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IMMUNOLOGICAL STUDIES ON THE PROTEIN IN HUMAN FETAL RED BLOOD CELLS

A Thesis Presented By

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For the Degree

M A S T E R O F S C I E N C E

in the

INSTITUTE OF GENETICS

GLASGOW UNIVERSITY

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Purpose of Investigation

This research project involves the raising of antibodies to the antigens in fetal red cell lysates with two aims:

1. The investigation of red blood cell soluble proteins as a model for tissue proteins generally, and

2. To look for novel 'fetal' proteins in red cells by immunological methods.

Red blood cells have the advantage of being commonly available and having a high concentration of soluble intracellular protein that can be very simply extracted. A large number of enzymes have been identified in red cells and polyacrylamide gel electrophoretic analysis of protein from fetal and term cord lysates indicates a number of non-haemoglobin protein bands.

It was decided to look for fetal specific proteins because very few are known in the haemopoietic system, although they have been demonstrated in other fetal tissues and in sera. The recurrence of 'fetal-type' proteins in certain cancers is well documented. This does, however, present certain interesting problems in the case of red cells, since the appearance of 'fetal' characteristics is also associated with severe anaemia where there is an increase of immature cell types, as opposed to the appearance of abnormal cells containing the 'fetal' proteins.

SUMMARY

This thesis is a report of immunological studies on the soluble protein inside human red blood cells. The differences shown by adult and fetal red cells in their physical structure, general metabolism and haemoglobin biochemistry were considered, together with the possibility of using red cells with their readily extractable intracellular soluble protein as an immunological model for tissue cells in general. Thus the two-fold aim of this research project was to investigate red cell protein as a tissue model and to look for novel fetal proteins in red cells by immunological methods.

Antisera were prepared using red cell lysate protein as an immunogen. The antibody responses were determined principally by gel precipitation techniques, although some affinity chromatography (with analysis by polyacrylamide/SDS slab gel electrophoresis) and haemagglutination studies were also involved. Three sources of protein were used as immunogens in adult rabbits: unfractionated fetal lysate, unfractionated term cord lysate, and term cord lysate fractionated by ion-exchange chromatography so as to maximise recovery of non-haemoglobin red cell protein. Fetal lysate protein was also used to immunise two rabbits which had been neonatally tolerised to adult serum and red cells.

In general, the results of the antibody analysis by gel precipitation could be divided into three categories of response: to contaminating serum proteins, to haemoglobin, and to other non-haemoglobin red cell proteins. Animals immunised with unfractionated lysates had a limited response to serum proteins whereas the ion-exchange fractionation concentrated certain serum proteins and this was reflected in an increased response to serum proteins. All the antisera showed a weak response to adult haemoglobin and a much stronger response to fetal haemoglobin possibly involving further antigenic determinants. A β mobility antigen was detected in adult and fetal red cell lysates by two antisera; and an α_2 mobility antigen, with erratic electrophoretic variants in fetal lysates, by a third.

Cont'

The existence of any novel non-haemoglobin protein in fetal red cells was not proved however.

The results showed a rather limited antibody response to the many proteins known to be present in red blood cells. It was concluded that this could have been due to the inherent lack of immunogenicity associated with tissue cells, to the manner in which the immunogen was presented, or to the insensitivity of the methods used to detect the immune response.

LIST OF ABBREVIATIONS

Red Cell Lysate	RCL
Haemoglobin	Hb
Adult Haemoglobin	HbA
Fetal Haemoglobin	HbF
Reduced Haemoglobin Red Cell Protein	rHbRCP
Pooled Adult Serum	PAS
Haptoglobin	Hp
Immunoglobulin	Ig
Antibody-Antigen Crossed Electrophoresis	AACE
One-Dimensional Antibody-Antigen Crossed Electrophoresis	1D AACE
Phosphate Buffered Saline	PBS
2,3 Diphosphoglycerate	2,3 DPG

