The development of ultrasensitive immunological methods for the detection of protein polymorphisms.

by

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Abstract.

This thesis describes the development of ultrasensitive methods for the immunological detection of two highly polymorphic human proteins, group-specific component (GC) and phosphoglucomutase-1 (PGM) following separation by high resolution isoelectric focusing (IEF) and transfer to immobilising membranes. All the steps involved in the immunoblotting procedure were investigated and the procedure optimised with a view to its application in the field of Forensic Science. In the case of the GC polymorphism, a 3000 fold increase in the sensitivity of detection was achieved compared with immunofixation methods. The improved immunoblotting procedure allowed the phenotyping of GC from forensic bloodstains, semen stains and post-coital vaginal swabs.

A high titre sheep anti-rabbit muscle PGM antiserum was produced that was capable of detecting both rabbit and human PGM1 isozymes on immunoblots, following sodium-dodecyl-sulphate gel electrophoresis or IEF, of tissues expressing high levels of the enzyme. This antiserum was used successfully for the detection of the human PGM1 polymorphism in fetal tissue from forensic casework samples, and the detection of low levels of PGM activity in rabbit haemolysates but was not suitable for screening human haemolysate PGM. The antiserum did not bind either PGM2 or PGM3 isozymes, but recognised the milk (PGM4) isozymes. This led to detailed studies of the biochemical and genetical features of the milk PGM isozymes and a novel method for screening the PGM2 isozyme polymorphism.

The sheep anti-rabbit PGM was used to purify small amounts of human PGM1 from skeletal muscle for use as an immunogen. An alternative method for producing the PGM protein, using cloned protein DNA sequence and a lambda gt11 expression vector was also investigated.

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Contents:

1. Introduction.

1.1 Aim.

This thesis is concerned with the development of ultrasensitive immunological techniques for the detection of genetic variation in man, with particular reference to forensic biology. Two highly polymorphic well characterised protein systems, the serum protein group specific-component (GC) and the enzyme phosphoglucomutase-1 (PGM1), were employed as model systems. Resolution of the phenotypic patterns and the detection of sub-nanogram amounts of these proteins was achieved using a combination of isoelectric focusing (IEF) and immunological detection systems.

1.2 Human Individuality.

This section of the introduction describes the multitude of techniques used over the years to reveal individuality in human populations ranging from the observation of morphological differences, such as individual facial characteristics, to the recognition of specific changes in the nucleotide sequence of a length of DNA.

1.2.1 Morphological Characteristics.

The variable nature of morphological characters within species has been noted since time immemorial. During the late 19th to early 20th century detailed human population studies were carried out on morphological characters including body weight, height and skin pigmentation (Christian *et al.* 1974; Kempthorne & Osborne, 1961; Harrison & Owen, 1964). All these morphological characters display continuous variation consistent with the influence of many genes (polygenic characters). The studies on fingerprints,

carried out by Francis Galton in 1892, were of particular importance in the demonstration of the degree of individuality inherent in the human population. He concluded from population studies that no two individuals could possess the same fingerprint pattern. This deduction was later greatly exploited in criminal investigations (Simpson & Knight, 1986).

1.2.2 Serological Variation.

The ABO blood group antigens (Landsteiner, 1901) were the first example of a serologically detected polymorphic trait which followed Mendelian-type inheritance. The discovery of the ABO blood group system paved the way for the identification of other highly polymorphic blood group antigens such as the MNSs (Landsteiner & Levine, 1927), Rhesus (Landsteiner & Wiener, 1940) and Lewis (Grubb, 1951) systems. There are currently twenty four well defined and genetically independent polymorphic blood group systems (P.Tippett, personal communication).

The highest degree of variability in serologically defined polymorphisms is displayed by the major histocompatability complex (MHC) cell surface human leucocyte antigens (HLA). The expression of these antigens is controlled by four closely linked gene loci situated on chromosome 6: HLA-A, HLA-B, HLA-C and HLA-Dr. To date, over fifty HLA alleles and a very large number of haplotypes have been identified (Roychoudhury & Nei, 1988), making this system an extremely important marker system in cases of disputed paternity (Terasaki *et al.* 1978), tissue donor screening prior to organ transplantation (Oliver *et al.* 1972), bone marrow

transplantation (Gale & Champlin, 1986) and forensic serology (Rittner & Waiyawuth, 1974, 1975). Currently, HLA analysis is carried out using DNA techniques which are discussed later in this chapter.

1.2.3 Inborn Errors of Metabolism.

Contemporary with the early work on blood groups, others were making observations on inborn errors of metabolism. Garrod (1909) deduced that the rare condition, alkaptonuria, was caused by the lack of an enzyme involved in the catabolism of the amino acids phenylalanine and tyrosine. Pedigree studies revealed alkaptonuria to be a recessive hereditary trait. It was concluded from this analysis that the absence of enzyme activity needed for the normal functioning of the biochemical pathway was due to the presence of an abnormal gene. This work formed the basis of the classical one gene:one enzyme or one polypeptide hypothesis. The work pioneered by Garrod created a great deal of interest in a wide range of other metabolic disorders such as phenylketonuria and galactosemia (Scriver *et al.* 1989). The investigation of these classical examples of inborn errors in metabolism gave an insight into the great of amount variation detectable in human gene products. Most of these conditions are relatively rare however, but some pharmacogenetic traits were found to be more common, contributing significantly to human individuality. For instance, some patients treated with the drug suxamethonium (a muscle relaxant used in anaesthetics) showed prolonged apnoea and reduced levels of serum cholinesterase (Bourne *et al.* 1952; Lehmann & Ryan, 1956) while the administration of primaquine produced acute haemolysis in males with glucose-

6-phosphate dehydrogenase deficiency (Hockwald *et al.* 1952; Carson *et al.* 1956). Both of these traits were found to be genetically determined.

1.2.4 Protein Electrophoresis.

Electrophoretic separation is a very powerful method for the detection of protein polymorphisms. The first example of protein variation identified in this way was the difference in mobility displayed between normal haemoglobin (HbA) and sickle cell haemoglobin (HbS) on a paper medium (Pauling, 1949). The introduction of starch led to significant improvements in the resolving power of electrophoresis. This is the result of separation by protein size due to molecular sieving effects of starch (Smithies, 1955). The subsequent analysis of serum proteins and red cell enzymes by starch gel electrophoresis led to the identification of an unexpected number of polymorphic protein systems, such as haptoglobin (hp) (Smithies 1955), GC (Parker *et al.* 1963), red cell acid phosphatase (EAP) (Hopkinson *et al.* 1963) and PGM1 (Spencer *et al.* 1964). There are also numerous rare variants associated with these and other enzyme systems (Harris 1980).

1.2.5 Isoelectric Focusing.

Isoelectric focusing (IEF) involves the separation of proteins in a pH gradient which is formed from a mixture of many different amphoteric buffers called polyampholytes. When an electric current is applied, the polyampholytes migrate to their respective isoelectric points (pI), where the net charge of each ampholyte is zero and migration ceases. As a result, a stable pH gradient is formed between

the anode and cathode. Proteins subjected to an electric current in this pH gradient migrate along the gradient until their respective pI positions are reached. Having reached its pI, the protein concentrates as a sharply resolved band (Svensson, 1961).

IEF is responsible for revealing many additional phenotypes such as the subtypes of the PGM1 marker (Bark *et al.* 1976), Transferrin (Kühnl & Speilmann, 1978, 1979), GC (Constans & Viau,1977) and orosomucoid (Thymann & Eiberg, 1986), which were not resolved by zone electrophoresis.

The introduction of chemical spacers, such as 4-aminobutyric acid and 6-amino-n-hexanoic acid, into the IEF gel matrix (Caspers *et al.* 1977; Ngugen & Chambach, 1980; Låås & Olsson, 1981) allowed the expansion and flattening of various regions of the pH gradient and increased the resolving power of IEF further still. This led to an improved separation of many protein systems such as the PGM1, GC and EsD subtypes (Gill & Sutton 1985; Edwards, 1986; Gill, 1985) and an increased confidence in subtyping the alpha-1-antitrypsin (PI) heterozygotes PIM3M1 and PIM3M2 (Weidinger *et al.* 1982).

The apparent lack of detectable neutral amino acid substitution variants would suggest a certain difficulty in their electrophoretic separation, as suggested by the charge state model proposed by Marshall and Brown (1975). However, such substitutions can now be detected if IEF gels containing urea and a detergent (Saglio *et al.* 1979) or Immobilised pH gradients (IPG) in polyacrylamide gels are

used (Cossu *et al.* 1986). The introduction of IPGs has been well received and has led to the identification of new variants in the PI system in the Japanese population. For example, PI Mtoyoura (Yuasa *et al.* 1988). Nevertheless, IPGs have yet to be accepted as a superior alternative to IEF in forensic studies, and this is mainly due to problems with Immobiline buffer instability (Rabilloud *et al.* 1987).

1.2.6 Two Dimensional Electrophoresis.

Two dimensional electophoresis (2D-E) combines the high charge-resolving capacity of IEF with high resolution SDS-PAGE separation of proteins with different molecular sizes (O'Farrell, 1977). A multitude of studies have been carried out to investigate the degree of protein variation using this technique (Goldman & Merril, 1983; Wanner, Neel & Meisler, 1982; Rosenblum, Neel & Hanash, 1983; Rosenblum *et al.* 1984). However, two dimensional electrophoresis has so far revealed fewer new protein polymorphisms than one might have expected (Neel, 1984).

1.2.7 DNA Polymorphisms.

Simple detection of genetic variation in both the coding and non-coding regions of the human genome was first made possible with the identification of restriction fragment length polymorphisms (RFLPs) (Southern, 1975).

RFLPs exhibit similar levels of heterozygosity to protein markers (Botstein *et al.* 1980; Allen *et al.* 1990). They have proved to be extremely useful for linkage analysis and for the identification of point mutations in DNA sequences, such as those identified in the globin genes (Kan & Dozy, 1978),

including the identification of gene cluster haplotypes (Wainscoat *et al.* 1983) and for the prenatal diagnosis of various diseases, such as severe combined immunodeficiency (SCID) (Goodship, Levinsky & Malcolm, 1989).

Greater degrees of heterozygosity were observed following the identification of hypervariable sequences of human DNA, consisting of short tandem repeats. These repeat sequences were first identified in the "core myoglobin gene sequence" and became known as VNTRs, variable numbers of tandem repeats (Jeffreys *et al.* 1985.a). Typical multiple locus probes such as 33.15 and 33.6, led to the detection of complex patterns of DNA fragments, each being unique to every individual in the human population, with the exception of monozygotic twins (Jeffreys *et al.* 1985.a.b). This procedure rapidly became known as "DNA fingerprinting" and was was applied to the analysis of samples in forensic cases (Gill, *et al.* 1985; Gill & Werrett, 1987; Helminen, *et al.* 1988).

Probes which possess both the core tandem repeat sequence plus the flanking sequence specific to a particular locus produce a restriction fragment pattern specific to a single locus (Fowler *et al.* 1988). These hypervariable repeat (HVR) or VNTR probes show high degrees of polymorphism and less complex band patterns than their counterpart multi-locus probes. For this reason many forensic and paternity laboratories are adopting panels of single locus probes rather than the multi-locus probes for their studies (Allen *et al.* 1989, 1990; Yokoi *et al.* 1990; Evett & Gill, 1991; Jeffreys *et al.* 1991.a; Schneider *et al.* 1991).

The next technological step in the analysis of DNA sequence variation, was the introduction of the polymerase chain reaction (PCR), an *in vitro* method for the enzymic synthesis of specific DNA sequences, using oligonucleotide primers chosen to hybridise the complementary opposite strands of DNA flanking the region of interest. The PCR reaction mixture undergoes a series of repetitive cycles involving template denaturation, primer annealing and extension, which results in an exponential amplification of the target DNA fragment (20 cycles produces an approximate amplification of 2^{20}; Mullis & Faloona, 1987). The amplification of DNA via PCR quickly found many applications in research, prenatal diagnosis and forensic biology (Abbott *et al.* 1988; Wright & Wynford-Thomas, 1990; Balnaves *et al.* 1990; Reynolds *et al.* 1991).

The simplified analysis of very short DNA sequence repeat blocks, such as CA repeat sequences or "microsatellites" (another pool of highly polymorphic DNA markers) and the easier assessment of polymorphic DNA markers, such as the beta-globin gene, HLA-DQ alpha alleles and many others using allele-specific or sequence-specific oligonucleotide probes, is the result of the successful application of PCR (Saiki, *et al.* 1986; Weber & May, 1989; Litt & Lutty, 1989). Furthermore, PCR facilitated the introduction of a wide range of techniques for the detection of point mutations. These procedures include single-strand conformation polymorphism (SSCP) analysis (Orita, *et al,* 1989; Demers *et al.* 1990), denaturing gradient gel electrophoresis (DGGE) (Abrams *et al.* 1990; Uitterlinden & Vijg, 1991) and allele-

specific amplification (Saiki *et al.* 1986, 1988).

1.2.8 The Application of Polymorphic Markers to the Identification of Individuals in Forensic Biology.

There are many ways of calculating the level of variation or information content supplied by a polymorphic marker. These include the heterozygosity index (Harris, 1980), the discrimination potential (PD) or paternity index (PI) (Sensabaugh, 1982), the probability of exclusion (Ito, *et al.* 1985) and polymorphism information content (PIC) (Botstein *et al.* 1980). Many of the calculations carried out on the protein polymorphisms used the heterozygosity index (Harris, 1980; Neel, 1984; Rosenblaum *et al*, 1984). However, the calculation of the degree of variability displayed by a polymorphic marker, whether a protein locus or a VNTR DNA locus, in a paternity or forensic investigation, is generally measured by the probalility of exclusion or likelihood ratio (Yokoi *et al.* 1990; Evett *et al.* 1989; Evett *et al.* 1991). In this section, I have used the probability of exclusion as a measure of the usefulness of a marker in forensic biology, due to the availability of figures from serological, protein and DNA marker sources.

Serological systems such as ABO, rh, MNSs, Kidd, Duffy, Kell and Lutheran were widely used in forensic biology for screening suspect bloodstains, semen and saliva. However, such large panels of serological markers are now rarely used, except in cases of disputed paternity, where their importance is still undeniable, as indicated by the 75% cumulative probability of exclusion (the probability of a wrongly named suspect being excluded) obtained using the

combination of markers described above (figures obtained between 1987 and 1988 from the Royal London Hospital Medical College) (figure 1.1). The high cumulative probability of exclusion (90-96%) obtained for the HLA system emphasises the discrimination potential of this serological system alone. (Royal London Hospital Medical College 1987-1988; Baird *et al*, 1986).

Zone electrophoresis and IEF were also quickly adopted by forensic biologists in both criminal cases and paternity suits. This additional genetic information gave considerable improvements in the probabilities of exclusion obtained per case. Figure 1.2 shows the effect of substituting the separation of six polymorphic protein systems by starch gel electrophoresis with high resolution IEF. As a direct result, the cumulative probability of exclusion increases from 58% to 84%. With the application of twelve different polymorphic protein systems using a combination of zone electrophoresis and IEF, the cumulative probability of exclusion rises to approximately 93% (table 1.3). The cumulative probability available when the seven serological (excluding HLA) and twelve electrophoretic systems described above, are used in combination, is approximately 98% (table 1.1) (all figures obtained from the Royal London Hospital Medical College 1987-1988).

Since the recognition of the highly polymorphic nature of the blood group antigens and red cell and serum proteins, the analysis of these systems at the DNA level has been of the upmost importance. The MHC has become the focus of much work on the possible correlation between the multitude of

Marker	Percentage exclusion rate
MNSs	32.1%
Rhesus	28.0%
Kidd	18.7%
Duffy	18.3%
ABO	17.6%
Kell	4.5%
Lutheran	3.7%

Cumulative exclusion rate for serological systems 75.3%.

Cumulative exclusion rate for the electrophoretic markers (illustrated in figure 1.3) and the above serological systems 98.1%.

The exclusion rate calculated for the HLA system 90-96%.

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Figure 1.1

Exclusion rates calculated from a panel of serological markers and the cumulative rates of exclusion calculated using the illustated panels of electrophoretic and serological protein markers

Marker	Exclusion rate (%) after separation by starch gel electrophoresis	Excluion rate (%) after separation by IEF
Haptoglobin	18.1%	32.4%
Transferrin	16.4%	17.1%
Group-specific component	16.3%	31.4%
Phosphoglucomutase-1	14.6%	32.2%
Esterase D	9.4%	10.7%
Alpha-1-antitrypsin	5.9%	31.1%

Cumulative exclusion rate for the above protein markers following starch gel electrophoresis 58.2%

Cumulative exclusion rate for the above protein markers following separation by IEF 84%

Figure 1.2

Increased rates of exclusion as a function of the electrophoretic method of protein separation used.

Protein marker	Exclusion rate (%) starch	Exclusion rate (%) IEF
Phosphoglucomutase-1		32.2%
Group-specific component		31.4%
Alpha-1-antitrypsin		31.1%
Erythrocyte acid phosphatase		23.7%
Glyoxalase I	18.7%	
Alpha-2-HS glycoprotein		18.4%
Haptoglobin	18.1%	
Plasminogen		17.4%
Transferrin		17.1%
Esterase D		10.7%
Adenosine deaminase	4.4%	
Adenylate kinase	3.4%	
Cumulative exclusion rate for electrophoretic systems 92.5%		

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Figure 1.3

Exclusion rates calculated using a panel of protein markers separated by starch gel electrophoresis or IEF.

known HLA variant antigens and RFLPs obtained from the DNA sequences at the different HLA gene loci. Correlation does exist between known protein variants and some of the RFLPs used, however certain RFLPs do not correspond to any of the known protein variants, suggesting the possibility of a greater degree of detectable variation at the DNA level than possible at the protein level (Summers, 1987).

The first DNA probes used in the forensic field were multi-locus VNTR probes such as 33.6 and 33.15 (Jeffreys *et al.* 1985.b; Gill *et al.* 1985; Gill & Werrett, 1987). Although many workers reported high probabilities of exclusion, in excess of 99.9% using these probes, problems have been encountered with the interpretation of the results due to the complexity of the band patterns produced (Gill & Werrett, 1987; Helminen *et al*, 1988; Tonelli *et al*, 1990; Markowicz *et al*, 1990; Norman, 1989; Lewin, 1989; Roberts, 1992). As a consequence of the simplified fragment patterns obtained when using single locus probes, their application to criminal investigations is a more attractive choice than the interpretation of the complex band patterns associated with the multi-locus probes. The use of several VNTRs to improve the cumulative probability of exclusion has become common practice in paternity testing and forensic biology. A typical panel used for paternity testing includes Muc7, MR24, YNH24 and TBQ7 probes which give an approximate cumulative probability of exclusion of 99.98% (figures obtained from the Royal London Hospital Medical College 1990) (table 1.4).

DNA probe	Rate of exclusion (%)
MUC7	80%
MR24	94%
YNH24	86%
TBQ7	91%

Cumulative exclusion rate calculated using the above single locus DNA probes 99.98%.

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Figure 1.4

Exclusion rates and cumulative exclusion rates calculated for the use of a panel of four single locus DNA probes.

1.3 Group-specific component.

Human group-specific component (GC) is a highly polymorphic glycoprotein which is synthesised by the liver and has an average plasma concentration of 40mg per 100ml (Kitchin & Bearn, 1965; Kueppers & Harpel, 1979). GC is monomeric, consists of 458 amino acid residues (51,335 mwt.) (Cooke *et al.* 1985; Yang *et al.* 1985) and functions primarily as the major vitamin-D binding protein (Daiger *et al.* 1975). The expression of this protein is controlled by a single gene locus located on chromosome 4 (Mikkelsen *et al.* 1977; Yang *et al* 1985). Its highly polymorphic nature makes GC an extremely useful protein marker for the determination of genetic variation in the human population.

1.3.1 Tissue Distribution.

GC is expressed at high levels in adult human liver and is secreted into the blood plasma. It is also found in extracellular fluids such as cerebrospinal fluid, urine and ascitic fluid (Putnam, 1977). The development of more sensitive protein detection systems have resulted in the identification of low levels of GC on the surfaces of leukocytes (Petrini *et al.* 1983), in seminal plasma and vaginal secretions (Horscroft & Westwood, 1986; Pflug *et al.* 1988).

Studies on the levels of RNA contained in a selection of rat tissues by Northern blot analysis revealed, as expected, high levels of mRNA in the adult liver. Low concentrations of the message were also detected in adult kidney, testis, abdominal fat and 18-day fetal yolk sack, suggesting that the expression of the GC protein may be more widespread than

first thought (McLeod & Cooke, 1989).

1.3.2 The Biological Functions of GC.

GC possesses three independent biological functions: a strong binding affinity for vitamin-D_3 and also for actin and the ability to reactivate the chemotactic properties of complement component C5a des Arg.

The best known property of the GC protein is its high affinity for vitamin-D_3 and derivatives. Vitamin-D is mainly manufactured in the skin by exposure to ultraviolet light but is converted to its more active form, 1,25-dihydroxycholecalciferol, in the liver. Vitamin-D increases the absorption of calcium and phosphate ions from the gut which are essential in bone metabolism, therefore an efficient form of vitamin-D transportation is needed (Frazer, 1980). Studies carried out by Daiger *et al* (1975) suggest that the GC protein is involved in the *in vivo* transport mechanism of vitamin D.

Variation between the binding affinities of the various common GC isoforms for vitamin-D and its derivatives has also been noted (Constans *et al.* 1980), but the reasons for these differences remain unclear.

GC has a strong binding capacity for monomeric actin or G-actin (Van Baelen *et al.* 1980). Due to tissue death, actin is released into the extracellular fluid in the form of G-actin or fibrous F-actin. The F-actin is fragmented or depolymerised by the action of the protein gelosin (Korn, 1982; Pollard & Cooper, 1986) into G-actin and subsequently complexes with GC. These complexes are ultimately eliminated

from the circulation by tissue uptake (Lind *et al.* 1986; Goldschmidt-Clermont *et al.* 1988). GC plays an important role in the clearance of cellular actin from the extracellular space. However, the fate of the GC-actin complexes after uptake remains unclear.

The formation of GC-actin complexes are of further interest because of their effect on the electrophoretic pattern of the GC isoforms, described later in this section.

The third biological function of GC is less clearly understood. It is capable of forming complexes with the complement components C5a and C5a des Arg (Perez *et al.* 1986). C5a is a potent chemotactic factor for neutrophils and is rapidly converted to a less active but more stable form, C5a des Arg, by the enzyme carboxypeptidase N (Fernandez *et al.* 1978). It was observed however, that at non-chemotatic concentrations, the C5a des Arg became reactivated in the presence of an anionic polypeptide (Perez *et al.* 1980). SDS-PAGE, western blotting and antibody subtraction studies revealed the C5a des Arg activator to be serum GC (Perez *et al.* 1988; Kew & Webster, 1988). The mechanism of action is unclear, however cell surface GC has been reported on several types of leukocytes, which suggests enhancement of C5-derived peptides via attachment to neutrophil membrane-bound GC (Petrini *et al.* 1984). GC appears to be the major serum-enhancing factor for complement chemotaxis and probably acts in the recruitment of neutrophils to sites of inflammation in the body.

1.3.3 Electrophoretic Variation.

The GC polymorphism was first identified after immunoelectrophoretic studies of human serum which revealed differences in the mobility of the alpha-2 globulin fraction of the serum (Hirschfield, 1959). Three common phenotypes were identified: GC1, GC2 and GC2-1, which were thought to be controlled by two autosomal alleles GC*1 and GC*2 (gene frequencies in the European population are 0.73 and 0.26, respectively). Separation by high resolution IEF revealed further heterogeneity (Constans & Viau, 1977). The GC1 phenotype could be resolved into fast and slow components of equal intensity, corresponding to the phenotypes GC 1F and GC 1S. This gave rise to six possible GC phenotypes which are controlled by three alleles GC*1S, GC*1F and GC*2 (gene frequencies in the European population are 0.592, 0.144 and 0.262, respectively).

Over 120 rare GC variants have been recognised by immunoblotting using an anti-human GC polyclonal antiserum (Cleve & Constans, 1988) and more recently using human GC monoclonal antibodies (Pierce *et al.* 1985; Hoffmann *et al.* 1990). One of the monoclonal antibodies recognises the common human GC phenotypes, a limited number of GC variants and GC in the great apes and old world monkeys but no other mammals (Hoffmann *et al.* 1990).

1.3.4 Analysis of the GC Polymorphism at the DNA Level.

Recently, the cDNA sequences of the GC phenotypes 1 and 2, determined by Cooke *et al* (1985) and Yang *et al* (1985) respectively, were used for phenotyping GC using the polymerase chain reaction (PCR) (Reynolds & Sensabaugh,

1990.a.b). There are four regions of base changes in the cDNA sequences from these two GC types. The crucial differences occur at positions 152, 310, 311, 416 and 420. Digestion of genomic DNA PCR products from the three homozygous types 1F, 1S and 2 with HaeIII and StyI endonucleases, reveals three distinct patterns which are directly related to the protein variants and also the base changes at positions 416 and 420. The GC 1F PCR product is not cut by either HaeIII or StyI. The GC 1S PCR product shows HaeIII digestion while the GC 2 product is cut by StyI. All six common GC types are easily distinguished in this way. DNA from individuals exhibiting rare GC protein variants were also investigated using this PCR technique, but they could not be distinguished from the common alleles.

1.3.5 The Albumin Gene Family.

A further feature of the GC protein is its high degree of sequence homology with albumin (ALB) and alpha-fetoprotein (AFP) at both the protein and cDNA nucleotide sequence levels. Cooke and David (1985), isolated a near full-length cDNA and were able to determine the entire primary structure of the encoded protein. They confirmed the close genetic linkage between GC, ALB and AFP. The maximum distance between the three genes is approximately 1.5 centimorgans, within the region 4q11 to 4q22 (Harper & Dugaiczyk, 1983).

All three proteins possess a high cysteine residue content and show a high degree of conservation in their amino acid number and position. A comparison between the three amino acid sequences revealed that GC has 24% identity with ALB and 19% identity with AFP, compared to 39% identity between

ALB and AFP (Morinaga *et al.* 1983). All three proteins possess a triplicated internal domain structure, although in the case of GC the third domain has been truncated due to its smaller size.

In view of these findings, it has been suggested that these serum proteins have evolved from a common precursor gene, and GC is now regarded as the third member of the albumin gene family.

1.3.6 The Application of GC in Forensic Investigations.

The highly polymorphic nature and stability of the GC protein in stains of biological fluids makes it an important protein marker in both forensic and paternity investigations. The analysis of GC alone by IEF produces a probability of exclusion of 31.4% (Royal London Hospital Medical College 1987-1988) or a discrimination potential (the probability of two random individuals in a population possessing a different phenotype) of 0.74 (Westwood *et al.* 1984). However, since actin is present in blood platelet membranes, the GC present in blood stains is largely in the form of a GC-actin complex. This gives rise to an anodal shift in the electrophoretic pattern of the GC isoforms. Unless the stains are treated with 4M guanidine/HCl (which dissociates the GC-actin complex), reliable phenotyping is impossible (Kimura *et al.* 1983). Even better results can be obtained by using 6M urea, which enables the phenotyping of GC in bloodstains up to a year old (Westwood, 1984; Horscroft & Westwood, 1986).

GC can also be detected in normal human semen, seminal fluid from vasectomised men and semen stained material. Extremely

low levels of GC are also present in vaginal secretions, but the phenotype is rarely distinguishable. Therefore, interpretation of the GC pattern from vaginal swabs after intercourse does not tend to confuse the contaminating male's phenotype (Pötsch-Schneider & Klein, 1988; Pflug, 1988.a; Pflug *et al.* 1988). This makes the GC protein an extremely useful marker in cases of suspected rape as well as other violent crimes.

1.4 Phosphoglucomutase.

Phosphoglucomutase (PGM) [E.C.5.4.2.2] is a highly polymorphic enzyme which catalyses the conversion of glucose-1-phosphate (G1P) to glucose-6-phosphate (G6P), during glycolysis. Human PGM is encoded by three well characterised autosomal unlinked structural gene loci: PGM1, PGM2 and PGM3 (Spencer *et al.* 1964; Hopkinson & Harris, 1965; Hopkinson & Harris, 1968; Parrington *et al.* 1968) located on chromosomes 1, 4 and 6 respectively (McAlpine *et al.* 1990). The PGM isozymes are monomeric and their molecular sizes range between 51,000 to 61,000, as determined by gel filtration (McAlpine *et al.* 1970.a). There is evidence for the existence of a fourth, less well characterised gene locus (PGM4), which is expressed in human milk (Cantu & Ibarra, 1982). At the time of writing, the structure and primary amino acid sequence of human PGM1 has not been determined. However, rabbit PGM1 has been purified and sequence data indicates a molecular weight of 61,600, consisting of 561 amino acid residues (Ray *et al.* 1983).

1.4.1 Tissue Distribution.

PGM is ubiquitously distributed in human tissues and body fluids. The PGM activity exhibited in various tissues is predominately due to PGM1, ranging between 85% in placentae and 95% of the total in skeletal muscle (McAlpine *et al.* 1970.b). The remaining PGM activity is mainly the result of the PGM2 isozymes, between 2% in skeletal muscle and 15% in placentae, with the final 1 to 2% being determined by PGM3 (McAlpine *et al.* 1970.b). Exceptions are found in some tissues. Equal amounts of PGM1 and PGM2 activity are found in red blood cells. Higher amounts of PGM3 are found in placentae and fibroblasts, ranging between 3% in placentae and 7% of the total PGM activity in fibroblasts (McAlpine *et al.* 1970.b). In addition, trace amounts of PGM3 activity have been detected in hair roots (Sutton *et al.* 1982). PGM4 activity appears to be confined to milk (Cantu & Ibarra, 1982).

1.4.2 The Biological Functions of PGM.

The mechanism of action of PGM was proposed by Ray & Peck (1972), however direct evidence for the catalytic mechanism was not available until structural studies were carried out on rabbit PGM (Ray *et al.* 1983). The catalytically active rabbit phosphoenzyme has a phosphate group attached to the serine residue at position 116 and a bivalent metal ion activator (Mg^{2+}) is bound nearby. This has been defined as the active site of PGM. The enzyme donates its phosphate group to G1P, producing glucose-1,6-diphosphate (G1,6diP). The dephospho form of PGM subsequently accepts the phosphate group from the 1 position of the substrate, converting the substrate to G6P and restoring the active phospho form of

the enzyme.

The primary function of PGM appears to be conversion of G1P to G6P, however studies have revealed that different human PGM isozymes possess different substrate specificities. Experiments using a five carbon sugar, ribose-1-phosphate as an alternative substrate, resulted in the discovery of strong phosphoribomutase activity displayed by the PGM2 isozymes. The other PGM isozymes (PGM1 and PGM3) showed little or no such activity (Quick *et al.* 1972). More detailed kinetic studies (Quick *et al.* 1974) revealed that the hexomutase activity of PGM2 is dependent on the cofactor G1,6diP concentration. PGM2 is more able to catalyse the interconversion of G1P and G6P with increasing cofactor concentration. The interconversion of R1P and R5P was relatively unaffected. From these observations it was concluded that PGM1 is the major phosphoglucomutase. PGM2 is capable of catalysing the conversion of both G1P and R1P, but is mainly a phosphopentomutase. PGM3 is a weak phosphoglucomutase and shows no phosphopentomutase activity (Harris, 1980). PGM4 possesses no phosphopentosemutase activity (Cantu & Ibarra, 1982).

1.4.3 Electrophoretic Variation.

Human PGM was initially characterised in red cell lysates after separation by starch gel electrophoresis (Spencer *et al.* 1964) and two sets of enzymes were identified, PGM1 and PGM2. Subsequent screening of a tissue panel revealed the existence of other isozymes PGM3 and PGM4, both of which display independent genetic variation, as indicated by their characteristic electrophoretic

mobilities (Hopkinson & Harris, 1966, 1968; Cantu & Ibarra, 1982).

1.4.3.1 PGM1.

PGM1 shows the greatest degree of electrophoretic variation and tissue distribution of the human phosphoglucomutases, as well as good stability in stain material. Consequently, PGM1 is the only mutase which is extensively used as a protein marker in forensic biology.

The polymorphic nature of PGM1 was first revealed after starch gel electrophoresis of human red cell lysates (Spencer *et al*, 1964). Three phenotypes were identified, which were considered to be controlled by two autosomal alleles, PGM1*1 and PGM1*2 (gene frequencies in the European population are 0.76 and 0.23, respectively). The introduction of IEF, allowed further sub division of the PGM1 isozymes, into ten phenotypes controlled by four autosomal alleles, PGM1*1A, PGM1*1B, PGM1*2A and PGM1*2B (gene frequencies in the European population are 0.63, 0.11, 0.18 and 0.064, respectively) (Bark *et al.* 1976; Kühnl *et al.* 1977; Dykes *et al.* 1985). Thermostability studies carried out on the PGM1 subtypes revealed still further heterogeneity. For each PGM1 subtype resolved by IEF, heat treatment revealed temperature sensitive and temperature resistant forms. Thus there are thirty six possible phenotypes under the control of eight common alleles (Scozzari *et al.* 1981).

PGM1 or PGM1-like activity is present in a very wide range of mammals. Most mammalian PGM1 patterns are simpler than the

human and appear to show no polymorphic tendencies apart from cow, horse, pig and mouse (Pflug, 1988.b; Shows *et al.* 1968).

1.4.3.2 PGM2.

Separation of PGM2 by starch gel electrophoresis normally reveals three bands of enzyme activity slightly more acidic than the PGM1 isozymes. PGM2 is not polymorphic, the common allele at the PGM2 locus being the PGM2*1 (gene frequency in the European population is 0.985). A considerable number of rare variants however have been identified within the European population, such as PGM2*2 or the "Atkinson" phenotype. In a number of other populations, such as the African, Pygmie and South American populations, PGM2 does exhibit polymorphism (Hopkinson & Harris, 1966; Roychoudhury & Nei, 1988). The low level of variation displayed by PGM2 in Europeans renders it unhelpful as a protein marker in most population or forensic studies.

1.4.3.3 PGM3.

PGM3 is another polymorphic locus from this gene family. The isozymes migrate considerably more anodally than the PGM1 and PGM2 components when subjected to starch gel electrophoresis. Three commonly occurring phenotypes are recognised which are controlled by two autosomal alleles PGM3*1 and PGM3*2 (gene frequencies in the European population are 0.74 and 0.26, respectively) (Hopkinson & Harris, 1968). However, the overall usefulness of PGM3 is somewhat limited due to its restricted tissue distibution, low enzymic activity and increased lability compared with PGM1 and PGM2. As a result, it is not used in the forensic

investigation of biological samples.

It is of interest to note that it is possible to detect PGM3 protein from human fibroblasts by non-specfic protein silver staining after separation by 2-Dimensional gel electrophoresis (Goldman *et al.* 1985; Whitehouse & Hopkinson, 1985). Therefore, the PGM3 isozymes would appear to be present at high concentrations in fibroblasts, but display relatively low levels of PGM activity. These observations support the kinetic studies of the PGM3 isozymes which suggest that they are not good phosphoglucomutases.

1.4.3.4 PGM4.

The PGM isozymes in human milk possess different electrophoretic mobilities from those displayed by the three well characterised glucomutases. Eight phenotypes have been distinguished by starch gel electrophoresis, which are possibly controlled by four alleles designated PGM4*1, PGM4*2, PGM4*3 and PGM4*4 (gene frequencies in the Mexican population are 0.346, 0.475, 0.114 and 0.065, respectively). Further studies have revealed that PGM4 isozymes are restricted to the secreted milk and are not found in extracts of mammary tissue from non-lactating women, which shows only PGM1 and PGM2 isozyme activity. Other mammals such as swine and rat also display PGM activity in milk. However, little is known about the PGM4 locus or the reason for its restricted expression in the lactating mammary gland. More recent work on PGM4 in human milk (Kvitko & Weimer, 1990) appears to support the original findings of Cantu and Ibarra. However, this study revealed that the PGM4

isozyme phenotypes obtained from colostrum samples did not concur with the phenotypes obtained from the analysis of mature milk. The overall effect was to reduce the incidence of heterozygous phenotypes and the distribution obtained in population studies revealed a divergence from the Hardy-Weinberg expectation. It was suggested that the changed pattern represented *in vivo* post-translational modification during the earlier stages of lactation.

1.4.4 Evolutionary Basis of the PGM Polymorphism.

The members of the PGM gene family, PGM1 to PGM4, are identified by their ability to display phosphoglucomutase activity. However, all four gene products show variations in their substrate specificity (Quick *et al*, 1972, 1974), tissue distribution (McAlpine *et al*, 1970.b), thermostability (McAlpine *et al*. 1970.c), electrophoretic mobility (Spencer *et al*, 1964; Hopkinson & Harris, 1965, 1968; Cantu & Ibarra, 1982) and molecular weight (McAlpine *et al*, 1970.a), as well as different chromosome locations for PGM1, PGM2 and PGM3 (McAlpine *et al*. 1990). Thus, the structural homology between the phosphoglucomutases is uncertain and the evolutionary basis for the existence of four PGM loci is unknown.

A study of the PGM1 polymorphism was carried out in primates and man in order to discover its evolutionary basis (Carter *et al*. 1979). The PGM1*1A allele or a product which resembles the electrophoretic mobility of PGM1*1A was found to be common to both man and primates. Therefore it was postulated that the PGM1*1B and PGM1*2A alleles were the result of two independent mutations of the PGM1*1A gene,

assuming this to be the ancestral gene, and the PGM1*2B allele was the result of an intragenic crossover.

Further studies, carried out on the Japanese population, extended this theory to include four rarer alleles found using IEF: PGM1*3A and 3B and PGM1*7A and 7B (Takahashi *et al.* 1982). This extended theory assumes that the PGM1*1A allele is the ancestral gene from which three initial nucleotide substitutions, followed by four intragenic recombinations, gave rise to the eight PGM1 alleles in the Japanese population. Evidence for intragenic recombination at the PGM1 locus has been produced during extensive paternity studies (Wetterling, 1990). From the examination of 16,463 parent-child pairs, eight possible cases of recombination were observed. These were the result of the lack of inheritance of the maternal alleles by the child. In all cases the recombination appears to have occurred during oogenesis. Further clarification of these nucleotide substitutions and recombination events will only occur after the examination of the full nucleotide sequences of the PGM1 alleles.

1.4.5 The Application of PGM Analysis in Forensic Investigations.

The importance of PGM1 in forensic investigations has been recognised ever since the polymorphism was first identified by starch gel electrophoresis. The more recent identification of the PGM1 subtypes by IEF, led to a significant increase in the discrimination potential of the system (from 0.56 to 0.76 in the European population). The detection of the thermostability alleles, in principle would

increase the efficency of the PGM system still further. For example, in the case of an eight allele system with a theoretical maximum of thirty six phenotypes, the discrimination potential would be in the region of 0.85, in the European population. In practice however, the routine analysis of thermostability differences would be difficult and even under ideal conditions only a proportion of all of the phenotypes could be identified with confidence.

The usefulness of PGM1 as a polymorphic protein marker in forensic investigations is not only measured by the degree of variation it displays, but also the ability to detect the enzyme in stain material. PGM1 remains stable in bloodstained material from two to three months (Oepen, 1988), and is found at phenotypable levels in seminal plasma, vaginal secretions, saliva and hair roots (Budowle *et al.* 1986; Coosemans & Hoste, 1988; Divall, 1986; Gambel *et al.* 1987). Although several observations have been made concerning changes in the normal electrophoretic patterns of the PGM1 isozymes when mixtures of body fluids are under analysis, (Sensabaugh *et al.* 1980; Divall, 1986; Coosemans & Hoste, 1988) there is no denying the overall usefulness of PGM1 as a marker for application in forensic biology and paternity investigations.

1.5 Methods of protein detection.

1.5.1 General Methods of Detection.

The visualisation of electrophoretically separated proteins is an essential step in the detection of genetic variation. Many proteins present in red cell lysates, plasma and tissues, need special methods of localisation. Coloured

proteins such as haemoglobin are easily localised. Other abundant proteins such as group-specific component (GC), the transferrins and alpha-1-antitrypsin, are localised after simple precipitation (Kühnl *et al.* 1978) followed by visualisation with general protein stains such as amido black, coomassie blue or a silver staining system (Parker *et al.* 1963; Wilson, 1979; Switzer *et al.* 1979; Westwood, 1985).

1.5.2 Specific Methods of Detection.

Less abundant proteins and enzymes may be detected by a number of more specific methods. Functional staining methods that utilise catalytic activity are normally used for detecting enzymes (Harris & Hopkinson, 1976). Radiolabelling and autoradiography can also be used to visualise some proteins (Cavalli-Sforza *et al.* 1977; Daiger *et al.* 1975; Daiger & Wildin, 1981). A large number of immunological detection systems, such as immunofixation and immunoblotting are also available for the detection of a wide range of proteins (Grabar & Williams, 1953; Hirshfield, 1959; Johnson, 1978; Towbin *et al.* 1979; Renart *et al.* 1979). The availability, high sensitivity and ease of use of these immunological detection systems has led to their widespread application in genetical and forensic studies (Kishi *et al.* 1988.a.b; Whitehouse *et al.* 1989.a).

The procedure of protein detection by immunoblotting provides a considerable increase in sensitivity and a reduction in costs, compared with other immunological detection systems such as immunofixation since the amounts of antiserum required are much less (Whitehouse & Putt,

1983; Mills, 1986).

1.5.3 Immunoblotting.

Immunoblotting involves the transfer of protein from the electrophoretic separation matrix by diffusion (passive blotting) or electrophoresis (electroblotting), to an immobilising membrane such as nitrocellulose (n/c) and subsequent detection using specific antisera (Renart *et al.* 1979; Towbin *et al.* 1979). An important practical feature of protein detection by immunoblotting, is the quenching of non-specific binding by reagent antibodies to the nitrocellulose, using either a protein solution such as bovine serum albumin (BSA), or a detergent such as Tween 20.

The methods of blotting, choice of transfer buffer, immobilising membrane, blocking agent, and final visualisation system, are devised to give optimal sensitivity and detection for each individual protein under investigation. Many researchers have devised or acquired methods of preference, a large number of which have been reviewed (Gershoni & Palade, 1983; Towbin & Gordon, 1984; Beisiegel, 1986).

1.5.3.1 Methods of Protein Transfer.

a) Passive blotting.

The passive blotting of polyacrylamide gels greater than 1mm thick, follows the reservoir technique devised by Southern for blotting nucleic acids (1975). The proteins are drawn out of the gel and onto the immobilising membrane by capillary action. However, this method is not particularly efficient, and extended blotting times of up to twelve hours are needed. The transfer of high molecular weight proteins

is particularly inefficient. Passive blotting from thinner gels such as IEF gels (0.15 to 0.5mm thick) requires considerably shorter transfer times, as little as thirty minutes (Whitehouse & Putt, 1983; Thomas *et al*. 1989). Nevertheless, considerable amounts of protein are left behind after passive transfer (Beisiegel, 1986). With the hope of improving protein transfer, vacuum blotting (in which capillary and suction forces are combined to draw proteins from the gel onto the immobilising membrane) has been investigated. However, the transfer efficiencies remained comparable with those obtained by passive transfer (Beisiegel 1986).

b) Electroblotting.

The electrophoretic method of protein transfer, "electroblotting" or "tank electroblotting", provides superior protein transfer coupled with greatly reduced blotting times (Towbin *et al*, 1979). As suggested by the name, tank electroblotting takes place in a large volume of transfer buffer in a tank which is fitted with platinium electrodes. During electroblotting at basic pH, the majority of proteins move towards the anodal face of the apparatus.

More recently, Kyhse-Andersen (1984) introduced another electroblotting technique which utilises minimal amounts of transfer buffer, hence the name "semi-dry electroblotting". This involves the horizontal transfer of proteins between two electrodes such as graphite, graphite based polymers, surface-conductive glass or stainless steel (Kyhse-Andersen, 1984; Millipore clinical news 1988; Svoboda *et al*. 1985). Semi-dry electroblotting is theoretically more efficient

than tank electroblotting since the inter-electrode distances are greatly reduced (approximately 1cm compared with 10cm inter-electrode distance in the tank apparatus) allowing lower operating voltages. This permits steeper voltage gradients, and less heating effects than the tank electroblotting method. Comparisons between these two electroblotting methods have confirmed that semi-dry electroblotting is indeed superior to conventional tank electroblotting for SDS gels. Greater amounts of protein can be transferred in considerably reduced times (from four hours to less than thirty minutes) (Tovey & Baldo, 1987). Further comparisons between these blotting systems have also revealed that high molecular weight proteins which are transferred with difficulty using the conventional method (Starita-Geribaldi *et al.* 1988), are more efficiently transferred by semi-dry electroblotting (Svoboda *et al.* 1985). On the other hand, electrotransfer of proteins from thinner polyacrylamide gels, such as IEF gels have been less well investigated.

1.5.3.2 Other Factors.

a) Transfer Buffers.

The most commonly used transfer buffer is the Tris/glycine/methanol (TGM) system devised by Towbin *et al* (1979). However, certain proteins have specialised transfer buffer requirements. Basic proteins require acidic transfer conditions, such as 0.7% acetic acid (Towbin *et al*, 1979; Biorad, transblot-cell booklet, 1983), while borate buffer systems are used for the transfer of high molecular weight glycoproteins, such as Apolipoprotein B (Myrseth *et al.*

1989). In addition, many other buffer systems have been adopted by different research groups, although it is seldom obvious why a particular buffer has been selected and to what advantage (Gershoni & Palade, 1983; Towbin & Gordon, 1984; Beisiegel, 1986).

b) Membranes.

The type of immobilising membrane normally chosen for immunoblotting studies is nitrocellulose due its high protein binding capacity (80-100$\mu g/cm^2$) and ease of use (Towbin *et al*, 1979; Andrews, 1987; Albaugh *et al*. 1987). However, other membranes such as activated diazobenzyloxymethyl-paper (Renart *et al*, 1979; Bittner *et al*. 1980), activated glass membranes (Aebersold *et al*. 1986), polyvinylidene difluoride (PVDF) membranes (Gültekin & Heermann, 1988) and nylon membranes (Miribel & Arnaud, 1988) have also been tried. Nitrocellulose membranes remain the most popular immobilising matrix.

c) Blocking.

Once protein transfer has occurred, the next step in the immunoblotting process is the choice of blocking agent and its working concentration. Certain proteins require very specific blocking conditions in order to obtain optimum results (Batteiger *et al*. 1982) and a great variety of protein solutions and detergents are available for these purposes. Care must be taken when employing certain reagents for blocking. For instance, at low concentrations, detergents can often eliminate non-specific background signals, but at high concentrations there may be some loss of transferred protein from the membrane surface (Hoffman &

Jump, 1986). Other blocking agents, such as dried skimmed milk, may also cause protein loss from the immobilising membrane (DenHollander & Befus, 1989).

1.5.3.3 Detection systems.

A vast number of schemes are available for the immuno-localisation of proteins including radiolabelled, enzyme-linked, biotinylated and colloidal gold labelled antibodies (Howe & Hershey, 1981; O'Connor & Ashman, 1982; Takatori & Tsutsubuchi, 1986; Moeremans *et al.* 1984). Whilst it is quite feasible for the primary antibody to be labelled, more commonly, double antibody systems are used, which are based on the solid phase enzyme-linked immunosorbent assays (ELISA) (Engvall & Perlmann, 1972; Voller *et al.* 1978). These often provide signal amplification and hence great sensitivity.

Radiolabelled antibody systems, although extremely sensitive, are expensive, require extended development times and special handling facilities (Beisiegel, 1986). More favourable means for the detection of blotted proteins are the highly sensitive non-radioactive antibody probes. These include the enzyme-linked antibodies and antibodies coupled to other reagents, such as colloidal gold.

Antibodies linked to enzymes such as horseradish peroxidase (HRP) and alkaline phosphatase (ALP) can be used in conjunction with a large variety of chromogenic substrates. For example, HRP conjugated antibodies can be visualised using hydrogen peroxide and compounds such as 3,3'-diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole. These reagents were first used in cellular ultrastructural studies

(Graham & Karnovsky, 1966; Graham *et al.* 1965). Alkaline phosphatase (ALP) conjugates can be visualised using nitrotetrazolium blue (NBT) (Blake *et al.* 1983) or amplified using NADP in the presence of diaphorase to precipitate the tetrazolium salt, INT-violet (Self, 1985). Both the HRP and ALP systems are capable of detecting between 10,000 and 30pg of antigen (Beisiegel, 1986; Iida *et al.* 1990; Blake *et al*, 1984; Doa, 1985), compared with the detection of (over a) picogram levels of antigen using radiolabelled antibodies (Beisiegel, 1986).

Chemiluminescent substrates used in conjunction with both HRP and ALP-linked antibodies have led to further increases in the sensitivity of enzyme-labelled ligands. Early work with chemiluminescence revealed great sensitivity when screening antigens by ELISA. However, the duration of the signal was extremely short and not particularly well suited to the detection of immunoblotted proteins (Whitehead *et al.* 1983; Maly *et al.* 1989). Enhancers such as firefly luciferin, substituted phenols, 6-hydroxybenzothioles and others, extended emission times and gave up to one thousand fold signal amplification (Thorpe *et al.* 1984; Thorpe *et al.* 1985.a.b).

Luminol-based systems give high sensitivity, fast development times and ease of use (Whitehouse & Hopkinson, 1985; Laing, 1986; Schneppenheim *et al.* 1991). With this in mind, Amersham International have produced an enhanced chemiluminescent kit which is claimed to detect femtomolar concentrations of immobilised horseradish peroxidase. This is made possible because of the flexibility

of the system's incubation times, which can vary from 5 seconds to 60 minutes (Amersham ECL booklet, 1992). Furthermore, an ALP-based chemiluminescent detection system developed by Tropix Inc, is claimed to give even greater sensitivity, detecting attomolar concentrations of immobilised alkaline phosphatase (Voyta *et al.* 1988; Bronstein *et al.* 1988; Bronstein & McGrath, 1989).

The other category of non-radioactive detection which has been extensively studied with particular relevance to electron microscopy, is the colloidal gold system (Hainfield, 1988). Gold particles are visualised via a reductive process which causes the precipitation of silver ions about the gold particles immobilised to the second antibody. Studies have shown that this system when applied to immunoblotted proteins, is capable of detecting approximately 50 pg of nitrocellulose-bound antigen (Moeremans *et al.* 1984, 1987).

Further enhancement of the double antibody systems is possible by the addition of a third ligand, such as the peroxidase-anti-peroxidase (PAP), alkaline phosphatase-anti-alkaline phosphatase (APAP) biotinylated antibodies, which allow the production of larger reporter molecule complexes (Hsu & Raine, 1981; Rohringer & Holden, 1985; Takatori & Tsutsubuchi, 1986; Elias *et al.* 1988). These systems utilise the high avidity of streptavidin for the vitamin biotin (k_d= 10^{-15}M). Each molecule of streptavidin, isolated from the bacterium *Streptomyces avidinii*, binds four molecules of biotin. Biotinylated antibodies incubated with biotin-streptavidin complexes conjugated to peroxidase, for

example, result in the formation of large biotin-streptavidin complexes which possess higher densities of signal molecules than can be obtained by conventional means (Weber *et al.* 1989), see figure 3.18.

1.6 Aims of this project and thesis structure.

The main objectives of this thesis include the developmemt of immunological methods for the detection of genetic variation in human proteins after electrophoretic separation. Ways of improving the sensitivity of the standard immunoblotting methodology (Towbin *et al.* 1979) are examined in detail using two polymorphic proteins, GC and PGM, as working and experimental systems respectively. Once the optimal conditions of immunodetection are established, these experimental procedures are applied to the detection of protein polymorphisms in forensic material, supplied by the Home Office laboratories.

This thesis is organised into eleven chapters, seven of which are results chapters. Chapter 3 describes the detailed examination of the steps involved in the electrophoretic separation and immunodetection of the human GC protein. Chapter 4 describes studies on the binding properties of the sheep anti-rabbit PGM antiserum. Chapter 5 describes the step by step examination of the electrophoretic separation and detection of the PGM1 polymorphism by immunoblotting. Chapter 6 reports a novel use of the sheep anti-rabbit PGM antiserum for the examination of PGM2 isozymes after IEF of red cell lysates. Chapter 7 describes the further characterisation of PGM4 in human milk samples, using both the sheep anti-rabbit PGM antiserum and enzyme activity

staining. Chapter 8, describes a method for the immunopurification of human PGM1 from skeletal muscle using the IgG fraction of the sheep anti-rabbit PGM antiserum, its use as an immunogen and the characterisation of the binding properties of the rabbit anti-human PGM antiserum subsequently produced. An alternative method for the large scale production of the purified rabbit PGM protein is also reported in this chapter. The final results chapter 9, describes the application of experimental data obtained for the ultra-sensitive immunodetection of the GC and PGM1 polymorphisms, reported in chapters 3 and 5 to the immunological visualisation of these proteins in forensic material. An overview of all the reported data is provided in the main discussion, chapter 10 and the cited references are listed in chapter 11.

Chapter 2.

Materials and methods.

2.1 Sheep immunisation protocol.

On day one, a single male sheep was immunised intramuscularly with 1 mg of rabbit skeletal muscle phosphoglucomutase (Boehringer mannheim) (specific activity 200 units/mg) in 1ml of Freund's complete adjuvant and sterile saline. Thirty days later, 1 mg of rabbit PGM in incomplete Freund's adjuvant was injected subcutaneously and after another thirty days 1 mg of rabbit PGM, in sterile saline, was given intravenously (following the administration of 20 mg of promethazine to prevent anaphalaxia). Four days after the third injection, and on several subsequent occasions, blood was collected by venipuncture. The whole blood was allowed to clot and subsequently ringed. The clot was allowed to retract at 37°C for several hours, prior to centrifugation at 300 x g. The serum was collected and stored at -70°C. An IgG fraction of this antiserum was prepared by protein G sepharose chromatography as described in section 2.2.3.1.

2.2 Sample preparation.

Human plasma was separated from red cells by the centrifugation of whole EDTA blood at 300 x g. The red cells were washed three times with isotonic saline and haemolysates prepared by freezing and thawing.

Aqueous tissue extracts were prepared by homogenising 1g of tissue with 2ml of chilled distilled water. These samples were centrifuged at 8,000 x g for 10 mins for the placentae and 38,000 x g for 30 mins for skeletal muscle. The clear

supernatant was used in a variety of studies.

Human milk samples were obtained from mothers who had recently delivered in the maternity wards at University College Hospital. Milk samples between 1ml and 20ml were expressed into sterile universal tubes and these were stored at room temperature or 4°C. Small fingerprick bloodspots were also obtained from the mothers and were soaked onto small squares of Whatman 3MM paper which were later extracted in an identical fashion to the stain extractions in section 2.2.1.

