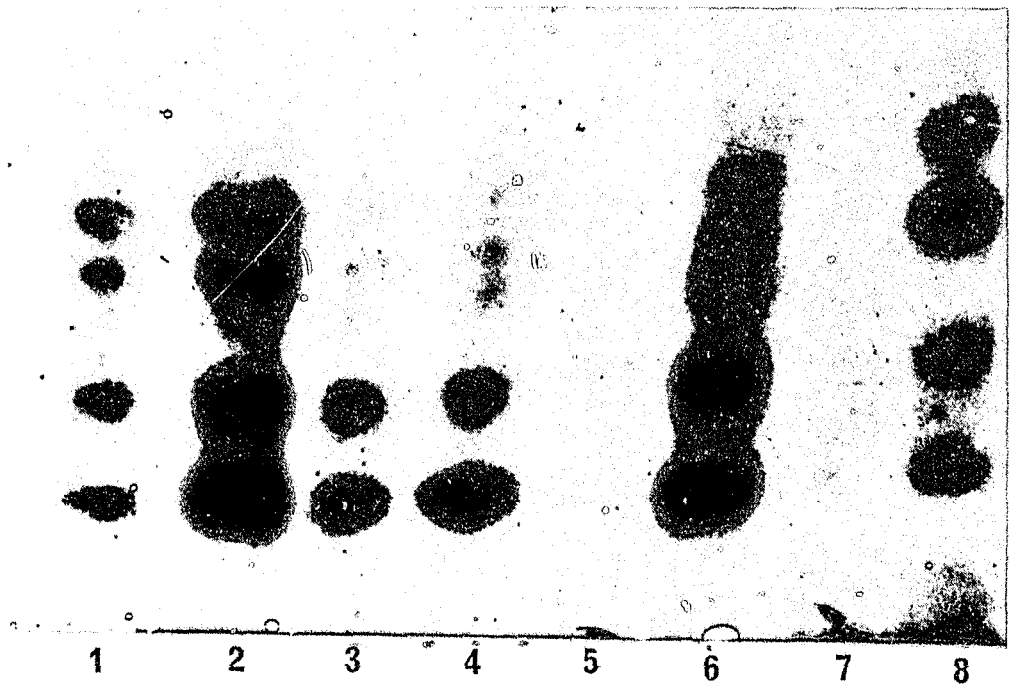

Figure 3-10. Loss of Activity and Development of a Diffuse Band in Stored Blood Samples.

The pseudo-'d' band is weaker and more diffuse than the normal 'd' band. It only has an effect on the typing of PGM 1, since the 'd' band is already present in PGM 2 and 2 - 1. The effect is to make a PGM 1 confusable with a PGM 3-1, which under normal fresh blood conditions has bands 'a', 'c' and 'c/d' (this latter band occurs between the normal 'c' and 'd' bands). This c/d is a normal clean-cut band, not a diffuse one

With storage at room temperature, a whole blood sample will develop either effect (a) or (b), generally the former. It has been known, however, for a sample with enhanced activity to finish up with no activity at all. Lysis, of course, occurs before any of these stages is reached. There seems, at this moment, no predictability as to which effect will be found or how long before these stages are reached.

Storage of the samples as haemolysates helps to prevent loss of activity and, therefore, storage at 0 °C as a lysate considerably slows down the effects found at room temperature. Storage at -20 °C as lysates preserves the normal patterns for a considerable period. These have been satisfactorily typed after 6 to 12 months storage. Effect (c) occasionally occurs here, particularly on samples that are repeatedly thawed and frozen. Experience to date shows that storage of the lysate or the red cells in liquid nitrogen preserves the activity and the pattern indefinitely.

The effects of age on blood stains are similar to those on blood samples except that effect (a) rarely occurs and effect (c) occurs more often. Only once has the enhancement effect (a) been seen in the Metropolitan Police Laboratory. This was on a bloodstained newspaper that had been outdoors in a wet environment. How long the blood on it had remained wet or only partially dried is unknown. It was dry when brought to the laboratory.

It is quite a frequent occurrence, however, to find the effect (c) in bloodstains of type PGM 1 which have been overdeveloped. As the stain becomes older this diffuse band increases in width until it embraces all the remaining bands. This appears as a long blue smear making typing of the stains impossible. The time taken to reach this stage is variable and depends entirely on storage conditions.

Inactivity has been found in stains one week old; on the other hand bloodstains over three months old have been typed quite satisfactorily.

Both these occurrences are rare and experience indicates that the probabilities of being able to type bloodstains are favorable up to one month old and unfavorable after two months.

As with all work on blood samples and bloodstains they should be typed as soon as possible.

3.1.7 EFFECT OF THE STAIN SUBSTRATE

Most clothing textiles have no effect on the ability to determine a PGM type. Some, however, are more difficult to handle than others. Very light thin material, such as nylon stockings, does not easily yield a single thread containing enough dried blood. It may be necessary, therefore, to make up several fine threads into one insert for a standard thin gel. Other materials may be of coarse springy fibres which are difficult to insert into the gel slot. These usually become manageable if they are soaked in a minimum of gel buffer.

In the case of a non-spun material, coarse carpet or rope fibres for example, sufficient fibres are removed and placed separately in the gel slot. With hard substrates, such as glass or metal, the stain is swabbed from the article with a standard cotton insert, which is then put in the gel as described under general methods.

Stains have been typed satisfactorily from many clothing materials (wool, cotton, fur, viscose acetate, nylon, acrylics, polyesters, PVC, leather, etc.) and from iron, brass, galvanized iron, steel, copper, glass, painted surfaces, wood, hardboard, paper, tarmac, vegetation, bricks, and soil.

3.1.8 EFFECTS OF VARIOUS CHEMICALS

Mercaptoethanol has no effect on the pattern of the enzyme. According to Dawson and Mitchell[43], iodine produces no change of total activity but increases the mobility of rabbit muscle enzyme. P-mercuribenzoate also produces an increase

in mobility with a reduction in total activity. The latter change was reversible with mercaptoethanol, whereas the iodine treatment was not reversible.

Enzyme activation by prior incubation with cisteine, imidazole and other metal-binding agents has been dealt with earlier. Excess glucose-1-phosphate and glucose-1:6-diphosphate have no effects.

The presence of fluoride as a preservative in blood samples has no effect on the ability to determine the PGM type providing the cells can be washed. If the blood sample is lysed, the markedly increased ionic strength of the sample distorts the patterns on that part of the gel. This results in a lower mobility of the component isozymes but with experience the type can still be determined.

This effect can be seen in Figure 3-11—1+F, PGM 1 with fluoride; 1, PGM 1 (without fluoride); 2-1+F, PGM 2-1 with fluoride; 2-1, PGM 2-1 (without fluoride). (Note the distortions that can occur.)

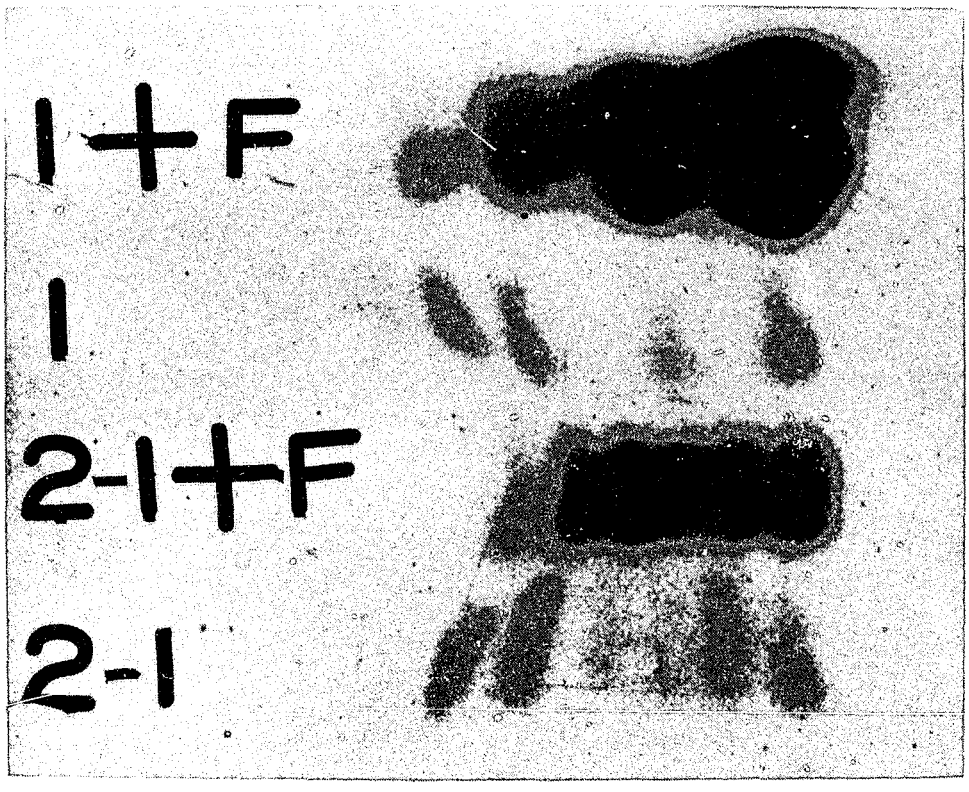

Figure 3-11. Effect of Fluoride

3.1.9 METHOD

Several methods have been described for the determination of PGM types[59, 53, 62]. Most workers use a thick starch gel such as that originally described by Smithies[58], used first for PGM by Spencer, Hopkinson and Harris[59] for lysates and Culliford[40] for bloodstains. Thin starch gel, Wraxall and Culliford 1968[62]; Agar gel, Monn 1968[53]; Acrylamide[42]; and cellulose acetate have all been tried.

Wraxall and Culliford's method has proved most satisfactory for forensic use, being specifically designed for work in this field. The sample preparation is minimal. For blood samples a cotton thread soaked in lysate is used as the insert. For bloodstains, a bloodstained thread is soaked in a minimum of gel buffer for 5 to 10 minutes before it is inserted into the gel.

The thin layer technique should be used as described in paragraph 1.4, using 10 percent starch and the following buffers:

a. Tank Buffer (pH 7.4)

TRIS (Trishydroxymethyl-aminoethane)	12.11 g
Maleic acid	11.62 g
EDTA (Ethylene-diaminetetracetic acid)	2.92 g
Magnesium chloride	2.03 g
Water (distilled)	to 1 litre

Adjust to pH 7.4 with 40 percent sodium hydroxide.

b. Gel Buffer

Tank buffer diluted 1:15.

Electrophoresis is carried out at 0 to 4 °C for 16 to 17 hours at 4 to 5 volts/cm.

The activity is developed using the agar overlay technique described earlier in paragraph 1.2.

c. Overlay

Glucose-1-phosphate containing 1 percent glucose-1:6-diphosphate	35 mg
Magnesium chloride	20 mg

Glucose-6-phosphate dehydrogenase	0.4 to 1.0 units
NADP (nicotinamide adenine dinucleotide phosphate)	1 mg
MTT (tetrazolium)	1 mg
PMS (phenazine methosulphate)	1 mg
Buffer TRIS 0.06 M	10 ml

(The buffer composition is TRIS 3.64 g in 500 ml of water adjusted to pH 8.0 with HCl.)

This solution is brought to 37°C and mixed with 10 grams of 2 percent agar in distilled water at 50 to 55°C and immediately poured onto the starch gel in a position from the origin to 8 cm towards the anode. When the overlay is set, it is covered with a glass plate and incubated in darkness at 37°C. With this reaction mixture the glucose-6-phosphate produced by PGM activity is used as a substrate for glucose-6-phosphate dehydrogenase; concomitant with the action of this latter enzyme, NADP is reduced. Using phenazine methosulphate as a transfer agent MTT tetrazolium is reduced to a blue colored formazan. This mechanism is summarized and illustrated in Figure 3-12.

The areas of activity are seen as blue bands on a yellow background in about one-half to two hours, depending on the activity of the samples or stains used in the starch plate.

After a little experience it is normally simple to interpret the results with respect to the usual variant types, so, unlike some other enzyme systems, no known control sample is put on the plate. Some of the rarer forms could be difficult to interpret in the absence of the correct reference sample. One or two lysates are usually put in each plate of stains in case of total failure of the stain samples from difficult case material.

The difficulties encountered with interpretation of stain material or old blood samples are discussed in paragraph 3.1.6.

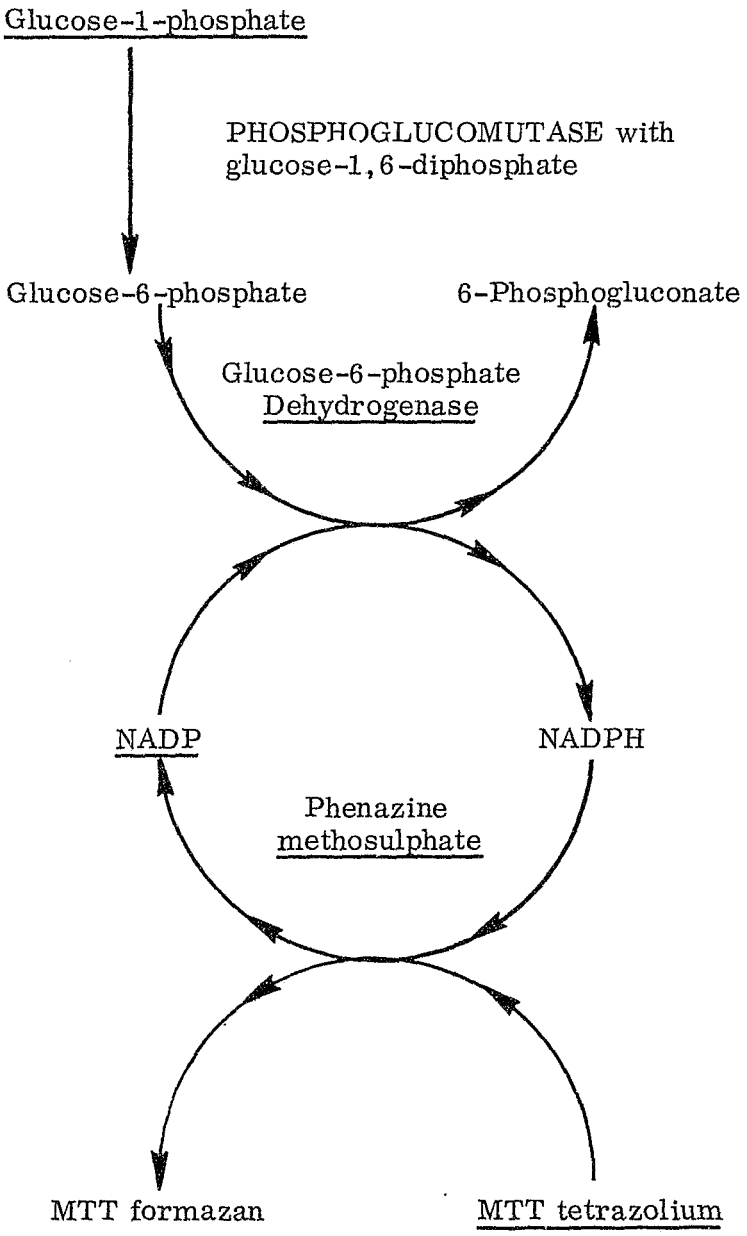

Figure 3-12. Sample Mechanism for Determining PGM Types

The assessment of the quality of the plate can be made by the appearance of the bands, which should be clear, clean, and straight, and there should be clear separation of the 'b' and 'c' bands in a PGM 2-1.

3.1.10 PROBLEMS

The 'd' band in stains has already been described. With fresh buffer, the haemoglobin moves slightly towards the cathode (about 1 to 2 cm from the origin). As the gel buffer deteriorates, the haemoglobin remains at the origin or later begins to move toward the anode. Shortly after this, the separation of the 'a'-'b' bands deteriorates.

The batch of buffer should be replaced before this latter effect occurs. It is usually a gradual process, which may occur within a week, or on the other hand a batch may last 2 to 3 months. If the buffer is replaced before the haemoglobin moves towards the anode, unsatisfactory separations caused by buffer deterioration may be avoided.

The use of disinfectants, such as Dettol, or strong soap solution either when taking a blood sample or attempting to clean bloodstaining, causes severe interference with PGM typing. The particular sample may give no reaction and the presence of these materials in the gel may interfere with adjacent samples as well.

The distribution of the various PGM types in the population is included in Table 3-1. A comparison is shown in Table 3-2.

Table 3-1

Distribution of PGM Types in Various Populations
(Taken from R. Stedman 1970)

Population	Number Tested	Expected Phenotypes %			Unusual Phenotypes		Gene Frequency		Reference
		PGM-1	PGM-2-1	PGM 2	No.	Type	PGM1[1]	PGM1[2]	
EUROPE									
England	2115	58.37	36.10	5.52	1 ea	3-1, 4-2, 7-1, 7-2, 5-1, 1 Palmer	0.764	0.235	66
Ireland	106	74.51	23.62	1.87			0.863	0.137	63
Germany	1016	58.86	35.70	5.42			0.767	0.233	73
Norway	2674	60.22	34.76	5.02	4	7-1	0.776	0.224	69
Sardinia	633	58.06	36.28	5.66			0.762	0.238	68
Cyprus-Turks	243	48.72	42.28	9.00	1	6-1	0.698	0.300	66
Turkey	274	45.83	43.72	10.43			0.677	0.323	73
AFRICA									
England-Negro	103	61.78	33.64	4.58	2	1At	0.786	0.214	66
Nigeria-Yoruba	153	57.46	36.23	5.71	1 ea	1At, 2-1At, 6-1	0.758	0.239	66
Mozambique	318	61.00	34.20	4.80	13 5 1	1At 2-1At 1 Moz	0.781	0.219	44
S. Africa-Bantu	99	62.73	32.00	4.08	5 1	1At 6-2	0.792	0.202	66

Table 3-1

Distribution of PGM Types in Various Populations (Continued)
(Taken from R. Stedman 1970)

Population	Number Tested	Expected Phenotypes %		Unusual Phenotypes		Gene Frequency			Reference
		PGM 1	PGM 2-1	No.	Type	PGM 2	PGM_1^1	PGM_1^2	
ASIA									
Iraq-Jews	69	45.43	43.94			10.63	0.674	0.326	66
Israel-Habbanite	222	18.49	49.02			32.49	0.430	0.570	70
Korea	72	83.91	15.38			0.70	0.916	0.084	63
Chinese (misc.)	427	56.25	36.30	4 2 1	6-1 7-1 6-2	5.86	0.750	0.242	67
Japan	932	59.75	35.09			5.16	0.773	0.227	71
AMERICA									
U.S.A.-White	508	56.55	37.30			6.15	0.732	0.248	49
U.S.A.-Negro (Seattle)	654	65.45	30.89	4 1	2-1 At 1 Palmer	3.65	0.809	0.191	49
U.S.A.-Negro (Ann Arbor)	202	70.73	26.74	1	1 At	2.53	0.841	0.159	64
Dominica-Carib Indian	99	63.68	32.24			4.08	0.798	0.202	65

Table 3-2

Comparison of Frequencies Between an English Population and a Forensic Sample

Sample	Number Tested		Phenotypes %				Gene Frequency		Reference
			PGM 1	PGM 2-1	PGM 2	Others	PGM_1^1	PGM_1^2	
England	2115	No.	1237	754	118	1ea 3-1, 4-2			
		obs.	58.48	35.67	5.58	5-2, 7-1	0.7645	0.2355	66
		exp.	58.37	36.11	5.52	7-2, 1 Palmer			
M.P.L.	2777	No.	1626	983	165	2 3-1			
		obs.	58.55	35.39	5.94	1 6-1	0.7633	0.2367	
		exp.	58.27	36.13	5.60				

3.2 ADENYLATE KINASE

3.2.1 PHYSICAL DATA

Experience at the Metropolitan Police Laboratory with the human red cell enzyme andenylate kinase (AK) indicates that it is a hardy enzyme, able to withstand very poor conditions yet still maintain a reasonable activity. The molecular weight of the human red cell enzyme is quoted by Giblett[44] as 24,000.

Adenylate kinase catalyzes the transfer of a high energy phosphate group from one molecule of adenosine diphosphate to another producing one molecule of each of the triphosphate and one molecule of the monophosphate (see Figure 3-13).

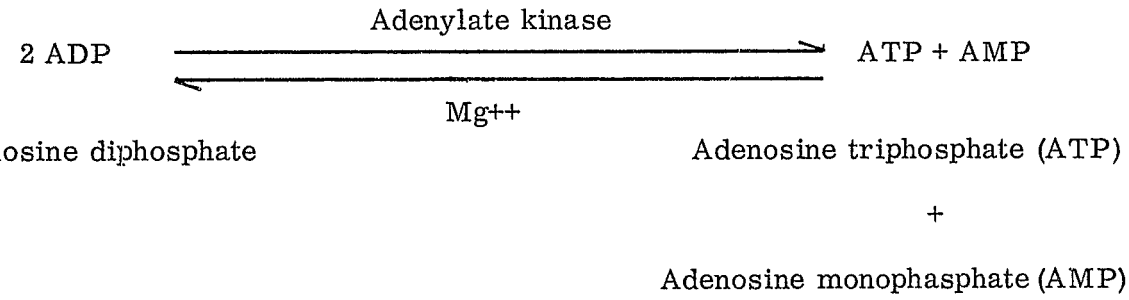

Figure 3-13. Adenylate Kinase Reaction

Adenylate kinase purified from rabbit muscle is reported by Colowick et al.[78] as remarkably stable to changes in pH and to heat. The pH optimum is quoted as 7.5 by Brewer et al.[75].

The equilibrium of the reaction catalyzed by adenylate kinase is dependent on the concentration of magnesium ions.[75] There is a significant increase in the equilibrium constant with the increase in the magnesium ion concentration. This is thought to be caused by changes in the binding constants of the Mg-ATP and Mg-ADP complexes.

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Wherever ATP is used in the body, adenylate kinase is likely to be found. Some of the enzymes dealt with in these sections are involved with glycolysis; therefore, two of AK's contributions to glycolysis via ATP are illustrated in Figure 3-14. The first of these reactions is that used in the detection of adenylate kinase on the electrophoresis plates.

3.2.2 ACTIVATORS AND INHIBITORS

Magnesium ions activate the enzyme and are included in the detection mixture. Fluoride and citrate are reported to inhibit AK. Inactivation can occur with oxidizing agenst (e.g., H_2O_2) which is reversible with treatment with glutathione or cysteine.

Adenosine monophosphate is also reported as having inhibitory effects[79, 84]. Since this AMP is one of the products of the reaction, the concentration of which gradually increases, non-linearity of the reaction rate is to be expected.

During experimental buffer trials, designed to separate and detect multiple enzyme systems from a single electrophoresis plate, it was found that succinic acid has an apparently strong activating effect on adenylate kinase. This is further described in paragraph 3.2.9.

Phosphate appears to inhibit AK activity and this inhibition appears to be relatively greater for the '2' band enzyme than for the major '1' band enzyme. This is an apparent effect on gels when using phosphate buffers instead of the histidine normally used. (100 percent activity was arbitrarily taken as the activity at .01 M phosphate buffer at pH 6.8). (See Figure 3-15)

3.2.3 COENZYMES

There is no requirement for any coenzyme with adenylate kinase. As with many of the enzymes associated with phosphate transfer, there is a requirement of magnesium ions. Other di-valent ions are not as efficient as magnesium.

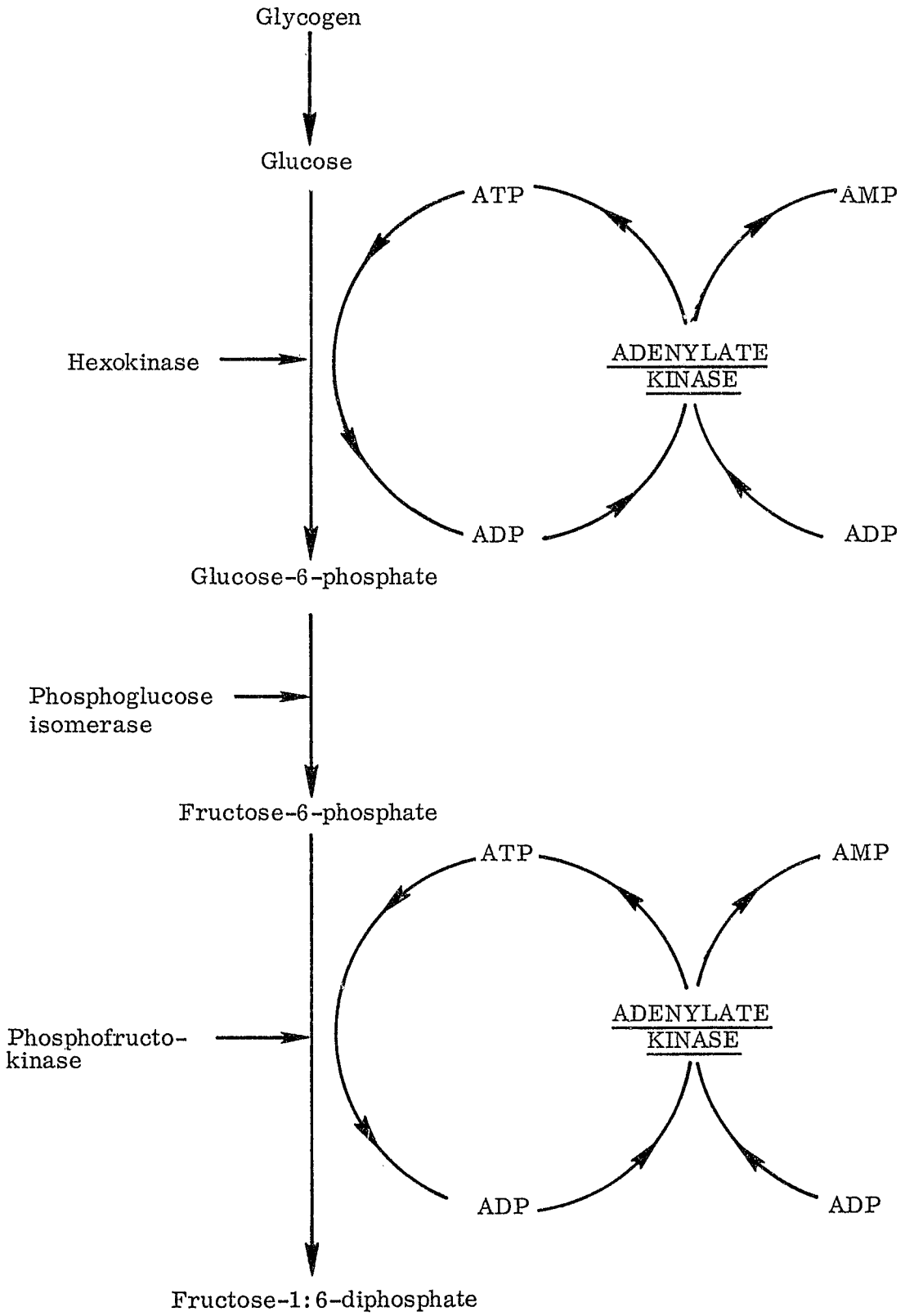

Figure 3-14. Two AK Contributions to Glycolysis via ATP

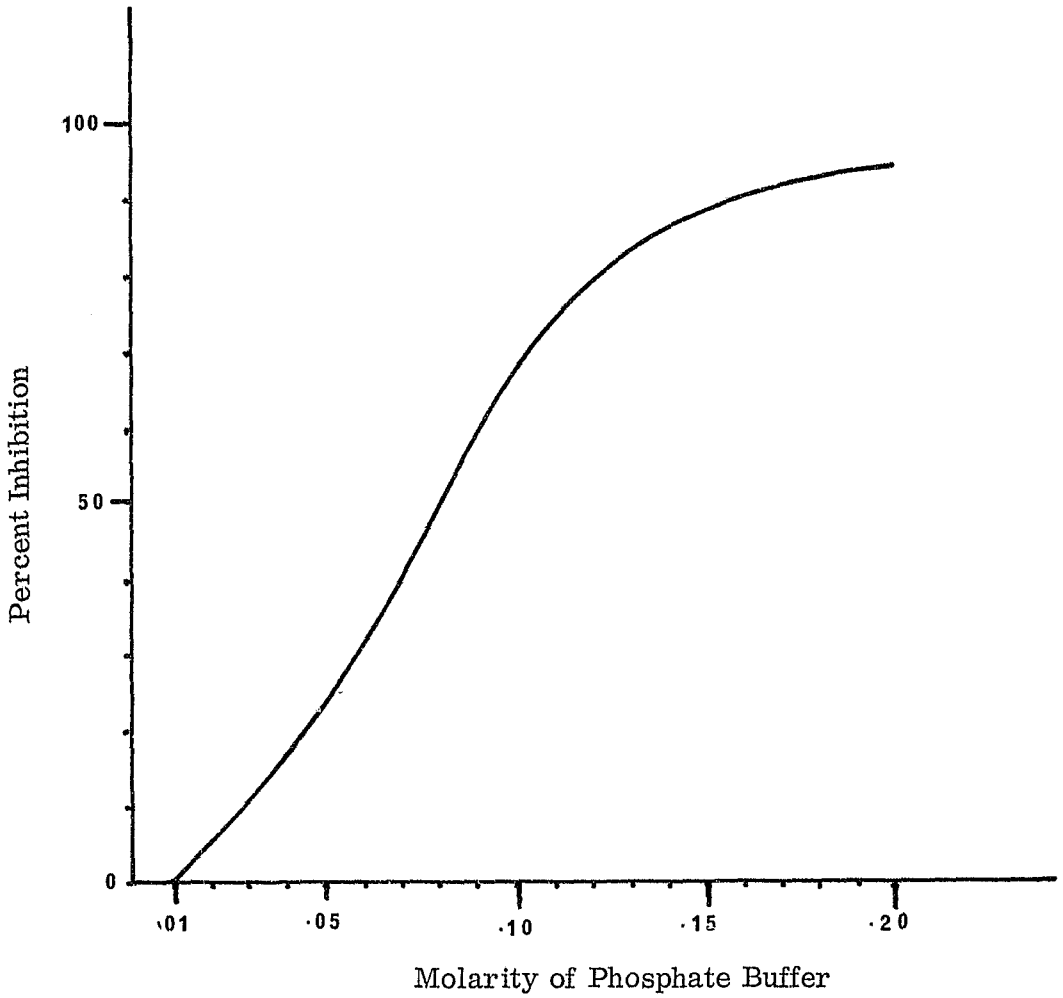

Figure 3-15. Inhibition of AK with Increasing Molarity of Phosphate

3.2.4 POLYMORPHISM

The polymorphism in human red cell adenylate kinase was first described by Fildes and Harris in 1966[81]. The genetics of AK in human red cells is, to date, a simple system of multiple alleles at a single locus. There are two common forms of the enzyme, AK1 and the heterozygote AK 2-1. The homozygote AK2 is rare, and even rarer are the other forms AK 3-1[76] and AK 4-1[83]. AK 3-1 has been described using a different buffer system (phosphate) from that originally used by Fildes and Harris. Figure 3-16 is diagrammatic representation of the AK phenotypes separated in histidine buffer. Figure 3-17 is diagrammatic representation of the AK phenotypes AK1 and AK3-1

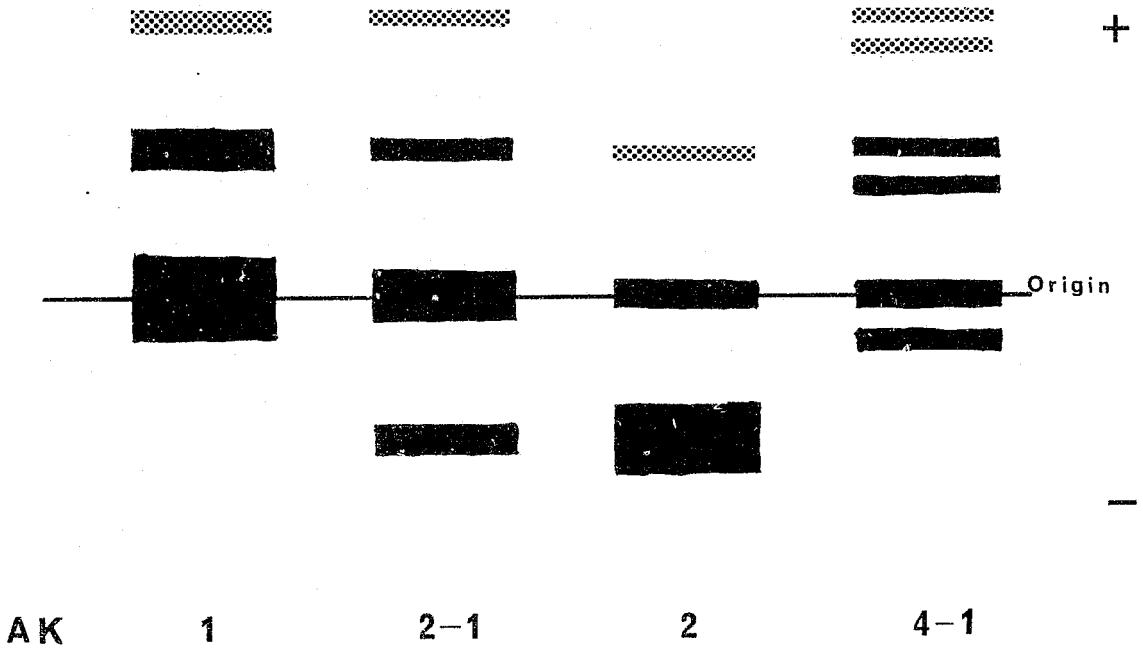

Figure 3-16. AK Phenotypes in Histidine Buffer

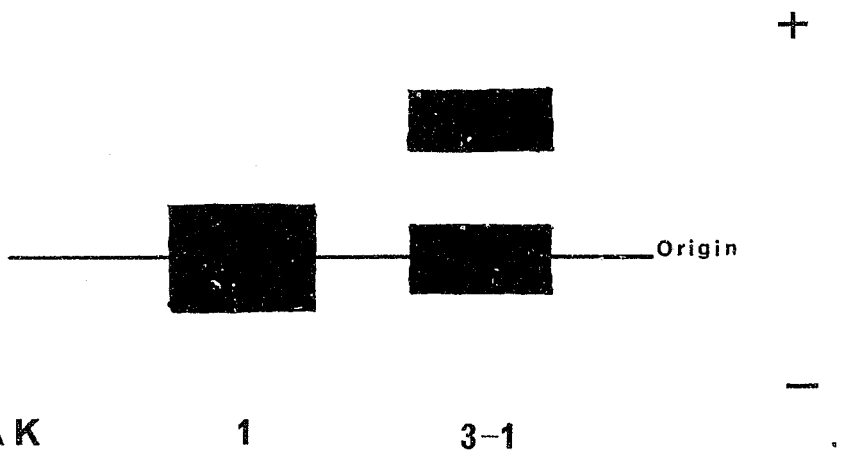

Figure 3-17. AK Phenotypes AK1 and AK3-1 in Phosphate Buffer

separated in phosphate buffer (drawn from photographs of Bowman et al.[76]. Figure 3-18 is separation of AK types using the succinic buffer system. Figure 3-19 is separation of AK types using the histidine buffer system.

The AK^2 gene appears to be appreciably more frequent in Indians and Pakistanis residents in England[83] and very low in negro populations[76,44,83].

Rapley et al.[83] in 1967 and Westkamp et al.[85] in 1969 investigated the possible linkage of AK with other group systems and found that there was a linkage of AK with ABO groups. They say that further studies are needed.

This is not a linkage to particular ABO groups but purely a linkage preventing segregation at meiosis. There is no correlation of AK types with ABO groups on a population basis. The percentage occurrence of AK 2-1 is the same whatever the ABO group.

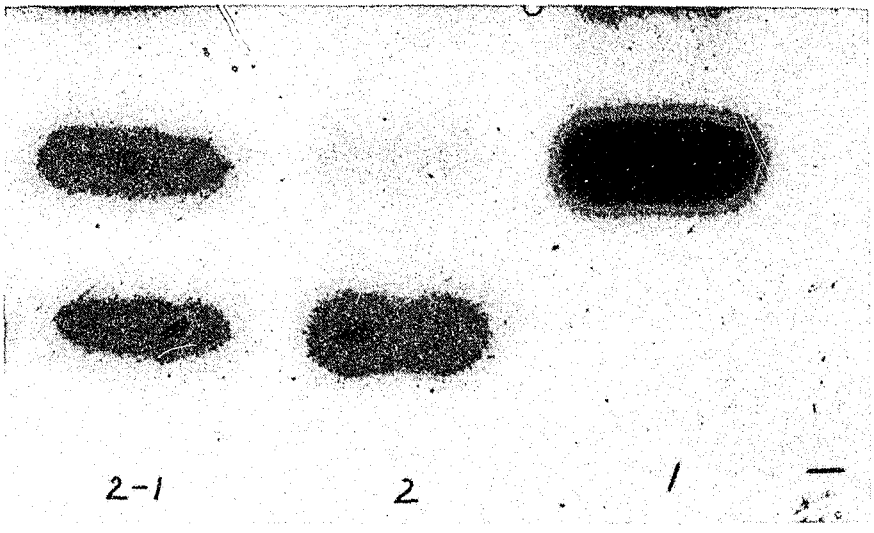

Figure 3-18. Separation of AK Types Using the Succinic Buffer System

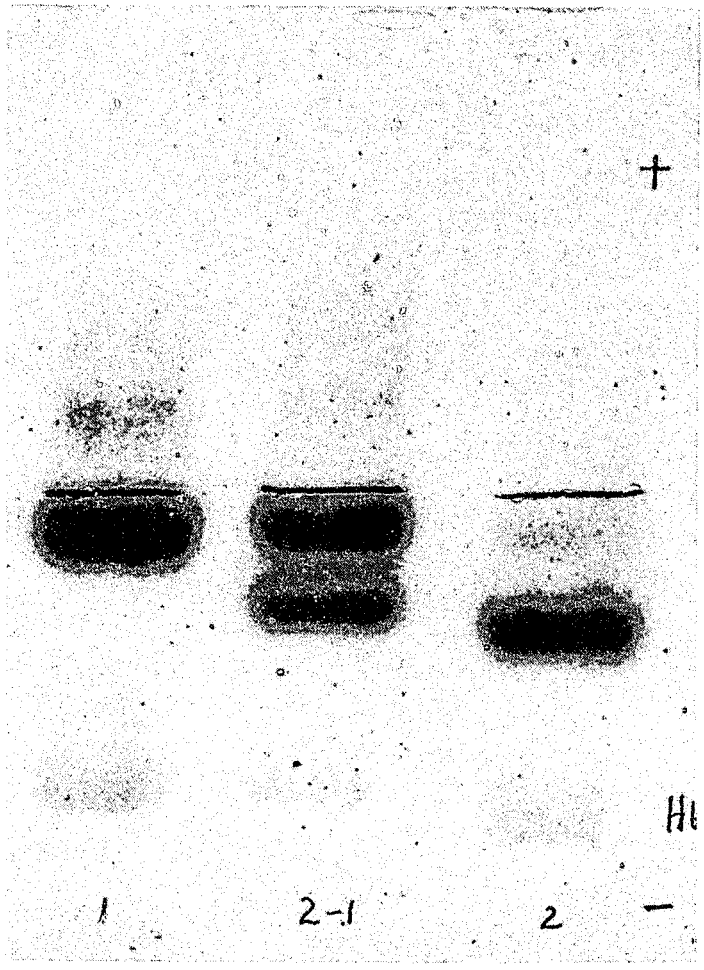

Figure 3-19. Separation of AK Types using the Histidine Buffer System

3.2.5 AK IN BLOOD AND OTHER TISSUES

Adenylate kinase is found in most tissues, particularly where there are high energy requirements, such as muscle. This enzyme was originally known under the name of Myokinase.