INTRODUCTION

Table 1 Some Physical Characteristics of Adult and Fetal Red Blood Cells

	FETUS	ADULT	REFERENCE
Mean Cell Volume	191 μ^3	90 μ^3	Breathnach, 1962
Mean Cell Diameter	8.0 μ	7.5 μ	Moore, 1968
Life Span	70 days	120 days	Pearson, 1967
Membrane Thickness	33% thicker than adult		Barton and Brown, 1964
Membrane Permeability		50% more permeable than fetus	Barton and Brown, 1964
Deformability	Greatly reduced		Gross and Hathaway, 1972
Occurrence of Abnormal Erythrocyte Shapes	Higher incidence of spherocytes, pyknocytes and elliptocytes		Pearson, 1967
Distribution of Membrane Lipids	Higher sphingomyelin content	Higher lecithin content	Crawly and Ways, 1964; Moore, 1968

A. THE RED BLOOD CELL

1. The Comparative Physical Characteristics of Adult and Fetal Red Cells

Red blood cells or erythrocytes are among the most highly adapted in the human body. Their principal function is the transport of oxygen from where it enters the body to the tissues where it is utilised in respiration. Oxygen is reversibly bound to the red cells' major protein: haemoglobin.

The characteristic symmetrical biconcave shape of the human erythrocyte, which permits efficient diffusion, is essentially very stable (Adams, 1973; Reeves and Whitmore, 1977) although flexible enough to deform when required to withstand the rigours of splenic microcirculation (see Gross and Hathaway, 1972). There are many slight differences in the physical characteristics of adult and fetal red cells and some of these are summarised in Table 1.

It appears that fetal erythrocytes are larger, thicker walled, and have certain membrane characteristics which reduce deformability or induce formation of abnormal cell shapes. This results in a shortening of the circulatory life span of red blood cells in the fetus compared with adults.

2. Erythropoiesis

The pattern of red cell production or erythropoiesis alters during human embryonic development. Haemoglobin-containing cells are first produced in the mesenchyme of the yolk sac at about 2 weeks gestation. From 6 weeks gestation, distinct cellular erythropoiesis begins in the liver and spleen (Oski and Naiman, 1972) with the bone marrow becoming the major site of erythropoiesis in the third trimester (Yoffey, 1971).

The blood cells develop as far as the nucleated reticulocyte stage before being released into the blood stream. After approximately 24 hours in the circulation, the nucleus ceases to function and breaks down: the cells are then fully developed erythrocytes. The loss of the nucleus enables the volume of haemoglobin and hence the amount of oxygen transported to be increased. Since approximately 1% of the red cell mass is replaced each day, the normal reticulocyte count is about 1% in adults.

In the fetus however, there is a higher proportion of circulating reticulocytes (reduced to 4-6% at term) because rapidly increasing body growth and blood volume at this time, together with the reduced circulatory life span (see Table 1) 'stresses' red cell production so that more cells are released prematurely (Zipursky, 1965).

Production of red cells involves an ordered sequence of differentiation regulated by erythropoietin, a specific erythroid stimulating hormone (see Paul, 1976; Peschle and Condorelli, 1976). This hormone is largely produced in the kidneys of adults and the liver and spleen in the fetus (Zanjini et al., 1974). Synthesis and rate of secretion is under a feedback control involving the effects of hypoxia on erythroid precursors and is also modified by the availability of iron and certain vitamins like B12 and folic acid (Gordon et al., 1973).

The principal action of erythropoietin is to induce the differentiation of primitive stem cells (Gordon, 1970) although the details of the erythropoietin control mechanism are unclear (see Benz and Forget, 1974; Hunt, 1976). A valuable tool in this field of investigation has been the discovery of a transformed cell line of mouse erythroleukaemic cells, named Friend cells (Friend, 1971). These cells are erythropoietin-responsive and can be induced to commence haemoglobin production, thus providing large quantities of pure cells with synchronised protein synthesis (Kabat et al., 1975; Peterson and McConey, 1976).