Serial milk samples were obtained from two mothers who had recently delivered. Ten milk samples at 27 days post-partum (pp), 28 days pp, 39 days pp, 40 days pp, 41 days pp, 42 days pp, 43 days pp, 44 days pp, 55 days pp and 62 days pp were obtained from the first mother. Twelve consecutive milk samples between 141 and 152 days pp were obtained from the second mother.

Raw sheep and cow milk was obtained from the Royal Veterinary College (London) and two mouse milk samples were obtained from Dr.Archibald (Edinburgh University).

Milk fat was separated from the aqueous fat globule layer by centrifugation at 12,000 x g, and the lower aqueous layer was stored at -70°C or 4°C.

2.2.1 Stain preparation and extraction.

Blood and semen stains were prepared by the Home Office Forensic Science Service as follows: Approximately 200µl of the body fluid was applied to squares of boiled white cotton and air dried for approximately 2 hours at room

temperature before storage at -70°C.

Bloodstains measuring approximately 20mm² and semen stains measuring 24mm² were extracted at room temperature for 1 hour in 0.5ml Eppendorf tubes (Scotlab) using either 25µl of 6M urea (Westwood, 1986) or 6M urea plus 0.5% bovine serum albumin (BSA; grade v, ICN Biochemicals) (Pflug, 1988.a). A small hole was made in each extraction tube prior to the extraction procedure and the extract was recovered into a larger 1.5ml Eppendorf tube by centrifugation at 12,000 x g for 30 seconds.

2.2.2 Sample diluents.

Tissue extracts, plasma samples and stain extracts were diluted using a series of diluents. These included: distilled water, phosphate buffered saline (PBS) (10 times stock solution: 1.37M NaCl, 0.02M KCl, 0.08M $Na_2HPO_4.2H_2O$, 0.015M KH_2PO_4 (pH 7.4); PBS plus 0.5% BSA; PBS plus 0.5% haemoglobin (Sigma); PBS plus 0.5% powdered skimmed milk (Marvel) and PBS plus 0.5% gelatin (Sigma).

2.2.3 Sample treatment.

2.2.3.1 Protein G immunoaffinity purification of whole antisera from various animal species.

An affinity purification column was prepared using protein G sepharose 4 fast flow (Pharmacia). The sepharose was stored at 4°C in 20% methanol. This was washed several times on a sintered glass filter with 50ml of the starting 20mM Sodium phosphate buffer, pH 7, resuspended and allowed to pack on the column for one hour. Following preparation, the column was washed with 25 ml of the phosphate buffer prior to the

purification steps. 5 ml of the whole antiserum was loaded onto the column and recirculated slowly four times at room temperature. The column was subsequently washed with approximately 80ml of phosphate buffer before elution with 25ml of ice cold 0.1M glycine/HCl, pH 2.7. 1 ml elution fractions were collected into 63µl of 1M Tris/HCl, pH 9, which gave a final pH of 7.3. The column was washed with 25ml of the phosphate buffer, prior to storage at 4°C in phosphate buffer plus 0.1% Sodium azide. Finally, the optical density (od) of the elution fractions was checked by spectrophotometry at 280 nanometres. Samples showing high od were pooled, the IgG concentration calculated (4-6mg of IgG/ml) and they were stored at -70°C as 1 ml aliquots.

2.2.3.2 Iodination of rabbit PGM.

Purified rabbit PGM (BCL) was labelled with ^{125}I by Amersham International using the chloramine-T method (Hunter & Greenwood, 1962) (5 microcuries per 100µg of rabbit PGM). Following IEF and electrotransfer of the iodinated rabbit PGM in the normal fashion (sections 2.5.2 and 2.6.3), the dried blotted gel and the corresponding nitrocellulose filter were exposed to X-ray film in an autoradiography cassette for four days at -70°C. The films were developed for approximately two minutes at room temperature (section 2.7.5(f)).

2.2.3.3 Neuraminidase treatment.

Milk samples were treated with neuraminidase (type v and vi, 1.8 units/mg and 3 units/mg respectively, BCL) diluted in the neuramindase buffer (0.01M Na_2HPO_4 and saturated citric acid, pH 5), to give a specific activity of 50 units/ml. 2µl

of the diluted neuraminidase was used to treat 25µl of sample, overnight at 37°C.

2.3 Ouchterlony double diffusion analysis.

Ouchterlony plates were poured using 1% HSA agarose (Park Scientific Ltd) to which 3% polyethylene glycol 6,000 (PEG) was added. Seven wells were cut and 10µl of each sample was loaded, with the central well used for the antiserum. The samples were left to diffuse overnight in a moist chamber at 4°C and were then pressed onto agarose gel bond (FMC Bioproducts) for one hour, dried and washed in PBS for 2 to 4 hours and finally stained in filtered 0.1% Coomasie blue R250 (National Diagnostics) in water: methanol: glacial acetic acid (5:5:2 by volume) for five minutes and destained in 10% acetic acid.

2.4 Dot immunobinding analysis.

Rabbit skeletal muscle phosphoglucomutase was applied to dry nitrocellulose in a series of 1µl spots (2.5ng of PGM/µl). The filter was allowed to air dry for approximately 30 minutes. The rest of the immunological procedure was carried out as described in section 2.6.3, except for the use of dilutions of the primary antiserum as low as 1 in 372,000.

2.5 Electrophoretic separations.

2.5.1 Starch gel electrophoresis.

11% starch gels (Cannaught Labs. Ltd) were made using a 1 in 10 dilution of the TEMM bridge buffer (0.1M Tris, 0.1M maleic acid, 0.01M $MgCl_2.7H_2O$, adjusted to pH 7.4 with NaOH). Samples were soaked into No.17 Whatman paper squares, which were pushed into the gel. Electrophoresis was carried out for 17 hours at 100 to 110 volts and 60 to 70 mA. The

gel was subsequently sliced and incubated in the PGM activity staining reaction mixture at 37°C for approximately 1 hour (see section 2.6.2) (Spencer *et al.* 1964).

2.5.2 Isoelectric focusing.

2.5.2.1 PGM.

Polyacrylamide gel isoelectric focusing of PGM was performed on gels measuring 240 x 100 x 0.4mm, prepared from the following mixture: 2ml 87% glycerol; 1.5ml acrylamide/bis-acrylamide 29:1 (Biorad); 6 µl N,N,N',N'-tetramethylethylene-diamine (TEMED) (Biorad); 170 µl of 3% ammonium persulphate (AMPS) (Biorad), and approximately 2.5% w/v Ampholytes (LKB) (pH range 5 to 7 for human samples, 6 to 8 for rabbit tissue and broad range 3.5 to 10, in some instances). The volume was made up to 15ml with deionised water.

Electrode contact with the gel was established using strips of Whatman No.17 MM paper soaked in 1M H_3PO_4 (Sigma) (anode) and 1M NaOH (cathode). Gels were prefocused with a limiting voltage of 300 V for 30 mins. 5 µl aliquots of haemolysates, tissue extracts and stain extracts were either applied directly to the gel 1cm from the anode, or soaked onto Whatman No.3 filter papers measuring 6 x 4mm. Focusing was carried out at 300 V for 30 mins after which time the settings were increased to 2000 V, 8 mA and 10 W for 3.5 hours. The sample application papers were removed from the gel ३० mins after this voltage increase. Following separation, PGM was either subjected to enzyme activity staining, as described in 2.6.2. or immunoblot analysis as described in section 2.6.3.

2.5.2.2 GC.

GC IEF gels were prepared from the following mixture: 2ml 87% glycerol; 1.5ml acrylamide/bis-acrylamide 29:1 (Biorad); 6 µl TEMED; 170µl 3% ammonium persulphate (AMPS); 0.375g 3-[N-morpholino] propanesulphonic acid (MOPS) (Sigma); 0.125g N-2-hydroxyethylpiperazine N-2-ethanesulphonic acid (HEPES) (Sigma) and approximately 2.5% w/v of Pharmalytes (Pharmacia) (pH range 4.5 to 5.4). The volume was made up to 15ml with deionised water.

Following the prefocusing step as described for PGM, 5µl aliquots of diluted plasma or stain extracts were either directly applied onto the gel at 1cm from the cathode or by filter paper application, as described previously. Focusing was carried out at 300 V for 30 mins after which time the settings were increased to 1800 V, 15 mA and 10 W for 3 hours 15 mins. The sample application papers were removed from the gel 30 mins after this voltage increase. After focusing the gel was immunoblotted, as described in section 2.6.3.

2.5.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Polyacrylamide 5-15% gradient and fixed 7.5% sodium dodecyl sulphate (SDS) gel electrophoresis was carried out as described by Karlsson *et al.* (1983), in conjunction with the discontinuous buffer system devised by Laemmli (1970). The resolving part of the 5-15% gel was made as follows: a) The 5% acrylamide solution consisted of 2.8ml acrylamide: bis-acrylamide 37.5:1 (Biorad); 3.3ml 1.87M Tris/HCl, pH 8.8; 15µl TEMED; 200µl 10% SDS; 100µl 10% AMPS; 10.5ml

distilled water.

b) The 15% acrylamide solution consisted of 1.8ml glycerol; 8.4ml acrylamide: bis-acrylamide 37.5:1; 3.3ml 1.87M Tris/HCl, pH 8.8; 15µl TEMED; 200 µl 10% SDS; 50µl 10% AMPS; 3ml distilled water.

These solutions were made separately and poured into the gel mould using a double compartment gradient mixer. Once poured a layer of distilled water was pipetted on top of the gel until set. The was subsequently discarded and the stacking gel pipetted on top of the set resolving gel. The stacking gel consisted of the following: 2.5ml acrylamide: bis-acrylamide 37.5:1; 2.5ml 1M Tris/HCl, pH 6.8; 20µl TEMED; 200µl 10% SDS; 100µl 10% AMPS; 15ml distilled water.

The 7.5% acrylamide gel consisted as follows: The stacking gel was the same as described previously. The resolving gel consisted of 9.4ml acrylamide: bis-acrylamide 37.5:1; 7.5ml 1.87M Tris/HCl, pH 8.8; 20µl TEMED; 400µl 10% SDS; 100µl 10% AMPS; 20.3ml distilled water.

The gel dimensions were 15 x 13 x 0.1 cm, and the tank buffer was composed of 0.025M Tris, 0.192M glycine and 0.1% SDS, pH 8.3. Samples were prepared prior to electrophoresis by boiling in 2% SDS, 10% glycerol, 5% mercaptoethanol (Sigma) and a trace amount of bromophenol blue in 0.064M Tris/HCl, pH 6.8.The gradient gels were run vertically at a constant current of 20mA for approximately 17 hours at room temperature, until the dye front reached the bottom of the gel. The 7.5% gels were run at a constant current of 90 mA for 6.5 hours at room temperature, and the dye front was

allowed to run off the gel. The separated proteins were subsequently subjected to either Coomassie blue protein staining or immunoblot analysis, as described in sections 2.3 and 2.6.3, respectively. Coloured molecular weight markers (200-14.3 Kda.) were supplied by Amersham International.

2.6 Protein detection systems.

2.6.1 Non-specific protein staining.

As described in section 2.3.

2.6.2 PGM enzyme activity staining.

The constituents of the PGM enzyme activity staining system as described by Spencer *et al.* (1964) were as follows: 10ml 0.5 M Tris, pH 8; 1ml 0.2 M $MgCl_2.7H_2O$; 1ml NADP (5 mg/ml) (BCL); 50mg glucose-1-phosphate (G1P) (grade vi) (Sigma); 100µl glucose-6-phosphate dehydrogenase (G6PD) (BCL) 1 in 10 dilution in saturated ammonium sulphate; 2 ml tetrazolium salt, (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml) (Sigma); 0.5ml phenazine methosulphate (PMS) (5 mg/ml) (Sigma); 10ml 2% agar noble (Difco labs.).

2.6.3 Standard immunoblot analysis.

2.6.3.1 IEF gels.

Following IEF, the gel was peeled off the casting plate using a dry sheet of Whatman 3MM paper. A piece of nitrocellulose pre-wetted with the transfer buffer was placed on top of the gel, and a cut was made in one corner to provide orientation. A piece of wet Whatman 3MM paper was placed on top of the nitrocellulose/gel layers, which was sandwiched between two soaked scotchbrite pads and placed in

a blotting cassette. The cassette was then placed in the buffer-filled Biorad Trans-blot tank with the nitrocellulose facing the anode. Electrotransfer was made towards the anode at limiting current (0.6 A) for 1 hour. Subsequently, the membrane was separated from the gel and subjected to a blocking step for 30 minutes. Subsequently, the membrane was incubated in the primary antiserum overnight at 4°C with agitation, given 4 X 10 minute washes with the blocking agent, and incubated in the secondary conjugated antiserum for one hour at room temperature with agitation. After washing as before the membrane bound antigens were visualised with one of many enzyme linked detection systems.

2.6.3.2 SDS gels.

The SDS gel was separated from the glass plate, and the nitrocellulose Whatman paper sandwich was arranged as for IEF gels. Great care was taken to exclude any air bubbles from the apparatus by assembling the sandwich completely submerged in the transfer buffer (0.025M Tris, 0.192M glycine, 20% methanol, pH 8.3). No other transfer buffer was used for the transfer of proteins from SDS-gels. Electrotransfer was carried out overnight at limiting current (0.15 A) with cooling. The subsequent steps were carried out as described for IEF gels.

2.6.3.3 Other methods of protein transfer.

a) Passive transfer.

This simple diffusion method of protein transfer was only applied to IEF gels. The gel was kept fixed to the glass plate during the transfer. To ensure optimal passive transfer results, the gel was kept wet during the whole

transfer procedure. The nitrocellulose was placed on top of the gel, followed by three pieces of buffer wetted Whatman 3MM papers and two dry ones. A glass plate was placed on top of the assembly which was then wrapped in clingfilm and compressed with a 1kg weight. The transfer took place at room temperature for 1 hour.

b) Semi-dry electrotransfer.

The efficiency of protein transfer was examined using two semi-dry electroblotting apparatus: The LKB semi-dry blotter, consisting of graphite electrodes, and the Millipore semi-dry electroblotter (SDE), consisting of tough plastic polymer electrodes. Transfers were carried out either using a continuous buffer system such as TGM, or a discontinuous buffer system such as that proposed by Kyhse-Andersen (1984), see figure 2.2.2. The transfer assembly consisted of nine pieces of Whatman 3MM either side of the gel and nitrocellulose membrane. All these papers were soaked in the same buffer for the continuous buffer system transfer. Only one discontinuous buffer system was examined (Kyhse-Andersen, 1984), and was assembled as follows: nine cathodic papers soaked in 0.040M 6-amino-n-hexanoic acid (Sigma)/0.025M Tris/20% methanol, pH 9.4; polyacrylamide gel; nitrocellulose; three anodic papers soaked in 0.025M Tris/20% methanol, pH 10.4; and six anodic papers soaked in 0.3M Tris/20% methanol pH 10.4. Transfer was carried out for 1 hour at room temperature with no cooling at limiting current, 0.58 A.

2.7.1 Immobilising membranes.

All membranes used in this work are listed in figure 2.1 and had a pore size of 0.45μm. All membranes were used as described above (2.6.3.1), except the polyvinylidene difluoride membrane (PVDF) (Millipore), which was prewetted with methanol and then equilibrated with the transfer buffer for 5 minutes prior to use.

2.7.2 Blotting buffers.

The different transfer buffers used for the blotting procedures are listed in figures 2.2.1 and 2.2.2.

2.7.3 Blocking agents.

The following blocking agents were examined: BSA, Tween 20 (Sigma), Nonidet-P40 (NP-40) (Sigma), Sarkosyl (Sigma), gelatin (Sigma), Skimmed milk (Marvel).

2.7.4 Antibody incubations.

The sheep anti-rabbit PGM antiserum was used at a working dilution of 1 in 500 in 3% BSA (10μg/ml). The goat anti-human GC antiserum (Atlantic antibodies) was used as recommended at a dilution of 1 in 1500. Rabbit anti-goat HRP conjugated antiserum (Dakopatts) was used as recommended at a 1 in 1500 dilution. The ALP linked antiserum was used at a 1 in 1000 dilution (Serotec).

The biotinylated antibodies were used as follows:
a) The vectastain ABC kit (Vectastain): incubation in biotinylated antibody for three hours at room temperature (two drops biotinylated antiserum in 20 ml of the blocking buffer). The filter was washed in the blocking buffer as described previously, followed by incubation in reagents A

Name of immobilising membrane	Manufacturer	Composition
Nitrocellulose	Schleicher & Schull	Nitrocellulose
Supported nitrocellulose	Schleicher & Schull	Supported nitrocellulose
Hybond C	Amersham International	Nitrocellulose
Hybond super C	Amersham International	Supported nitrocellulose
Hybond N	Amersham International	Nylon
Gene screen	Amersham International	Nylon
Zetaprobe	Biorad	Nylon
PVDF	Millipore	Polyvinylidene difluoride

Figure 2.1 Table listing some immobilising membranes, manfacturers and composition

Mode of protein transfer	Transfer buffer
Passive blotting	0.02M Tris / 0.152mM glycine (pH 8.3) (modified from Miribel & Arnaud, 1988) 0.02M Tris / 0.152M glycine + 20% methanol (pH 8.3) (modified from Towbin *et al.* 1979) 0.02M Tris / 0.152M glycine + 20% methanol +0.1% SDS (pH 8.3) (modified from Beisiegel, 1986) 0.1M Sodium citrate (pH 5.0) 3mM di-Sodium carbonate / 10mM Sodium Hydrogen carbonate + 20% methanol (pH 9.9) (Dunn, 1986) 40mM boric acid / 10mM Sodium tetraborate (pH 9.2) (Myrseth *et al.* 1989) 50mM Tris / 150mM Sodium Chloride (pH 10.3) Thomas *et al.* 1989)

Figure2.2.1 Transfer buffers used for the passive transfer of proteins onto nitrocellulose membranes.

Mode of protein transfer	Transfer buffer
Tank electroblotting	0.02M Tris / 0.152M glycine (pH 8.3). 0.02M Tris / 0.152 glycine + 20% methanol (pH 8.3) 0.02M Tris / 0.152M glycine + 20% methanol + 0.1% SDS (pH 8.3) 3mM di-Sodium carbonate / 10mM Sodium Hydrogen carbonate + 20% methanol (pH 9.9) 40mM boric acid / 10mM Sodium tetraborate (pH 9.2) 0.7% acetic acid (pH 5.0) (Blot towards cathode) (Towbin *et al.* 1979) 0.1M Sodium citrate (pH 5.0) (Blot towards cathode)
Semi-dry electroblotting	40mM 6-amino-n-hexanoic acid / 25mM Tris / 20% methanol. (pH 9.4) / 0.3M Tris / 20% methanol . (pH 10.4) / 25mM Tris / 20% methanol (pH 10.4). (Kyhse-Andersen, 1984) 0.02M Tris / 0.152M glycine (pH 8.3) 0.02M Tris / 0.152M glycine + 20% methanol (pH 8.3) 0.02M Tris / 0.152M glycine + 10% methanol (pH 8.3) (modified from Towbin *et al.* 1979).

Figure 2.2.2 Transfer buffers used for the electrotransfer of proteins onto nitrocellulose membranes.

and B (four drops of each in 20 ml of blocking solution) for 2 hours at room temperature. The filter was washed and stained.

b) The HRP and ALP Amersham kit: The filter was incubated in a 1 in 500 dilution of biotinylated antibody for 20 minutes. The filter washed as before and incubated in a 1 in 1000 dilution of the streptavidin complex for 20 minutes.

The filter was washed again and stained.

2.7.5 Detection systems.

a) Conventional HRP/DAB stain: 25ml PBS, 1mg 3,3'-diaminobenzidine (DAB) (Sigma isopac, 100mg) dissolved in 100ml of PBS (0.1% solution), 12.5µl 30% hydrogen peroxide (H_2O_2) (Sigma) (Graham & Karnovsky, 1966).

b) HRP/DAB enhancement: The filter was washed in 0.5% Cobalt Chloride for 5 minutes prior to staining in the normal fashion. 25ml PBS, 1mg DAB, 12.5µl H_2O_2 (Adams, 1981).

c) HRP/3-amino-9-ethylcarbazole: 30ml PBS, 25mg carbazole (Sigma) dissolved in methanol and 30µl H_2O_2 (Graham *et al.* 1965).

d) HRP/tetrazolium: 20ml PBS, 40mg NADH (BCL), 4mg phenol (BDH), 6mg NBT (Sigma), 12.5µl H_2O_2 (Taketa *et al.* 1986).

e) HRP/DAB-NAP mixture: 30mg 4-chloro-1-naphthol (NAP) (Sigma) dissolved separately in 5ml methanol, 2mg DAB, mixed together in 40ml PBS plus 12.5µl H_2O_2 (Young, 1989).

All the above reaction mixtures were incubated between 20 to 30 minutes at room temperature.

f) HRP/luminol: 2ml glycerol, 16ml 0.01M Tris/HCl pH 8, 4mg luminol (Sigma), 25µl H_2O_2. The reaction mixture was incubated with the filter for approximately 1 minute, discarded, and the excess solution allowed to drip from the filter surface. The membrane was wrapped in saran wrap and fixed to the inside of an X-ray cassette. ECL hyperfilm (Amersham) was exposed to the filter at room temperature for 1 to 5 minutes. It was developed in phenisol (Ilford) for approximately 2 minutes, stopped and fixed (Ilford) for approximately 1 minute.

g) HRP/luminol/p-iodophenol: 16ml 0.01M Tris/HCl pH 8, 4mg luminol, 100µl 20mM p-iodophenol (Sigma), 25µl H_2O_2. Hyperfilm exposure time between 1 and 5 minutes.

h) HRP/ECL: This was used in kit form, supplied by Amersham International. The nitrocellulose filter was incubated in an equal volume of reagents A and B for 1 minute, then drained and assembled in the cassette as before. Hyperfilm exposure times varied from 5 seconds to 30 minutes.

i) Alkaline phosphatase stain (from the Amersham ALP antibody kit): 20ml diethanolamine buffer (100mM diethanolamine, 5mM $MgCl_2.6H_2O$, pH 9.5), one drop of nitro-blue tetrazolium (NBT) in 70% dimethylformamide and one drop of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 100% dimethylformamide. This reaction mixture was either applied in liquid form or as a 2% agar overlay. Both systems were allowed to develop for 20 to 30 minutes at 37°C.

2.8 PGM subtraction experiments.

PGM subtraction experiments were carried out on samples including human skeletal muscle extracts, placental extracts, red cell lysates and human and mouse milk. Subtraction was carried out by two methods:

a) The sample was incubated with the sheep anti-rabbit PGM IgG fraction for 1 hour at room temperature, followed by electrophoretic separation and visualisation by activity staining.

b) The sample was applied to a protein G sepharose column, previously loaded with the sheep anti-rabbit PGM antiserum, as described in section 2.2.3.1. The sample was passed through the column, collected and was used for electrophoretic analysis. No elution step was required.

As a control, aliquots of the untreated sample were retained for comparison with the treated sample in each case.

2.9 PGM degradation experiments.

A set of rabbit and human tissue extracts were made. The extracts were divided into 1ml aliquots and stored at room temperature and 37°C from 17 hours to 22 days. Each sample was transferred from the degradation chamber and to a -70°C freezer. Once the series of degradations were completed, all the samples were thawed out and analysed simultaneously.

2.10 The purification of human PGM.

2.10.1 Protein G immunoaffinity chromatography.

The sheep anti-rabbit PGM antiserum was passed through a protein G column as described in section 2.2.3.1. The column was subsequently washed with 80ml of isotonic saline,

and loaded with 5ml of a 1 in 10 dilution of human skeletal muscle extract, which was recirculated three times through the column. The recirculated muscle extract was kept for later analysis. The column was washed again with isotonic saline, prior to elution with 0.1M glycine/HCl (pH 2.7). 1ml fractions were collected into 63µl of 1M Tris/HCl (pH 9.0). Aliquots of the human muscle extract taken before and after passage down the column, wash fractions collected during the purification and the acid elution fractions, were all analysed by SDS-PAGE.

2.10.2 Preparation of purified human PGM antigen for immunisation.

After the analysis and positive identification of human PGM1 from selected elution fractions, those eluates containing PGM1 were pooled and applied to a 7.5% SDS-polyacrylamide curtain gel. After separation and electroblotting onto nitrocellulose was carried our as described in sections 2.5.3 and 2.6.3, Strips from each end of the filter were taken and probed with the sheep anti-rabbit PGM IgG fraction in order to locate the position of the purified human PGM1 and an area measuring approximately 10 x 0.5cm, corresponding to this band, was cut from the immunoblot. Nitrocellulose strips from several such immunopurifications were pooled to obtain 25mg dry weight of immobilising membrane. The strips were dissolved in 1ml of dimethylsulphoxide (DMSO) (Sigma) and sterile 0.08M Sodium carbonate buffer (pH 9.0) was added in a dropwise manner to this solution, while vortexing constantly, until a 2:1 ratio of carbonate buffer to DMSO was reached. The addition of carbonate buffer in this fashion, caused the n/c to

precipitate as a fine powder, which was harvested by centrifugation at 12,000 X g for 5 minutes. The supernatant was discarded and the pellet was washed three times in sterile isotonic saline prior to injection through a size 21g needle.

2.10.3 The rabbit immunisation schedule.

The first immunisation consisted of three 0.3ml subcutaneous injections of n/c powder in sterile isotonic saline, at different sites on the back. The animal was bled from the ear 37 days later and the antiserum was tested. The same week, two intramuscular injections of nitrocellulose powder were administered, 0.7ml in the left leg and 0.8ml in the right leg. The rabbit was bled again 37 days later and the antiserum tested.

2.11 Preparation of recombinant rabbit PGM from a lambda gt11 recombinant lysogen.

10ml of LM medium (1% tryptone, Difco Labs; 0.5% yeast extract, Difco Labs; 80mM NaCl; 8mM $MgSO_4.7H_2O$, plus 0.2% maltose), was innoculated with one colony of *E.coli* Y1089 and incubated overnight with agitation at 37°C. 100µl of Y1089 cells were infected with 100µl of amplified lambda stock of rabbit cDNA. Y1089 cells were also infected with lambda gt11 which acted as a control. The infected cells were incubated at 32°C for 20 minutes, and the following dilutions were made: (a) 1µl of infected cells plus 1ml of phage storage medium (PSM) (0.1M NaCl, 8mM $Mg_2SO_4.7H_2O$, 0.05M Tris, 2% gelatin, pH 7.5), whirlimixed.

b) 20µl of (a) plus 1ml PSM, whirlimixed.

c) 100µl of (b) and plated onto LM agar plates (1% tryptone;

0.5% yeast extract; 80mM NaCl; 8mM $MgSO_4.7H_2O$; 1.5% agar, Difco Labs).
Subsequently, the plates were incubated at 32°C overnight.

Thirty five colonies from the clone and the control plates were picked, and plated out in a grid pattern using cocktail sticks on LM agar plates in duplicate. One set was incubated at 32°C and the other at 42°C, overnight.

The lysogens which contained the recombinant lambda were identified by growth at 32°C but not at 42°C. Two colonies from each 32°C plate were picked and grown in 7ml LM medium at 32°C with agitation for 3 to 4 hours, until the optical density at 600nm reached 0.4 to 0.5. Subsequently, the cultures were incubated at 43°C for 20 minutes. 350µl of 200mM isopropyl beta-D-thiogalactosidase (IPTG) (BCL) was added, and the cultures immediately returned to the shaker for 1 hour at 37 to 38°C. The cells were centrifuged at 1000 X g for 5 minutes at room temperature, the supernatant discarded and the tubes allowed to drain for a couple of minutes. 300µl of lysis buffer was added to each pellet (Lysis buffer, 50mM Tris, 1.5% SDS and 4M urea), and stored at 4°C until ready for analysis by SDS-PAGE. Dithiothreitol (DTT) (Sigma) was added to a concentration of 7.5mg/ml prior to electrophoretic analysis. (Method obtained from Huynh *et al.* 1985).

Unless stated otherwise, all reagents mentioned in this chapter were analytical grade and obtained from BDH (Poole, UK).

Chapter:3

The detection of human group-specific component by immunoblotting.

This chapter is concerned with the transfer and detection of the GC protein polymorphism. The aim is to increase the sensitivity of immunological detection by the investigation and refinement of all the steps involved in the immunoblotting process. Each step in the procedure, shown in figure 3.1, can be varied to a greater or lesser degree in order to obtain optimal conditions. A similar series of experiments is reported for the PGM1 isozyme polymorphism in chapter 5.

3.1 Sample preparation.

The detection of doubling dilutions of GC in human plasma was carried out using a standard immunoblotting procedure, illustrated in figure 3.2. Figure 3.3(a) depicts human GC in plasma diluted with distilled water and separated by IEF on a 4.5 to 5.4 pH gradient. Track 1, which contains a 1 in 128 dilution of plasma and corresponds to approximately 8ng of GC protein (average plasma GC concentration, 20mg per 100ml of plasma) displays a strongly stained GC band pattern. Track 2 is equivalent to approximately 4ng of GC and as expected shows approximately half the signal strength of track 1. Track 3, which has been loaded with approximately 2ng of GC protein, shows an extremely low signal which does not correspond to a doubling dilution. The same result was obtained in numerous experiments and only rarely was the limit of GC detection improved below 2ng. The reason for this cut off point in GC immunodetection was investigated further.

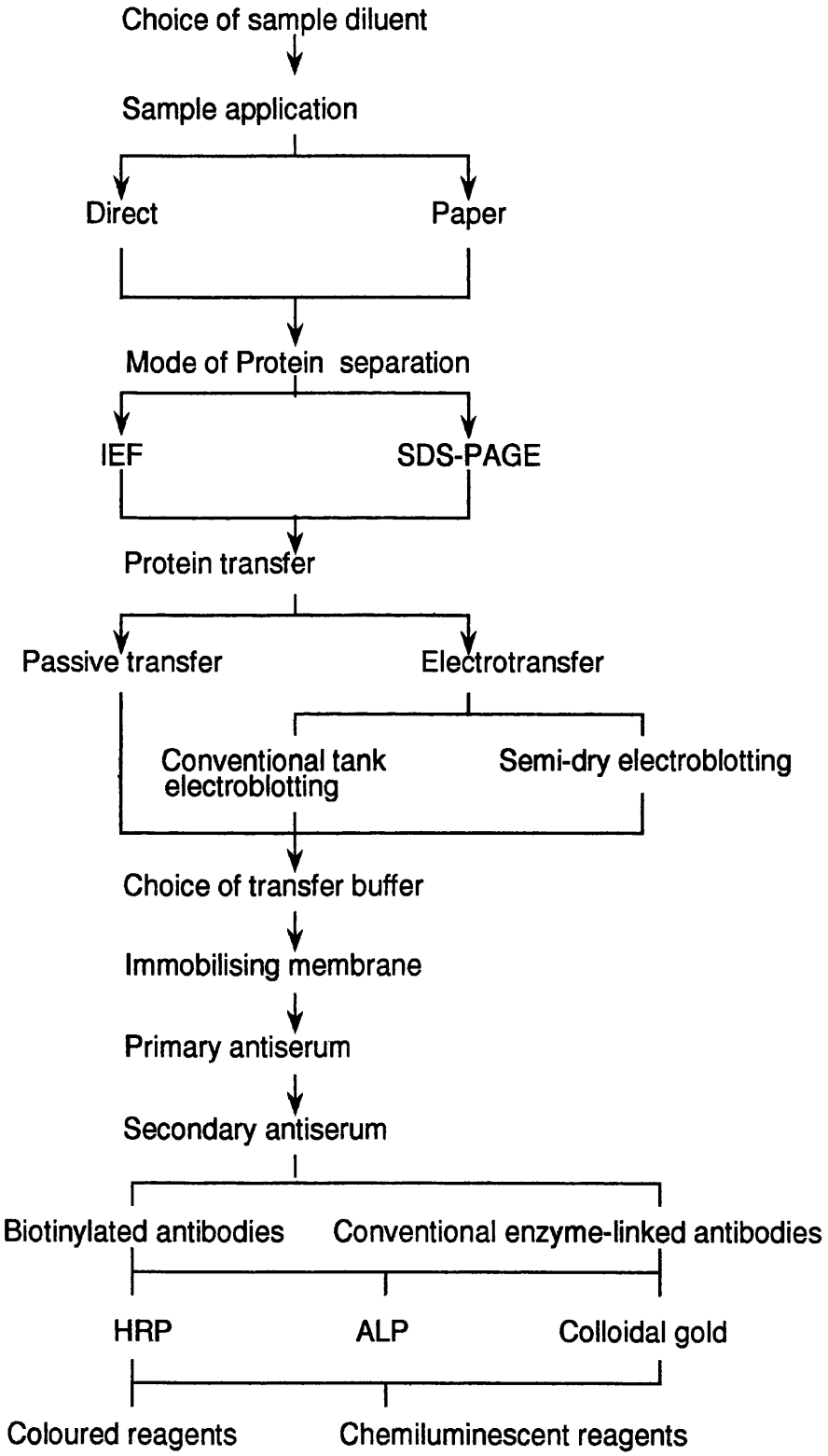


Figure 3.1 Steps which effect the detection of proteins by immunoblotting.

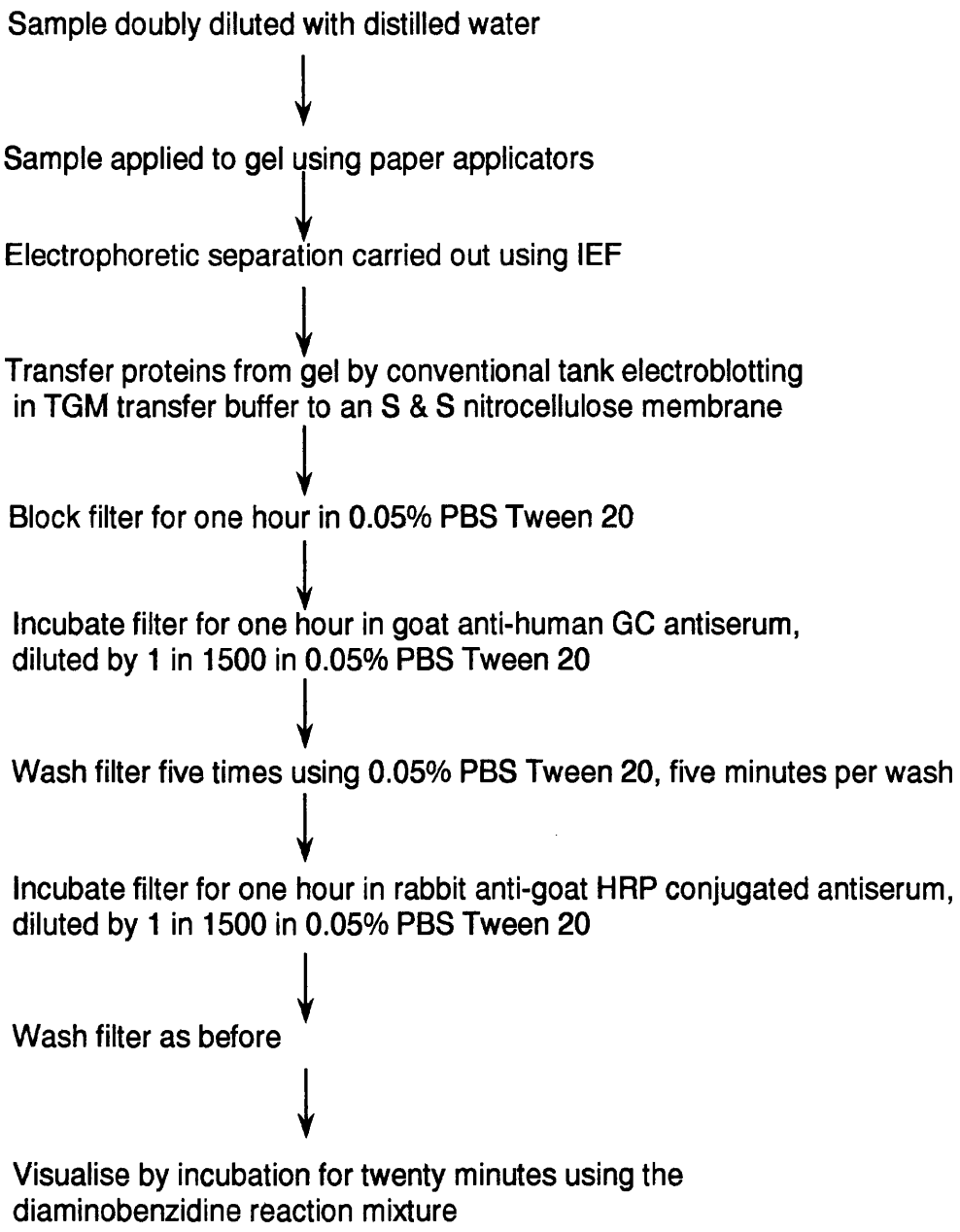


Figure 3.2 The standard procedure for the immunodetection of proteins by immunoblot analysis

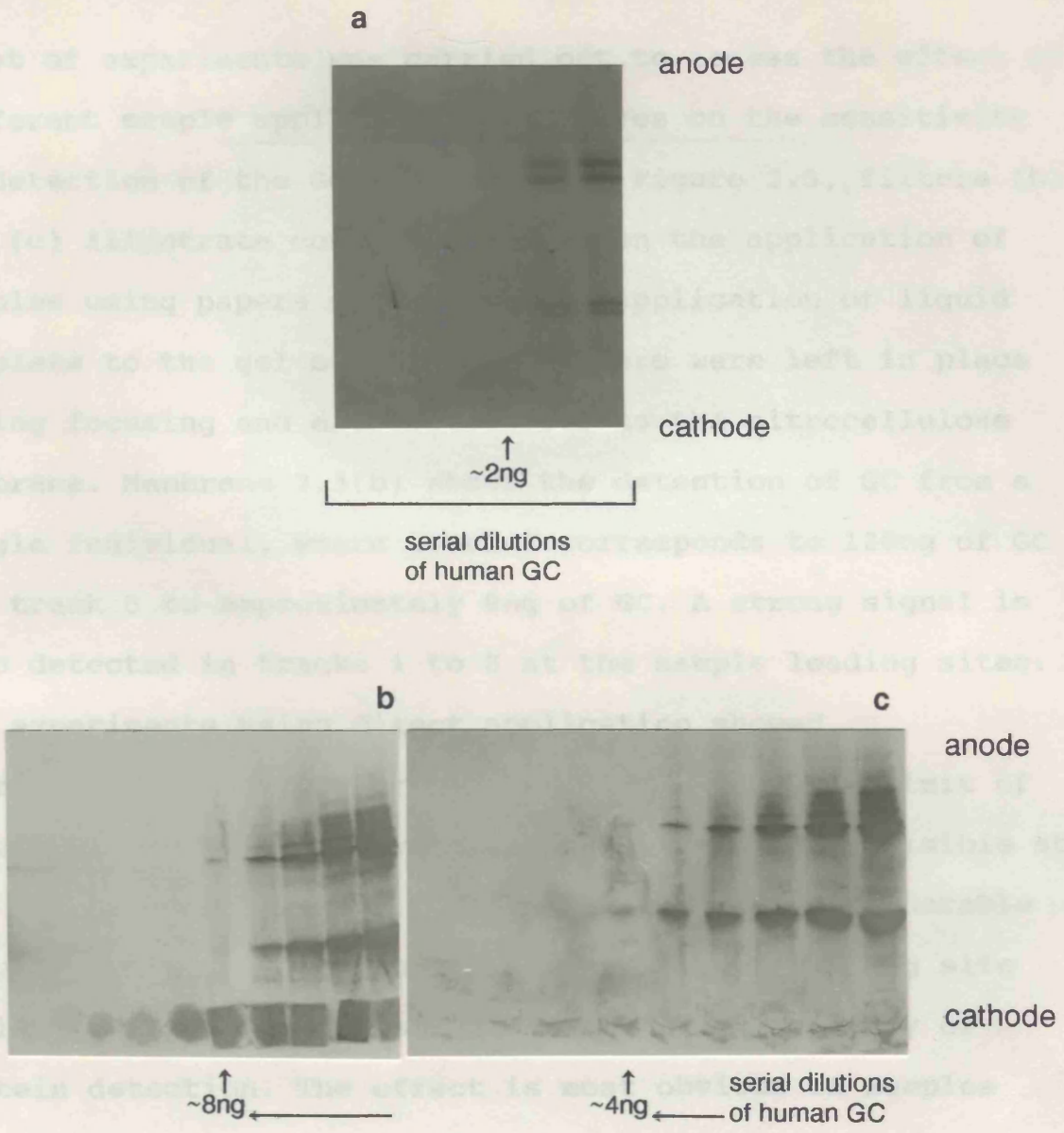


Figure 3.3
Immunological detection of human plasma GC following IEF on a 4.5-5.4 pH gradient and electrotransfer to a nitrocellulose membrane. Serial dilutions of plasma GC using water as the diluent (a). The effect of sample application using Whatman 3MM paper (b) and direct gel surface application (c), on the sensitivity of the immunological detection of GC.

A set of experiments was carried out to assess the effect of different sample application procedures on the sensitivity of detection of the GC polymorphism. Figure 3.3, filters (b) and (c) illustrate comparisons between the application of samples using papers and the direct application of liquid droplets to the gel surface. The papers were left in place during focusing and electrotransfer to the nitrocellulose membrane. Membrane 3.3(b) shows the detection of GC from a single individual, where track 1 corresponds to 128ng of GC and track 5 to approximately 8ng of GC. A strong signal is also detected in tracks 1 to 8 at the sample loading sites. The experiments using direct application showed approximately two fold greater sensitivity in the limit of detection. However, GC immunoreactivity was again visible at the sample loading sites. This indicates that considerable amounts of protein are not released from the loading site during the focusing, thus decreasing the sensitivity of protein detection. The effect is most obvious in samples with low protein concentrations.

Figure 3.4 compares the effects of two loading buffers on sensitivity. Panel (a) (PBS) illustrates the reduction of sensitivity of the immunoblotting system in comparison with figure 3.4(b), which shows the effect of adding 0.5% BSA. This results in a 16 fold increase in the sensitivity of detection of the GC isoforms, and a concurrent decrease in the amount of immunoreactive material at the sites of sample application.

Further experiments were performed to evaluate the

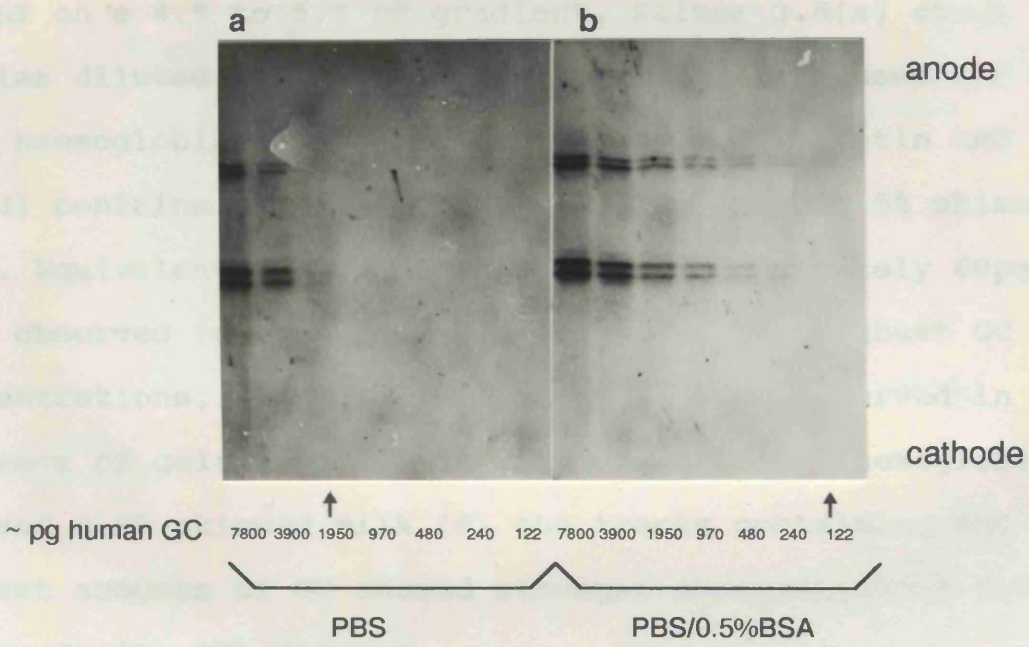


Figure 3.4

The immunological detection of human GC in plasma following separation by isoelectric focusing on a 4.5-5.4 pH gradient and electroblotting in the standard fashion. a) serial dilutions of GC 1F-1S plasma made with PBS. b) an identical series of dilutions made with PBS plus 0.5% BSA. The arrow indicates the highest dilution phenotyped in each panel.

properties of other proteins: haemoglobin, gelatin and skimmed milk. Figure 3.5 illustrates four GC blots separated by IEF on a 4.5 to 5.4 pH gradient. Filter 3.5(a) shows samples diluted with PBS plus 0.5% BSA, 3.5(b) uses PBS plus 0.5% haemoglobin, 3.5(c) uses PBS plus 0.5% gelatin and 3.5(d) contains samples diluted with PBS plus 0.5% skimmed milk. Equivalent limits of detection (approximately 60pg) were observed in (a) and (c) although at the highest GC concentrations, stronger immunostaining was observed in the presence of gelatin (c). Similarly, with 0.5% haemoglobin (b) and 0.5% skimmed milk (d) the tracks containing the highest amounts of GC showed stronger immunostaining than observed with BSA diluent.

These results indicate that a fixed concentration of a diluent protein, such as BSA, appears to aid the migration of the sample into the gel by decreasing the amount of sample retention at the application site. It was concluded that BSA was the best diluent protein for the analysis of low concentrations of GC by IEF.

3.2 Protein transfer.

These experiments assess the efficiency of GC transfer from IEF gels to nitrocellulose membranes by passive protein transfer and by two methods of electrotransfer: conventional tank electroblotting and semi-dry electroblotting.

3.2.1 Passive transfer and conventional tank electrotransfer.

Figure 3.6 shows the separation of doubling dilutions of GC in plasma by IEF on a 4.5 to 5.4 pH gradient. Filters 3.6(a) and 3.6(b) compare the amounts of GC transferred to

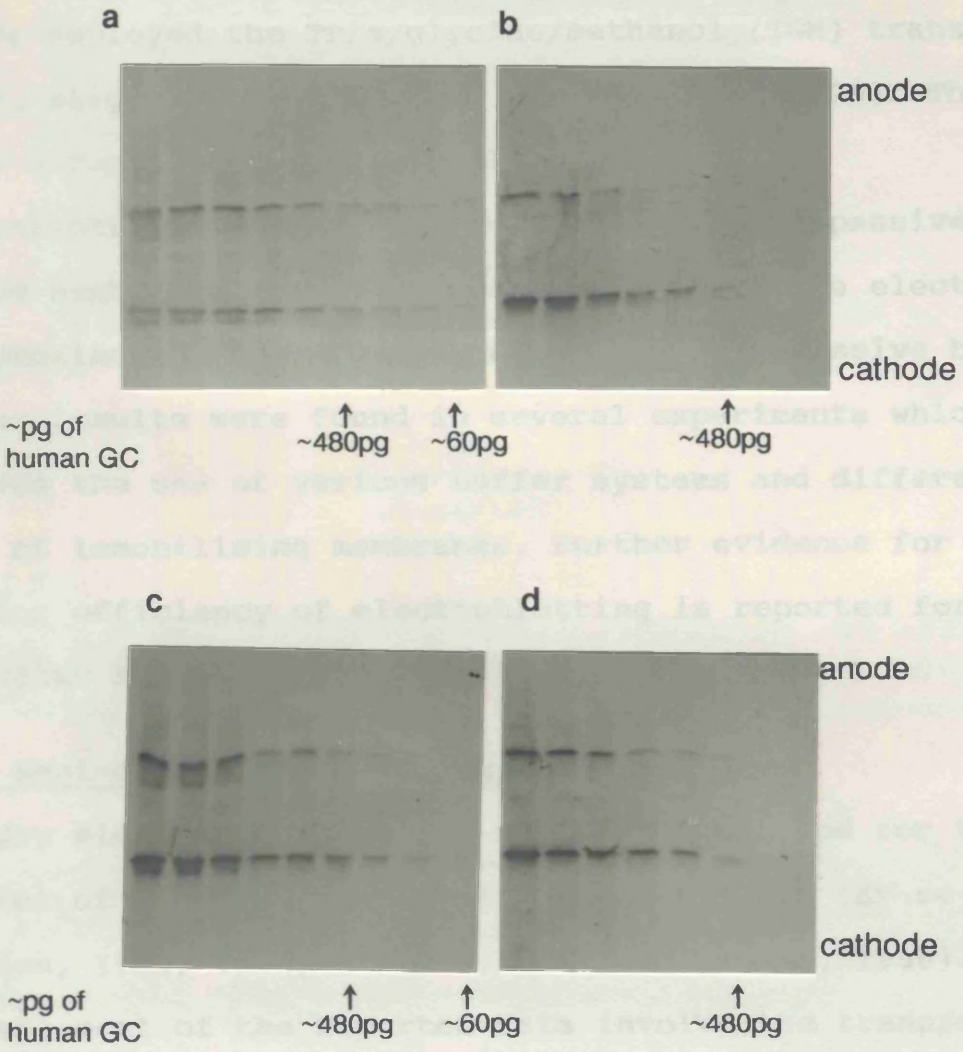


Figure 3.5

Immunological detection of human plasma GC following separation by IEF on a 4.5-5.4 pH gradient and electrotransfer to nitrocellulose. The effect of different sample loading buffers on the sensitivity of GC detection. Panel (a) utilises PBS/0.5%BSA, (b) PBS/0.5% haemoglobin, (c) PBS/0.5% gelatin and (d) PBS/0.5% skimmed milk.

nitrocellulose membranes after conventional tank electroblotting and passive transfer, respectively. Both methods employed the Tris/glycine/methanol (TGM) transfer buffer, slightly modified from Towbin *et al* (1979). The signal strength is substantially higher after electroblotting for one hour compared with the passively blotted membrane. The overall sensitivity of the electroblot is approximately eight fold greater than the passive blot. Similar results were found in several experiments which involved the use of various buffer systems and different types of immobilising membranes. Further evidence for the superior efficiency of electroblotting is reported for PGM in chapter 5 (see figure 5.6).

3.2.2 Semi-dry electroblotting.

Semi-dry electroblotting is a widely used method for the transfer of proteins to immobilising membranes (Kyhse-Andersen, 1984; Tovey & Baldo, 1987; Beiseigel, 1986). However, most of the reported data involve the transfer of proteins from SDS-polyacrylamide gels and there is relatively little information on the semi-dry transfer of proteins from IEF gels. In this section, the transfer efficiencies of two semi-dry blotting systems, manufactured by LKB and Millipore respectively, are compared with the Biorad Transblot cell tank electroblotting apparatus.

Figure 3.7, compares the transfer of GC using different blotting systems after separation by IEF on a 4.5 to 5.4 pH gradient. Filter (a), was subjected to conventional tank blotting in TGM transfer buffer at approximately 3mA per cm^2 of gel, and filter 3.7(b), was subjected to semi-dry

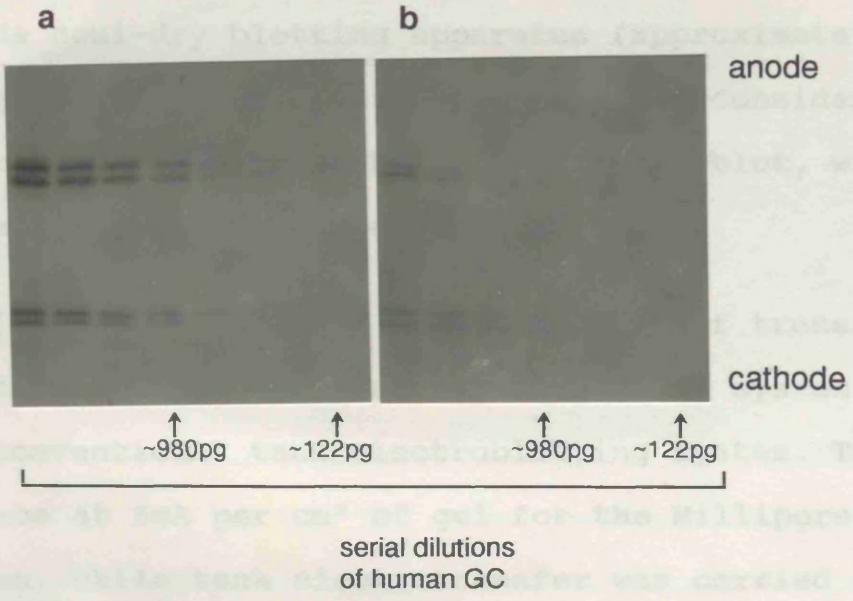


Figure 3.6
Immunological detection of human plasma GC following IEF on a 4.5-5.4 pH gradient. Serial dilutions of GC 1S-1F using PBS/BSA as diluent. Comparison between the transfer efficiency of tank electrotransfer (a) and passive transfer (b).

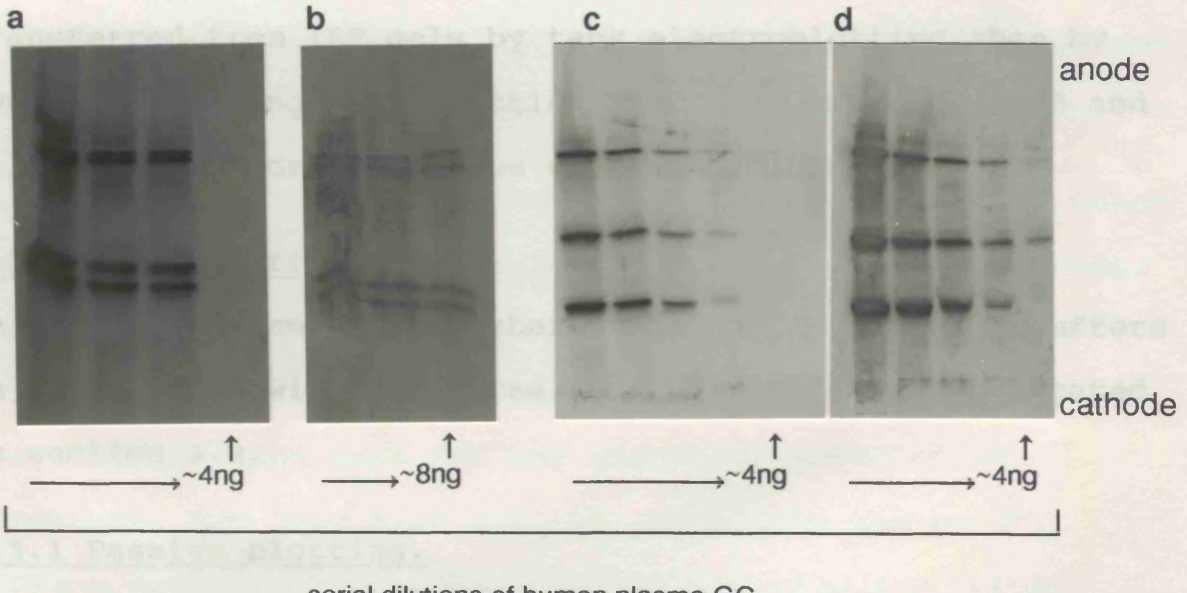


Figure 3.7
Immunological detection of human plasma GC following IEF on a 4.5-5.4 pH gradient. Serial dilutions of plasma GC using water as diluent. Comparison between the transfer efficiency of tank electrotransfer (a) & (d), and semi-dry electrotransfer, using the LKB apparatus (b) and the Millipore apparatus (c).

blotting in TGM transfer buffer, using an LKB graphite electrode semi-dry blotting apparatus (approximately 5mA per cm^2 of gel). The tank blotted filter shows considerably stronger signal strength than the semi-dry blot, with approximately two fold greater sensitivity.