If no blood in good condition is available from a cadaver, then muscle tissue can be used for the AK type determination. The AK activity of such material is high. Often muscle tissue can be found in good condition when other tissues have degenerated.

The electrophoretic mobility of adenylate kinase varies slightly depending on what tissue it is derived from[74]. This does not interfere with the ability to determine the AK type.

3.2.6 EFFECTS OF AGE

AK is a very stable enzyme and refrigeration of a blood sample or a lysate is all that is required to keep good activity for several weeks. A lysate deep frozen (-20°C) keeps its activity satisfactorily for periods exceeding one year.

Dried bloodstains rarely give trouble from loss of activity up to one month old and stains 3 to 6 months old have been satisfactorily typed. After 3 to 4 weeks, the effects of loss of activity are seen and as time progresses this loss of activity becomes more and more apparent until at three months some samples will retain sufficient activity but many will not have sufficient residual activity to make typing possible.

The '2' band seems more sensitive to aging than the major '1' band, particularly if phosphate buffer is used for the investigation. If it is suspected that the sample is old or in a condition in which low activities might be expected the use of succinic buffers is recommended.

3.2.7 EFFECT OF THE BLOODSTAIN SUBSTRATE

The stain substrate appears to make no difference to the enzyme. Satisfactory typing has been performed on all types of textile materials, carpets, metal, glass, painted surfaces, and others. The usual techniques for dealing with various substrates apply with no difficulty.

3.2.8 EFFECT OF VARIOUS CHEMICALS

Mercaptoethanol has no effect at dilutions up to 1 percent. The effects of other chemicals have not been investigated in the Metropolitan Police Laboratory since this enzyme has not given the problems that need to be solved by this type of investigation.

3.2.9 SAMPLE PREPARATION

3.2.9.1 General

There is some disagreement on the nature of AK in the red cell. According to Kashket and Denstedt[82], an appreciable proportion of AK activity is associated with the red cell stroma. These authors[82] say that the major part of the activity is in the soluble fraction, but stroma prepared from the haemolysate and well washed with distilled water and isotonic KCl showed, as a suspension in KCl, an activity amounting to about one quarter of the total activity.

Cerletti and De Ritis[77] disagree with this conclusion. These authors state that freshly sedimented stroma from a haemolysate display AK activity. However, as shown, when the stroma are washed the activity consistently decreases and disappears after 5 to 6 washings and is found in the washing liquid.

This is not a point that is critical when examining forensic material which, as a bloodstain, is going to contain a mixture of haemolysate and stroma. It does, however, indicate that the presence of stroma will not have a deleterious effect on the AK enzyme, as in the case with some other enzymes.

From the above, and from the information that AK is an enzyme stable to heat and a wide range of pH, one can expect that minimum preparation of the sample will be necessary, and this has been found to be the case.

Blood samples can be dealt with as a simple lysate—that is, packed cells with a minimum of distilled water added. There is no need for further additions or centrifugation unless the lysate is going to be used for other enzyme examinations, such as ADA or G6PD.

Likewise, the preparation of a bloodstain for examination for AK is minimal. Soaking the bloodstained thread in a minimum of gel buffer for a few minutes (5 to 10 minutes) prior to its insertion into the gel is all that is required.

The activity of AK in bloodstains is reasonably high, and it is possible to overload the gel with enzyme if the thread that is used is thick and heavily bloodstained. This does not prevent type classification but tends to make a plate have a poor appearance; in addition the sample may spread and interfere slightly with adjacent samples.

3.2.9.2 Methods

Usually starch gel has been used in investigations of AK polymorphism and the original buffer system described by Fildes and Harris[81] is better than any other published method that has been tried. The determination of AK types in bloodstains was reported by Culliford and Wraxall[80] in 1968. It has been found that a succinic buffer is also excellent and both will be described in the following paragraphs.

In an effort to reduce the amount of bloodstain required for a completed enzyme investigation, an attempt was made to combine AK with 6-phosphogluconate dehydrogenase on a phosphate buffer gel, developing one part of the plate for 6-phosphogluconate dehydrogenase and the cathodal part for AK. The results for lysates and strong fairly fresh bloodstains were satisfactory and correct typing was obtained. It was found, however, that stains of low activity, either because of the age of the stain or because of the small amount of blood in the stain, gave weak AK 1 results when in fact they were AK 2-1. The '2' band did not stain with the reaction mixture as it should have done.

When the same stains were run with histidine buffer, the '2' band showed clearly. Therefore, the phosphate buffer was dropped and all AK work is now done using the histidine buffer or the succinic buffer.

The thin layer starch gel technique is used and this can be done in a cold room 0°C or using a cooling plate below the gel at 6 to 8°C. At room temperature too much diffusion occurs.

3.2.9.3 Histidine Buffer System

Starch gel at a concentration of 13 g percent is made in the gel buffer adjusted to pH 7 with 2N sodium hydroxide and composed of:

Histidine (0.005 M)	0.79 g
Distilled water	1 litre

The tank buffer, adjusted to pH 7 with 2N sodium hydroxide, is composed of:

Citric acid (0.41 M)	86.1 g
Distilled water to	1 litre

The inserts are put into the gel at the centre of the gel. Electrophoresis is performed for four hours at 10 to 12 volts/cm or overnight (17 hours) at 3 volts per cm at 0°C.

After electrophoresis, a reaction mixture agar overlay is poured onto the starch gel 5 cm towards both the anode and the cathode from the origin.

3.2.9.4 Succinic Buffer System

Starch gel at a concentration of 13 g percent is made in the gel buffer, adjusted to pH 5 with saturated succinic acid solution if necessary, and which is composed of:

Succinic acid	1.84 g
TRIS (Tris-hydroxymethylamino-ethane)	2.236 g
Distilled water to	1 litre

The tank buffer, adjusted to pH 5 with 40 percent sodium hydroxide and make up to 1 litre, is composed of:

Citric acid	86.1 g
Distilled water	800 ml

The inserts are put into the gel towards the anode end of the plate, about one-third of the distance from the anode to the cathode. Electrophoresis is performed for 4 hours at 12 volts/cm.

After electrophoresis, a reaction mixture agar overlay is poured onto the starch gel from 2 cm on the anodic side of the origin to 7 to 8 cm to the cathodic side of the origin.

3.2.9.5 Development of the Gels

The AK detection is accomplished by using the ATP formed by AK from ADP to phosphate glucose in the presence of hexokinase. The glucose-6-phosphate is then ozidized by G-6-P dehydrogenase and NADP forming NADPH, which, through the usual PMS and MTT system forms the blue bands at the positions of AK activity. This mechanism is illustrated in Figure 3-20.

The reaction mixture is as follows:

Buffer 0.1 M TRIS (12.14 g TRIS in 500 ml distilled water adjusted to pH 8 with HCl).

Buffer	10 ml
Glucose	18 mg
Magnesium chloride	40 mg
Adenosine diphosphate	5 mg
Nicotinamide adenine dinucleotide diphosphate	3 mg
Phenazine methosulphate	2 mg
MTT tetrazoleum	2 mg
Hexokinase	0.8 units
Glucose-6-phosphate dehydrogenase	0.4 units

This mixture is brought to 37 °C in the incubator and mixed with 10 g of 2 percent agar in distilled water at 50 to 55 °C and immediately poured onto the gel.

When set it is covered with a glass plate and incubated at 37 °C for one-half to one hour; the bands of activity are seen, as illustrated in Figures 3-18 and 3-19.

3.2.9.6 AK and Pseudocholinesterase C_5 from the Same Electrophoresis Plate

Using the acrylamide/starch method described under pseudocholinesterase C_5, AK and C_5 typing can both be obtained from one plate and one insert. This system

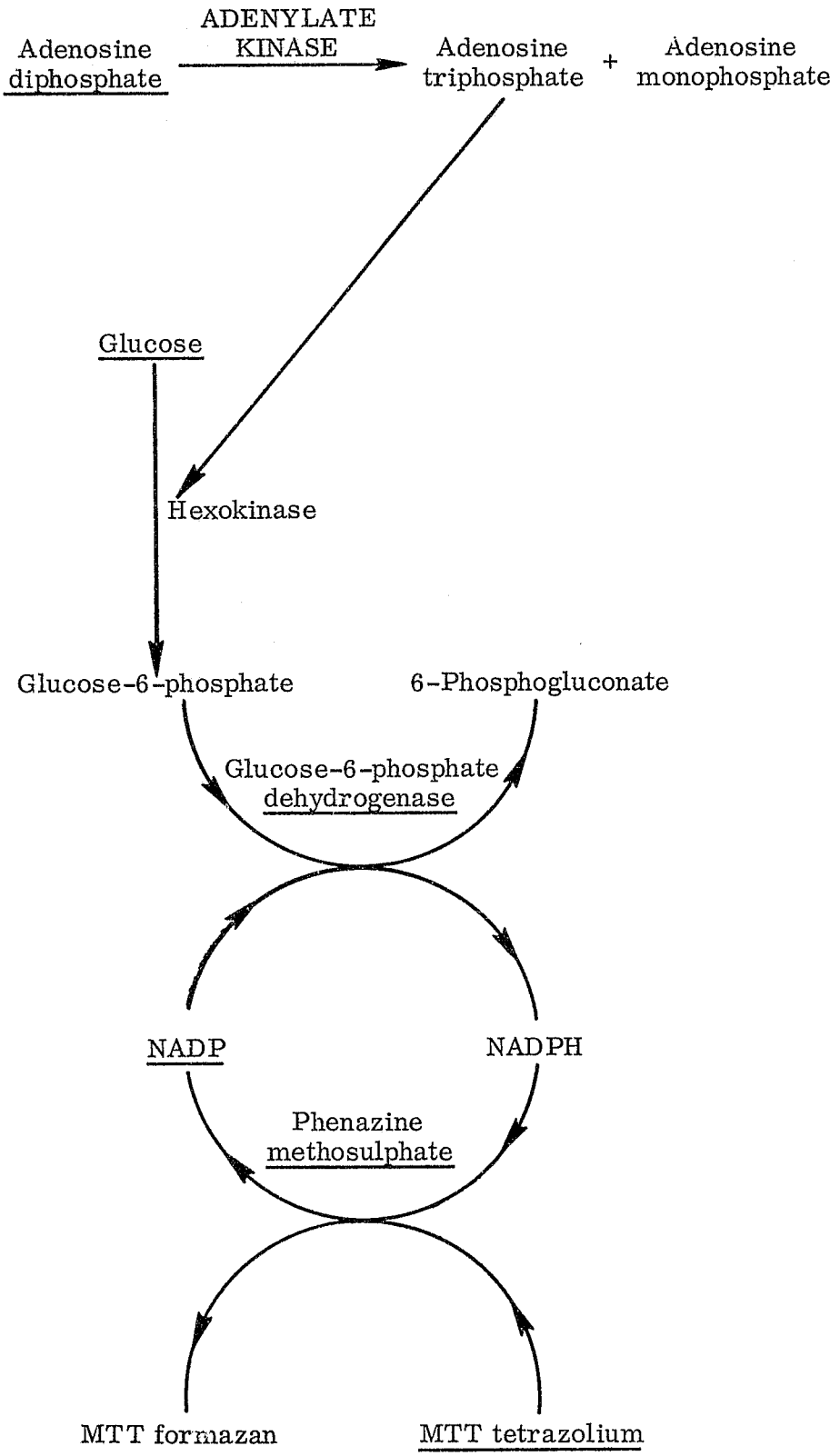

Figure 3-20. Mechanism for Detecting AK

uses the clarity obtained with succinic buffers and the distinctness of acrylamide in the banding. A photograph of AK types separated by this technique is also illustrated in paragraph 3.6, Pseudocholinesterase.

3.2.10 PROBLEMS WITH THE AK SYSTEM

When the histidine buffer system is used, as bloodstains age, the AK_2 band weakens and the AK_1 band tends to widen and become diffuse. These can make interpretation difficult in old stains, particularly if the activity of the sample is low.

In cases where only an indistinct pattern can be seen, no interpretation should be given. There is considerable reduction of these aging effects if the succinic buffer system is used.

Occasionally, with fresh blood samples, a weak shadow band between AK_1 and AK_2 bands can be seen. This appears to occur when the red cells are incompletely lysed. Care should be taken to ensure complete lysis of the sample.

In general, very few difficulties are encountered with this group system.

The population distribution of AK types is shown in Table 3-3. Comparison of frequencies is given in Table 3-4.

Table 3-3

Distribution of AK Types in Various Populations
(Taken from R. Stedman 1970)

Population	Number Tested	AK Phenotypes %					Gene Frequency		Reference
			AK 1	AK2-1	AK2	-	AK^1	AK^2	
EUROPE									
England	1887	obs. exp.	91.15 91.24	8.74 8.56	0.10 0.20		0.9552	0.0448	83
Ireland	114	obs. exp.	80.70 76.18	13.15 22.19	6.14 1.62		0.8728	0.1271	63
Finland (Lapps)	307	obs. exp.	99.02 99.02	0.97 0.97	0.00 0.01		0.9951	0.0049	83
Finland	77	obs. exp.	92.21 92.35	7.79 7.50	00.00 0.15		0.9610	0.0390	83
Germany	2415*	obs. exp.	92.79 93.11	6.91 6.73	0.04 0.12		0.9649	0.0349	91, 93
Italy	841	obs. exp.	92.62 92.64	7.25 7.20	0.12 0.14		0.9625	0.0374	90
Sardinia	1033	obs. exp.	97.19 97.12	2.71 2.86	0.10 0.02		0.9855	0.0145	89
AFRICA									
Ghana/Nigeria	800	obs. exp.	100.0 100.0	0.00 0.00	0.00 0.00		1.000	0.0000	76
Nigeria	153	obs. exp.	100.0 100.0	0.00 0.00	0.00 0.00		1.000 1.000	0.0000 0.0000	83
Congo-Baginga pygmy	300	obs. exp.	100.0 100.0	0.00 0.00	0.00 0.00		1.000 1.000	0.0000 0.0000	89
Mozambique	318	obs. exp.	99.37 99.36	0.63 0.62	0.00 0.01		0.9968	0.0031	44
S. Africa-Bantu	100	obs. exp.	98.00 98.01	2.00 0.98	0.00 0.01		0.9900	0.0100	88
S. Africa-White	100	obs. exp.	92.00 91.20	7.00 8.60	1.00 0.20		0.9550	0.0450	88

* 6 AK 3-1 observed.

Table 3-3

Distribution of AK Types in Various Populations (Continued)
(Taken from R. Stedman 1970)

Population	Number Tested	AK Phenotypes %					Gene Frequency		Reference
			AK 1	AK2-1	AK2	-	AK^1	AK^2	
ASIA									
Iraq-Jews	139	obs. exp.	90.65 90.86	9.35 8.90	0.00 0.22		0.9532	0.0467	83
Iran-Moslem	322	obs. exp.	90.06 90.31	9.93 9.42	0.00 0.25		0.9503	0.0496	87
Pakistani (England)	54	obs. exp.	74.07 76.74	25.92 22.56	0.00 1.68		0.8703	0.1296	83
Indian (England)	132	obs. exp.	81.06 81.27	18.18 17.74	0.75 0.97		0.9015	0.0984	83
India - South	246	obs. exp.	84.14 82.90	13.82 16.28	2.03 0.80		0.9105	0.0894	86
Malayan (S. Africa)	100	obs. exp.	93.00 93.12	7.00 6.76	0.00 0.12		0.9650	0.0350	88
Thailand	201	obs. exp.	95.51 95.57	4.47 4.36	0.00 0.05		0.9776	0.0223	44
Korea	75	obs. exp.	89.33 87.10	8.00 12.45	2.67 0.45		0.9333	0.0667	63
China and Taiwan	227	obs. exp.	99.55 99.54	0.44 0.44	0.00 0.01		0.9977	0.0022	92
AMERICA									
U.S.A. - White	1740	obs. exp.	91.72 91.73	8.10 8.09	0.17 0.18		0.9577	0.0422	76,64,44
U.S.A. - Negro	1424	obs. exp.	98.59 98.60	1.40 1.39	0.00 0.01		0.9930	0.0070	76,64,44
West Indies	85	obs. exp.	98.82 98.82	1.17 1.17	0.00 0.01		0.9941	0.0059	83

Table 3-4

Comparison of Frequencies Between an English Sample and a Forensic Sample

Sample	Number Tested	AK Phenotypes %					Gene Frequency		Reference
			AK-1	AK 2-1	AK 2	-	AK^1	AK^2	
ENGLAND	1887	No.	1720	165	2				
		obs.	91.15	8.74	0.11		0.9552	0.0448	83
		exp.	91.24	8.56	0.20				
M.P.L.	2511	No.	2322	189	0				
		obs.	92.47	7.53	0.00		0.9623	0.0377	
		exp.	92.60	7.25	0.15				

451-769 O - 72 - 10

3.3 6-PHOSPHOGLUCONATE DEHYDROGENASE (PGD)

3.3.1 PHYSICAL DATA

Kazazian[119] reports a molecular weight of 79,000 ± 8000 for 6-phosphogluconate dehydrogenase in both man and drosophila. Parr and Fitch[126] suggest that the molecule is a dimer. A heterozygote would, therefore, produce, by random combination of 'A' and 'C' chains, for example, three types of molecule- 'AA', 'AC', and 'CC'. In the A/C heterozygote three bands are in fact seen. The 6-phosphogluconate dehydrogenase reaction is shown in Figure 3-21.

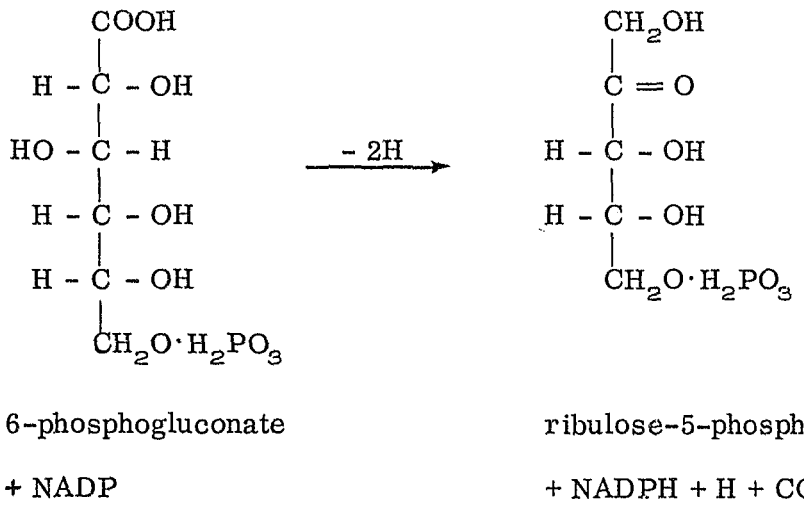

Figure 3-21. 6-Phosphogluconate Dehydrogenase Reaction

3.3.2 ACTIVATORS AND INHIBITORS

The enzyme from yeast is reported to be inactivated by heavy metals, but metal binders will reactivate the enzyme. Manganese and magnesium activate the red cell enzyme and the latter is included in the developing solution. An excess is reported as inhibitory[99]. Fluoride, malonate and cyanide are reported not to be inhibitory[102]. Phosphate at concentrations over 0.1 m have been shown to be inhibitory[108] (see paragraph 3.3.11).

The White chapel variant is reported as unstable in the absence of the usual type PGD^A. That is, the stability of the homozygous $PGD^W \cdot PGD^W$ is poor but that of the Dalston variant $PGD^W \cdot PGD^A$ is satisfactory.

The heat stability of the enzyme at 37°C in human lysates is poor and variation between the normal and the less stable variant types was described by Parr and Parr[114].

3.3.3 COENZYMES

NADP is required as a coenzyme (hydrogen accepter). There is an optimum concentration since, as will be described later, NADP can have an apparently inhibitory effect.

3.3.4 POLYMORPHISM

The polymorphism of PGD was attributed originally[102,103,104] to two alleles, alpha and beta, giving three possible variations, the homozygous types for alpha and beta alleles and the heterozygous type, with one of each allele.

The symbols alpha and beta were later thought to be inappropriate, since they are generally accepted as representing polypeptide chains that may be synthesized by genes at different loci (e.g., the alpha and beta chains of haemoglobin). Since Arabic numbers had from the start been used to denote the phosphogluconate dehydrogenase electrophoretic components, it seemed quite appropriate to use the same numbers (where applicable) for the genes responsible for these components.

To avoid this confusion, the alleles were subsequently renamed PGD^A and PGD PGD^{C}[98,113], thus giving three (electrophoretic) phenotypes: the A (or usual) type, with an 'a' electrophoretic band; the C (or canning) variant, with a dominant 'c' band; and the heterozygous CA type (the so-called common variant), with both 'a' and 'c' electrophoretic bands and also a strong hybrid 'b' band.

Since then, various rare alleles have been described—Richmond and Hackney in 1966[109]; Ilford, Dalston, Whitechapel, and Newham[113]; Thai[116], Friendship[101], and Elcho[94]. Other alleles have been discovered but details have not yet been published (Neath variant, Freiberg variant[110]). See Figure 3-22.

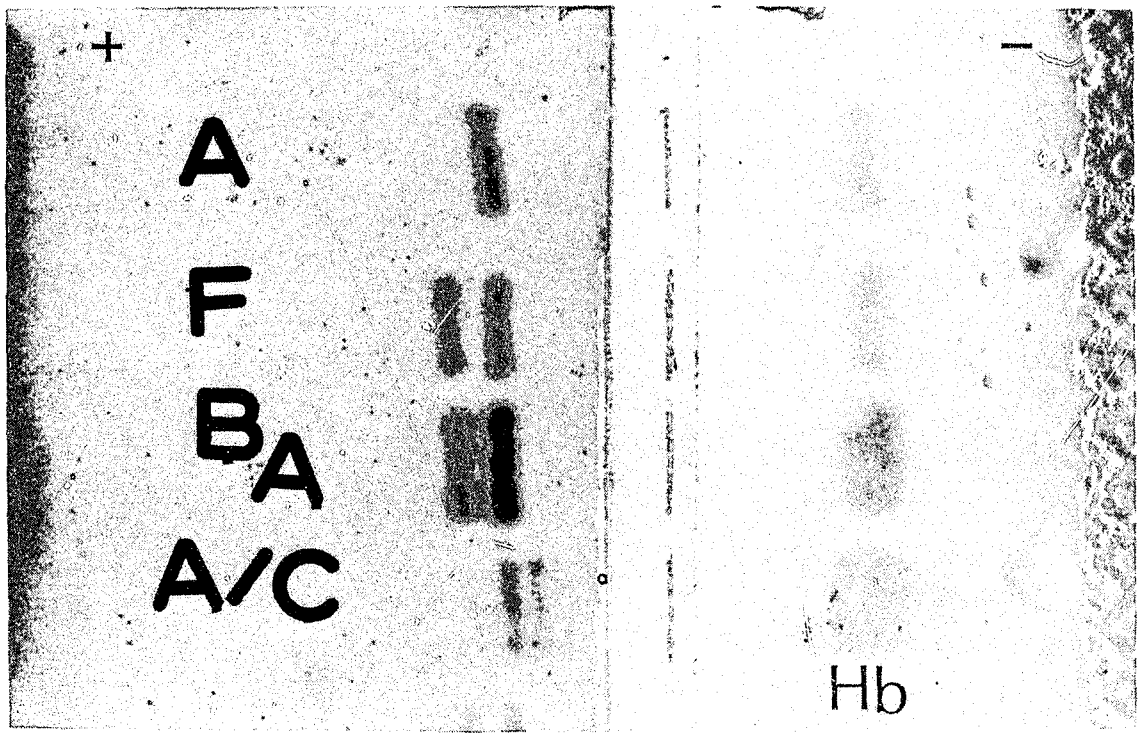

Figure 3-22. Other Alleles
(A, Normal Type; F, Freiberg; BA, Richmond; A/C, Common Variant; Hb, Haemoglobin)

The activities of some of these rare variants are less than that of the normal type of enzyme. Some are slightly less than normal; some are half activity, for example the Ilford type (genotype PGD^{A} PGD^{Ilford}) with a 'silent' gene; and one is deficient in red blood cells, the Whitechapel type.

So in this system of enzyme variants there are qualitative (i.e., electrophoretic) and quantitative (i.e., activity) variability.

Parr, et al[114] have suggested that those half activity forms should be named similarly to the electrophoretic variants to avoid any confusions that might arise from the earlier half-activity nomenclature.

Throughout this paragraph Parr's recent nomenclature and notation have been adopted, since this is clearly more satisfactory in use than the earlier systems.

It was reported[99] that the drosophila enzyme of M.W. 80,000 was composed of two sub-units each of about 40,000. Parr and Fitch[113] suggest a similar structure to explain the triple-banding of the heterozygous common variant. For details, refer to this and other papers by Parr. See Figure 3-23 for a table of enzyme activity.

3.3.5 PGD IN BLOOD AND OTHER TISSUES

The assay of total activity in red cells is dependent on the PGD phenotype present. The average assay on the usual type is 136 ± 24 μ Moles NADPH per gram, haemoglobin per hour according to Brewer and Dern[96] or 3.08 ± 0.33 units per gram Hb according to Parr and Fitch[112] (the latter result is 185 ± 19.8 μ Moles NADPH per gram haemoglobin per hour).

Gibbs[105] reported the 6PGD activity in vaginal secretion. Unfortunately Gibbs states that the first action is to make up the sample of vaginal secretion to 3 ml from an unknown original amount. Therefore, the relationship of enzyme activity to original secretion cannot be determined from the figures he quotes. No PGD activity was found in human semen.

3.3.6 EFFECTS OF AGE

The effects of storage are connected with those of stability in the usual, common variant, and canning types.

The development of anodal bands, the so-called 'storage bands' or 'satellite bands', occurs with the A or usual type associated with some loss of activity. Anodal band formation also occurs with the CA and the C types but the storage stability of these is lower than that of the A type and loss of activity is more marked.

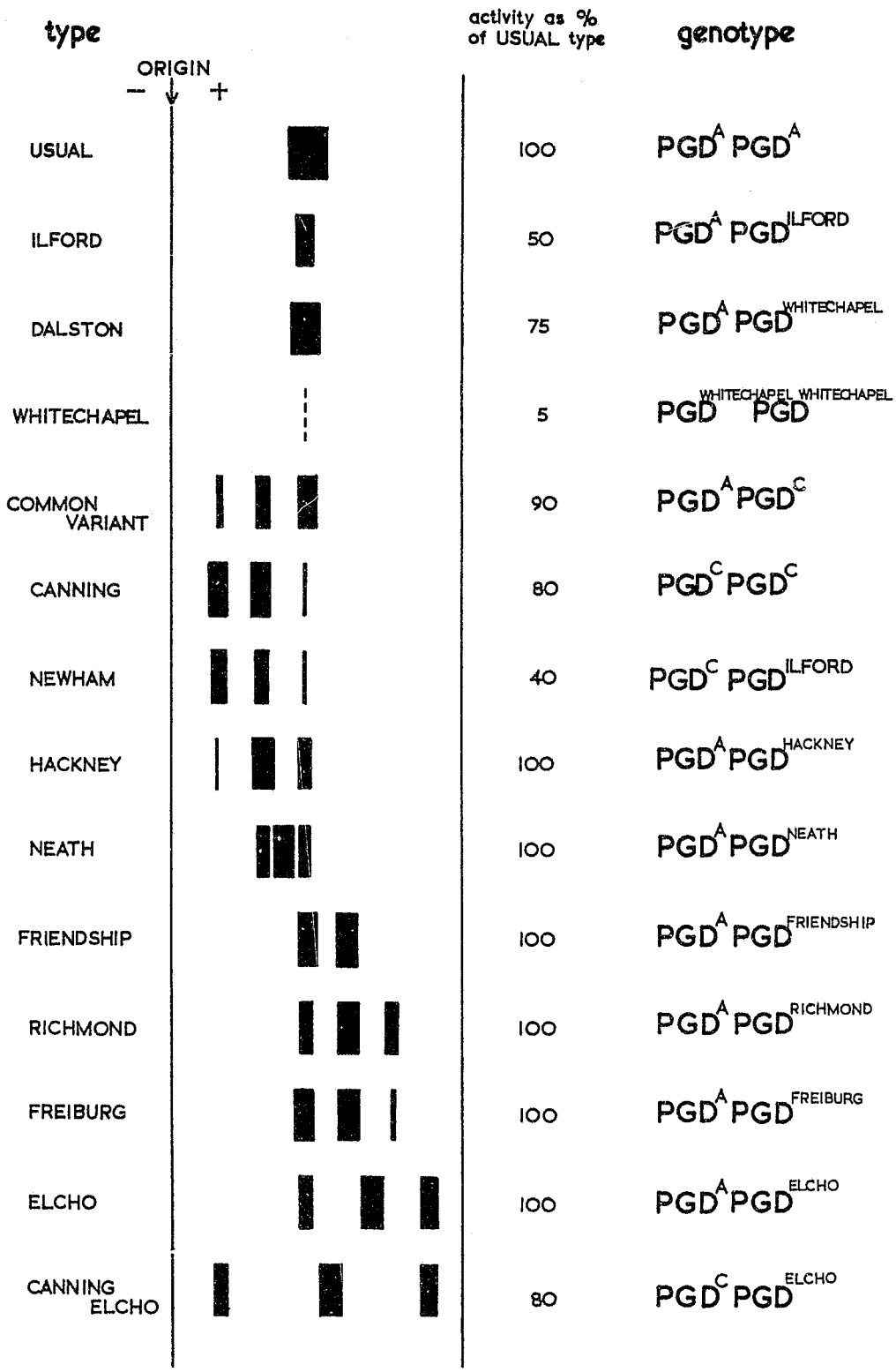

Figure 3-23. Table of Enzyme Activity

In Figure 3-24, storage bands are shown in the two centre samples (marked sb). The samples are, from the top, A/C lysate fresh; bloodstain type A (marked S); A, lysate old; A, lysate fresh.

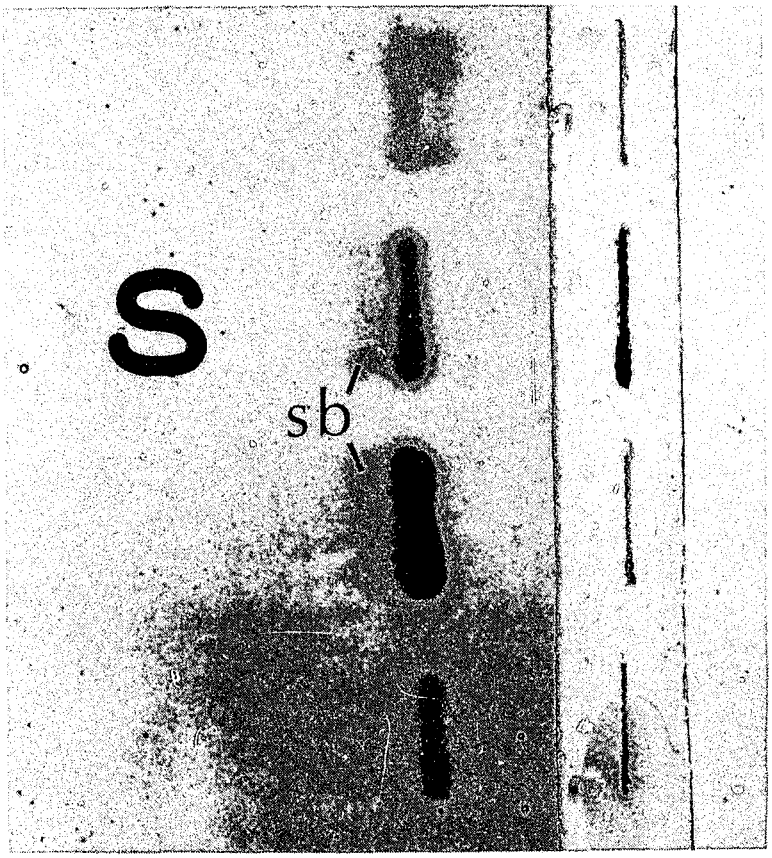

Figure 3-24. PGD After Storage

These bands have never interfered with the interpretation of the pattern as seen on the electrophoresis plate.

Carter, et al.[98] report the production of these anodal bands in the presence of NADP. The Forensic Science Laboratory also found this to occur. NADP, either as a pre-treatment to the sample or when added to the starch gel, causes a reduction in the activity shown by the enzyme bands on development of color at the end of electrophoresis.

Storage or incubation leads to loss of activity. Parr and Parr[114] report a rapid loss of activity dependent on enzyme type after 15 minutes incubation at 37°C.

		Mean Loss of Activity (%)
(AA)	Usual	27
(CA)	Common Variant	49
	Newham	70
	Ilford	35
(CC)	Canning	62

Storage of blood samples at room temperature leads to loss of PGD activity at a slower rate than this. Storage at 0°C delays this but loss of activity is apparent in two to three weeks. Excellent preservation according to Azewedo and Yoshida is obtained by storage at -70°C.

The Forensic Science laboratory found that at -20°C, unsatisfactory storage of A/C is obtained beyond one week, if it is thawed and frozen each day, while storage in liquid nitrogen has been found to be excellent. At present, no very long terms of storage have been tried. To date, all samples stored in nitrogen that have been tested have been satisfactory to periods of over one year.

The storage factor with bloodstains at normal temperatures is similar to that of other enzymes. During a short period of storage—up to three weeks—satisfactory results can be obtained by electrophesis. The loss of activity is faster than that of PGM, AK, or ADA, but sufficient activity persists for a time, which makes this system useful in most instances of bloodstain examination.

3.3.7 DEFICIENCY

As has been mentioned, genetically controlled partial deficiency can also occur associated with several forms of the enzyme, e.g., where a 'silent' gene PGD^{Ilford} is present. For example, PGD^{A} PGD^{Ilford} is a half activity usual electrophoretic type (the Ilford type). The deficient types can be assayed readily in fresh samples of lysates and their activity expressed in units per gram Hb or as a ratio of 6PGD to G6PD activities. G6PD can also be deficient and Brewer and Dern[96] showed that individuals can be deficient in both enzymes.

In bloodstains, loss of activity with time is normal so that an expression of activity as units per gram Hb is more likely to be an expression of the condition of a normal enzyme in a stain rather than any genetic deficiency.

Enzymes degrade at different rates in bloodstains and in some cases unpredictably. G6PD loss is faster than that of 6PGD and therefore the ratio 6PGD:G6PD is also a function of bloodstain condition and time.

As a result, partially deficient forms of PGD cannot at the moment be used for forensic purposes when bloodstain material is involved. Some better method will have to be devised before this becomes a practical possibility.

3.3.8 SPECTROPHOTOMETRIC METHODS

The usual, common variant, and canning types have different stabilities not only to heat, as previously mentioned, but also to various inhibitors.

Carter, et al.[117] (1966) showed that the residual activity after incubation at 25°C with urea (1.5 m) and iodoacetate (0.01 m) was markedly different.

	Residual Activity (% ± SD)	
	1.5 M Urea	Iodoacetate
Usual	63 ± 10	80 ± 4
Common Variant	30 ± 7	50 ± 3
Canning	5 ± 6	20 ± 3

This technique using iodoacetate has potential in forensic problems, where a dilute solution of blood or a dilute stain extract is all that can be obtained. It is also much more rapid than the electrophoretic technique. With microcells in the spectrophotometer, less material is needed than for the electrophoretic method. It does not, however, distinguish adequately the other variants, which, while rare in occurrence, do occur in case material and are extremely valuable evidence when they do occur.

Teepol has also been used by Parr[111] as a selective inhibitor. In the Forensic Science Laboratory, this technique was found to be difficult to work with satisfactorily. It does, however, point out the necessity of adequately cleaning glassware prior to its use in such assays and ensuring complete freedom from detergents.

3.3.9 EFFECTS OF STROMA

Carson, et al.[97] reported the effects of stroma on the activity of G6PD, PGD, and GR in lysates. The assay of the activity of PGD varies with (a) the presence or absence of NADP, (b) the electrophoretic type of the enzyme, and (c) the presence or absence of stroma in the lysate. These authors found that after two hours' incubation at 45°C in the absence of stroma, the usual enzyme gave a 97 percent residual activity without NADP and 96 percent residual activity with 10^{-4} M. NADP.

In the presence of stroma and the absence of NADP, there was 82 percent residual activity, but in the presence of both stroma and 10^{-4} M. NADP only 55 percent of the original activity remained after the incubation. This seems surprisingly high when compared with the results of incubation reported by Parr and Parr[114].

If the PGD type is other than the usual type, i.e., common variant or canning, then there is a much lower residual activity after incubation, which agrees with the relative stability findings of Parr and Parr[114]. These findings have relevance in the examination of bloodstains, for in these it is not possible to remove stromal debris during the ageing of the stain and during the preparative stages prior to electrophoresis. Upon electrophoresis, the enzyme is separated from the stromal debris.

It is disadvantageous to have NADP in the gel or as a pre-treatment to the stain prior to electrophoresis. NADP is, however, required as a coenzyme and obviously the concentration of this during the development stage after electrophoresis should be kept to an amount that will give the optimum results, unlike other enzymes where an excess is not deleterious.

Unfortunately, most of these findings with regard to PGD are reversed when considering G6PD. This leads to some difficulty in getting good results for both enzymes from one electrophoresis plate when dealing with the more critical bloodstain material. Results can be obtained from lysates in this way, but they are not the optimal, high-quality results obtainable by using separate gels for each system.