Erythropoiesis has also been studied by observing the protein patterns following transplantation of haematopoietic stem cells in mice (Renoricca et al., 1976) or bone marrow in human patients (Alter et al., 1976); where it appears that regenerating bone marrow initially produces a partial fetal-type pattern of protein production, with levels of fetal haemoglobin increasing from 0.5% to 10-20%, high titre fetal i antigen and fetal-type enzyme levels. However, all of these characteristics had declined by 200 days from the bone marrow transplant. Erythropoietin mediated control of erythropoiesis is operative in both the neonate and fetus (Kazazian, 1974); with maximum responsiveness (as indicated from the amount of haemoglobin produced by stimulated fetal liver and spleen cells in culture) during 14-18 weeks gestation (Basch, 1972). Erythropoietin is responsive

Table 2 Metabolic Characteristics of Neonatal Erythrocytes Compared with Age-Matched Adult Cells

(Based on Oski and Komazawa, 1975)

Glycolytic Enzymes	
Increased activity of	phosphoglucose isomerase
	glyceraldehyde-3-phosphate dehydrogenase
	phosphoglycerate kinase
	enolase
Decreased activity of	phosphofructokinase
Non-Glycolytic Enzymes	
Decreased levels of carbonic anhydrase isoenzymes	
Decreased activity of	NADP-dependent methemoglobin reductase
	catalase
	adenylate kinase
	glutathione synthetase and peroxidase
	methemoglobin diaphorase
Phosphate Metabolism	
Decreased inorganic phosphate uptake	
Slower incorporation into ATP and 2,3 DPG	
Increased instability of 2,3 diphosphoglycerate	
Isoenzyme Distribution	
Fetal:	Hexokinase I and II, Enolase I predominates, Aldolase A and C
Adult:	Hexokinase I and III, Enolase III predominates, Aldolase A, B and C
Other	
Increased methemoglobin content and tendency to form Heinz bodies	
Increased Glutathione instability	
More rapid assumption of altered morphological forms on storage or incubation	
Increased potassium efflux and greater degree of hemolysis on storage	

to changes in tissue oxygenation, and in the relatively hypoxic environment of intrauterine life, erythropoietin levels are high and erythropoiesis is active. However, when the arterial oxygen saturation rises at birth, erythropoietin activity drops and is barely detectable after the first week of extra-uterine life (O'Brien and Pearson, 1971). Erythropoiesis recommences at 2-3 months of age, after the haemoglobin concentration has fallen from approximately 300 mg/ml to 120 mg/ml (Stockman, 1975).

3. The Function of Adult and Fetal Red Cells

a) General Metabolism

Although loss of the nucleus makes the red cell a more perfect vessel for oxygen transport, it does impose on the cell a finite life span, for it cannot replace or repair its vital enzymes as it is unable to synthesise protein. However, the mature enucleated red cell is not metabolically inert and the levels of important metabolic organic phosphates such as 2,3 diphosphoglycerate and adenosine triphosphate are actively maintained within the cell. Energy is also needed to maintain haem iron in the reduced form since methaemoglobin (which contains oxidised haem iron) does not bind oxygen effectively.

While considering a possible role for 'fetal specific' non-haemoglobin proteins in red cells it was discovered that there are a number of differences in general cell metabolism between fetal and adult red cells. Some of these are summarised in Table 2.

The characteristics shown in Table 2 appear to be unique to fetal and neonatal erythrocytes since normal adult cells do not have these properties. Using radioactive labelling to monitor newly released cells, differential ultracentrifugation was used to separate the cells into the bottom 10% (oldest cells) and top 10% (youngest cells) so that adult and fetal red cells were age-matched (Oski and Smith, 1968). Certain parameters (e.g. increased activity of aldolase, glucose-6-phosphate dehydrogenase and hexokinase) were originally thought to be characteristic of fetal cells, but these are now known to be related to red cell

age rather than to inherant differences between fetal and adult cells (Oski and Komazawa, 1975).

Total glucose consumption by fetal erythrocytes is higher than that of normal adult cells but young red cells generally consume more glucose. However, when neonatal red cells are compared with adult cells of a similar age, the rate of glucose consumption appeared to be less than expected (Oski and Smith, 1968). This reduction in glucose consumption is probably due to an impairment in glycolysis at the rate limiting phosphofructokinase or hexokinase steps. Phosphofructokinase activity is lower in fetal than adult red cells and is inhibited by adenosine triphosphate (ATP) at pH 7.2. In vitro incubation of neonatal red cells with higher pH or increased phosphate concentrations which increase phosphofructokinase activity also result in greatly increased glucose consumption (Oski and Travis, 1972). This suggests that the relative deficiency of phosphofructokinase might be responsible for the impairment of glycolysis in fetal cells. Further evidence comes from the observation that glucose consumption is also increased if phosphofructokinase is by-passed by incubating term cord red blood cells in 10^{-6}M methylene blue which causes glucose to be metabolised oxidatively in the pentose phosphate pathway (Oski and Komazawa, 1975).