Panels (c) and (d) compare the efficiency of transfer of GC using the Millipore semi-dry blotting (SDE) system with that of the conventional tank electroblotting system. Transfer took place at 5mA per cm^2 of gel for the Millipore apparatus, while tank electrotransfer was carried out as before. Both systems show 4ng GC detection limits, but the tank blotted filter (d) shows considerably greater signal strength than the semi-dry blot (c).

These results show that considerably more GC protein is transferred from IEF gels by tank electroblotting than by semi-dry blotting. The blotting efficiencies of the LKB and Millipore semi-dry apparatus were not compared.

3.3 Transfer buffer.

This section examines a number of different transfer buffers in conjunction with the three blotting methods investigated in section 3.2.

3.3.1 Passive blotting.

The passive blotting system was examined using seven different buffer systems (figure 2.2.1). Each was assessed for the degree of GC transfer efficiency, as measured by the limit of detection.

Figure 3.8 shows doubling dilutions of plasma GC separated

by IEF on a 4.5 to 5.4 pH gradient followed by passive transfer to nitrocellulose filters. Filter 3.8(a) was blotted using a Tris/glycine buffer (TG), 3.8(b) using a Sodium carbonate buffer, 3.8(c) using TGM and 3.8(d) using the borate buffer. Filters (a) and (b) show poor sensitivity, only detecting approximately 16ng of transferred GC. Filters (c) and (d) however, showed considerably better results, detecting very faint GC isoform bands from a 2-1S individual, in samples containing approximately 4ng of GC. The other buffers listed in figure 2.2.1 gave much poorer results and were considered no further. In conclusion, the best results for the transfer of GC from IEF gels by passive blotting, were obtained using either TGM or the borate buffer system.

3.3.2 Tank electroblotting.

Figure 2.2.2 lists a total of seven different buffer systems used in conjunction with the tank electroblotting apparatus.

In these experiments TGM was used as the standard with which each of the other buffer systems were compared. The results obtained using acidic buffers such as 0.7% acetic acid and 0.1M citrate buffer, were extremely poor. More commonly, basic buffers are used for the electrotransfer of GC. Therefore, TGM plus 0.1% SDS, TG, carbonate and borate buffers were examined. The transfer efficiencies for all these buffers, except the borate buffer, were comparable with the standard TGM buffer. The borate buffer showed marginally better transfer efficiency than the TGM buffer. Figure 3.9 illustrates the transfer efficiencies of these two buffers. Filter (b), electroblotted using the borate

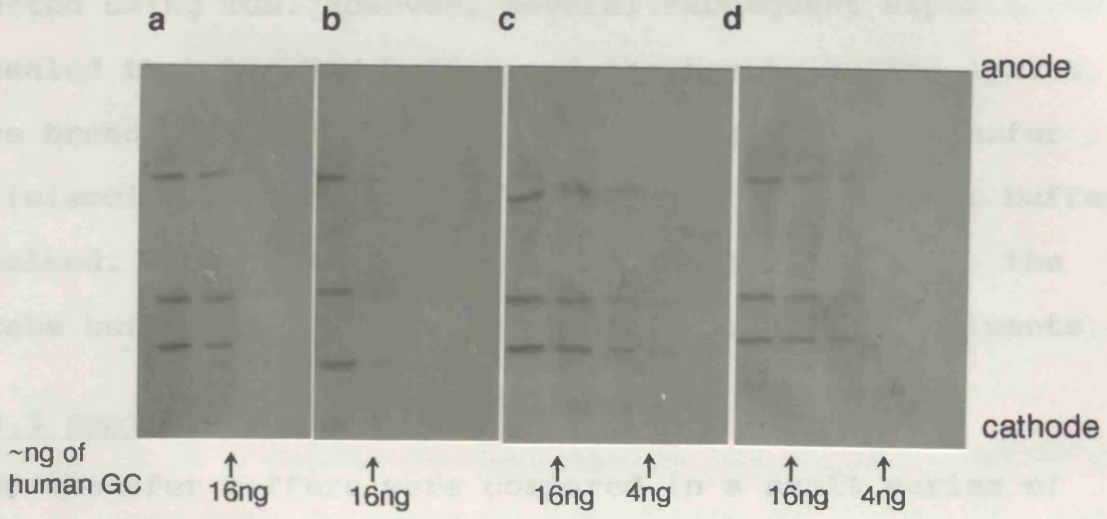


Figure 3.8
Immunological detection of human plasma GC following separation by IEF on a 4.5-5.4 pH gradient and passive transfer to nitrocellulose. The transfer efficiency of four transfer buffers are examined. (a) Tris/glycine (TG), (b) carbonate, (c) TGM and (d) borate buffer.

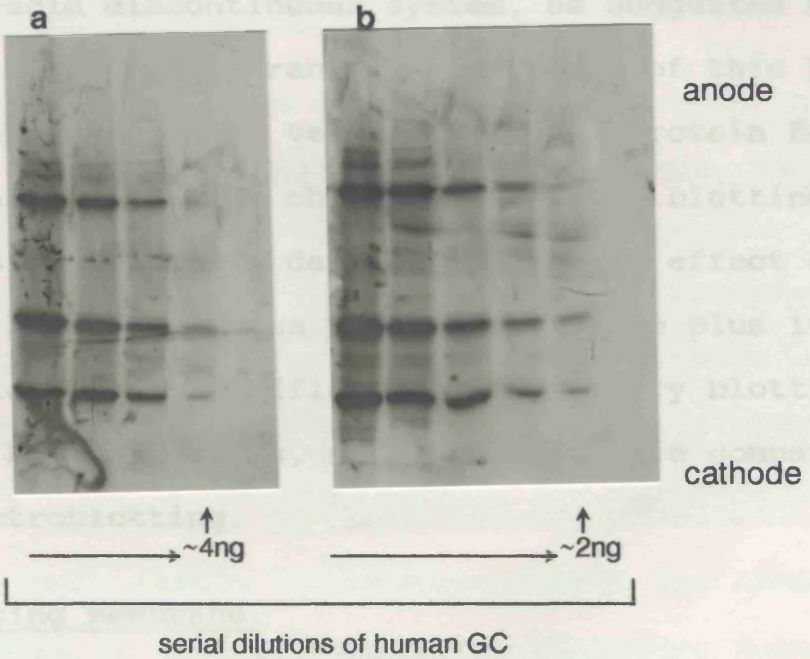


Figure 3.9
Immunological detection of human plasma GC following IEF on a 4.5-5.4 pH gradient and tank electrotransfer to nitrocellulose using the TGM transfer buffer (a) and the borate buffer (b).

buffer, shows two fold better sensitivity than filter (a), blotted using TGM. However, several subsequent experiments revealed that the TGM buffer and the borate buffer system, give broadly comparable results, although their transfer efficiencies were consistently better than the other buffers examined. Due to the lower cost of the constituents, the borate buffer was preferred for the remaining experiments.

3.3.3 Semi-dry electroblotting.

Four transfer buffers were compared in a small series of semi-dry electroblotting experiments due to the restricted loan of the Millipore SDE system.

The buffer system normally used for the semi-dry transfer of proteins from SDS gels is the Tris/glycine/6-amino-n-hexanoic acid discontinuous system, as suggested by Kyhse-Andersen (1984). The transfer efficiency of this buffer system was assessed as very poor for GC protein following IEF, regardless of the choice of semi-dry blotting apparatus. Preliminary data assessing the effect of other blotting buffers such as TGM, Tris/glycine plus 10% methanol and TG alone, on the efficiency of semi-dry blotting, gave somewhat better results, although none were comparable with tank electroblotting.

3.4 Blotting membrane.

A large number of protein immobilising membranes are available from various manufacturers. Figure 3.10 illustrates a comparative study carried out on the binding efficiency of GC to five different immobilising membranes, listed in figure 2.1.

Figure 3.10 illustrates the results using five immobilising membranes: the Schleicher and Schull (S&S) supported, panel (a) and unsupported nitrocellulose membranes, panel(b), Millipore polyvinylidene difluoride membranes (PVDF), panel (c) and the Amersham Hybond C supported, panel (d) and unsupported nitrocellulose membranes, panel (e). Sample dilution was carried out using PBS and tank electrotransfer of the GC was carried out using the standard TGM buffer system. It is possible to distinguish the GC isoforms on all five filters down to track 4, equivalent to 2ng of GC. All five filters show very little background staining and the signal strengths of the dilutions are largely comparable. The quality of the transfers was highly reproducible for the nitrocellulose membranes, but PVDF was less consistent in this respect.

Other membranes such as Zetaprobe, gene screen and Amersham Hybond-N nylon membranes were also evaluated. These membranes produced consistently inferior results to those described in figure 3.10. Particularly noteworthy, was the high background staining and the need for extended blocking times experienced with nylon membranes. As a consequence, the examination of these membranes was not pursued.

There was very little difference between the binding efficiencies of the nitrocellulose membranes examined in figure 3.10. In some experiments, marginally greater sensitivity was obtained with using the Amersham Hybond nitrocellulose membranes, but overall the results were comparable. It must be added however, that the increased durability of the supported n/c membranes relative to the

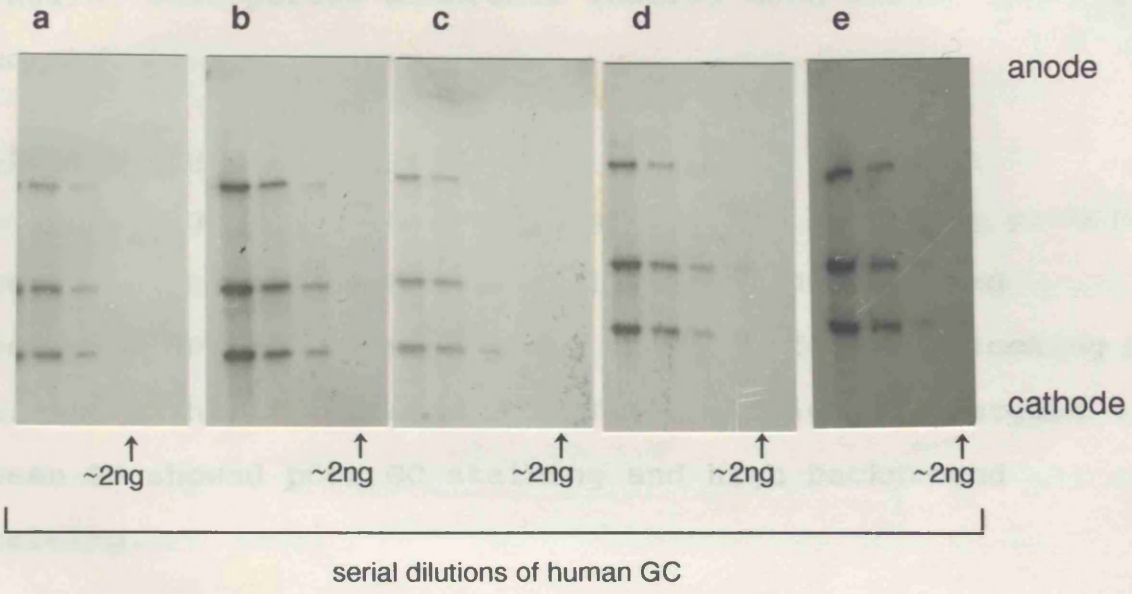


Figure 3.10
Immunological detection of human plasma GC following IEF on a 4.5-5.4 pH gradient and electrotransfer to supported Schleicher & Schull (S&S) nitrocellulose (a), unsupported S&S nitrocellulose (b), Polyvinylindene difluoride membrane (PVDF) (c), supported Amersham nitrocellulose (d) and unsupported Amersham nitrocellulose (e).

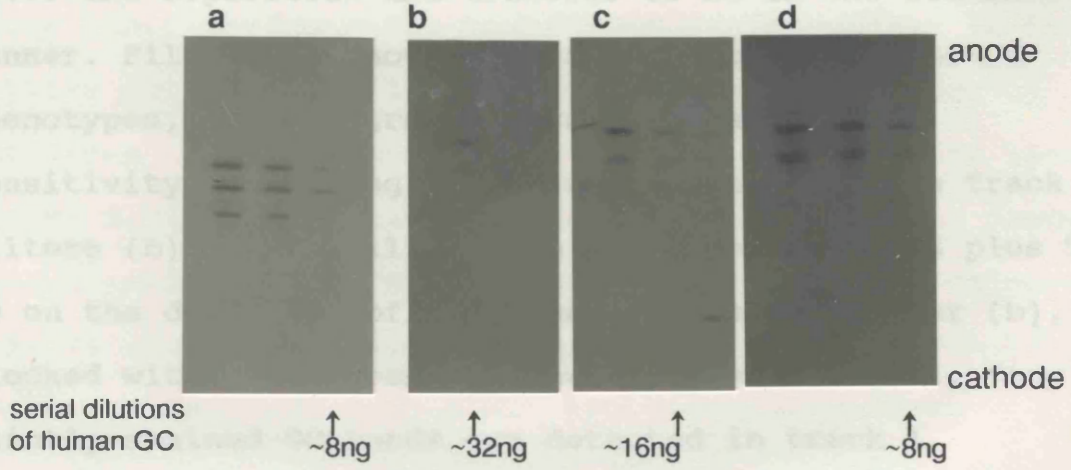


Figure 3.11
Immunoblot analysis of human plasma GC following separation by IEF on a 4.5-5.4 pH gradient. The effect of different blocking solutions at different concentrations on the sensitivity of GC detection. Panel (a) blocked with 3% PBS/BSA, (b) 0.5% Tween 20, (c) 0.15% Tween 20 and (d) 0.05% Tween 20.

fragile unsupported membranes enables much easier handling and storage.

3.5 Blocking buffer.

The blocking of the immobilising membrane following protein transfer, is an essential step in the immunoblotting procedure. Preliminary findings on the effect of blocking GC filters with a 0.15% solution of the non-ionic detergent Tween 20 showed poor GC staining and high background staining.

Experiments testing the effect of various concentrations of Tween 20 revealed that higher concentrations had a marked effect on the adherence of GC to the immobilising membrane. Figure 3.11 compares the blocking efficiencies of PBS plus 3% BSA, panel (a), and PBS plus Tween 20 at three different concentrations 0.5%, 0.15% and 0.05%, panels (b) to (d), after the separation and transfer of GC in the standard manner. Filter (a) shows good resolution of the GC phenotypes, low background signal and acceptable sensitivity, detecting approximately 8ng of GC in track 3. Filters (b) and (c) illustrate the effects of PBS plus Tween 20 on the detection of dilutions of the GC. Filter (b), blocked with 0.5% Tween 20 shows little GC signal. Very faintly stained GC bands are detected in track 1, approximately 32ng of GC. Filter (c), blocked with 0.15% Tween 20 shows slightly better sensitivity than filter (b), displaying faintly stained GC bands in the 16ng track. Filter (d), blocked using 0.05% Tween 20, showed considerably better results. Strong signal strength in tracks 1 and 2 and the detection of 8ng of focused GC in

track 3.

High concentrations of Tween 20, between 0.5 and 0.15%, cause a considerable decrease in the sensitivity of GC detection. It would appear that these concentrations interfere with the interaction between GC and the membrane in an undetermined fashion, causing the release of significant amounts of the GC from the membrane. This effect does not appear to occur when low concentrations (0.05%) of Tween 20 are used.

Direct comparison between panels (a) and (d) reveal comparable sensitivities of GC detection. However, in the long term, better signal strength was obtained with Tween 20, and consequently Tween 20 at low concentration was the preferred blocking agent.

3.6 Detection system.

After the blocking step, the filter was incubated in the primary antiserum followed by the secondary conjugated antiserum, used as recommended by the manufacturers (see figure 3.2). This final step in the immunoblotting process, visualisation of the membrane bound antigen, was compared using a variety of different detection systems.

3.6.1 Horseradish peroxidase-linked antibodies.

Many chromogenic substances that become insoluble when oxidised are available for the detection of horseradish peroxidase (HRP) linked antibodies. The two most commonly used are 3,3'-diaminobenzidine (DAB) and 4-chloro-1-naphthol (NAP) (Graham & Karnovsky, 1966; Hawkes, Niday & Gordon, 1982). The tetrazolium dye DAB was used as the standard in

these studies.

Disappointing results were obtained using the following reagents: the soluble reagent tetramethylbenzidine (TMB) (Bos *et al.* 1981), 3-amino-9-ethylcarbazole (Graham, Lundholm & Karnovsky, 1965) and cobalt chloride as a signal enhancer in the standard DAB method (Adams, 1981). TMB was considerably less sensitive than DAB, difficult to precipitate at the antibody binding site and also subject to fading. TMB is commonly used in conjunction with ELISA systems, which normally require highly soluble dyes. The carbazole reagent was approximately four times less sensitive than DAB, and the cobalt chloride enhancement method for DAB, led to no noticeable improvement in the sensitivity of the DAB system.

More encouraging results were obtained using a combination of DAB and NAP (Young, 1989). Figure 3.12, shows dilutions of plasma GC detected using the DAB system (a), compared with the DAB/NAP mixture (b). Although the sensitivities of detection are comparable, marginally greater signal strength is observed at higher GC concentrations in filter (b).

Another reagent which gave good results was tetrazolium (Taketa *et al.* 1986). Figure 3.12, panel (c) shows the sensitivity of the standard DAB method, compared with the tetrazolium system, which precipitates at the site of antibody/antigen binding to give an intense purple colour (d). Again, the sensitivities of the two systems were comparable but the contrast displayed by the tetrazolium filters was better than obtained with the DAB filters. Overall, none of the HRP-linked reagents examined gave a

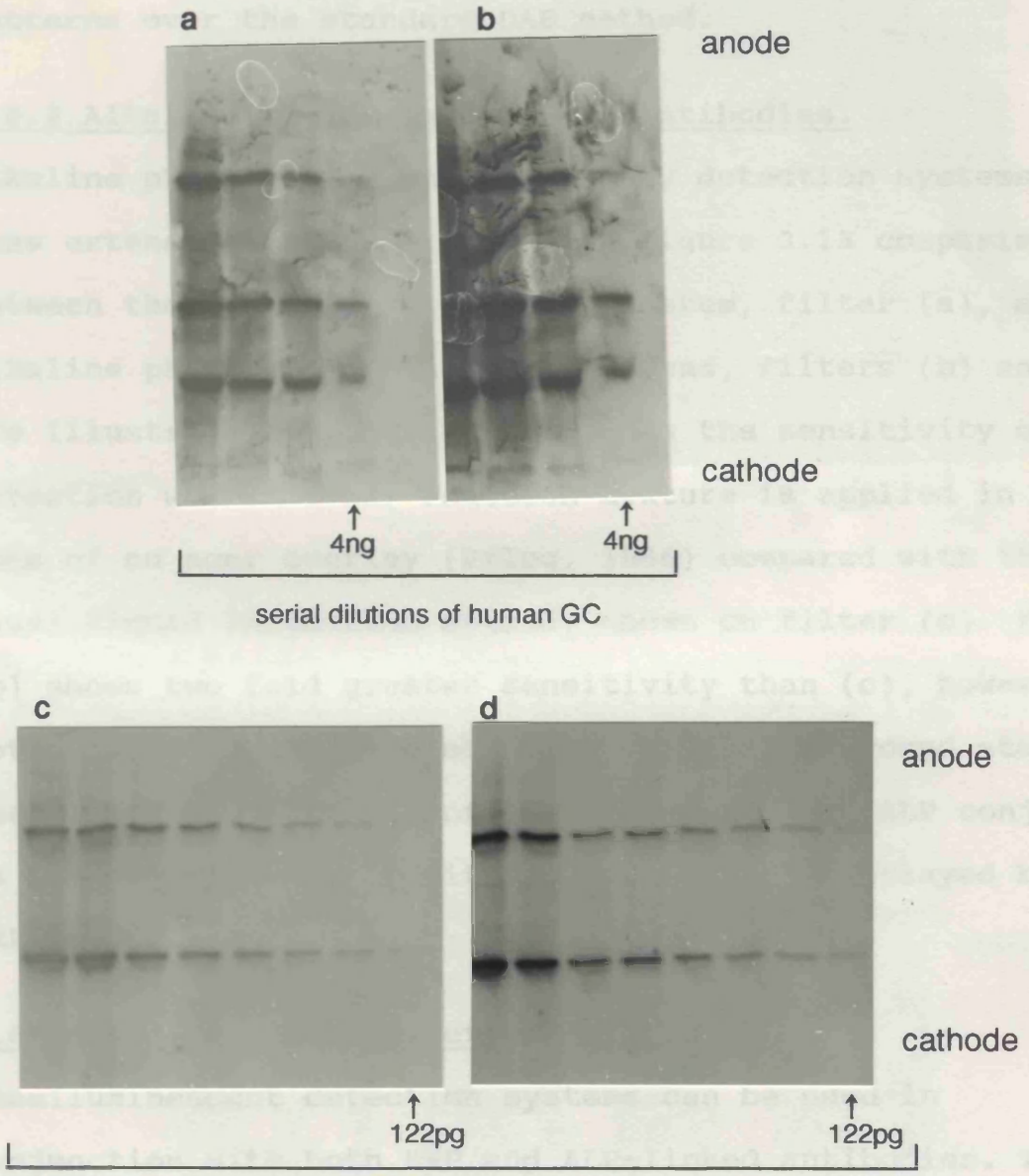


Figure 3.12

Immunological detection of human plasma GC following separation by IEF on a 4.5-5.4 pH gradient and electrotransfer to nitrocellulose. All the filters are incubated with the HRP-linked antiserum, but visualised using diaminobenzidine (DAB), filters (a) & (c), a mixture of DAB and 4-chloro-1-naphthol (NAP), filter (b), and tetrazolium, filter (d).

marked improvement in the sensitivity of detection of GC patterns over the standard DAB method.

3.6.2 Alkaline phosphatase-linked antibodies.

Alkaline phosphatase-linked antibody detection systems were less extensively investigated. In figure 3.13 comparisons between the conventional HRP/DAB system, filter (a), and alkaline phosphatase detection systems, filters (b) and (c) are illustrated. Filter (b) displays the sensitivity of GC detection when the ALP reaction mixture is applied in the form of an agar overlay (Pflug, 1986) compared with the more usual liquid incubation method, shown on filter (c). Filter (b) shows two fold greater sensitivity than (c), however both ALP filters show unacceptably high background staining. The overall sensitivity of GC detection using ALP conjugates is comparable to the limit of GC detection displayed by the HRP conjugates (a).

3.6.3 Chemiluminescent detection systems.

Chemiluminescent detection systems can be used in conjunction with both HRP and ALP-linked antibodies. This section is concerned with the detection of HRP-linked antibodies using the chemiluminescent substrate luminol. Oxidation of luminol in the presence of HRP and hydrogen peroxide results in elevation of electrons from the ground energy state to a higher energy, excited state. On returning to the ground state, electrons release energy as photons, which can be recorded on film. The signal produced can be intensified by the addition of chemical enhancers such as p-iodophenol, which work by increasing the number of photons emitted and by extending the emission time of the substrate.

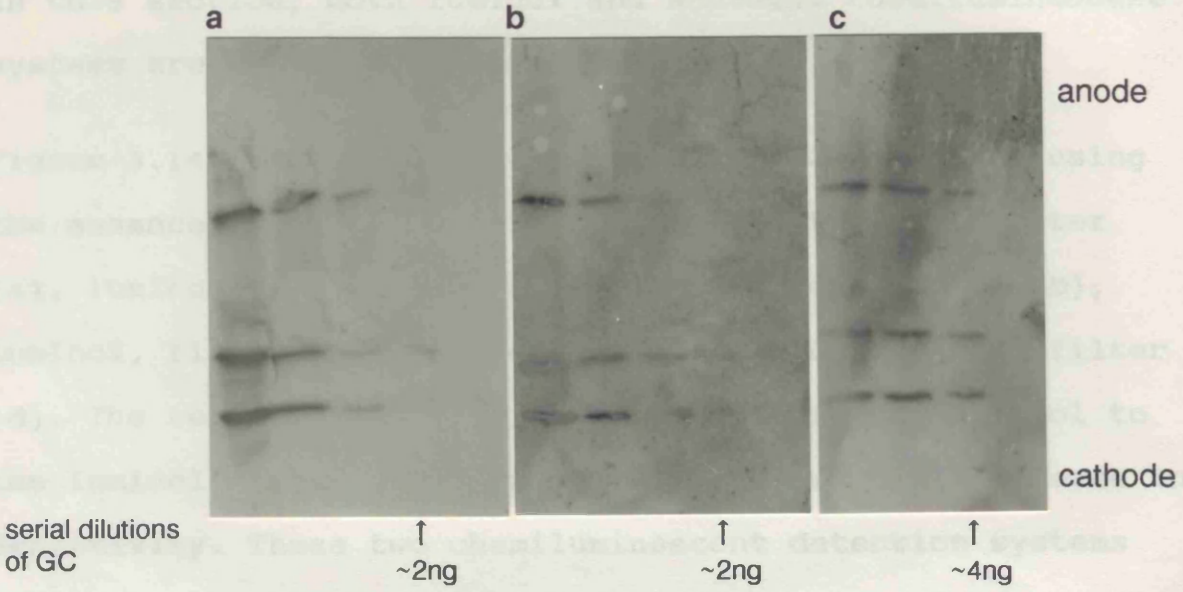


Figure 3.13
Immunological detection of serial dilutions human plasma GC following separation by IEF on a 4.5-5.4 pH gradient. Comparison between the sensitivity of the HRP/DAB detection system (a) and an ALP-linked antibody system applied as an agar overlay (b), and more conventionally as a liquid medium (c).

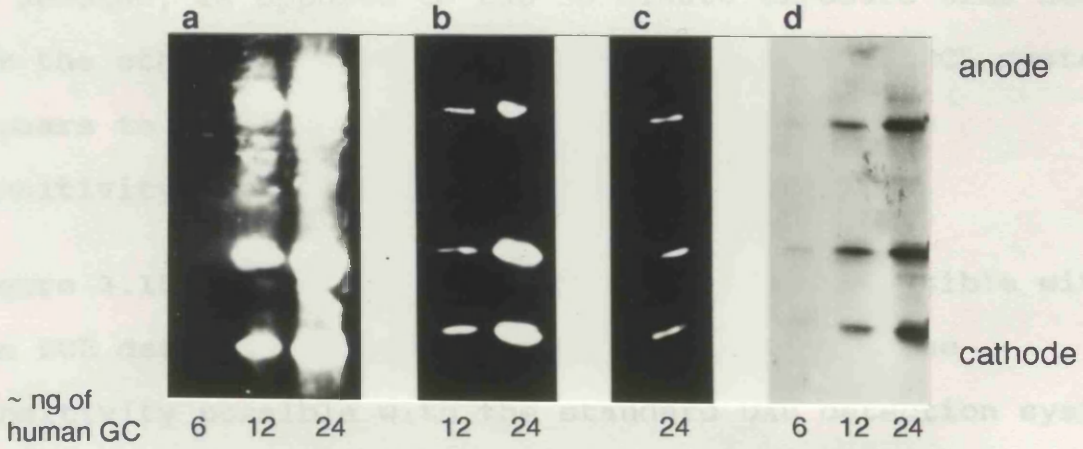
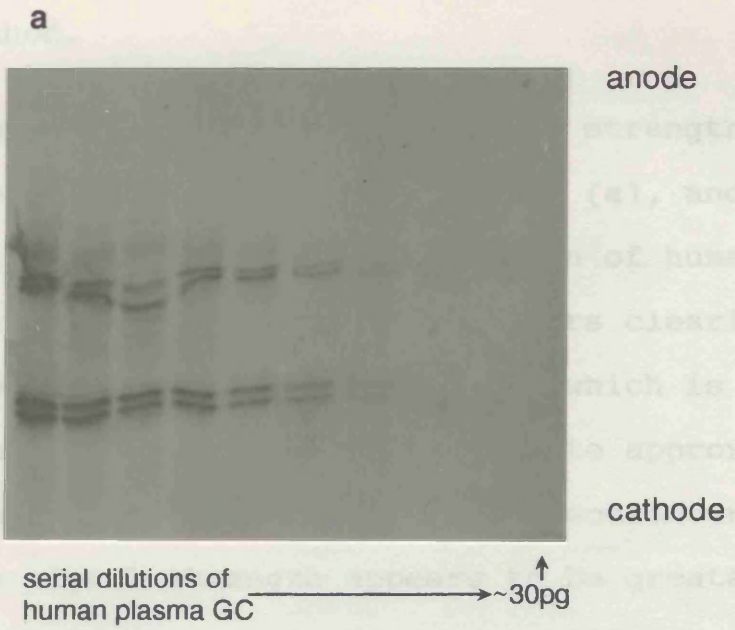


Figure 3.14
The immunological detection of human GC in plasma following separation by isoelectric focusing on a 4.5-5.4 pH gradient and electrotransfer to nitrocellulose. Serial dilutions of GC 21-S plasma, visualised with four HRP-linked detection systems:
a) ECL chemiluminescence; b) luminol plus enhancer p-iodophenol; c) luminol; d) DAB coloured product.

In this section, both luminol and enhanced chemiluminescent systems are assessed for sensitivity.

Figure 3.14 shows doubling dilutions of GC, detected using the enhanced Amersham chemiluminescent kit (ECL), filter (a), luminol plus the enhancer p-iodophenol, filter (b), luminol, filter (c) and the standard HRP/DAB system, filter (d). The results show that the addition of p-iodophenol to the luminol reaction mixture leads to a two fold increase in sensitivity. These two chemiluminescent detection systems however, are considerably less sensitive than standard DAB staining. Filter (a), the ECL filter, shows equivalent sensitivity with the DAB-stained filter, and considerably greater signal strength at the high GC concentrations. Since the ECL results were obtained after an exposure time of only 15 seconds, as opposed to the 30 minute exposure time needed for the other two chemiluminescent systems, the ECL system appears to be a good candidate for increasing the sensitivity of GC detection after immunoblotting.

Figure 3.15 illustrates the detection limits possible with the ECL detection system. Filter (a) indicates the sensitivity possible with the standard DAB detection system, approximately 30pg, and filter (b) shows the overall sensitivity of the ECL detection system, approximately 8pg. By increasing the film exposure times to 30 minutes, slight increases in sensitivity of the ECL system were attained, but this was also associated with a considerable increase in the background signal. These results show that the detection of immunoblotted plasma GC using the Amersham ECL kit is at least four times more sensitive than the DAB detection



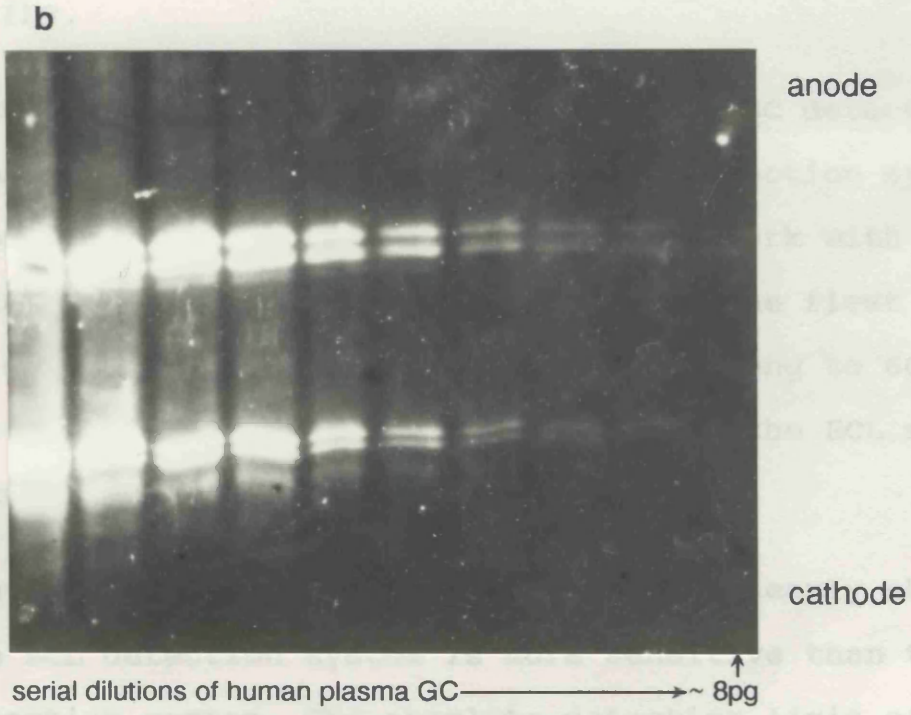


Figure 3.15

Immunological detection of human GC in plasma following separation by isoelectric focusing on a 4.5-5.4 pH gradient. Serial dilutions of GC detected using two HRP-linked detection systems, DAB, (a) and the chemiluminescent ECL system (b).

method.

Figure 3.16 compares the signal strengths obtained between the standard DAB system, filter (a), and the ECL system, filter (b), following separation of human plasma GC by SDS gel electrophoresis. Both filters clearly show the electrophoresis of diluted GC, which is identified as a single component corresponding to approximately 50,000 mwt. Comparable sensitivities of detection are observed. However, the signal strength appears to be greater in filter (b), obtained after only 15 seconds film exposure time. The results obtained for the detection of GC after separation by SDS-PAGE are comparable with those obtained after separation by IEF.

Figure 3.17 shows the absolute limit of GC detection after separation by SDS-PAGE using the ECL detection system (obtained as a result of collaborative work with Amersham International). Filter (a) illustrates the first part of the GC dilution series, from approximately 40ng to 600pg. Filter (b) shows the lower detection limits of the ECL system, between 310pg and 10pg.

The result of using chemiluminescence, clearly shows that the ECL detection system is more sensitive than the DAB detection system. The absolute detection limit of this system is in the region of 8pg of GC protein, approximately four times more sensitive than any results obtained with the DAB system.

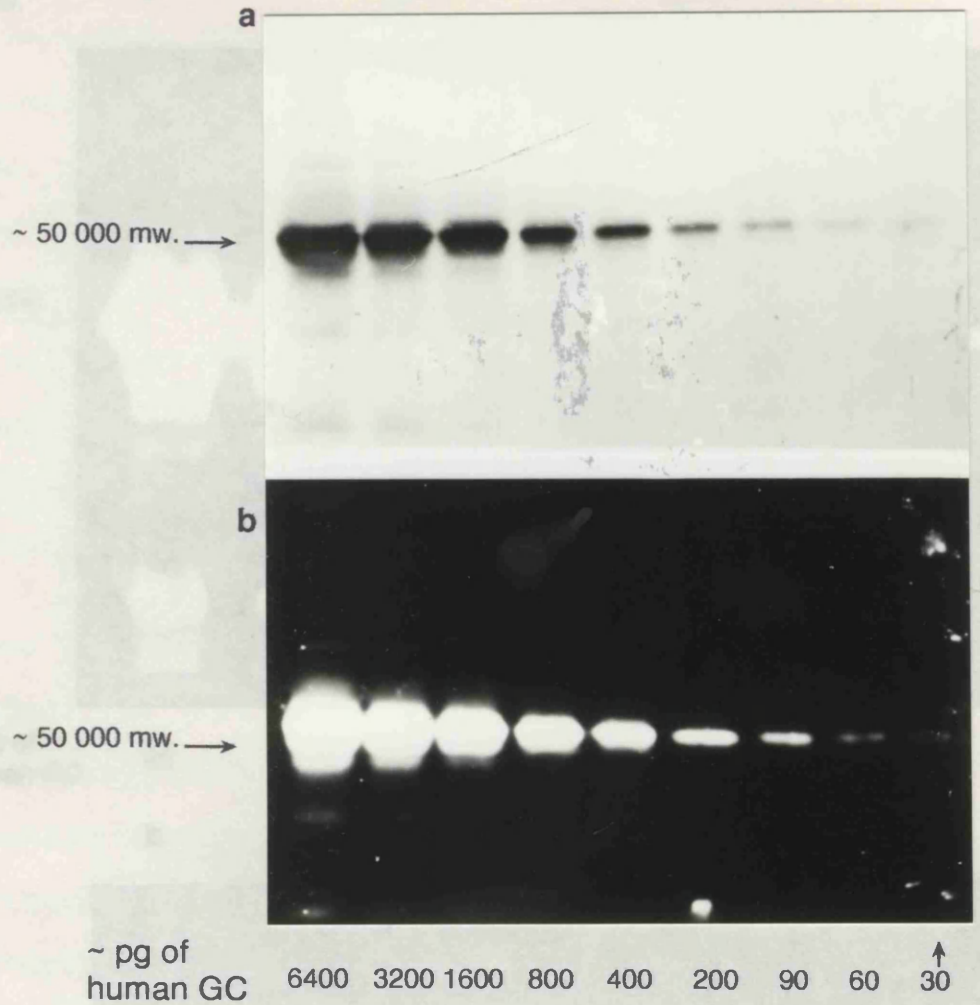


Figure 3.16

The immunological detection of human GC in plasma following separation by SDS gel electrophoresis, comparing the sensitivity of the coloured HRP-linked reagent , DAB (a) with the HRP-linked chemiluminescent ECL detection system (b).

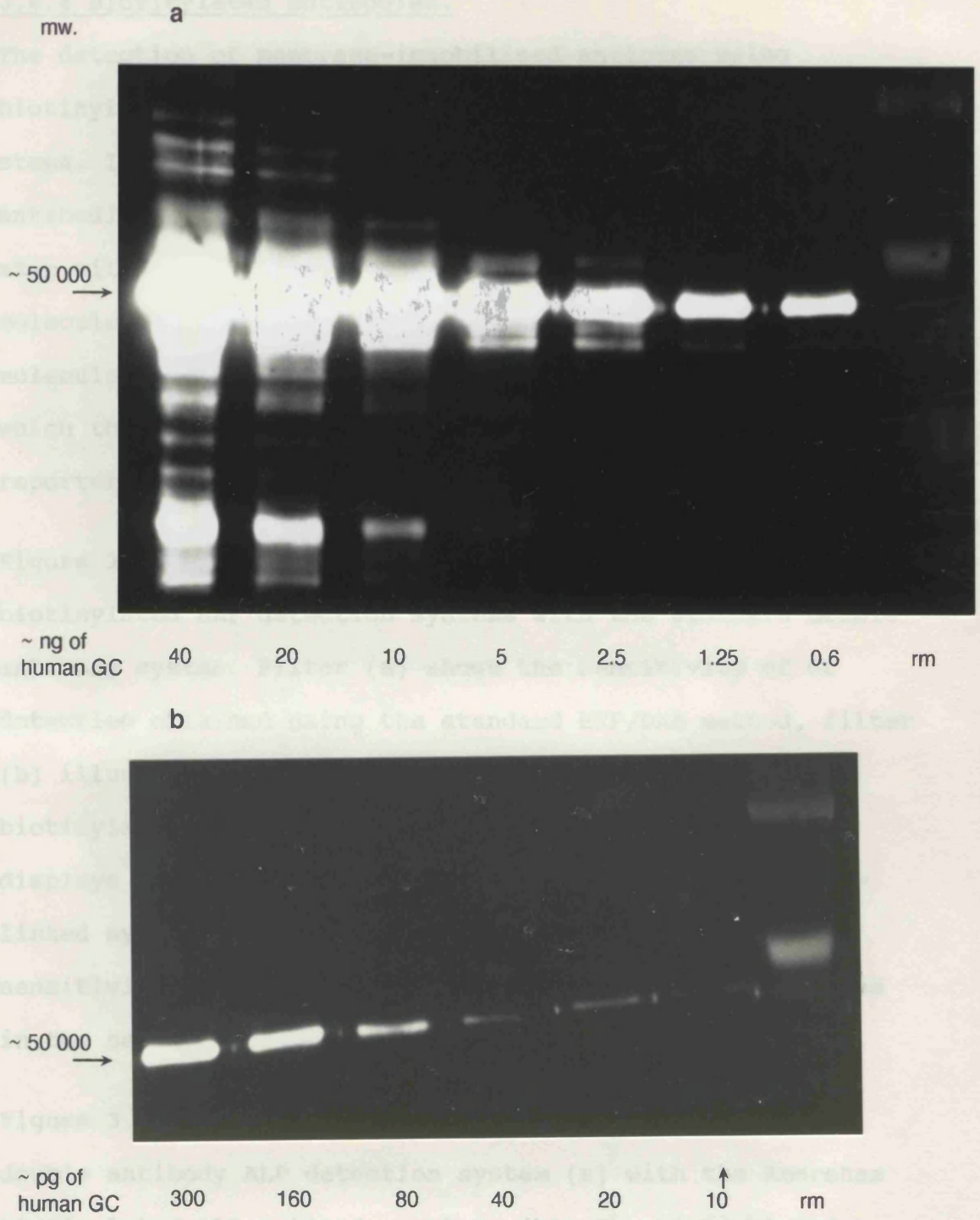


Figure 3.17

The immunological detection of human GC in plasma following separation by SDS gel electrophoresis. Serial dilutions of plasma detected using HRP-linked chemiluminescent ECL system. The arrow indicates the highest dilution visible. Molecular weight marker tracks are identified by (rm).

3.6.4 Biotinylated antibodies.

The detection of membrane-immobilised antigens using biotinylated antibodies usually involves three incubation steps. Incubation in the primary antibodies, the secondary antibodies (conjugated to biotin) and a third incubation step with biotin-streptavidin complexes linked to a reporter molecule, such as HRP or ALP (see figure 3.18). Each molecule of streptavidin binds four molecules of biotin which theoretically concentrates a greater number of reporter molecules at the antigen binding site.

Figure 3.19 compares the sensitivity of two different biotinylated HRP detection systems with the standard double antibody system. Filter (a) shows the sensitivity of GC detection obtained using the standard HRP/DAB method, filter (b) illustrates the sensitivity of the Vectastain biotinylated HRP-linked antibody system and filter (c) displays the sensitivity of an Amersham biotinylated HRP-linked system. Filters (a) and (b) show comparable sensitivity while filter (c) indicates a two fold increase in the sensitivity of GC detection.

Figure 3.20 compares the sensitivity of the conventional double antibody ALP detection system (a) with the Amersham biotinylated ALP antibody system, (b). The biotinylated system, shows sixteen fold better sensitivity than filter (a).

Figure 3.21 illustrates the limit of GC detection attainable with the biotinylated ALP system. The results show that the biotinylated ALP detection system is approximately four fold more sensitive than the standard HRP/DAB detection system,

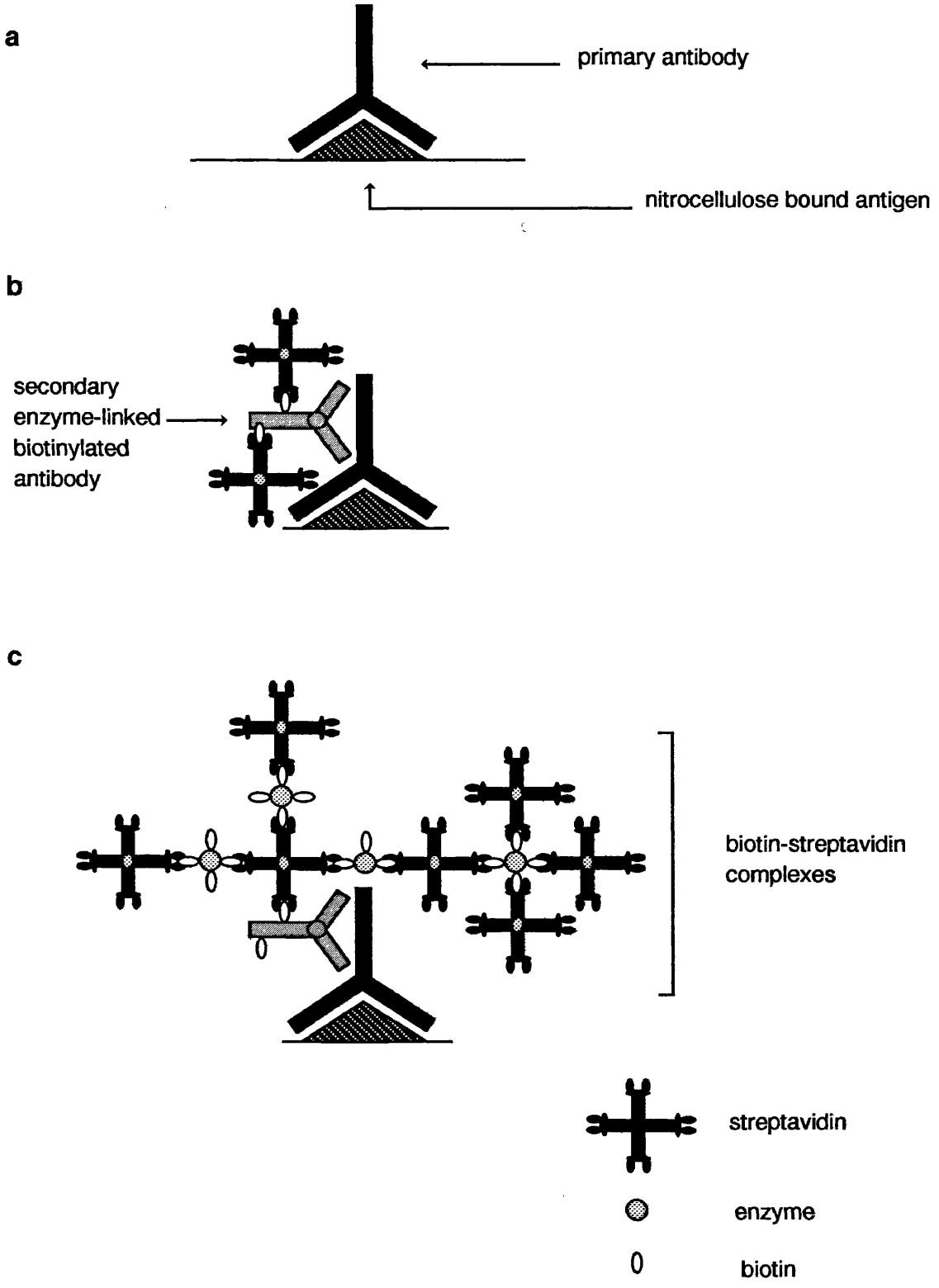
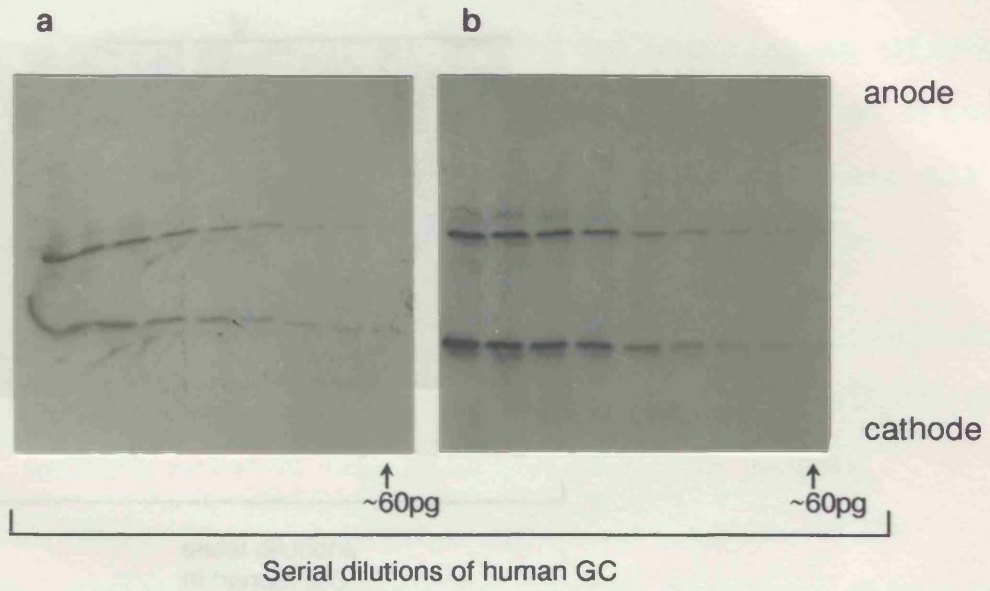


Figure 3.18
Schematic diagram illustrating the construction of biotin steptavidin complexes and the resulting amplification of the signal of the enzyme-linked signal. (a) primary antibody binding the nitrocellulose bound antigen, (b) binding of the enzyme-linked secondary biotinylated antibodies and (c) the building of the enzyme-linked biotin-streptavidin complexes.



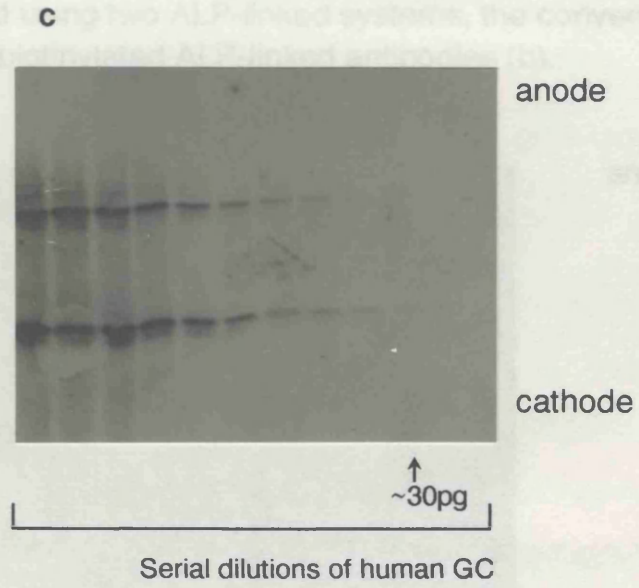


Figure 3.19

Immunological detection of human plasma GC following separation by IEF on a 4.5-5.4 pH gradient and electrotransfer to nitrocellulose. Serial dilutions of GC visualised using three HRP-linked antibody systems, conventional (a), the biotinylated ABC kit (b) and the Amersham biotinylated system (c).

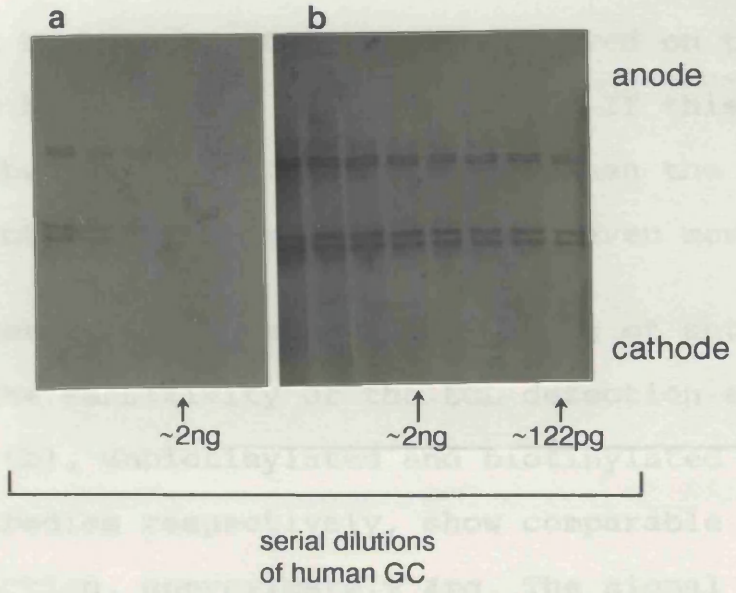


Figure 3.20
Immunological detection of human plasma GC following separation by IEF on a 4.5-5.4 pH gradient and electrotransfer to nitrocellulose. Serial dilutions of GC detected using two ALP-linked systems, the conventional ALP-linked antibodies (a) and biotinylated ALP-linked antibodies (b).

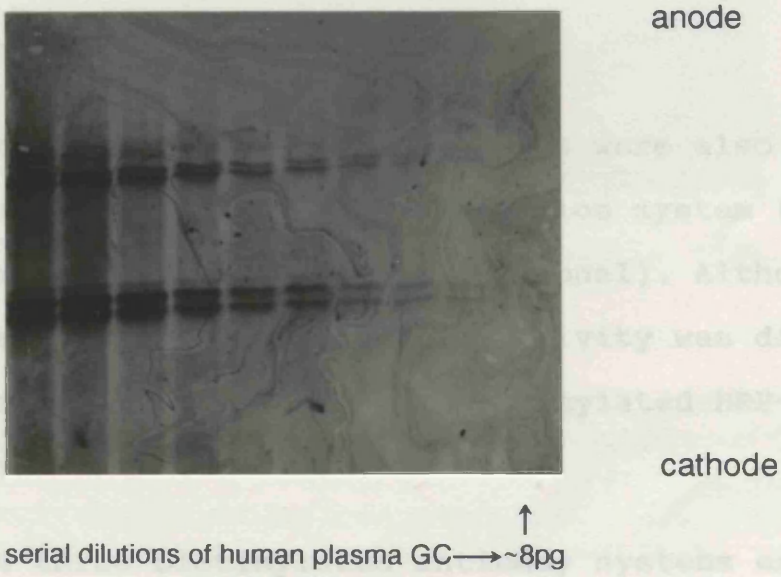


Figure 3.21
Immunological detection of human plasma GC following separation by IEF on a 4.5-5.4 pH gradient and electrotransfer to nitrocellulose. The limit of GC detection using the biotinylated ALP-linked antibody system.

illustrated in figure 3.15 (a). At lower GC concentrations a high background staining is observed on the filters probed with biotinylated ALP antibodies. If this background signal can be substantially decreased, then the biotinylated ALP detection system may prove to be even more sensitive.

Filter 3.22 displays the influence of antibody biotinylation on the sensitivity of the ECL detection system. Filters (a) and (b), unbiotinylated and biotinylated HRP linked antibodies respectively, show comparable limits of GC detection, approximately 4pg. The signal strength obtained after probing with the biotinylated antibodies appears slightly greater than obtained on filter (a), but greater background chemiluminescence is observed on filter (b). These results suggest that no great improvement in the chemiluminescent detection of GC is obtained using biotinylated antibodies.