3.3.10 STAIN SUBSTRATE

The substrate material that is bloodstained appears to have no effect on the ability to determine the type of PGD. Cotton, wool, and synthetic fibres, glass, metal, and other substrates have been investigated and found satisfactory.

3.3.11 EFFECTS OF CHEMICALS

Mercaptoethanol leads to a diminution or loss of activity depending on the concentration used. It is not, therefore, employed to remove storage bands as with some other enzyme systems.

In lysed blood samples, which have been preserved with fluoride, there is a marked diminution or complete loss of activity. In normal blood samples, cells preserved with fluoride should be well washed prior to use.

3.3.12 METHODS

3.3.12.1 General

Numerous methods have been described for the determination of PGD types. The method first described was by starch gel electrophoresis[103]. Bowman, et al.[95] used starch with a different buffer system. Khan and Rattazzi[107] described the use of cellulose acetate gel. Cellulose acetate membranes also have been tried[42]. Peebles et al.[115] used acrylamide gel (for the drosophila enzyme).

All of these techniques have been tried but the one found most satisfactory for forensic work on bloodstains was based on the thin layer starch gel technique[62]

with a single cooling plate underneath at 6 to 8° C. The following buffer system is used:

Tank Buffer: 0.2 M phosphate

a.	Disodium Hydrogen Phosphate (anhydrous)	28.4 g
	Water to	1 litre
b.	Sodium Dihydrogen Phosphate ($2H_2O$)	31.2 g
	Water to	1 litre

b. is added to a. until pH 6.8 is reached.

Gel Buffer

1/15 dilution of tank buffer.

The bloodstained thread is soaked in a minimum of gel buffer for 15 to 30 minutes prior to insertion into the gel. Only just sufficient buffer to moisten the stained thread should be used without any excess. During this soaking period the samples should be kept in a moist chamber.

A lysate or a stain eluate or a liquid sample of any sort may be used undiluted or, if somewhat too concentrated (e.g., lysate), it can be diluted with gel buffer. Clearer bands of activity are seen if the activity of the sample is not too high.

A standard control sample of the common variant (A/C) should always be included in the plate as a marker. The A/C control lysate should not be diluted. Since there is loss of activity in storage, it has been found most satisfactory to use a fresh lysate for this. Rather than store this as a lysate and have to continually freeze and thaw (which also decreases activity), it has been found best to store the control as a cell suspension at 0 to 4° C and lyse a small amount of this immediately before use.

3.3.12.2 Electrophoresis

Electrophoresis is carried out at 9 volts/cm for 4 to 5 hours with a cooling plate at 6 to 8° C. Overnight runs at reduced voltage give diffuse bands and poorer separation.

3.3.12.3 Development

After electrophoresis is completed, development of the PGD bands is accomplished by the agar overlay technique. Fitch and Parr described a painting technique for this, but this was found to be less satisfactory than the agar overlay method when dealing with bloodstains.

The reaction buffer is 12.14 g TRIS dissolved in 450 ml of distilled water and adjusted to pH 8 with 2N HCl. It is made up to 500 ml.

The reaction mixture includes:

Buffer	10 ml
Magnesium chloride	40 mg
6-Phosphogluconate (trisodium salt)	3 mg
Phenazine methosulphate	1 mg
MTT	2 mg
NADP	2 mg

The above mixture is brought to 37°C and mixed with 10 g 2 percent agar in distilled water and melted and cooled to 50 to 55°C and immediately poured onto the starch plate from the origin to 7 cm towards the anode. When set, it is covered with a glass plate supported on 3-mm thick glass edge strips as described in paragraph 1.2. Incubation in the dark can be at room temperature, but 37°C is preferable. The blue bands of PGD activity are seen 15 to 60 minutes later. See Figure 3-25 of a diagrammatic representation of the detection mechanism.

3.3.13 PROBLEMS

The only problem which has arisen, apart from loss of activity, is the appearance of a fast anodal band of activity in blood samples that are in bad condition and in some old bloodstains. When this band occurs, it replaces the bands in the normal positions and is non-specific with reference to the original type of enzyme present, and the sample cannot be typed. Storage bands are present normally with stains and this should not be confused with the Richmond type. Table 3-5 shows the population distribution for PGD types. Table 3-6 is a comparison of frequencies.

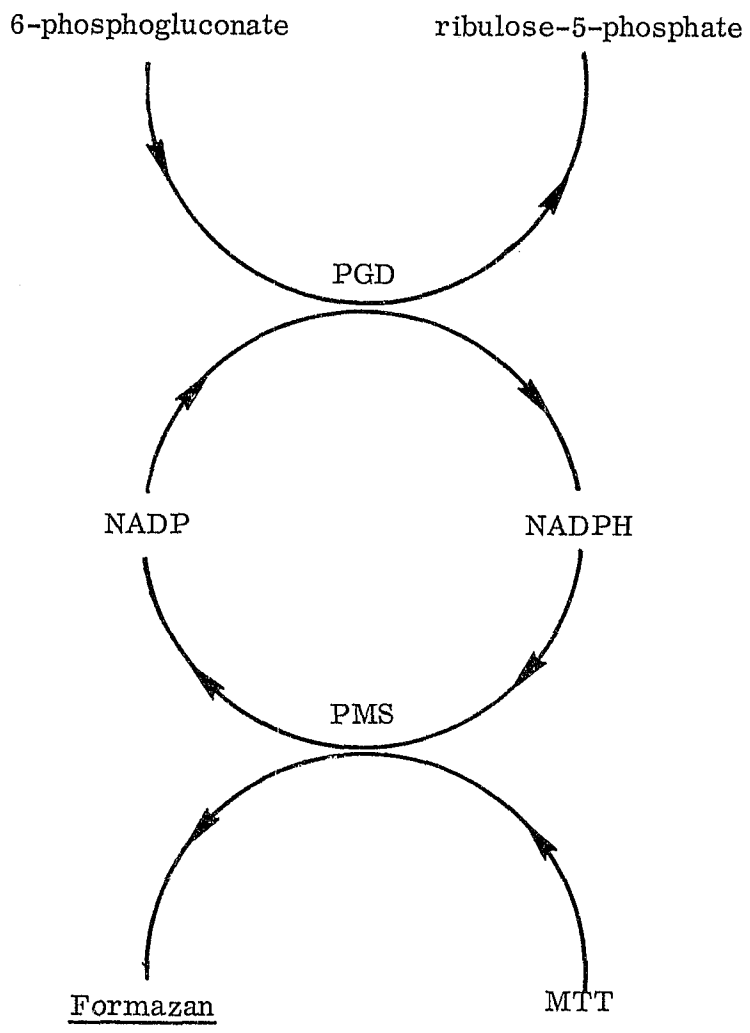

Figure 3-25. Diagrammatic Representation of the Mechanism for Detecting PGD

Table 3-5

Population Distribution for PGD Types

Population	Number Tested	PGD Phenotypes (%)					Gene Frequency		Reference
			PGD^A	PGD^{AC}	PGD^C	–	PGD^A	PGD^C	
EUROPE									
England	4558	Obs	95.79	4.13	0.08		0.9786	0.0214	98
		Exp	95.77	4.19	0.04				
Germany (Southwest)	404	Obs	94.06	5.69	0.25		0.9691	0.0309	102
		Exp	93.92	5.98	0.10				
Greece	128	Obs	92.19	7.81	0.00		0.9610	0.0390	98
		Exp	92.35	7.50	0.15				
AFRICA									
Nigeria/Uganda	209	Obs	88.53	11.47	0.00		0.9426	0.0574	103
		Exp	88.85	10.82	0.33				
Mozambique	318	Obs	82.08	17.61	0.31		0.9086	0.0914	42
		Exp	82.56	16.61	0.83				
South Africa—Bantu	200	Obs	71.50	26.50	2.00		0.8474	0.1526	101
		Exp	71.81	25.86	2.33				
South Africa—Cape Coloured	200	Obs	91.00	9.00	0.00		0.9550	0.0450	101
		Exp	91.20	8.60	0.20				
South Africa—White	200	Obs	93.50	6.50	0.00		0.9674	0.0326	101
		Exp	93.59	6.31	0.10				
ASIA									
Israel—Habbanite	499	Obs	85.98	13.22	0.80		0.9260	0.0740	98
		Exp	85.75	13.70	0.55				
Iran	322	Obs	94.41	5.59	0.00		0.9721	0.0279	97
		Exp	94.50	4.72	0.78				
India—South	267	Obs	95.88	4.12	0.00		0.9795	0.0205	94
		Exp	95.94	3.64	0.42				
Bhutan	95	Obs	54.74	41.06	4.20		0.7525	0.2475	98
		Exp	56.63	37.25	6.12				
Thailand	441	Obs	85.92	14.08	0.00		0.9296	0.0704	42
		Exp	86.41	13.10	0.49				
Malay (South Africa)	100	Obs	95.00	5.00	0.00		0.9750	0.0250	101
		Exp	95.06	4.32	0.62				
China	228	Obs	87.30	12.26	0.44		0.9342	0.0658	104
		Exp	87.28	12.29	0.43				
Japan	180	Obs	86.10	13.90	0.00		0.9305	0.0695	42
		Exp	86.58	12.94	0.48				
AMERICA									
USA—White	2624	Obs	94.93	4.92	0.15		0.9739	0.0261	96,99,42
		Exp	94.85	5.08	0.07				
USA—Negro	2444	Obs	92.31	7.53	0.16		0.9607	0.0393	96,99,100
		Exp	92.29	7.55	0.15				
Brazil—Indian	181	Obs	100.0	0.00	0.00		1.0000	0.0000	105
		Exp	100.0	0.00	0.00				
Venezuela—Indian	283	Obs	99.65	0.35	0.00		0.9979	0.0021	95
		Exp	99.58	0.38	0.04				
Mexico—Yucatan	85	Obs	100.0	0.00	0.00		1.0000	0.0000	96
		Exp	100.0	0.00	0.00				

Table 3-6

Comparison of Frequencies Between an English Population and a Forensic Sample

Sample	Number Tested		PGD Phenotypes (%)				Gene Frequency		Reference
			PGD^A	PGD^{AC}	PGD^C	Others	PGD^A	PGD^C	
London, England	4558	No.	4361	188	4	2 Hackney 2 Richmond 2 Whitechapel			
		Obs.	95.79	4.13	0.08		0.9786	0.0214	98
		Exp.	95.77	4.19	0.04				
M.P.L.	653	No.	610	40	1	1 Hackney 1 Richmond			
		Obs.	93.28	6.12	0.20		0.9677	0.0322	60
		Exp.	93.64	6.23	0.10				

3.4 GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD)

3.4.1 INTRODUCTION

Since such a large number of papers have been written and such a large mass of information has been accumulated about glucose-6-phosphate, this paragraph will cover in detail only those aspects which, it is thought, can be of use in bloodstain examination to the forensic scientist.

The WHO report[118] in 1967 quoted five major parameters and some minor ones desirable for the identification of G6PD variants from red cells. These were:

a. Activity.
b. Electrophoresis.
c. Michaelis constant for G6P.
d. Relative rate of utilization of 2 desoxy G6P.
e. Thermal stability.

The electrophoresis should be in two different buffers: TRIS chloride and EDTA boric TRIS.

As minor parameters, activity/pH curves, activation energy, differential inhibition, fingerprinting, column chromatography, immunological techniques, micropurification, and hybridization are mentioned.

This is obviously impractical for the forensic scientist dealing with bloodstain material, so largely the concern must be with the major electrophoretic variants only at the moment.

In view of this, the background to the subject will be markedly restricted, particularly from any clinical point of view, so the reader is recommended to obtain this background elsewhere. (WHO report no. 366[129], Lehmann and Huntsman[125], Giblett[121], Harris[122], and the bibliographies given in these.)

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3.4.2 PHYSICAL DATA

The molecular weight of the enzyme has been variously estimated between 105,000[123] and 190,000[119]. These were early estimates. More recently Yoshida[130,131] reported a molecular weight of 240,000 formed as a hexamer of six identical units. The enzyme has a pH optimum between pH 8-9. The reaction is shown in Figure 3-26.

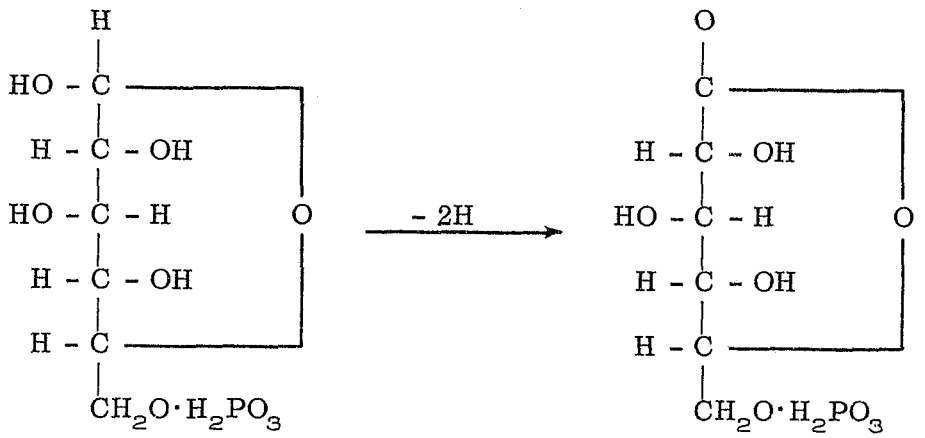


Figure 3-26. Glucose-6-Phosphate Dehydrogenase (G6PD) Reaction

3.4.3 ACTIVATION AND INHIBITION

NADP is bound to the enzyme strongly[119,123]—two molecules of NADP being bound to each molecule of enzyme. If NADP is removed from the enzyme, it becomes inactive and the molecular structure drops to subunits of a molecular weight of 40,000.

Carson et al.[118] described the effect of stroma and NADP on G6PD incubated at 40°C. The results on 6PGD in respect of this have already been mentioned. In the presence of stroma and without NADP the activity fell to 5 percent or below its original value. Whereas in the presence of NADP (10^{-4}M) and without stroma the activity was maintained at 86 to 94 percent.

Phosphate inhibits G6PD particularly at concentrations above 0.1 M.

3.4.4 COENZYMES STABILITY

NADP is required as a coenzyme in this system. It is bound to the active hexameric enzyme molecule.

Some of the rarer variants are labile, particularly those associated with congenital non-spherocytic haemolytic disease.

The normal forms of the enzyme 'A' and 'B' are similar in their thermostability.

3.4.5 POLYMORPHISM

Multiple forms of variation occur in G6PD—electrophoretic variation and deficiency. In this it is similar to PGD.

A large amount of work has been done on G6PD deficiency both partial and total. This work originated from the finding that a small proportion of people were sensitive to the 8-aminoquinoline anti-malarial drugs (e.g., primaquine). This deficiency occurs in the Negro, Mediterranean, Caucasian, and other races, but is rare in the northern European Caucasian.

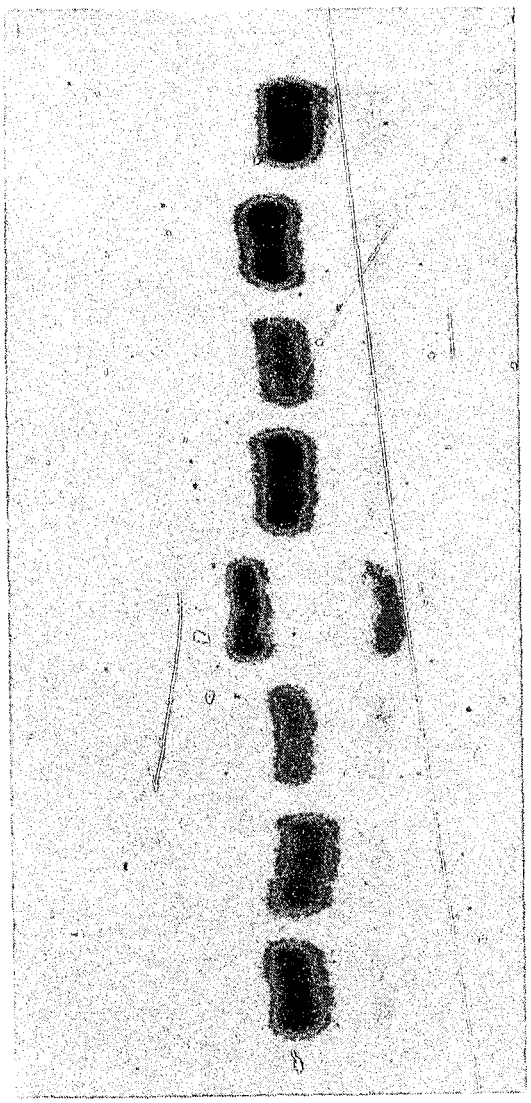
For full details of G6PD deficiency and its distribution, the reader is referred to the WHO report 1967,[129] Giblett[121], etc.

Since forensic work must deal with blood in the form of dried stains of sometimes unknown age, the determination of G6PD deficiencies is difficult and fraught with possible error. The enzyme may have deteriorated or have been inhibited by some factor of its environment as a stain and hence results that would appear to indicate a deficiency may not be accurate.

Figure 3-27 shows G6PD $^{A\&B}$ blood samples. Figure 3-28 is a diagrammatic representation of various G6PD phenotypes.

The electrophoretic variants can be used. The type 'B' is the normal form of the enzyme giving a single electrophoretic band of activity. Type 'A' gives a single band that runs faster than 'B' and is common in Negro populations

+ve



-ve

$G6PD^{A}$

$G6PD^{B}$

Hb

Figure 3-27. $G6PD^{A\&B}$ Blood Samples

(The Fifth Sample from the Top is $G6PD^{A}$. The Remaining Samples are $G6PD^{B}$. The Position of the Haemoglobin is Marked Hb)

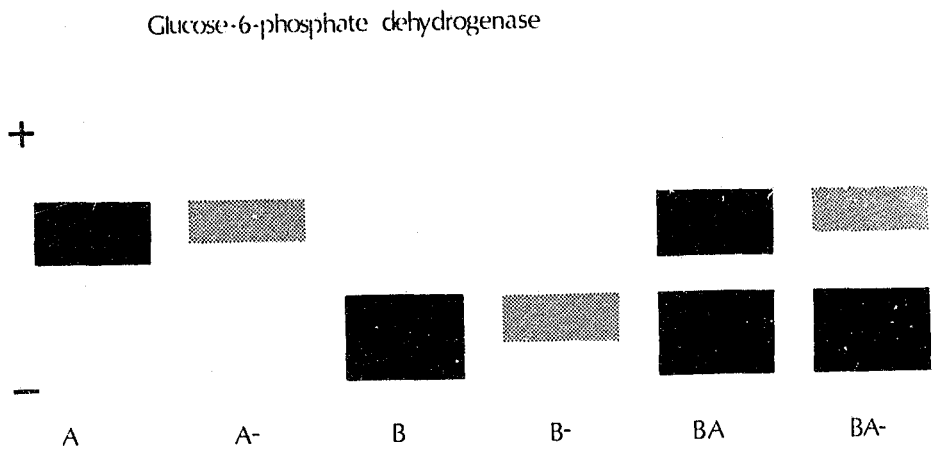


Figure 3-28. Diagrammatic Representation of Various G6PD Phenotypes

(Table 3-7), but is not found in Caucasians. Similarly the type A^-, a deficient form, runs similarly to 'A' electrophoretically but has only 8 to 20 percent of the activity of the normal 'B', while 'A' has about 80 percent of the activity of the 'B' type. The heterozygous form AB or A-B can only occur in females since this enzyme determinant is sex-linked on the X chromosome.

Gd Greece and Gd Mediterranean are deficient forms which electrophoretically run like the normal 'B' type. Gd A^-, Gd Greece and Gd Mediterranean are distinguishable from the A and B types on DEAE Sephadex or calcium phosphate gel.

Yoshida [130 and 131] showed that the difference between the A type and the B type was in a single amino acid substitution—B type asparagine being replaced by aspartic acid in the A type enzyme. The A^-, Greece and Mediterranean types, have also been shown to be structurally different forms.

In addition to those types already mentioned, there are over 30 other types of this enzyme. These are of rare occurrence and in many cases require techniques other than activity assay and electrophoresis to distinguish one from another.

In terms of the type of sample dealt with in forensic science, it is impractical at present to consider variants other than electrophoretic ones.

3.4.6 ACTIVITY OF THE ENZYME IN BLOOD

The accurate determination of the activity of this enzyme in crude mixtures, such as blood, can give rise to more problems than most enzyme assays.

The methods are based on the production of reduced coenzyme, NADPH. The product of G6PD is gluconolactone which is converted to 6-phosphogluconate, the substrate for 6-phosphogluconate dehydrogenase, which is also NADP coupled.

Temperature has the effect of increasing the activity but to a much less extent than with other enzymes. The WHO bulletin[129] quotes an increase in activity of 6 percent per 0°C. In trials at Metropolitan Police Laboratory this figure seemed to be rather high.

Tan and Whitehead[128] reported the activity of the enzyme in normal type B human red cells as 62 to 102 μMoles of NADPH generated per minute per 100 mls packed cells. Zinkham et al.[139] reported the activity as 4.5 to 6.5 units per 100 mls red blood cells where 1 unit equals an optical density change at 340 nm of 2.07 absorbance units per minute per 3 ml of reaction mixture which contained 0.1 haemolysate (made from 0.2 ml packed red cells with 3.8 ml water) at 26°C.

The WHO bulletin[129] gives no absolute figures. It may be that a comparative ratio would be more useful for forensic purposes.

3.4.7 EFFECTS OF STORAGE

The effects of storage on G6PD are dependent upon the condition of the blood stored. The effects are those which Carson described as caused by stromal NADP-ase. If a lysate free from stroma or intact cells is stored, the loss or activity is less than if a lysate with stroma present is stored.

The temperature of storage has the usual effect, in other words, the colder the better. A lysate free from stroma with NADP added to about 10^{-4}M will keep its activity for several months at -20°C. At room temperature there is rapid loss of activity.

Storage of a bloodstain has similar but slower effects to storage of a blood sample. Since stroma are present in the stain, fairly rapid loss of activity can be expected and is in fact found. Treatment of the stain or stain extract with 10mM NADP improves the quality of the result. It is, however, much more difficult to get satisfactory levels of activity of G6PD from bloodstains than most other enzymes that have been tested.

3.4.8 EFFECT OF CHEMICALS

One of the major causes of the intense investigation of G6PD in recent years has been the effects of a wide variety of drugs when abnormal G6PD is present. These have been fully reported and it would serve no useful purpose to summarize these here. (See the WHO bulletin and other papers and books already mentioned.)

Mercaptoethanol when in the presence of NADP appears to have desirable effects in helping to prevent inactivation of this enzyme.

3.4.9 BLOODSTAIN SUBSTRATE

So far there has been inadequate experience of the variety of substrates but to date nothing stands out as a distinctly poor or inhibitory substrate for G6PD determination in a bloodstain.

3.4.10 SAMPLE PREPARATION

The WHO bulletin recommends the use of very dilute lysates, approximately 1:100 dilution of packed red cells. Metropolitan Police Laboratory has found stronger lysates to be better suited to its methods.

Lysis should be brought about using the recommended lysing solution, (NADP 10 μM, beta-mercaptoethanol 7 nm and sodium EDTA, pH7, 2.7 nm), then centrifuged to remove stroma for assay work. For electrophoresis, cells freshly lysed with a minimum of water and/or sonicated are quite satisfactory.

Bloodstained threads are soaked in a minimum of gel buffer for a few minutes immediately prior to insertion into the gel.

3.4.11 METHODS

Like 6PGD numerous methods for the electrophoresis and assay of G6PD have been described. Cellogel was used by Rattazzi et al. in 1967[126], cellulose acetate by Sparks et al. in 1969[127], but starch gel is usually employed, with a starch concentration increased above the usual 10 percent. The concentration preferred for forensic work is 15 percent.

Various buffers have been suggested; the WHO report 1967 recommends two.

a. TRIS chloride buffer method (from Kirkman and Hendrickson)[124]

Gel buffer solution: 150 ml of 0.5 M TRIS chloride at pH 8.8 (25°C) and 15 ml of 0.27 M sodium EDTA at pH 7.0 are added to 1350 ml of H_2O.

Reservoir solution: One litre of gel buffer solution plus 3 g sodium chloride.

Running time: 14 to 16 hours at 2°C.

Gradient: 4 volts per cm

b. EBT (EDTA, boric acid, TRIS system)

Stock buffer solution:	EDTA (acid form)	0.02 M
	Boric acid	0.5 M
	TRIS	0.9 M

Gel buffer solution: Dilute stock buffer solution 1:20

Reservoir solutions:

For cathodic chamber: dilute stock buffer 1:7
For anodic chamber: dilute stock buffer 1:5

Running time: 12 hours at 2 to 4 °C
Gradient: 4.5 volts per cm

Parr, Giblett and others use a phosphate buffer of the same constitution as that for 6PGD. This did not, when tried at the Metropolitan Police Laboratory, give good results; the separation between the A and B bands were very small. The EBT system of the WHO report is the one found most satisfactory.

The starch gel is made of 15 percent starch using the thin gel system, adding 2 mg of NADP per 40 ml of starch gel after boiling the starch and before de-gassing.

Electrophoresis is carried out for about 17 hours at 4 to 5 volts/cm at 0-2 °C.

The enzyme bands are detected with an agar gel overlay, the reaction mixture being:

Buffer (0.1 M TRIS/HC1, pH 8.0)	10 ml
Magnesium chloride	40 mg
Glucose-6-phosphate	10 mg
MTT	2 mg
PMS	1.5 mg
NADP	4 mg

This mixture is put in the incubator and brought to 37 °C. It is then mixed with 10 ml of 2 percent agar in distilled water, melted and cooled to 50 to 55 °C.

This agar mixture is immediately poured onto the starch gel surface from the position of the haemoglobin to 6 cm towards the anode. After covering the agar layer with a glass plate, it is incubated at 37 °C for 30 to 60 minutes, after which the results may be read. See Figure 3-29.

The detection mechanism is shown below.

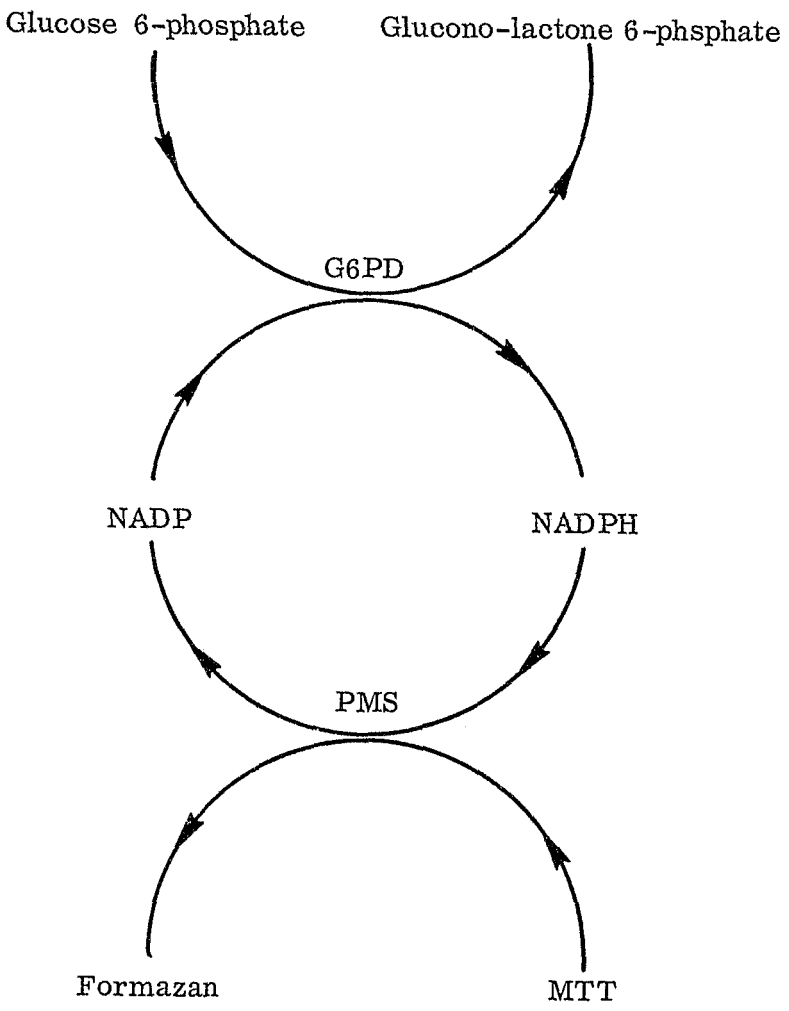


Figure 3-29. Mechanism for Determining G6PD

3.4.12 G6PD AND PGI ON THE SAME GEL

Phosphoglucose isomerase (PGI), under the conditions described here for G6PD, runs towards the cathode whereas G6PD runs towards the anode. Therefore, the cathodic side of the gel from the origin can be developed for one enzyme (PGI) and the anodic part for another enzyme (G6PD).

PGI has rare variants associated with it[120]. There are about ten variants that together occur in about 1 percent of the population. While it would be uneconomic to use this polymorphic system on its own, if it can be conveniently developed with G6PD it becomes worth while. An example of this is shown in Figure 3-30, showing on the left side G6PD variants, and on the right side variants of PGI.

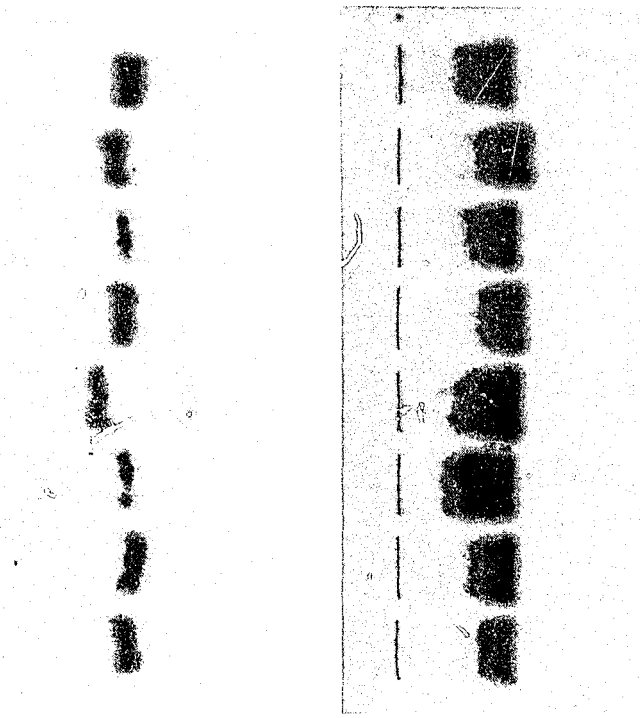


Figure 3-30. G6PD and PGI Variants

The reaction mixture for PGI is as follows:

Reaction buffer (as described for 6PGD)	10 ml
2 Percent agar in distilled water	10 g
NADP	2 mg
MTT	2 mg
PMS	1 mg
Fructose-6-phosphate	5 mg
Glucose-6-phosphatedehydrogenase (diluted as for use in PGM)	150 µl

The commonest variant is the PGI 3-1 shown third from the bottom in Figure 3-30. Asiatics show the highest variance.

3.4.13 PROBLEMS

There is a rapid loss of activity in stains, which is the cause of most of the problems. Grouping of stains up to 3 to 4 weeks old can be attempted, after this time there is usually little point. The other main problem is one of technique in obtaining an adequate separation between the A and B types.

3.4.14 POPULATION DATA

Very little population data on the electrophoretic variant is available. Various studies on negro populations have been done and some of the gene frequencies in males are as follows:

Population	No.	Gd^B	Gd^A	Population Data Reference
America	311	0.66	0.34	134
	135	0.65	0.35	124
	1941	0.71	0.29	136
Africa				
Nigeria (Yoruba)	141	0.56	0.44	134
Bantu	90	0.82	0.18	133
Negroes	150	0.85	0.15	88
Mozambique (S.E.Bantu)	315	0.64	0.36	135

3.5 ADENOSINE DEAMINASE (ADA)

3.5.1 PHYSICAL DATA

The calf mucosa enzyme has a pH optimum of 7 to 7.4[140]. The human red cell enzyme appears to have a rather broad pH optimum curve and has a good activity below 7. The reaction is shown in Figure 3-31.

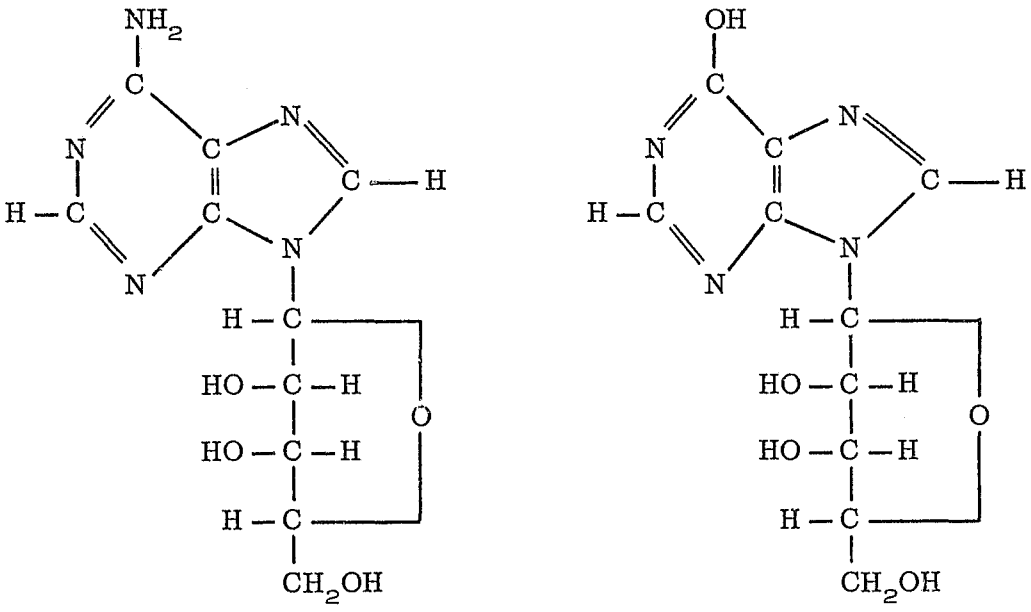


Figure 3-31. ADA Reaction

3.5.2 ACTIVATORS AND INHIBITORS

Little is known of these at the moment. Oxidation and reduction are dealt with later. Heavy metals and alkaline earths are reported as strongly inhibitory[138].

3.5.3 COENZYMES

There is no coenzyme required for the adenosine deaminase reaction.

3.5.4 POLYMORPHISM

Polymorphism of human erythrocyte ADA was described by Spencer, Hopkinson and Harris (1968)[141] as being determined by two alleles ADA^1 and ADA^2 at a single locus. This yields three phenotypes, ADA 1, ADA 2, and the heterozygote ADA 2-1. ADA^1 is much more common than ADA^2. The homozygote ADA 2 is rare, Spencer, et al. describing only two, one found among 580 English, and one among 213 Indians.

A rare allele, ADA 3-1, has been described by Dissing and Knudsen[137] and Hopkinson et al[139].

Figure 3-32 shows various examples of types ADA 1 and 2-1.

3.5.5 ASSAY AND OCCURRENCE

ADA is found in most tissues of the body and can be satisfactorily determined from some of these tissues in the absence of a blood sample, as has been described under PGM. The assay of ADA is not easy and at present different methods so far published and tried appear to give markedly different results.

3.5.6 EFFECTS OF AGE

Spencer, et al. (1968)[141] describe the effects of storage on lysates at 4°C. Under these conditions, the anodic bands become stronger in staining reaction and the cathodal ones correspondingly weaker. Identical results have been found at the Forensic Science Laboratory. If the lysate is stored at -20°C, this effect is negligible over short periods (a few weeks). In liquid nitrogen no storage changes have been noticed.

These storage changes appear to be the result of oxidation and can be induced by treatment of the lysate with oxidized gluthathione and can be reversed by beta-mercaptoethanol (Spencer, et al.)[141].

The condition of ADA in bloodstains is similar to that obtained under storage, in that oxidation has occurred. When comparing oxidized and reduced results there is a possibility of confusion between an oxidized ADA 2-1 and an untreated or

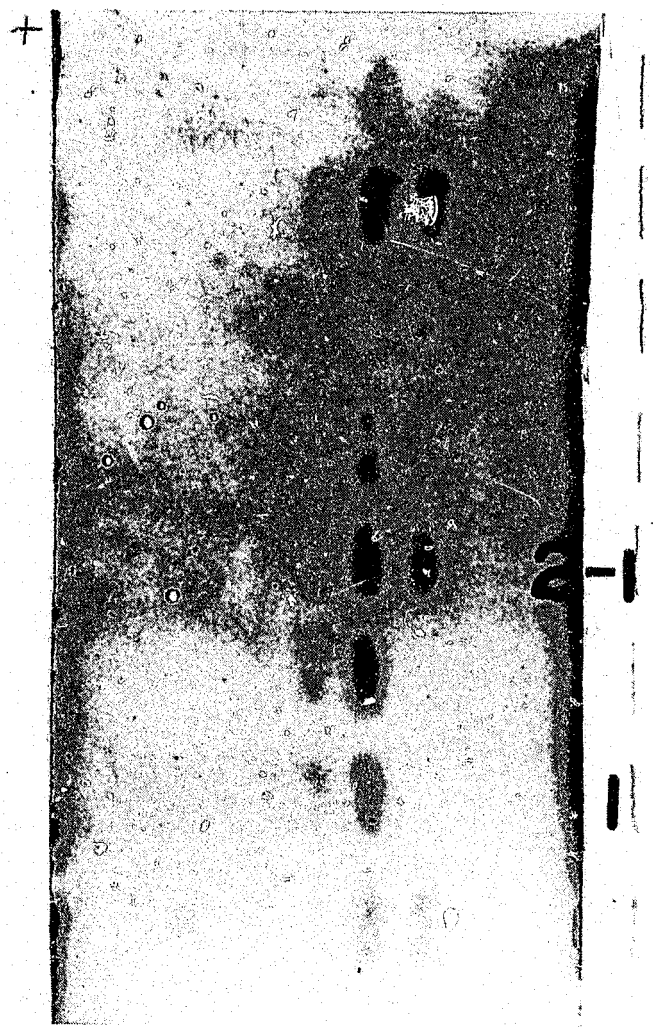


Figure 3-32. Various Examples of Types ADA 1 and ADA 2-1

reduced ADA 1. The Forensic Science Laboratory therefore decided, since the degree of oxidation was completely unknown in most samples, to standardize on either fully oxidized or fully reduced material. On trial it was decided that the reduced form was easier to obtain and read unequivocal results from. Hence, all samples are treated with beta-mercaptoethanol prior to electrophoresis.