It is thought that hexokinase, the other rate-limiting enzyme in glycolysis is not directly implicated in the reduced glucose consumption of fetal erythrocytes because the concentration of hexokinase is very similar in adults and neonates and is under a direct feedback control from its metabolic product glucose-6-phosphate (Oski and Komazawa, 1975). Several enzymes in the glycolytic pathway including hexokinase and phosphofructokinase are inhibited by 2,3,diphosphoglycerate (2,3DPG) but it would appear that this inhibition is not responsible for the difference in glucose consumption in adults and neonates as the concentration of 2,3 DPG is the same for both. The rate of 2,3 DPG turnover in the neonate is six times greater than in adults (Schroter and Winter, 1967). This is because 2,3DPG is less firmly bound to fetal than adult haemoglobin due to amino acid substitutions in the binding region (White, 1972), and because of higher 2,3DPG enzymatic breakdown in the fetal red blood cell (Oski, 1974). The reason for the increased breakdown is not clear but it has

been suggested that a possible cause of this is a unique fetal 2,3DPG phosphatase with increased affinity for its substrate (Oski and Komazawa, 1975).

Apart from fetal haemoglobin, the extent and importance of 'fetal type' proteins in the initiation and maintenance of patterns of metabolism in fetal red blood cells is unclear. It is known, however, that several enzymes in the red cell, including hexokinase, enolase and aldolase exist as isoenzymes with particular forms occurring either specifically or in higher concentration in the fetus. The fetal hexokinase isoenzyme, for example, has kinetic differences which affects the rate of feedback inhibition, and therefore may contribute to the altered pattern of glycolysis in the fetus (Oski and Smith, 1968).

Fetal erythrocytes have higher adenosine triphosphate (ATP) levels and greater ATP instability than normal adult erythrocytes (Oski and Naiman, 1965). They also have significantly less ouabaine sensitive membrane ATPase and thus less of the Na^+/K^+ activated membrane transport system than adult red cells with a comparable reticulocyte count (Whaun and Oski, 1969). This reduced capacity for active transport may make fetal erythrocytes more vulnerable to membrane damage or malfunction (Blum and Oski, 1969). Fetal erythrocytes are also characteristically susceptible to biochemical oxidation. They tend to more readily form methaemoglobin with oxidised haem, probably due to a level of methaemoglobin diaphorase, the concentration of which is only 60% of normal adult values (Ross, 1963) until two months after birth (Ross and Ciccarelli, 1962).

Fetal erythrocytes have a greater tendency than adult cells to accumulate hydrogen peroxide (Bracci et al., 1970) which is the major cause of the oxidation damage to haemoglobin resulting in the formation of Heinz bodies. Although fetal haemoglobin is more prone to denaturation, there is apparently no relationship between the fetal haemoglobin content of a cell and its tendency to form Heinz bodies (Vlukutlu et al., 1966). Detoxification of hydrogen peroxide is dependent on glutathione peroxidase, glutathione reductose and glucose-6-phosphate dehydrogenase (Bienzle et al., 1976), and the activity of these enzymes is lower in fetal than adult cells (Whaun and Oski, 1970) yet there does not appear to be a relationship between these relative deficiencies and the