A restricted number of experiments were also carried out on an immunogold biotinylated detection system (carried out as recommended by Amersham International). Although good contrast was obtained, the sensitivity was disappointing, only about half that of the biotinylated HRP-linked antibody system.

Of the three biotinylated antibody systems examined, only the biotinylated ALP system is more sensitive than the standard HRP/DAB system (by a factor of four). The chemiluminescent ECL detection system is the most sensitive method of immobilised antigen detection examined here. It proves to be at least two fold more sensitive than the

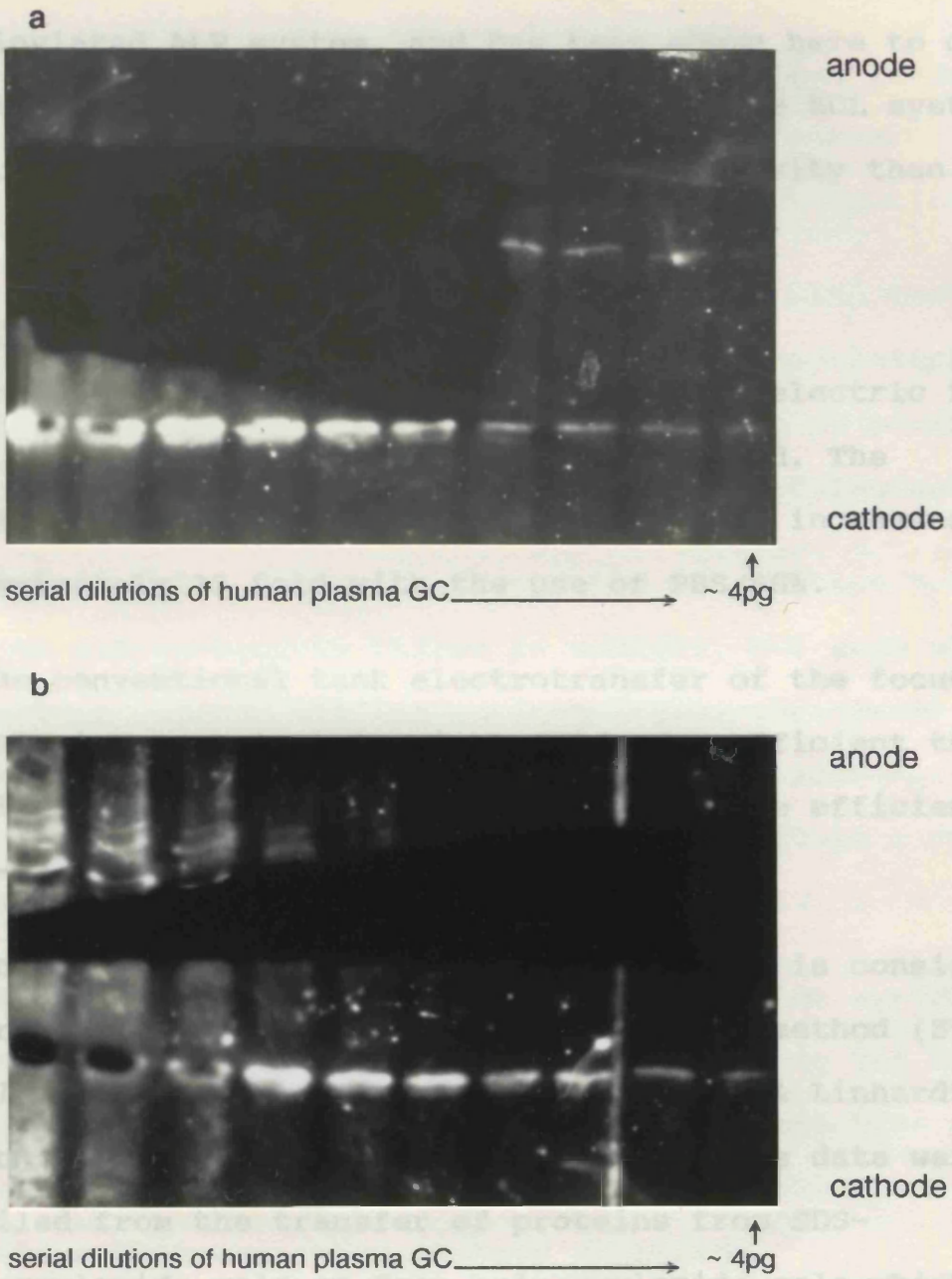


Figure 3.22
Immunodetection of human GC following separation by isoelectric focusing on a 4.5-5.4 pH gradient. Serial dilutions of GC 1S plasma detected using HRP-linked antibodies (a) and biotinylated HRP-linked antibodies (b) with the chemiluminescent ECL system. The arrow indicates the highest dilution visible in each panel.

biotinylated ALP system, and has been shown here to detect as little as 4pg of focused GC. Overall, the ECL system is about two fold better, in terms of sensitivity than the next best system examined in this thesis.

3.7 Summary and conclusions.

1) The entry of of human plasma GC into isoelectric focusing gels is dependent on the sample diluent used. The sensitivity of immunodetection of human GC, increases by approximately 33 fold with the use of PBS/BSA.

2) The conventional tank electrotransfer of the focused GC protein is approximately eight fold more efficient than passive blotting, and at least two fold more efficient than semi-dry electroblotting.

In the literature, semi-dry electrotransfer is considered superior to the conventional tank transfer method (Svoboda *et al.* 1985; Tovey & Baldo, 1987; Al-Hakim & Linhardt, 1990; Jacobson & Kårsnäs, 1990), however all these data were compiled from the transfer of proteins from SDS-polyacrylamide gels or from polyacrylamide gels which are considerably thicker than the IEF gels used for the comparative studies reported here.

3) The efficiency of GC transfer and binding to the immobilising membrane is not significantly affected by the choice of transfer buffer or transfer membrane used.

However, several recommendations were made as a direct consequence of the results obtained here. The TGM transfer buffer, conventionally used in protein transfers (Towbin *et al.* 1979) was replaced by a borate buffer (Myrseth *et al.*

1989) due to its cheaper components. Reinforced immobilising membranes were also recommended for their superior durability (Schleicher & Schull, Amersham International).

4) Optimal blocking conditions are obtained with low concentrations of Tween 20 in PBS. High concentrations of Tween 20 should not be used for blocking the GC protein, as this leads to elution of GC from the immobilising membrane.

The way in which Tween 20 affects the interaction between GC and the nitrocellulose filter is unclear, but much of the literature reports similar antigen losses when Tween 20 and other detergents are used (Batteiger *et al.* 1982; Lin & Kasamatsu, 1983; Spinola & Cannon, 1985; Hoffman & Jump, 1986; DenHollander & Befus, 1989).

5) Of the multitude of immunological detection systems examined, the chemiluminescent ECL system gave the highest sensitivity of GC detection, enabling the detection of approximately 4 pg of GC, between two and four fold more sensitive than any other systems examined.

Other researchers (Brönstein *et al.* 1988; Voyta *et al.* 1988) have reported the development of an alkaline phosphatase-linked chemiluminescent detection system which has been reported to detect sub-attomolar concentrations of immobilised alkaline phosphatase. The ECL chemiluminescent results reported in this chapter, demonstrate that this system is quite capable of detecting attomolar concentrations of GC. Following separation by IEF, immunoblot analysis and antigen visualisation using the ECL system, the detection of as low as 80 attomoles of GC is

possible.

To conclude, the optimised method for the immunological detection of the GC polymorphism developed during this thesis: using a combination of PBS/BSA as the loading buffer, conventional tank electroblotting, 0.05% Tween 20 as the blocking agent, and the ECL chemiluminescent detection system, leads to a 3000 fold increase in sensitivity over the immunofixation method used by the Forensic Science Service (Westwood, 1985).

The only constraints on the use of the more sensitive ECL system appears to be the availability of dark room facilities and greater cost incurred per test, as the DAB detection system is only one quarter of the cost. Therefore, the optimal immunoblotting procedure deduced from the results illustrated in this chapter, with regard to the overall cost of each test, require the incorporation of the standard HRP/DAB detection system in lieu of the ultrasensitive chemiluminescent ECL detection system. This still gives at least 250 fold greater sensitivity than the old method. These data have been published as an internal Home Office report to replace the method of GC detection by immunofixation for use in the routine examination of the human GC polymorphism in forensic material (Drago *et al.* 1990, CRSE report no:718).

Chapter 4:

Phosphoglucomutase.

This chapter describes the detection of phosphoglucomutase-1 (PGM1), a polymorphic enzyme, using polyclonal antisera raised against a commercial preparation of purified rabbit muscle PGM1 in two different animal species. The binding properties of these antisera were investigated and the detection of the human and rabbit PGM1 isozymes was carried out by immunoblot analysis.

4.1 Characterisation of the anti-rabbit PGM antisera.

4.1.1 Guinea pig antiserum.

Guinea pig anti-rabbit PGM antiserum was analysed by Ouchterlony double diffusion, dot immunobinding and immunoblotting. Neat guinea pig antiserum was tested on Ouchterlony plates against the neat purified rabbit PGM. No precipitin arcs were detected after staining for protein with Coomassie blue. Dot immunobinding was carried out by spotting a dilution series of purified rabbit PGM onto nitrocellulose (n/c), using a 1 in 600 dilution of the guinea pig anti-rabbit PGM antiserum as the primary antiserum, followed by the normal double antibody incubation procedure. The results showed the detection of approximately 9ng of rabbit PGM, but immunoblot analysis of purified rabbit PGM, separated by SDS-PAGE, gave negative results. No further characterisation of the guinea pig anti-rabbit PGM antiserum was carried out.

4.1.2 Sheep antiserum.

Undiluted sheep antiserum, obtained 30 days after the first immunisation was tested using Ouchterlony plates against the neat rabbit PGM. A single precipitin line was obtained. No precipitation was obtained when preimmune and non-immune control sera were tested. The antiserum was also found to be effective after considerable dilution. For instance, dot immunobinding, using a constant concentration of the antigen (2ng of rabbit PGM), revealed the detection of purified rabbit antigen by anti-rabbit PGM immune serum, diluted 1 in 372,000. A negligible titre of 1 in 12,000 was obtained on the control filters probed with pre-immune sera.

Ouchterlony analysis of the sheep anti-rabbit PGM antiserum 30 days after a second immunisation with 1mg of purified rabbit PGM, resulted in two precipitin lines (figure 4.1). The stronger line was located midway between the antiserum and antigen wells, and the weaker line was closer to the antiserum well. Ouchterlony analysis of human skeletal muscle homogenate showed a weak line which fused with the stronger rabbit PGM precipitin line, figure 4.1. No spurs were observed. These experiments show that the sheep antiserum binds to more than one component in the purified rabbit PGM, and also cross-reacts with a component found in aqueous human skeletal muscle extracts.

The following experiments serve to identify the component or components bound by the sheep anti-rabbit PGM antiserum in purified rabbit PGM and human skeletal muscle. Figure 4.2 shows four identical blots of purified rabbit PGM and human skeletal muscle extract (tracks 2 and 3) separated by SDS-

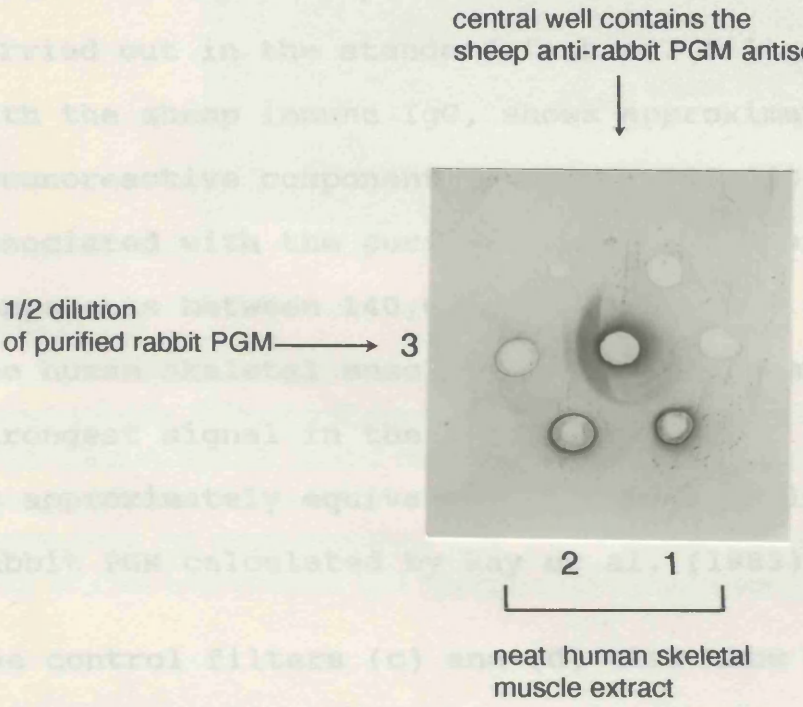


Figure 4.1

Ouchterlony double diffusion plate. Central well loaded with the the sheep anti-rabbit PGM antiserum, wells 1 and 2 contain neat human skeletal muscle extracts and well 3 a 1 in 2 dilution of the purified rabbit PGM.

PAGE and analysed by immunoblotting. Filter (a) was incubated in the absence of a primary antiserum, filter (b) was incubated with the sheep anti-rabbit PGM immune IgG fraction, filter (c) was incubated in whole non-immune serum and filter (d) in the IgG fraction of the sheep preimmune serum. The other steps in the immunoblotting process were carried out in the standard fashion. Filter (b), incubated with the sheep immune IgG, shows approximately twenty immunoreactive components, between 180,000 and 22,000 mw. associated with the purified rabbit PGM sample, and eighteen components between 140,000 and 14,000 mw. are detected in the human skeletal muscle extract. Both samples show the strongest signal in the 60,000 mw. region of the gel, which is approximately equivalent to the molecular weight of rabbit PGM calculated by Ray *et al.* (1983) (61,600 mw).

The control filters (c) and (d) also show some immunoreactivity. Five components between 60,000 and 32,000 mw. are detected in purified rabbit PGM on both control filters. The human skeletal muscle extract shows between twelve and fifteen immunoreactive bands ranging from 140,000 to 14,000 mw., on filters (c) and (d) respectively. Filter (a) shows no immunoreactive components, which proves that none of the above cross-reactivity shown on filters (b), (c) and (d) is due to non-specific binding of the HRP-conjugated antibody. Furthermore, none of the control filters show any immunoreactive bands which correspond to the major 60,000 mw. component detected on filter (b) in either the rabbit or the human samples.

Figure 4.3 compares the components detected in purified

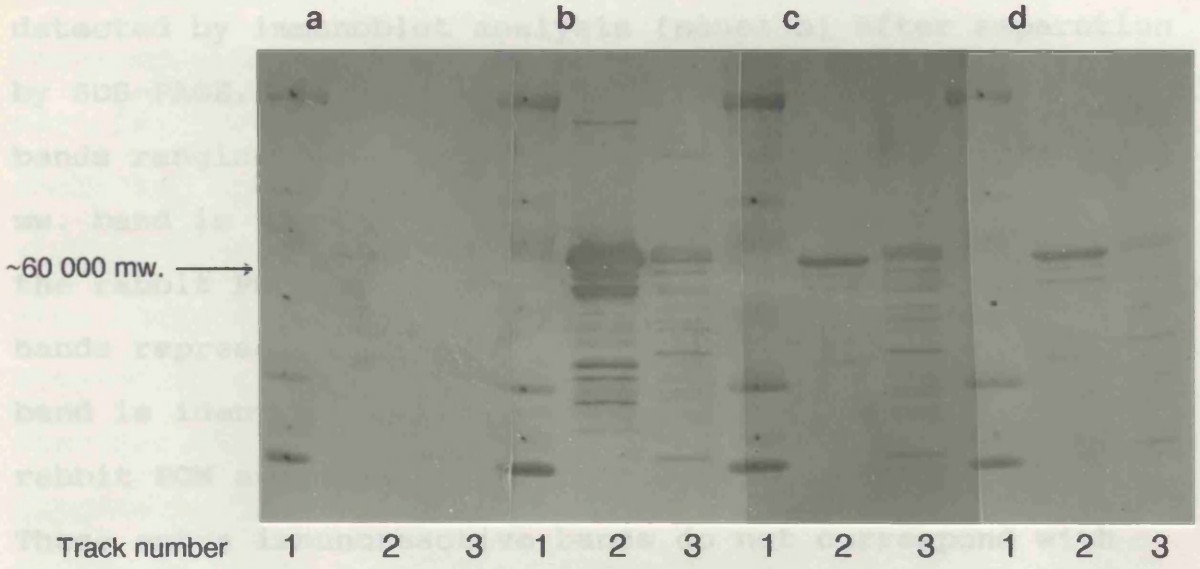


Figure 4.2

Immunoblot analysis of rabbit and human muscle extracts, tracks 2 and 3 respectively, following separation by SDS gel electrophoresis. Track 1 contains standard molecular weight rainbow markers. No primary antiserum control panel (a), incubation with the immune IgG fraction of the sheep anti-rabbit PGM antiserum (b), cross-reactivity with non-immune IgG fraction of sheep serum (c) and the pre-immune IgG fraction of the sheep serum (d).

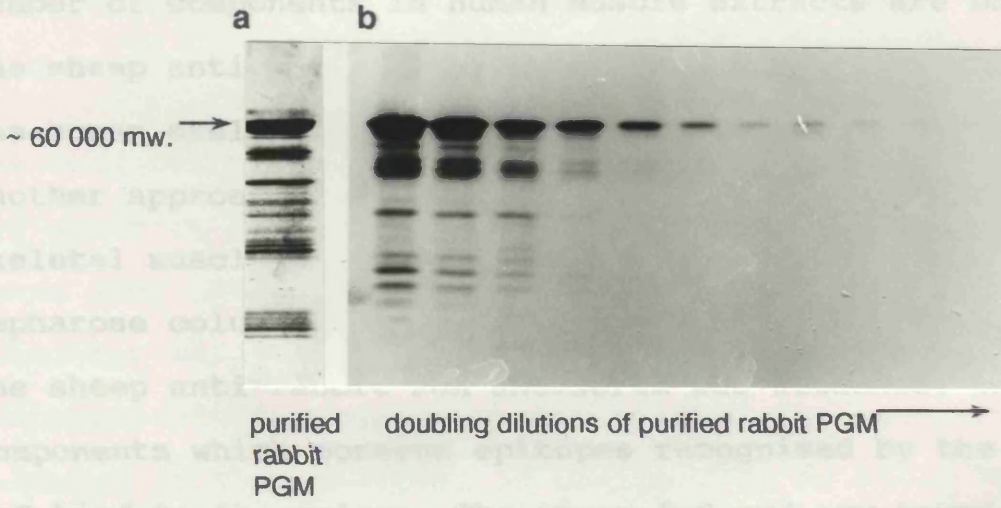


Figure 4.3

SDS gel electrophoresis of phosphoglucomutase: (a) Purified rabbit muscle PGM stained with Coomassie blue; (b)Immunodetection of dilutions of purified rabbit mucle PGM to the visual limit.

rabbit PGM using protein staining (panel a) with those detected by immunoblot analysis (panel b) after separation by SDS-PAGE. The protein stained profile consists of several bands ranging from 60,000 to 15,000 mw., of which a 60,000 mw. band is the major component. This suggests that either the rabbit PGM is partially purified or the faster migrating bands represent degradation products. The major PGM protein band is identified by the IgG fraction of the sheep anti-rabbit PGM antiserum, together with several minor bands. These extra immunoreactive bands do not correspond with those detected by protein staining. However, after serial dilution of the purified rabbit PGM however, the minor immunoreactive bands disappear, leaving a single component of approximately 60,000 mw., which shows close agreement with the molecular weight determination of rabbit PGM.

Previous experiments illustrated in figure 4.2, show that a number of components in human muscle extracts are bound by the sheep anti-rabbit PGM IgG fraction. To further examine the human skeletal muscle components bound by the sheep IgG, another approach was used. A 1 in 10 dilution of human skeletal muscle homogenate was passed through a protein G sepharose column (Pharmacia) to which the IgG fraction of the sheep anti-rabbit PGM antiserum was attached. The muscle components which possess epitopes recognised by the sheep IgG bind to the column. The sheep IgG and any bound human muscle components are then co-eluted using 0.1M glycine/HCl (pH 2.7). Figure 4.4 shows three sets of samples separated using SDS-PAGE and visualised by immunoblotting or protein staining the gel: the starting material, unbound muscle components and the eluted IgG-bound components. Filter (a)

shows an immunoblot of the column fraction components detected with the sheep anti-rabbit PGM IgG. The starting material (track 3) shows many human muscle components including the strongly stained 60,000 mw. component. The eluate, containing the unbound column material (track 4) also shows several components, but the 60,000 mw. band and several of the smaller components are absent from this fraction. The IgG bound fractions eluted with the acidic glycine buffer, shown in tracks 5 to 10, contain the major 60,000 mw. band along with large amounts of IgG heavy and light chains. The latter mask the expected positions of the smaller eluted components. The control filter (b), which was not treated with the primary sheep anti-rabbit PGM IgG, showed no immunoreactivity in the 60,000 mw. region of the gel, only the presence of large quantities of IgG heavy and light chains, which were visualised by the binding properties of the rabbit anti-goat HRP conjugated antibody used as the second antibody.

The band patterns visualised on the gel by protein staining are shown in figure 4.4 panel (c). The starting material shows many intensely stained bands some of which have a molecular weight of approximately 60,000. Considering the comparatively low concentration of PGM1 in the body, protein detected at this position is unlikely to be human PGM1 alone. The most striking observation was the lack of differences between the protein patterns observed in the untreated skeletal muscle extract (track 3) and the eluate (track 4) after passage through the column. The acidic elution fractions (tracks 5 to 10) revealed only the heavy

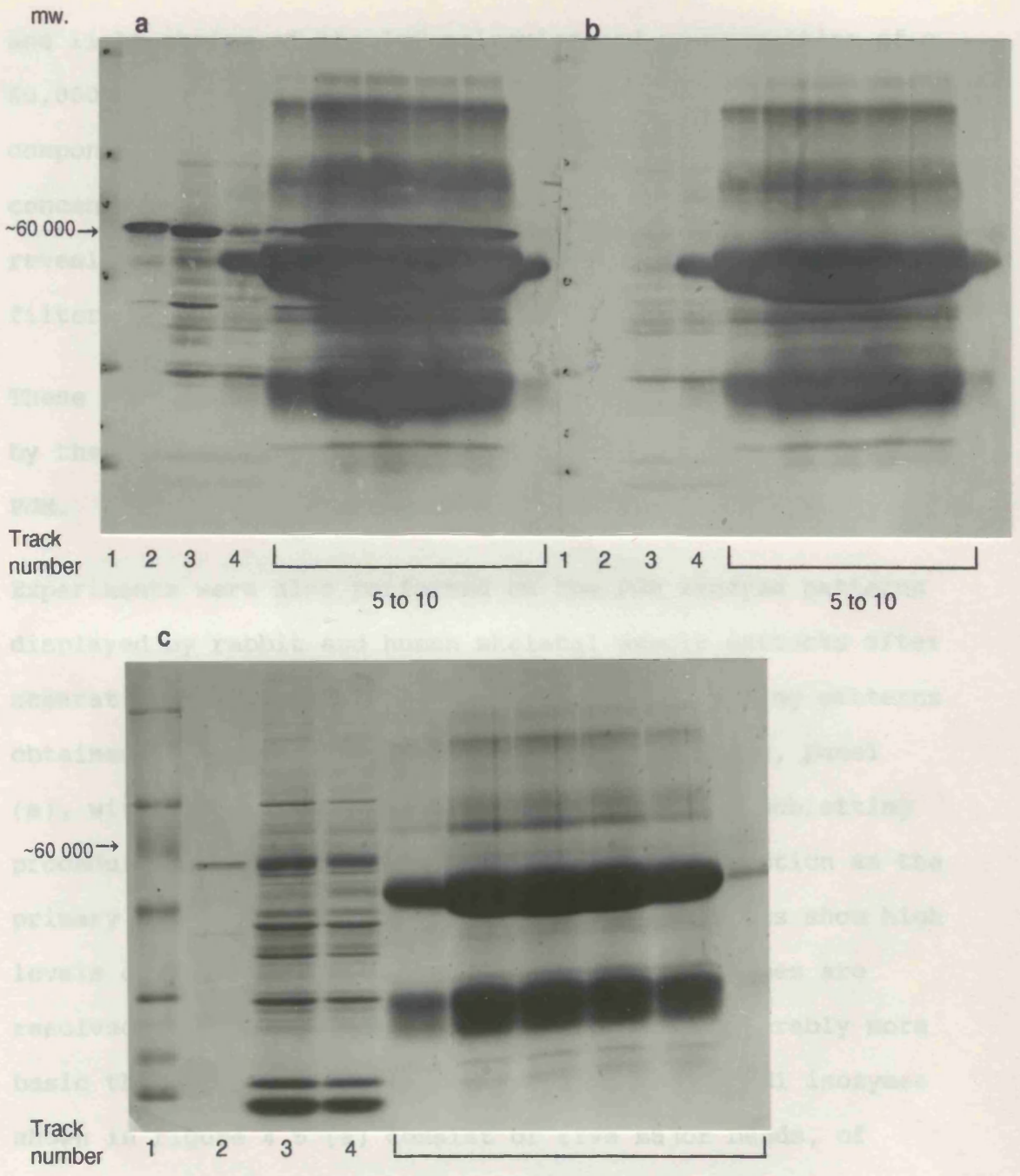


Figure 4.4

SDS gel electrophoresis of immunoaffinity purified column fractions. Track 1, standard molecular weight rainbow markers. Track 2, purified rabbit PGM. Track 3, 1 in 10 dilution of human skeletal muscle extract prior to column purification. Track 4, human muscle extract after purification. Tracks 5 to 10, first 6 elution fractions. Panels (a) and (b) are subjected to immunoblot analysis, (a) incubated in the sheep anti-rabbit PGM IgG fraction, (b) incubated in the secondary HRP-linked antibody alone. Panel (c) illustrates the protein stained gel.

and light chains of the IgG molecules and no suggestion of a 60,000 mw. band. The complete absence of any other components, is probably due to their relatively low concentrations. Only more sensitive detection methods would reveal the column-bound components, as shown in figure 4.4, filter (a).

These results indicate that the 60,000 mw. component bound by the sheep anti-rabbit PGM IgG fraction is probably human PGM.

Experiments were also performed on the PGM isozyme patterns displayed by rabbit and human skeletal muscle extracts after separation by IEF. Figure 4.5 compares the focusing patterns obtained by conventional enzyme activity staining, panel (a), with those obtained using the standard immunoblotting procedure with the sheep anti-rabbit PGM IgG fraction as the primary antibody, panel (b). Both sets of isozymes show high levels of enzyme activity. The rabbit PGM1 isozymes are resolved as four major bands of activity, considerably more basic than the human PGM1 isozymes. The human PGM1 isozymes shown in figure 4.5 (a) consist of five major bands, of which the most basic are the primary PGM1*1A and 1B isozymes. The antiserum detects two of the major rabbit PGM isozymes detected by activity staining and four of the major human PGM1 isozymes, panel (b). As a result, the PGM1 phenotype of this human skeletal muscle extract is easily determined by the sheep anti-rabbit PGM antibody.

Further studies comparing the isozymes detected in other sources, such as red cell lysates, by conventional enzyme activity staining with those bound by the sheep anti-rabbit

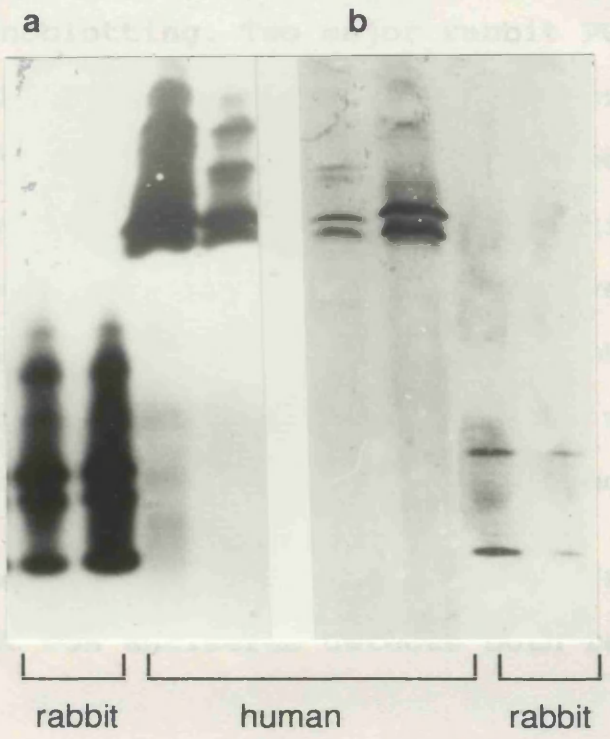


Figure 4.5
Isoelectric focusing of human and rabbit skeletal muscle extracts on a pH 6-8 gradient using enzyme activity staining (a) and immunodetection following electroblotting using anti-rabbit PGM antibodies (b).

PGM antibody are illustrated in figure 4.6. Panel (a) shows the rabbit PGM isozyme pattern detected using enzyme activity staining and panel (b) displays the pattern detected by immunoblotting. Two major rabbit PGM isozymes are detected by both systems. However, the isozymes detected by enzyme activity staining are considerably weaker than those revealed by the binding of the sheep anti-rabbit PGM antibody. This suggests that extremely low levels of enzyme activity are possessed by the PGM found in rabbit red cells, and that the detection of rabbit red cell PGM using the sheep antibody is superior to activity staining.

These results show conclusively that the IgG fraction of the sheep anti-rabbit PGM antiserum detects both rabbit and human PGM1.

4.2 PGM locus specificity.

In this section, experiments were carried out in order to evaluate whether the sheep anti-rabbit PGM antibody shows cross-reactivity with the PGM isozymes determined by the three PGM loci: PGM1, PGM2 and PGM3. The investigation of the PGM isozymes in human milk (PGM4) using conventional enzyme activity staining and the sheep anti-rabbit PGM IgG are described separately in chapter 7.

In the following investigations, fresh red cell lysates were used as a source of PGM1 and PGM2 isozymes and placental homogenates as a source of PGM1, PGM2 and PGM3 isozymes. Figure 4.7 shows the separation of the human PGM isozymes in red cell lysates and placental extracts by starch gel electrophoresis using the TEMM buffer (pH 7.4). The samples

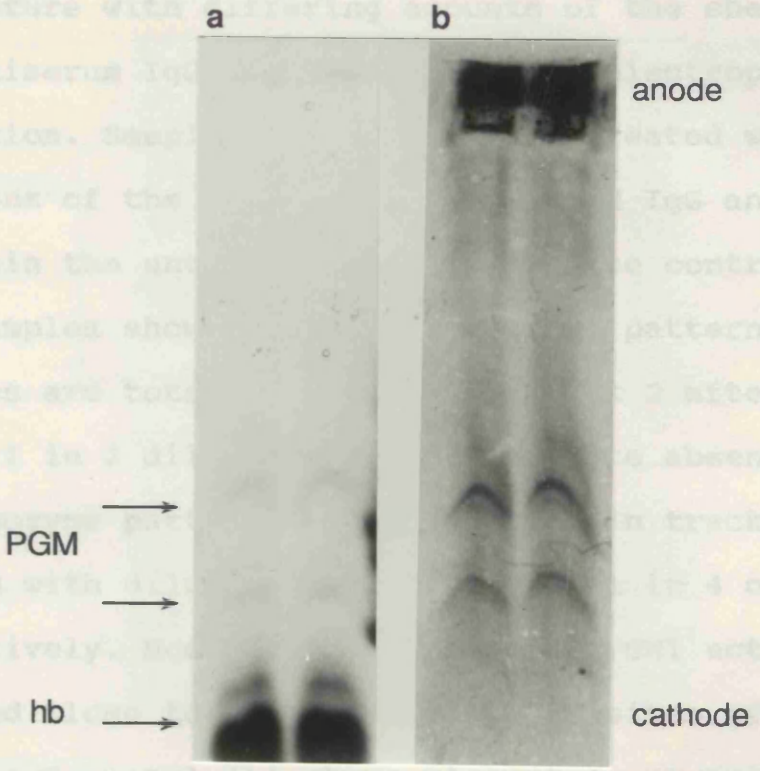
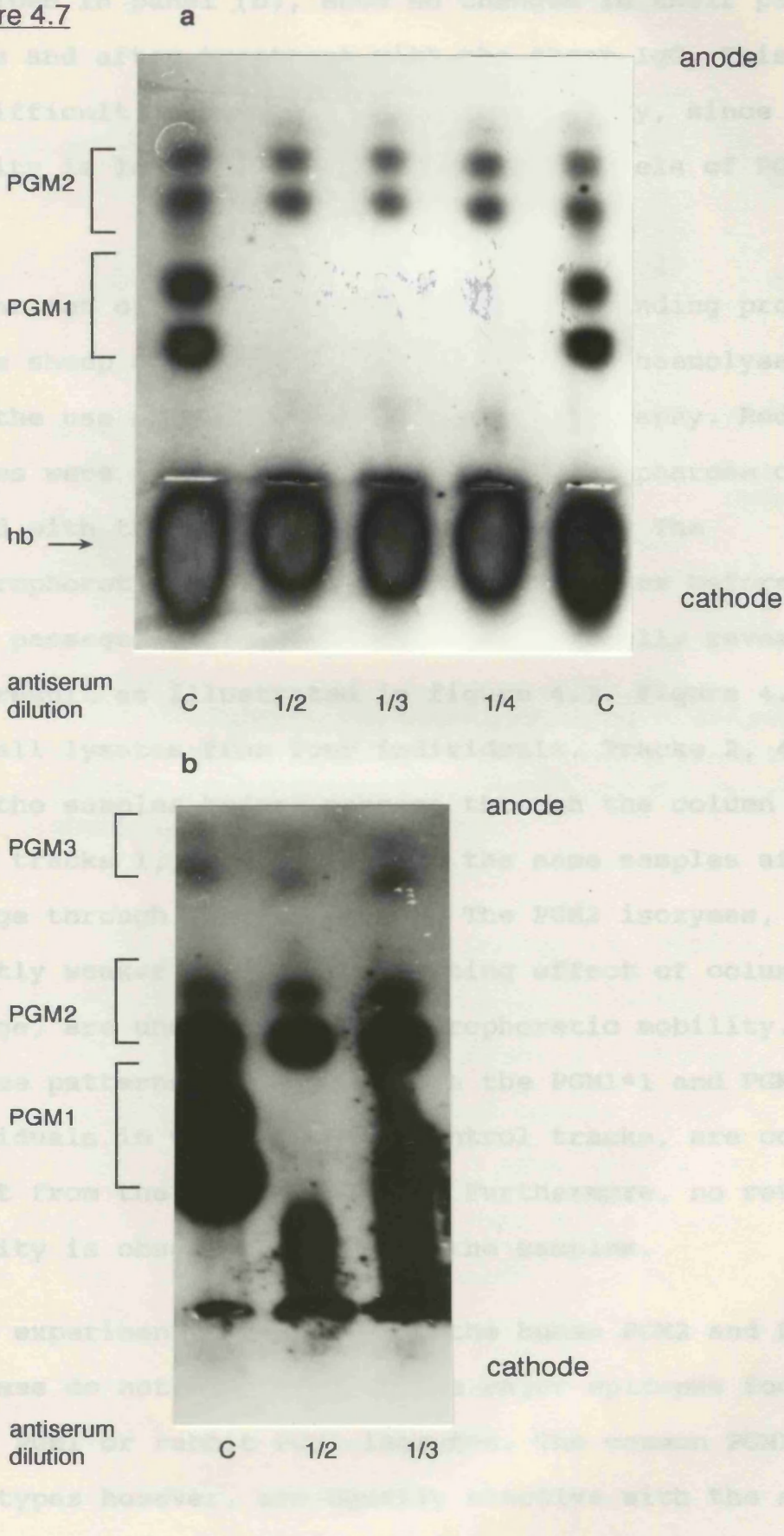


Figure 4.6
The detection of rabbit red cell PGM using enzyme activity staining (a) and immunoblot analysis, after electroblotting in the standard fashion following IEF on a pH 6-8 gradient (b).

shown in figure 4.7 were incubated for 1 hour at room temperature with differing amounts of the sheep anti-rabbit PGM antiserum IgG fraction, prior to electrophoretic separation. Samples 2, 3 and 4 were treated with different dilutions of the sheep anti-rabbit PGM IgG and tracks 1 and 5 contain the untreated red cell lysate control samples. All five samples show normal PGM2 isozyme patterns, but the PGM1 isozymes are totally absent from track 2 after treatment with a 1 in 2 dilution of IgG. Complete absence of normal PGM1 isozyme patterns was also noted in tracks 3 and 4, treated with dilutions of 1 in 3 and 1 in 4 of IgG respectively. However, low levels of PGM1 activity were observed close to the sample loading sites of these tracks. Figure 4.7, panel (b) shows placental extracts treated in the same fashion as panel (a). The IgG treated samples are shown in tracks 2 and 3 and track 1 contains the untreated control sample. Once again, IgG treatment does not affect the mobility or enzyme activity of the PGM2 isozymes but does result in the complete disappearance of normal PGM1 isozyme patterns. As with the red cell lysates, The placental samples showed some retarded PGM1 enzyme activity, close to the sites of sample application. Due to the higher levels of PGM1 found in placental tissue, the amount of retarded material displaying PGM activity was greater than that seen with the red cell lysates. The reduced mobility and enzyme activity displayed by PGM1 after treatment with the sheep anti-rabbit PGM IgG is the result of the formation of antibody-PGM complexes which still possess some enzyme activity.

As with PGM2 isozymes, the placental PGM3 isozymes,

Figure 4.7


a
anode
PGM2
PGM1
hb
cathode
antiserum dilution
C
1/2
1/3
1/4
C
b
anode
PGM3
PGM2
PGM1
cathode
antiserum dilution
C
1/2
1/3

described in panel (b), show no changes in their pattern before and after treatment with the sheep IgG. This result was difficult to reproduce photographically, since PGM3 activity is low compared with the high levels of PGM1 and PGM2.

Another set of experiments examined the binding properties of the sheep anti-rabbit PGM IgG to human haemolysate PGM with the use of immuno-affinity chromatography. Red cell lysates were passed through a protein G sepharose column, linked with the sheep anti-rabbit PGM IgG. The electrophoretic analysis of red cell lysates before and after passage through this column essentially reveal the same result as illustrated in figure 4.7. Figure 4.8 shows red cell lysates from four individuals. Tracks 2, 4, 6 and 8 show the samples before passage through the column (-), while tracks 1, 3, 5 and 7 show the same samples after passage through the column (+). The PGM2 isozymes, although slightly weaker due to the diluting effect of column passage, are unchanged in electrophoretic mobility. The PGM1 isozyme patterns associated with the PGM1*1 and PGM1*2-1 individuals in the untreated control tracks, are completely absent from the column eluates. Furthermore, no retarded PGM activity is observed in any of the samples.

These experiments suggest that the human PGM2 and PGM3 isozymes do not share any of the major epitopes found on the human PGM1 or rabbit PGM1 isozymes. The common PGM1 phenotypes however, are equally reactive with the sheep anti-rabbit PGM antiserum. Lastly, on a point of methodology, it is clear that incubation of blood or tissue

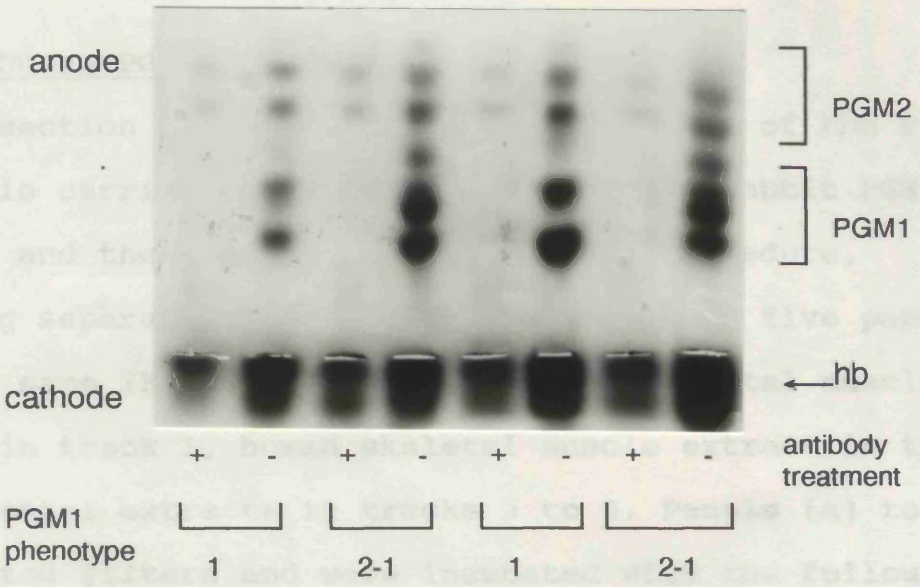


Figure 4.8

Starch gel electrophoresis showing the human PGM isozyme patterns from four haemolysates detected using activity staining, before (-) and following (+) immunoaffinity chromatography using the sheep anti-rabbit PGM IgG fraction.

samples with the sheep IgG fraction give more reliable results than the protein G method.

4.3 The human PGM1 polymorphism.

In this section a more detailed investigation of the PGM1 alleles is carried out using the sheep anti-rabbit PGM antibody and the standard immunoblotting procedure, following separation by IEF. Figure 4.9 shows five panels from the same IEF gel containing rabbit skeletal muscle extract in track 1, human skeletal muscle extract in track 2 and placental extracts in tracks 3 to 5. Panels (a) to (d) are blotted filters and were incubated with the following soluions: panel (a), untreated; panel (b), the IgG fraction of the sheep anti-rabbit PGM immune serum; panel (c), the IgG fraction from a non-immune serum and panel (d) with the IgG fraction from the preimmune serum. Panel (e) shows an PGM enzyme activity stained section of gel.

Incubation with the immune IgG, (panel b), displays strong immunoreactivity with all of the samples examined. The rabbit muscle consists of a multitude of immunoreactive components, the strongest of which appear to be the most basic isozymes. The human samples show considerably simpler immunoreactive isozyme patterns. The human skeletal muscle sample consists of five immunoreactive bands, two of which possess the same isoelectric points as the most basic rabbit PGM components. The most acidic component detected in the human skeletal muscle corresponds to the primary PGM1 isozyme, the PGM1A phenotype. The human placental extracts display immunoreactivity with the primary PGM1 isozymes alone, corresponding with the PGM1 phenotypes PGM1A and

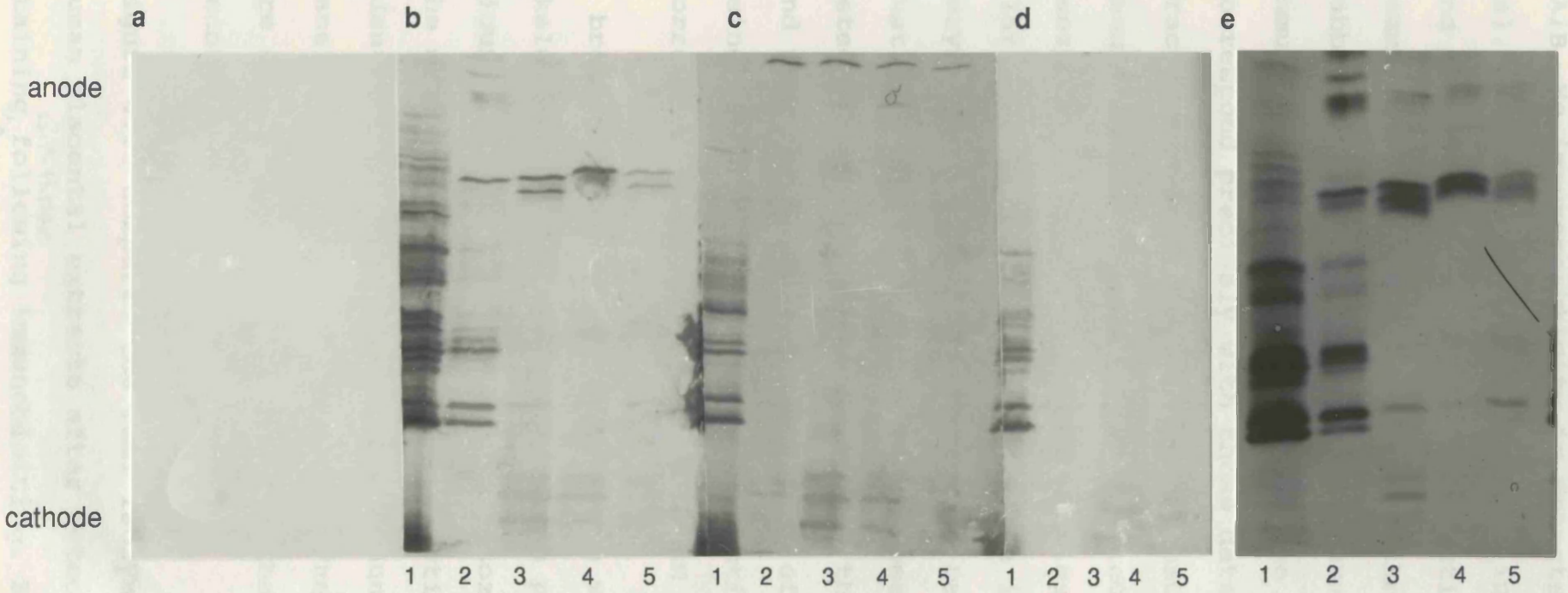


Figure 4.9

Immunological detection and enzyme activity staining of rabbit muscle PGM (track 1) , human skeletal muscle PGM (track 2) and human placental PGM (tracks 3 to 5), after separation by IEF on a 6-8 pH gradient. Panels a to d were subjected to immunoblot analysis, specifically, panel (a) incubated in the secondary HRP-linked antibody alone, panel (b) illustrates the cross-reactivity with the IgG fraction of the sheep anti-rabbit PGM immune serum, panel (c) incubation in non-immune sheep IgG, and panel (d) incubation in preimmune sheep serum. Panel (e), shows the components from the same samples displaying PGM enzyme activity.

1A1B. No immunoreactivity is detected on the control filter (a). However, panels (c) and (d), incubated in non-immune and preimmune IgG fractions respectively, show a number of immunoreactive components in all the samples examined. The rabbit sample shows the greatest number of non-specific immunoreactive bands, although none of these components correspond precisely with those detected by the immune IgG fraction. The non-immune and preimmune IgG do not reveal the immunoreactive components associated with the major PGM1 isozymes in the human tissue samples examined. This is clarified further by comparing the immunoblots with the PGM1 enzyme activity patterns shown in panel (e). Panel (e) shows that there is no relationship between the components detected by activity staining and those using the non-immune and preimmune IgG fractions. Many of the components detected using the sheep anti-rabbit PGM antibody, on the other hand, correspond to those detected by PGM activity staining.

A broader range of PGM1 phenotypes were examined from human skeletal muscle extracts and human placental extracts in figures 4.10 and 4.11. The PGM1 isozyme patterns detected in the skeletal muscle extracts by activity staining are identical to those detected by immunoblotting, figure 4.10, panels (a) and (b) respectively. The primary PGM1 isozymes are easily identified along with the more faintly stained secondary isozymes.

Figure 4.11 compares the PGM1 isozyme patterns obtained from human placental extracts after detection by enzyme activity staining, with those following immunoblotting. Both the primary and secondary PGM1 isozymes are easily identified from the

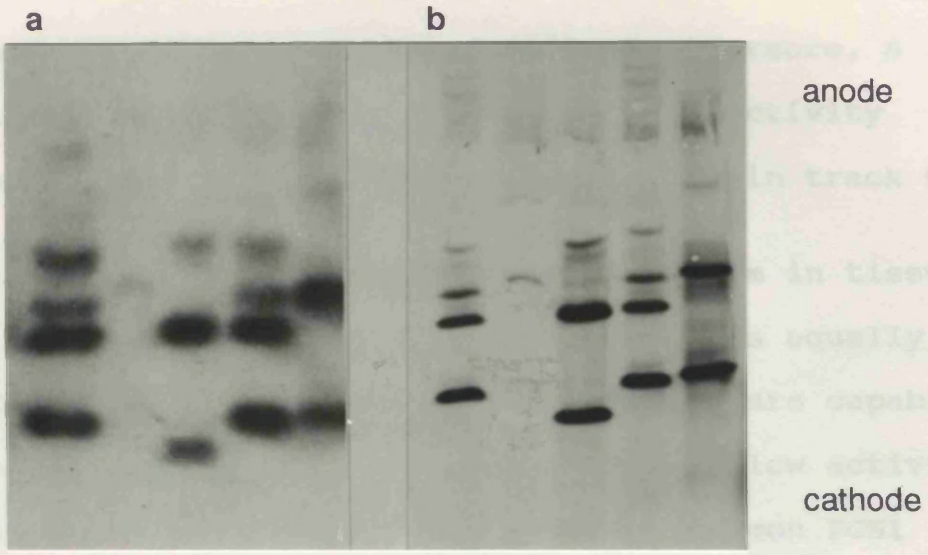


Figure 4.10

Isoelectric focusing of human skeletal muscle PGM1 phenotypes on a pH 5-7 gradient detected using enzyme activity staining (a) and immunodetection using anti-PGM antibodies following passive transfer to nitrocellulose (b).

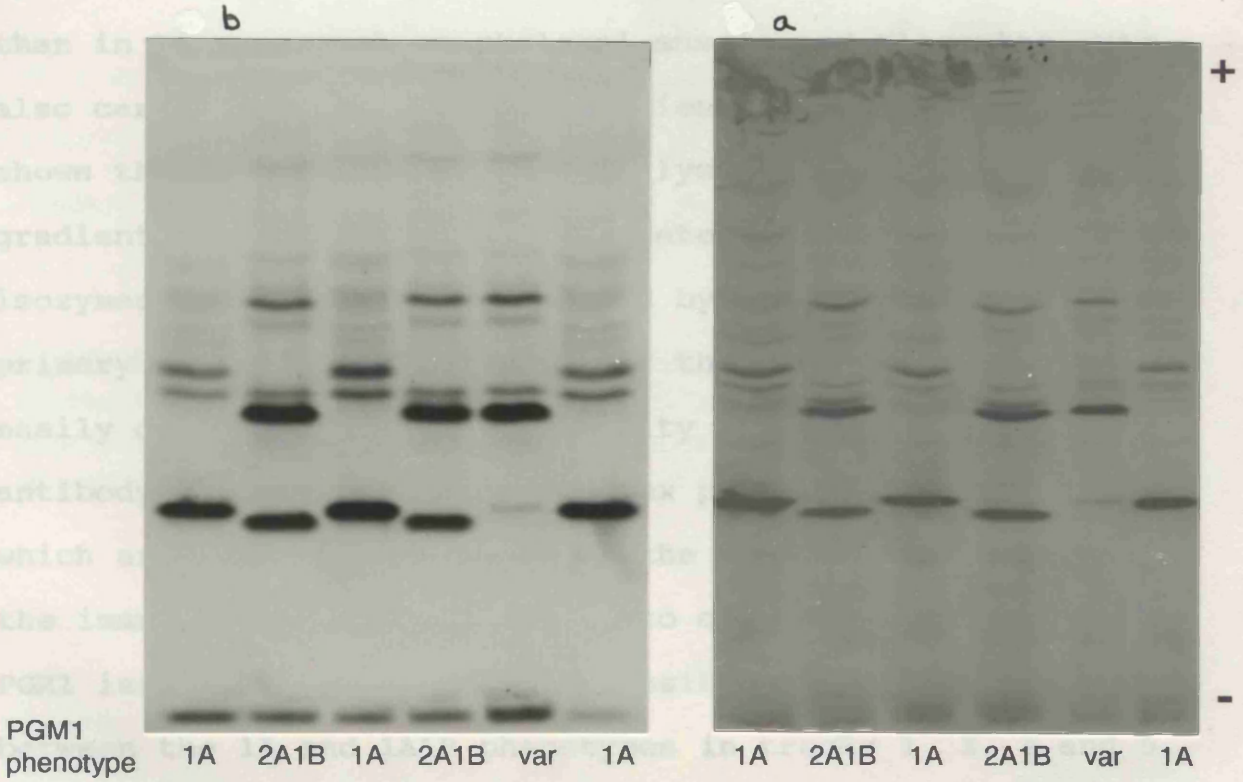


Figure 4.11

Immunological detection (a) and enzyme activity staining (b) of a selection of human placental extracts following separation by IEF on a 5-7 pH gradient. Two common PGM1 phenotypes are identified along with a possible PGM1 variant in track 5.

common phenotypes PGM1*1A, 2A1A and 2A1B. Furthermore, a possible rare variant is identified using both activity staining and the sheep anti-rabbit PGM antibody in track 5.

These results indicate that the PGM1 polymorphism in tissues containing high levels of PGM1 enzyme activity is equally well detected using either method. Both methods are capable of detecting a possible rare PGM1 variant. This low activity variant must share several epitopes with the common PGM1 proteins, since the intensity of the immunostained bands correspond to those observed with activity staining.