The activity of ADA remains high in stains up to three to four weeks old and has been determined satisfactorily in stains up to three months old. At this age, however, the activity has become low and larger inserts may be required.

ADA is reasonably resistant to change on drying into a stain and presents few difficulties, adequate activity being found in most casework material.

Figure 3-33 shows ADA Types 1 and 2-1 stain with and without mercaptoethanol.

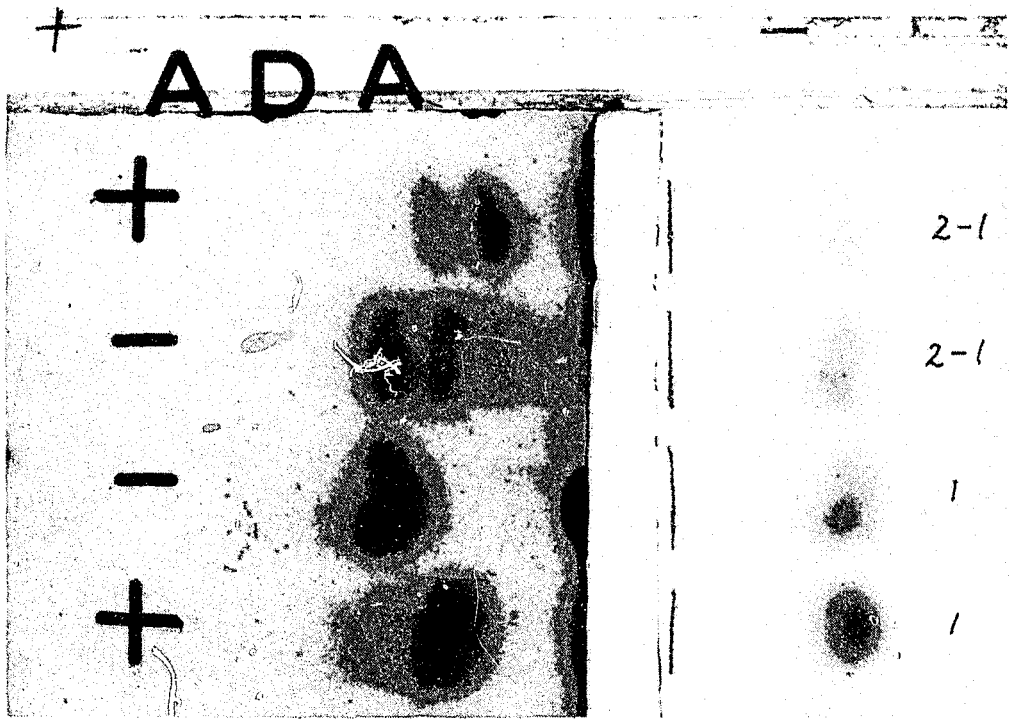


Figure 3-33. Showing ADA Types 1 and 2-1 From Bloodstains With (+) and Without (-) Mercaptoethanol

3.5.7 SUBSTRATE

So far no difficulties have been encountered with any particular stain substrate. Textiles, metals, glass, etc., have been tried without trouble.

3.5.8 EFFECT OF CHEMICALS

The effect of oxidizing and reducing agents have been described. The effects of other chemicals, fluoride, etc., are similar to those obtained with some other enzymes (e.g., PGM).

3.5.9 PREPARATION OF SAMPLES

If lysates are fresh, no preparation is needed. If they have been stored for some time prior to electrophoresis, then in view of the decision to standardize on the reduced form of the enzyme in work on stains, the same decision must hold here. This is achieved by mixing four or five drops of lysate with one drop of 10 percent beta-mercaptoethanol in gel buffer at least 15 minutes before use. The treated lysate is kept at room temperature during this time.

With bloodstains, the stained thread is soaked in a minimum of gel buffer containing 1 percent beta-mercaptoethanol solution for a similar time prior to its insertion into the gel. The amount of solution used should be just sufficient to make the thread wet without any excess and it should be placed in a moist chamber until required.

The starch gel technique described by Spencer, et al. (1968)[141] is excellent, but for forensic purposes takes too much material. The thin-layer starch gel technique therefore is used with a cooling plate below the gel. Tap water can be used through the cooling plate but water at 6-8°C is preferred.

Electrophoresis is carried out overnight for 16 to 18 hours at 3 to 3.5 volts/cm or for a shorter daytime run 4 hours at 8 to 9 volts/cm.

The starch gel should be about 10 percent in 0.01 phosphate buffer pH 6.5.

a.	NaH_2PO_4	15.601 g
	Water	to 1 litre
b.	Na_2HPO_4	14.196 g
	Water	to 1 litre

a. is added to b. until pH is 6.5.

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This is the 0.1 M tank buffer. The gel buffer in this tank buffer is diluted 1:10.

At the end of electrophoresis, an agar gel overlay containing the reactants is poured over the surface of the starch gel from the origin to a line 10 cm towards the anode. The reaction mixture is made up in phosphate buffer 0.025 M pH 7.5.

Using the previously described phosphate solutions, A is added to B until pH 7.5 is reached. The resulting solution is diluted 1 in 4. This forms the reaction buffer.

Buffer	10 ml
Adenosine	15 mg
MTT	2 mg
PMS	2 mg
Xanthine Oxidase (Boehringer)	10 μl (= 0.03 units)
Nucleoside Phosphorylase [Boehringer diluted 1/10 with $(NH_4)_2SO_4$]	10 μl (= 0.3 units)

This mixture is brought to 37°C and mixed with 10 g of 2 percent agar in distilled water at 50 to 55°C and immediately poured on the starch gel. When set, the agar is covered with a glass plate to prevent evaporation and incubated at 37°C for 30 to 60 minutes, at which time bands of activity will be seen. The detection mechanism for ADA is shown in Figure 3-34.

3.5.10 PROBLEMS

Two problems may occur, the first of these in relation to the oxidized or the reduced form of the enzyme. If insufficient beta-mercaptoethanol is used then one may get a mixture of reduced and oxidized enzyme, which gives a confusing picture. If too much beta-mercaptoethanol is used, then partial destruction of the enzyme may occur.

The second problem is in relation to old lysed and bacteria-infected blood samples. In some of these, instead of losing the enzyme completely, a single slow

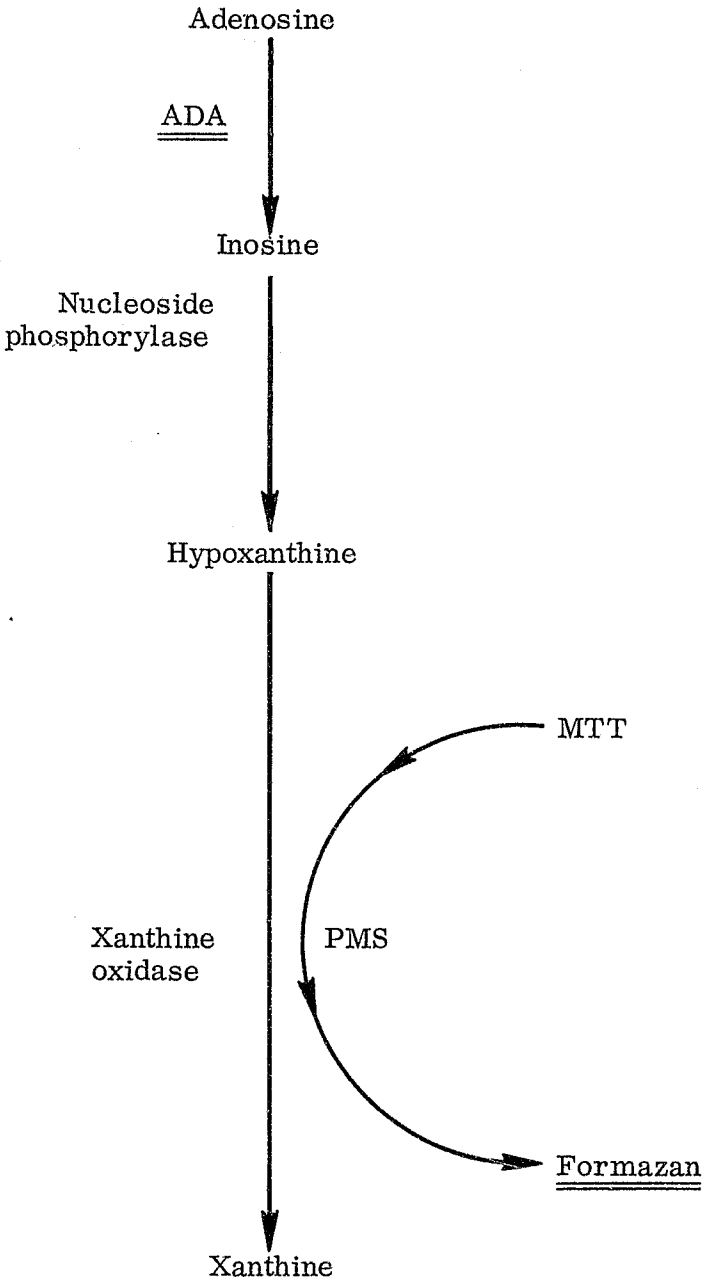

Figure 3-34. Mechanism for Determining ADA

band of activity is produced which is more cathodic than the normal ADA 2 band seen in the 2-1 and the 2 types. This is illustrated in Figure 3-35. In Figure 3-35 between lysates of types ADA 1 and ADA 2-1 is the slow band, which is occasionally shown by old bacterially infected blood samples.

Table 3-7 shows the population distribution. Table 3-8 shows the comparison of frequencies.

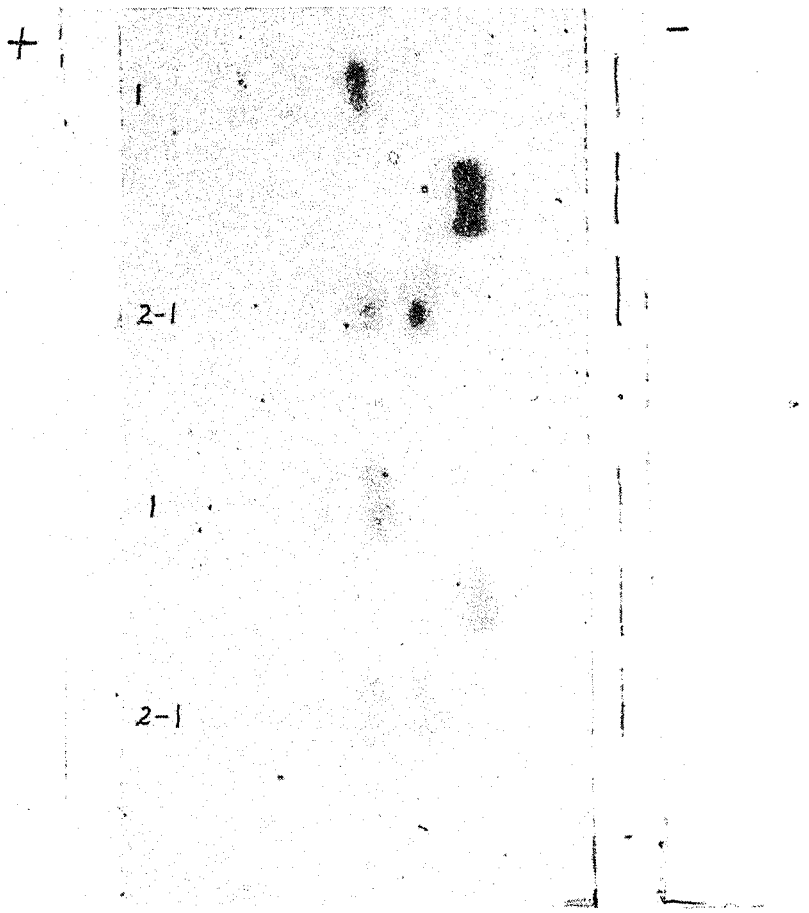

Figure 3-35. Old Lysed and Bacteria-Infected Blood Samples

Table 3-7

Distribution of ADA Types in Various Populations[142]

Population	Number Tested		ADA Phenotypes %				Gene Frequency		Reference
			ADA 1	ADA 2-1	ADA 2	—	ADA^1	ADA^2	
EUROPE									
England	1353	obs	90.40	9.38	0.22		0.9508	0.0491	137
		exp	90.40	9.34	0.24				
Germany	302	obs	85.09	14.57	0.33		0.9238	0.0761	139
		exp	85.34	14.06	0.58				
AFRICA									
Negro	300	obs	94.34	5.66	0.00		0.9716	0.0283	137
		exp	94.40	5.50	0.08				
ASIA									
India	460	obs	77.38	21.74	0.87		0.8826	0.1173	137
		exp	77.90	20.70	1.38				

Table 3-8

Comparison of Frequencies Between an English Population and a Forensic Sample[142]

Population	Number Tested		ADA Phenotypes %				Gene Frequency		Reference
			ADA 1	ADA 2-1	ADA 2	—	ADA^1	ADA^2	
England	1,353	No.	1223	127	3		0.9508	0.0491	139
		obs	90.40	9.38	0.22				
		exp	90.40	9.34	0.24				
Metropolitan Police Laboratory	410	No.	373	37	0		0.9548	0.0451	142
		obs	90.97	9.02	0.00				
		exp	91.16	8.61	0.23				

3.6 PSEUDOCHOLINESTERASE (PCE)

3.6.1 PHYSICAL DATA

Esterases are often difficult to classify, but cholinesterases fall into two classes:

a. Those that hydrolyse acetyl-choline rapidly and other choline esters slowly.
b. Those that hydrolyse choline esters other than acetyl-choline rapidly.

It is to the latter class (b) that pseudocholinesterase belongs. True cholinesterase [the (a) type] occurs within the red cell, while pseudocholinesterase occurs in the serum.

The function of true cholinesterase in nerve tissues is well known, but the physiological function or functions of pseudocholinesterase remain obscure at the moment.

The trivial names that have been applied to this enzyme have included serum cholinesterase, S-type cholinesterase, cholinesterase II, and pseudocholinesterase. The latter name has been chosen as suitable for the purposes of the Metropolitan Police Laboratory and will be used with the abbreviation PCE. The reaction is shown in Figure 3-36.

3.6.2 ACTIVATORS AND INHIBITORS

There are many compounds that affect the activity of PCE. Some of those that inhibit activity are:

a. Quaternary ammonium compounds.
b. Fluorides.
c. Chlorides.
d. Ethyl alcohol.
e. Dibucaine.
f. Solanin.
g. Organo-phosphates (nerve gases and insecticides).
h. Heavy metals.

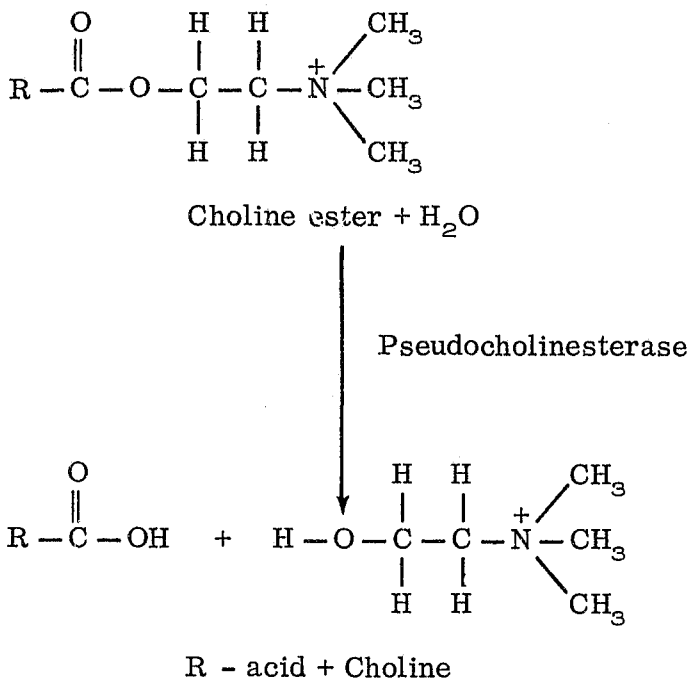

Figure 3-36. Pseudocholinesterase Reaction

Disturbance of activity levels occurs with or without the presence of dibucaine as an inhibitor in high dilution of the enzyme. This is dealt with in more detail later.

3.6.3 COENZYMES

There is no requirement of coenzymes with pseudocholinesterase; neither is there any metal ion requirement, such as is found with many enzymes.

3.6.4 POLYMORPHISMS

The polymorphism of PCE first came to notice when suxamethonium (succinyldicholine) started to be used as a muscle relaxant of very short duration. This short duration was caused by the hydrolysis of this compound by PCE. In a few individuals this hydrolysis did not occur at the normal rate and the result was apnoea lasting some hours. It was found that these individuals had an unusually low level of PCE. It was later found[150,151,152] that this was a genetically controlled variant enzyme.

The most satisfactory way of defining this variant was using the selective inhibition of PCE by dibucaine. The usual form of the enzyme was inhibited about 80 percent whereas the atypical form was inhibited about 20 percent. A third type was also found which was inhibited about 60 percent. This it became apparent was the heterozygote.

The degree of inhibition was expressed as a Dibucaine Number (DN) as presented in Table 3-9.

$$DN = \frac{\text{activity uninhibited} - \text{activity inhibited}}{\text{activity uninhibited}} \times 100$$

Table 3-9
Variance DN

Enzyme	DN
Usual Type UU	80 ± 2
Heterozygote UA	62 ± 4
Atypical AA	20 ± 3

The variation appeared to be caused by a simple form of inheritance of two autosomal genes.

It was next found that fluoride produced similar results to dibucaine; but on a few rare occasions an enzyme was found that was inhibited by dibucaine but was not inhibited to the same extent by fluoride, showing that a third allele might exist.

Various workers have investigated other inhibitors and showed variation of this enzyme using dibucaine[151], fluoride[148], chloride[144,148], alcohol[159], each producing results not quite identical with the others, thus showing a more complicated polymorphism of rare alleles at a single locus.

This polymorphism can be expressed as in Table 3-10 where only dibucaine, fluoride, and silent gene variants are considered.

Table 3-10

Polymorphism of Four Allelic Genes (E_1^u, E_1^a, E_1^f, and E_1^s) at Locus E_1

Phenotype		Genotype
Usual	U	E_1^u, E_1^u E_1^u, E_1^s
Usual/Fluoride	UF	E_1^u, E_1^f
Intermediate	I	E_1^u, E_1^a
Fluoride	F	E_1^f, E_1^f E_1^f, E_1^s
Intermediate/Fluoride	IF	E_1^a, E_1^f
Atypical	A	E_1^a, E_1^a E_1^a, E_1^s
Silent	S	E_1^s, E_1^s

In forensic work, the dibucaine inhibition has been found to work well and reliably, whereas fluoride inhibition is a less reliable technique yielding little more information than dibucaine. Therefore, from a practical viewpoint, there are only two allelic genes at the E_1 locus, E_1^u—producing the usual enzyme, and E_1^a—producing the atypical enzyme. These yield the three types $E_1^uE_1^u$(usual), $E_1^uE_1^a$(intermediate), and $E_1^aE_1^a$ (atypical) forms.

The silent gene[154] E_1^s also can occur at the E_1 locus in combination with E_1^a or E_1^u. In this instance the activity of the serum is reduced. The homozygote $E_1^sE_1^s$ has also been found where there were no detectable amounts of PCE in the serum.

At a separate locus E_2 independent of the previously described polymorphism, there is a second system of polymorphism exhibiting electrophoretic variation[146,157]. The previous polymorphs at the E_1 locus have the same electrophoretic mobilities in starch gel.

Upon electrophoresis, most sera exhibit four areas of PCE activity plus a fifth in the albumin region. The albumin region and the anodal three bands, designated C_1, C_2, and C_3, are all very weak in their activity, the most cathodal band, C_4, exhibiting about 90 percent of the total activity.

In a small proportion of sera, there is a further more cathodic band, C_5, the bulk of the activity in these sera being divided between bands C_4 and C_5. This latter variation has been designated C_5^+ while the usual form is C_5^-. The quantitative expression of this C_5 band when compared with the C_4 band can vary considerably from being very weak to very strong. The presence of the C_5 band can mean an increase in total activity of up to 30 percent, and C_5^+ types generally have a higher activity than the C_5^- types. The variation in the intensity of the C_5 band could be caused by variable gene expressions such as is seen in the heterozygous haptoglobin Hp 2-1.

Figure 3-37 illustrates the main bands of the C_5^- and the C_5^+ pseudocholinesterase variants. Figure 3-38 illustrates the storage bands that develop in bloodstains of the C_5^- and the C_5^+ variants. These storage bands are removed by treatment with mercaptoethanol.

A great deal more work is needed before this C_5^+ problem is elucidated genetically. This does not, however, preclude its use as a means of identification in forensic work.

3.6.5 PCE IN BLOOD AND OTHER TISSUES

Kalow and Genest[151] quote the usual type of enzyme as having an activity of 207 ± 46 units. The unit is defined as μ Moles of acetylcholine hydrolysed by 1 ml serum in one hour at 37 °C.

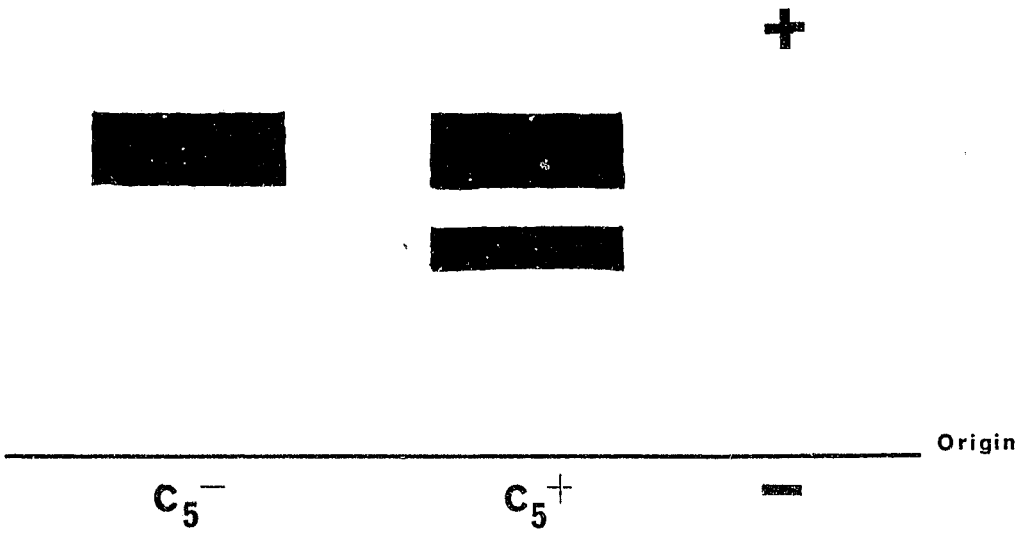

Figure 3-37. Main Bands of the C_5^- and the C_5^+ Pseudocholinesterase Variants

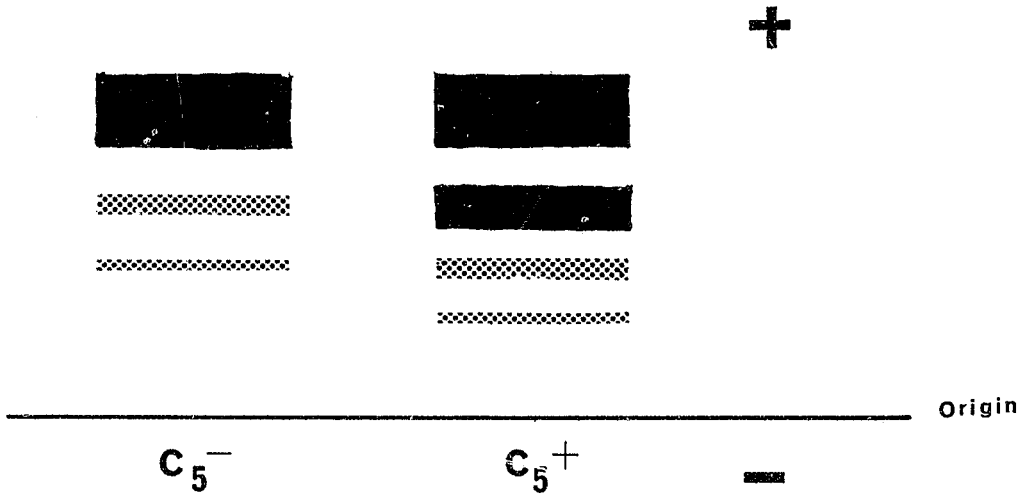

Figure 3-38. Storage Bands That Develop in Bloodstains of the C_5^- and the C_5^+ Variants

In addition to its presence in serum, the enzyme has also been found in heart muscle, skin, glia, intestines, and liver. There are no detectable amounts of PCE in semen.

In the absence of a satisfactory blood sample from a cadaver, it is doubtful if a satisfactory alternative tissue source could be found.

3.6.6 EFFECTS OF AGE

Up to 30 percent loss of activity on a single freezing and thawing has been reported. The experience of the Metropolitan Police Laboratory has been that the losses are much less than this.

Storage of serum samples of -20°C has yielded samples that are still satisfactory after repeated freezing and thawing for over three years, freezing and thawing occurring at least once per week.

There is an appreciable loss of activity when a bloodstain dries, but good results have been obtained from bloodstains up to three months old. After this the results tend to become weak and indistinct, very little activity remaining after six months for determining the inhibition variant.

Bloodstains and stored serum samples develop storage bands that can be quite strong and, unless the correct techniques are used, they may be confused with the C_5 band. C_5^+ sera also develop a storage band cathodal to the C_5 band. These storage effects are removed by treatment with mercaptoethanol.

The age of the person from whom the sample has come also appears to affect the activity. The older the donor, the lower the activity.

3.6.7 EFFECTS OF BLOODSTAIN SUBSTRATE

Bloodstains from numerous substrates, including most textiles, metals, glass, etc., have been examined and none has so far given any difficulties that were directly attributable to the substrate.

3.6.8 EFFECTS OF VARIOUS CHEMICALS

Most of the effects have been dealt with under information on inhibitors. Mercaptoethanol has no effect on the enzyme except to remove storage band effects, as previously described.

3.7 PSEUDOCHOLINESTERASE E_1 LOCUS VARIANTS

3.7.1 SPECTROPHOTOMETRIC METHOD

3.7.1.1 General

Dibucaine number variants are practical determinations on bloodstains as has been shown by Lehmann[153] and Parkin[155]. The determination of fluoride or alcohol variants is considered an uneconomic use of effort in a routine forensic laboratory in view of their low occurrences and the amount of additional work required to detect them. Hence, the only inhibition variants to be considered here are the dibucaine ones.

The original method of determining dibucaine numbers, described by Kalow and Genest[151], is the most reliable method for work on bloodstains and is the one which should be used to give final answers. There are, however, various difficulties and a variety of ways of solving each. The first difficulty is that a bloodstain extract in buffer or water contains a large amount of haemoglobin which makes the extract optically very dense at 240 nm.

When determination of the dibucaine number of a fresh unhaemolysed serum is performed, the serum is diluted approximately 1:50 to 1:100 with buffer. Hence, a fairly dilute extract of a bloodstain approximately equivalent to a 1/50 dilution of serum is required.

The density of the extract may still be too high for a programmed slit width, and it is found with the Beckman DB-G that opening the slit width to 1-2mm improves the signal-to-noise ratio to acceptable levels. Even so, some extracts of stains are still optically dense producing an unacceptable signal-to-noise ratio which then gives poor or wrong results when determining inhibited activity. This difficulty has been overcome by visually monitoring the signal-to-noise ratio by connecting an oscilloscope across the photomultiplier output. If an unsatisfactory trace on the oscilloscope is seen, then the sample is diluted to give a satisfactory signal. This dilution must be done with care since overdilution leads to the other major problem.

At a serum dilution somewhere in excess of one in four to six hundred or the equivalent dilution of a stain extract, the degree of inhibition by dibucaine is upset, and the dibucaine numbers obtained no longer correlate with their proper values. To overcome this, a purely empirical decision was made that if an uninhibited sample produced a decrease in OD_{240} of less than 0.09 absorbance units per five minutes, the result was rejected. Bearing these conditions in mind the following method is used.

The spectrophotometer used in the Metropolitan Police Laboratory was a Beckman DB-G with a five-inch recorder and scale expansion accessory. To this was added an oscilloscope to monitor signal-to-noise ratio and a thermostat bath at 25°C to keep the cuvette chamber at constant temperature. Temperature fluctuation without this was found to be troublesome. Other spectrophotometers are equally satisfactory particularly if there is scale expansion facility. Quartz cuvettes are required, the standard 1 cm are suitable unless there is insufficient material when micro-cuvettes are necessary.

3.7.1.2 Solutions Required

The required solutions for this method include:

a. Buffer M/15 phosphate pH 7.4

Hydrogen di-sodium phosphate (anhydrous)	7.078 g
Potassium di-hydrogen phosphate	1.816 g
Distilled water	to 1 litre

b. Benzoyl-choline chloride

Benzoyl-choline chloride	12.4 mg
Buffer	250 ml

c. Inhibitor-dibucaine hydrochloride

Dibucaine hydrochloride	4.16 mg
Buffer	250 ml

d. Enzyme solution

0.1 ml serum is diluted with 9.9 ml of buffer; or, when dealing with a bloodstain, an extract of bloodstain in buffer approximating a 1/50 dilution of whole blood.

3.7.1.3 Procedure

The reference cell was maintained the same for readings of uninhibited and inhibited reactions. The quantities of each solution are given in Table 3-11.

If a non-recording machine is used, readings should be taken every thirty seconds and plotted, and the straight-line reaction should be taken over at least a three minute period. If a recording instrument is used, the best straight-line over a period of at least three minutes should be taken.

Table 3-11
Solutions Used in Dibucaine Determinations

	Cuvette		
	Reference	Uninhibited	Inhibited
Bloodstain extract or diluted serum	1 ml	1 ml	1 ml
Buffer	1 ml	0.5 ml	-
Benzoyl-choline chloride	-	0.5 ml	0.5 ml
Dibucaine hydrochloride	-	-	0.5 ml

In most cases the scale expansion accessory of the Beckman DB-G was found to be essential working at ×5 or ×10 on a percent transmission mode; the transmission figures being converted to absorbance by means of tables prior to calculation of the dibucaine number.

The calculation of the dibucaine number (DN) is:

$$DN = \text{percent inhibition}$$

$$= \frac{OD_{240} \text{ change uninhibited} - OD_{240} \text{ change inhibited}}{OD_{240} \text{ change uninhibited}} \times 100$$

The change in OD_{240} uninhibited and inhibited must be taken as an OD_{240} change in a given time. What this time is, is irrelevant providing the same time is taken for the inhibited and the uninhibited reactions. The usual time taken varied between three minutes and ten minutes depending on the quality of the trace on the chart and on the overall rate of reaction.

Enzyme Type	Dibucaine Number
Usual	80 ± 2
Intermediate	60 ± 4
Atypical	20 ± 3

3.7.2 SCREENING TECHNIQUE FOR SERA

3.7.2.1 General Approach

In any laboratory dealing with large numbers of blood samples and casework stains, the time occupied in spectrophotometric determination of all samples would be unduly time-consuming. In the Metropolitan Police Laboratory, all blood samples from victims or suspects are examined for PCE variants. Bloodstains are not examined for these variants unless it is found that an atypical or intermediate is involved in the case from an examination of the blood samples. The only exception is where bloodstains are examined in the absence of any blood samples from suspects or victims. The blood samples or stains are examined by a screening procedure. Table 3-12 presents a distribution of PCE types in various populations.

The first of these screening tests was described by Harris and Robson[147]; this test relies on the diffusion of the sample into agar gels, one containing inhibitor and the other not containing inhibitor. Upon using a staining reaction, the relative size and intensity of the inhibited and uninhibited reactions are compared. The color of the reaction is red-brown and is severely interfered with by the presence of haemoglobin which has a similar color. A variety of methods have been tried to remove the haemoglobin, such as the absorption of Hb on CM Sephadex[155], electrophoresis in starch[145], and electrophoresis on paper[155].

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The most satisfactory, rapid method requiring the least amount of sample is the differential removal of Hb by means of CM Sephadex which is the only screening method which will be described in detail. It should be noted that ALL SAMPLES believed, as a result of this screening test, to be intermediate or atypical or those which are of doubtful interpretation are checked by spectrophotometer and for which the dibucaine number is determined.

3.7.2.2 Method for Screening Serum Samples

Petri dishes or plastic boxes are filled to a depth of about 3 mm with the gels and marked appropriately "Control" and "Inhibited". Small wells 4 mm in diameter are punched in the gels (a cork borer connected to a water pump vacuum is used). These should be about 3 cm apart as in Figure 3-39.

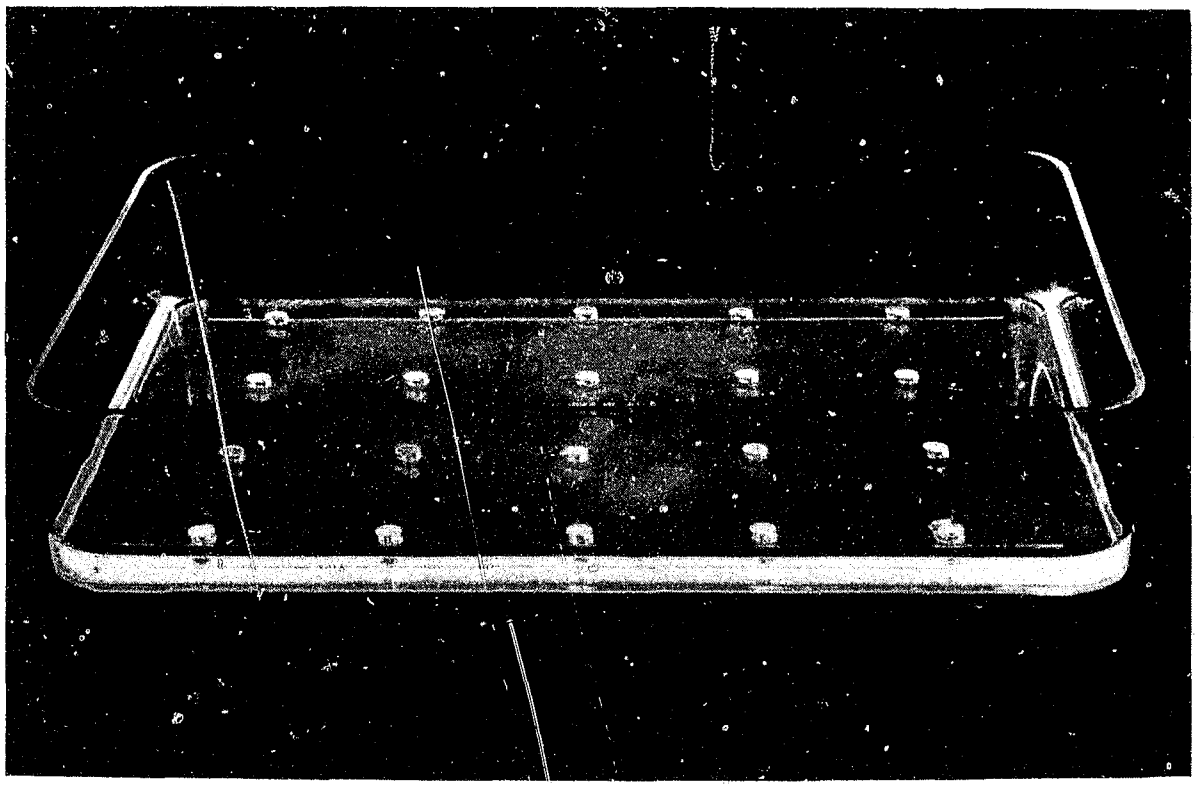


Figure 3-39. Tray of Agar with Wells Prepared for Diffusion Screening Technique

The concentration of inhibitor was chosen to produce about 10 percent of the activity of the atypical or intermediate forms in the control plate. Hence, by making the sera in the inhibited plate four times as concentrated as that in the control plate, the stained-up diffusion zones developed around the atypical or intermediate types in the inhibited plate would be as great as, or even greater than, the corresponding sera in the control plate. On comparison, the usual type of sera at the same concentrations would have a far weaker zone of color in the inhibited plate than in the control plate.

The serum dilutions actually chosen for the most convenient results were 1 in 8 for the inhibited plate and 1 in 32 for the control plate. The dilutions were carried out in a depression tile, using the TRIS/hydrochloric acid gel buffer, by counting the drops from a Pasteur pipette. The diluted sera were pipetted into the wells of the appropriate boxes, and their positions were noted. The lids were replaced to prevent the gels from drying, and the boxes were left to diffuse for 17 hours in an incubator at 37°C.

3.7.2.3 Reaction Mixture

The diffusion zones of each gel were developed by flooding with 50 ml of the following reaction mixture:

20 mg fast red TR salt (5-chloro-o-tolidine).
2 ml 1 percent α -naphthyl acetate in 50 percent aqueous acetone.
100 ml 0.2M phosphate buffer, pH 7.1.

The buffer that was used was prepared as indicated below:

Disodium hydrogen phosphate	107.4 g
Distilled water	1.5 litres
Sodium dihydrogen phosphate	31.2 g
Distilled water	1.0 litre

The sodium dihydrogen phosphate solution was added to the disodium hydrogen phosphate solution to give a pH of 7.1.

The α -naphthyl acetate was used as a substrate for the diffused enzyme, PCK, since it was non-specific cholinesterase, it hydrolysed the α -naphthyl acetate

at pH 7.1, releasing naphthol. The naphthol reacted with the fast red TR salt, a diazo reagent, yielding a red colored product.

The usual type of PCE was inhibited in the inhibited plate, generating a color which was weak, pink, and diffuse due to a reduced amount of liberated naphthol; but the atypical and intermediate types at this concentration were stained up strongly with red, well-defined diffusion zones. The diffusion zones of the intermediate form were roughly the same size and concentration as the corresponding control sample, but the atypical type produced a diffusion zone larger than the control sample. However, the difference between the intermediate and atypical forms was so slight that this could not be regarded as a reliable method of differentiating them.

Although the results could usually be read after one hour, the reaction was allowed to continue for two hours for optimum color development and then stopped by flooding with the alcohol/acetic acid "wash" solution. The usual type could then be easily distinguished from the intermediate and atypical types.