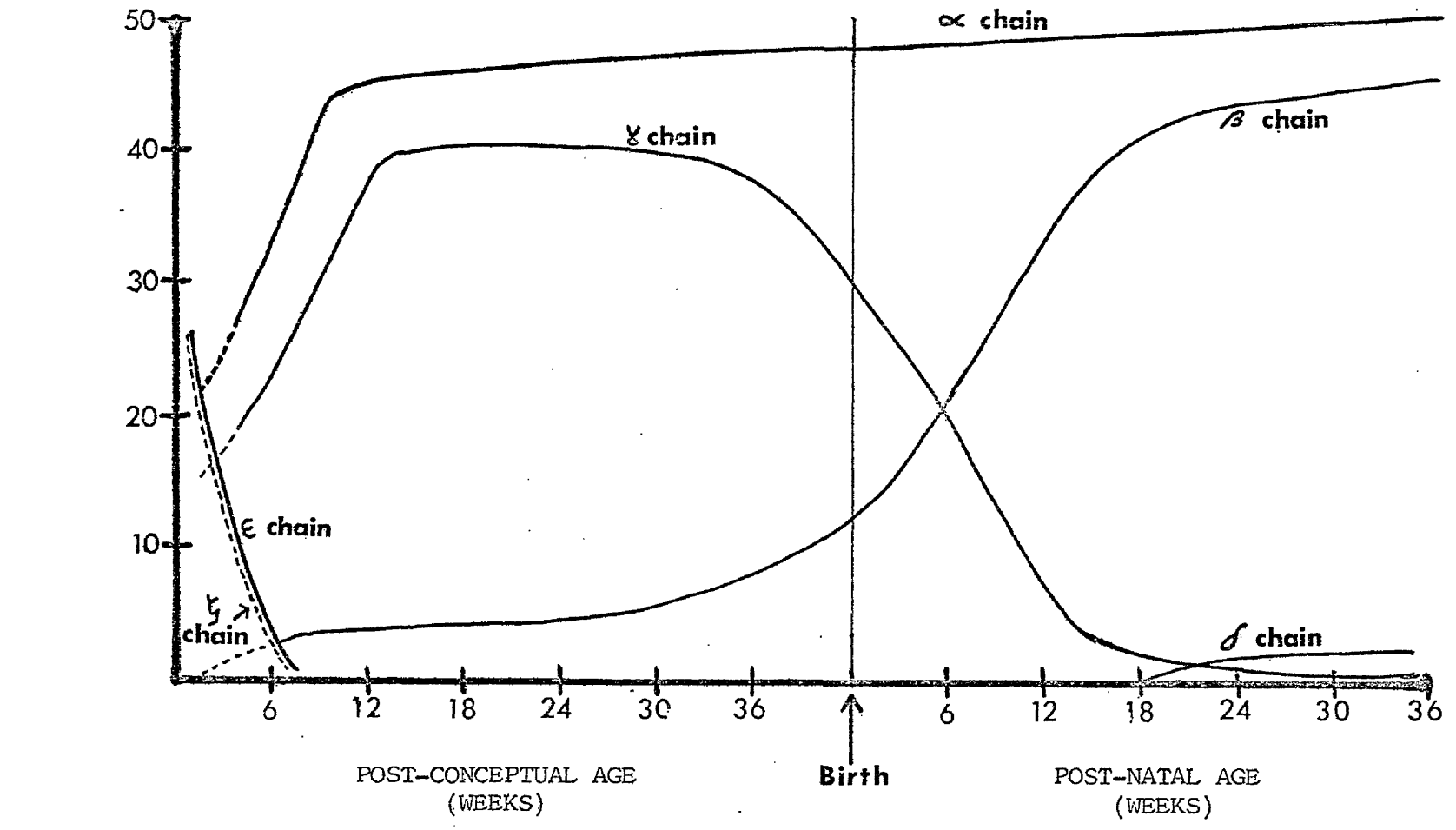
Table 3 Haemoglobin Chains During Normal Fetal Development

Hb Gower 1	ε_4 or $\zeta_2\ \varepsilon_2$	Huehns et al., 1961, 1964 Hecht et al., 1966 Huehns and Farooqui, 1975
Hb Gower 2	$\alpha_2\ \varepsilon_2$	Huehns et al., 1964
Hb Portland 1	$\zeta_2\ \gamma_2$	Kaltsoya et al., 1966 Capp et al., 1967
Fetal Haemoglobin	HbF $\alpha_2\ \gamma_2$	Körker, 1866
Adult Haemoglobin	HbA $\alpha_2\ \beta_2$ $HbA_2\ \alpha_2\ \delta_2$	Kunkel and Wallenius, 1955 Muller and Jonxis, 1960

γ chain may have glycine or alanine in position 136 of the polypeptide chain (Schroeder et al., 1968). In normal infants and fetuses, the ratio of $^G\gamma$ to $^A\gamma$ is 3:1 (Nute et al., 1973) whereas in adults this ratio changes to 2:3 (Schroeder et al., 1970).