The identification of the PGM1 polymorphism in red cell lysates, where the enzyme activity is considerably lower than in tissues such as skeletal muscle and placentae, was also carried out using two detection systems. Figure 4.12 shows the separation of red cell lysates on a pH 5-7 gradient IEF gel, comparing the detection of the PGM1 isozymes by activity staining and by immunoblotting. The primary and secondary isozymes of the PGM1 phenotypes were easily distinguished after activity staining. However, the antibody identifies a very complex pattern of bands, most of which are much more acidic than the PGM1 isozymes. Some of the immunoreactive bands appear to correspond to the primary PGM1 isozymes, making it just possible to distinguish between the 1A and 1A1B phenotypes in tracks 1, 2, 4 and 5. However, the 2A1A heterozygote in track 3, is not identifiable by immunoblotting. It was concluded that the detection of red cell PGM1 using the sheep anti-rabbit PGM antiserum is less satisfactory than conventional enzyme activity staining. This is due to the low levels of PGM1 in

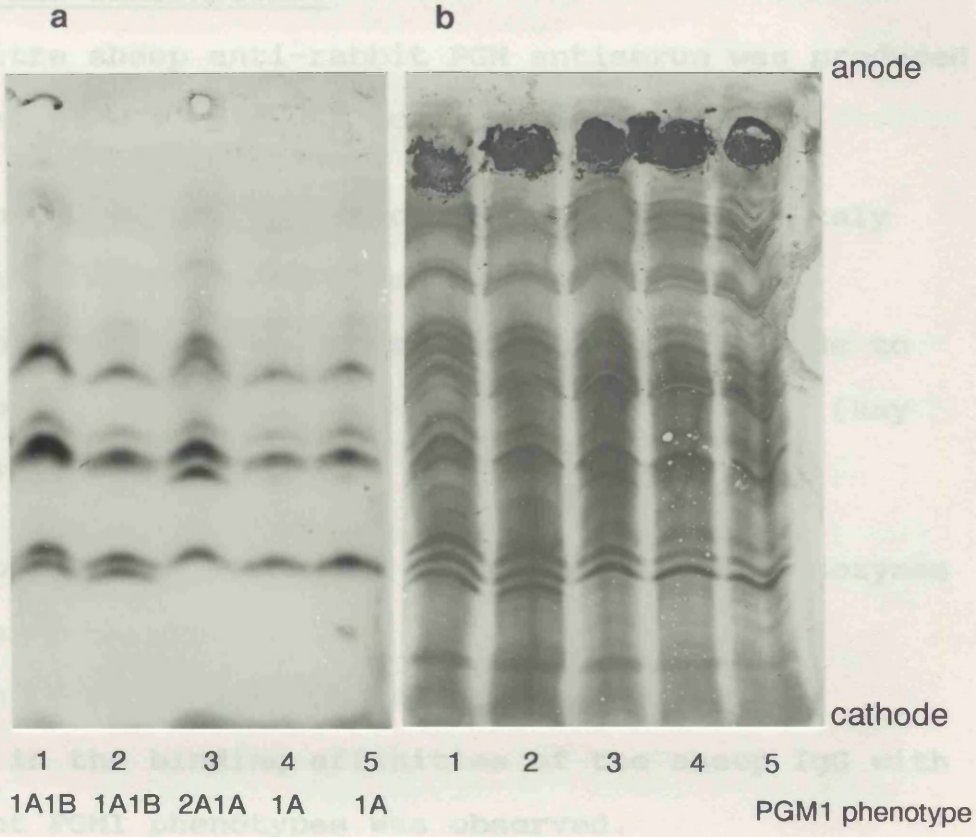


Figure 4.12
Enzyme activity staining (a) and immunological detection (b) of the PGM1 phenotypes displayed by a selection of human haemolysates following separation by IEF on a 5-7 pH gradient.

human red cells relative to placenta and skeletal muscle and the large number of extraneous highly immunoreactive bands that were not seen in the other tissues.

4.4 Summary and conclusions.

1) A high titre sheep anti-rabbit PGM antiserum was produced which cross-reacts with and precipitates human PGM1.

2) The sheep IgG identifies a component of approximately 60,000 mw. in rabbit and human muscle extracts after separation by SDS-PAGE which approximately corresponds to the known molecular weight of rabbit PGM, 61,600 mw. (Ray *et al.* 1983).

3) The sheep IgG identifies the focused rabbit PGM isozymes and the human PGM1 polymorphism in tissue extracts containing high concentrations of the protein. No differences in the binding affinities of the sheep IgG with the different PGM1 phenotypes was observed.

4) PGM isozyme subtraction experiments using the sheep IgG show the disappearance of the PGM1 isozymes from red cell lysates and both placental and skeletal muscle extracts after treatment. The PGM2 and PGM3 isozymes remain unchanged.

5) The sheep anti-rabbit PGM IgG also recognised a possible PGM1 variant. No family studies were possible to confirm the basis of this variant phenotype.

The sheep anti-rabbit PGM polyclonal antibody shows good cross reactivity with the rabbit antigen and binds specifically to human PGM1 alone. The high titre of this

antibody enables the immunodetection of extremely low levels of PGM present in the rabbit haemolysates. Thus, anti-human PGM antibodies may be helpful for the detection of lower levels of human PGM present in blood samples and forensic material.

Chapter 5:

The detection of PGM by immunoblotting.

This chapter covers the detection of rabbit and human PGM by immunoblot analysis and highlights the steps in the procedure which lead to greater sensitivities of detection. The basic approach is similar to that used for the immunodetection of the GC polymorphism, described in chapter 3.

5.1 Sample preparation.

Figure 5.1, compares the application of purified rabbit PGM to IEF gels using Whatman 3MM paper with direct application to the gel surface. The activity stained gel shows high levels of PGM remaining on the application papers in panel (a), restricting the detection limit to approximately 780ng. Panel (b) demonstrates direct application of the sample increases the sensitivity by approximately 520 times. The immunodetection of rabbit PGM, using the sheep anti-rabbit PGM IgG characterised in chapter 4, also shows similar increases in sensitivity of detection. Direct application of the rabbit PGM improves the sensitivity approximately 65 fold (panel d) compared with paper application (panel c). It is therefore likely that purified rabbit PGM has a high binding affinity for Whatman paper. This seriously diminishes the sensitivity of both enzyme activity staining and immunodetection after IEF.

Figure 5.2 shows that the method of sample application also has a profound effect on the sensitivity of PGM1 detection in dilutions of human skeletal muscle extract. Panel (a) shows the paper application of doubling dilutions of human

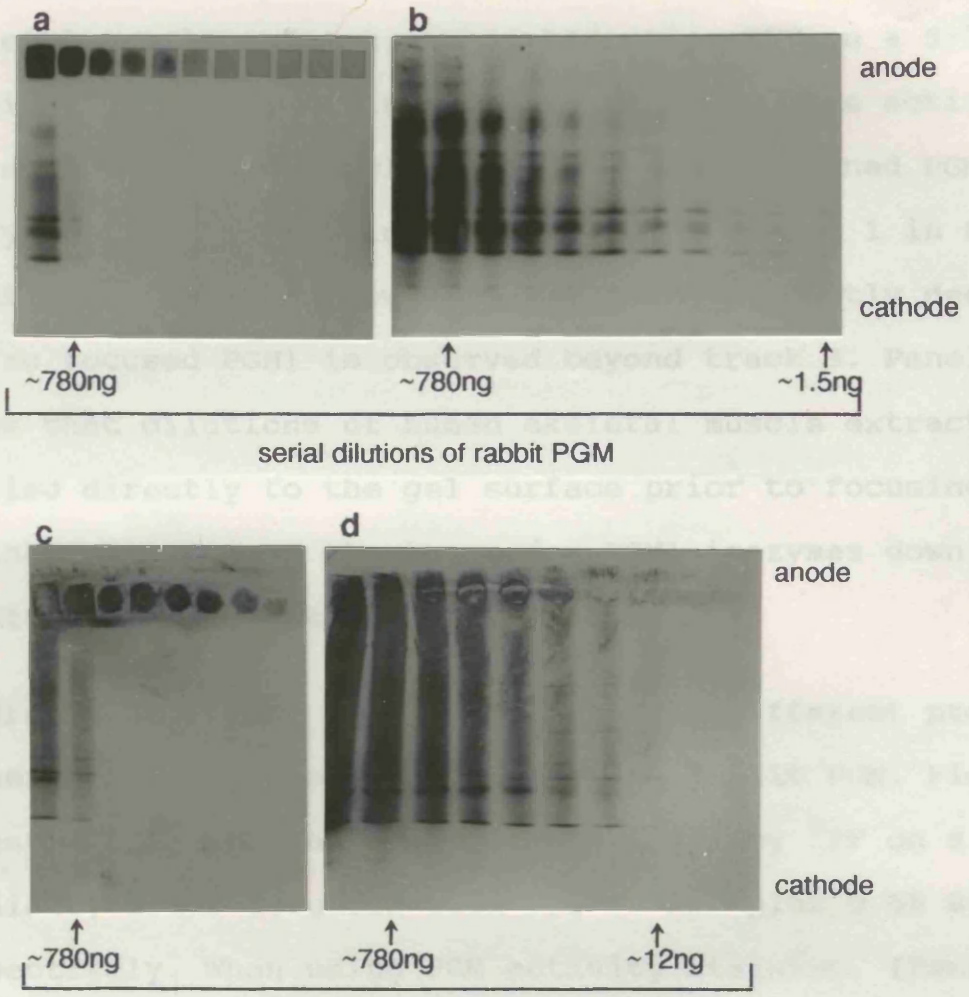


Figure 5.1

Serial dilutions of purified rabbit PGM detected using enzyme activity staining (a) and (b) or immunoblot analysis, (c) and (d), following separation by IEF on a 6-8 pH gradient. The samples are applied to the gels either using paper applicators, as illustrated in panels (a) and (c) or loading directly onto the gel surface (b) and (d).

skeletal muscle extract, separated using IEF on a 5-7 pH gradient. Activity staining shows strong enzyme activity at the sites of sample application. Strongly stained PGM1 isozymes are observed in track 1, containing a 1 in 8 dilution of extract. However, the signal abruptly decreases, and no focused PGM1 is observed beyond track 3. Panel (b) shows that dilutions of human skeletal muscle extract applied directly to the gel surface prior to focusing, lead to the detection of the two major PGM1 isozymes down to a dilution of 1 in 2048.

Figures 5.3 and 5.4 show the effects of different protein diluents on the detection of purified rabbit PGM. Figure 5.3 illustrates the separation of rabbit PGM by IEF on a 6-8 pH gradient, after dilution with PBS or PBS plus 0.5% BSA respectively. When using PGM activity staining, (Panels a and b), the addition of BSA to the loading buffer (b) results in over 60 fold greater sensitivity of rabbit PGM detection but only a 4 fold increase in sensitivity by immunoblot analysis (Panels c and d).

Figure 5.4 illustrates experiments with other sample diluents: 0.5% haemoglobin, 0.5% gelatin and 0.5% skimmed milk in PBS. Rabbit PGM separated by IEF using 6-8 pH gradients is detected by activity staining in panels (a) to (c) and by immunoblotting in panels (d) to (f). Of the diluents examined in this set of experiments, haemoglobin gives good signal intensity of the PGM1 isozymes, even after considerable dilution (panels a and d). Furthermore, there was a complete lack of enzyme activity and immunoreactivity at the sample application sites. Panel b (gelatin) shows

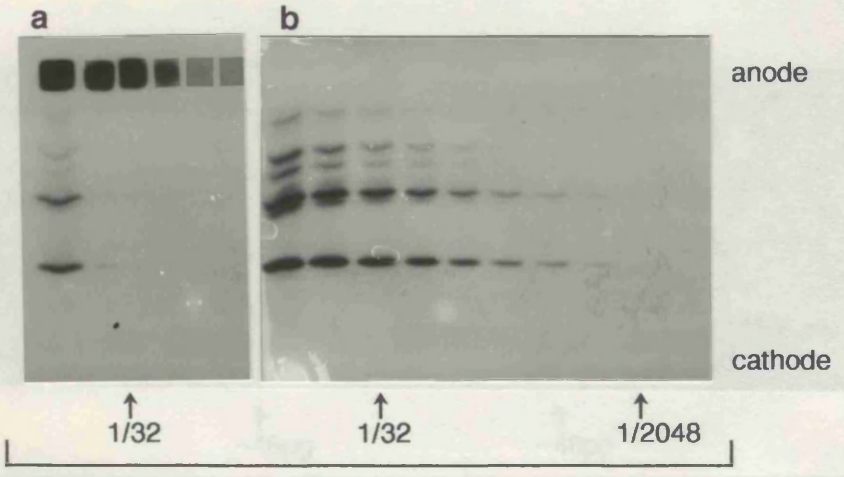


Figure 5.2
Detection of serial dilutions of human skeletal muscle PGM using enzyme activity staining following separation by IEF on a 5-7 pH gradient.The effect of paper applicators (a) vs direct sample application (b) on the sensitivity of human PGM detection.

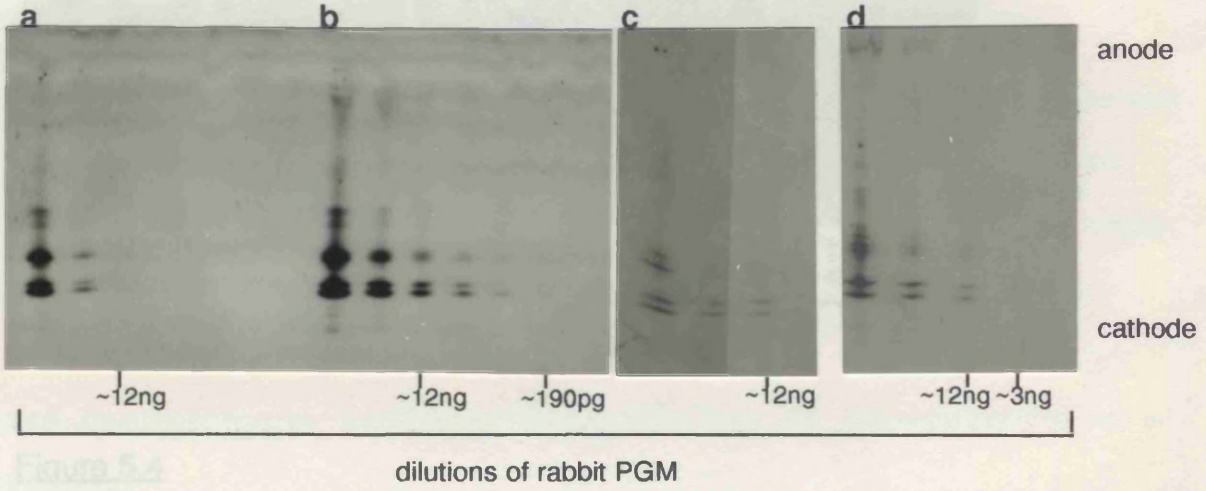
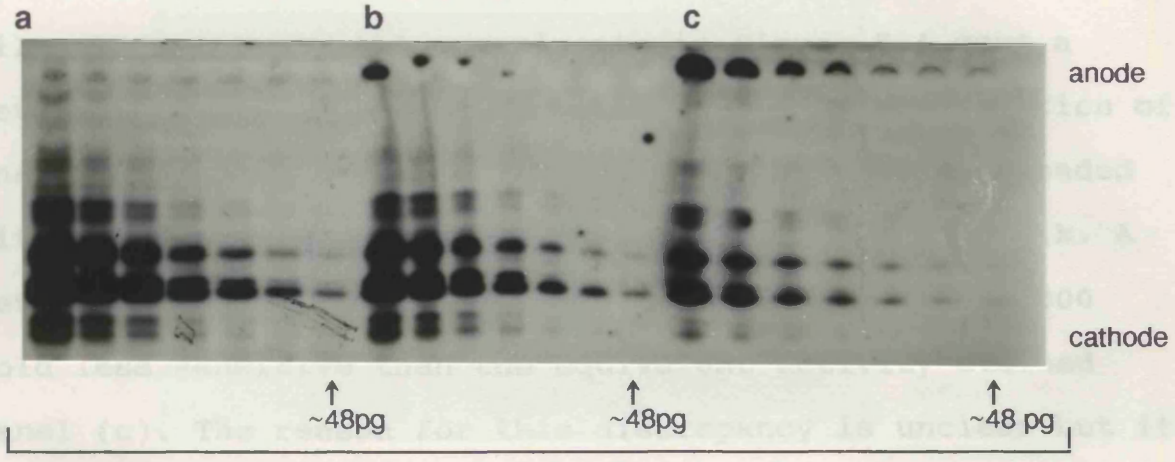


Figure 5.3
Dilutions of purified rabbit PGM detected using enzyme activity staining (a) and (b) or immunoblot analysis (c) and (d), following separation by IEF on a 6-8 pH gradient. Panels (a) and (c) display the sensitivity of PGM detection following dilution in PBS, and panels (b) and (d) following the use of PBS/0.5% BSA as the sample diluent.



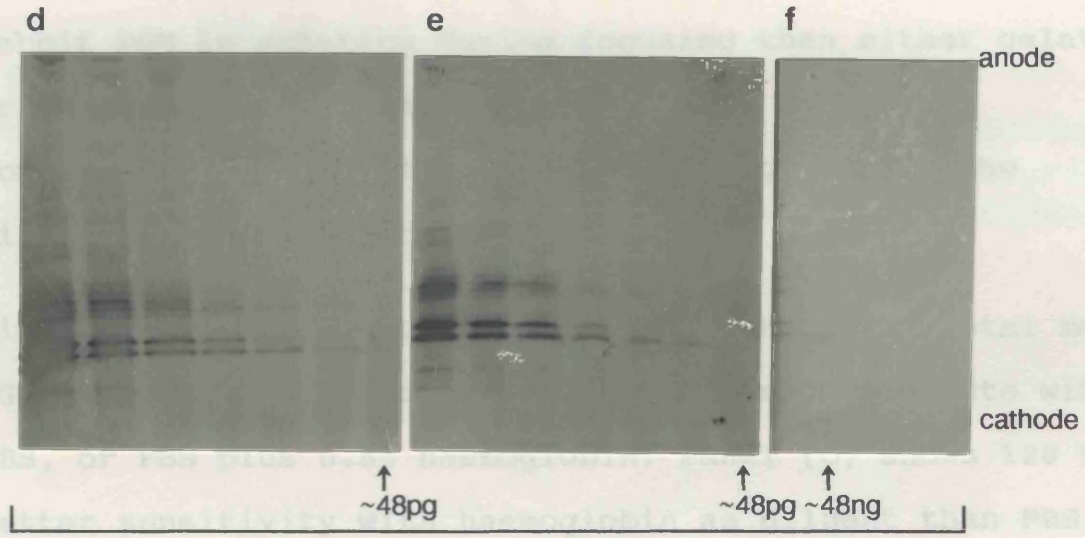


Figure 5.4

Serial dilutions of rabbit PGM using different loading buffers, PBS/0.5% haemoglobin (a) and (d), PBS/0.5% gelatin (b) and (e), and PBS/0.5% skimmed milk (c) and (f). The rabbit PGM isozymes were separated by IEF on a 6-8 pH gradient, and subjected to enzyme activity staining, panels (a) to (c) or immunological detection, panels (d) to (f).

small amounts of unfocused protein, while high levels of unfocused protein were found when skimmed milk was used as diluent (panel c). All experiments in figure 5.4 gave a detection limit of 48pg of rabbit PGM, with the exception of the immunoblot shown in panel (f). Here the gel was loaded with rabbit PGM diluted with PBS plus 0.5% skimmed milk. A detection limit of approximately 48ng was recorded, 1000 fold less sensitive than the equivalent activity stained panel (c). The reason for this discrepancy is unclear but it may be due to reduced efficiency of protein transfer. These results show that skimmed milk is less efficient at keeping rabbit PGM in solution during focusing than either gelatin or haemoglobin. Optimal focusing of low rabbit PGM concentrations are obtained using haemoglobin as the diluent, panels (a) and (d).

Figure 5.5 compares the sensitivity of human skeletal muscle PGM1 detection after dilution of the muscle extracts with PBS, or PBS plus 0.5% haemoglobin. Panel (b) shows 128 fold better sensitivity with haemoglobin as diluent than PBS alone, panel (a). Thus, detection of human muscle PGM is also considerably improved with the use of haemoglobin as a diluent.

5.2 Method of protein transfer.

This section describes a set of experiments carried out to investigate the efficiency of PGM transfer from IEF gels. Comparisons are made between passive transfer, conventional tank electroblotting and two semi-dry electroblotting systems.

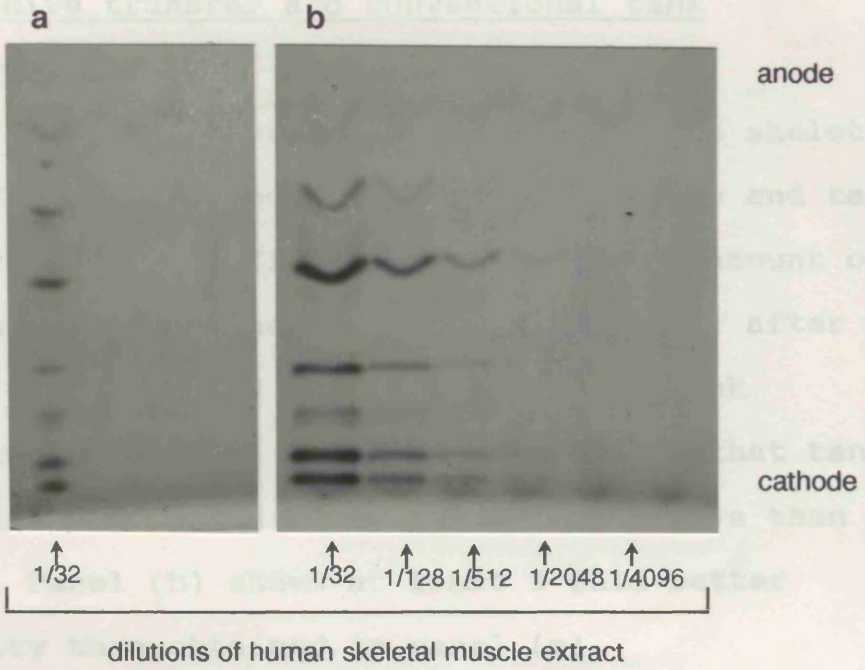


Figure 5.5

Detection of dilutions of human skeletal muscle PGM using enzyme activity staining following separation by IEF on a 5-7 pH gradient. The samples loaded in panel (a) were diluted with PBS alone and the samples loaded in panel (b) were diluted with PBS/0.5% haemoglobin.

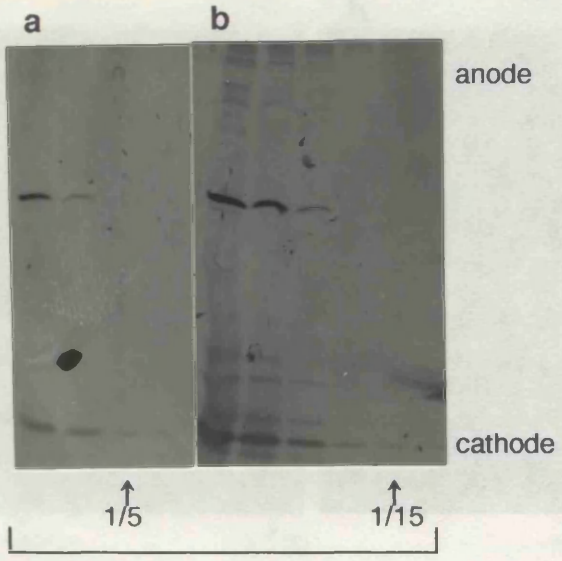
5.2.1 Passive transfer and conventional tank electrotransfer.

Figure 5.6 displays serial dilutions of human skeletal muscle PGM1 detected before and after passive and tank electrotransfer. The figure illustrates the amount of human PGM1 transferred to the nitrocellulose filter after passive transfer (panel a) and after conventional tank electroblotting (panel b). The results show that tank electroblotting is considerably more effective than passive blotting. Panel (b) shows at least 3 fold better sensitivity than obtained in panel (a).

Panel (e) shows a section of gel stained for PGM activity after passive transfer. The results show that considerable amounts of human PGM remain untransferred, whereas panel (f), shows the complete absence of PGM activity in the gel after tank electrotransfer. No significant changes in the amount of PGM activity are displayed after incubation in the TGM transfer buffer for 1 hour, panel (d), compared with the untreated, unblotted gel, panel (c). It is estimated that more than 90% of the human PGM is transferred by conventional tank electroblotting.

5.2.2 Tank electroblotting and semi-dry electroblotting.

Figure 5.7 compares the efficiency of rabbit PGM transfer from IEF gels by conventional tank electroblotting, panel (a), with semi-dry electroblotting using the LKB semi-dry blotting apparatus, panel (b). The sensitivity of rabbit PGM detection is approximately 16 fold better after tank electroblotting than semi-dry transfer with the LKB unit.



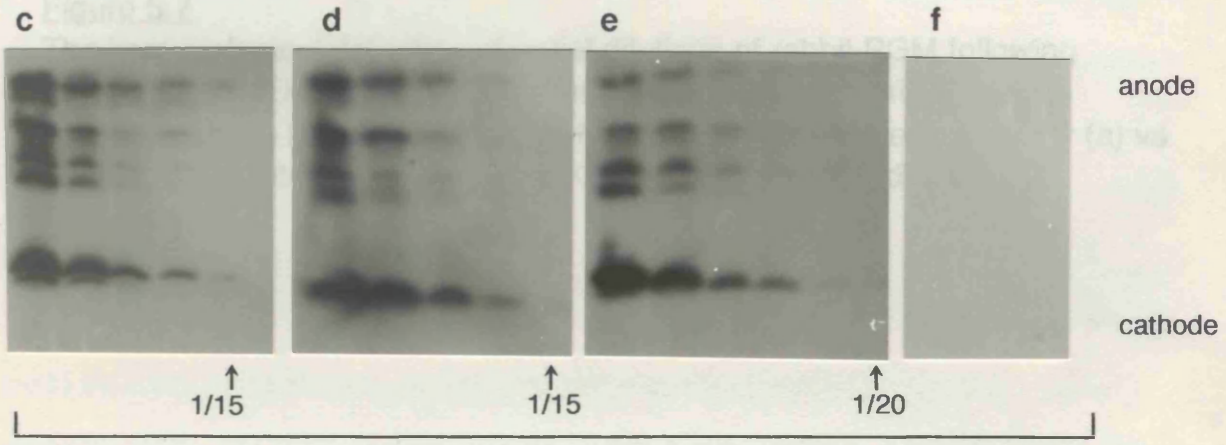


Figure 5.6

Dilutions of human skeletal muscle PGM detected by immunoblot analysis (panels a and b) or enzyme activity staining (panels c to f), following separation by IEF on a 6-8 pH gradient. Panels (a) and (b) display the sensitivity of the immunological detection of human PGM1 following passive transfer and conventional tank electrotransfer to nitrocellulose using the TGM transfer buffer. Panel (c) displays the human PGM1 isozymes detected in the gel prior to transfer, panel (d) shows the effect of one hour incubation in theTGM transfer buffer, panel (e) displays the amount of human PGM1 remaining within the gel after passive transfer and panel (f) displays the human PGM1 remaining in the gel after electrotransfer.

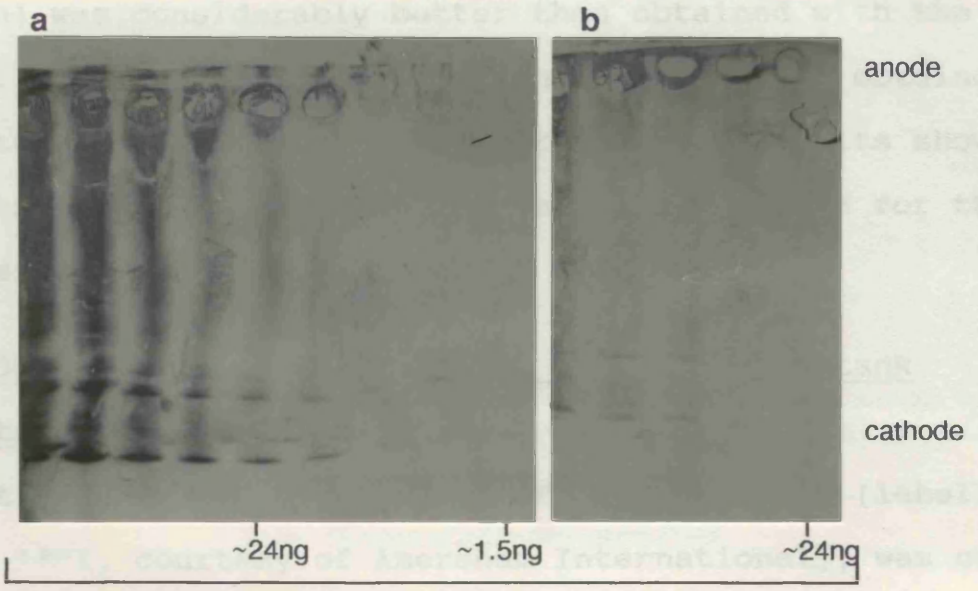


Figure 5.7

The immunological detection of serial dilutions of rabbit PGM following separation by IEF on a 6-8 pH gradient and transfer to a nitrocellulose membrane. The transfer efficiency of conventional tank electrotransfer (a) vs semi-dry electrotransfer using the LKB apparatus (b) are displayed.

The transfer obtained with the Millipore system (results not shown) was considerably better than obtained with the LKB unit. However 2 fold better transfer was still obtained with the tank electroblotting apparatus. These results show tank electroblotting to be the most efficient method for the transfer of rabbit PGM from IEF gels.

5.2.3 The transfer of iodinated rabbit PGM by tank electroblotting.

A set of experiments using purified rabbit PGM (labelled with ^{125}I, courtesy of Amersham International), was carried out in order to assess further the efficiency of protein electrotransfer. Figure 5.8 illustrates the separation of iodinated rabbit PGM using a 6 to 8 pH gradient. In panels (a) and (b), the samples were doubly diluted using 0.5% haemoglobin in PBS as the diluent, and loaded directly onto the gel surface. Track 1 contains approximately 20ng of iodinated PGM, equivalent to 125 nanoCuries (nCi) of gamma radiation, which is diluted to approximately 40pg, equivalent to 240 picoCuries (pCi) of gamma radiation, in track 10. Following focusing and electroblotting, the gel was dried onto Whatman paper and together with the nitrocellulose filter was exposed to X-ray film for four days at -70°C. The autoradiograph revealed focused rabbit PGM isozymes down to approximately 290 pg of starting material (panel a, track 6). No focused isozymes were observed on the blotted gel, panel (b). Although the samples were diluted with 0.5% haemoglobin in PBS, which is the optimal diluent for PGM (see section 5.1), large amounts of iodinated PGM remain at the site of application in both panels. However, all the focused protein appears to have

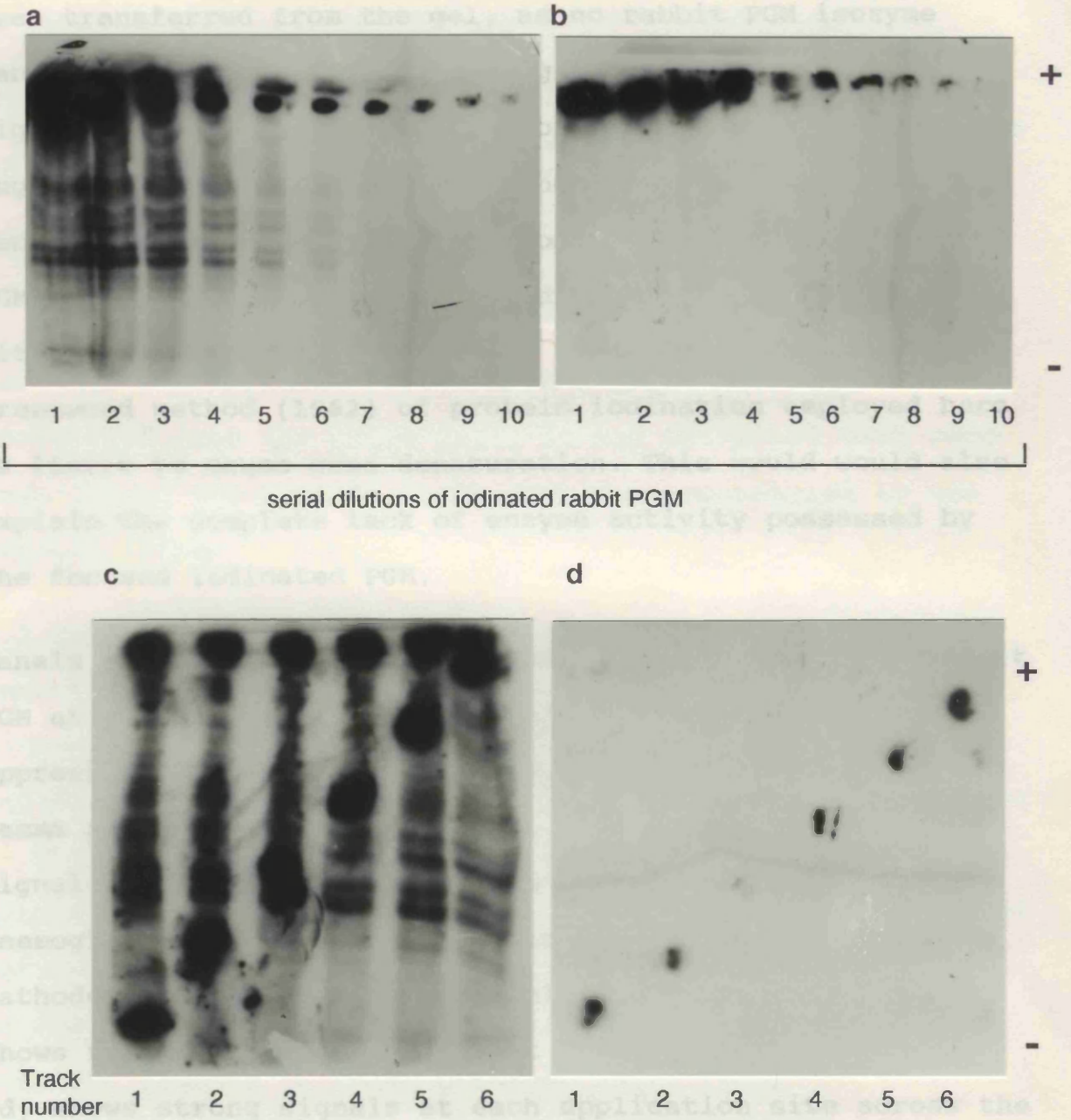


Figure 5.8

The detection of radioactively labelled purified rabbit PGM following separation by IEF on a 6-8 pH gradient and electrotransfer to nitrocellulose with the TGM transfer buffer. Serial dilutions of the iodinated rabbit PGM detected after transfer to the immobilising membrane, panel (a), and detection of the rabbit PGM remaining within the gel after transfer (b). Fixed concentration of iodinated rabbit PGM applied diagonally, from cathode to anode, detected on the immobilising membrane after electrotransfer (c) and the detection of the labelled PGM remaining within the gel after the transfer process (d).

been transferred from the gel, as no rabbit PGM isozyme bands are observed after blotting. The intensity of the signal observed at the application sites of the blotted gel suggests that at least half of the unfocused protein remained untransferred. The reason for the large amounts of PGM at the application site would appear to be connected with the iodination process. For instance, the Hunter & Greenwood method (1962) of protein iodination employed here is liable to cause some denaturation. This would would also explain the complete lack of enzyme activity possessed by the focused iodinated PGM.

Panels (c) and (d), show the application of iodinated rabbit PGM at different sites on the gel. The sample containing approximately 5ng of purified rabbit PGM, about 31 nCi of gamma radiation, panel (a) track 3, was selected for good signal strength on the film. The PGM was diluted in 0.5% haemoglobin as before, but applied in a diagonal line from cathode to anode. The nitrocellulose filter, panel (c), shows focused patterns of rabbit PGM in all tracks. Panel (d) shows strong signals at each application site across the gel. Of some interest is the very intense component which migrates towards the anode, independently of the site of application (eg. panel c). This component remains incompletely resolved near the anode and may be a degradation product resulting from the iodination method used here.

In panel (d), the blotted gel shows none of the rabbit PGM isozymes but some iodinated PGM at the sites of sample application. There is a slight decrease in the amount of PGM

present in track 3 where the sample was applied to the centre of the pH gradient and slightly higher amounts of iodinated sample are observed at the most acidic application site, track 6. No trace of the unresolved acidic component is observed in panel (d) and it is therefore assumed that complete transfer of the component has taken place.

Although the efficiency of transfer could not be accurately quantified from the above data, it was estimated that more than 90% of the protein in the gel was transferred to the nitrocellulose filter.

5.3 Blotting membrane.

Immobilising membranes such as Schleicher & Schull (S&S) nitrocellulose, Amersham nitrocellulose, Amersham Hybond-N and Millipore PVDF were assessed for their abilities to bind PGM after separation by IEF. All the membranes gave comparable transfer except for Amersham Hybond-N which gave very poor transfer and a much higher background signal. In several experiments, the Millipore PVDF membrane showed superior results to those obtained using (S&S) nitrocellulose. Figure 5.9 compares the sensitivity of detecting purified rabbit PGM after electrotransfer to S&S nitrocellulose (a), with that transferred to the PVDF membrane panel (b). The PVDF membrane, shows 2 fold higher sensitivity than the nitrocellulose filter. However, the increase in sensitivity obtained with the PVDF membranes was found to be far from consistent. This is in contrast to the highly reproducible results obtained using the S&S nitrocellulose membranes which were the preferred choice for subsequent experiments.

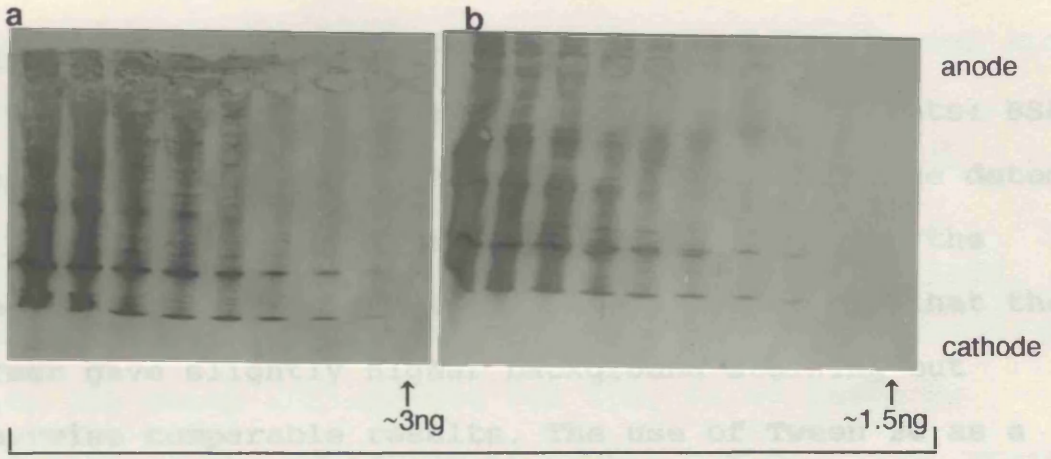


Figure 5.9
Immunological detection of serial dilutions rabbit PGM following separation by IEF on a 6-8 pH gradient and electrotransfer to an immobilising membrane. The rabbit PGM has been transferred to two different types of immobilising membrane, conventionally used S & S nitrocellulose , panel (a) and polyvinylidene diflouride (PVDF) membrane, panel (b).

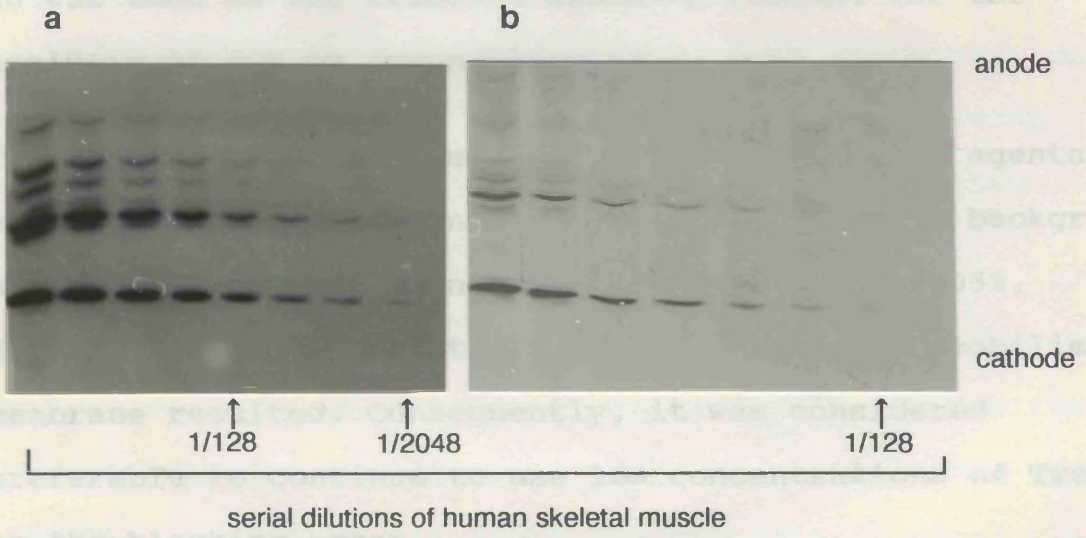


Figure 5.10
The detection of PGM1 isozymes in serial dilutions of human skeletal muscle extract following separation by IEF on a 5-7 pH gradient and enzyme activity staining (a) or immunological detection with the sheep anti-rabbit PGM IgG fraction, following electrotransfer to nitrocellulose (b).

5.4 Blocking reagent.

In this section the effect of the blocking reagents: BSA, Tween 20, Nonidet-P40 (NP-40) and sarkosyl, on the detection of PGM were examined. Preliminary data comparing the blocking properties of BSA with Tween 20 showed that the former gave slightly higher background staining but otherwise comparable results. The use of Tween 20 as a blocking reagent has been well documented in the literature. Higher concentrations of this detergent, have been associated with the loss of membrane bound antigens. This phenomenon has also been reported for the GC protein in section 3.5 of this thesis. In contrast to GC, the binding of the rabbit and human PGM1 isozymes seems unaffected by Tween 20 concentrations as high as 0.5%. Consequently, Tween 20 was used as the standard blocking reagent for the analysis of PGM by immunoblotting.

The properties of sarkosyl and NP-40 as blocking agents were also examined (results not shown). Both gave low background staining but even at concentrations as low as 0.05%, differing degrees of antigen elution from the immobilising membrane resulted. Consequently, it was considered preferable to continue to use low concentrations of Tween 20 as the blocking agent.

5.5. Detection systems.

In this section several different enzyme-linked detection systems are assessed for the sensitivity of PGM detection. Comparative studies on the conventional PGM enzyme activity staining and immunoblot analysis using the HRP/DAB system of

antigen visualisation were carried out. Comparisons were also made between the sensitivities displayed by the ALP system, the HRP/ECL system and selected biotinylated systems.

5.5.1 Conventional enzyme staining and immunoblotting.

Figure 5.4 illustrates that the sensitivity of detection using the sheep anti-rabbit PGM IgG, is at least as sensitive as the detection of rabbit PGM by enzyme activity staining. Furthermore, experiments on the detection of PGM1 in rabbit red cell lysates, described previously (section 4.1.2), indicate the superior sensitivity of the sheep anti-rabbit PGM antibody used in conjunction with the HRP/DAB detection system (see figure 4.6).

Experiments carried out on the detection of human muscle PGM, reveal a different story. Figure 5.10 illustrates the separation of doubling dilutions of human skeletal muscle homogenate, by IEF on a 5-7 pH gradient. The activity stained panel (a) shows approximately 16 fold better sensitivity than the immunoblotted panel (b).

The primary antiserum used in these experiments was raised against to the rabbit PGM1. Previous results showed that although the sheep anti-rabbit PGM IgG cross-reacts well with the human PGM1, it lacks the specificity shown for the rabbit antigen. The results in this section show that human muscle PGM1 can be detected at much lower concentrations by activity staining than by immunoblot analysis with the antiserum. In contrast, low concentrations of the rabbit antigen are detectable by both the enzyme activity stain and the sheep anti-rabbit PGM antibody.

5.5.2 Different enzyme conjugated antibodies.

A direct comparison was made between the sensitivities of the HRP and the ALP conjugated antibody systems. Figure 5.11 illustrates the immunoblot analysis of human placental PGM1 isozymes separated by IEF on a 5-7 pH gradient. The PGM1 phenotypes of the placental extracts are easily distinguished in both panels, but the HRP probed panel (a) shows slightly stronger signals than the ALP panel (b). Minor placental components, visualised with the HRP/DAB system, are undetectable using the ALP detection system. No cross-reactivity with either conjugated antibody system is observed when tested against the placental extracts in the absence of the primary antiserum, figure 4.9 and other results (not shown). Therefore, the minor components detected in panel (a) are specifically bound by the primary sheep anti-rabbit PGM IgG. These results indicate that the HRP conjugated antibody system used in conjunction with DAB, is more sensitive than the ALP conjugated antibody system.

A detailed study of these two conjugated antibody systems was carried out on the GC protein, as reported in chapter 3. These results also showed that the HRP conjugated antibodies are more sensitive and easier to use than the ALP conjugated antibodies.

5.5.3 The ECL chemiluminescent detection system.

The majority of the work carried out to determine the most sensitive visualisation systems and their optimal working conditions was reported previously for the GC protein in chapter 3. This section aims to apply those findings to the

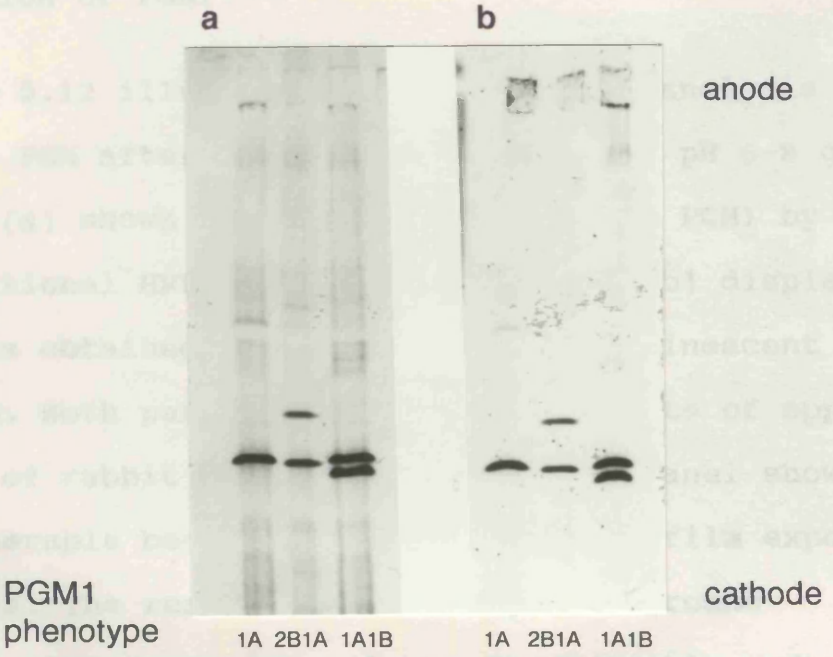


Figure 5.11

Immunodetection of human placental PGM1 phenotypes after IEF on a pH 5-7 gradient and electroblotting, using anti-rabbit PGM antibodies. The second antibody is conjugated to horseradish peroxidase (HRP) (panel a) and conjugated to alkaline phosphatase (ALP) in panel (b).

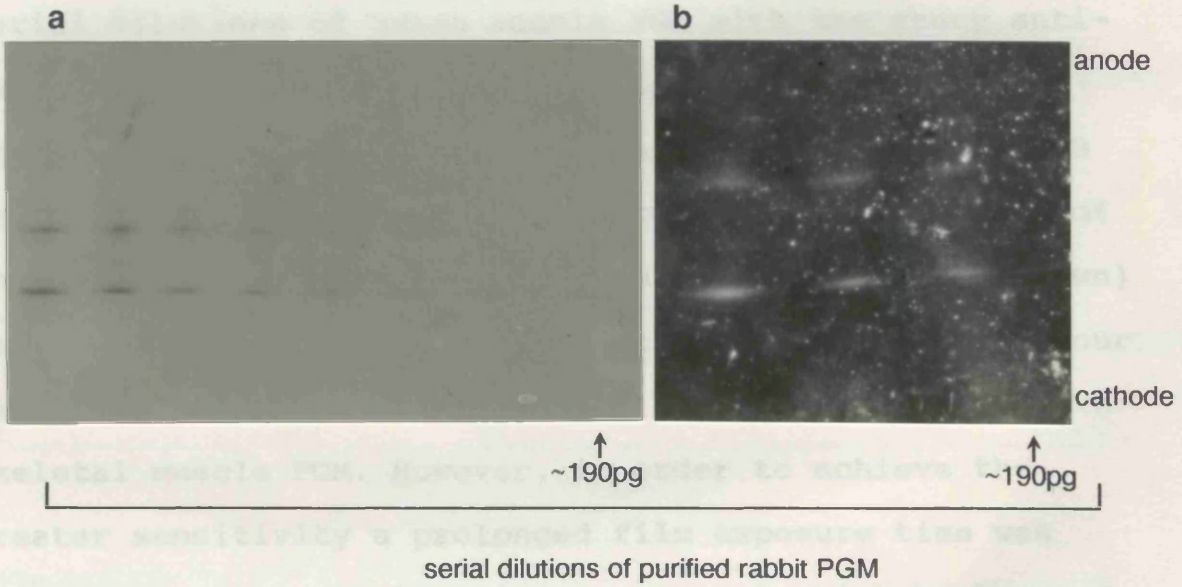


Figure 5.12

Immunodetection of serial dilutions of purified rabbit PGM using the HRP/DAB detection system (a) and the chemiluminescent ECL detection system (b) following separation by isoelectric focusing on a 6-8 pH gradient and transfer to an immobilising membrane.

detection of PGM1.

Figure 5.12 illustrates the immunoblot analysis of purified rabbit PGM after separation by IEF on a pH 6-8 gradient. Panel (a) shows the detection of rabbit PGM1 by the conventional HRP/DAB system and panel (b) displays the results obtained using the ECL chemiluminescent detection system. Both panels show detection limits of approximately 190pg of rabbit PGM. However, the ECL panel shows considerable background signal after a film exposure of 30 minutes. The reason for this high background chemiluminescence is unclear. Considerable reduction of the background signal is possible with shorter film exposure times, but this also decreases the sensitivity of antigen detection.

Figure 5.10 panel (b), displays the sensitivity of detecting serial dilutions of human muscle PGM with the sheep anti-rabbit PGM IgG following separation by IEF, immunoblot analysis and visualisation with the conventional HRP/DAB detection system. A comparison between the sensitivity of this system and the HRP/ECL system (immunoblots not shown) revealed that the chemiluminescent system is at least four times more sensitive, detecting a 1/512 dilution of human skeletal muscle PGM. However, in order to achieve the greater sensitivity a prolonged film exposure time was necessary, which resulted in an extremely high background signal.

Figure 5.13 displays the detection of human placental PGM1 phenotypes from six unrelated individuals by immunoblotting after separation by IEF on a 5-7 pH gradient. Panel (a) is

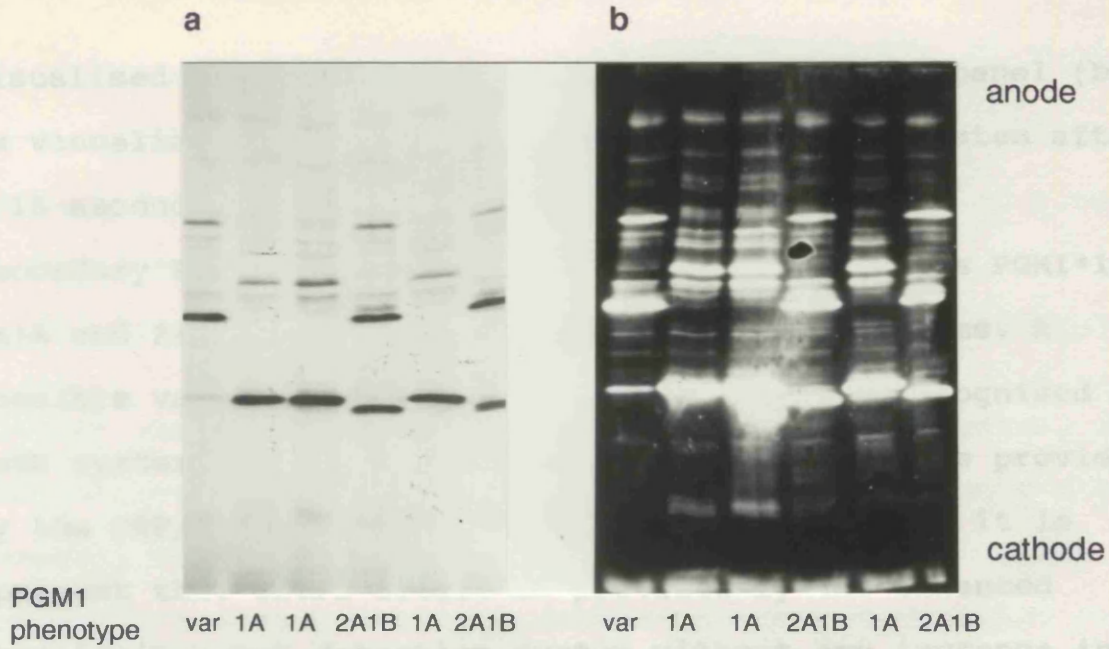


Figure 5.13
Immunodetection of human placental PGM1 phenotypes using the sheep anti-rabbit PGM antibodies, after IEF on a pH 5-7 gradient and electroblotting to a nitrocellulose filter. HRP-linked second antibodies are used to visualise the antigens in conjunction with the diaminobenzidine (DAB) coloured reagent (a) and the ECL chemiluminescent detection system (b).

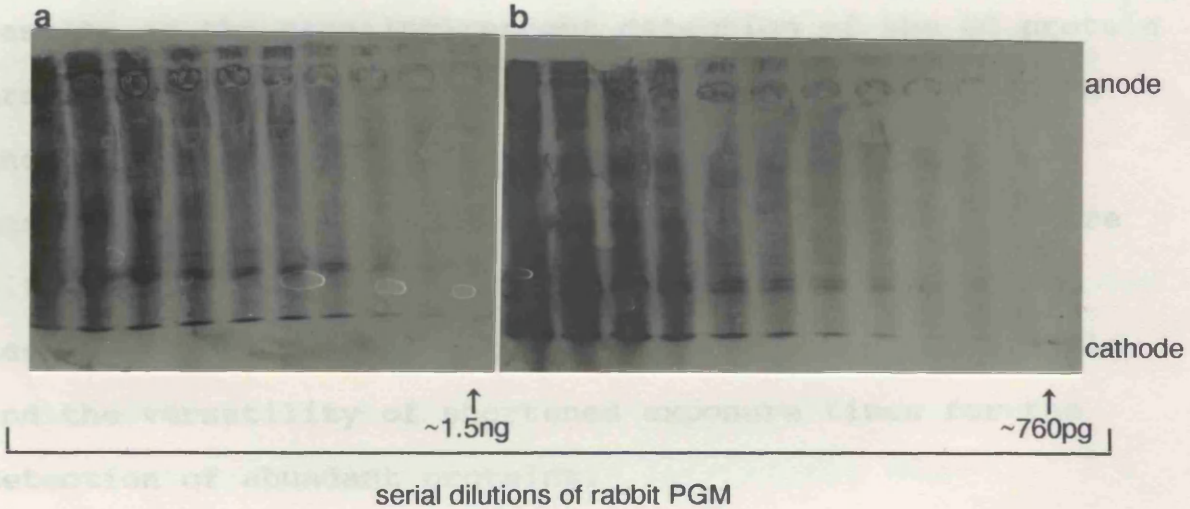


Figure 5.14
Immunological detection of rabbit PGM following separation by IEF on a 6-8 pH gradient and electotransfer to nitrocellulose with TGM as the transfer buffer. Serial dilutions of rabbit PGM visualised using conventional HRP-linked antibodies (a) and biotinylated HRP-linked antibodies (b).

visualised using the DAB reaction mixture, while panel (b) is visualised using the Amersham ECL detection system after a 15 second film exposure time. The primary and secondary PGM1 isozymes from the common phenotypes PGM1*1A, 2A1A and 2A1B are easily identified by both systems. A possible variant observed in track 1, is also recognised by both systems. Comparing the quality of the results provided by the HRP/DAB and the HRP/ECL detection systems, it is apparent that extra detail is provided by the enhanced chemiluminescent detection system without any increase in background signal. Many bands which are not visible after staining the filter with DAB, are clearly visible using the ECL detection system.