When the results were read, the inhibited plate was always checked against the corresponding sera in the control plate with special reference to the discreetly stained light-red diffusion zone and the innermost fine dark-red circle which marked the intermediate and atypical forms. The "usual" zone in the inhibited plate was weakly stained and diffused as shown in Figure 3-40.

The strength of the color zones on the control plate also gave a rough guide to the relative level of PCE activity in the various sera used.

3.7.3 SCREENING OF BLOODSTAINS

3.7.3.1 General Application

Where there is plenty of bloodstain material, the screening technique just described can work well. More bloodstain is needed for this method than in determining the dibucaine number.

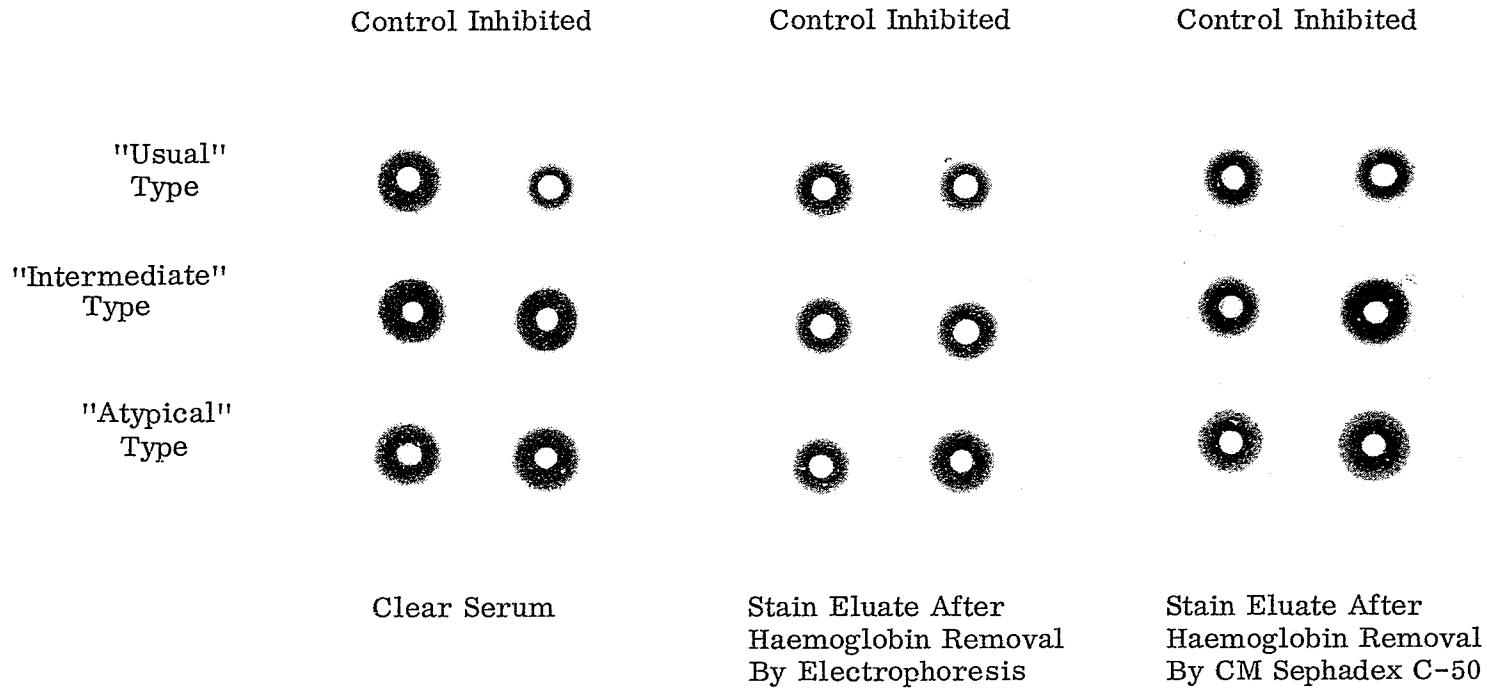


Figure 3-40. Results of Agar Diffusion Test

The color developed in this test is red-brown and is markedly obscured by haemoglobin from a bloodstain extract. Haemoglobin must, therefore, be removed prior to testing. The following techniques were developed by B. H. Parkin[155] of the Metropolitan Police Laboratory.

3.7.3.2 Method of Haemoglobin Removal

A portion of bloodstain, about 1 cm square, is cut up into fragments in a small test tube with about 1 ml of buffer. This buffer, with a pH of 6.5 is composed of:

Disodium hydrogen phosphate	0.8875 g
Sodium dihydrogen phosphate	4.875 g
Water	1 litre

The stain is well soaked and mashed with a glass rod. The eluate is then pipetted off into a small tube. A small quantity of dry CM Sephadex C-50 is added to the eluate; it swells rapidly absorbing buffer. In its swollen state, it should occupy about half to two-thirds of the volume of the eluate in the tube. This is allowed to stand with occasional agitation for about thirty minutes. The slurry is centrifuged, and the supernatant liquid is used as it is in the inhibited gel and is diluted one in four for the uninhibited gel.

After incubation for 17 hours at 37°C, it is stained and evaluated in exactly the same manner as serum samples. The estimation of the quantity of stain required may cause difficulty; about 1 cm square of well stained cotton cloth is sufficient, but weak blood smearing or thinner material will require a greater area of cloth. An area of bloodstaining on an item of clothing may be eluted without cutting the cloth but by moistening and squeezing out the extract. In this instance the eluate may be greater in volume and of low concentration. If this is so, more dry Sephadex is added to concentrate the eluate.

The problems which arose early in the trials of this method were associated with impure distilled water which still contained sufficient contaminants to inhibit the enzyme; and the use of certain plastic boxes which absorbed materials from the gels, which resulted in eventual inhibition by their repeated use. Once these initial difficulties had been overcome no further problems have been found.

3.7.4 ELECTROPHORETIC SCREENING METHOD

3.7.4.1 General

Another screening technique has been developed recently in the Metropolitan Police Laboratory by Wraxall [62]. It is more rapid than others, but it requires an accuracy of technique greater than the other methods, and it has not yet had the long-term trials of other methods. In spite of this, it could become the standard method for bloodstains or lysed blood samples because of the simplicity of the haemoglobin removal stage.

3.7.4.2 Method

Pour 7 ml of 1.5 percent Difco Noble agar in the gel buffer (below) onto a 2 inch by 3 inch glass slide and allow to set. This will be the uninhibited plate. A second plate is made with the addition of 0.2 ml of inhibitor RO.2-0683 per 50 ml of agar. The inhibitor solution contains:

TRIS	12.1 g
Water	800 ml

Gel and tank buffer are the same and are adjusted to pH 7.0 with HCl and made up to one litre.

Small wells 1.5 to 2 mm diameter are punched in each plate in a regularly spaced pattern.

The sera are diluted as before—1:8 for the inhibited plate and 1:32 for the uninhibited plate and are put in the appropriate wells.

Using a precipitin type tank and set-up (see paragraph 1.4), electrophoresis is performed at 50v for 45 minutes. The plates are then developed in the same solution as for the previous screening test, and the results are interpreted in the same way (Figure 3-41).

The advantages of this method are its speed and the fact that there is simultaneous removal of the haemoglobin from the sample towards the cathode so that it is outside the enzyme reaction area.

It seems probable that this technique will supercede the earlier gel diffusion technique.

In Figure 3-41, the upper slide (C) is not inhibited, the lower contains inhibitor. On comparison of the comparable wells on the upper and lower slides it can be seen that the upper row third from the left and the lower row left end are Intermediate types. The remainder are usual types.

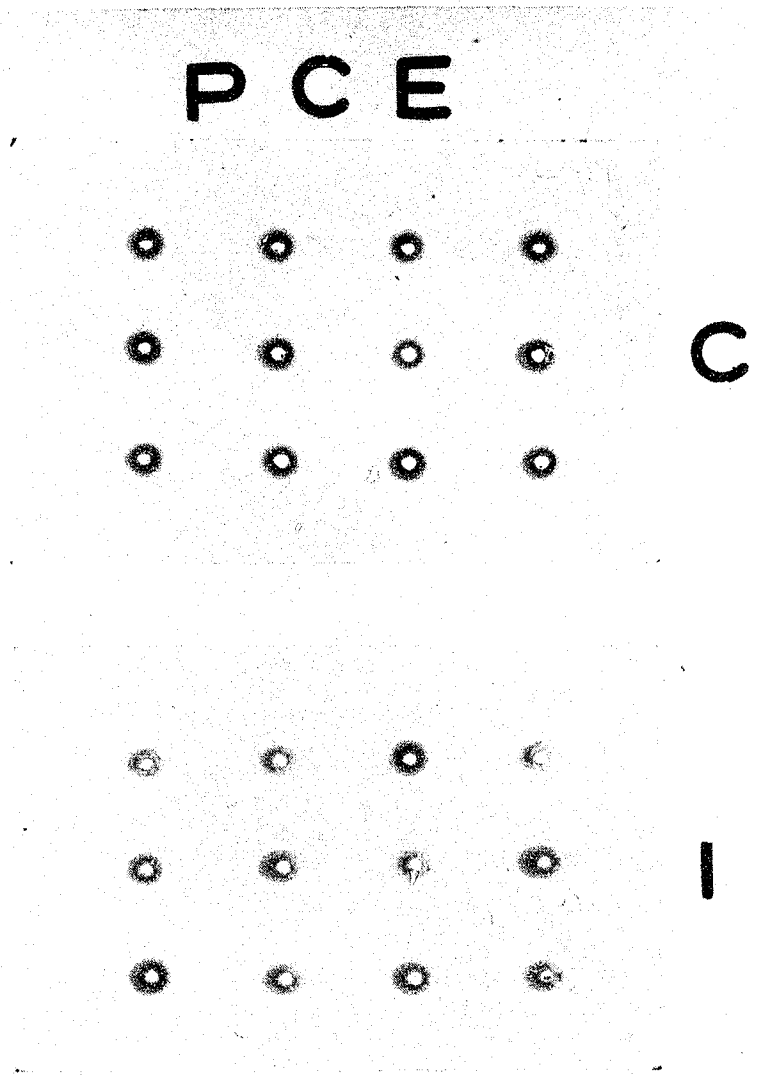


Figure 3-41. Results of Electrophoretic Screening Method of Sera

Table 3-12

Distribution of PCE Types in Various Populations

Population	Number Tested		PCE Phenotypes %				Gene Frequency		Reference
			$E_1^uE_1^u$	$E_1^uE_1^a$	$E_1^aE_1^a$	—	E_1^u	E_1^a	
EUROPE									
United Kingdom	1493*	Obs	96.31	3.35	0.00		0.9832	0.0168	167,173
		Exp	96.67	3.30	0.03				
Germany	8314	Obs	96.79	3.17	0.04		0.9837	0.0163	160
		Exp	96.76	3.20	0.03				
Czechoslovakia	180	Obs	93.33	6.67	0.00		0.9667	0.0333	160
		Exp	93.45	6.44	0.11				
Yugoslavia	363	Obs	97.52	2.47	0.00		0.9876	0.0123	164
		Exp	97.53	2.43	0.01				
Italy	382	Obs	95.81	4.19	0.00		0.9790	0.0210	173
		Exp	95.84	4.11	0.05				
Portugal	179	Obs	96.64	3.36	0.00		0.9832	0.0168	167
		Exp	96.67	3.30	0.03				
Greece	561	Obs	97.13	2.85	0.00		0.9857	0.0143	163
		Exp	97.16	2.82	0.02				
AFRICA									
Gambia-Negro	103**	Obs	98.05	0.97	0.00		0.9950	0.0050	173
		Exp	99.00	0.99	<0.01				
Nigeria-Ibo	69	Obs	100.00	0.00	0.00		1.0000	0.0000	173
		Exp	100.00	0.00	0.00				
Congo-Negro	460	Obs	99.78	0.22	0.00		0.9989	0.0011	169
		Exp	99.77	0.22	<0.01				
ASIA									
Israel-Ashkenazi	923	Obs	96.75	3.14	0.11		0.9832	0.0168	172
		Exp	96.67	3.30	0.03				
Israel-non-Ashkenazi	1340	Obs	95.30	4.47	0.23		0.9753	0.0247	167,172
		Exp	94.12	4.82	0.06				
Israel-Arab	110	Obs	98.18	1.82	0.00		0.9909	0.0091	172
		Exp	98.19	1.80	0.01				
Pakistan	121	Obs	97.52	2.48	0.00		0.9876	0.0124	170
		Exp	97.53	2.45	0.02				
Thailand	723	Obs	100.00	0.00	0.00		1.0000	0.0000	160
		Exp	100.00	0.00	0.00				
Korea	115	Obs	100.00	0.00	0.00		1.0000	0.0000	162
		Exp	100.00	0.00	0.00				
Taiwan-Chinese	340	Obs	99.70	0.30	0.00		0.9985	0.0015	169
		Exp	99.70	0.29	<0.01				
Japan	371	Obs	100.00	0.00	0.00		1.0000	0.0000	160
		Exp	100.00	0.00	0.00				

*Includes 6 other genotypes without atypical gene.
**Includes 1 other genotype without atypical gene.

Table 3-12

Distribution of PCE Types in Various Populations (Continued)

Population	Number Tested		PCE Phenotypes %				Gene Frequency		Reference
			$E_1^uE_1^u$	$E_1^uE_1^a$	$E_1^aE_1^a$	—	E_1^u	E_1^a	
AMERICA									
U.S.A.-White	246	Obs Exp	96.75 96.76	3.25 3.20	0.00 0.03		0.9837	0.0163	169
U.S.A.-Negro	666	Obs Exp	98.95 98.94	1.05 1.05	0.00 <0.01		0.9947	0.0053	169
U.S.A.-Oriental (mainly Japanese)	566	Obs Exp	99.29 99.28	0.71 0.71	0.00 <0.01		0.9964	0.0036	169
Canada	2017	Obs Exp	96.28 96.25	3.67 3.71	0.05 0.04		0.9811	0.0189	166
Mexico-Indian	377	Obs Exp	98.14 98.15	1.86 1.84	0.00 0.01		0.9907	0.0093	168
Brazil-Mixed	2100	Obs Exp	97.14 97.10	2.81 2.87	0.05 0.03		0.9854	0.0146	161
Australia	98	Obs Exp	98.97 98.96	1.03 1.02	0.00 <0.01		0.9948	0.0052	165
Philippines	427	Obs Exp	99.53 99.52	0.47 0.47	0.00 <0.01		0.9976	0.0024	169

3.8 PSEUDOCHOLINESTERASE E_2 LOCUS ELECTROPHORETIC VARIANTS

3.8.1 METHODS

The original method of Harris, et al[146], improved by Robson and Harris[158] used starch gels at acid pH's. The development of this method for use with bloodstains was described by Parkin[155] and later adapted to the thin layer technique[62].

Subsequently Parkin[156] developed a greatly improved technique involving starch and acrylamide. The major part of the gel is 7 percent acrylamide with a strip of 7 percent starch at the origin. In a thin layer technique, small pore acrylamide is less simple to prepare at the origin, and agar is less satisfactory.

The acrylamide gel is made as a 1 mm thick flat plate using the same mould as in the thin layer starch technique (paragraph 1.2).

3.8.2 MATERIALS

The materials and their preparations are:

a. Acrylamide Gel Buffer

Succinic acid 0.0156 M	1.84 g
TRIS 0.0184 M	2.23 g
Water	125 ml

Adjust to pH 4.8, if necessary, with saturated succinic acid solution.

b. Starch Gel Buffer—Acrylamide gel buffer diluted 1/8 with distilled water.

c. Tank Buffer Stock Solution

Citric acid 0.05 M	86.16 g
Water	800 ml

Adjusted to pH 4.8 with 40 percent sodium hydroxide and made up to 1 litre.

This is diluted 1/8 for use.

d. Acrylamide Stock Solution

Acrylamide	28 g
Bis-acrylamide	0.735 g
Water	to 100 ml

e. Activator

Ammonium persulphate	0.07 g
Water	25 ml

(this must be freshly prepared)

f. To Make the Gel

Acrylamide gel buffer	6.5 ml
Acrylamide stock solution	13 ml
Distilled water	6.5 ml
Activator (ammonium persulphate)	26 ml

3.8.3 PROCEDURE

The above solutions are mixed thoroughly and de-gassed with the aid of a water pump. The mixture is then poured into a gel mould 1 mm thick by 22 cm by 15 cm. A glass plate 22 by 15 cm is carefully laid over the gel mould excluding all air, and the whole is transferred to an incubator at 55 to 60°C for 30 minutes in which time gelling will occur. If the de-gassing has been inadequate, numerous small air bubbles will appear at this stage, and the gel should be discarded.

The upper glass plate should be carefully removed. The origin slot is cut in the center of the plate with a sharp blade; a slight sawing action of the blade is helpful to get a clean cut edge. This slot should be made by removing a strip of acrylamide gel about 5 to 7 mm wide and extending to about 5 mm from each side of the plate. The slot thus formed is filled with 7 percent starch gel made in the usual manner with the starch gel buffer.

The inserts are standard cotton threads about 1 cm long which are put into slots made in the center of the starch gel strip. A plate prepared in this way is shown in Figure 3-42.

Figure 3-42. Acrylamide Plate with Starch Strip and Samples Inserted—before Electrophoresis

With serum samples, no prior preparation is required unless they are old and have developed storage bands. With bloodstains, the thread taken from the stain should be soaked in a minimum of starch gel buffer containing 1 percent mercaptoethanol for about 10 to 30 minutes prior to insertion into the gel. This treatment removes storage bands that may have developed in the bloodstain material.

Electrophoresis is carried out overnight (16 to 17 hours) at 7 volts/cm with a cooling plate at 6 to 8° C. Line 'A' (Figure 3-43) shows 1, 2, and 4 C_5^- and 3 C_5^+ samples untreated with mercaptoethanol; 5, 7, and 8 C_5^- and 6 C_5^+ samples treated with mercaptoethanol. The lack of mercaptoethanol in samples 1 through 4 shows the interference of storage bands. Line B shows the same samples as line A but inserted directly into acrylamide without starch at the origin.

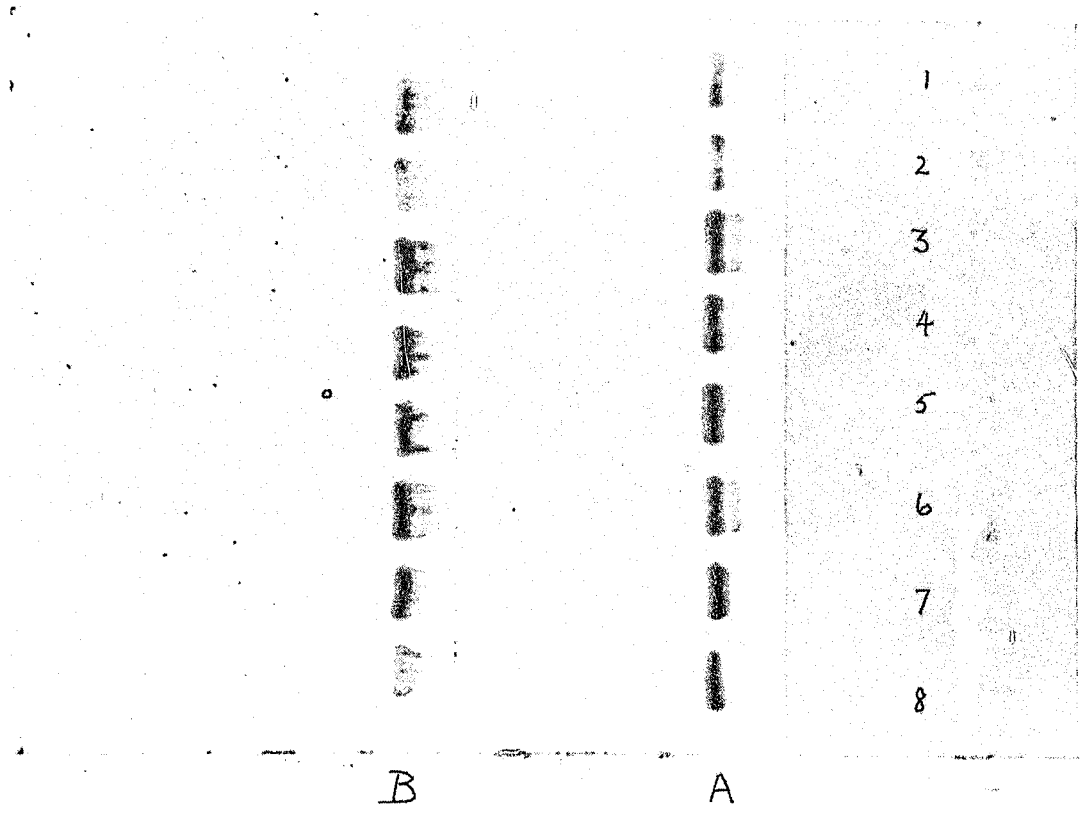


Figure 3-43. Samples Treated and Untreated with Mercaptoethanol

The zones of PCE activity are between the origin and the anode so this area is flooded with the detecting reaction mixture.

3.8.4 REACTION MIXTURE

The buffer which has pH 7.1 is prepared as follows:

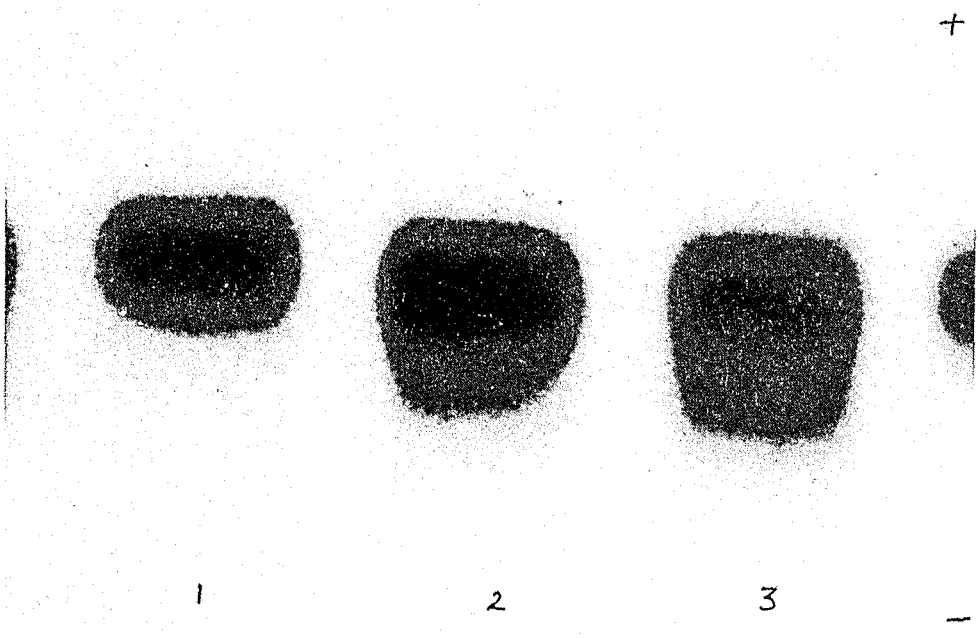
a.	Sodium dihydrogen phosphate	31.2 g
	Water	1 litre
b.	Hydrogen disodium phosphate: $12H_2O$	107.4 g
	(anhydrous	42.59)
	Water	1 litre
	Add a to b until pH 7.1 is reached.	
c.	Buffer (as above)	10 ml
	1 percent -naphthyl acetate in 50 percent aqueous acetone	0.4 ml
	Fast Red TR salt	4.0 mg

This is mixed with 10 grams of 2 percent agar in distilled water which has been melted and cooled to 50 to 55°C. This agar mixture is immediately poured over the acrylamide gel from the origin towards the anode. The reaction is allowed to proceed at room temperature for 30 to 60 minutes when the red-brown areas of activity will be seen (refer to Figure 3-44).

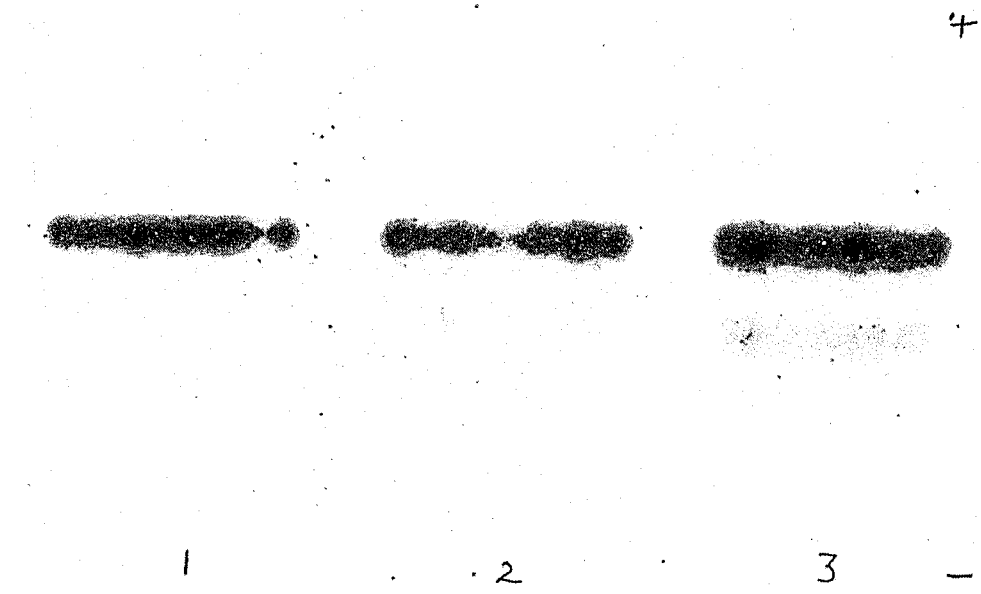
3.8.5 C_5 AND AK TYPES FROM SINGLE SAMPLES IN ONE GEL

With this acrylamide/starch (AK) system of electrophoresis, adenylate kinase types are also separated and appear on the cathodic side of the origin[157]. This cathodic area of the acrylamide gel may be developed in place or removed and developed for AK types. The development of these is identical to the reaction system described under AK using thin starch technique.

The succinic buffer system appears to enhance the AK activity, and good clear results have been obtained from stains over six months old using this technique. The minor banding of AK is also more apparent than in the starch technique (refer to Figure 3-45). The diluted succinic may also be used to replace the histidine buffer in the AK thin layer starch method. The C_5 types in various populations are listed in Table 3-13.

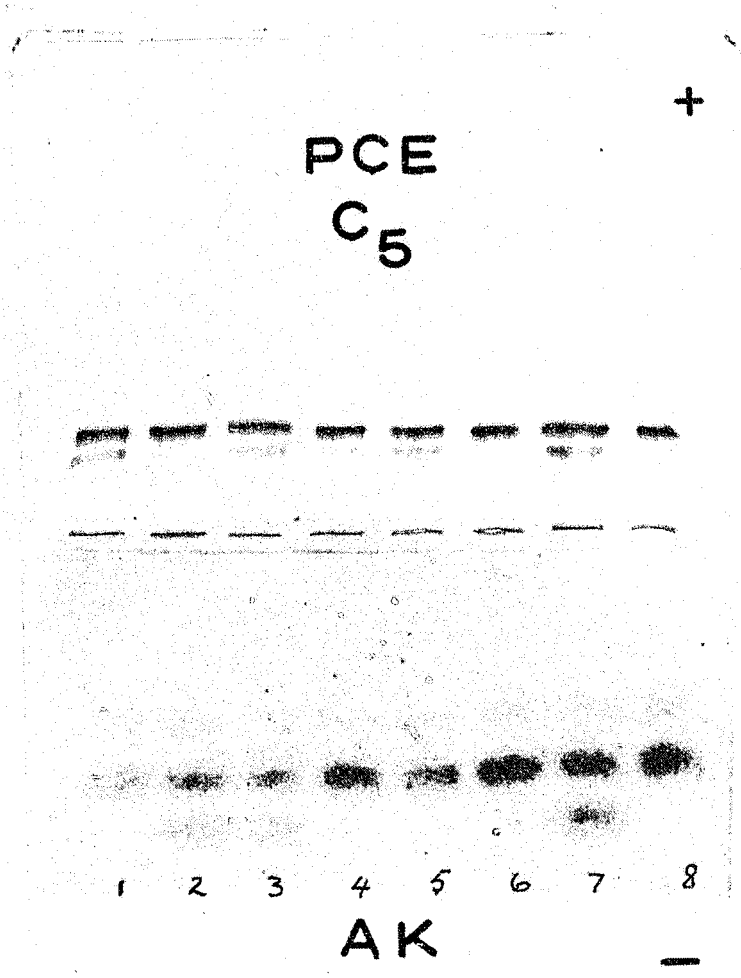


a. Sample 1 is a C_5^-, sample 2 is a C_5^T (weak), and sample 3 is a C_5^+ as determined in starch gel.



b. The same samples as in plate 3 determined by the acrylamide/starch method.

Figure 3-44. Samples as Determined in Starch Gel and in Acrylamide/Starch



Samples 1 and 5: - AK 1, C_5^+;
Sample 2: - AK2-1, C_5^-;
Samples 3 and 7: - AK2-1, C_5^+;
Samples 4, 6, and 8: - AK 1, C_5^-.

Figure 3-45. C_5 and Adenylate Kinase Types

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Table 3-13

C_5 Types in Various Populations

Population	Number Tested	C_5 Phenotypes %			Reference
			C_{5-}	C_{5+}	
EUROPE England	1941	No obs.	1752 90.26	189 9.74	171
AMERICA U.S.A.-Negro	417	No obs.	399 95.68	18 4.31	161,171
Brazil-mixed	2102	No obs.	1932 91.90	170 8.10	161
Dominica- Carib Indians	99	No obs.	90 9.91	9 9.09	165

SECTION 4

POLYMORPHIC PROTEIN SYSTEMS

4.1 HAPTOGLOBIN

4.1.1 INTRODUCTION

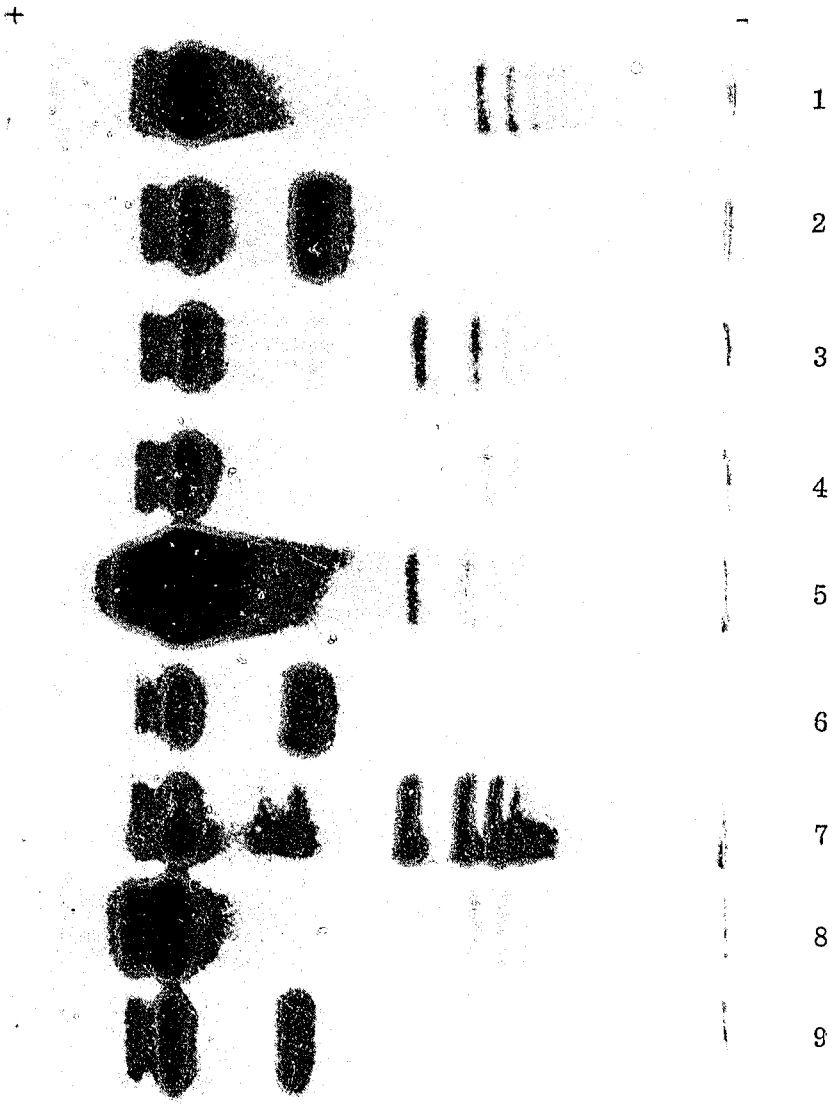
Haptoglobin is an alpha$_2$ globulin that binds free haemoglobin when it is released into the serum by red cell breakdown. The haemoglobin is removed from the serum after this transport as a haemoglobin/haptoglobin complex.

More has been written over the years about haptoglobin than any other single serum protein and no attempt is made to summarize all the available information here. The following references will provide an excellent background and detailed information about haptoglobin: Kirk[177], Smithies[58], Allison[174], and their bibliographies. There are also many other treatises on haptoglobins.

4.1.2 POLYMORPHISM

Haptoglobin was the first of the serum proteins to be described as a polymorphic system under genetic control. Smithies[180], in 1955, described the electrophoresis of serum proteins in hydrolysed starch gel and showed the three main types of Hp. There are shown in Figure 4-1.

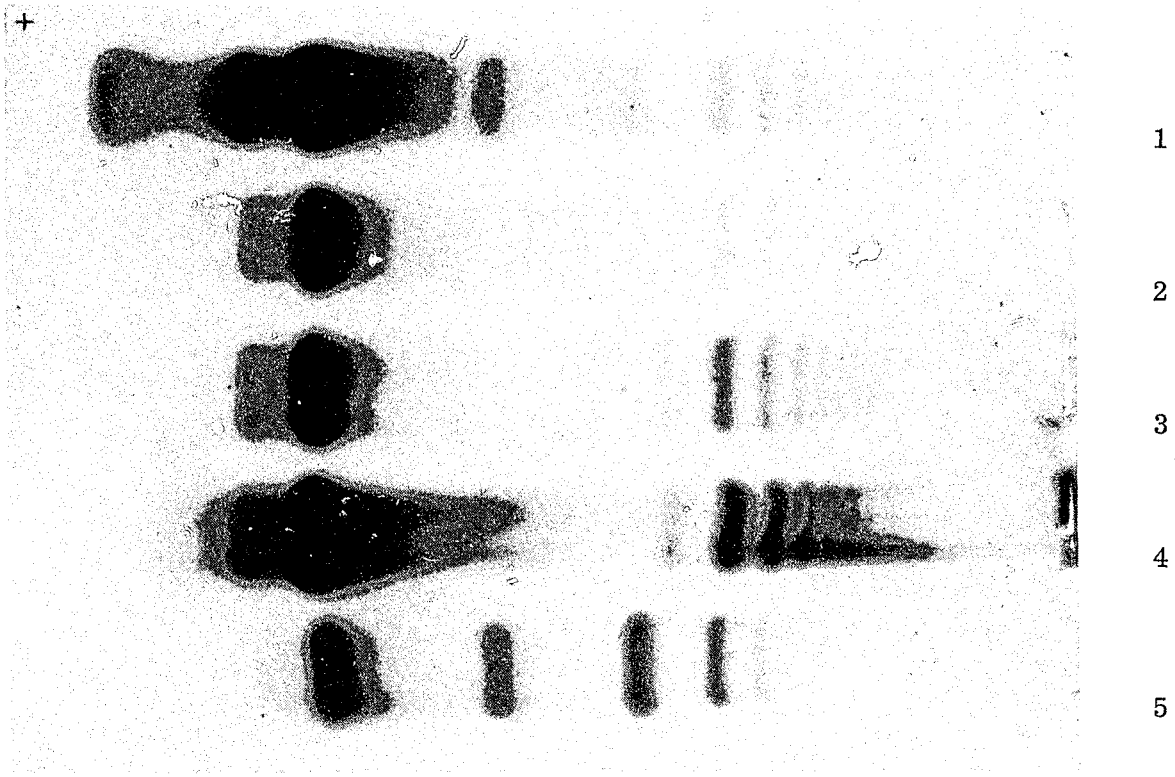
These three Hp types were shown to be two alleles at a single locus. These were denominated Hp 1 and Hp 2, giving the homozygotes Hp 1-1 and Hp 2-2 and the heterozygote Hp 2-1. The quantitative expression of these genes also varies markedly. The Hp 1-1 types have, on average, the highest concentration of haptoglobin, and Hp 2-2 types the lowest. The number of multiple bands shown by the types Hp 2-1 and Hp 2-2 can vary considerably, particularly in the heterozygote. This has been described in detail as variable gene expression by Sutton and Karp[181,199].



Slots 2, 6 and 9, Hp 1-1; Slots 3, 5 and 7, Hp 2-1; Slots 1, 4 and 8, Hp 2-2

Figure 4-1. Three Main Types of Hp

Various rare alleles have been subsequently described, and these are illustrated in Figure 4-2. The commonest of these is the Hp 2-1 M (2-1 modified) which occurs particularly in Negro populations, some of which show a high frequency of this.



Slots 2, 3 and 4, Hp 2-2; Slot 1, Hp 2-1; Slot 5, Hp 2-1 M

Figure 4-2. Variations of Intensity of Hp

A further polymorphism of the polypeptide chains of the Hp 1-1 type was described by Connel, et al[175], and Nance and Smithies[178]. This separated the Hp 1 gene into two forms, a fast running (F) or a slow running (S) chain. This produces the three forms in Hp 1-1 and two forms in Hp 2-1.

Hp 1-1 Forms	Hp 2-1 Forms
Hp 1^F-1^F	Hp 2-1^F
Hp 1^F-1^S	Hp 2-1^S
Hp 1^S-1^S	

The cleavage of the haptoglobin was brought about using urea and mercapto-ethanol. The quantity of serum required was large because purification of the haptoglobin from the other serum proteins was necessary. It is, therefore,

unsuitable for work on bloodstains for forensic purposes, and the method will not be described.

4.1.3 DETERMINATION OF Hp TYPES IN SERUM

4.1.3.1 General Description

The preparation of the thick starch gel has been described in the chapter "Techniques".