Fig. 1 Haemoglobin Chain Production in Fetal and Neonatal Life

(After Wood, 1976)



oxidant vulnerability (Bienzle et al., 1976). There is also no apparent relationship between the low levels (one-tenth adult values) of fetal carbonic anhydrase isoenzymes B and C and the Respiratory Stress Syndrome in neonates (Mondrup and Anker, 1975). Carbonic anhydrase catalyses the reversible hydration of carbon dioxide in blood cells and it was felt that the reduced levels of these isoenzymes might aggravate the carbon dioxide retention and acidosis of the Respiratory Stress Syndrome.

It would therefore appear that while being essentially similar to adult cells there are certain anomalies in fetal red cell metabolism; most of which are attributable to 'fetal type' enzyme activities. Since the contribution made by 'fetal type' proteins has not been fully assessed, it is possible that the immunological characterisation of such proteins might provide further information. This might be gained through the direct monitoring of fetal proteins during gestation, or by investigating the effect of specific antibodies to isolated 'fetal' enzymes in the assays of a particular metabolic pathway.

b) Haemoglobin

The mammalian haemoglobin molecule is a tetramer of four monomeric peptide chains, normally two α and two non-α chains. The function of the molecule - the reversible carriage of oxygen - depends upon its four haem groups, one attached to each of the four globin chains. Pairs of chains interact and undergo conformational changes during oxygenation and deoxygenation with the result that any variation in chains (i.e. 4 non-α chains instead of 2 α and 2 non-α) or in the amino acids in the regions of contact between the chains results in decreased efficiency of oxygen transport.

The sequence of haemoglobin chain formation during embryonic and fetal life is summarised in Table 3 and Fig. 1, and has been the subject of several recent reviews: Cooper and Hoagland (1972), Weatherall et al. (1974), Weatherall (1976). The timing of different globin chain production in the embryo, fetus and neonate coincides with changes in erythropoietic site: from yolk sac to liver to bone marrow; and suggests a close relation between the two events (Lucarelli et al., 1968).
It appears, however, that although haemoglobin synthesis may be site restricted in the embryonic to fetal change-over, it is not