The results described in this section show that the ECL system is more sensitive than the DAB system, reflecting the results of the chemiluminescent detection of the GC protein presented in chapter 3. However, results in this section show that extended film exposure times may lead to unacceptable background signals, as illustrated in figure 5.12. Nevertheless, the ECL system provides both great sensitivity for the detection of trace amounts of protein and the versatility of shortened exposure times for the detection of abundant proteins.

5.5.4 Biotinylated antibodies.

The aim of these experiments was to determine whether the use of biotinylated antibodies significantly increases the sensitivity of PGM detection. The following results were obtained using the ABC Vectastain kit (Vector laboratories). Figure 5.14 shows the immunoblot analysis of doubling

dilutions of purified rabbit PGM separated by IEF on a 6-8 pH gradient. Panel (a), probed with normal unbiotinylated HRP conjugated antibodies shows a detection limit of 1.5ng purified rabbit PGM, compared with a 2 fold higher sensitivity obtained with the biotinylated antibodies (b).

Further analysis using the biotinylated HRP antibodies, in conjunction with the ECL chemiluminescent detection system, revealed no improvement in the sensitivity of PGM detection compared with the double antibody technique.

The application of the vectastain biotinylated antibody system to the detection of PGM does not lead to any long term improvement in sensitivity. A more detailed study of the evaluation of biotinylated antibody systems carried out on the GC protein in chapter 3, section 3.6.4, also suggests that the Vectastain kit is unreliable. More reliable results were obtained with an Amersham biotinylated HRP antibody system (data not shown).

5.6 Summary and conclusions.

1) The method of sample application is a restricting factor in the sensitivity of PGM detection. Direct sample application in PBS/haemoglobin loading buffer, gives approximately 16,000 fold better sensitivity than conventional sample application papers and PBS as the diluent.

Montgomery and Fu (1988), reported that certain proteins which possess no detectable cellulase activity, possess a high affinity for the cellulose found in Whatman paper. Treatment of the proteins with SDS significantly reduced

this cellulose binding behaviour, but this type of treatment cannot be used for the separation of proteins in their native state. However, it is possible that non-ionic detergents such as Tween 20 and NP-40 could be used to reduce the cellulose binding properties of PGM and other proteins. This would be an interesting avenue to pursue in future investigations.

The effect of protein-based diluents on the behaviour of PGM and GC during focusing is unclear. It is apparent that the focusing process has a profound effect on the solubility of these proteins. Large amounts of both PGM and GC appear to precipitate at the site of application when diluted with protein-free solutions. The loading buffers used may act as carrier solutions which help to keep both the PGM and GC in solution while focusing, enabling the detection of considerably lower concentrations.

2) The use of conventional tank electroblotting for the transfer of focused PGM to N/C is between 2 and 4 fold more efficient than passive or semi-dry electrotransfer. Similar results were also obtained for the GC protein, chapter 3.

3) The efficiency of protein transfer was investigated further by following the progress of iodinated rabbit PGM during the transfer process after separation by IEF. All the focused rabbit PGM isozymes were completely transferred, leading me to believe that the transfer efficiency of tank electroblotting approaches 100%. Problems were encountered with the precipitation of rabbit PGM at the sites of sample application which could still be detected in the gel matrix

after transfer. This probably stems from the method of PGM iodination used, since the method of sample application and choice of sample diluent were both eliminated. The Chloramine-T oxidation method for protein iodination (Hunter & Greenwood, 1962) although simple, may lead to protein damage and loss of enzyme activity, which was indeed noted in the case of rabbit PGM. The use of a less harsh enzyme labelling technique utilizing lactoperoxidase to catalyse the oxidation of iodide may be a preferable alternative for labelling rabbit PGM (Marcholonis, 1969).

4) The conditions required for blocking PGM filters were found to be more flexible than those required for the GC protein, chapter 3. Concentrations of Tween 20 as high as 0.5% were used with no detrimental effects. In order to keep the immunoblotting procedure as uniform as possible, low concentrations of Tween 20, as determined in chapter 3 were used.

5) The immunodetection of rabbit PGM is slightly more sensitive than conventional activity staining. However, the sheep anti-rabbit PGM IgG detects human PGM less efficiently than enzyme activity staining.

6) The ECL chemiluminescent detection system is approximately 4 times more sensitive than the conventional HRP/DAB immunological detection of PGM.

To conclude, the detection of rabbit PGM by the immunoblotting method developed during this thesis, using PBS/haemoglobin as the loading buffer, direct sample application to the gel surface, conventional tank

electroblotting, 0.05% Tween 20 as the blocking agent, and the ECL chemiluminescent detection system, leads to a 16,250 fold increase in sensitivity, compared with the sensitivity of this system at the initiation of this work.

Chapter 6:

6.1 A novel technique for the examination of polymorphism at the PGM2 locus.

The analysis of the PGM2 isozymes by starch gel electrophoresis shows that the PGM2 gene locus is not polymorphic within the European population, (Hopkinson & Harris, 1966; Henke & Hoffmann, 1988). In addition to the common PGM2 phenotype, rare variants are also identified such as the PGM2*2 phenotype which exists more frequently in the African population (Lie-Injo, 1966). Since the identification of this rare variant, many more variants associated with the PGM2 gene locus have been identified, several of which show appreciable frequencies in certain isolated populations, for instance the central African Pygmie population, 0.067 gene frequency; the American Surinam Indian population, 0.098 gene frequency; and the Daga Papua New Guinea population, 0.097 gene frequency (Roychoudnury & Nei, 1988).

In later studies, the PGM2 isozymes were examined by isoelectric focusing, with the hope of revealing further electrophoretic variation. Problems with the PGM2 enzyme visualisation system, described by Hopkinson and Harris (1966) and the cofocusing of the PGM2 isozymes with the secondary PGM1 isozymes (Sutton, 1979), made the detection of further variation impossible by this method.

The experiments described here utilised a novel method for screening a population for PGM2 isozymes following separation by high resolution IEF. The sheep anti-rabbit PGM IgG, described in chapter 4, was used to remove the PGM1

isozymes from red cell lysates, allowing the examination of the unmasked PGM2 isozymes. Preliminary experiments on the subtraction of PGM1 from red cell lysates were carried out by passing lysates down a protein G sepharose column to which the IgG fraction of the sheep anti-rabbit PGM antiserum was attached. Illustration of the resulting subtraction after separation by starch gel electrophoresis is shown in figure 4.8. All four samples analysed in this figure, show the complete absence of the PGM1 isozymes following column treatment.

Figure 6.1 shows the focusing of red cell lysates from eight unrelated individuals, before and after column treatment, on a pH 5-7 gradient. The tracks containing the untreated samples are identified by (-), and the adjacent tracks are the column treated samples (+). The results show that column treatment removes the focused PGM1 isozymes. The remaining isozyme patterns are those of the focused PGM2 isozymes, allowing the unambiguous examination of the focused PGM2 isozyme pattern. This consists of four weakly stained isozymes, considerably weaker than the PGM1 isozymes observed before treatment.

In later experiments, the method of PGM1 elimination from red cell lysates was modified. The lysates were incubated with the sheep anti-rabbit PGM IgG fraction for 1 hour at room temperature. This leads to the precipitation of immunoreactive material, including the PGM1 enzyme. Haemolysates from a panel of fifty three individuals, thirty two of which are unrelated, were treated using this immunoprecipitation method.

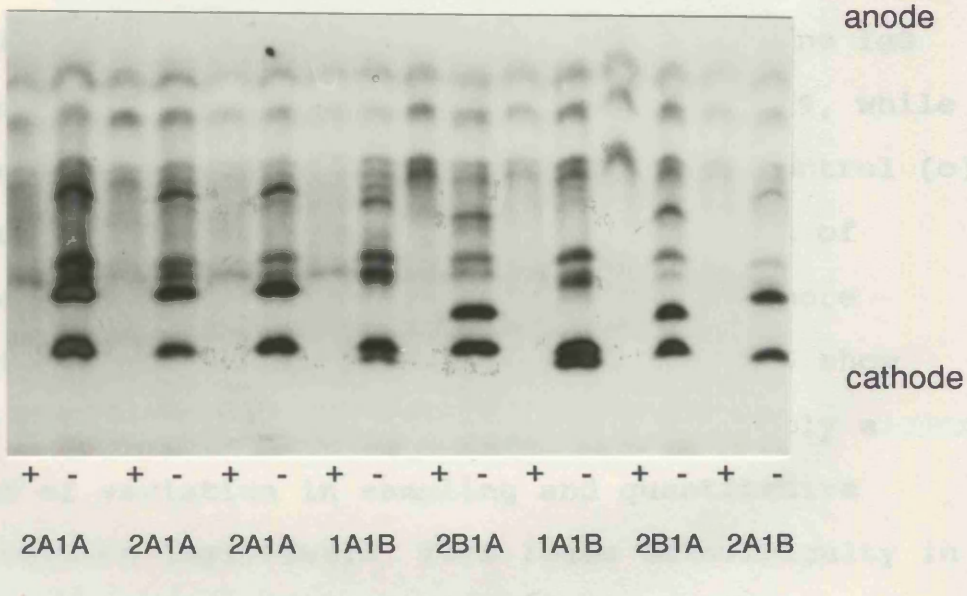


Figure 6.1

Isoelectric focusing of erythrocyte PGM from eight unrelated individuals on a 5-7 pH gradient before (-) and after (+) treatment with a 1 in 5 dilution of the sheep anti-rabbit PGM IgG fraction. Three common PGM1 subtypes are illustrated.

The PGM2 isozymes, detected following separation by IEF on a 5-7 pH gradient, by conventional PGM enzyme activity staining are shown in figure 6.2(a). A panel of nine IgG treated red cell lysates are shown in tracks 1 to 9, while track 10 contains the untreated red cell lysate control (c). The focused PGM2 patterns in tracks 1 to 9 consist of approximately four isozymes, two of which appear more strongly stained than the rest. Samples 2, 5 and 6 show weaker PGM activity than the rest, which is probably a combination of variation in sampling and quantitative variation between individuals. This leads to difficulty in the interpretation of the weaker acidic PGM2 isozymes.

This preliminary study revealed no electrophoretic variation at the PGM2 locus after separation by IEF, except for the possible identification of a PGM2 variant in track 6, identified by (*). In this case, the more acidic component of the two most basic PGM2 isozymes, shows a higher isoelectric point than normally observed, which is more clearly shown in track 2 of the schematic diagram, figure 6.2(b). In contrast, the other components show no change in electrophoretic mobility. The variation displayed by the sample in track 6, may be of genetical origin, but it is not possible to verify this for certain until family studies are carried out. The sample size restricted analysis to IEF alone, therefore confirmation of a previously identified PGM2 variant by starch gel electrophoresis was not possible.

It is possible that this Northern European individual is a PGM2 heterozygote. A prediction may be made on the likely nature of the PGM2*2-1 and PGM2*2 isozyme patterns, assuming

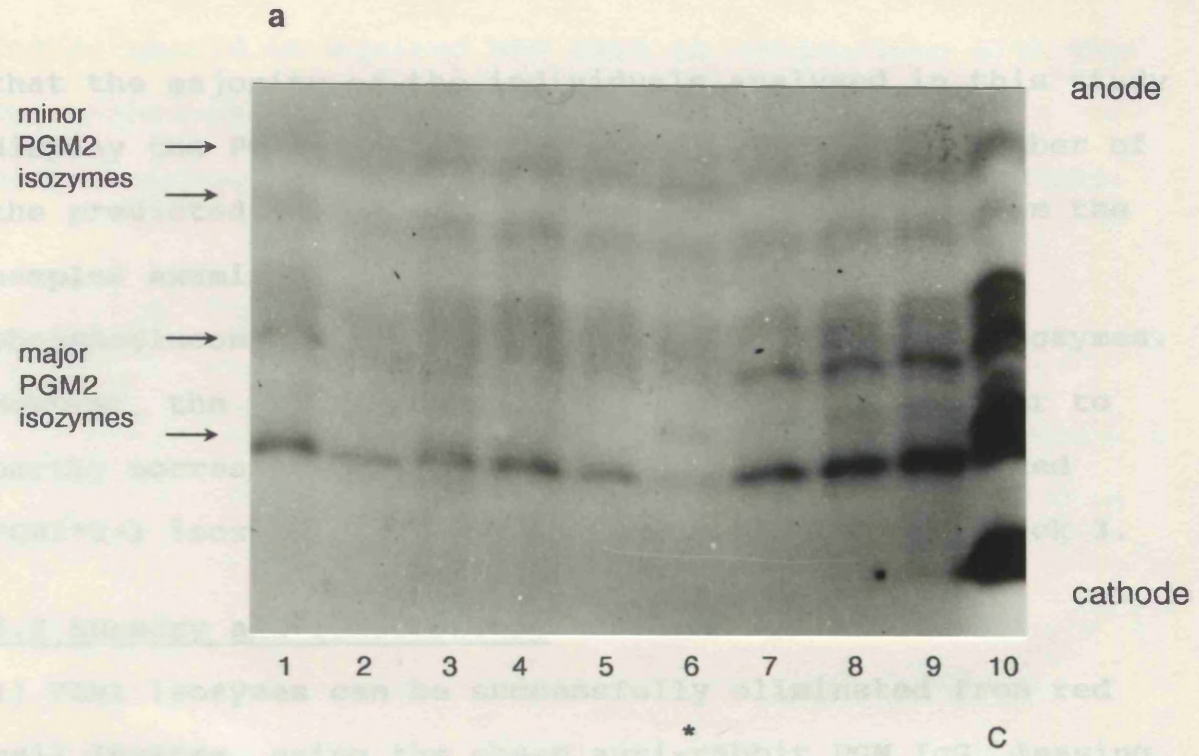


Figure 6.2(a)

Isoelectric focusing on a pH 5-7 gradient showing erythrocyte PGM2 isozyme patterns from unrelated individuals following removal of PGM1 activity by immunoaffinity chromatography with the sheep anti-rabbit PGM IgG fraction. A possible variant is identified by (*). An untreated control sample (c), PGM 1A1B phenotype is included for comparison.

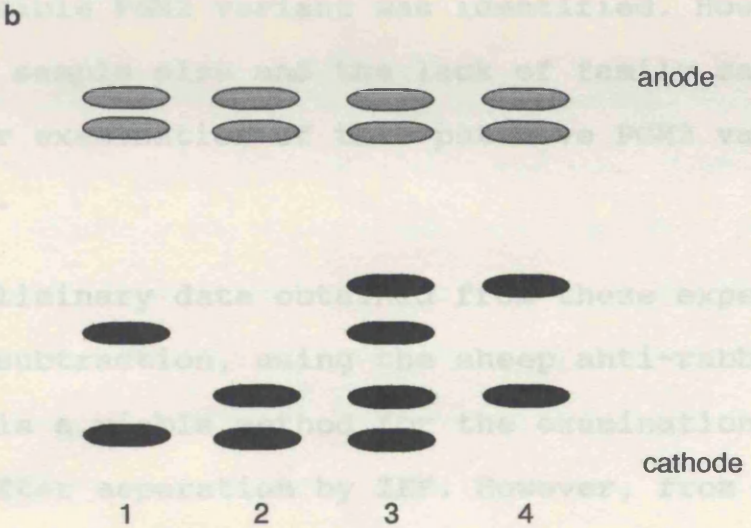


Figure 6.2 (b)

Schematic diagram showing the erythrocyte PGM components remaining after incubation with the sheep anti-rabbit PGM antiserum, following IEF on a pH 5-7 gradient. Pattern 1 corresponds to the PGM2 isozyme pattern displayed by all samples with one exception, pattern 2, which corresponds to the PGM2 isozyme pattern displayed by sample 6, panel (a). Pattern 3, corresponds to the expected PGM2* 2-1 heterozygote and pattern 4 the PGM2*2 homozygote.

that the majority of the individuals analysed in this study display the PGM2*1 homozygote isozyme pattern. A number of the predicted PGM2 isozymes appear to be missing from the samples examined, probably a result of the low phosphoglucomutase activity displayed by the PGM2 isozymes. However, the PGM2 isozymes observed in track 6 appear to partly correspond with those predicted for the focused PGM2*2-1 isozyme pattern shown in figure 6.2(b), track 3.

6.2 Summary and conclusions.

1) PGM1 isozymes can be successfully eliminated from red cell lysates, using the sheep anti-rabbit PGM IgG, leaving the PGM2 isozymes alone.

2) Considerable quantitative variation of the PGM2 isozymes was observed.

3) One possible PGM2 variant was identified. However, restricted sample size and the lack of family samples, made any further examination of this putative PGM2 variant impossible.

4) The preliminary data obtained from these experiments show that PGM1 subtraction, using the sheep anti-rabbit PGM antibody, is a viable method for the examination of PGM2 isozymes after separation by IEF. However, from the limited data available, it is uncertain whether IEF will be successful in revealing further microheterogeneity at the PGM2 locus. Nevertheless, these results indicate that a larger population study is worth undertaking.

The anti-human PGM2 antiserum produced by Piatti *et al*

(1990) should be aquired and used in conjunction with the sheep anti-rabbit PGM IgG to examine possible microheterogeneity of the focused PGM2 in greater detail.

Chapter 7.

7.1. Milk phosphoglucomutase.

Milk phosphoglucomutase activity was first identified in human, swine and rat milk by Cantu and Ibarra in 1982.. Following these preliminary findings, a small human population study was carried out. The electrophoretic mobilities of the milk PGM isozymes identified were shown to differ considerably from the PGM isozymes associated with the well characterised PGM1, PGM2 and PGM3 gene loci. Consequently, Cantu and Ibarra proposed the existence of a fourth PGM gene (PGM4), expressed solely in milk. Four alleles PGM4*1, PGM4*2, PGM4*3 and PGM4*4, were proposed to explain the degree of electrophoretic heterogeneity displayed by human milk PGM. The estimated gene frequencies of these four putative alleles were as follows: 0.346, 0.475, 0.114 and 0.065 respectively, appearing to fit the phenotypic Hardy-Weinberg expectation.

Further analysis of the milk PGM isozymes showed that they possess no pentosemutase activity, and the milk isozymes are more thermolabile than either the PGM1 or PGM2 isozymes. This evidence added weight to the argument for the existence of a PGM4 gene. However, examination of tissue extracts from the non-lactating mammary gland revealed the presence of only PGM1 and PGM2 isozymes, with no trace of the PGM4 isozymes. The reason for this observation is unclear, although Cantu and Ibarra have proposed that the presence of PGM4 in milk may be the result of the activation of the PGM4 gene with the simultaneous inactivation of the PGM1 and PGM2 genes, during the lactation process.

Since these early studies, the pathway for the generation of lactose within the mammary gland has been proposed (Casey & Hambridge, 1983). As suspected, PGM was found to play an important role in lactose synthesis (figure 7.1).

Most recently, the milk PGM polymorphism was examined in a larger human population study, consisting of over 600 lactating Brazilian women (Kvitko & Weimer, 1990). The colostrum samples examined showed varying levels of PGM activity, ranging from complete absence to levels equivalent to those obtained from weak red cell lysates. The gene frequency calculations showed an excess of the common homozygote classes, 4*1, 4*2 and 4*3. When milk samples instead of colostrum were tested, the recalculated gene frequencies did not deviate significantly from the Hardy-Weinberg expectation. The results showed that approximately 36% of the milk samples tested displayed a different electrophoretic pattern to those found in the corresponding colostrum samples. Kvitko and Weimer suggested that the change in distribution of the phenotypes was the result of *in vivo* post-translational modification during the early stages of lactation.

Furthermore, the method of sample storage was shown to have a profound effect on the qualitative variation displayed by the PGM4 isozymes. Freezing of samples led to changes in the isozyme patterns compared with those stored at 4°C. The reason for this was also uncertain but was attributed to an *in vitro* post-translational modification process.

The series of experiments carried out in this chapter examine the findings previously reported in the literature.

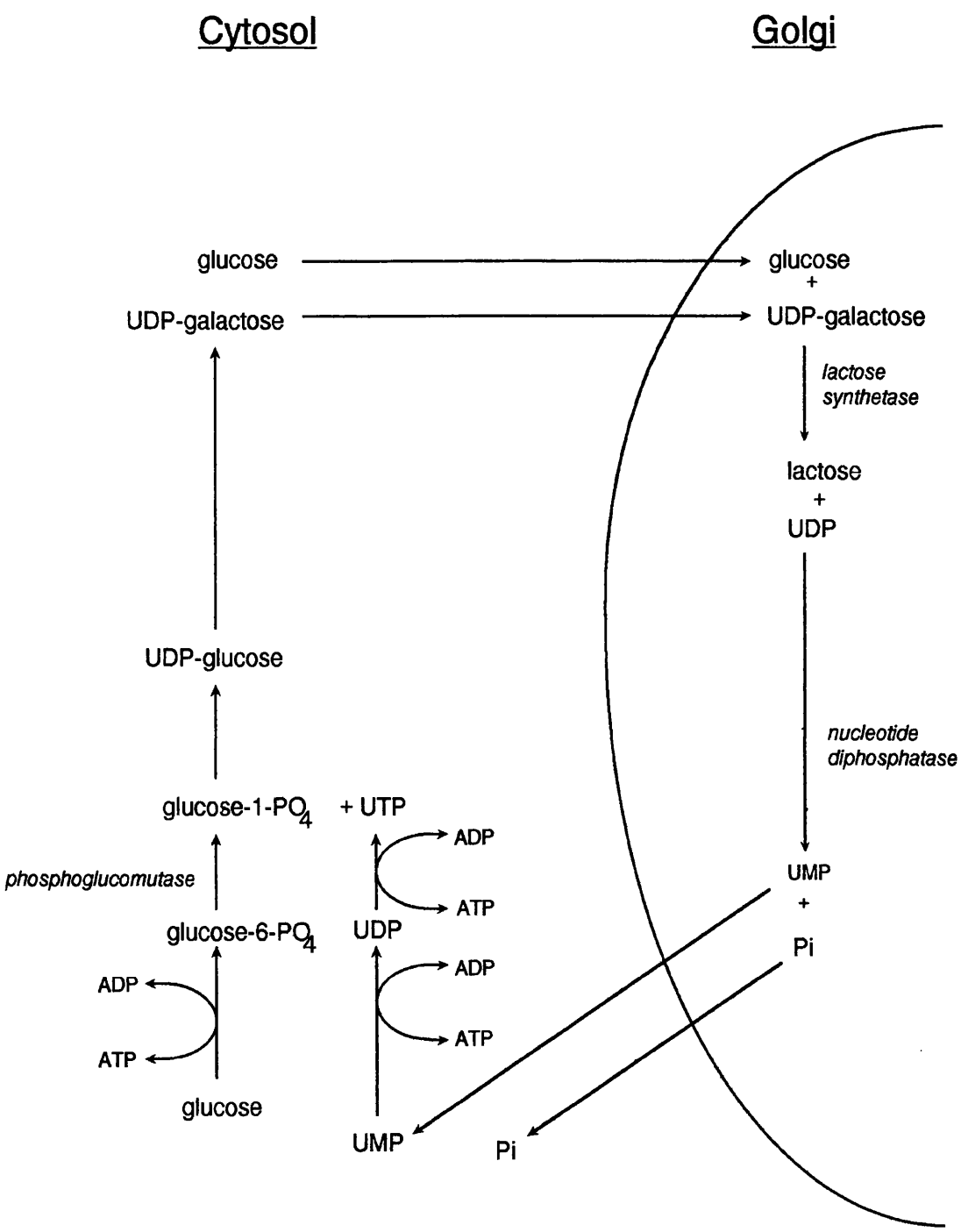


Figure 7.1 Lactose synthesis within the mammary gland and substrate transport across the golgi membrane.

These investigations are extended to include the examination of changes in milk PGM patterns from the same individual over the course of several weeks, the analysis of milk PGM with the sheep anti-rabbit PGM antibody and the examination of *in vivo* and *in vitro* modifications to the milk PGM isozymes.

7.1.1 The analysis of the PGM4 polymorphism.

The milk PGM isozyme patterns revealed by electrophoresis were examined in samples collected from twenty lactating human mothers. Blood samples from the milk donors were also obtained and PGM1 subtyped.

Figure 7.2 shows the milk PGM isozymes displayed by eight individuals, after separation by starch gel electrophoresis. Tracks 2 to 9 contain the milk samples, while tracks 1 and 10 contain red cell lysate controls from two PGM1*1 individuals. The milk samples show considerable quantitative and qualitative diversity, which is simplified in the schematic diagram, figure 7.3. The milk PGM phenotypes shown in figure 7.2, were compared with those observed by Cantu and Ibarra (1982), and the observations summarised in figure 7.4. All the milk samples which displayed PGM activity, with one exception, show some similarities to the electrophoretic patterns observed by the previous workers.

The milk PGM isozymes displayed in figure 7.2, were also compared with the mobilities of the PGM1 and PGM2 isozymes shown in the control samples in tracks 1 and 10, tabulated in figure 7.5. All the milk samples examined in this small study, possess certain PGM isozymes which share

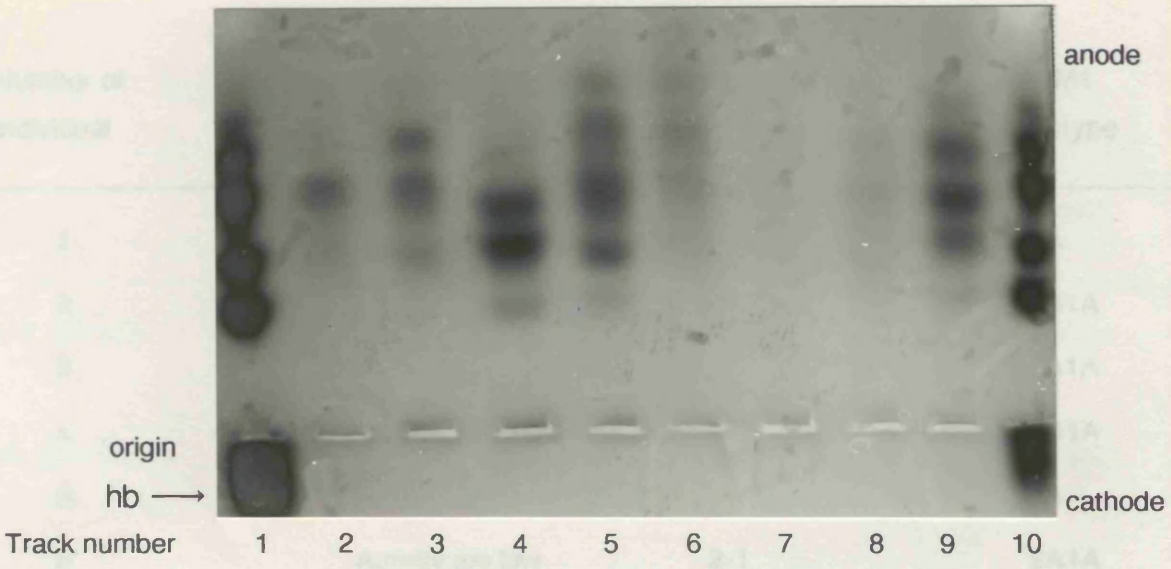


Figure 7.2

The detection of PGM4 isozymes in human milk samples from eight unrelated individuals (tracks 2 to 9) following separation by starch gel electrophoresis and conventional PGM activity staining. Red cell haemolysate controls from two PGM1*1 individuals are shown in tracks 1 and 10.

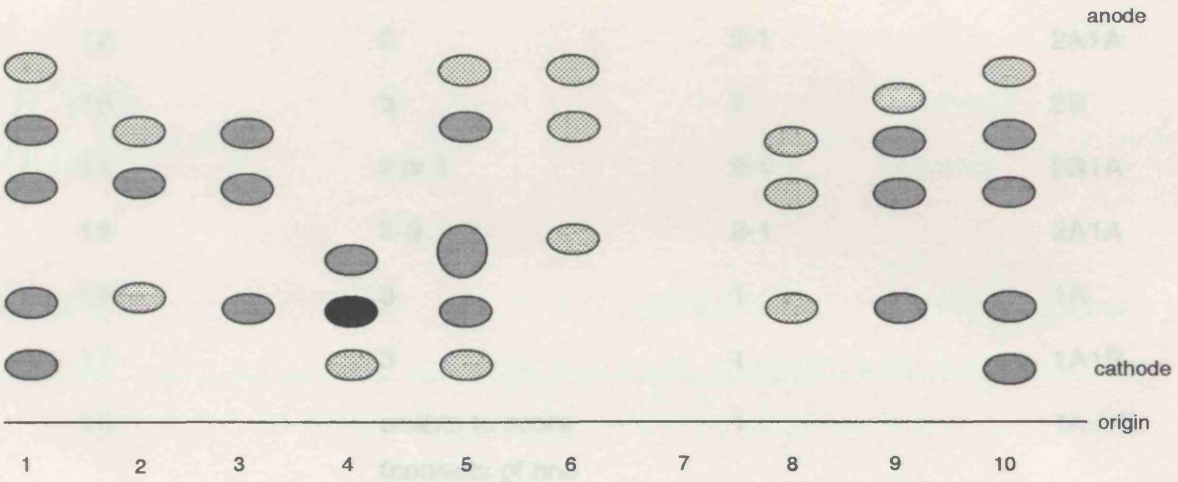


Figure 7.3

Schematic diagram of milk PGM isozymes identified in figure 7.2, following separation by starch gel electrophoresis. Tracks 1 and 10 contains haemolysate controls. Tracks 2, 3, and 8 are possible PGM4*2 homozygotes.Track 4 is a possible PGM4*1 homozygote.Track 5, contains a possible PGM4*3-1 heterozygote. Track 6, a possible PGM4*3 homozygote. Track 9, a possible PGM4*2-3 heterozygote and track 7 contains no PGM activity.

Number of individual	PGM4 phenotype	PGM1 phenotype	PGM1 subtype
1	Weak 2	1	1A
2	2	2-1	2B1A
3	2-3	2-1	2A1A
4	1	2-1	2B1A
5	1-3	1	1A
6	Activity too low	2-1	2A1A
7	No PGM activity	2-1	2A1B
8	Very weak 3	2-1	2A1A
9	Very weak 2	1	1A1B
10	Activity too low	2-1	2A1A
11	3	1	1A1B
12	2	2-1	2A1A
13	3	2	2B
14	2 or 3	2-1	2B1A
15	2-3	2-1	2A1A
16	3	1	1A
17	3	1	1A1B
18	unable to score (consists of one band only)	1	1A /1B
19	2-3	-	-
20	2-3	-	-

Figure 7.4

Tabulation of the putative milk PGM4 phenotypes identified from each individual used this study, and their corresponding erythrocyte PGM1 phenotypes.

Individuals	PGM4 phenotype	Number of milk isozymes	Mobility sharing between PGM1, PGM2 and PGM4 isozymes
1, 2, 9, 12 & 14	2	3	Two PGM2 isozymes
3, 15, 19 & 20	2-3	4	One PGM1 and two PGM2 isozymes.
4	1	3	Three PGM1 isozymes.
5	1-3	5	Three PGM1 and one PGM2 isozyme.
8, 11, 13, 14 16 & 17	3	3	Two PGM2 isozymes
18	-	1	One PGM1 isozyme.

Figure 7.5

Table showing the putative milk PGM4 phenotypes identified from this small population study, and the existence of any correlation between the positions of the milk isozymes and the corresponding erythrocyte PGM1 phenotypes, following separation by starch gel electrophoresis.

electrophoretic mobility with some of the PGM1 and PGM2 isozymes, but none of the common phenotypes associated with PGM1 or PGM2 are recognised.

The examination of this small group of milk samples, shows that milk PGM possesses a great deal of electrophoretic variation. This particular set of experiments however, do not help to clarify the existence of a fourth PGM gene locus. They only serve to show that human milk possesses PGM activity and displays electrophoretic variation which is unrecognisable as any of the common PGM1 or PGM2 phenotypes. An alternative means of detecting milk PGM would be useful in further studies.

7.1.2 The characterisation of PGM4 using the sheep anti-rabbit PGM antiserum.

Further work was carried out on the examination of the PGM polymorphism found in milk, using the sheep anti-rabbit PGM antiserum.

a) SDS-polyacrylamide gel electrophoresis.

The preliminary work on the binding properties between the IgG fraction of the sheep anti-rabbit PGM antiserum and milk PGM were carried out by immunoblot analysis after separation by SDS-PAGE. Figure 7.6(a) compares the immunoreactive components detected in extracts of human skeletal muscle and placental tissue with human milk from two individuals, using the sheep anti-PGM IgG. Much immunoreactivity is observed in the milk samples, but one component in particular corresponds to the molecular weight of human PGM1, detected in the tissue extracts (tracks 2 and 3). Furthermore, a high degree of cross-reactivity is observed between the secondary

HRP conjugated antibodies and a single milk protein of about 80,000 mw. (figure 7.6b). The identity of this component was not established but it could be lactoferrin, a protein that is present at high concentrations (2mg/ml) in human milk with a molecular weight of 76,000 (Neville *et al.* 1983).

b) Starch gel electrophoresis.

Figure 7.7 illustrates the subtraction of milk PGM using the sheep anti-rabbit PGM IgG, after separation by starch gel electrophoresis. Track 3, contains untreated milk from one individual, which consists of three bands of PGM activity, two of which possess approximately the same mobility as the PGM2 isozymes present in the red cell lysate control sample in track 1. Tracks 4, 5 and 6 show milk from the same individual treated with decreasing amounts of sheep IgG. IgG treatment results in the complete disappearance of the milk PGM isozymes. Track 7 contains an untreated milk sample from a second individual, which consists of four bands of milk PGM activity, three with strong enzyme activity and a fourth more acidic band showing weaker activity. Tracks 8 and 9 contain the IgG treated samples from this second individual. Both tracks are devoid of milk PGM activity.

Traces of PGM activity are observed at all the sites of sample application, appearing strongest in the tracks containing the IgG treated samples. This indicates that the IgG treatment leads to the precipitation of milk PGM at the origin of the gel.

From these results, it would appear that milk PGM possesses similar antigenic properties to the PGM1 isozymes in the

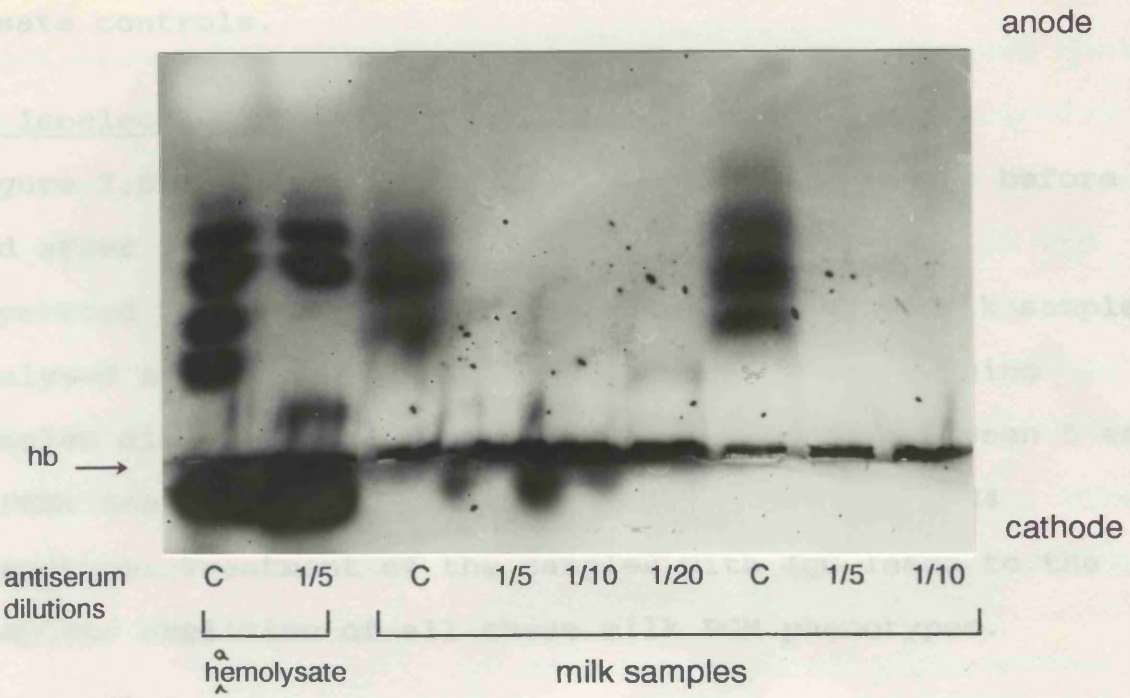


Figure 7.7
Starch gel electrophoresis showing PGM4 isozymes in untreated milk (c) and complete removal of PGM4 activity by immunoprecipitation using various dilutions of the sheep anti-rabbit PGM IgG. A haemolysate control before and after treatment with the sheep IgG is also illustrated.

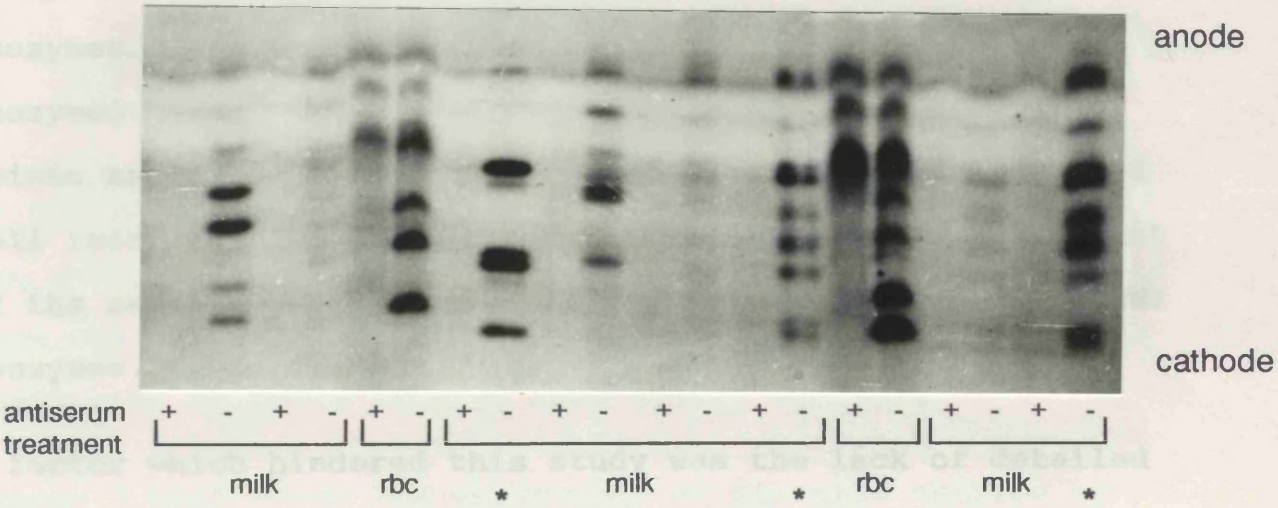


Figure 7.8
Isoelectric focusing on a 5-7 pH gradient showing the PGM4 isozyme patterns by enzyme activity staining, from eight unrelated individuals, before (-) and after (+) treatment with a 1 in 5 dilution of the sheep anti-rabbit PGM IgG. The samples labelled with the (*) are observed to show some similarity with the PGM1A and 1B isozymes. Two haemolysate controls are labelled (rbc).

lysate controls.

c) Isoelectric focusing.

Figure 7.8 shows the analysis of eight milk samples before and after treatment with sheep anti-rabbit PGM IgG, separated by IEF on a 5-7 pH gradient. Half the milk samples analysed show little or no PGM activity. The remaining samples display complex patterns consisting of between 5 and 8 PGM4 isozymes. Each sample displayed a unique PGM4 phenotype. Treatment of the samples with IgG leads to the complete abolition of all these milk PGM phenotypes.

Further examination revealed no consistency between the possible PGM4 phenotype detected after starch gel electrophoresis and the number of components identified after separation by IEF. However, some milk samples appear to show some electrophoretic identity with certain PGM1 isozymes. Tracks 8, 14 and 20, identified by (*), possess isozymes which share approximately the same isoelectric points as those displayed by the PGM1*1A and 1B primary red cell isozymes. The mobility of the milk isozymes in the rest of the samples bear no relationship with either PGM1 or PGM2 isozymes.

A factor which hindered this study was the lack of detailed information for some of the samples. Thus, in many cases, the number of days post-partum and the time of day the samples were collected were not recorded. Storage of the samples was also often suspect. Some samples were stored as requested at 4°C, whereas others were left at room temperature until collection. This made accurate comparative assessments of the samples extremely difficult. Despite

these drawbacks, this small population study indicates that a great deal of electrophoretic and quantitative variation is displayed by PGM4 isozymes. The isozyme patterns obtained by starch gel electrophoresis show some similarity to the phenotypes identified by Cantu and Ibarra. However, evidence for the existence of a closer electrophoretic relationship between the PGM1, PGM2 and PGM4 isozymes than that recorded by Cantu and Ibarra is reported here (see figure 7.5). Data obtained from the focusing patterns of the PGM4 isozymes do not support this observation.

7.1.3 The examination of PGM isozymes in milk samples collected over an extended time period.

Two lactating mothers who had recently delivered, each provided a series of milk samples, collected over prolonged periods of time. This provided the opportunity for investigating the stability of the PGM4 isozyme pattern in a more rigorous manner.

a) Analysis of the first individual.

Ten samples collected over a period of 35 days were obtained from the first individual. Due to feeding difficulties, the collection of these samples were highly variable.

Figure 7.9(a) shows the separation of six milk samples by starch gel electrophoresis. Track 2, contains the first milk sample obtained from this study, 27 days post-partum (pp) which consists of four milk PGM isozymes, a possible PGM4*2-3 phenotype. Tracks 3, 4, 5 and 7 possess the same number of components, but the most basic band identified in track 2 is replaced by a more acidic component. This acidic component

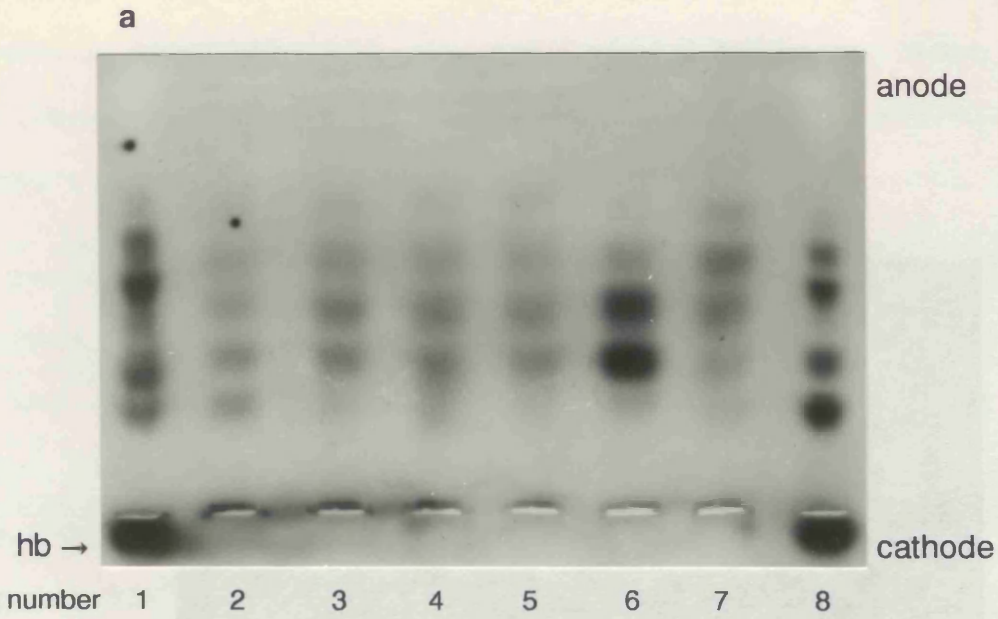


Figure 7.9 (a).

The detection of PGM4 isozymes from milk samples obtained at different times post-partum from one individual, following separation by starch gel electrophoresis and enzyme activity staining. Milk obtained 27 days post-partum (pp) track 2, 28 days pp. track 3, 39 days pp. track 4, 40 days pp. track 5, 41 days pp. track 6 and 42 days pp. track 7. Heamolysates from two PGM1*1 individuals are illustrated in tracks 1 and 8.

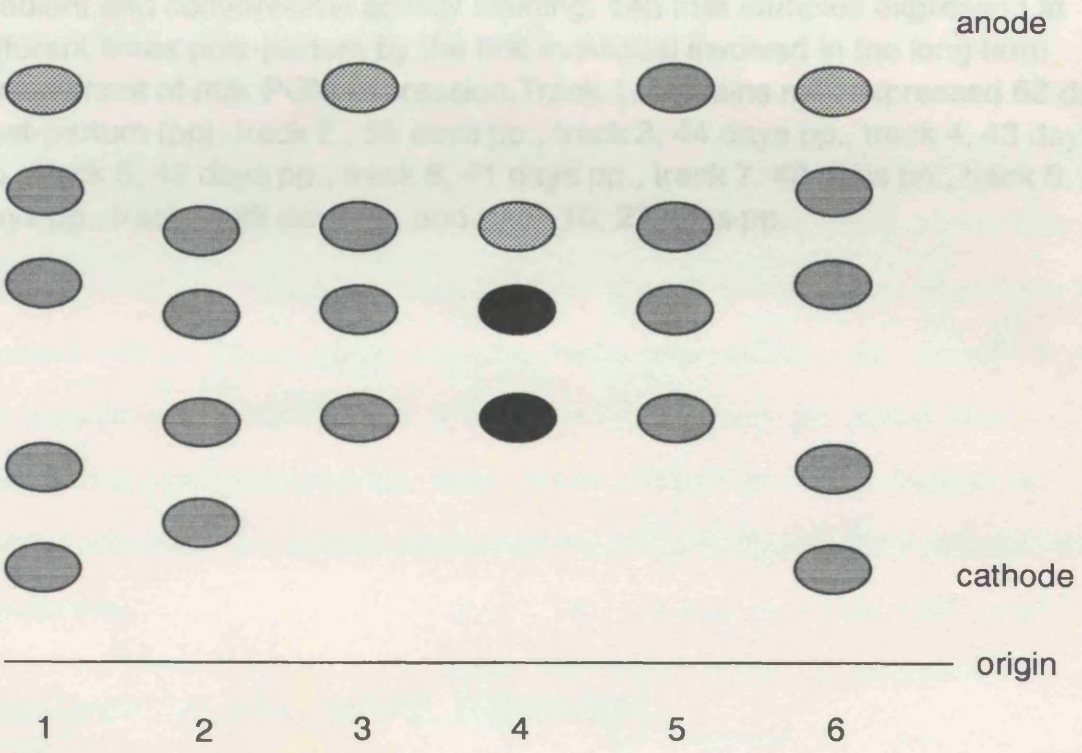


Figure 7.9 (b).

A schematic diagram illustrating the comparison between the electrophoretic mobilities of the PGM4 and PGM1 isozymes observed following separation by starch gel electrophoresis in figure 7.8(a). Track 2 corresponds to track 2 in panel (a), track 4 to track 6 in panel (a) and tracks 3 and 5 display the milk PGM patterns shown by the remaining milk samples. Tracks 1 and 6 display the PGM1 and PGM2 isozymes of the haemolysate control samples.

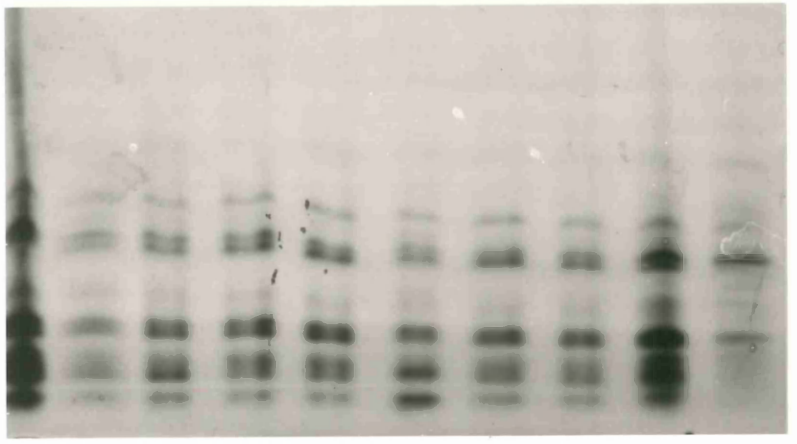


Figure 7.10

The detection of PGM4 isozymes following separation by IEF on a 3.5-7 pH gradient and conventional activity staining. Ten milk samples expressed at different times post-partum by the first individual involved in the long term assessment of milk PGM expression.Track 1, contains milk expressed 62 days post-partum (pp), track 2 , 55 days pp., track 3, 44 days pp., track 4, 43 days pp., track 5, 42 days pp., track 6, 41 days pp., track 7, 40 days pp., track 8, 39 days pp., track 9, 28 days pp. and track 10, 27 days pp.

possesses approximately the same mobility as the most acidic PGM2 isozyme displayed by the red cell lysate control, in tracks 1 and 8.

The sample in track 6 consists of only three milk PGM isozymes, both the most basic component observed in track 2 and the most acidic component observed in track 3 are absent from this sample. The two most basic components in this sample, show considerably higher PGM activity than observed in any of the other milk samples. These results are summarised more clearly in the schematic diagram 7.9(b).

Figure 7.10 shows the analysis of ten milk samples ranging from 27 days post-partum to 62 days post-partum, on a broad range IEF gel, pH gradient 3.5-7. The pattern observed in the majority of the samples consists of a total of eight isozymes, five of which show strong PGM activity. Track 10, sample collected 27 days post-partum, possesses a total of five milk isozymes, two of which show strong PGM activity. Tracks 9 and 6, samples collected 28 and 41 days post-partum respectively, also show slight heterogeneity. In track 6 the most basic component has a slightly higher pI than the equivalent components in the other samples, and track 9 shows a change in electrophoretic mobility of the basic milk components.

b) Analysis of the second individual.

The second mother used in this study donated a sample every day over a 12 day period between 7.30am and 12.15pm.

Figure 7.11(a) and (b) shows the analysis of this series of milk samples by starch gel electrophoresis. Track 2, sample

collected 141 days post-partum, consists of five PGM isozymes, the more basic of which is probably the result of contamination from track 1. Tracks 3 to 8, show milk samples which consist of four PGM isozymes. The first, located closest to the cathode, possesses approximately the same electrophoretic mobility as the second PGM1*1 haemolysate isozyme in track 1. Track 3 shows the highest milk PGM activity which appears to decrease steadily to track 8.

Figure 7.11(b) illustrates the electrophoretic separation of the rest of the samples in this series, 148 and 152 days post-partum respectively. Track 2 also shows contamination from the red cell lysate control track, but tracks 3 and 4 show further decreases in milk PGM activity compared with the last milk sample analysed in panel (a). The enzyme activity present in tracks 3 and 4, panel (b), is so low that the most acidic milk PGM isozyme present in the other samples is bearly visible. In track 5, panel (b), the level of PGM activity increases to approximately the same level as observed at the beginning of the study, figure 7.11 (a) tracks 3 and 4.

Figure 7.12, illustrates the separation of the complete series of milk samples from this individual by isoelectric focusing on a 5-7 pH gradient. The focused milk PGM pattern consists of eleven bands. The PGM activity is considerably reduced in tracks 7 to 10, which correspond to samples 7 and 8 in figure 7.11(a), and samples 2 and 3 in figure 7.11 (b). The samples either side of tracks 7 and 10, show considerably higher PGM activity. There is a steady reduction of PGM activity from track 4 to 9, followed by an

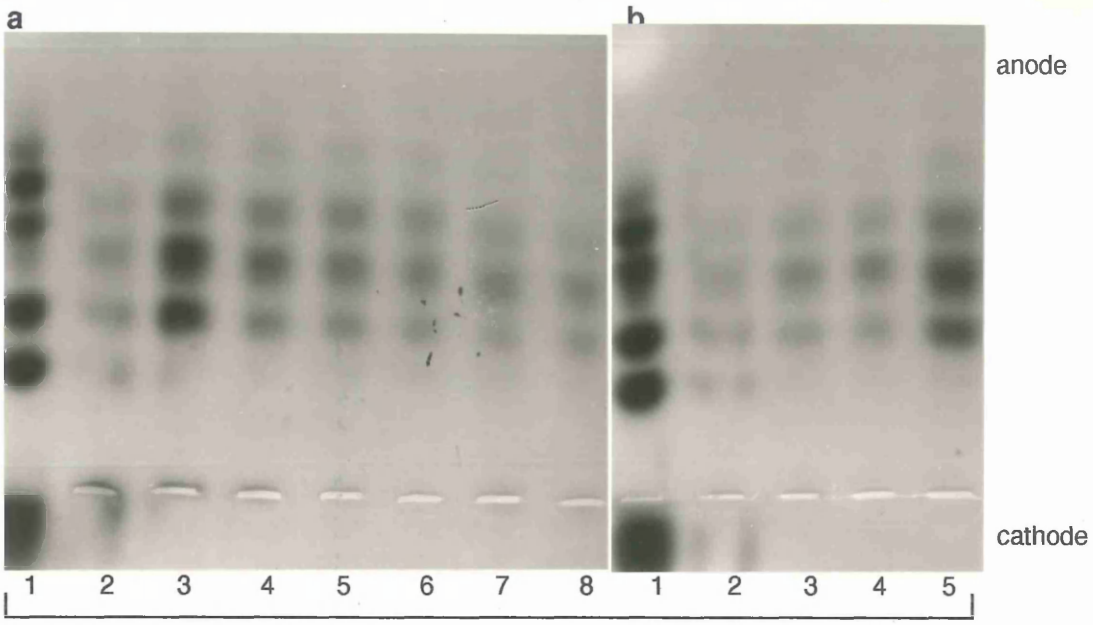


Figure 7.11

The detection of PGM4 isozymes following separation by starch gel electrophoresis and conventional enzyme activity staining. Panel (a) contains seven milk samples obtained from a second individual over a week's period, from 141 (track 2) to 147 days post-partum (track 8), with the haemolysate control track 1. Panel (b), the consecutive samples, 148 (track 2) to 152 days post-partum (track 5). Haemolysate control, track 1.