The buffers used are:[179]

Tanks: pH 7.9

Sodium hydroxide	2.0 g
Boric acid	18.6 g
Distilled water to	1 litre

Gel: pH 8.6

TRIS	9.2 g
Citric acid	1.05 g
Distilled water to	1 litre

4.1.3.2 Inserts

Pieces of Whatman 3 MM filter paper, 8 mm by 5 mm, are used for soaking in the serum sample.

4.1.3.3 Sample Preparation

The sera samples should have a small amount of haemoglobin A added until they are pink in colour.

The haptoglobin binds haemoglobin and is electrophoresed as the Hp/Hb complex so that subsequent detection can use the peroxidase activity of the haemoglobin in the complex. There should always be a slight excess of haemoglobin; otherwise, the electrophenogram produced is obscure, as can be seen in Figure 4-3. Sample 2 has no excess haemoglobin and is incompletely saturated. The run is overnight, from 15 to 17 hours, at about 60 volts at room temperature or 100 volts at 4°C.

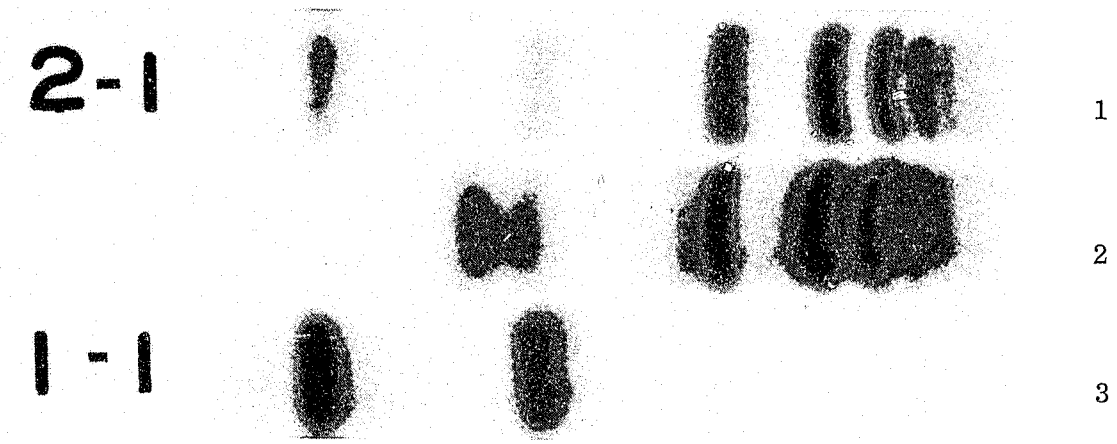


Figure 4-3. Electrophenogram of Haemoglobin

After electrophoresis, the filter paper inserts are removed and the gel is sliced horizontally to produce two parts each about 2 to 3 mm thick. This is done by removing the upper glass strips around the gel and leaving the lower strips in place. Then, using the lower strips as a guide level, a knife or fine wire is run through the gel from the anode to the cathode. Working from the anode towards the cathode avoids the possibility of fragments of the filter paper insert catching on the knife and tearing the gel surface. A piece of filter paper is laid over the surface of the gel, and the upper slice is carefully removed.

4.1.3.4 Development

The lower half of the gel is developed with a solution of o-tolidine in ethanol/acetic acid (about 50/50). The quantity of o-tolidine is not critical between about 0.1 percent to 1 percent. After two or three minutes, this is followed by 2 percent hydrogen peroxide (the hydrogen peroxide may be combined with the o-tolidine solution if desired) that stains the haemoglobin and the haptoglobin/haemoglobin complex bands so that the bands can then be interpreted.

If a stronger solution of hydrogen peroxide is used, catalase will produce bubbles of oxygen in the gel which will be disrupted and the bands of haptoglobin destroyed.

4.1.4 HAPTOGLOBIN TYPES IN BLOODSTAINS

4.1.4.1 General Description of Methods

The method used for serum samples has a very limited application to bloodstains[145]. Excess haemoglobin should be removed first, also the stain must be very fresh, that is, less than three days old. The pattern obtained from sera is lost when blood dries to a stain, but the relative mobility is retained for fast, intermediate, and slow types of 1-1, 2-1, and 2-2, respectively; therefore, an immuno-electrophoretic method was developed for their detection in stain material[176]. This method has been subsequently improved and is described below. Very recently, 1970[42], a different method has been developed which used the same or slightly more material, but the results from stains are considerably better than those using the immuno-electrophoretic technique. It is an expensive technique requiring specialized equipment which may not be available; therefore, both techniques are described.

4.1.4.2 Immuno-electrophoretic Method

4.1.4.2.1 Equipment and Buffers

Electrophoresis is performed on microscope slides using the Shandon immuno-electrophoresis equipment. Buffers as indicated below were used:

a. Tanks: pH 8.6

Sodium barbitone	8.76 g
Barbituric acid	1.38 g
Calcium lactate	0.38 g
Distilled water to	1 litre

b. Gel: pH 8.6

Sodium barbitone	7.00 g
Barbituric acid	1.10 g
Calcium lactate	1.00 g
Distilled water to	1 litre

4.1.4.2.1 Agar Stock

The agar stock is 2 percent Ionagar No. 2 in distilled water. Equal volumes of gel buffer and agar stock are melted, cooled to about 50 to 55°C, and poured over the microscope slide to give a layer of gel about 1 mm thick. Using the Shandon slide tray which takes eight slides 3 inches by 1 inch, about 40 ml of buffer/agar mixture is required.

When the get is set firm, the antigen slots are cut to the pattern as shown in Figure 4-4. Do not cut antiserum slots until AFTER the first electrophoretic separation. Cut out enough stain material to fill the slot, and soak it in gel buffer for ten minutes prior to insertion into the gel.

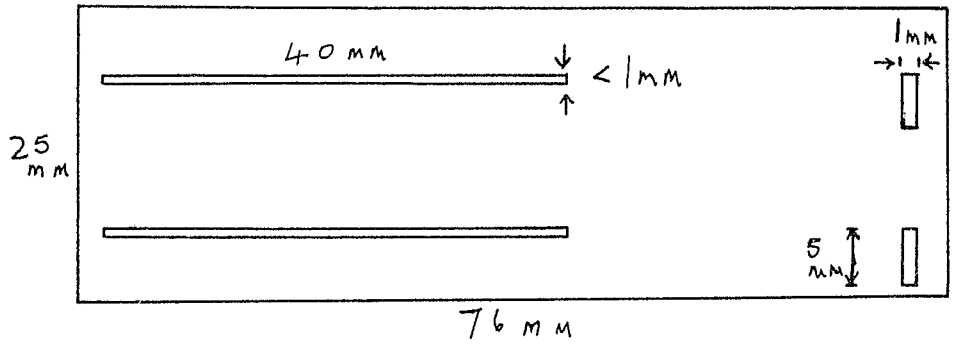


Figure 4-4. Pattern of Antigen Slot

4.1.4.2.2 Electrophoresis (1)

Use 160 v (7 v/cm) and current of 48 ma (constant) from 4 to 4-1/2 hours at room temperature, using gel contact troughs filled with 2 percent Ionagar in tank buffer. Cut antiserum troughs with Shandon gel cutter, and remove waste gel before filling troughs with antiserum.

4.1.4.2.3 Electrophoresis (2)

Perform electrophoresis at right angles to direction of first separation as in Figure 4-5. Use 160 v (7 v/cm) and constant current of 22 ma for 1 hour at room temperature. Use Spontex bridges held in place with a glass plate.

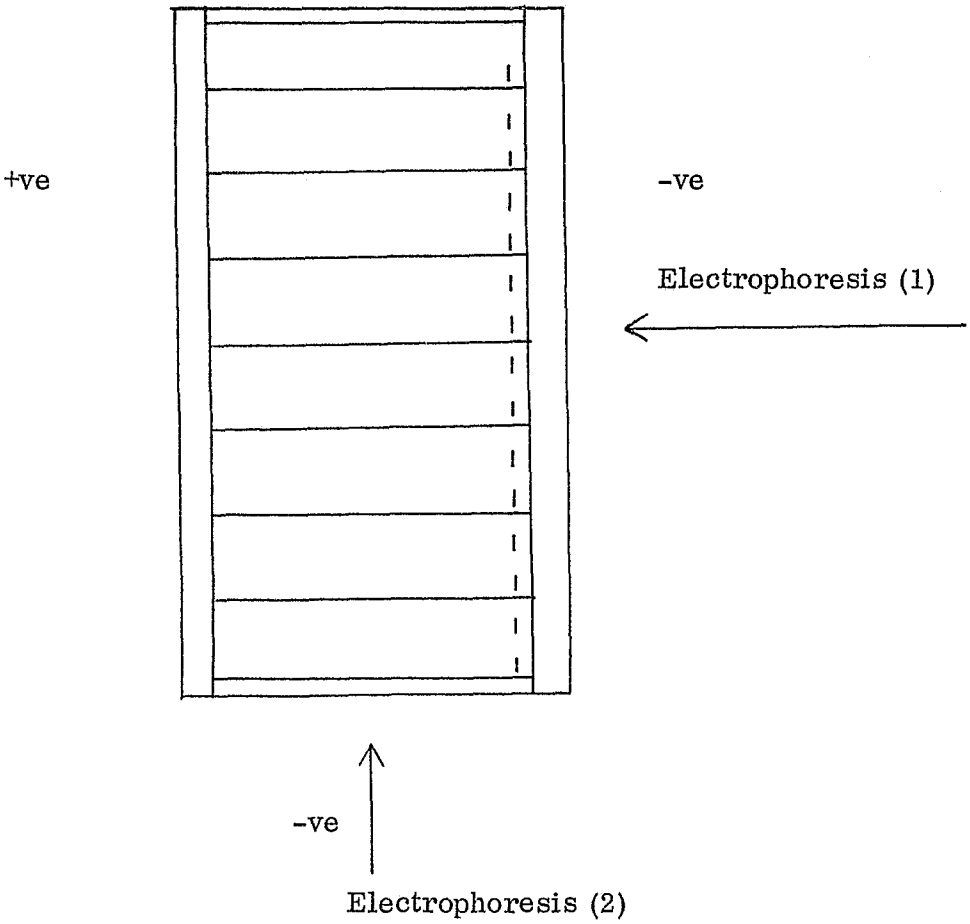


Figure 4-5. Tray and Slides

4.1.4.2.4 Washing

Place the tray in a large dish containing 1 M sodium chloride solution and leave overnight. Place it in distilled water for 1/2 hour before drying. Remove the individual slides, cover them with wet Whatman No. 1, and then dry at 56°C for 1/2 hour.

4.1.4.2.5 Staining

Use 0.1 percent Amido Black in alcohol/acetic acid/water (40/10/50) for about 15 to 30 minutes. Differentiate in the alcohol/acetic acid/water mixture. The results can then be read. A control or known stain of all three types should be included in each tray of slides.

The interpretation is exactly the same as the earlier diffusion technique[176] shown in Figure 4-6. (Arrowed are the peaks of the precipitation areas.)

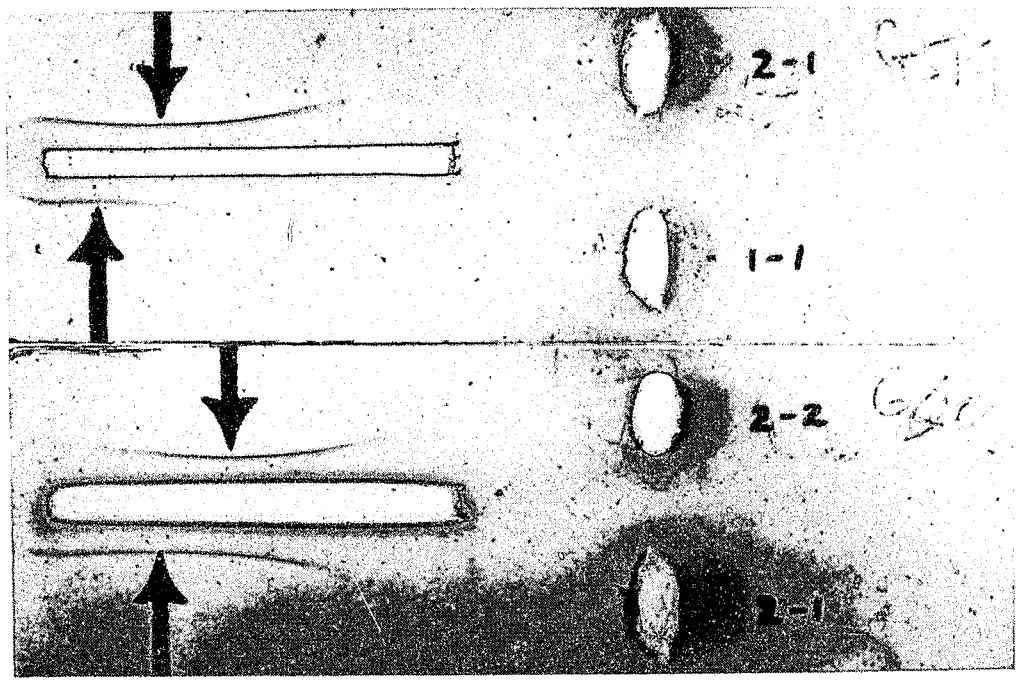


Figure 4-6. Immuno-electrophoresis of Bloodstains of the Three Major Types of Haptoglobin

4.1.4.3 Acrylamide Gradient Technique for Bloodstains

4.1.4.3.1 General Description

Haptoglobins may be separated by means of charge as in the immuno-electrophoretic technique, by a combination of charge and molecular size as in the starch gel technique for sera or by molecular size alone as in this technique.

An acrylamide gradient is created so that in vertical electrophoresis the top of the gel contains 5 percent acrylamide and the bottom of the gel contains 25 percent acrylamide, with a smooth gradient of concentration between these. Under these conditions the pore size of the gel gradually reduces, and electrophoresis

is performed until the mobility of the proteins is stopped by the molecular pore size of the gel becoming too small to allow the molecule further movement through the gel. Sample slots are cast into the top of the gel, and the sample is applied as a liquid extract.

4.1.4.3.2 Preparation of the Gel

The gel is made with a gradient former according to the manufacturer's instructions using buffer solution in one side of the gradient former and approximately 25 percent acrylamide in the other. The acrylamide is dissolved in buffer in both instances. The two solutions are as follows:

a. First Solution

Cyanogum 41 (BDH)	40 g
Ammonium persulphate 10 percent	4 ml
Tetramethylene-ethylene-diamine (TEMED)	20 µl
Buffer	160 ml

b. Second Solution

Ammonium persulphate 10 percent	4 ml
TEMED	250 µl
Buffer	160 ml

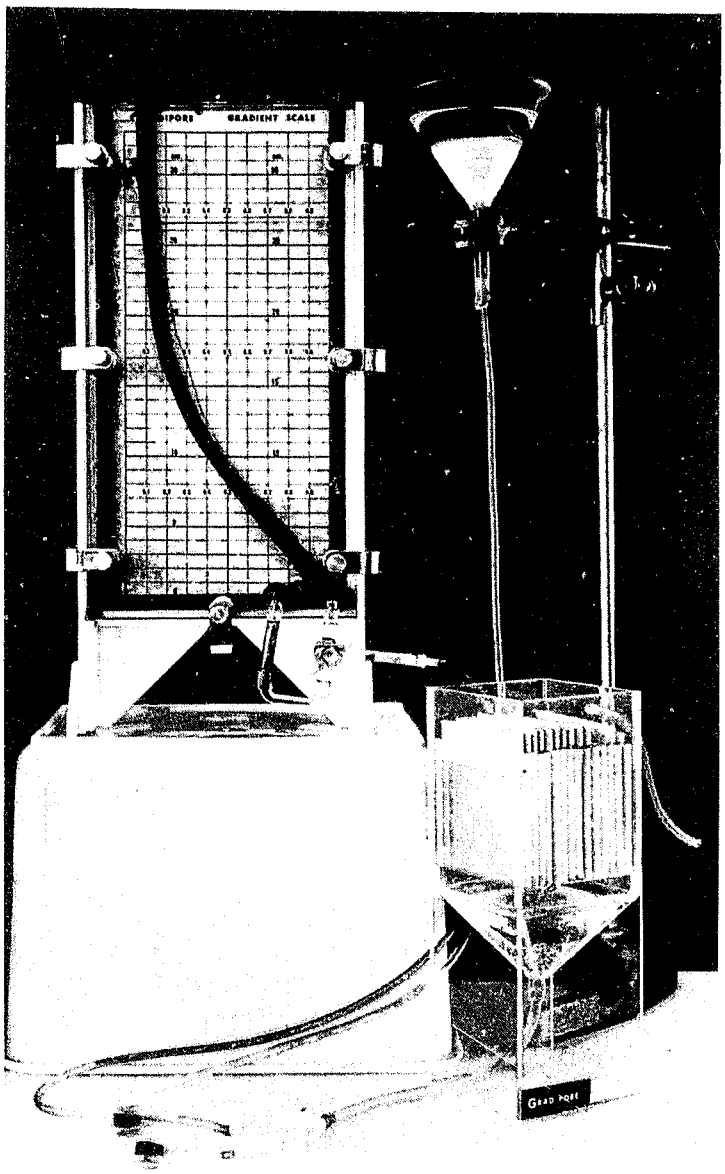
4.1.4.3.3 Buffers

Tank and gel buffers are as indicated below:

TRIS	10.75 g
Sodium EDTA	0.93 g
Boric acid	5.04 g
Distilled water to	1 litre

4.1.4.3.4 Apparatus

The manufacturer of this apparatus is Gradipore, and the various components are illustrated in Figures 4-7 through 4-10.



(Fourteen plates are ready to be formed in the tower on the right.)

Figure 4-7. The Acrylamide Gradient Forming Apparatus

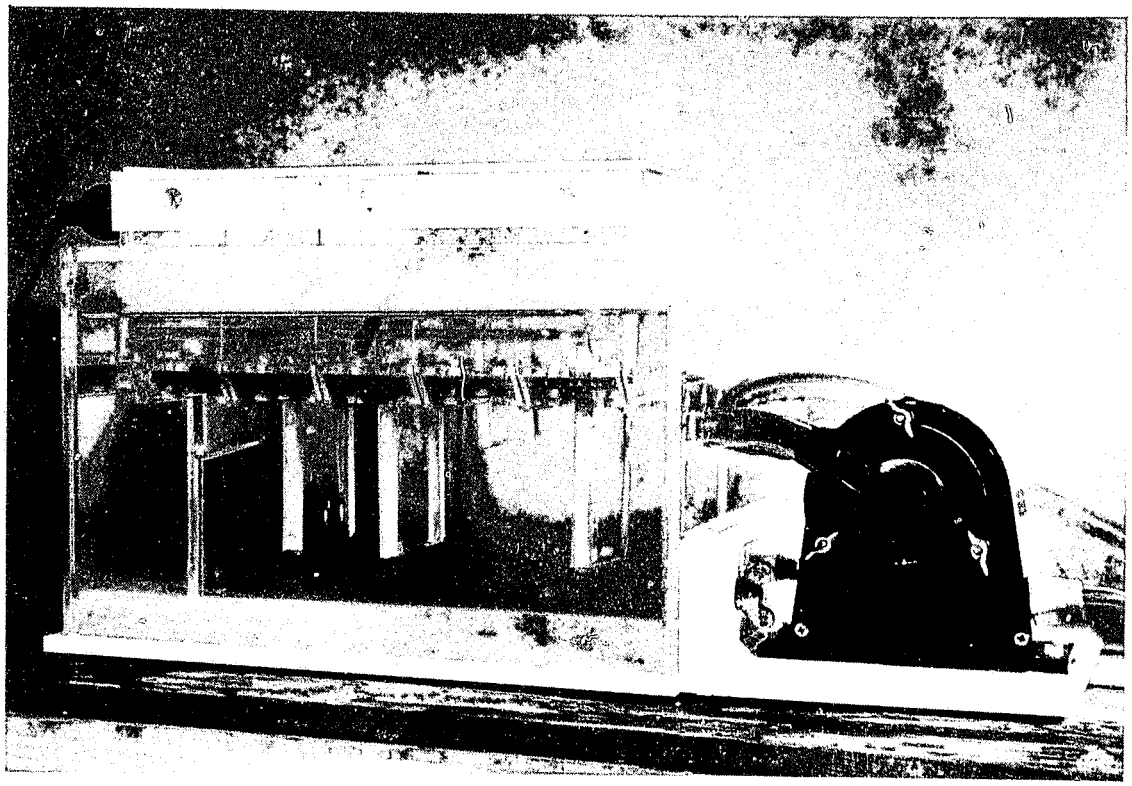
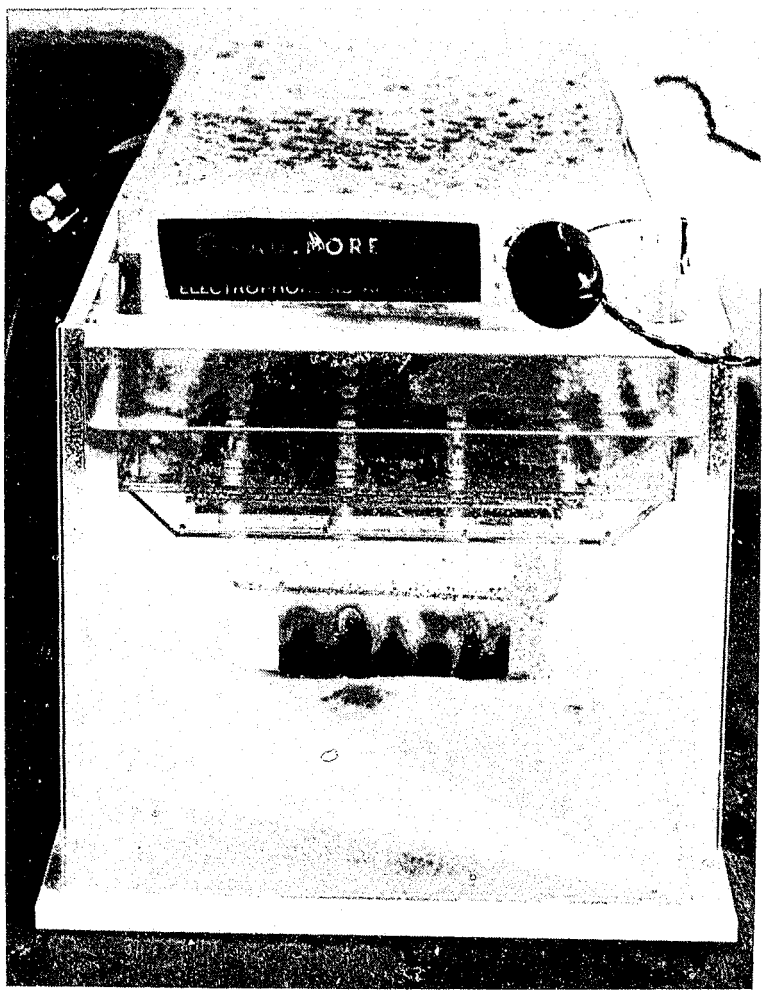
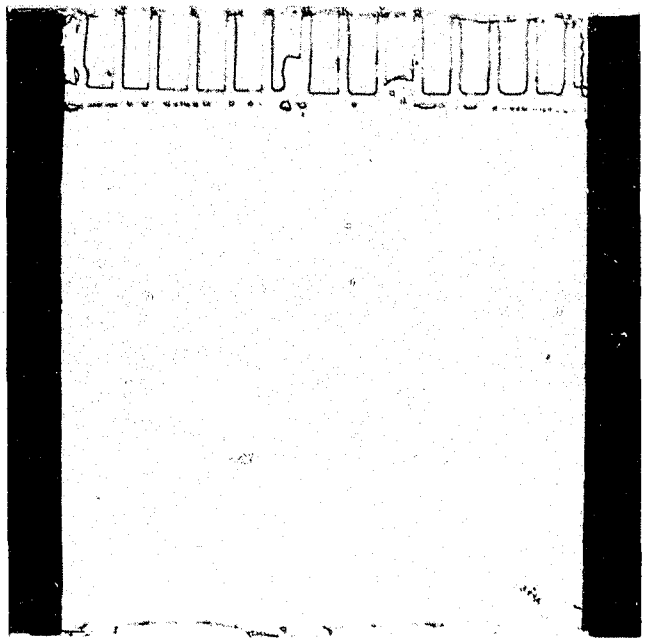


Figure 4-8. A Five-Unit Tank with Buffer Circulation Pump



(Haemoglobin in large excess can be seen at the bottom of the plate.)

Figure 4-9. An Acrylamide Plate Approaching Completion



(The concentration of acrylamide is 5 percent at the top and around the sample wells; 30 percent acrylamide at the lower edge.)

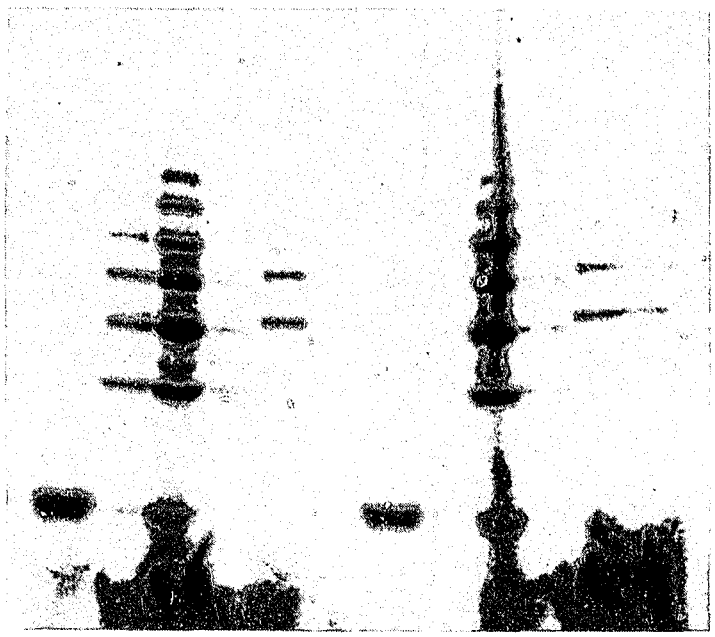
Figure 4-10. A Plate of Gradient Acrylamide with Sample Wells Pre-formed in the Top of the Gel

4.1.4.3.5 Procedure

The bloodstain, about 1/4 to 1/2 cm^2, is soaked in a minimum of buffer (to which may be added 10 percent sucrose) until maximum extraction of the stain is obtained. This is then placed in a gel slot by means of a fine Pasteur pipette. It has been found advisable to leave a space between each sample if using the standard well-forming combs. Electrophoresis is performed for one hour at 100 v prior to application of the samples, subsequently for 24 hours at 100 v.

The gel is then stained in the o-tolidine solution described under the starch gel technique for sera. It may alternatively or subsequently be stained with Amido Black (as described under the immuno-electrophoretic technique) and destained electrophoretically in the apparatus provided by the manufacturer.

The results obtained from stains repeat the original banded results that are obtained for sera using a starch gel and are illustrated in Figure 4-11. Results obtained by this technique are far easier to interpret, and sera and stains may be run together on one gel. This technique, if the apparatus is available, should supercede the immuno-electrophoretic one.



(A mixture of serum and bloodstain samples stained with the benzidine/peroxide mixture. Samples 1 and 6, Hp 1-1; Samples 2, 3, 4, 7 and 8, Hp 2-1; Samples 5, 9 and 10, Hp 2-2.)

Figure 4-11. A Gradient Acrylamide Plate at the End of Electrophoresis

4.1.5 PROBLEMS

The problems that arise with haptoglobins are associated with the immuno-electrophoretic technique and are largely those associated with this type of technique in general. The major additional difficulty is that a Hp 2-1 M can appear to be a slightly slow Hp 1-1. Variants from the main types cannot be distinguished. In any of these techniques, the fresher the bloodstain the more likely it is to give good readable unequivocal results.

A distribution of Hp types in various populations is presented as Table 4-1.

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Table 4-1

Distribution of Hp Types in Various Populations

Population	Number Tested		Hp Phenotypes (Percent)				Gene Frequency		Reference
			1-1	2-1	2-2	0	Hp^1	Hp^2	
EUROPE									
England	397	Obs	13.85	52.64	31.98	1.51	0.4079	0.5920	183, 200
		Exp	16.64	48.30	35.05				
Austria	621	Obs	16.10	48.15	35.74	0.00	0.4018	0.5982	from 203
		Exp	16.14	48.07	35.78				
Belgium	610	Obs	18.36	46.22	35.41	0.00	0.4147	0.5852	214
		Exp	17.20	48.54	34.25				
Denmark	2046	Obs	15.98	47.26	36.75	0.00	0.3961	0.6038	197
		Exp	15.69	47.84	36.46				
Finland	891	Obs	14.48	41.97	43.32	0.22	0.3554	0.6445	204
		Exp	12.63	45.81	41.54				
France	2156	Obs	15.44	47.40	36.82	0.32	0.3927	0.6072	from 203
		Exp	15.42	47.69	36.87				
Germany	26307	Obs	15.06	47.21	37.73	0.00	0.3866	0.6133	186
		Exp	14.95	47.42	37.62				
Italy	3714	Obs	13.49	46.23	40.28	0.00	0.3660	0.6339	212
		Exp	13.39	46.53	40.18				
Norway	5811	Obs	14.21	46.87	38.91	0.00	0.3765	0.6235	194
		Exp	14.17	46.95	38.87				
Poland	6017*	Obs	13.54	48.23	38.22	0.00	0.3766	0.6234	from 203
		Exp	14.18	46.95	38.86				
Portugal	1838*	Obs	15.67	47.28	37.05	0.00	0.3931	0.6069	189, 213
		Exp	15.45	47.71	36.83				
Spain	2763	Obs	16.32	48.24	35.43	0.00	0.4044	0.5955	209
		Exp	16.36	48.17	35.47				
Sweden	2282	Obs	15.07	46.49	37.95	0.48	0.3851	0.6149	from 203
		Exp	14.83	47.36	37.81				
Switzerland	920	Obs	15.22	48.80	35.97	0.00	0.3962	0.6038	193
		Exp	15.70	47.84	36.46				
Greece	2026≠	Obs	10.96	45.86	43.18	0.00	0.3395	0.6604	185
		Exp	11.53	44.84	43.62				
Cyprus	196	Obs	5.10	34.69	57.14	3.06	0.2315	0.7684	208
		Exp	5.36	35.57	59.04				

* — Includes 1 Hp 2-1 M
\+ — Includes 2 Hp 2-1 M
≠ — Includes 9 Hp 2-1 M

Table 4-1

Distribution of Hp Types in Various Populations (Continued)

Population	Number Tested	Hp Phenotypes (%) (Observed)					Gene Frequency		Reference
		1-1	2-1	2-2	2-1M	0	Hp^1	Hp^2	
AFRICA—NEGROES									
Senegal	393	37.37	35.61	11.86	11.11	4.04	0.6328	0.3671	206
Gambia	157	30.57	14.65	7.00	7.00	40.76	0.6989	0.3010	200
Liberia	329	41.94	17.93	9.42	10.94	19.75	0.7026	0.2973	207
Nigeria	477	36.68	25.57	4.61	2.09	31.02	0.7325	0.2674	183, 188, 192, 210
Uganda	273	25.27	34.80	7.32	4.76	27.84	0.6243	0.3756	182, 184
Tanzania—Bantu	60	16.67	35.00	23.33	5.00	20.00	-	-	184
Burundi	182	28.02	47.80	20.88	-	3.29	0.5369	0.4630	214
Kenya—Nilo-Hamite	50	20.00	44.00	24.00	2.00	10.00	-	-	184
Central African Republic	353	24.08	22.66	16.71	3.40	33.11	0.5550	0.4449	211
South Africa	674	30.41	35.90	20.92	7.27	5.49	0.5502	0.4497	from 203

Population	Number Tested	Hp Phenotypes (Percent)					Gene Frequency		Reference
			1-1	2-1	2-2	0	Hp^1	Hp^2	
AFRICA—OTHERS									
Egypt	219	Obs Exp	4.10 4.51	34.24 33.44	61.64 62.03	0.00	0.2123	0.7876	201
South Africa Cape Coloured	100	Obs Exp	16.00 15.21	46.00 47.58	38.00 37.21	0.00	0.3900	0.6100	199

Table 4-1

Distribution of Hp Types in Various Populations (Continued)

Population	Number Tested	Hp Phenotypes (Percent)					Gene Frequency		Reference
			1-1	2-1	2-2	0	Hp^1	Hp^2	
ASIA									
Middle East (Jews)	1594*	Obs	8.09	42.72	48.68	0.50	0.2960	0.7039	196
		Exp	8.76	41.67	49.55				
Pakistan	392	Obs	5.70	30.83	60.36	3.10	0.2179	0.7820	202
		Exp	4.75	34.08	61.15				
India	2011+	Obs	3.64	27.34	66.86	2.14	0.1770	0.8230	190, from 203
		Exp	3.13	29.14	67.73				
Ceylon	462	Obs	1.08	24.89	71.21	2.81	0.1392	0.8608	202
		Exp	1.94	23.96	74.10				
Thailand	472	Obs	5.29	35.38	57.63	1.69	0.2338	0.7661	202
		Exp	5.47	35.82	58.69				
Korea	115	Obs	10.43	42.61	46.95	0.00	0.3174	0.6826	187
		Exp	10.07	43.33	46.59				
Taiwan - Chinese	471	Obs	11.04	47.77	39.49	1.70	0.3552	0.6447	191, 195
		Exp	12.61	45.80	41.56				
Japan	1451	Obs	7.58	36.94	55.48	0.00	0.2605	0.7394	105, 71
		Exp	6.79	38.52	54.67				

Population	Number Tested	Hp Phenotypes (Percent)					Gene Frequency		Reference
			1-1	2-1	2-2	0	Hp^1	Hp^2	
AMERICA									
Dominica—Carib-Indians	99	Obs	55.55	34.34	8.08	2.02	0.7423	0.2577	65
		Exp	55.10	38.26	6.64				
USA—Whites	409	Obs	13.20	50.61	36.18	0.00	0.3851	0.6149	198
		Exp	14.83	47.36	37.81				

Population	Number Tested	Hp Phenotypes (%) (Observed)					Gene Frequency		Reference
		1-1	2-1	2-2	2-1M	0	Hp^1	Hp^2	
AMERICA									
USA—Negroes	1657	28.48	38.68	18.53	10.92	3.38	0.5515	0.4484	198

4.2 HAEMOGLOBIN

4.2.1 INTRODUCTION

Variation in the human haemoglobin is generally known as a haemoglobinopathy or Hb abnormality. There are, however, variants of adult haemoglobin which are a normal part of development and, therefore, are not abnormalities. These are the embryonic and foetal haemoglobins. The foetal haemoglobin is more likely to be encountered than the embryonic form and differs both in structure and electrophoretic mobility from adult haemoglobin. Together with the other variants of adult haemoglobin, these show that haemoglobin is polymorphic. This fact enables us to differentiate not only between individuals but between races.

Many treatises have been written on the structure of the haemoglobin molecule by Perutz[222], Perutz and Lehmann[223] and by other workers. No intent is made to discuss this work here, but merely to provide a little background information. For a full background on haemoglobin, suitable for the forensic scientist, the reader is referred to "Man's Haemoglobins" by Lehmann and Huntsman[220].

Haemoglobin is synthesized in the protoplasm of the developing red cell and consists of two pairs of polypeptide chains. These are known as alpha chains, containing 141 amino acid residues and beta chains containing 146. The sequence of these amino acid residues show marked differences between the alpha and beta chains, but their tertiary structure and the folding of the chains are very similar. The histidine group of the chain is attached by a covalent bond to the iron atom, and the haeme part of the molecule is between the loops of the polypeptide chain.

It has been reported by Lehmann and Huntsman[220] that the dimensions of the haemoglobin molecule are 64 by 55 by 50 angstrums and that the molecular weight is about 67000. If one amino acid is replaced by another in either of the alpha or beta chains this will give rise to a haemoglobin variant. Most variants are extremely rare so it is proposed to consider only the more common variants which are relevant to the forensic scientist.

4.2.2 HAEMOGLOBINS A AND F

There are at least three physiological haemoglobins of which adult haemoglobin (HbA) and foetal haemoglobin (HbF) are the most important. The main adult haemoglobin, HbA_1, consists of two alpha chains and two beta chains ($\alpha_2 \beta_2$) and comprises 98 percent of normal blood. The other 2 percent is known as HbA_2 and consists of one pair of alpha chains and one pair of delta chains. These differ from the beta chain amino acid sequence at 10 positions, $HbA_2 = \alpha_2 \delta_2$.

In very young embryos there are polypeptide chains called epsilon (ϵ) chains, but at approximately three months, this embryonic haemoglobin is replaced by foetal haemoglobin which contains gamma chains. These differ from the normal alpha chain at 39 positions, $HbF = \alpha_2 \gamma_2$. It is surprising that although HbA and HbF differ so widely in structure, the functional differences are very small, and an electrophoretic separation of the two is not easy. At birth, there is approximately 20 percent HbA present (although this is extremely variable) and this gradually increases so that at six months, in most cases, all the foetal haemoglobin has been replaced by adult. A decrease in the formation of the alpha and beta chains causes thalassemia (discussed later).

4.2.3 HAEMOGLOBIN S

Sickle cell haemoglobin (HbS) is found in two forms; the "disease" or homozygous form and the "trait" or heterozygous form. In the homozygous state, nearly all the haemoglobin is HbS, HbA_1 being absent and the balance consisting of HbA_2 and HbF. As anaemia is often severe and the patient is normally subjected to sickle cell crises, this type of individual usually does not occur in a forensic sampling. The heterozygote individual, however, being quite healthy does occur quite frequently, and here HbS accounts for approximately 45 percent of the total haemoglobin. The rest consists of HbA_1 and HbA_2. Sickling of the cells does not normally occur in vivo but if the oxygen tension is lowered, e.g., at high altitudes, or if the individual undergoes violent exercise or receives an emotional shock, then a sickle cell crisis can occur and can cause death.

There is no difference in the structure of the alpha chain between HbA and HbS but in the beta chain valine replaces glutamic acid at the sixth position. It is not

fully understood why such a simple amino acid substitution should make such a difference not only electrophoretically but in the solubility of deoxygenated HbS.

Haemoglobin S is also known to occur with other abnormal haemoglobins which only occur quite rarely. These are as follows:

a. Sickle Cell HbC Disease (S/C).
b. Sickle Cell HbD Disease (S/D).
c. Sickle Cell HbE Disease (S/E).
d. Sickle Cell Thalassemia.
e. Sickle Cell Associated with Foetal Haemoglobin Other Than During Infancy.

Carriers of the HbS gene are normally confined to Negro populations or persons of negroid ancestry. Being a carrier for the gene seems to afford the person protection against malignant malaeria. As can be seen in the population data, Table 4-2, the sickle cell gene has a wide geographical distribution, but all of these are areas where malignant malaeris was indemic.

4.2.4 HAEMOGLOBIN C

Unlike haemoglobin S, both the homozygote and heterozygote of haemoglobin C are found in healthy individuals. One homozygote C was found, during a survey[231], at a London railway station working as a porter. Although at Metropolitan Police Laboratory a number of haemoglobin A/C individuals have been found, no homozygote form has been encountered in a criminal case.

The homozygous state is reported to produce a mild anaemia or jaundice, but this is the exception rather than the rule. The heterozygote has not been reported as having produced any clinical symptoms. Similarly, those individuals which have a combination of sickle cell and HbC disease (S/C) seem to be perfectly healthy. These are even more rare than the homozygote C and have not been encountered in our survey. Like haemoglobin S, there is only a single amino acid substitution in the alpha chain, also occurring at the sixth position where the glutamic acid of HbA is replaced by lysine. This produces an even larger difference in the electrophoretic mobility.

The area of origin of HbC is confined to North and West Africa (Table 4-3) with a very rare incidence found in Italy. Obviously any country with immigrants from these areas will have individuals carrying the HbC gene. It is still surprising that it occurs in our forensic sampling as often as it does (Table 4-4).

4.2.5 HAEMOGLOBINS D, E, AND OTHERS

Both the homozygote and the heterozygote individuals for haemoglobins D and E were first found during the course of a survey[231], and so one can safely conclude that they enjoy reasonable health. Obviously it is the HbA/E or A/D forms which are the most likely forms to be encountered.

In haemoglobin D, there is only one amino acid substitution, i.e., glutamic acid to glutamine at position 121 in the chain. In haemoglobin E, lysine replaces glutamine at position 26. Unfortunately, although the substitutions are quite different from those of HbS and HbC, it is quite difficult to separate electrophoretically HbS from HbD and HbC from HbE at alkaline pH; however, this problem can be resolved (see paragraph 4.2.6.3).

Other abnormalities which may be encountered are the thallasemias. These are known as either alpha or beta thallasemia, depending on whether the alpha or beta chain production is affected. The beta thallasemia is the more common of the two. In the homozygote there is a complete absence of HbA_1, generally associated with high levels of HbA_2 and HbF. The heterozygote is found in conjunction with other abnormal haemoglobins.

The homozygote for alpha thallasemia shows a complete absence of HbA_1, HbA_2, and F and is, therefore, unable to sustain life. The heterozygote, however, is normally completely without symptoms and is very difficult to detect. Although we have not encountered any thallasemias, we have found the abnormal haemoglobins J and M both in conjunction with HbA.

Care must be exercised not to interpret a homozygote instead of a heterozygote combined with a thallasemia gene. The latter is normally found with high levels of HbA_2 and HbF; nevertheless, homozygotes should not be confirmed without family studies.

4.2.6 METHODS AND SAMPLE PREPARATION

4.2.6.1 General Description

Electrophoresis is the most useful method for differentiating between the haemoglobin variants in whole blood although the spectrophotometer, the sickling test, and peptide mapping can also be used.

Numerous papers have been written on the identification of haemoglobin variants in whole blood, but only three papers have been published on the determination of variants in bloodstains. Each author seems to have a preference for one particular buffer and media so it may be difficult to choose the best method. Lehmann and Ager[221] and Huntsman and Lehmann[218] used Whatman 3 MM paper in a barbiturate buffer at pH 8.6. In 1964, Culliford[216], working on bloodstains, recommended using cellulose acetate in the Poulik discontinuous buffer pH 8.6. Breen, et al[215], suggested cellulose acetate in a Tris Glycine buffer pH 9.3. All of these buffers are suitable for separating the common variants HbS and HbC from HbA, but not the other variants. Graham and Grunbaun[217] and Kohn[219] suggested a discontinuous buffer for the separation of HbA from HbF. This method was adapted for bloodstains by Wraxall[225] using the following buffers:

a. Tank Buffers

Anode: pH 9.1

TRIS Tris (hydroxymethyl methylamine)	25.2 g
EDTA (ethylene diamine tetra acetic acid)	2.5 g
Boric Acid	1.9 g
Distilled water to	1 litre

Cathode: pH 8.6

Sodium diethyl barbiturate	5.15 g
Diethyl barbituric acid	0.92 g
Distilled water to	1 litre

b. Strip Buffer

Equal volumes of anodic and cathodic buffer.

Besides the media described, starch, agar, and acrylamide have been used, but the cellulose acetate membrane was found to be the simplest and quickest. Several brands have been compared, and it was found that Sartorius acetate strips gave the best resolution.

4.2.6.2 Sample Preparation

Haemolysates are prepared by washing cells three times in physiological saline and lysing by freezing or sonication. If the lysate is required as a standard control sample, then a small amount of carbon tetrachloride should be added as described by Lehmann and Ager[221]. Stain extracts are prepared by soaking a small piece of bloodstain (2-1/2 by 1 mm—more if on thin material) in a minimum amount of distilled water for ten minutes. If the stain is on a hard surface (e.g., knife, stone, etc.), the blood should be carefully scraped off and as strong a solution as possible should be made.

4.2.6.3 Method

The 5 cm-wide cellulose acetate strips are soaked in strip buffer prior to use. Two 5 by 2 cm strips are also soaked in buffer to be used as "edge strips". After soaking, the strips are blotted to remove excess buffer and are supported in the tank across a 4.5 cm-wide gap between bridges of three layers of Whatman 3 MM paper. The "edge strips" are butted onto each end to minimize any distortion of the samples at the ends of the strip during electrophoresis. The samples are applied to the cellulose acetate, midway between the anode and cathode, using fine drawn glass capillary tubes. The application lines should be at least 1 cm in length and applied using a straight edge as a guide supported 2 to 3 mm above the surface of the cellulose acetate (Figure 4-12).

Electrophoresis is carried out at room temperature at 160 v (10 v/cm) for sixty minutes (Figure 4-13). The strip is then stained in 0.15 percent Ponceau S. in 3 percent trichloroacetic acid and differentiated in 5 percent acetic acid.

The results seen in Figure 4-14 show that it is possible to separate not only HbA from HbS and from HbC, but also these from HbF. Bloodstains up to three months old have been typed successfully using only small amounts of stain.

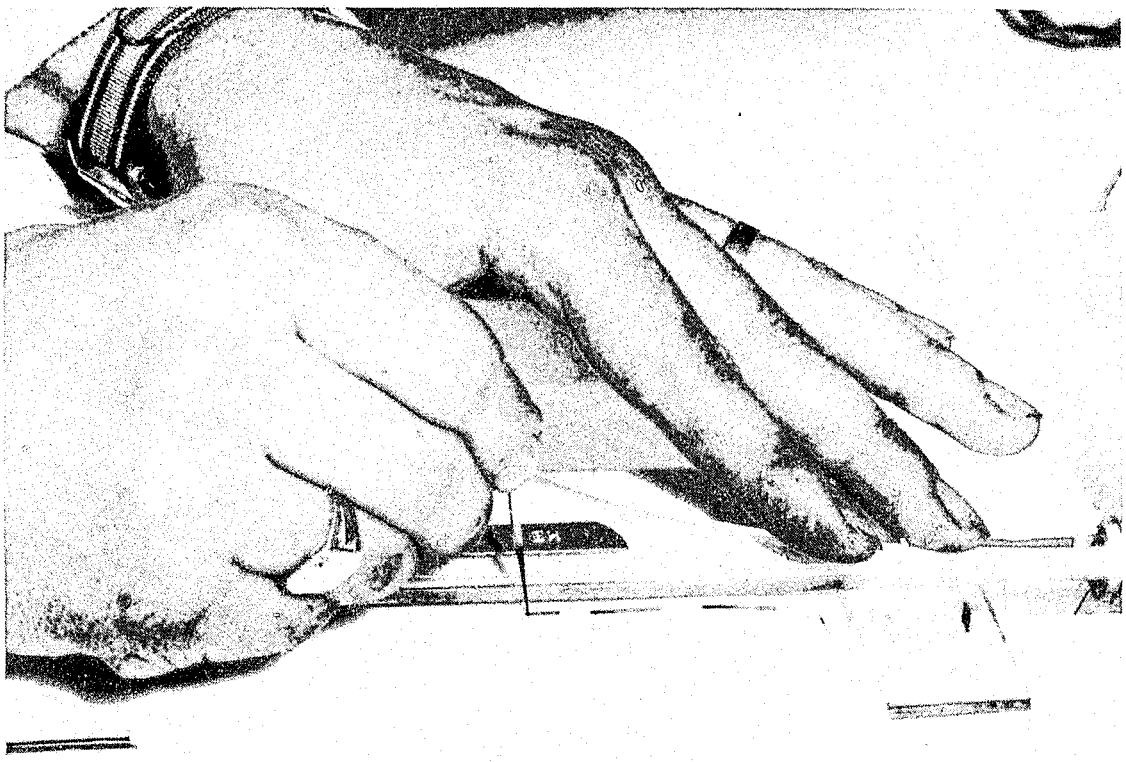

Figure 4-12. Applying the Hb Sample

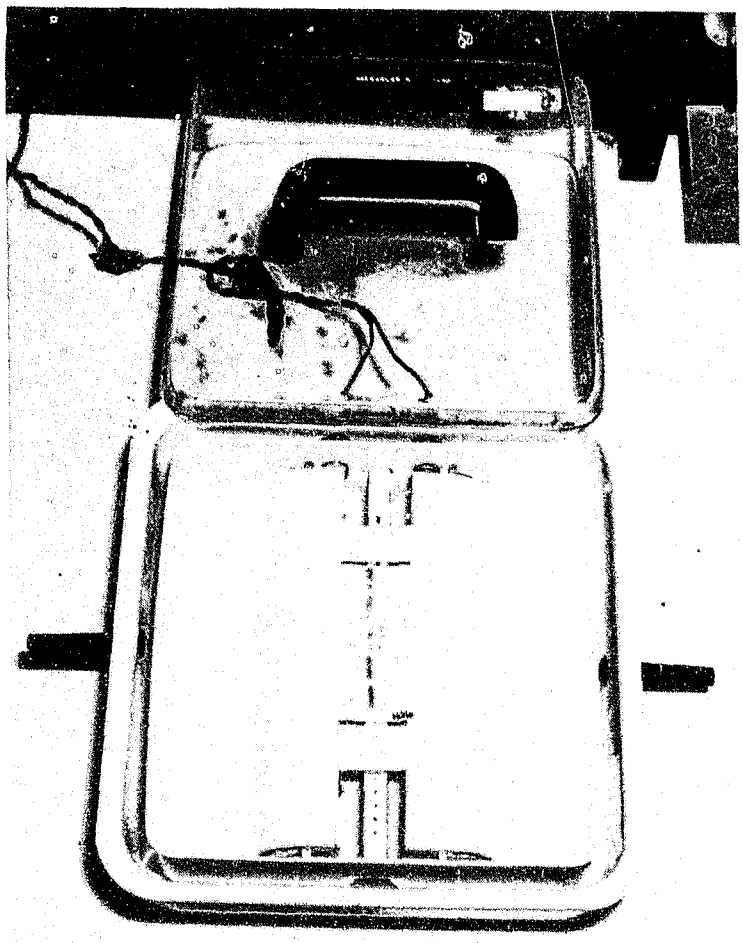

Figure 4-13. Hb Samples after Electrophoresis

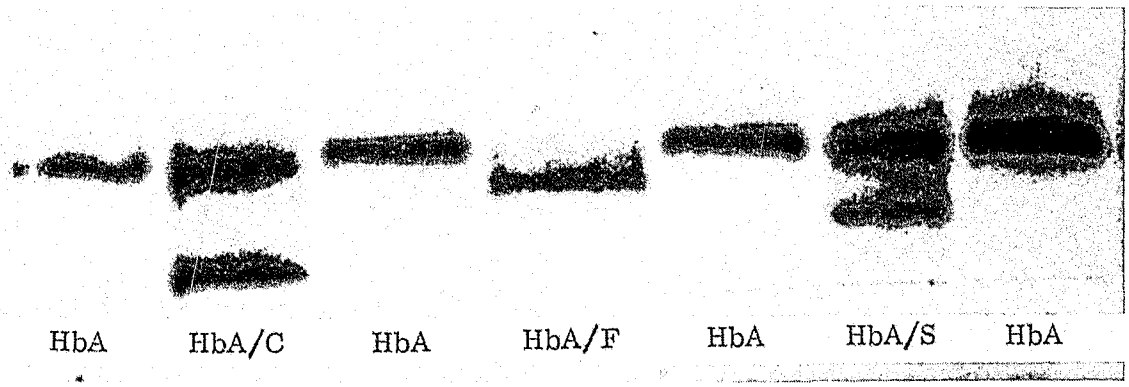

Figure 4-14. Hb Separations of A, A/C, A/F, and A/S

Unfortunately, using the buffer described, it is not possible to distinguish HbS from HbD and HbC from HbE. Lehmann and Huntsman[218] describe a method for separating these haemoglobins using a citrate buffer in agar gel. This method requires a current of 25 ma for 16 hours. At Metro Police, it was found to be unsatisfactory; however, using cellulose acetate with a more dilute strip buffer, a good separation of HbS from HbD was obtained in 15 minutes (Figure 4-15).

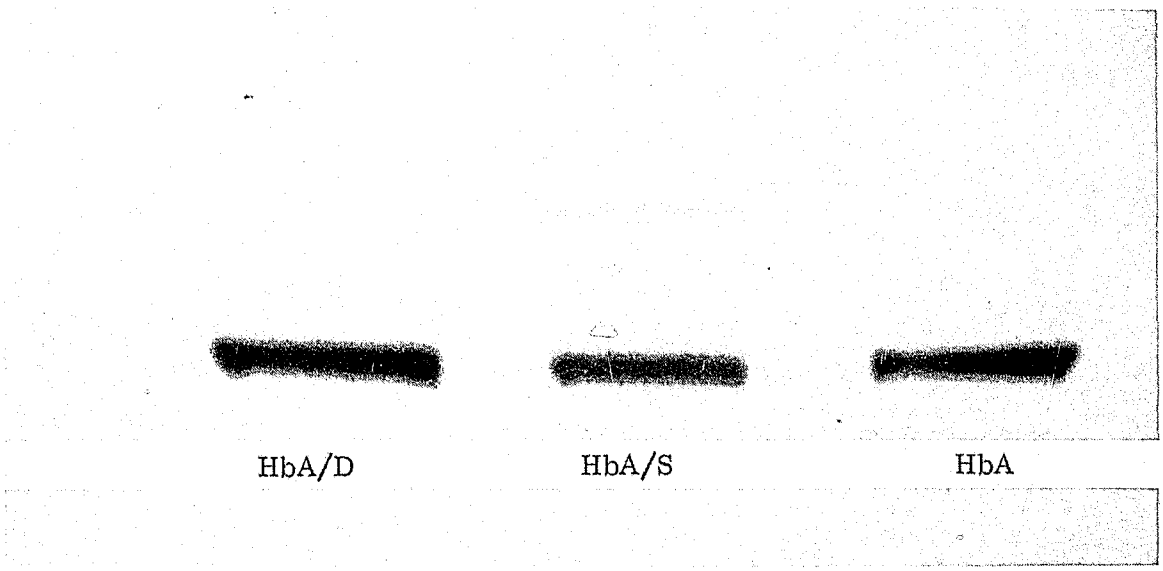

Figure 4-15. Hb Separations of A/D, A/S, and A

The buffers used are as follows:

a. Tank Buffer

Sodium citrate	73.5 g
Distilled water	500 ml

Add 34.0 ml of 0.5 M citric acid (10.5 g/100 ml) and make up to 1 litre with distilled water—pH 6.2.

b. Strip Buffer

Tank buffer diluted 1:20.

This buffer should also separate HbC from E.

Another method for separating HbS from D is by the sickling test although this method cannot be used for bloodstains. All suspected HbS or A/S individuals should be confirmed using this test.

4.2.6.4 Sickling Test

It is preferable to use clotted blood, a small drop of which is mixed with 2 to 3 drops of 2 percent sodium metabisulphite ($Na_2 S_2 O_5$). It is essential that the solution is freshly prepared and that the chemical has not become oxidized due to long storage. The experiment is carried out on a microscope slide, covered with a coverslip and incubated at 37°C in a moist chamber. After 20 minutes, it is examined under low power for sickling (Figure 4-16); however, if the blood sample is old, the sickling may not occur for at least three hours. Care should be taken not to allow the cells to dry out as intracellular haemoglobin crystallisation may be mistaken for sickling.

4.2.6.5 Alkali Denaturation

Since von Korber in 1866 described "the increased resistance of foetal haemoglobin to alkali", several different procedures have been described for the quantitative determination of haemoglobin F. Singer, et al[224], and Lehmann and Ager[221] have described methods for HbF identification in whole blood. Similarly, Huntsman and Lehmann[218] and Culliford[216] have described the identification of foetal haemoglobin in bloodstains using this technique.

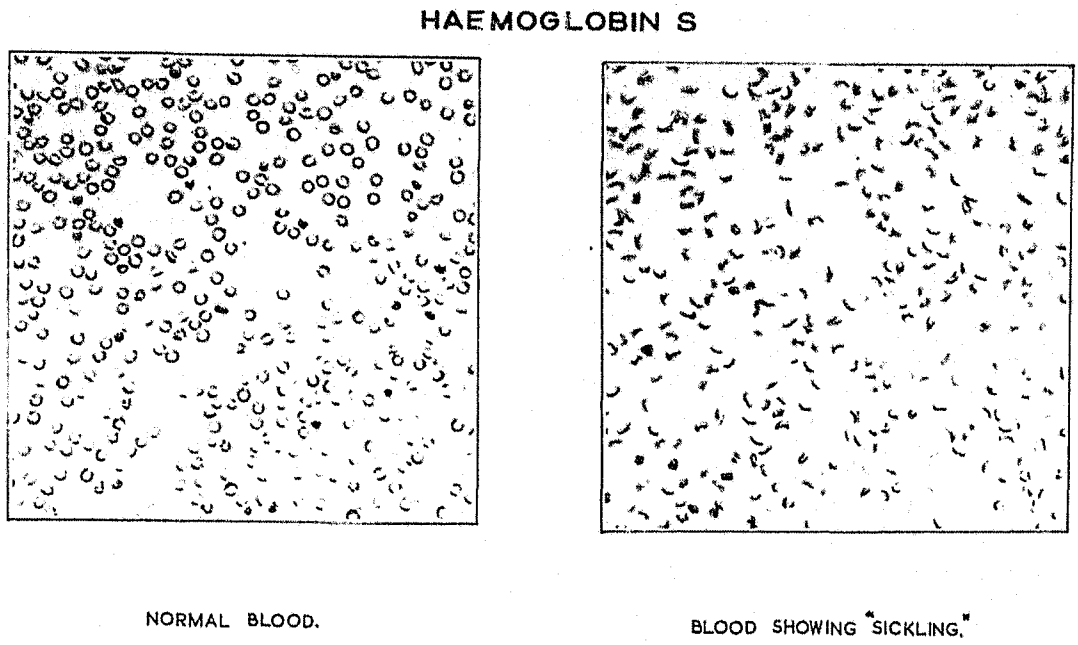

Figure 4-16. Sickling Test

This involves the measurement of the absorption curve in visible light before and after the denaturation of haemoglobin by alkali. When considering whole blood samples, a haemolysate is diluted with distilled water until a rose pink colour is obtained. A 1-ml aliquot of this solution is mixed with an equal volume of distilled water, and a spectrum absorption curve is prepared by scanning the solution between 450 and 600 mμ using water as a reference. The second aliquot is added to 1 ml of M/12.5 NaOH and the sample scanned with M/25 NaOH in the reference cell. The results are shown in Figure 4-17.

When dealing with bloodstains, a portion of the stain, approximately 5 by 2 mm (larger if on thin material), is cut out and extracted into 2 ml of distilled water. The solution is divided into 1 ml aliquots, and the procedure is carried out as above.

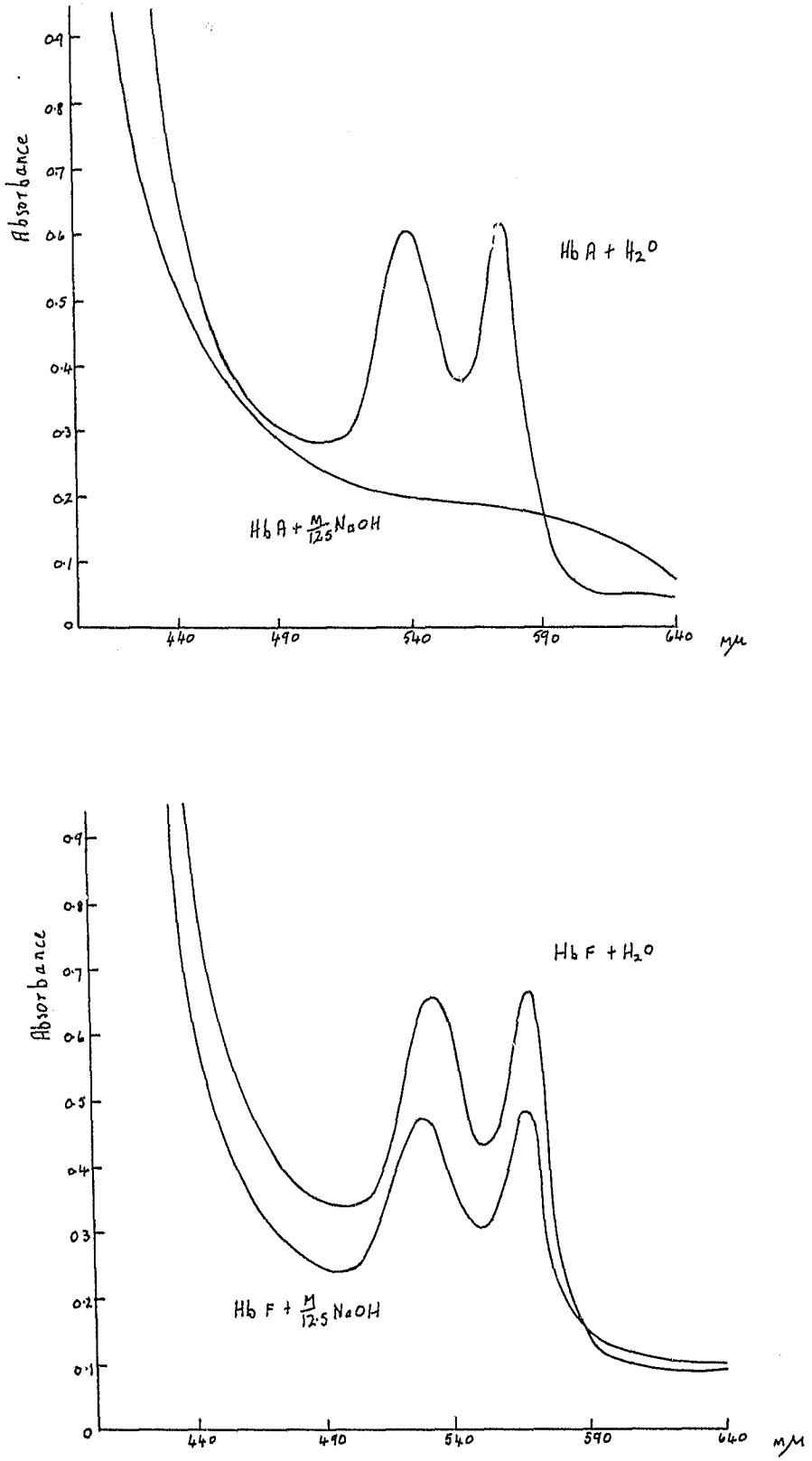

Figure 4-17. Alkali Denaturation Results

Problems with bloodstains do not arise until the stain is 4 to 5 days old, when, at approximately 500 mμ, a third peak appears. This is methaemoglobin, and interpretation becomes difficult when comparing the two absorption curves. Figure 4-18 shows traces from bloodstains of different ages.

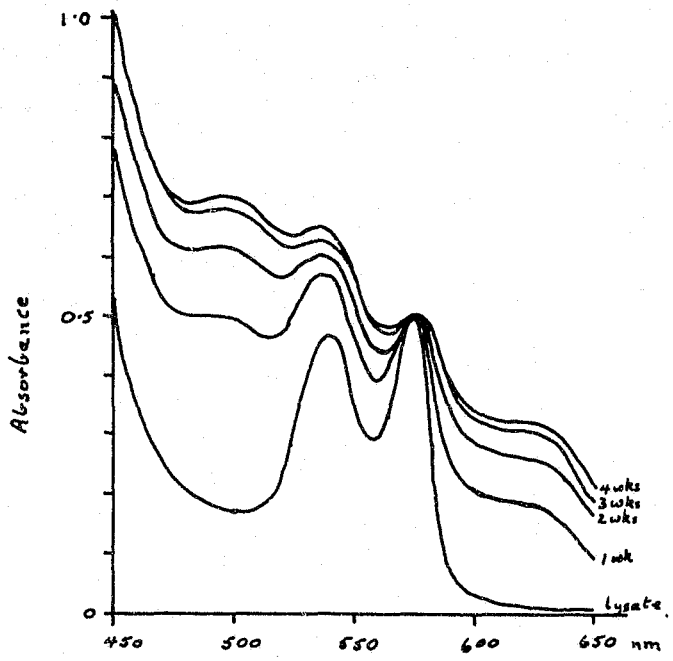

Figure 4-18. Absorption Curve of Stain 1, 2, 3, 4 Weeks Old

The addition of 2-mercaptoethanol removes the methaemoglobin but renders the HbA resistant to alkali denaturation. A further difficulty arises with old stains. The HbA resists the complete denaturation by NaOH and two small peaks appear on the trace at 538 mμ and 576 mμ; therefore, the solution must be allowed to stand for at least thirty minutes to allow complete denaturation to occur.

4.2.6.6 Peptide Mapping

This method should be used to positively identify any abnormal haemoglobin other than the common variants. It involves the partial hydrolysis of the globin; the resultant mixture of peptides is resolved into a two-dimensional pattern or peptide map using paper electrophoresis and chromatography. When the peptide maps of a normal and variant haemoglobin are compared, it is possible to

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distinguish a difference in structure to one amino acid residue. Unfortunately, the amount of sample required is quite large, and so far, no work has been carried out on bloodstains.

4.2.7 PROBLEMS

All blood samples received in the laboratory are routinely screened for haemoglobin variants by the electrophoresis procedure described. Any variants found are checked against known controls to positively identify the variant. These controls, after prolonged freezing and thawing, tend to produce a brown precipitate. This can be avoided by adding carbon tetrachloride as described.

Foetal haemoglobin can be confirmed using the spectrophotometer, but in bloodstains more than a week old caution should be exercised when using this technique.

Fluoride, as with other systems, tends to affect the electric field and therefore should be avoided.

Table 4-2

Sickle Cell Trait in Various Populations

Population	Number Tested	Sickle Cell Trait		Reference
		No.	Percent	
EUROPE				
Greece	11,285	1161	10.29	239
AFRICA				
Congo—Bantu	2,459	627	25.50	239
Rwanda Burundi—Hamite	3,798	298	7.84	239
Uganda, Kenya, Tanzania—Bantu	7,353	1209	16.44	241
Uganda, Kenya, Tanzania—Nilote	1,628	392	24.07	241
Uganda, Kenya, Tanzania—Hamite	883	93	10.05	241
Uganda, Kenya, Tanzania—Nilo-Hamite	919	12	0.13	241
Gabon	2,161	267	12.35	238,229
South Sudan—Nilote	964	56	5.82	236
—Nilo-Hamite	922	53	5.74	236
Zambia—Bantu	2,465	358	14.51	241
Portugese East Africa—Bantu	150	42	28.00	236
Tanzania, Malawi, Rhodesia, Portugese East Africa—Bantu	2,742	131	4.77	241
Botswana—Mixed	1,738	5	0.29	239
Southwest Africa—Bushmen	618	0	0.00	241
South Africa—Bantu	2,527	5	0.19	239
—Cape Coloured	1,555	9	0.58	234
ASIA				
Israel, Jordon—Arab	537	2	0.37	241
India—All Castes	32,141	1522	4.73	239
—Bengal	12,018	0	0.00	239
Ceylon	2,060	0	0.00	252
AMERICA				
USA—White	1,729	0	0.00	241
Central America, West Indies	1,595	154	9.66	245
Panama—Coloured	2,170	151	6.96	241
Curacao—Negro	2,499	266	10.68	243
Columbia—Coloured	489	46	9.41	240
Surinam—Creole	789	88	11.15	232
Brazil—Negro	1,138	118	10.38	241
—Coloured	678	47	6.93	241
—White	184	0	0.00	241
—Indian	670	0	0.00	244
AUSTRALASIA				
Queensland—Aborigine	574	0	0.00	239

Table 4-3

Hb Variants in Various Populations

Population	Number Tested	Hp Phenotypes (Percent)										Reference
			S	AS	SC	AC	C	AD	AK	AG	Others	
AFRICA												
Algeria	15,564	No. Obs.	13	67 0.43	11	65 0.42	7	39 0.25	3	-	AI5 AJ2	231
Cape Verde Island	1,682	No. Obs.	-	74 4.40	-	-	-	-	-	-	—	247
Senegal	1,385	No. Obs.	2	178 12.85	-	17 1.23	1	-	14 1.01	2	—	239
Gambia	1,663	No. Obs.	2	236 14.19	-	29 1.74	-	-	-	-	—	226
Liberia	2,453	No. Obs.	5	213 8.68	-	17 0.69	1	1	2	-	AN10	207
Ivory Coast	468	No. Obs.	6 1.28	33 7.05	-	13 2.77	-	-	-	-	—	239
Upper Volta	3,467	No. Obs.	13 0.37	200 5.77	22 0.63	594 17.13	51 1.47	-	27 0.77	3	—	242
Ghana	4,936	No. Obs.	7	726 14.71	46 0.93	598 12.11	40 0.81	-	3	1	—	239
Nigeria—Hausa	308	No. Obs.	-	63 20.45	-	4 1.29	-	-	-	-	—	239
Nigeria—Fulani	343	No. Obs.	1	64 18.65	-	3 0.87	-	-	-	-	—	233
Nigeria—Yoruba	16,019	No. Obs.	41 0.25	3704 23.12	96 0.60	792 4.94	31 0.19	-	-	-	SG1	239
Nigeria—Ibo	1,240	No. Obs.	-	287 23.14	-	6 0.48	-	-	-	1	—	239
Nigeria—Mixed	22,163	No. Obs.	81 0.36	5052 22.79	147 0.66	941 4.24	36 0.16	-	-	1	SG1	239
Chad	162	No. Obs.	-	20 12.26	-	-	-	-	-	-	—	230
Cameroun	1,621	No. Obs.	5	363 22.39	-	18 1.11	-	-	-	-	—	239
Sudan	2,230	No. Obs.	8	42 1.88	-	-	-	-	-	-	AO1	249
Sierra Leone	2,256	No. Obs.	647 28.67		-	10 0.44	-	-	-	-	—	239
Angola	3,885	No. Obs.	-	965 24.84	-	-	-	4	3	-	—	239

Table 4-3

Hb Variants in Various Populations (Continued)

Population	Number Tested	Hb Phenotypes (Percent)										Reference
			S	AS	SC	AC	C	AD	AK	AJ	Others	
AMERICA												
USA—Negroes	20,240	No. Obs.	129 0.63	1969 9.69	21 0.10	410 2.02	13	23	–	2	AG1, AL1 SJ1	239
Canada	13,450	No. Obs.	1	1	–	2	–	–	26 0.19	–	—	250
British Honduras	724	No. Obs.	–	164 22.65	1	18 2.48	–	–	–	–	—	235
Puerto Rico	1,102	No. Obs.	3	37 3.37	4	9 0.82	1	–	–	–	—	239
Jamaica	1,018	No. Obs.	2	106 10.41	2	30 2.94	–	–	–	–	—	251
St. Vincent	748	No. Obs.	–	65 8.70	–	20 2.67	–	–	–	–	—	227
Dominica	664	No. Obs.	–	63 9.48	–	10 1.50	–	–	–	–	—	227
Guadeloupe	1,000	No. Obs.	5	92 9.2	–	27 0.27	–	1	–	–	—	229
Martinique	324	No. Obs.	–	28 8.64	–	12 3.70	–	–	–	–	—	239
Barbados	913	No. Obs.	–	64 7.01	–	42 4.60	–	–	–	–	—	227
St. Lucia	825	No. Obs.	–	115 13.93	–	31 3.75	–	–	–	–	—	227
Trinidad	204	No. Obs.	–	19 9.31	–	6 2.94	–	–	–	–	A[illegible] AN-1	237
			S	AS	AD	AE	AJ	AK	AL	AQ	Others	
ASIA												
India	9,824	No. Obs.	20 0.20	350 3.56	16 0.16	49 0.50	3	9	3	4	E-2	239
Malaya—Malays	9,968	No. Obs.	–	–	7	452 4.53	2	8	–	–	E-7	248
Malaya—Chinese	10,031	No. Obs.	–	–	1	27 0.27	3	–	–	3	AG1	247
Malaya—Indians	6,647	No. Obs.	–	–	30 0.45	30 0.45	2	10	4	–	—	248

Table 4-4

Hb Variants in a Forensic Sample

Hb	M.P.L.	2,338		A	A/S	A/C	F
			No. Obs.	2286 97.73	30 1.28	13 0.55	9

SECTION 5

FUTURE OF BLOODSTAIN GROUPING SYSTEMS

5.1 INTRODUCTION

There are many polymorphisms of human blood which have not been dealt with in detail in the previous chapters. Only those which have been fully investigated in bloodstains and are now in routine use have been considered in depth. Many, however, have had some work done on them, and the results so far are listed below. When these investigations are complete, the full results will be published in the journals.

5.2 RED CELL ACID PHOSPHATASE

This, at first glance, is an excellent polymorphic system for forensic work. It was one of the first to be discovered and was the first to be investigated. It was found that when blood dried into a stain, it very rapidly lost its activity to the substrate, phenlophthalein phosphate. It was not until recently that it was decided to re-investigate this enzyme, when it was found that although phenolphthalein diphosphate was useless as a substrate, the enzyme remained active to other phosphate substrates after having dried into a bloodstain.

The most satisfactory of these substrates was 4-methyl-umbelliferyl-phosphate. Using this, it has been found possible to type stains satisfactorily up to a few weeks old. Based on present work, a confident prediction can be made that within a year, it will be possible to use red cell acid phosphatase as a routine enzyme typing method. Of the common phenotypes, the population is divided as follows:

Phenotype	A	BA	B	CA	CB
Percentage	11.2	47.7	33.5	2.5	5.2

5.3 PHOSPHO-GLUCOSE ISOMERASE

This enzyme has been found to be very resistant to drying into a bloodstain, and results are easy to obtain. The big disadvantage with this polymorphism is the

low frequency of the variants. About ten variants have been described which together form only about 1 percent of the population. This enzyme if treated on its own would head strongly towards uneconomic effort. It can, however, be combined with a number of other enzyme systems and be detected on the same starch gel plate as these. The most satisfactory combination so far is with G6PD. The development of PG1 on G6PD plates is described in paragraph 3.4.

5.4 PEPTIDASES

There are four major peptidases in the red cell, denominated A, B, C, and D according to their mobility and their substrate specificity. Each of these has variant forms or polymorphism associated with it. So far, it has been shown that the peptidases survive drying into a bloodstain and retain a good activity. A great deal more work is required, and it would seem probable that 12 to 18 months will be required to develop better techniques before peptidase typing can be routinely used. Some of the polypeptide substrates used are rather expensive at present, and with a fairly low level of variants it may be thought initially uneconomic of time and money—this remains to be seen.

5.5 SERUM AMYLASES

Work on these enzymes has proved to be very difficult, and erratic results at present seem commonplace. This is a system where no two workers seem to be in agreement, and at the present time with the difficulties which have been found, it holds little hope of use in forensic work.

5.6 SERUM ALKALINE PHOSPHATASE

This is another rather difficult system with which to work. It is partly a qualitative and partly a quantitative variation, dependent on factors other than genetic in its quantitative aspect. The activity of the enzyme in bloodstains has been found to be very low, and the results so far have been largely unsatisfactory. It is not a system for which there can be much future in forensic work.

5.7 OTHER ENZYMES

Other enzyme polymorphisms are also being investigated such as glutathione reductase, galactose-1-phosphate uridyl transferase, etc., but this work is at an early stage, and it is not possible yet to predict its potential usefulness in this field. Many other enzyme variations are known and documented, but the rarity of the variant forms makes the screening for these very uneconomic unless some blanket screening techniques can be developed.

5.8 Gc GROUPS

The polymorphic α_2 protein Gc has been investigated for use in bloodstain typing in Denmark, Germany, and England. So far no satisfactory technique has been developed for bloodstain work if the stains are more than a few days old. Stains in good condition and fairly new state can be typed by the method described originally by Hirschfeld or by the technique described in paragraph 4.1 on haptoglobins for typing by immuno-electrophoresis. Reliability for any but the best of bloodstains is at present suspect.

5.9 TRANSFERRINS

Due to the low frequency of transferrin variants in European populations, no hard effort has been put into this polymorphism. There should be no reason why this should not be possible if the effort required is thought to be justified. Immune techniques appear to be useless. Starch or acrylamide gel possibly using a radioactive detection method seems theoretically the best chance.

5.10 OTHER POLYMORPHIC PROTEINS

Either rarity of variants or lack of work on some of the more recently discovered polymorphisms has so far led to insufficient information to be able to judge the value of these polymorphisms.

5.11 Gm AND Inv SERUM GROUPS

The complexity of the Gm system is such that, if it was possible to carry out full genotyping on dried bloodstains, it would be more useful than any single system. In practise, the quality of many of the antisera is inadequate for use in

stain grouping. Gm(1) and Gm(2) can be easily detected by absorption-inhibition, and this is routine in some European countries. Preliminary work in the Metropolitan Police Laboratory has shown that it is also possible to detect Inv types in dried bloodstains.

5.12 OTHER RED CELL ANTIGEN SYSTEMS

It is highly likely that, in time, given adequate quantities of bloodstain and good quality antisera, it will be possible to detect most, if not all of these in dried bloodstains.