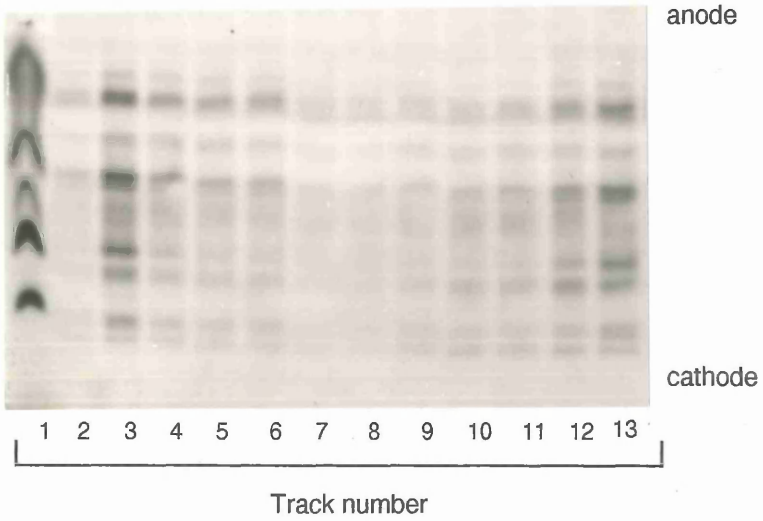


Figure 7.12

The detection of PGM4 isozymes following separation by IEF on a 5-7 pH gradient and conventional enzyme activity staining. Twelve milk samples obtained from the second individual, between 141 and 152 days post-partum, tracks 2 to 13 respectively. Haemolysate control, track 1.

equivalent increase in activity from track 10 to 13, with the one exception in track 2, which contains considerably lower levels of PGM activity than expected.

The reduced resolution of the milk PGM isozymes in tracks 2, 7, 8 and 9, combined with the low levels of enzyme activity in these samples makes the number and mobility of the milk PGM components difficult to assess. However, it would appear that the focused milk PGM isozyme pattern remains constant over the twelve day collection period.

This extended study has revealed the following. Samples from the first individual show that the electrophoretic mobility of the milk PGM isozymes changes with time. The milk samples from this individual however were collected at irregular times, and the mother in question was finding breast feeding a rather traumatic experience. Therefore, it is conceivable that a considerable number of factors such as stress and the hormone status of this individual are influencing the quantitative and qualitative expression of milk PGM.

The second individual shows no qualitative variation over the twelve day study period but considerable quantitative variation is observed. The reason for this lack of qualitative variation is unclear, but this individual may be less influenced by the stress associated with the weaning of a new born infant as this mother was considerably more experienced. These reduced stress factors may contribute to the uniformity of the milk PGM isozyme patterns reported.

7.2 Further experimentation on human milk PGM.

7.2.1 The effect of storage on milk PGM.

Kvitko and Weimer (1990) reported changes in the electrophoretic pattern displayed by milk PGM when stored at different temperatures. In this section the method of storage was examined.

Figure 7.13 compares the effect of two different storage temperatures, 4°C and -70°C respectively, on the electrophoretic mobility of the milk isozymes. Samples 1 and 2 were analysed by starch gel electrophoresis, panel (a), and samples 3 and 4 were analysed by IEF, panel (b). Sample 1, stored at 4°C, consists of five milk PGM isozymes. This is reduced to four isozymes by freezing, as seen in track 2. Although this experiment was repeated a number of times, this change in isozyme pattern was not always observed.

Tracks 3 and 4, panel (b), contain the same milk sample analysed by IEF, stored at -70°C and 4°C respectively. Track 3 consists of eleven milk PGM isozymes, while track 4 shows the disappearance of the most basic component. A change in the mobility of the four most basic bands is also associated with freezing the milk sample. The rest of the isozymes detected in this sample appear unaffected.

The reason for the change in electrophoretic mobility of the milk PGM isozymes after one round of freezing is unclear, but it is interesting to note that subsequent freezing and thawing of the milk samples results in no further qualitative or quantitative variation.

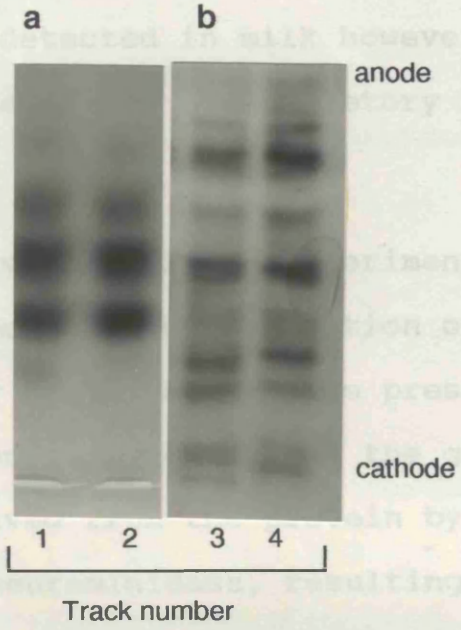


Figure 7.13

The detection of PGM4 isozymes by conventional enzyme activity staining, following separation by starch gel electrophoresis (panel a) and IEF on a 5-7 pH gradient (panel b). Milk samples from the same individual subjected to different storage temperatures, 4°C, tracks 1 and 4, and -70°C, tracks 2 and 3.

7.2.2 The effect of neuraminidase treatment on milk PGM.

Phosphoglucomutase is a cytosolic enzyme and no experimental data exists to suggest that PGM becomes glycosylated during post-translational processing (Whitehouse *et al.* 1992). The phosphoglucomutase detected in milk however may be influenced by the secretory and excretory nature of the mammary gland.

This section reports preliminary experimental data on the assessment of the possible glycosylation of PGM in the mammary gland prior to secretion. The presence of terminal sialic acid residues attached during the glycosylation process can be cleaved from the protein by the action of the hydrolytic enzyme neuraminidase, resulting in a basic shift in electrophoretic mobility of the glycoprotein.

Pilot treatments on milk PGM using grade v neuraminidase at 50 units/ml, proved unsuccessful, but subsequent treatments with purer preparations of neuraminidase, grade vi, adjusted to the same units of activity, gave some interesting results. Figure 7.14(a) shows the analysis of milk from two individuals by starch gel electrophoresis before and after neuraminidase treatment. Track 1 contains an untreated milk sample (c-) which possesses five milk isozymes. Track 2, contains the same milk sample diluted in the neuraminidase buffer (b-), which has the effect of considerably reducing PGM activity of the sample and causing the disappearance of the most basic and acidic components. Track 3, shows the same milk sample treated with grade vi neuraminidase in neuraminidase buffer (n+). This results in a significant decrease in PGM activity and the disappearance of the most

acidic isozyme present in this sample.

The untreated milk sample from a second individual, track 4, consists of four milk PGM isozymes. The sample diluted in the treatment buffer (track 5), as before shows a decrease in PGM activity and the disappearance of the most acidic isozyme. Track 6, the neuraminidase treated sample, also displays an isozyme pattern consisting of three isozymes of reduced enzyme activity. Furthermore, the central isozyme is slightly more acidic than the same component identified in tracks 4 and 5.

Figure 7.14(b), shows the effect of neuraminidase treatment on milk samples from two more individuals after starch gel electrophoresis. Again the same effect of the neuraminidase buffer on the milk PGM activity is observed. In track 2 the disappearance of the most acidic band, observed in the untreated sample, track 1, is noted. The neuraminidase treated sample, track 3, shows a change in the mobility of all four milk PGM isozymes towards the cathode. Panel (b), track 6, which shows the neuraminidase treated sample from a fourth individual also displays a shift of all the milk isozymes towards the cathode, compared to the untreated sample in track 4.

In figure 7.15, milk samples from the same four individuals are analysed using IEF on a 5-7 pH gradient. Tracks 1, 4, 7 and 10 contain the untreated milk samples, identified by more intense staining of the milk PGM isozymes. Neuraminidase treatment, tracks 6 and 12, produces a marked change in the milk PGM isozyme patterns, compared with their respective control milk tracks, 4 and 10. Tracks 2, 5, 8 and

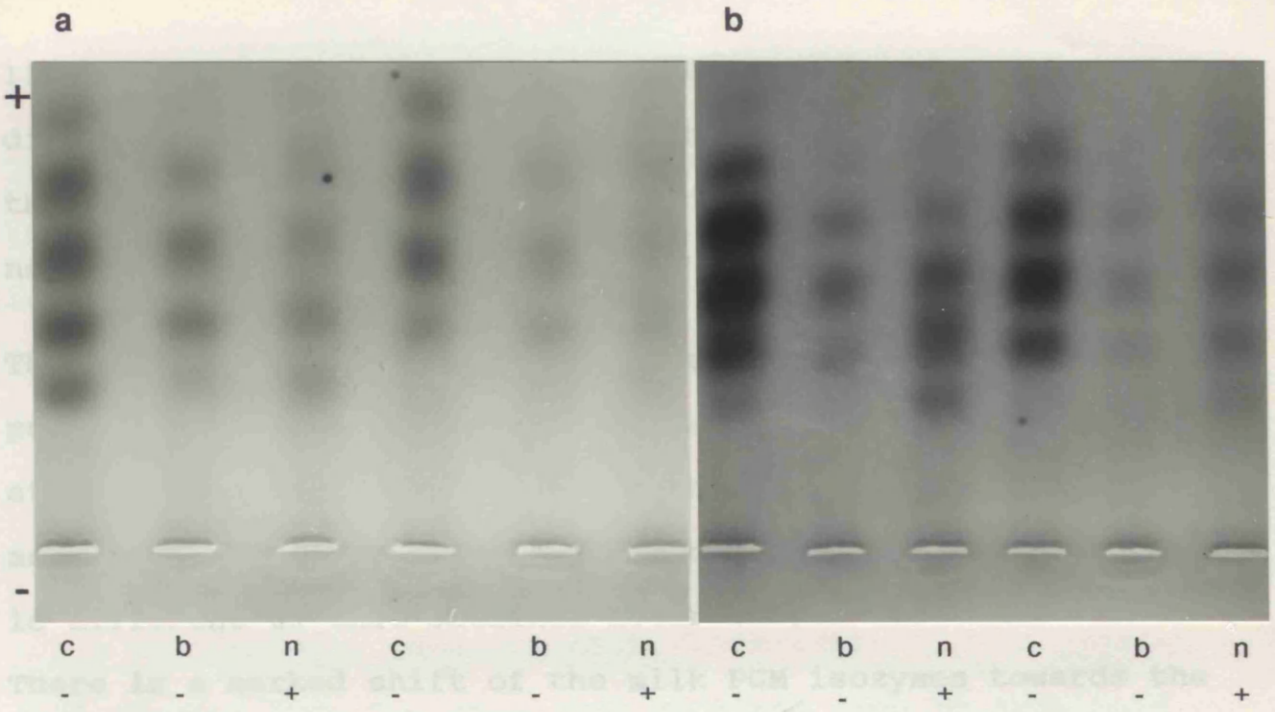


Figure 7.14

The detection of PGM 4 isozymes following separation of human milk by starch gel electrophoresis and conventional enzyme activity staining. Panels a and b display milk samples from four unrelated individuals following treatment with neuraminidase (n +), the acidic neuraminidase buffer minus the neuraminidase enzyme (b -) and dilution with water (c).

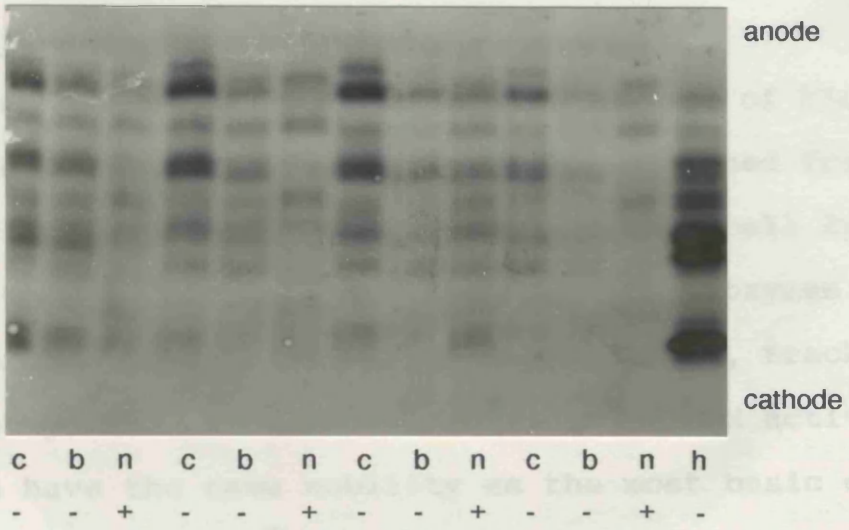


Figure 7.15

The detection of PGM4 isozymes following the separation of human milk samples following IEF on a 5-7 pH gradient and conventional enzyme activity staining. Milk samples from the same four individuals examined above, were treated with neuraminidase (n +), the acidic neuraminidase buffer minus the neuraminidase enzyme (b -) and dilution with water (c). The haemolysate control is shown by (h).

11, containing the neuraminidase buffer diluted samples, display milk isozyme patterns which are different from both those displayed in their corresponding untreated and neuraminidase digested samples.

The changes in the normally observed milk PGM isozyme patterns detected by starch gel electrophoresis and IEF are attributable to a combination of the neuraminidase buffer and the hydrolase, as the pattern obtained after digestion is different to that obtained after buffer treatment alone. There is a marked shift of the milk PGM isozymes towards the cathode as a result of neuraminidase treatment, which is consistent with the addition of sialic acid residues during the glycosylation process. Treatment with neuraminidase however, does not cause the milk PGM isozymes to adopt any recognisable PGM1 isozyme patterns.

7.3 The examination of PGM4 in animal milk.

7.3.1 Domesticated species.

Figure 7.16 shows the starch gel analysis of blood and milk samples from several cows and sheep obtained from the Royal Veterinary College (London). The cow red cell lysate, track 2 in this figure, consists of five PGM isozymes and shows higher PGM activity than the sheep lysate, track 1. The sheep lysate consists of four bands of PGM activity, two of which have the same mobility as the most basic cow PGM components. Tracks 3 to 8 contain milk samples from three sheep and three cows respectively. All the milk samples show a uniform pattern of poorly resolved bands of weak PGM activity. The most basic milk components displayed appear to possess the same mobility as the second most basic component

identified in both the sheep and cow haemolysates.

The conversion of G6P to G1P by PGM4 is an essential step in the lactose synthesis pathway and the concentration of lactose in milk may thus be dependant on the level of PGM4 activity. For instance, cow milk contains almost half the lactose concentration of that found in human milk (Oftedal, 1984), which may explain the higher levels of PGM activity present in human milk.

7.3.2 PGM activity in mouse milk.

Milk samples from two mice were obtained from Dr.Archibald (Edinburgh University). The samples were analysed by IEF and compared with the PGM isozyme patterns displayed by mouse kidney extracts in figure 7.17. The mouse kidney extracts, tracks 2 and 3, consist of six major PGM isozymes with high enzyme activity. The mouse milks, tracks 6 and 7, consist of three well resolved PGM isozymes which possess approximately the same isoelectric points as three of the major kidney PGM isozymes. Tracks 1, 4 and 5 contain a human placental extract and two milk samples respectively. Several of the human milk PGM isozymes appear to share approximately the same mobility as the mouse milk PGM isozymes.

Further analysis of PGM in mouse milk was not possible due to the small volume of sample. However, the sheep anti-rabbit PGM antibody was used to assess the binding properties of PGM expressed in mouse tissue extracts.

Figure 7.18(a) shows a set of human and mouse tissue extracts subjected to PGM subtraction by incubation with the sheep anti-rabbit IgG, and separation by IEF on a 5-7 pH

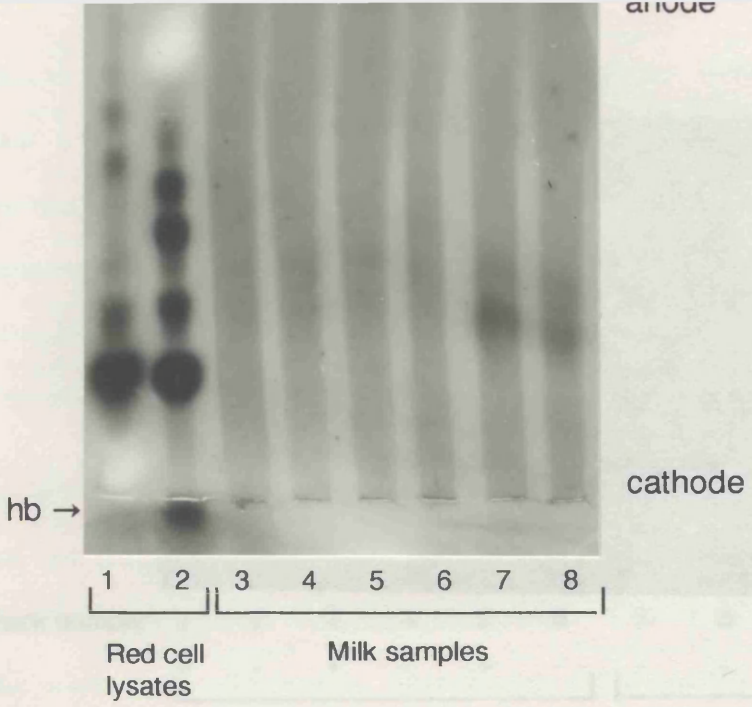


Figure 7.16

The detection of PGM isozymes by conventional enzyme activity staining following separation by starch gel electrophoresis of red cell lysate and milk samples from several sheep and cows. Tracks 1 and 2 contain sheep and cow haemolysates respectively, and tracks 3 to 8 contain milk samples from three different sheep and three different cows respectively.

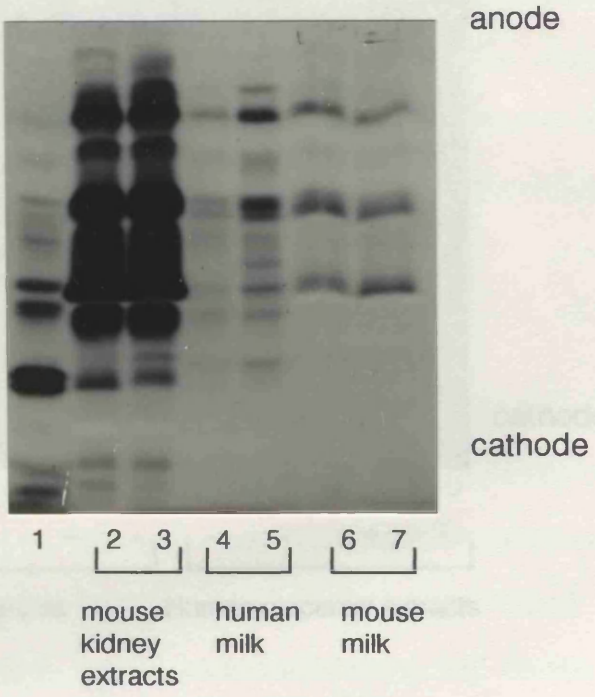


Figure 7.17

The detection of PGM isozymes by conventional enzyme activity staining and following separation by IEF on a 5-7 pH gradient . Human placental extract, track 1, kidney extracts from two unrelated mice, tracks 2 and 3, human milk samples from two unrelated individuals, tracks 4 and 5, and mouse milk samples from different individuals, tracks 6 and 7.

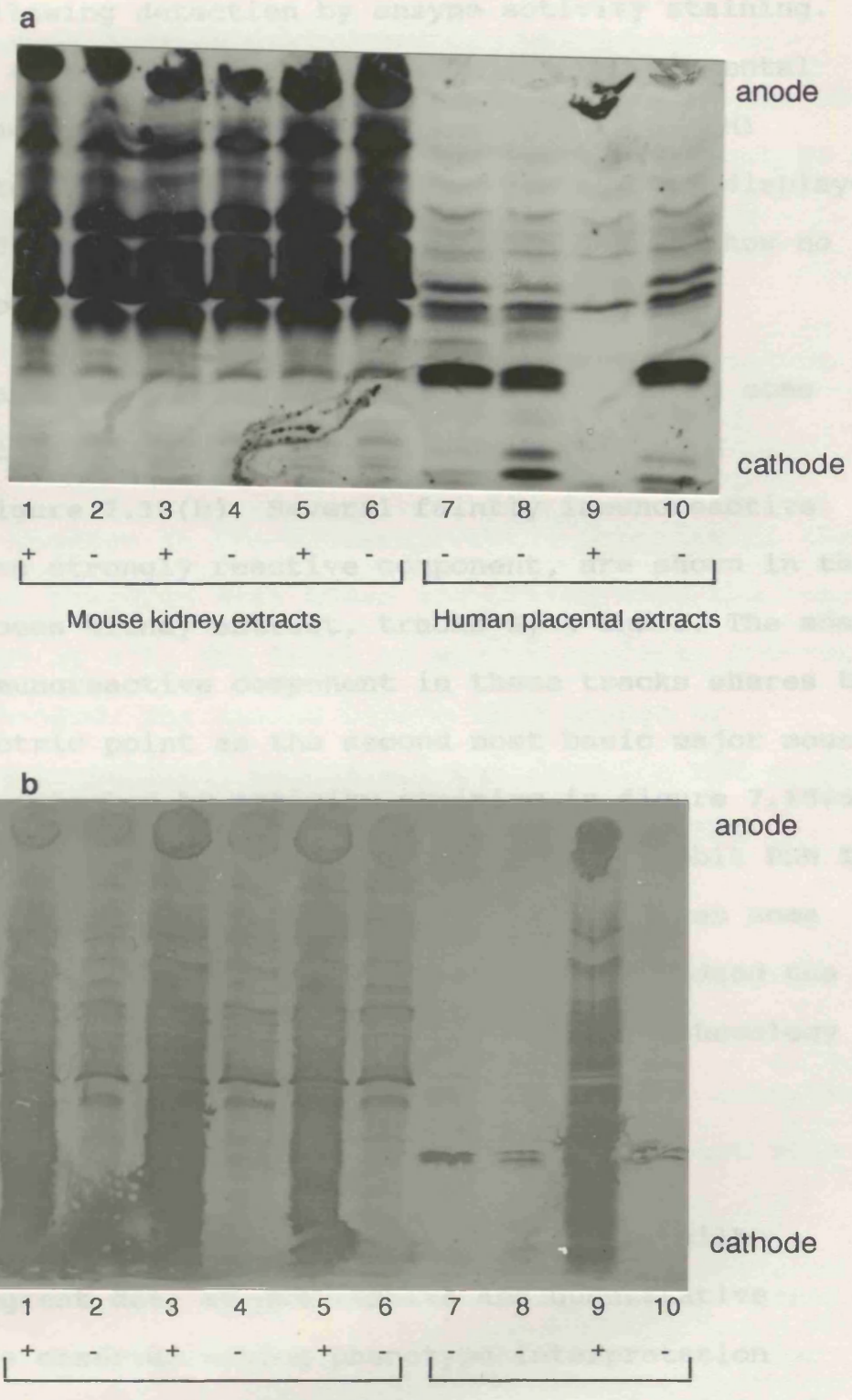


Figure 7.18

The separation of PGM isozymes following IEF on a 5-7 pH gradient and detection by enzyme activity staining (panel a) and immunoblot analysis (panel b). Mouse kidney extracts are displayed before (-) and after (+) treatment with the sheep anti-rabbit PGM antiserum in tracks 1 to 6. Human placental extracts are displayed before (-) and after (+) treatment with the sheep anti-rabbit PGM antiserum in tracks 7 to 10.

gradient following detection by enzyme activity staining. Tracks 1 to 4, the treated and untreated human placental extracts, show the expected disappearance of the PGM1 isozymes after treatment. The PGM isozyme patterns displayed by mouse kidney extracts, tracks 5 to 10 however, show no difference before and after IgG treatment.

Immunoblot analysis of the mouse PGM however, shows some cross reactivity with the sheep anti-rabbit PGM IgG fraction, figure 7.18(b). Several faintly immunoreactive bands and one strongly reactive component, are shown in the untreated mouse kidney extract, tracks 2, 4 and 6. The most strongly immunoreactive component in these tracks shares the same isoelectric point as the second most basic major mouse PGM isozyme detected by activity staining in figure 7.18(a). Although cross-reactivity with the sheep anti-rabbit PGM IgG is weak, mouse PGM in the tissue extracts possesses some epitopes in common with human PGM1. If this is indeed the case, mouse milk PGM may possess some structural homology with both the mouse kidney PGM and human PGM1.

7.4 Summary and conclusions.

1) PGM enzyme activity can be detected in human milk samples. A great deal of qualitative and quantitative variation is observed making phenotype interpretation difficult. However, the milk PGM phenotypes identified in this study did resemble some of those reported by Cantu and Ibarra (1982).

2) The examination of milk PGM from the same individual over an extended period, shows that milk PGM can vary both

quantitatively and qualitatively over a period of a week. When examined on a daily basis however, considerable variation in the levels of milk PGM are observed, while the PGM4 phenotype remains constant. Therefore, there must be a point during such a study when the change in the PGM4 isozyme pattern is observed.

3) As first reported by Kvitko and Weimer (1990), storage temperature of human milk was shown to have a marked qualitative effect on the PGM4 isozymes. Milk stored at -70°C shows changes in the electrophoretic mobility of the PGM4 isozymes compared with those identified from the same sample stored at 4°C. The level of enzyme activity however remains unchanged. The reason behind this change of electrophoretic mobility is unclear but freezing the sample does appear to change the charge on certain milk PGM isozymes.

4) Examination of the possible glycosylation of milk PGM, indicates that neuraminidase treatment changes the PGM4 isozyme pattern. An electrophoretic shift of some isozymes towards the cathode is observed, which is consistent with the cleavage of sialic acid residues.

5) The most important finding in this study is the shared binding properties between PGM1 and PGM4. Taken together with the similar catalytic properties and the similar molecular size, the immunological results suggest that there may be considerable sequence homology between PGM1 and PGM4. It is possible therefore, that the PGM4 isozymes may not originate from a fourth PGM gene, but rather as post-translationally modified forms of the PGM1 proteins.

6) A small study of animal milk shows that the levels of milk PGM in ruminants is extremely low. High levels of PGM activity are detected in mouse milk. The mouse milk PGM appears to be a distinct subset of those isozymes identified in the mouse kidney extracts. Mouse kidney PGM shows some degree of immunoreactivity with the sheep anti-rabbit PGM antiserum, suggesting that mouse pgm2 shares a high degree of structural homology with both the human PGM1 and putative PGM4 proteins. It should be noted that these results were obtained from a small number of animals. A larger study should be undertaken before firm conclusions can be drawn.

The results reported in this chapter suggest that the PGM found in milk is closely related to PGM1. The reason for the electrophoretic mobility differences observed between the PGM1 proteins and the milk PGM isozymes is still uncertain, but this could be the result of the glycosylation of the protein within the mammary gland. Thus, PGM4 may be actively secreted into the milk where it may have a distinct, but as yet unknown, biological function.

The occurrence of greater lactose concentrations in human milk compared with ruminant milk (Casey & Hambridge, 1983; Oftedal, 1984), may be one of the major factors contributing to the higher amounts of PGM activity in human milk. Furthermore, this would indicate the importance of PGM in the lactose production. The discovery of high levels of PGM activity in mouse milk, may make the biochemical and genetical analysis of milk PGM easier, providing that some

heterogeneity distinct from that associated with the mouse pgm2 locus is observed (Shows *et al.* 1968).

Chapter 8.

The production of anti-human PGM antiserum.

The sheep anti-rabbit PGM antiserum described in chapter 4, cross-reacts with human PGM1 and can be used to identify the PGM1 phenotypes in tissues containing high levels of the enzyme by immunoblotting. However, the anti-human activity of this antiserum is not sufficient to allow the recognition of the human PGM1 phenotypes from sources of low PGM1 activity such as haemolysates, semen or saliva, material frequently encountered in forensic analyses, whereas a high titre anti-human PGM1 may enable the detection of the PGM1 phenotypes in this type of material. Attempts were therefore made to purify human PGM as a first step in the production of a specific anti-human PGM1 antiserum.

8.1 The immunological purification of human PGM.

Human PGM1 was purified from skeletal muscle extracts by protein G sepharose column chromatography. Sheep anti-rabbit PGM IgG was attached to the column as described in section 2.2.3.1 of the materials and methods. Human skeletal muscle extract was subsequently passed down the column and the human PGM/sheep anti-rabbit PGM IgG complexes eluted and collected for analysis.

Figure 4.4 shows the immunodetection of the protein G column fractions following SDS-PAGE. Panel (a) is visualised using the sheep anti-rabbit PGM IgG, while panel (b) has the primary antiserum omitted from the immunodetection procedure. Tracks 3 and 4, contain the human skeletal muscle extract before and after passage down the column, and tracks 5 to 9 contain the acid eluted column fractions. The results

show that the major PGM component, approximately 60,000 mw., is completely absent from the sample in track 4, along with another strongly immunoreactive 40,000 mw. component. These two components reappear in the first elution fraction along with large quantities of heavy and light chain fragments of the sheep IgG. They are also observed in the other tracks but less easily seen, due to the masking effect of the large quantities of IgG present. These observations are reinforced by the results from the control, no first antibody filter (b). The 60,000 and 40,000 mw. components bound by the primary antiserum are not visible on this filter. The 50,000 and 25,000 mw. components, corresponding to the sheep IgG protein chains are strongly immunoreactive. This is the result of their cross-reactivity with the secondary rabbit anti-goat conjugated antiserum.

Figure 4.4(c) shows the electrophoretic pattern displayed by the same samples after protein staining. No difference is observed between the starting material (track 3) and this material after passage down the column (track 4). No muscle specific proteins are detected in the column eluate, tracks 5 to 10.

This set of results indicates a high level of human PGM1 purification by a simple protein G sepharose procedure. However, there was insufficient human PGM in a single batch of skeletal muscle extract to be detected by protein staining. Therefore, it was decided to use the same small column repeatedly, in order to generate a pool of purified material, which could then be used for immunisation.

8.2 The preparation of the purified human PGM for immunisation.

A major disadvantage of using protein G sepharose is the co-purification of the sheep IgG with the human PGM1 protein. Thus the final step of purification must involve the separation of the major human PGM1 component from the sheep IgG heavy chain (50,000 mw.). This was successfully achieved by acrylamide gel electrophoresis using a 7.5% gel and a separation time of six and a half hours (figure 8.1).

Figure 8.1 shows the outer segments of the nitrocellulose filter prepared from an experiment using the full width sample comb to apply the sample of PGM/IgG mixture obtained from one protein G sepharose column purification. Rabbit PGM is the flanking marker. The human PGM1 is resolved as a sharp band at 60,000 mw. and the 50,000 mw. sheep IgG heavy chain runs more anodally and is considerably less well resolved. Minor components associated with the sheep IgG are also seen in the higher molecular weight region of the filter. The separation of 0.7 to 0.8 cm between the major band of human PGM1 and sheep IgG is sufficient to allow the excision of a nitrocellulose strip containing only the PGM1 component. The central portion of the filter corresponding to the position of the PGM1 band, was dissolved in dimethylsulphoxide (DMSO) and treated with 0.08M Sodium carbonate buffer (pH 9.0) to precipitate the nitrocellulose as a finely divided powder that was resuspended in saline and used directly for immunisation. Material from two to three separate gels was used for each injection. In preliminary experiments, these final stages of purification

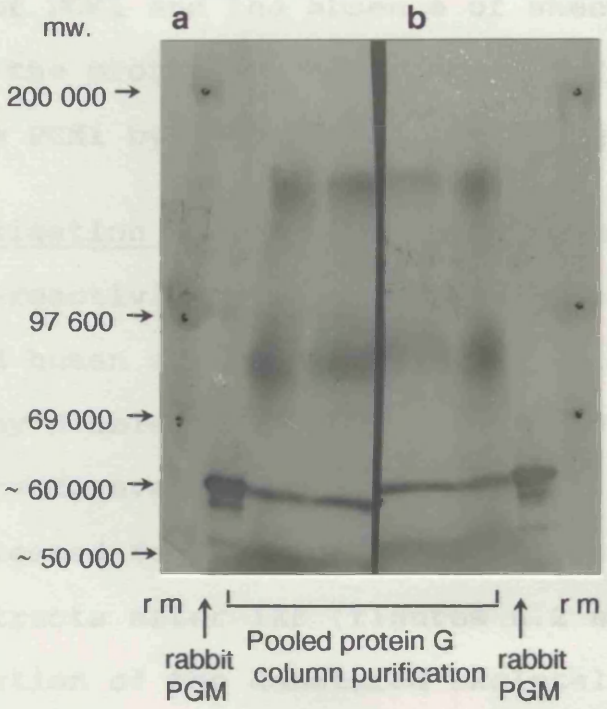


Figure 8.1

Immunological detection of PGM following separation by SDS gel electrophoresis and electrotransfer to nitrocellulose. The bracketed part of the filter shows the human skeletal muscle PGM1 protein (~ 60 000 mw.) following passage down a protein G column to which the sheep anti-rabbit PGM IgG fraction was bound. The centre part of the filter contained the material which was used for immunisation. Purified rabbit PGM was run each side of the column purified human PGM as visual markers. Molecular weight markers are identified by (r m).

were monitored by immunodetection. This confirmed the presence of PGM1 and the absence of sheep IgG but at no stage was the protein concentration sufficiently high to detect the PGM1 by non-specific staining.

8.3 Investigation of the rabbit anti-human PGM antiserum.

The cross-reactivity of the rabbit immune serum against the rabbit and human skeletal muscle PGM was first examined by Ouchterlony double diffusion analysis. No precipitin arcs were observed, even by protein staining. The IgG fraction was also tested for the detection of native PGM in skeletal muscle extracts after IEF (figures 8.2 and 8.3), and by the identification of the denatured skeletal muscle protein after separation by SDS-PAGE (figure 8.4).

Figure 8.2 shows the separation of undiluted human skeletal muscle extract by isoelectric focusing on a 5-7 pH gradient. Filter 1 was incubated in the preimmune rabbit IgG, filter 2 in the rabbit anti-human PGM IgG obtained from the second immunisation and filter 3 incubated in the sheep anti-rabbit PGM IgG. A number of components are detected by both the sheep and rabbit antibodies which possess approximately the same isoelectric points as the primary and secondary isozymes of a PGM1*1A individual. However, the major components detected by the rabbit IgG are also detected by the preimmune rabbit IgG fraction.

Figure 8.3 shows the separation of human placental extracts by IEF on a 5-7 pH gradient. Filters 1, 3 and 5 were incubated in the sheep IgG fraction, and filters 2, 4, 6 and 7 in the rabbit anti-human IgG fraction. The rabbit IgG detects between 13 and 16 components, whereas the preimmune

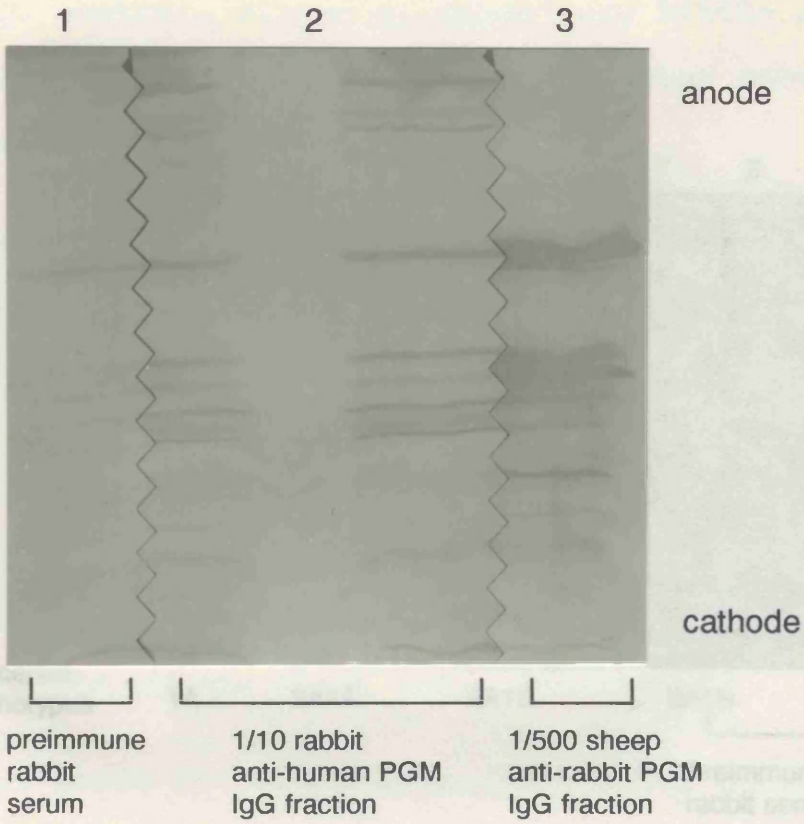


Figure 8.2

The immunological detection of human skeletal muscle PGM following separation by IEF on a 5-7 pH gradient and electrotransfer to nitrocellulose. Panel 1 is incubated in preimmune rabbit serum, panel 2 in a 1 in 10 dilution of the rabbit anti-human PGM IgG serum fraction and panel 3 in a 1 in 500 dilution of the sheep anti-rabbit PGM IgG serum fraction.

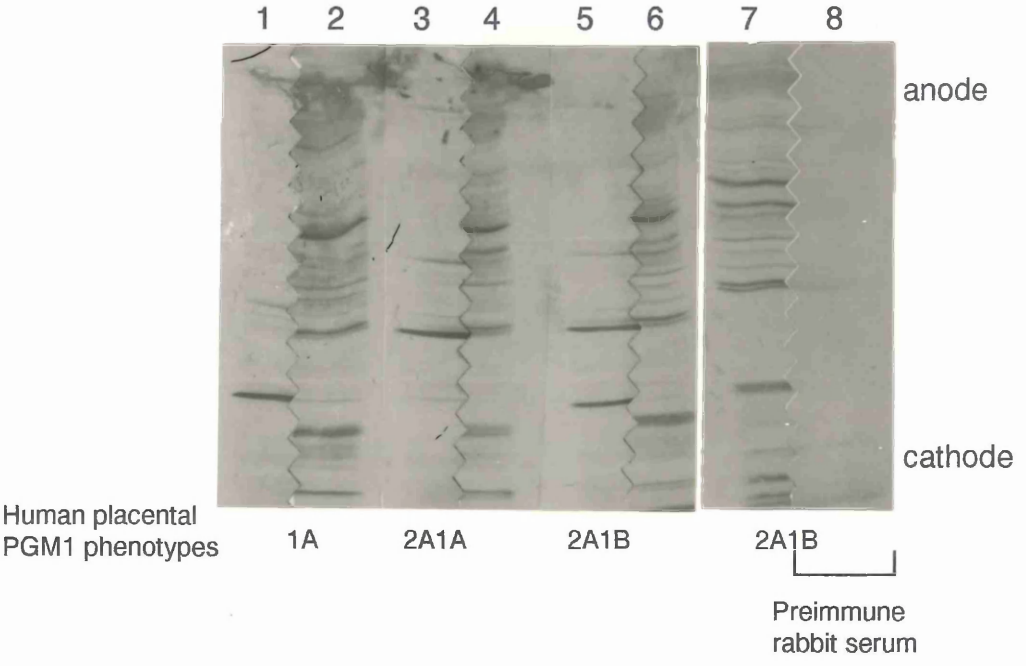


Figure 8.3

The immunological detection of placental PGM1 phenotypes following separation by IEF on a 5-7 pH gradient and electrotransfer to nitrocellulose. Panels 1, 3 and 5 display the immunoreactivity of the sheep anti-rabbit PGM IgG fraction with placental extracts from three unrelated individuals, panels 2, 4, 6 and 7 display the immunoreactivity of the rabbit anti-human PGM antibodies with the same samples,and panel 8 displays the cross-reactivity of the preimmune rabbit serum with a placental extract from a PGM1*2A1B individual.

rabbit IgG control, filter 8, shows very little cross-reactivity with the human placental proteins focused in this pH gradient.

Weakly immunoreactive components, possessing similar isoelectric points to some of the major PGM1 isozymes are visible on the anti-human PGM IgG filters 2, 4, 6 and 7. Some of these bands exactly match the PGM1 components detected by the sheep anti-rabbit PGM IgG on the adjacent filters (1, 3 and 5). However, the rabbit anti-human PGM1 IgG is not specific enough to allow the positive identification of the human PGM1 phenotypes.

Figure 8.4 shows the SDS-PAGE separation of purified rabbit PGM and a crude human skeletal muscle extract, followed by immunoblot analysis. Panel (a) is incubated with the rabbit anti-human PGM1 IgG fraction and panel (b) with the preimmune rabbit IgG fraction. A component with approximately the same molecular weight as PGM1 is detected by both the immune and preimmune IgG fractions and there was no difference in the number of muscle components detected by either immunological reagent.

8.4 The production of recombinant PGM protein.

During the final stages of this project it was possible to consider an alternative approach for the production of anti-human PGM1 antibodies. This was made possible by the successful identification of rabbit PGM clones and subsequently human PGM clones. Since the rabbit PGM was cloned first, the production of rabbit PGM in the form of a recombinant fusion protein was attempted. The methodology of

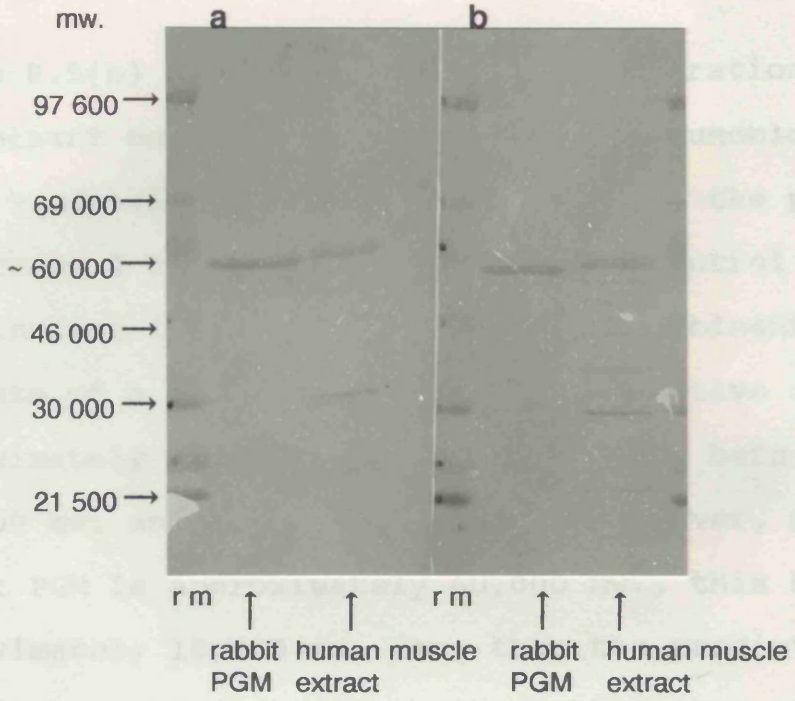


Figure 8.4

The immunological detection of muscle proteins following separation by SDS gel electrophoresis and electrotransfer to nitrocellulose. Tracks 1, 2 and 3 contain the standard molecular markers (r m), purified rabbit PGM and human muscle extract respectively. Panel (a) was incubated in the rabbit anti-human PGM IgG fraction and panel (b) in the preimmune rabbit serum.

this procedure is described in chapter 2 (Huynh *et al.* 1985).

Figure 8.5(b) displays the SDS-PAGE separation of recombinant rabbit PGM protein after immunoblot analysis. Track 2 contains a 1 in 1024 dilution of the purified rabbit PGM, track 3 the infected lambda gt11 control and track 4 contains a 1 in 10 dilution of the recombinant protein. It consists of a single strongly immunoreactive component, of approximately 160,000 mw. made up of the beta-galactosidase, 116,000 mw. and rabbit PGM protein. However, given that rabbit PGM is approximately 60,000 mw., this band is approximately 18,000 mw. less than the predicted fusion protein band of 176,000 mw. The rabbit cDNA used for these experiments is in frame, and corresponds to the full length rabbit DNA sequence reported by Whitehouse *et al* (1992). Therefore, expression of the complete recombinant protein should take place. The degree of error exhibited by SDS-PAGE molecular weight determinations may explain the discrepancy between expected and observed molecular weights. It is also possible that proteolytic degradation of the fusion protein has taken place within the bacterial cells. However, evidence for this suggestion is not forthcoming as no smaller immunoreactive fragments are detected with the sheep anti-rabbit PGM IgG.

Figure 8.5(a) shows the separation of the same samples, visualised by general protein staining. Tracks 3 and 4 show large numbers of strongly stained components. The band patterns are identical, apart from a faintly stained component observed in track 4, which has the same

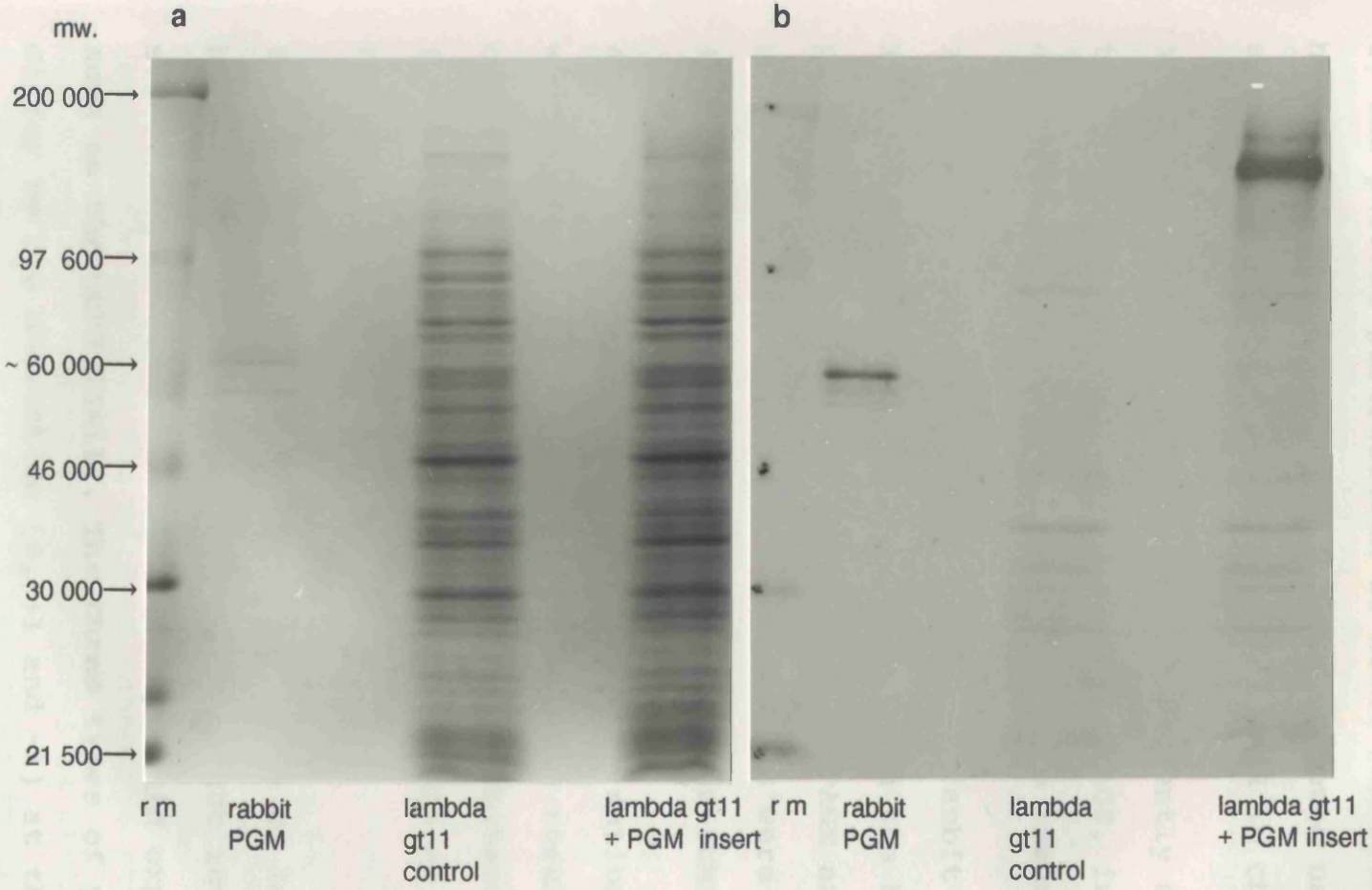


Figure 8.5

The analysis of the production of recombinant rabbit PGM plus controls following separation by SDS gel electrophoresis and Coomasie blue protein staining (a) and immunological detection with the sheep anti-rabbit PGM IgG (b) following electrotransfer to nitrocellulose. Tracks 1, 2 , 3 and 4 contain standard molecular weight markers (r m), purified rabbit PGM control, the lambda gt11 control, and the recominant proteins expressed from the rabbit PGM cDNA inserted into lambda gt11 respectively.

electrophoretic mobility as the rabbit fusion protein observed in figure 8.5(b).

8.5 Summary and conclusions.

1) The partial purification of human PGM from an aqueous human skeletal muscle extract was achieved using the sheep anti-rabbit PGM IgG fraction, described in chapter 4.

2) The purified human PGM1 was subsequently separated from the sheep anti-rabbit PGM IgG by SDS-PAGE, immunoblotted and a nitrocellulose suspension was used to immunise a rabbit.

3) Subsequent characterisation of the rabbit anti-human PGM IgG revealed weak cross-reactivity with the human muscle proteins after separation by both SDS-PAGE and IEF. However, none of these immunoreactive components were positively identified as the gene products of human PGM1.

4) Therefore, an alternative method of antibody production was investigated which gave encouraging results. The complete rabbit PGM cDNA, isolated by Whitehouse *et al* (1992), was used to express large amounts of the recombinant rabbit PGM in E.*coli*.

5) A better approach for the production of large quanties of human PGM1 for use as an immunogen, might involve the subcloning of the human cDNA into plasmid expression vectors such as the pUEX family. The three types of pUEX vector differ by one nucleotide (0, +1 and -1) at the cloning site, ensuring that the recombinant cDNA will be in its correct translational reading frame in one of the vector types (Stanley & Luzio, 1984).

Chapter 9:

The immunodetection of GC and PGM in Forensic Material.

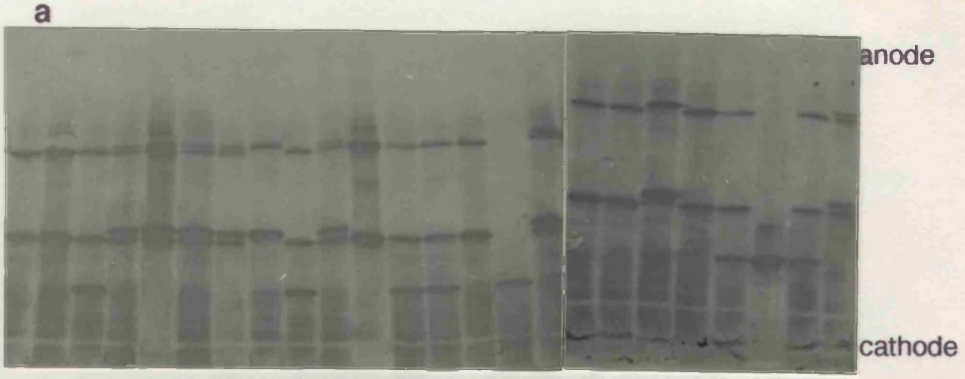
This chapter is concerned with the identification of the GC and PGM polymorphisms in forensic samples. Biological material such as bloodstains, semen stains, vaginal swabs and products of conception were provided by the Home office CRSE for analysis by conventional immunoblotting.

9.1. Group-specific component.

Figure 9.1 illustrates the immunodetection of the GC phenotypes from a selection of twenty one bloodstains and three plasma samples, after separation by IEF on a 4.5 to 5.4 pH gradient. The stains (20 mm^2), were extracted in 6M urea in order to dissociate the GC from the actin present in the stain material. Good resolution of the GC isoforms was obtained. It was estimated that the amount of free GC present in 5µl of each stain extraction was 320ng (assuming the average level of free GC is 80ng/mm^2 of bloodstain) (Westwood, 1985).

Figure 9.2 shows the immunoblot analysis of three different sources of biological material after separation by IEF on a 4.5 to 5.4 pH gradient, three plasma samples, five vaginal swabs and five semen stains. Vaginal swabs were taken from the same woman at different intervals after two separate intercourse events. The male GC phenotype attributable to the presence of semen was identified as 1F-1S in tracks 4 and 8, 84 and 36 hours after intercourse respectively. The semen free control (*) shows no detectable GC, as do tracks 2 and 3.

a



Track number 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

C C C

b

Track number	GC phenotype
1	1S
2	1S
3	2-1S
4	1F-1S
5	1F-1S
6	1F-1S
7	1F-1S
8	1F
9	2-1S
10	1F-1S
11	1S
12	2-1S
13	2-1S
14	1S
15	2
16	1F-1S
17	1S
18	1S
19	1F
20	1S
21	2-1S
22	2
23	2-1S
24	1F-1S

Figure 9.1

The immunological detection of human plasma GC in bloodstains and plasma controls (c), following separation by IEF on a 4.5-5.4 pH gradient and electrotransfer to a nitrocellulose membrane (a). Table (b) displays the GC phenotypes of all the samples illustrated in (a).