SECTION 6

REFERENCES

1. Bier, M., Electrophoresis, Theory, Methods and Applications, Academic Press, Vol. 1, 1959, Vol. 11, 1967.

2. Grabar, P. and Burtin, P., Immuno-electrophoretic Analysis, Applications to Human Biological Fluids, Elesevier Publishing Co., 1964.

3. Gordon, A. H., Electrophoresis of Proteins in Polyacrylamide and Starch Gels, North Holland Publishing Co., 1969.

4. Nerenberg, S. T., Electrophoresis, A Practical Laboratory Manual, F. A. Davies Co., Philadelphia 1966.

5. Van Deen, "Tinctura guajaci und ein ozontrager, ais reagens aus sehr geringe blut mengen," Arch. sur die Hollandische bei trage zur Nature. Herklunde (Utrecht) 1861, Vol. 3 p. 228 - 231.

6. Adler, O. and Adler, R., Zeitschr. F. Physill, Chimie, 1904, 41: 59.

7. Glaister, J., A Textbook of Medical Jurisprudence and Toxicology, 5th ed. 1931.

8. Hunt, A. C., Corby, C., Dodd, B.C. and Camps, F., J. For. Med., 1960, 7: No. 2.

9. Gradwohl, R.B.M. (Editor), Legal Medicine, 1954.

10. Gonzales, T. A. Vance, M., Helpern, M. and Umberger, C. J., Legal Medicine, Pathology and Toxicology, 2nd ed. 1954.

11. Smith, S., Taylors Principles and Practice of Medical Jurisprudence, 10th ed., 1948.

12. Nickolls, L. C., Scientific Investigation of Crime, 1956.

13. Fiori, A., Minerva medicolegale e arch. antropol. criminale, 1957, 77: 84.

14. Hirschfield, J., Acta p. Pathol. Microbiol. Scand., 1959, 47: 160, (also in Science Tools, 1960, 7: No. 2).

15. Coombs, R. R. A. and Dodd, Barbara E., Med. Sci. Law, 1961, 1: 357.

16. Douglas, R. and Staveley, J. M., J. Forens. Sci., 1969, 14: 255.

17. Lincoln, P. J. and Dodd, Barbara E., Med. Sci. Law, 1968, 4: 288.

18. Fiori, A., Marigo, M. and Benciolini, P., J. For. Sci., 1963, 8: 419.

19. Kind, S. S., Nature Lond., 1960, 185: 397.

20. Kind, S. S., Nature Lond., 1960, 187: 789.

21. Kind, S. S. and Cleevely, Rosalyn H., J. For Sci. Soc., 1969, 9: 131.

22. Hayward, J. W., J. For Sci. Soc., 1969, 9: 147.

23. Howard, H. D. and Martin, P. D., J. For. Sci. Soc., 1969, 9: 28.

24. Nickolls, L. C. and Pereira, Margaret, Med. Sci. Law, 1962, 2: 172.

25. Outteridge, R. A., Nature Lond., 1962, 194: 385.

26. Outteridge, R. A., J. For Sci. Soc., 1965, 5: 22.

27. Pereira, M., to be published later.

28. Poon, W. L. and Dodd, Barbara, Med. Sci. Law, 1964, 4: 258.

29. Yada, S., Japan J. Leg. Med., 1962, 16: 290.

30. Allen, F. H., Corcoran, P. A. and Ellis, F. R., Vox Sangirirus, 1960, 5 : 224.

31. Hirsch, W., Moores, P., Sanger, R. and Race R. R., J. Haemet, 1957, 3: 134.

32. Pereira, Margaret, Med. Sci. Law, 1963, 3: 268.

33. Uhlenbruk, G., Proc. 10th Congr. Int. Soc. Blood Transf., Stockholm, 476, 1965.

34. Bargagna M. and Pereira, Margaret, J. For. Sci., 1967, 7: 123.

35. Goldsmith, K., Lancet, 1955, 1: 76.

36. Marsh, W. L., Automation in Analytical Chem. Technicon Symposium, 1967, 2: 47.

37. Pereira, M., Dodd, B. E., and Marchant, J. V., Med. Sci. Law, 1969, 9: 116.

38. Bocchini, V., Ahoto, M. A., and Najjar, V., Biochemistry, 1967, 6: 313.

39. Cori, G. T., Colowick, S. P., and Cori, C.F., J. Biol. Chem., 1938, 123: 375.

40. Culliford, B. J., J. Forensic Science Soc., 1967, 7: 131.

41. Culliford, B. J., 5th Internation Meeting in Forensic Sciences, Toronto, 1969, (printed in International J. Legal Medicine Vol. 4).

42. Culliford, B. J., Unpublished Data.

43. Dawson, D. M., and Mitchell, A., Biochemistry, 1969, 8: 609.

44. Giblett, E. R., Genetic Markers in Human Blood, Blackwell Scientific Publishers, 1969.

45. Gounaris, A. D., Horton, H. R., and Koshland, D. E., Biochem. Biophys. Acta, 1967, 132: 41.

46. Harris, H., Hopkinson, D. A., Luffman, J. E., and Rapley, S., City of Hope Symposium Series, Vol. 1, Grune and Strutton, 1968.

47. Hopkinson, D. A., and Harris, H., Nature, 1965, 208: 410.

48. Hopkinson, D. A., and Harris, H., Ann. Hum. Genet, 1966, 30: 167.

49. Hopkinson, D. A. Personal Communication.

50. Joshi, J. G., Kuwaki, T., Swanson, J. R., Sukaradh, T., and Handler, P., Proc. Natn. Acad. Sci., 1967, 57: 1482.

51. Lie-Injo Luan Eng, Nature, 1966, 210: 1183.

52. Monn, E., Acta. Genet., 1968.

53. Monn, E., Vox. Sang., 14: 70.

54. Najjar, V. A., in Methods in Enzymology, Vol. 1, Academic Press, 1955.

55. Najjar, V. A., in Mechanism of Enzyme Action, 1954.

56. Najjar, V. A. and Pullman, M.E., Science, 1954, 119: 631.

57. Passoneau, J. V., Lowry, O. H., Schulz, D. W., and Brown, J. G., J. Biol. Chem., 1969, 244: 4, 902.

58. Smithies, O., Biochem. J., 1955, 61: 629.

59. Spencer, N., Hopkinson, D. A., and Harris, H., Nature, 1964, 204: 742.

60. Stedman, R., J. Forensic Science Soc., 1970.

61. Sutherland, E. W., J. Biol. Chem., 1949, 180: 1279.

62. Wraxall, B. and Culliford, B.J., J. Forensic Science Soc., 1968, 8: 81.

63. Bajatzadeh, M., Walter, H., and Palsson, J., Humangenetik, 1969, 7: 353.

64. Brewer, G. J., Bowbeer, D. R., and Tashian, R. E., Acta Genet., 1967, 17: 97.

65. Harvey, R. G., Godber, M. J., Kopec, A. C., Mourant, A. E., and Tills, D., Hum. Biol., 1969, 141: 342.

66. Hopkinson, D. A. and Harris, H., Ann. Hum. Genet., 1966 30: 167.

67. Lie-Injo, L. E., Lopez, C. G., and Poey-Oey, H. G., Am. J. Hum. Genet., 1968, 20: 101.

68. Modiano, G., Scozzari, R., Gigliani, F., Filippi, G., and Latte, B., Rend. Acc. Nat. Lincei., 1967, 42: 906.

69. Monn, E., Hum. Hered., 1969, 19: 274.

70. Mourant, A. E. and Tills, D., Nature, 1967, 214: 810.

71. Shinoda, T., Natn. Inst. Genet., Japan, Ann. Rpt., 1968, 19: 89.

72. Wille, B., Schmidt, E. and Ritter, H., Humangenetik, 1968, 5: 271.

73. Hummel, K., Pulverer, G., Schaal, K., and Weidtman, V., Humangenetik, 1970, 8: 330.

74. Bockelmann, W. and Ritter, H., Humangenetik, 1968, 6: 373.

75. Bowen, W. I. and Kerwin, T. D., Arch. Biochem. Biophys., 1954, 49: 149.

76. Bowman, J. E., Frischer, H., Ajmar, F., Carson, P. E., and Gower, M. K., Nature, 1967, 214: 1156.

77. Cerletti, P. and De Ritis, G., Clin. Chim. Acta, 1962, 7: 402.

78. Colowick, S. P. and Kalclar, H. M., J. Biol. Chem., 1943, 148: 117.

79. Colowick, S. P. and Kaplan, N. O., in Methods in Enzymology, Vol. 11, 1955.

80. Culliford, B. J. and Wraxall, B. G. D., J. Forensic Science Soc., 1968, 8: No. 2.

81. Fildes, R. A. and Harris, H., Nature, 1966, 209: 261.

82. Kashket, S. and Denstedt, O. F., Canad. J. Biochem. Physiol., 1958, 36: 1057.

83. Rapley, S., Robson, E. B., Harris, H., and Smith, S. M., Ann. Hum. Genet., 1967, 31: 237.

84. Slater, E. C., Nature, 1950, 166: 982.

85. Westkamp, L. R., Sing, C. F. Schneffler, D. C., and Guttarmsen, S. A., Ann. Hum. Genet., 1969, 21: 600.

86. Ananthakrishnan, R. and Kirk, R. L., Ind. J. Med. Res., 1969, 57: 1011.

87. Bowman, J. E and Ronaghy, H., Am. J. Phys. Anthrop., 1967, 27: 119.

88. Gordon, H., Vooijs, M., and Keraan, M. M., S. Afr. Med. J., 1966, 40: 1031.

89. Modiano, G., Scozzari, R., Gigliani, F., Filippi, G., and Latte, B., Rend. Acc. Naz. Lincei, 1967, 42: 906.

90. Modiano, G., Scozzari, R., Gigliani, F., and Santolamazza, C., Humangenetik, 1969, 8: 253.

91. Radam, G. and Strauch, H., Humangenetik, 1968, 6: 90.

92. Shih, Ling-Yu, Hsia, D., Bowman, J. E., Shih, Shu-Chuang, and Shih, Ping-Long., Am. J. Hum. Genet., 1968, 20: 474.

93. Willie, B. and Ritter, H., Humangenetik, 1968, 5: 278.

94. Blake, N. M. and Kirk, R. L., Nature, 1969, 221: 278.

95. Bowman, J. E., Carson, P. E., Frischer, H., and de Garay, A., Nature, 1966, 210: 811.

96. Brewer, G. J. and Dern, R. J., Am. J. Human Genet, 1964: 16: 472.

97. Carson, P. E., Ajmar, F., Hashimoto, F., and Bowman, J. E., Nature, 1966, 210: 813.

98. Carter, N. D., Fildes, R. A., Fitch, L. I., and Parr, C. W., Acta. Genet. Basel, 1968, 18: 109.

99. Colowick and Kaplan, Methods in Enzymology.

100. Culliford, B. J., 5th International Conference in Forensic Science, Toronto, 1969.

101. Davidson, R. G., Am. Human Genet, 1967, 30: 355.

102. Fildes, R. A. and Parr, C. W., Proc. Sixth Int. Cong. Biochem., New York, 229.

103. Fildes, R. A. and Parr, C. W., Nature, 1963, 200: 890.

104. Fitch, L. I. and Parr, C. W., Biochem. J., 1966, 99: 20p.

105. Gibbs, D. F., J. Clin. Path., 1968, 21: 189.

106. Kazazia, H. H., Nature, 1966, 212: 197.

107. Khan, P. M. and Rattazzi, M. C., Biochem. Genetics, 1968, 2: 231.

108. Negelein, E. and Haas, E., Biochem. Z., 1935, 282: 206.

109. Parr, C. W., Nature, 1966, 210: 487.

110. Parr, C. W. Personal communication.

111. Parr, C. W., 1968, Personal communication, (Hanshaw, E. and Parr, C. W.), Trans. R. S. Trop. Med. Hyg., 1969, 63: 119.

112. Parr, C. W. and Fitch, L. I., Biochem. J., 1964, 93: 28c.

113. Parr, C. W. and Fitch, L. I., Ann. Hum. Genet., 1967, 30: 339.

114. Parr, C. W. and Parr, I.B., Biochem. J., 1965, 95: 16p.

115. Peebles, E. E., Barnett, D. R., and Oliver, C. P., J. Heredity, 1967, 58: 243.

116. Tuchinda, S., Rucknagel, D. L., Na-Nakorn, S., and Wasi, P., Biochemical Genetics, 2: 253.

117. Carter, N. D., Gould, S. R., Parr, C. W., and Walter, P. H., Biochem J., 1966, 99: 17p-18p.

118. Carson, P. E., Ajmar, F., Hashimoto, F., and Bowman, J. E., Nature, 1966, 210: 814.

119. Chung, A. E. and Langdon, R. G., J. Biol. Chemistry, 1963, 238: 2309.

120. Detter et al., Ann. Hum. Genet., 1968, 31: p. 329.

121. Giblett, E. R., Genetic Markers in Human Blood, Blackwell Scientific Publications, 1969.

122. Harris, H., The Principles of Human Biochemical Genetics, North-Holland Publishing Co., 1970.

123. Kirkman, H. N. and Hendrikson, E. M., J. Biol. Chemistry, 1962, J. Biol. Chemistry, 237: 2371.

124. Kirkman, H. N. and Hendrikson, E. M., Amer. J. Hum. Genet., 1963, 15: 241.

125. Lehmann, H. and Huntsman, R. G., Man's Haemoglobins, North-Holland Publishing Co., Amsterdam, 1966.

126. Rattazzi, M. C. Bernini, L. F., Fiorelli, G., and Manucci, P. M., Nature, 1969, 213: 79.

127. Sparks, R. S., Baluda, M. C., and Townsend, D. E., J. Lab. Clin. Med., 1969, 73: 531.

128. Tan, I. K. and Whitehead, T. P., Clinical Chemistry, 1969, 15: 467.

129. World Health Organization, Technical Report Series No. 366, Standardization of Procedures for the Study of Glucose-6-Phosphate Dehydrogenase, 1967.

130. Yoshida, A., J. Biol. Chem., 1966, 241: 4966.

131. Yoshida, A., Biochem. Genet., 1967, 1: 81.

132. Zinkham, W. H., Lenhard, R. E., and Childs, B., Bull. Johns Hopkins Hosp., 1958, 102: 169.

133. Balinsky, D., and Jenkins, T., S. Afr. J. Med. Sci., 1966, 31: 124.

134. Porter, J. H., Boyer, S. H., Watson-Williams, G. H., Adam, A., Szeinberg, A., and Siniscalo, M., Lancet, 1964, 1: 895.

135. Reys, L., Manso, C., and Stamatoyannopoulos, G., Amer. J. Hum. Genet.,1970, 22: 203.

136. Yoshida, A., Stamatoyannopoulos, G., and Motulsky, A., Science, 1967, 155: 97.

137. Dissing, J. and Knudsen, J. B., Human Heredity, 1969, 19: 375.

138. Fielgelson, P. and Davidson, J., J. Biol. Chemistry, 1956, 223: 65.

139. Hopkinson, D. A., Cook, P. J. L., and Harris, H., Ann. Hum. Genet., 1969, 32: 361.

140. Kalckar, H. M., J. Biol. Chemistry, 1947, 167: 445.

141. Spencer, N., Hopkinson, D. A., and Harris, H., Ann. Hum. Genet., 1968, 32: 9.

142. Stedman, R., J. Forensic Sci. Soc., 1971.

451-769 O - 72 - 17

143. Tariwerdian, G. and Ritter, H., Humangenetik, 1969, 7: 176.

144. Callaghan and La Du, Fedn. Proc. Fedn. Am. Socs. Exp. Biol., 1965 24: 610.

145. Culliford, B. J., Nature, 1963, 198: 797.

146. Harris, H., Hopkinson, D. A., Robson, E., and Whittaker, M., Ann. Hum. Genet., 1963, 26: 359.

147. Harris, H. and Robson, E., Lancet, 1963, (i): 218.

148. Harris, H. and Whittaker, M., Nature, 1961, 191: 496.

149. Harris, H. and Whittaker, M., Ann Hum. Genet., 1963, 27: 53.

150. Kalow, Lancet, 1956, (ii): 576.

151. Kalow and Genest, K., Can. J. Biochem. Physiol., 1957, 35: 339.

152. Kalow and Lindsay, Can. J. Biochem. Physiol., 1955, 33: 568.

153. Lehmann, H. and Davies, Med. Sc. and Law, 1962, 2: No. 3.

154. Liddell, Lehmann, and Silk, Nature, 1962, 193: 561.

155. Parkin, B. H., M. Sc. Thesis, 1968.

156. Parkin, B. H., Biochem. J. to be published.

157. Parkin, B. H., Ph.D Thesis.

158. Robson, E. and Harris, H., Ann. Hum. Genet., 1966, 29: 403.

159. Whittaker, M., Acta Genet., 1968, 18: 325-340.

160. Altland, K., Epple, F., and Goedde, H. W., Human Genetik, 1967, 4: 127.

161. Ashton, C. C. and Simpson, N. E., Am. J. Hum. Genet., 1966, 18: 438.

162. Bajatzadeh, M., Neumann, S., and Walter, H., Humangenetik, 1969 7: 91.

163. Fraser, G. R., Stamatoyannopoulos, G., and Kattamis, C., Ann. N.Y. Acad. Sci., 1964, 119: 415.

164. Fraser, G. R., Grunwald, P., Kitchin, F. D., and Steinberg, A. G., Hum. Hered., 1969, 19: 57.

165. Horsfall, W. R., Lehmann, H., and Davies, D., Nature, 1963, 199: 1115.

166. Kalow, W. and Gunn, Dr. R., Ann. Hum. Genet., 1959, 23: 239.

167. Kattamis, C., Zannos-Mariolea, L. Franco, A. P. Liddell, J., Lehmann, H., and Davies, D., Nature, 1962, 196: 599.

168. Lisker, R., Del Morel, C., and Loria, A., Nature, 1964, 202: 815.

169. Motulsky, A. G. and Morrow, A. C., Science, 1968, 159: 202.

170. Neumann, S. and Walter H., Nature, 1968, 219: 950.

171. Robson, E. B. and Harris, H., Ann. Hum. Genet., 1966, 29: 403.

172. Szeinberg, A., Pipano, S., and Ostfeld, E., Acta Anaesth, Scand., Suppl., 1966, 24: 199.

173. Whittaker, M., Acta Genet., Basel, 1968, 18: 567.

174. Allison, A. C., Develop. Med. Child. Neurol., 1963, 5: 51.

175. Connell, G. E., Dixon, G. H., and Smithies, O., Nature, 1962, 193: 505.

176. Culliford, B. J., Nature, 1966, 211: 872.

177. Kirk.

178. Nance, W. E., and Smithies, O., Nature, 1963, 198: 869.

179. Poulik, M. D., Nature, 1957, 180: 1477.

180. Smithies, O., Nature, 1955, 175: 307.

181. Sutton, H. E. and Karp, G. W., Amer. J. Hum. Genet., 1964, 16: 419.

182. Allbrook, D., Barnicott, N.A., Dance, N., Lawler, S. D., Marshall, R., and Mungai, J., Hum. Biol., 1965, 37: 217.

183. Allison, A. C., Blumberg, B. S., and Ap Rees, W., Nature, 1958, 181: 824.

184. Allison, A. C., and Barnicott, N. A., Acta Genet., Basel, 1960, 10: 17.

185. Angelopoulos, B., Tsoukantas, A., and Danopoulos, E., J. Med. Genet., 1966, 3: 276.

186. Baitsch H., Liebrich, K. G., Pinkerton, F. J., and Mermod, L. E., Acta. Genet. Med. Gemell, 11: 308.

187. Bajatzadeh, M. and Walter, H., Hum. Hered., 1969, 19: 514.

188. Barnicott, N. A., Garlick, J. P., and Roberts, D. F., Ann. Hum. Genet, 1960, 24: 171.

189. Barros, F., IXth Congr. Soc. Europ. Haematol., Lisbon, 1963, 26.

190. Baxi, A. J. and Cameons, H., Hum Hered., 1969, 19: 65.

191. Blackwell, R. Q., Lin, T-Y, and Shiao, D. D. F., Nature, 1962, 193: 284.

192. Blumberg, B. S. and Gentile, Z., Nature, 1961, 189: 897.

193. Butler, R., Metaxas-Buhler, M., Rosin, S., and Wandrey, R., Schweiz, Med. Wschr., 1959, 89: 1041.

194. Fleischer, E. A. and Mohr, J., Acta Genet., Basel, 1962, 12: 281.

195. Fraser, G. R., Giblett, E. R., Lee, T-C, and Motulsky, A. G., J. Med. Genet., 1965, 2: 21.

196. Fried, K., Bloch, N., Sutton, E., Neel, J. V., Bayani-Sioson, P., Ramot, B., and Duvdevani, P., in Genetics of Migrant and Isolate Populations, 1963, edited by Goldschmidt, E., Williams and Wilkins, Baltimore, 266.

197. Galatius-Jensen, F., Acta Genet., Basel, 1958, 8: 232.

198. Giblett, E. R. and Brooks, L. e., Nature, 197: 576.

199. Gordon, H., Robertson, M., Blair, J. M., and Vooijs, M., S. Afr. Med. J., 1964, 38: 734.

200. Harris, H., Robson, E. B., and Siniscalco, M., in Biochemistry of Human Genetics, 1959, edited by Wolstenholme, G. E. W. and O'Connor, C. M., Churchill, London, 151.

201. Hashem, N., Kamel, K., and Hammond, E. I., J. Med. Genet., 1966, 3: 279.

202. Kirk, R. L. and Lai, Y. C., Acta Genet, Basel, 1961, 11: 97.

203. Kirk, R. L., The Haptoglobin Groups in Man, Karger, Basel, 1968.

204. Makela, O., Eriksson, A. W., and Lehtovarra, R., Acta Genet., Basel, 1959, 9: 149.

205. Matsumoto, H., Jap. J. Leg. Med., 1964, 18: 380.

206. Moullec, J., Fine, J. M., and Linhard, J., Rev. Haemotol., 1960, 15: 174.

207. Neel, J. V., Robinson, A. R., Zuelzer, W. W., Livingston, F. B., and Sutton, H. E., Am. J. Hum. Genet., 1961, 13: 262.

208. Plato, C. C., Rucknagel, D. L., and Gershowitz, H., Am. J. Hum. Genet, 1964, 16: 267.

209. Planas, J., Fuste, M., Vinas, J., and Irizar, J. L., Acta Genet, Basel, 1966, 16: 371.

210. Shim, B-S. and Bearn, A. G., Am. J. Hum. Genet., 1964, 16: 477.

211. Spendini, G., Riv., 1961, 68: 145.

212. Spendini, G., Acta. Genet. Med. Gemell., 1964, 13: 388.

213. Torrinha, J. A. F., Acta Genet.,Basel, 1967, 17: 74.

214. Van Ros, C., Van Sande, M., and Druet, R., Ann. Soc. Belge. Med. Trop., 1963, 43: 511.

215. Breen, M., Weinstein, H. G., and Schap, F. D., Biochem. Med., 1968, 2: 35-40.

216. Culliford, B. J., J. For. Sci. Soc., 1964, Vol. 4, No. 3.

217. Graham, J. L. and Grunbaum, B. W., Amer. J. Clin. Path., 1963 Vol. 39, No. 6, 567-78.

218. Huntsman, R. G. and Lehmann, H., Med. Sci. and Law, 1963, 3: 59.

219. Kohn, J., J. Clin. Path., 1969, 22: 109-10.

220. Lehmann, H. and Huntsman, R. G., "Mans Haemoglobins", North-Holland Publishing Company, Amsterdam, 1966.

221. Lehmann, H. and Ager, J., Ass. of Clin. Path. Broadsheet No. 33, 1961.

222. Perutz, M. F., Scientific American, Nov. 1964.

223. Perutz, M. F. and Lehmann H., Nature, 1968, 219: 902-9.

224. Singer, K., Chernoff, A. I., and Singer, L., Blood, 1951, 6: 413-428.

225. Wraxall, B. G. D., J. For. Sci. Soc., 1971, to be published.

226. Allison, A. C., Ann. Hum. Genet., 1956, 21: 67.

227. Allison, A. C., Cold Spring Harbor Symposia on Quantitative Biol. 1964, 29: 137.

228. Andre, L. and Andre-Gadras, E., Med. Trop., 1957, 17: 596.

229. Bideau, J. and Courmes, E., J. Med. Bordeaux, 1965, 142: 961.

230. Cabannes, R. and Ruffie, J., C. R. Soc. Biol., Paris, 1961, 155: 2449.

231. Cabannes, R., in Abnormal Haemoglobins in Africa, 1965, edited by Jonxis, J. H. P., Davis Philadelphia.

232. Collier, W. A. and Prarra, D. A. de la, Docum. Med. Geogr. Trop., 1952, 4: 221.

233. Edington, G. M., Ann. Rpt. Dept. of Path. Univ. Coll. Ibadan, 1959.

234. Esrachowitz, S. R., Friedlander, S., Radloff, G., and Saunders, S., S. Afr. Med. J., 1952, 26: 239.

235. Firschen, I. L., Am. J. Hum. Genet., 1961, 13: 233.

236. Foy, H., Kondi, A., Timms, G. L., Brass, G. W., and Bushra, F., Brit. Med. J., 1954, 1: 294.

237. Henry, M. U., Carib. Med. J., 1963, 25: 26.

238. Lalouel, J.F. B., Bull. Soc. Anthrop., Paris Ser. X, 1955, 6: 129.

239. Livingstone, F. B., Abnormal Haemoglobins in Human Populations, Aldine, Chicago, 1967.

240. Mera, B., Bol. Ofic. Sanit. Pan-Amer., 1943, 22: 680.

241. Mourant, A. E., The Distribution of the Human Blood Groups, Blackwell, Oxford, 1954.

242. Sansarricq, H., Marill, H., Portier, A., and Cabannes, R., Sang, 1959, 30: 503.

243. Sar, A. van der, Ned. Tijdschr. Geneesk., 1949, 93: 1867.

244. Silva, E. M. da, Mem. Inst. Osw. Cruz., 1948, 46: 125.

245. Tomlinson, W. J., Am. J. Med. Sci., 1945, 209: 181.

246. Trincao, C. and Martini de melo, J., Israel J. Med. Sci., 1965, 1: 772.

247. Vella, F., Acta Haematol, 1960, 23: 393.

248. Vella, F., Oceania, 1962, 32: 219.

249. Vella, F., in Abnormal Haemoglobins in Africa, Edited by Jonxis, J. H.P., Davis, Philadelphia, 1965, 339.

250. Vella, F., cited by Livingstone, F. B., 1966, (14).

251. Went, L. N., Nature, 1957, 180: 1131.

252. Wickremasinghe, R. L. and Ponnuswamy, N. E. L., Spol. Zeyland, 1963, 30: 149.

APPENDIX A

SAMPLE EQUIPMENT AND LIST OF CHEMICALS AND ANTISERA

After testing many makes of electrophoresis apparatus, the Shandon equipment was selected because it provides an almost pure direct current output. Tests on the output of almost all other power supplies measured on an oscilloscope demonstrated that the output was not filtered as well as the output of the Shandon power supply. If a person does not wish to employ cooling platens which must be provided with a circulating water pump such as RTE 3 Circulator, manufactured by Neslab Instruments Inc., Portsmouth, N.H. or any other make, it is possible to run the equipment where cooling is necessary inside a common refrigerator. The power supply is kept on top of the refrigerator, the connecting wires are thin enough to fit under the rubber seal of the refrigerator without difficulty.

Figure A-1 is used for precipitin tests. Figure A-2 illustrates the electrophoresis apparatus and its power supply. Figure A-3 is the cooled platen. A pair is required in each tank.

The complete Shandon apparatus, including power supply but not including cooled platens, is under $600.00.

A list of chemicals and antisera and their sources begins on page 268.

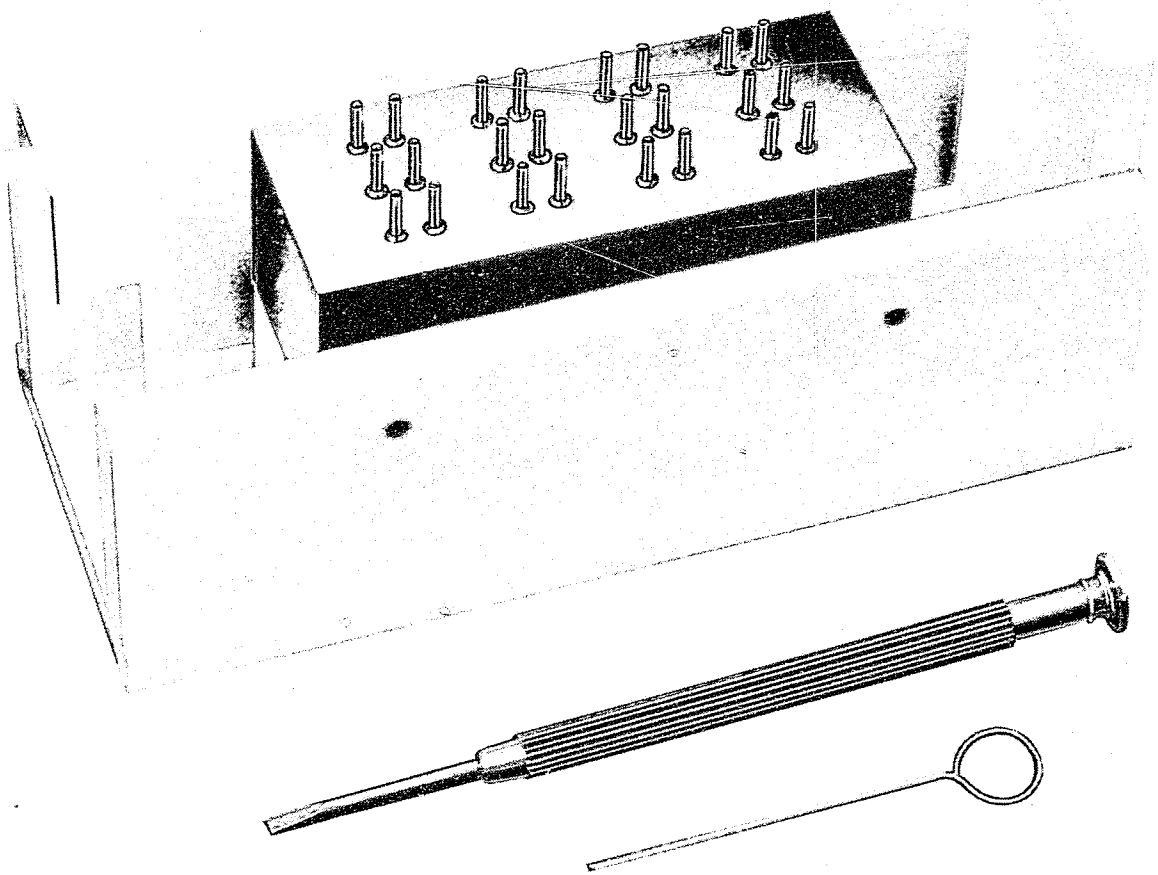


Figure A-1. Equipment Used for Precipitin Tests

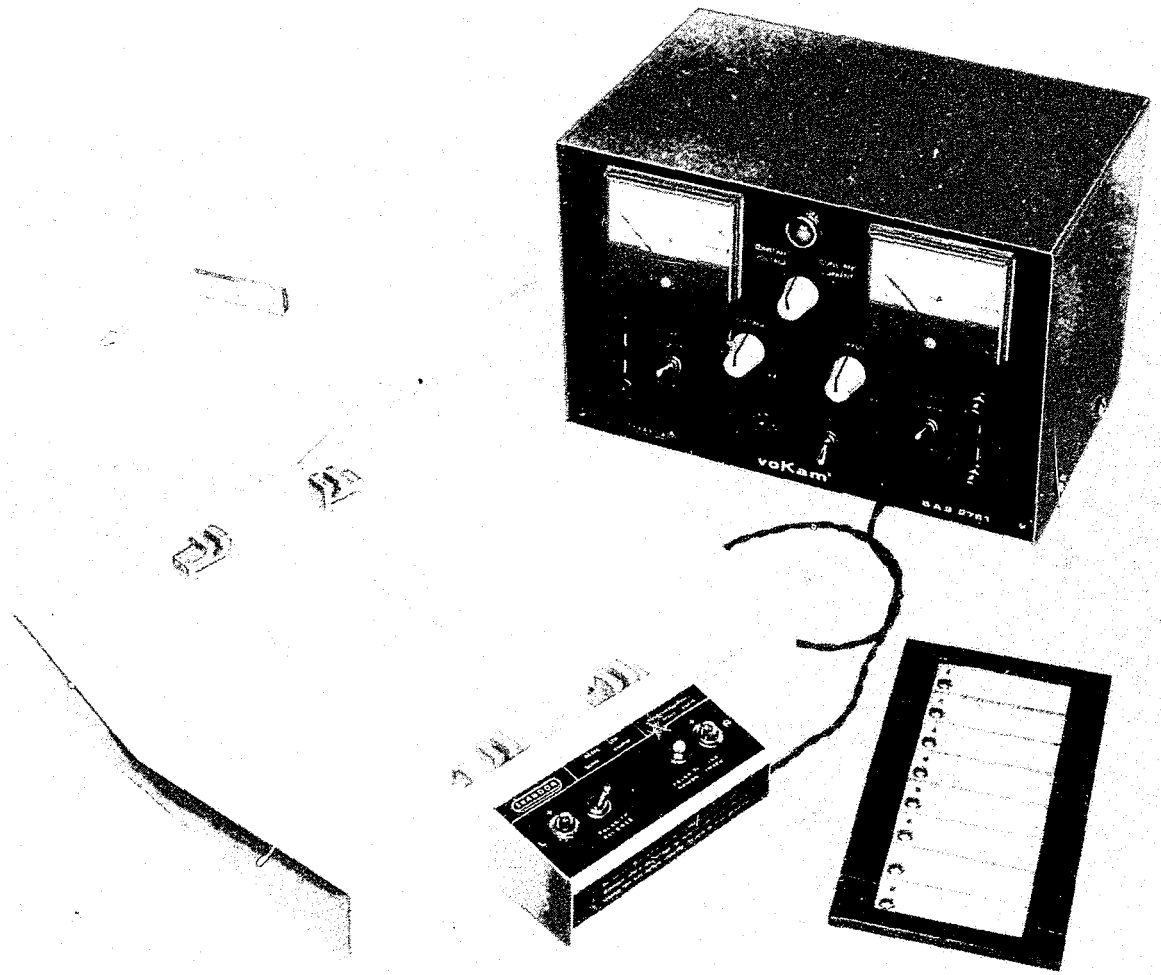
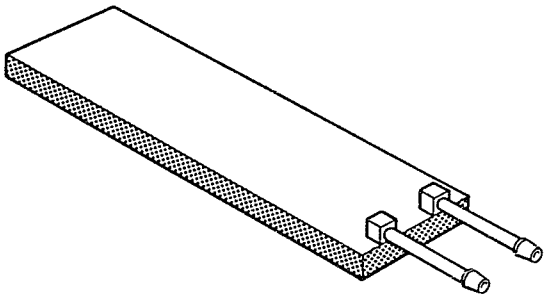


Figure A-2. The Elctrophoresis Apparatus and Its Power Supply



Cooled Platen

The Shandon Cooled Platen is strongly recommended for use when higher voltages are being employed in the interests of rapid results or when the nature of the sample demands protection against heat. Constructed in anodized aluminium, it incorporates a simple, but effective labyrinth water circulation system and requires only two easily-made connections, one to the water supply and the other to waste. A special lid with locating slots for the coolant connections is supplied with the platen.

SPECIFICATION

SAE-2697 COOLED PLATEN, including slotted lid for Electrophoresis Apparatus Model U77.

Figure A-3. Cooled Platen

Chemicals and Antisera—Sources

Catalog Number	Item	Company
103031	Anti-A serum	Behring Diagnostics, Inc. 400 Crossways Park Drive Woodbury, New York 11797 (516) 921-7430
054511	Anti-c Slide serum	
02-4522	Anti-hr (Anti-e) serum	
018550	Anti-Haptoglobin Antiserum (Rabbit)	
01-8549	Gc Globulin (Group Specific Component)	
01-8530	Gc-Globulin Type 1-1 Type 2-1 Type 2-2	
01-8568	Haptoglobin Type 1-1 Type 2-1 Type 2-2	
05-411	Anti-hr' (anti-little e)	
PX1565	Potassium Phosphate, Monobasic, reagent, crystals	Amend Drug and Chemical Co. P. O. Box 797 Hillside, N.H. 07205
020115	Anti CDE Typ sera	Hyland Div. Travenol Labs. 160 Lockwood Avenue Yonkers, New York 10710
070050	Anti Human Precipitin Serum	
023016	Q-Pak Grp A, Grp B Test Cells	
023018	Grp A and Grp B test Cells	
020015	Anti D Typ	
020035	Anti C Typ	
020055	Anti E Type	
020155	Anti c Typ	
020172	Anti e Typ	
020332	Anti M Serum	
020337	Anti N Serum	
021030	Anti A Typ	
021070	Anti B Typ	
021145	Anti H Lectin	

Chemicals and Antisera—Sources (Continued)

Catalog Number	Item	Company
023-016	Group A1 and Group B test	
	Cells, Prewashed (6 to 8 percent cell concentration)	
	Thiazolyl Blue (MTT) 3 (4,5-Dimethyl thiazolyl 2) 2, 5-Diphenyl 2 N tetrazolium bromide	K & K Lab. Inc. 121 Express Street Engineers Hill Plainview, New York 11803
	Adenosine 5 Diphosphate (Na) ADP	
	Benzoyl Choline Chloride	
	Agarose (Electrophoresis grade)	
20587	Xanthine Oxidase	
15545	Hexokinase, 100,000 units	
3034	Glucose-1-phosphate, diNa	
14866	Glucose-6-phosphate dehydrogenase, 140u per mg	
6469	Amido Black 10B	K & K Lab., Inc.
18133	Ponceau S	
11130	Dibucaine HC1	
16619	Methylphinazonium methosulfate	
22238	Nucleoside phosphorylase	
1126	Triphosphopyridine nucleotide	
12830	Nicotinamide adenine dinucleotide	
7498	N N methylene bis acrylamide	
19605	Tris (hydroxmethyl) aminomethane buffer	

Chemicals and Antisera—Sources (Continued)

Catalog Number	Item	Company
S-676	Starch hydrolyzed	Fisher Scientific Company 52 Fadem Road Springfield, New Jersey 07081
5521-X	Acrylamide	
A-682	Ammonium persulfate	
4196	2-Mercaptoethanol	

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