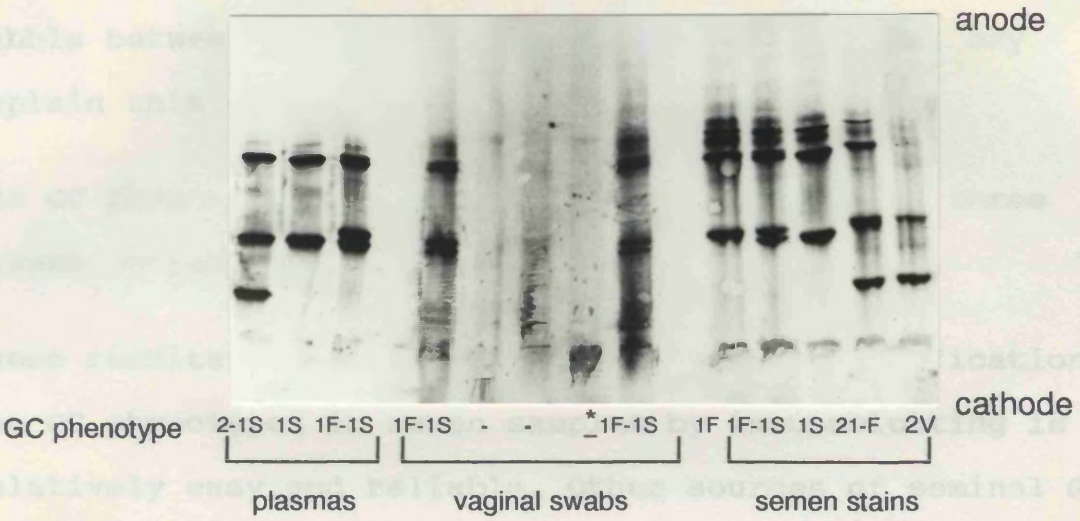


Figure 9.2

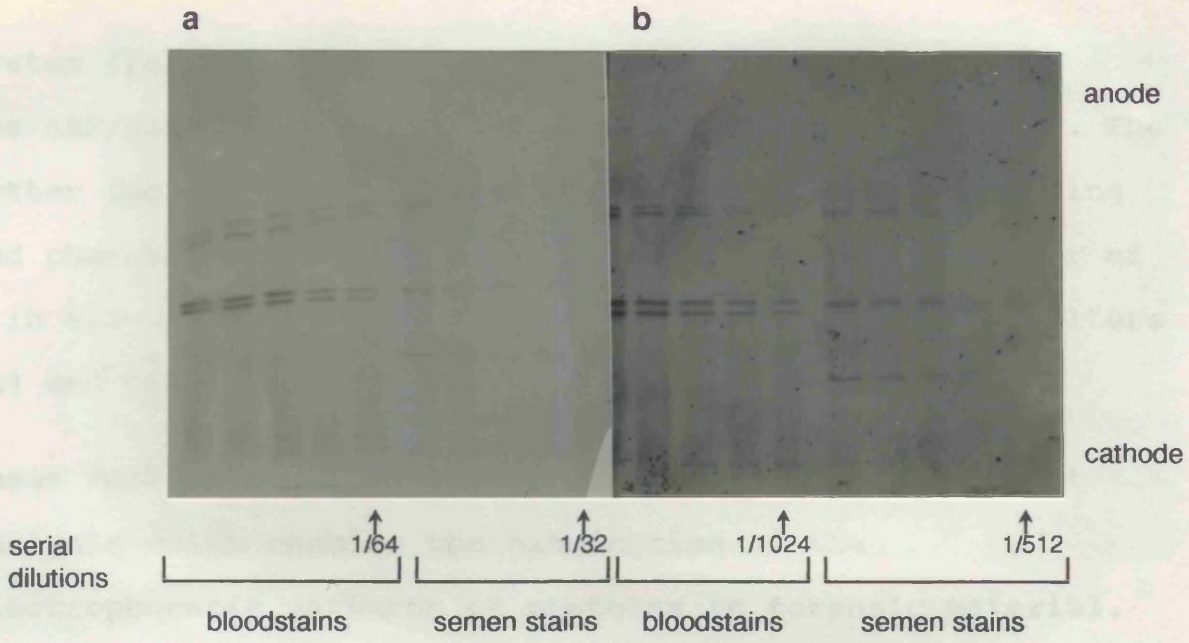
Immunoblot analysis of human GC in plasma, vaginal swabs and semen stains after separation by isoelectric focusing on a 4.5-5.4 pH gradient. Stain extraction was carried out in 0.5% PBS / 6M urea.The vaginal swabs from left to right are 84 h. time since intercourse (tsi); 36 h. tsi; 84 h. tsi; semen free control (*); 36 h. tsi. The semen stain sample labelled 2V, lacks a 1F component.

The GC phenotypes from all five semen stains are easily identified, apart from the sample in track 5. This sample displays the GC isoforms consistent with a common heterozygote individual, except one GC1 isoform is missing. A blotting inconsistency such as the formation of an air bubble between the transfer membrane and the gel, may explain this unusual isoform pattern.

The GC phenotypes are easily recognised from all three plasma control samples.

These results show that the detection and identification of the GC phenotypes in semen samples by immunoblotting is relatively easy and reliable. Other sources of seminal GC such as vaginal swabs, which are subject to degradation in the vaginal environment, were also phenotyped successfully. The method of immunofixation which was until recently the method of choice for the phenotyping GC in Forensic Science Service laboratories, is sensitive enough to give the reliable phenotyping of GC from bloodstains. However, it is unable to identify the GC phenotypes from body fluids such as semen, that contain significantly lower levels of GC (Westwood, 1985).

Figure 9.3 shows the application of three immunological detection systems to the identification of the GC polymorphism in dilutions of bloodstained and semen stained material. The HRP/DAB system (panel a) detects approximately 5ng of GC from bloodstained material, assuming the average level of free GC in bloodstained material is about 80 ng per mm^2 (Westwood, 1985). The analysis of bloodstain dilutions using the biotinylated ALP system (panel b) and the HRP/ECL



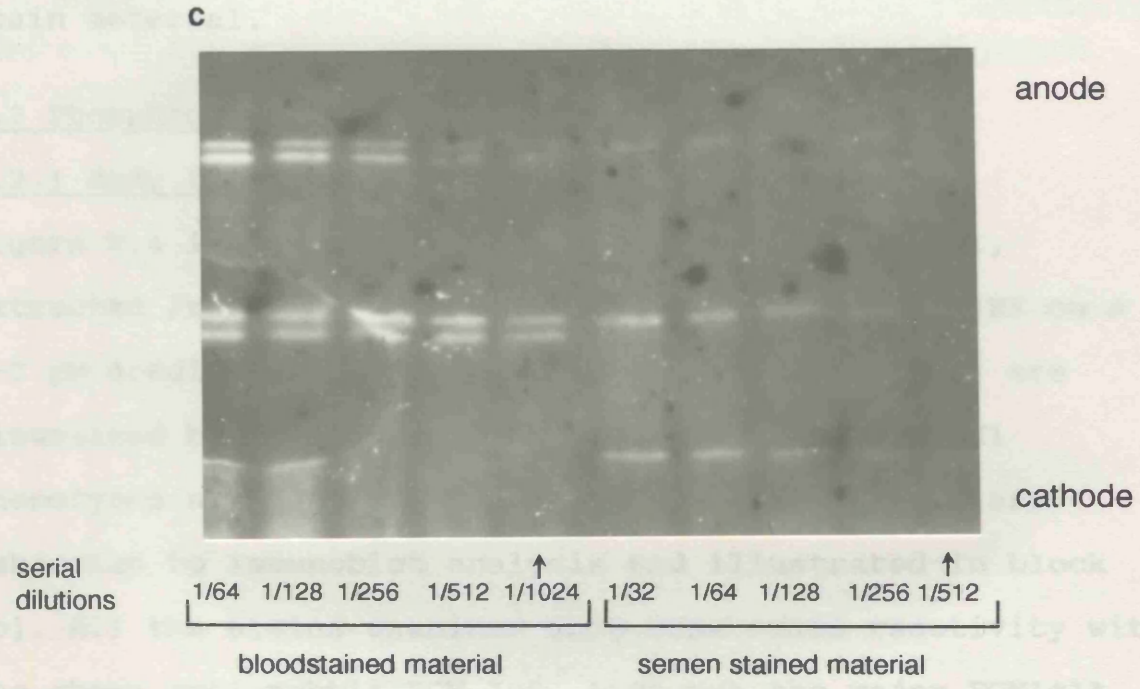


Figure 9.3

Immunoblot analysis of serially diluted bloodstained material from a GC1F-1S individual and semen stained material from a GC 2-1F individual, detected using three systems (a) HRP-DAB system (b) biotinylated ALP system and (c) HRP-chemiluminescent ECL system.

system (panel c) show 16 fold better sensitivity than the HRP/DAB system, detecting approximately 300pg of GC. The latter two detection systems are also capable of detecting and phenotyping GC from semen stains diluted by a factor of 1 in 512, as shown in the second block of samples on filters (b) and (c).

These results show the extreme sensitivity of immunoblot analysis which enables the examination of the electrophoretic patterns of proteins in forensic material. Taking into consideration the limits of GC detection reported in chapter 3, one can assume that lower picogram amounts of GC (between 10 and 50pg) may be detected in stain material.

9.2 Phosphoglucomutase.

9.2.1 Body fluids.

Figure 9.4 illustrates the separation of human PGM1, extracted from a selection of nine bloodstains by IEF on a 5-7 pH gradient. The samples displayed in block (a) are visualised by enzyme activity staining. All the PGM1 phenotypes are easily recognised. The same samples are subjected to immunoblot analysis and illustrated in block (b). All the stains examined show some cross-reactivity with the sheep anti-rabbit PGM IgG. Although the major PGM1*1A and 1B isozymes would appear to be identified by the sheep anti-rabbit PGM IgG, the 1A and 1B phenotypes cannot be reliably distinguished. Also the PGM1*2 alleles do not appear to show any cross-reactivity with the sheep IgG at all.

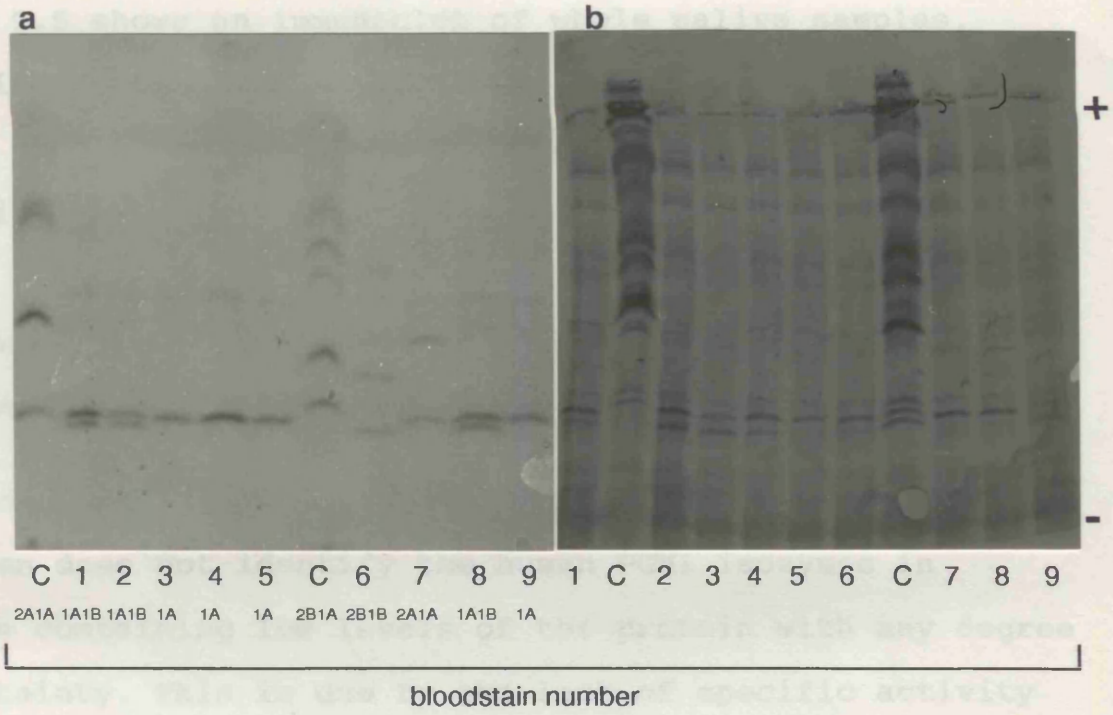


Figure 9.4

The detection of human PGM1 phenotypes from bloodstains obtained from nine unrelated individuals and two haemolysate controls (c), following separation by IEF on a 5-7 pH gradient, and conventional enzyme activity staining (a) or immunological detection with the sheep anti-rabbit PGM IgG (b).

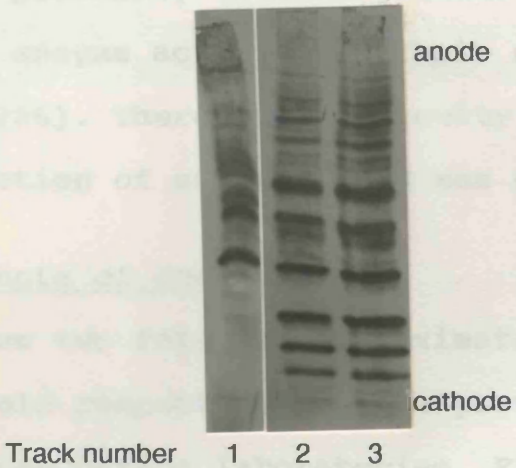


Figure 9.5

The immunological detection of PGM1 isozymes in human skeletal muscle from a PGM1*1A individual (track 1) and the identification of immunoreactive components in whole saliva from one individual (tracks 2 and 3), using the sheep anti-rabbit PGM IgG, following separation by IEF on a 3.5-10 pH gradient and electrotransfer to nitrocellulose.

Figure 9.5 shows an immunoblot of whole saliva samples, following separation by IEF on a 3.5-10 pH gradient. A series of nine intensely immunoreactive bands were detected in saliva samples from one individual using the sheep anti-rabbit PGM IgG. The isoelectric points of these salivary components do not correspond with those of the major PGM1 isozymes.

These results show that the sheep anti-rabbit PGM IgG fraction does not identify the human PGM1 isozymes in samples containing low levels of the protein with any degree of certainty. This is due to the lack of specific activity of this sheep anti-rabbit PGM antibody for the human enzyme. Therefore conventional enzyme activity staining is still the best method for the detection of the PGM1 polymorphism in red cell lysates, bloodstains and semen. The visualisation of the saliva PGM1 phenotypes by enzyme activity staining is not always possible, as a large number of individuals display no enzyme activity in their salivary secretions (Divall, 1986). Therefore difficulty with the immunodetection of salivary PGM was expected.

9.2.2 Products of conception.

Samples from two fetuses, approximately seven and nine to ten weeks old respectively, were provided for analysis by the CRSE Home Office laboratories. Figure 9.6 shows the analysis of these two specimens following separation by IEF on a 5-7 pH gradient. The focused PGM isozymes were visualised separately by conventional enzyme activity staining, panel (a), and immunoblot analysis, panel (b). The PGM1 phenotypes detected by enzyme activity staining

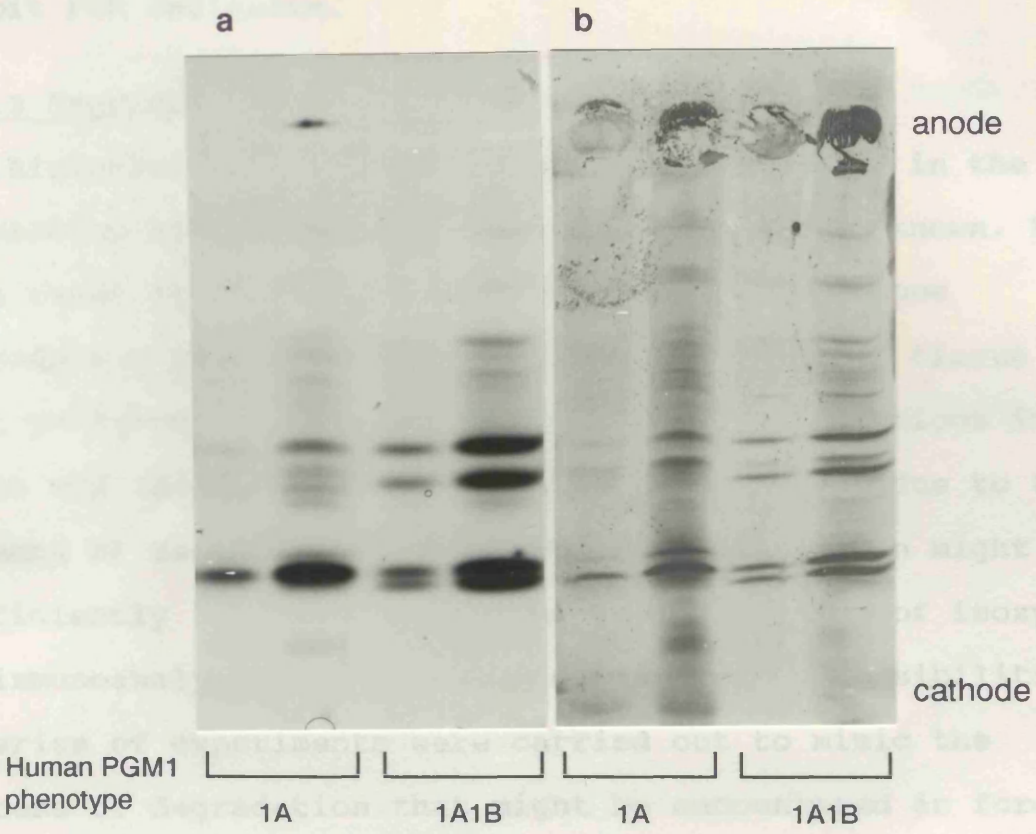


Figure 9.6

The detection of the PGM1 phenotypes of tissue obtained from two aborted fetuses, following separation by IEF on a 5-7 pH gradient, by conventional enzyme activity staining (a) and immunological detection (b) using the sheep anti-rabbit PGM antibodies, following protein transfer to a nitrocellulose membrane.

completely agree with those recognised by the sheep anti-rabbit PGM antiserum.

9.2.3 Degradation experiments.

The histories of the forensic specimens examined in the preceeding series of experiments were largely unknown. In some cases it was clear from the material that some degradation had occurred, but in other cases the tissue was well preserved. It is possible to envisage situations in which PGM isozyme activity might have been lost due to the effects of degradation, whilst the enzyme protein might be sufficiently intact to allow the identification of isozymes by immunoanalysis. In order to explore these possibilities, a series of experiments were carried out to mimic the process of degradation that might be encountered in forensic specimens.

Human placental tissue was subjected to storage at room temperature and 37°C, for between 17 hours and one week. Figure 9.7 displays the electrophoretic separation by IEF on a 5-7 pH gradient, of this tissue after homogenisation. Samples stored at room temperature are shown in panels (a) and (b), visualised by enzyme activity staining and immunoblot analysis respectively. Panels (c) and (d), contain the samples stored at 37°C. The results indicate that the phenotyping of the placental PGM1 is possible by both detection systems after storage at both temperatures for 24 hours. After this time period however, no PGM enzyme activity or immunoreactivity with the antiserum is observed.

Figure 9.8 shows the results of similar degradation experiments on rabbit and human skeletal muscle tissue.

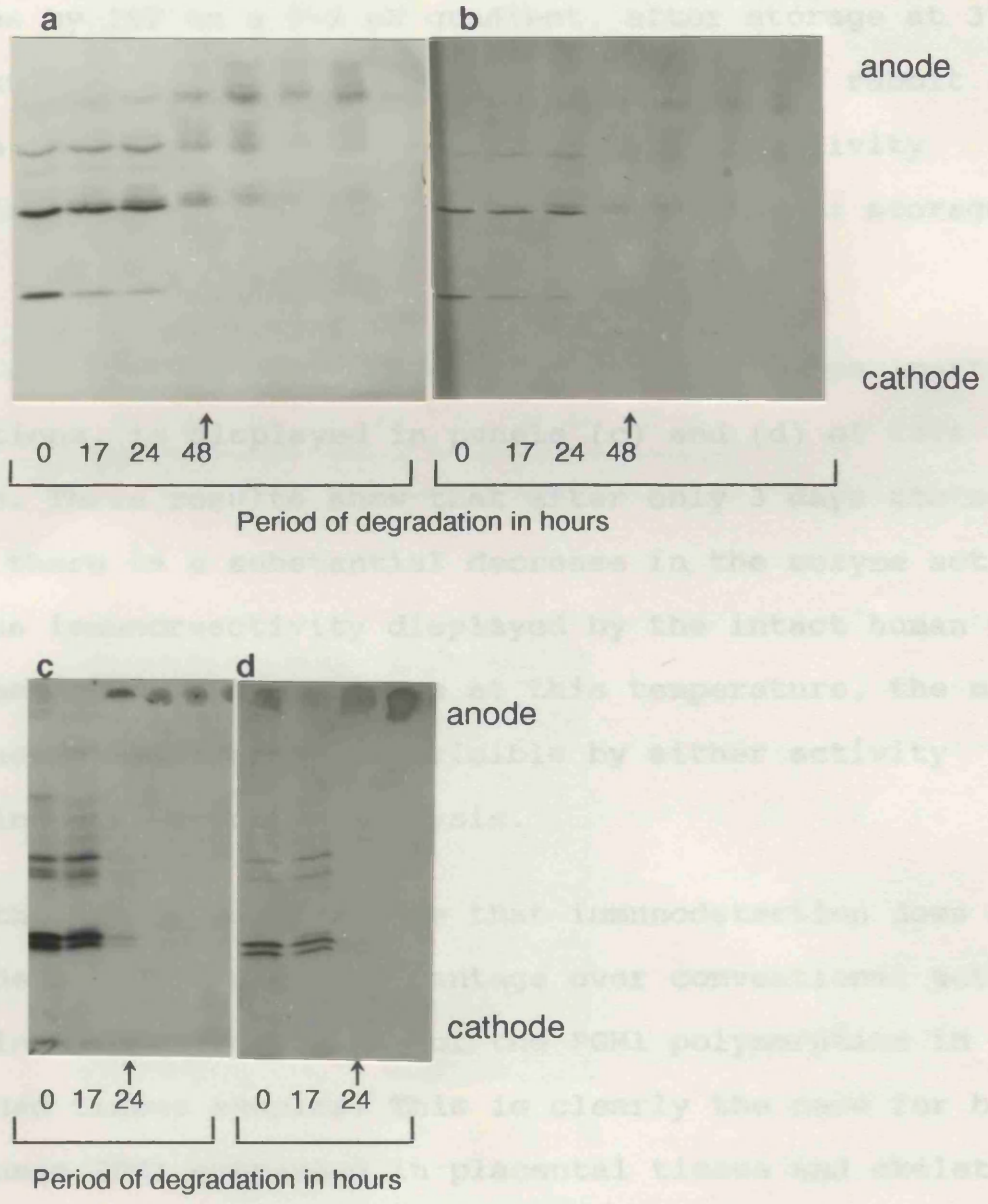


Figure 9.7

The detection of the PGM1 isozymes following separation by IEF on a 5-7 pH gradient and conventional enzyme activity staining (panels a and c), and immunological detection with the sheep anti-rabbit PGM antibody following electrotransfer to nitrocellulose (panels b and d). Panels (a) and (b) display the loss of enzyme activity and immunoreactivity of human placental extract with time at room temperature.Panels (c) and (d) display the loss of enzyme activity and immunoreactivity of human placental extract with time at 37^{o}C.

Panels (a) and (b) show the separation of the rabbit muscle samples by IEF on a 5-7 pH gradient, after storage at 37°C. The intact electrophoretic pattern displayed by rabbit muscle PGM is still detected by both enzyme activity staining (a) and the sheep IgG (b) after 22 days storage at 37°C.

Human skeletal muscle, subjected to the same experimental conditions, is displayed in panels (c) and (d) of this figure. These results show that after only 3 days storage at 37°C, there is a substantial decrease in the enzyme activity and the immunoreactivity displayed by the intact human PGM1 isozymes. By 12 days storage at this temperature, the major PGM isozymes are no longer visible by either activity staining or immunoblot analysis.

From these results, it seems that immunodetection does not provide any significant advantage over conventional activity staining for the detection of the PGM1 polymorphism in degraded tissue samples. This is clearly the case for both the human PGM1 expressed in placental tissue and skeletal muscle as well as rabbit PGM. The latter, which appears to be more stable, shows equivalent results between isozyme and immunostaining after prolonged storage (22 days at 37°C). Electrophoretic analysis after storage at a higher temperature (12 days at 55°C) also led to a coincident loss of isozyme staining and immunodetectable patterns (results not shown).

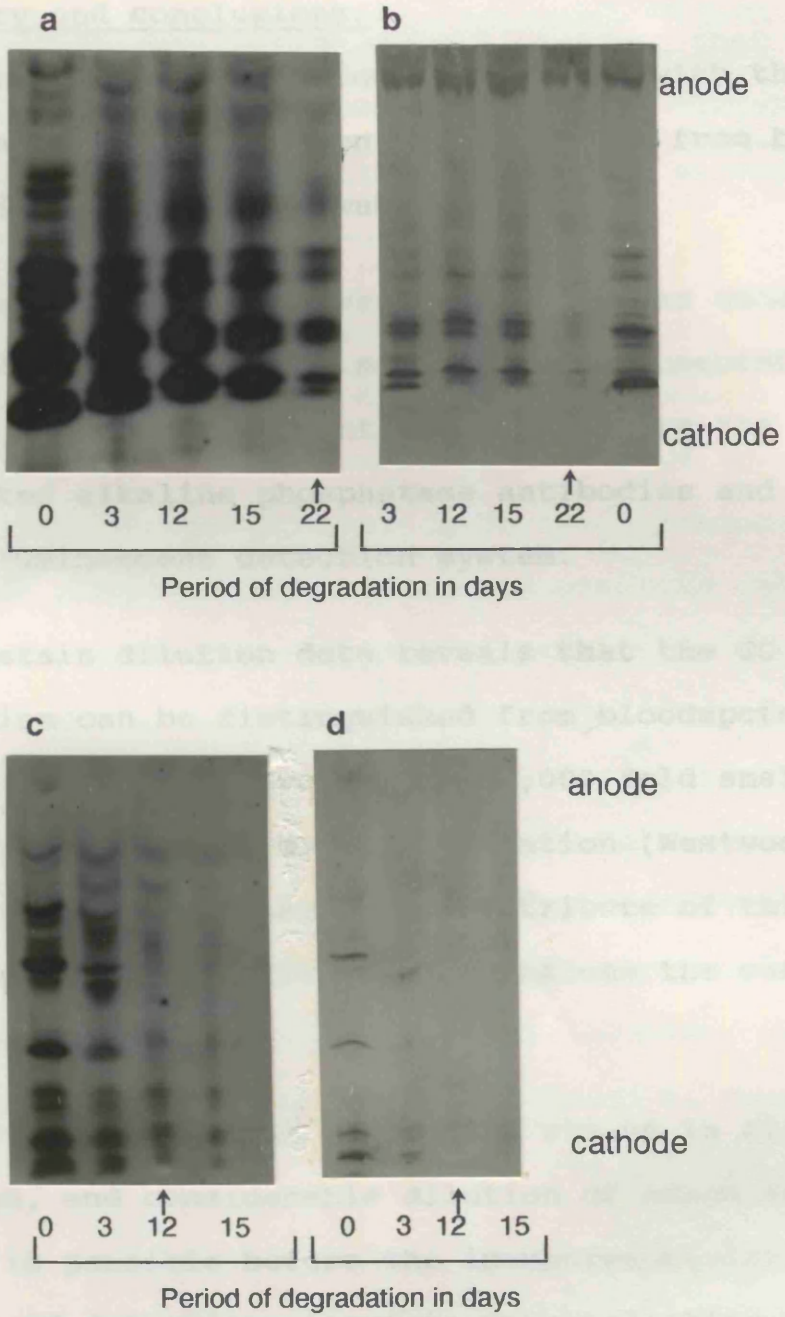


Figure 9.8

The detection of PGM isozymes following separation by IEF on a 6-8 pH gradient (panels a and b) and a 5-7 pH gradient (panels c and d), conventional enzyme activity staining (a and c) or immunological detection with the sheep anti-rabbit PGM IgG fraction following electrotransfer to nitrocellulose (b and d). Panels (a) and (b) display the reduction of enzyme activity and immunoreactivity from rabbit skeletal muscle extracts with prolonged storage at 37^{o}C. While panels (c) and (d) display the loss of enzyme activity and immunoreactivity from human skeletal muscle extracts with prolonged storage at 37^{o}C.

9.3 Summary and conclusions.

1) Immunoblot analysis successfully helps with the identification of the common GC phenotypes from bloodstains, semen stains and vaginal swabs.

2) Dilution experiments reveal that picogram amounts of GC can be detected from stain material by immunoblot analysis utilising signal enhancement systems such as the biotinylated alkaline phosphatase antibodies and the ECL chemiluminescent detection system.

The bloodstain dilution data reveals that the GC polymorphism can be distinguished from bloodspots as small as 4 μm^2, which are approximately 1,000 fold smaller than the bloodstains phenotyped by immunofixation (Westwood & Fryer, 1986). This is a most impressive attribute of this protein marker, since in many forensic situations the sample size is restricted.

The GC protein extracted from semen stains is also easily phenotyped, and considerable dilution of semen stain extracts is possible before the immunoreactivity falls below the level of detection. Accurate quantification of seminal GC is not possible due to a lack of such information. Tentative approximations however, have been made by Pflug (1988), who suggests that semen contains approximately 1,000 fold less GC than blood plasma. From the stain dilution data presented here, the extraction of semen stains measuring approximately 10 μm^2 in diameter should give positive identification of the GC phenotype. This would suggest that the seminal GC concentration is considerably higher than expected, considering the minimal bloodstain required for GC

phenotyping. It must be emphasised however, that these figures do not take into consideration the variables encountered between the efficiencies of GC extraction from both types of stain.

The final source of GC protein examined in this work was vaginal swabs taken before and after intercourse. The main concern of using an ultrasensitive technique for such investigations is the possible misinterpretation of the assailant's phenotype due to the simultaneous detection of the victim's phenotype. Vaginal GC protein has been detected before (Pflug *et al.* 1988) but this protein is present at considerably lower concentrations than seminal GC. Therefore, the detection of vaginal GC in such immunological investigations is rare. The results reported in this thesis show that the male GC phenotype is distinguished without any interference from that of the female. However, the screening of a larger panel of swabs is recommended to ensure the total absence of any contamination from the victim.

Some of the swabs examined here show no cross-reactivity with the anti-GC antiserum, implying that the detection of GC from post-coital samples is far from reliable. From this data it can be assumed that at least 50% of vaginal swab material will be phenotyped correctly for the GC polymorphism. Therefore, this system could be used to great effect as a primary screen, prior to the analysis of stains with more expensive techniques such as those associated with single locus DNA probes.

3) The sheep anti-rabbit PGM IgG fraction successfully

recognises the PGM1 phenotypes from fetal tissue, but enzyme activity staining remains the most reliable method for detecting low levels of PGM1 activity from stain material.

4) Experiments using degraded material from human and rabbit, indicate that the immunodetection of PGM with the currently available sheep anti-rabbit PGM antibody is not superior to enzyme activity staining.

The concurrent absence of enzyme activity and complete lack of immunoreactivity were recorded. From these findings, it was established that the conformational changes of the PGM protein which may have occurred during these experiments would appear to be totally different from those known to occur during SDS-PAGE analysis (Berzofsky, 1985). Earlier work has shown that the PGM1 protein retains immunoreactivity with sheep anti-rabbit PGM antiserum after SDS-PAGE analysis (Whitehouse *et al.* 1989). Therefore, we can assume that some if not all of the antibody binding sites situated on unravelled PGM protein remain intact and accessible to the antiserum.

These findings emphasise the need for a high titre anti-human PGM antiserum, which was in the process of being raised towards the end of this project. Another group have managed to produce an anti-human PGM antiserum during the course of this work. Cross-reactivity of this polyclonal antiserum with human PGM1 has been reported, but as yet no data concerning its possible application to forensic investigations have been published (Piatti *et al.* 1990).

In conclusion, immunological reagents for the reliable

detection of the PGM1 polymorphism from samples containing low levels of the enzyme are required. The production of a high titre anti-human PGM polyclonal antiserum would probably allow the detection of human PGM1 from red cell lysates, semen and stained material and possibly saliva.

In addition, several panels of monoclonal antibodies could be manufactured. These would include allele specific antibodies, which would enable the identification of the common PGM1 phenotypes by ELISA tests. The recognition of short PGM peptide sequences, corresponding to the the major epitopes on the PGM protein would also be possible. This would allow the construction of a detailed epitope map for the PGM protein, and could be instrumental in the recognition of the PGM1 phenotype in samples which have been severely degraded.

Chapter 10.

Discussion.

The main goal of this project was to develop ultrasensitive immunological methods for the detection of highly polymorphic proteins after separation by isoelectric focusing (IEF). The ultimate aim of this work is the application of this technology to the detection of protein polymorphisms in forensic material such as blood and semen stains. The work reported in this thesis concentrates on two polymorphic proteins, the serum protein human group-specific component (GC) and the red cell enzyme phosphoglucomutase (PGM). The employment of these two proteins as model systems serves to form the basis for the wider objectives outlined above.

The method of immunoblot analysis used to detect the intact electrophoretic pattern of a protein has been established for some time (Towbin *et al.* 1979), and since the introduction of the immunoblotting technique, many groups have applied this method to the detection of a multitude of proteins by 2-dimensional electrophoresis (Legocki & Verma, 1981; Goldman & Merril, 1983; Goldman *et al.* 1985). The assessment of factors which may be important in the immunoblotting procedure, such as the influence of different blotting systems on the efficiency of protein transfer and the effect of different blotting buffers, have subsequently been investigated in some depth (Gershoni & Palade, 1983; Towbin & Gordon, 1984; Beisiegel, 1986). However, it should be noted that most of these studies were carried out on denatured proteins separated by SDS-PAGE. More recently, there have been reports of several proteins separated by IEF

which have subsequently been visualised by immunoblotting; such as the sixth complement component (C6) (Whitehouse & Putt, 1983), alpha-2-HS-glycoprotein (alpha-2 HSG) (Boutin *et al.* 1985), haptoglobin (Teige *et al.* 1985) and alpha-1-antitrypsin (Whitehouse *et al.* 1989.a). In these cases and several others, the immunoblotting technique has replaced more complex or less sensitive existing methods. In spite of these successes, no systematic examination of the immunoblotting process has been carried out on native proteins separated by IEF. Thus the work reported in this thesis was initiated.

The two polymorphic proteins, human group-specific component (GC) and phosphoglucomutase (PGM), were chosen as models to follow and assess each stage of the immunoblotting process. Both of these systems have the advantage of being highly polymorphic and important in forensic analyses and cases of disputed paternity. Furthermore, GC is a serum glycoprotein, related to albumin and alpha fetoprotein, while PGM is a cytosolic enzyme which occurs in all tissues.

Historically, much of the research on the phenotyping of the GC polymorphism from forensic specimens has been carried out at the Home Office Research Establishment laboratories, and as this project was funded by the same Institute, this work was directed towards the development and application of techniques for use in the Forensic Science Service. When this work was initiated, the method currently being used in the Forensic Science Service to detect the GC polymorphism was immunofixation (Westwood, 1985; Westwood & Werrett, 1989). This is effective but relatively insensitive compared

with immunoblotting. Since a good commercially produced anti-human GC polyclonal antiserum was available and expertise on both the analysis of the GC protein and an abundance of knowledge on the collection and treatment of forensic samples was available from the Home Office laboratories, the GC protein was an ideal candidate to use for the in depth examination of the immunoblotting process (Drago *et al.* 1990).

PGM is also an important protein marker in the forensic field. Much research on the application of this enzyme polymorphism to forensic analyses have been carried out at both the Home Office laboratories and the Metropolitan Police laboratories (Gill & Sutton, 1985; Divall & Ismail, 1983). Closer to home however, much of the pioneering work on the PGM proteins was initiated at the MRC Biochemical Genetics Unit, where the current project was carried out.

Much expertise in the production and characterisation of antisera was available in this laboratory, along with a vast knowledge of the PGM proteins, therefore the production of an anti-PGM polyclonal antiserum for the immunodetection of the human PGM1 polymorphism in forensic samples was considered to be an ideal model system for this work. The production of an immunological reagent against PGM would also open up the exciting prospect of examining all the members of the PGM gene family, and perhaps provide some insight into the genetical origin of the PGM found in human milk, the so called PGM4 isozymes.

10.1 Immunoblotting.

The detailed examination of the detection of PGM and GC by immunoblotting has revealed that certain steps in the procedure are more critical to the final result than others. These include the method of protein transfer, the choice of blocking agent and the choice of detection system.

The method of sample application and the use of different loading buffers were found to be the two most important factors influencing the sensitivity of protein detection after separation by IEF. Ironically, these two features were almost overlooked, as the separation of proteins by IEF has been examined in considerable depth since its first application by Svensson (1961), and consequently the methodology is considered well established. In the case of the rabbit PGM, a 16,000 fold improvement in sensitivity was recorded as a result of changes in the sample loading technique.

Detailed examination of the steps involved in the immunoblotting procedure *per se*, revealed a considerable number of blotting conditions which match exactly the observations reported for the immunoblot analysis of proteins separated by SDS gels. Some disagreement concerning the choice of protein transfer method however, must be noted. Protein transfer from IEF gels by tank electroblotting gives consistently superior transfer compared with semi-dry electrotransfer; this is in complete disagreement with the data obtained from SDS gels (Tovey & Baldo, 1987). The reason for this is unclear. The voltage gradient, a measure of the voltage of the blotting system

and the distance between the blotting electrodes, is supposed to give a good indication of the efficiency of an electroblotting system. Both semi-dry blotting systems show approximately 3 times higher voltage gradients than the tank blotting system, yet the tank system still gives superior blotting efficiency. Other electrical readings such as the calculated current per cm^2 of gel was comparable for both blotting systems. Therefore it is uncertain why tank electroblotting gives superior protein transfer from IEF gels.

The utilisation of non-isotopic detection systems, one of the major features of the immunoblotting procedure examined in this thesis, also has a considerable influence on the overall sensitivity of the test. Of the systems examined here, the horseradish peroxidase-linked antibody system, has proved to be the most simple, sensitive and reliable to use. Good sensitivity is obtained with the coloured HRP/DAB system of visualising the peroxidase labelled antibodies, but the greatest sensitivity is obtained with a chemiluminescent detection system.

Much previous work with the detection of proteins by immunological means has been carried out at the Galton laboratory, including some work with chemiluminescent detection systems (Whitehouse & Hopkinson, 1985). The opportunity arose to combine the work that was already underway on the chemiluminescent detection of proteins in this particular project, with the development of the Amersham ECL, enhanced chemiluminescent detection system, first developed for the non-isotopic detection of DNA. In

fact this was to be the first in depth examination of polymorphic proteins using chemiluminescent detection systems. The examination of the ECL detection system revealed that it was considerably more sensitive than any of the other chemiluminescent systems I was using at the time, and at least 8 fold more senstive than the routinely used DAB detection system. This chemiluminescent detection system allowed the quick and easy visualisation of the GC phenotypes from sources of simulated forensic material such as blood and semen stains. Considerable dilution of these stain extracts was also possible before the results became ambiguous. Therefore, it is fair to conclude that the immunoblotting procedure described here, using the ECL chemiluminescent detection system is likely to be of considerable value in the detection of a wide range of polymorphic proteins. This significant step forward in sensitivity, together with the simplicity of the immunoblotting procedure make this method useful whether utilised for research purposes, paternity tests or in forensic science laboratories, where test material is often likely to be in very short supply.

10.2 Forensic Application.

Considering the current availability and use of multilocus and single locus VNTR DNA probes available for the identification of individuals from forensic samples, why should the development of ultrasensitive methods of protein detection warrant investigation? Conventionally used, multilocus or VNTR DNA probes are restricted in sensitivity and therefore not ideal for use in forensic investigations. Between 50 and 500ng of DNA is required for the use of these

probes, and positive results often take weeks to develop (Reynolds *et al.* 1991).

Protein technology, on the other hand is cheaper and more sensitive than the conventional use of DNA probes, which proves especially useful when the sample size is restricted. Non-isotopic detection systems are conventionally used for the visualisation of electrophoretically separated proteins, therefore no radioactive isotopes or facilities for the handling of such substances are needed. Furthermore, results recently published by the U.S government show that between 20 to 25% of DNA tests carried out remain unresolved, either as a result of insufficient amounts of DNA or other technical problems (Lewontin & Hartl, 1991).

Although, the probability of exclusion calculated for a multilocus DNA probe or a combination of single locus probes is superior to that calculated for a panel of protein markers, these systems can be used with great effectiveness in conjunction with DNA technology as a primary screening system. For example, 100 individuals tested using the PGM and GC polymorphic markers alone, show that the probability of excluding a wrongly named individual from the investigation is approximately 53%. Over half of the individuals tested in this manner are excluded from the investigation without the need for DNA techniques. The cumulative probability of exclusion calculated for larger panels of protein markers such as the serological and electrophoretic protein panels routinely used for paternity tests at the Royal London Hospital (see figures 1.1 and 1.3), indicate only 2% of the cases remain unresolved by

these means.

In fact, carefully selected panels of protein markers which display both high heterozygosity and stablity in forensic situations, are still routinely used in many British forensic laboratories, in conjunction with DNA profiling.

Currently the Forensic Science Services both here and abroad are caught up with the introduction of the polymerase chain reaction (PCR) (Mullis & Faloona, 1987), for the routine analysis of samples. The use of PCR enables the rapid examination of polymorphic gene markers from minute amounts of DNA, less than 1ng, using non-radioactive detection systems, overcoming the problems inherent in conventional DNA profiling (Reynolds *et al.* 1991). Since its introduction, PCR has been used in a multitude of genetical and medical investigations, as well as receiving a wide application in the forensic field. PCR has been used to amplify DNA from hair roots, bloodstains, single human sperm, saliva stains and pathological specimens of human remains (Higuchi *et al.* 1988; Jeffreys *et al.* 1988; Li *et al.* 1988; Higuchi, 1989; Pääbo, 1989; Reynolds *et al.* 1991).

Subsequently, more detailed studies on the application of PCR for looking at polymorphic markers, namely the HLA DQa alleles in forensic samples has been carried out (Westwood & Werrett, 1990; Budowle *et al.* 1991). The discriminating power of this HLA locus is far in excess of any single protein marker, approximately 94%, but certain problems have been encountered. These include, sample contamination, confused interpretation due to the sensitivity of the

detection system and difficulty in distinguishing between the male and female genotypes of DNA extracted from vaginal swabs after intercourse.

Recently, Jeffreys *et al* (1991.b), have published a technique called minisatellite variant repeat mapping (MVR), used in conjunction with PCR, which detects allelic variation within minisatellites. This technique not only increases the degree of variation displayed by selected minisatellites, but it is also applicable to forensic samples where the DNA may be degraded and rape cases where mixtures of material from both suspect and victim are present. Furthermore, standardisation of electrophoretic techniques is not so vital, since no gel distortion or band shifting is observed, no fragment length measurement errors are encountered and no side by side sample comparisons are required. MVR mapping would appear to be an extremely important development in DNA technology, and will have a considerable impact in the field of forensic science.

Although there is a great deal of interest and optimism surrounding MVR mapping, the current methods of DNA profiling have come under a great deal of scutiny since their introduction as evidence in court. Several cases examined by the American company, Lifecodes inc. have revealed far from satisfactory quality control assurance (Lander, 1989; Lewin, 1989; Norman, 1989). Since then, much time and effort has been spent on collating proper guidelines for DNA profiling which all institutes will follow (Ferrara *et al.* 1990; Kearney *et al.* 1991; Gill *et al.* 1991).

Most recently, the Forensic Science Services have been subjected to further critism concerning the use of biased reference population data for calculating their figures for presentation in court (Lewontin & Hartl, 1991). Lewontin & Hartl suggest that the reference data used does not take into consideration the genotypes possessed in small subpopulations. Earlier work on small inbred populations has revealed that DNA profiles do show an increased degree of band sharing (Bellamy *et al.* 1991). These observations, in conjunction with the calulations made by Lewontin & Hartl has led to a considerable amount of controversy. Chakraborty & Kidd (1991) however, strongly defend the reference population data currently used for the calculation of probabilities of indentity in forensic cases and suggested that the other group's figures are at fault. Consequently, these two publications have led to further lively debate on the subject (Willis *et al.* 1992; Roberts, 1992). These latest disagreements on the deviation and interpretation of the figures calculated from DNA evidence, serve to strengthen the case for the continued use of protein markers in forensic analyses.

10.3 The immunological examination of the PGM protein family.

Work carried out by various workers in the mid 60's to early 70's, led to the identification of a family of phosphoglucomutases, controlled by three unlinked structural gene loci: PGM1, PGM2 and PGM3 (Spencer *et al.* 1964; Hopkinson & Harris, 1965, 1968). Since these early human studies, several immunological reagents to PGM have been

produced, including a high titre sheep anti-rabbit PGM antiserum produced during this project. The use of this immunological reagent as a research tool, has resulted in some further clarification of the genetic basis of the PGM genes.

The immunological data compiled in this thesis and the attached publications, show that both the rabbit and human PGM1 are detected in their native and denatured states by the anti-rabbit PGM antiserum, after separation by IEF and SDS-PAGE respectively (Whitehouse *et al.* 1989.b; Drago *et al.* 1991). The strong cross-reactivity of the antiserum with both the rabbit and human protein suggests that the amino acid sequences of both enzymes are highly conserved. This has been confirmed by the derived amino acid sequences of rabbit and human PGM1, which show 97% homology (Whitehouse *et al.* 1992). Southern blot analysis of a wide range of vertebrate species revealed that the conservation of sequence homology at the PGM1 locus extends far into the evolutionary past from which the ancestral PGM gene probably arose.

Additional data gleaned from immunological detection of the human PGM1 polymorphism, indicates that the antiserum has no preferential binding affinity for any of the common PGM1 gene products. This suggests that the amino acid sequences of the common PGM1 proteins are highly conserved and the sequence variation in no way effects the binding properties of the sheep anti-rabbit PGM antiserum.

Assuming that the ancestral gene is the PGM1*1A allele, the proposed phylogeny to account for the PGM1 polymorphism

consists of two point mutations and a cross-over event (Carter *et al.* 1979; Takahashi *et al.* 1982) to generate the four common alleles. Since the formulation of this original hypothesis and its expansion to include other less common variants PGM1*3 and PGM1*7, evidence for the occurrence of intragenic recombination at the PGM1 locus in several paternity investigations has been reported (Wetterling, 1990). The lack of inheritance of the maternal PGM1 alleles by the child and more rarely non-paternity, was in direct conflict with the results obtained for the other protein markers tested. This phenomenon is thought to be the direct result of a recombination event occurring during oogenesis and more rarely during spermatogenesis.

The sheep anti-rabbit PGM antiserum shows no cross-reactivity with either the PGM2 or PGM3 gene products. These findings have been partly confirmed by the use of anti-human PGM1 and PGM2 antibodies produced and characterised by Piatti *et al* (1990), although the PGM3 isozymes were not examined by this group. Assuming that the PGM1, PGM2 and PGM3 loci have arisen as a result of gene duplication, a certain degree of sequence divergence must have occurred, accounting for the lack of cross-reactivity between these specific antisera and the other members of this protein gene family (Whitehouse *et al.* 1992).

10.4 The investigation of milk PGM.

PGM enzyme activity was first identified in milk by Cantu & Ibarra (1982). Later studies on the biochemistry of lactation and the composition of milk revealed that phosphoglucomutase plays an important role in lactose

synthesis (Casey & Hambridge, 1983; Neville *et al.* 1986). Such studies also revealed that the concentrations of the major components expressed in milk, such as fat, cholesterol and lactose varied quite significantly between species. Considering that PGM was found to play an important role in lactose synthesis, was there any relationship between the concentrations of milk lactose and levels of PGM activity found expressed in milk?

The concentration of lactose in human milk was found to be high, averaging 7g per 100ml of milk (Casey & Hambridge, 1983), compared with approximately 5g of lactose per 100ml of ruminant milk (Oftedal, 1984). If the levels of milk PGM expressed, were indeed a direct indication of the level of lactose synthesis, the expected PGM activity would be higher in human milk than the ruminant, which was indeed the case, as observed in the results reported in this thesis. In fact such low levels of PGM activity were detected in both cow and sheep milk that the milk PGM isozyme patterns were more or less undiscernable. These observations may lead us to form a tentative link between high levels of PGM activity and high milk lactose concentration. However, considerably more detailed examinations of both PGM enzyme expression and the other enzymes involved in the lactose biosynthesis pathway will be needed in order to fully assess the weight of these initial observations.

All of the previously published work on the biochemical properties and genetical basis of milk PGM was carried out on mature milk, a stage at which there is the least fluctuation in the expression of milk proteins (Cantu &

Ibarra, 1982; Kvitko & Weimer, 1990). Quantitative variation of the milk PGM isozymes was commonly observed between individuals, but no data was available from the extended study of milk PGM expression. Indeed, other milk constituents such as cholesterol (Prentice *et al.* 1981), fat (Blanc, 1981), selected proteins (Harzer *et al.* 1983) and vitamins (Udipi *et al.* 1987) have been shown to exhibit diurnal variation. Therefore, it was hardly surprising to find data which confirms that a high degree of quantitative variation of human milk PGM occurs on both a weekly and daily basis. It is uncertain whether these observed fluctuations in milk PGM activity coincide with changes in the levels of lactose synthesis in the mammary gland, but lactose concentrations have also been observed to vary on a daily basis in colostrum and mature milk (Mepham, 1987).

The most exciting observation which has resulted from these studies of milk PGM is that the human milk PGM isozyme pattern changes with time. Such qualitative variation, has been previously observed for another enzyme protein found in milk, lactate dehydrogenase (LDH), which showed a diurnal change in LDH phenotype (Hamosh *et al.* 1985). The reason for such qualitative changes in isozyme pattern however, have thus far remained unexplained.

The PGM activity identified in human milk is currently believed to be the result of the expression of an additional locus, PGM4. None of the milk PGM components identified by electrophoresis by Cantu & Ibarra (1982) appear to correspond with any of the previously identified PGM1, PGM2 or PGM3 isozymes. Their findings suggested a high degree of

electrophoretic heterogeneity displayed by the human milk PGM isozymes, which agreed with the Hardy-Weinberg expectation. However, it has not been possible to follow the predicted inheritance pattern of the PGM4 phenotypes or to examine sib-sib correlations because of the difficulties associated with family studies on isozymes restricted to the lactating breast. Thus, there is a certain degree of speculation concerning the genetical basis of the PGM4 polymorphism. The immunological analysis of milk PGM using the sheep anti-rabbit PGM antiserum has contributed to this debate: For example, immunoblot analysis of human milk separated by SDS-PAGE, revealed that human milk PGM has approximately the same molecular weight as the major 60,000 mw. PGM1 component identified from the preliminary studies on human skeletal muscle and placental extracts. Furthermore, the utilisation of the sheep anti-rabbit PGM IgG fraction for milk PGM isozyme subtraction studies, led to the complete disappearance of the milk PGM isozyme patterns.

This immunological data leads us to believe that milk PGM shares a high degree of protein sequence homology with PGM1 protein, since both of these sets of isozymes show good cross-reactivity with the sheep anti-rabbit PGM antiserum, which is in stark contrast with the PGM2 and PGM3 isozymes. Therefore, either milk PGM is indeed the product of an additional independantly acting PGM gene, which expresses gene products closely related to the PGM1 iosozymes, or the PGM isozymes identified in human milk are the product of post-translationally modified PGM1 isozymes.

Data obtained from the analysis of human milk PGM have revealed some preliminary evidence for the glycosylation of milk PGM. Treatment with neuraminidase caused some milk PGM isozymes to become more positively charged, suggesting that sialic acid residues had been removed. However, this treatment did not lead to the generation of recognisable PGM1 isozymes. The glycosylation of a number of other proteins such as lactoperoxidase, glycosyltransferase, lactoferrin and many more have also been reported prior to secretion from the mammary gland (Neville *et al.* 1986). Certain workers have suggested that the presence of high levels of enzymes such as lipoprotein lipase in milk is the result of cell damage incurred during suckling or forced expression of the milk (Hamosh *et al.* 1985). Although this was proved not to be the case, such alternatives should not be dismissed in the case of milk PGM. Unfortunately, this hypothesis was not tested in this work, but such an investigation could easily be carried out.

It is still uncertain whether the PGM enzyme activity found in milk is the result of the expression of a fourth locus. Evidence compiled within this thesis however, leads me to believe that milk PGM is subjected to one or more modifications which may occur post-translationally within the lactating mammary gland. One possible scenario, and perhaps the simplest interpretation, may involve the glycosylation of the normally expressed PGM1 gene product, accounting for the confused milk PGM isozyme patterns identified in human milk.

10.5 Future work.

The work carried out over the duration of this thesis has led to the ultrasensitive immunodetection of the GC and PGM1 polymorphisms, particularly the application of a working chemiluminescent detection system to the Forensic Science Service. The production of a high titre sheep anti-rabbit PGM antiserum has allowed the preliminary immunological analysis of the PGM protein family and will undoubtably be useful as a research tool in the further immunological analysis of the PGM proteins. Considerable new information has come to light concerning the relationship between the members of the PGM protein family, especially that associated with the putative PGM4 milk isozymes. As the work carried out in this thesis is the example of the sucessful immunological analysis of the PGM proteins, it is fair to say that much of the work performed here will form the basis of much of the future immunological analysis of the PGM protein family. The extension of this work into the future falls into four categories.

1) Monoclonals are already available to the GC protein (McLeod *et al.* 1986). These monoclonals and the production of allele-specific clones, could lead to the routine phenotyping of forensic material using enzyme-linked immunosorbent assay (ELISA) technology, in conjunction with the ultrasensitive chemiluminescent detection systems available. This could be carried out using an ultrasensitive chemiluminescent system, measured using the microplate luminometer recently developed by Berthold (1992).

2) The development of a panel of PGM1 allele specific monoclonal antibodies would also be a step toward the further immunological analysis of the PGM1 polymorphism, as is the case for the alkaline phosphatase polymorphism (Slaughter *et al.* 1981). Such monoclonals could be used both as routine practical tools in the forensic analysis of samples and as extremely powerful research tools.

The reliable phenotyping of the heat sensitive PGM1 alleles, which were first identified by Scozzari *et al.* (1981), may be possible with the production of such immunological reagents.

Peptide sequence specific monoclonals could be used for the construction of a complete epitope map of the protein, determining the major antigenic sites and the binding sites which enable the distinction between the PGM1 phenotypes. These studies could be further extended to include the immunological analysis of the of the rabbit PGM structure, which appears to consist of four structurally distinct domains, recently elucidated by Dai *et al.* (1992).

3) The problems encountered with the sib-sib investigation of the inheritance of the putative PGM4 polymorphism could be solved by using an animal model. Good candidates which may show electrophoretic heterogeneity of milk PGM include the swine and the mouse. The swine may prove the more useful of the two due the larger amounts of milk produced. It is uncertain whether milk PGM is expressed by any of the primate groups, but this would also be an interesting avenue to follow.

In addition, the aquisition and construction of cultured mammary cell lines for the comparison of the PGM protein within non-lactating and lactating mammary tissue may also prove useful in the future.

4) During the latter part of this thesis, the sheep anti-rabbit PGM antiserum was used to screen a rabbit muscle cDNA library, which resulted in the identification of the rabbit PGM cDNA. More recently the human PGM cDNA has been identified using the rabbit cDNA (Whitehouse *et al.* 1992). This has opened up the ongoing project considerably, since it provides a means of examining the PGM gene family at the molecular level. The further analysis of the PGM gene family should reveal considerable information concerning the evolutionary basis of the PGM genes, including the putative PGM4 locus, and uncover the reasons behind the extremely polymorphic nature of the PGM1 locus.

Chapter 11.

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