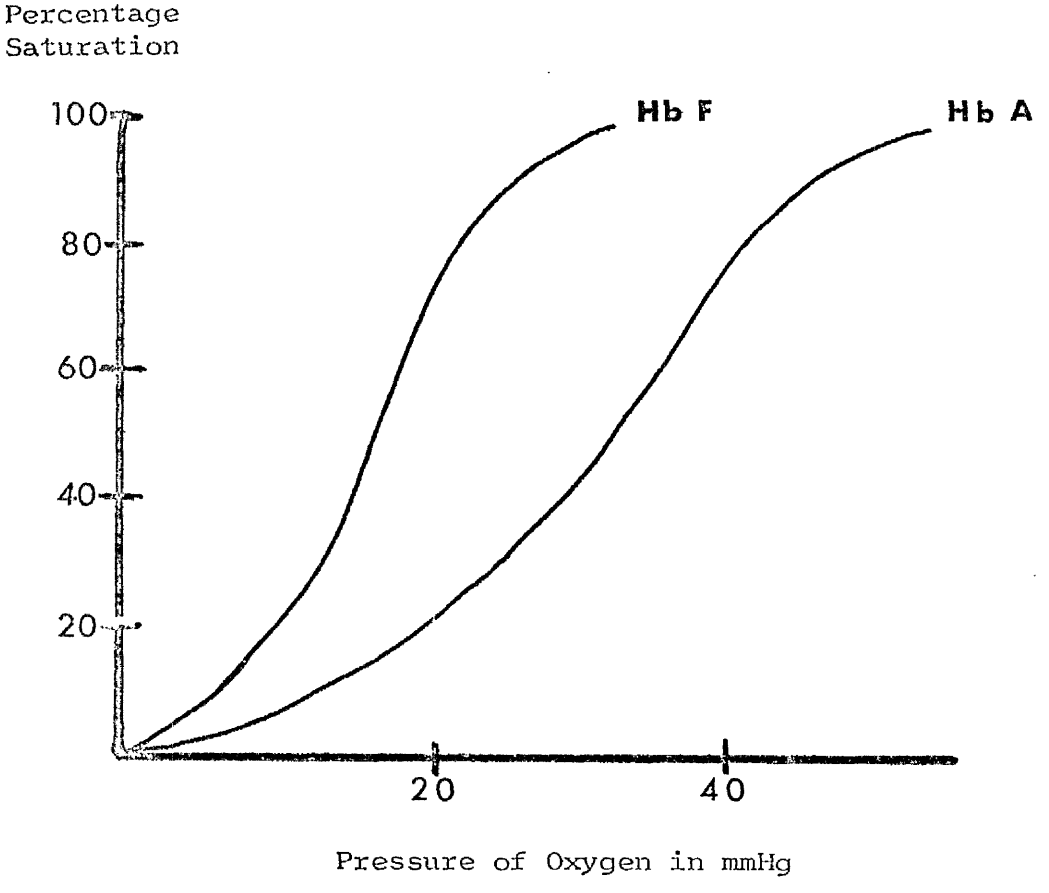
in the fetal to adult switch (Kazazian and Woodhead, 1973; Kazazian, 1974). The first demonstration that HbF and HbA synthesis was not restricted by the site of erythropoiesis was achieved by Thomas et al. (1960) who showed that liver erythroid cells from 9 - 17 week old fetuses synthesised both haemoglobins. This was confirmed and expanded by Wood and Weatherall (1973) who showed that in a series of fetuses of 13 - 34 weeks gestation there was no difference in the relative proportions of HbF and HbA synthesised by erythroid cells of liver, spleen or bone marrow origin, at any stage of development. Studies of single erythrocytes from term cord blood using differential acid elution (Kleihauer et al., 1957; Zipursky and Israels, 1960) and fluorescent antibody technique (Dan and Hagiwara, 1967) have demonstrated that HbF and HbA may be synthesised within the same cell.

Whilst the timing and rate of change over from HbF and HbA is fairly well described, knowledge of the mechanisms controlling the switch is more limited. This basically stems from the lack of human experimental material during the switching period. Human fetal liver, maintained in tissue culture is being examined as a model system for studying the factors involved in the switch from fetal to adult haemoglobin production (Shichory and Weatherall, 1975).

The continuation of fetal haemoglobin production into adulthood in Hereditary Persistence of Fetal Haemoglobin (HPFH) is due to deletions in various parts of the gene cluster which would otherwise lead in some way to the suppression of γ chain expression (see Weatherall and Clegg, 1972, 1976; Weatherall et al., 1976).

An increase in fetal haemoglobins also occurs in the Thalassaemias, which are a group of diseases caused by the deletion of one of the four major genes governing globin chain synthesis in adults (Weatherall and Clegg, 1972). In α Thalassaemia there is limited (heterozygotes) or no (homozygotes) α chain production and Hb Barts (γ_4) or HbH (β_4) are mainly produced. This is always fatal in the homozygous state since four similar haemoglobin chains are extremely inefficient at oxygen transportation. In β Thalassaemia there is limited (heterozygotes) or no (homozygotes) β chain production which results in increased

Fig. 2 Typical Oxygen Dissociation Curves of Fetal and Adult Haemoglobin



levels of HbA_2 ($\alpha_2\delta_2$) and HbF ($\alpha_2\gamma_2$) to compensate.

The Thalassaemia and Hereditary Persistance of Fetal Haemoglobin syndromes have a number of variants which appear when specific mutations of DNA in the vicinity of globin genes occurs. These mutations may alter the gene sequences directly, or affect the subsequent translation into globin chains. This is therefore a useful system in which to observe gene expression (Weatherall et al., 1975; Weatherall and Clegg, 1976; Wood et al., 1976, 1977). Experimental wheat germ culturing systems using messenger RNA prepared from the reticulocytes of patients with different types of Thalassaemias are now in use (Pritchard et al., 1976).

A major difference between adult and fetal red blood cells is the higher oxygen affinity of fetal haemoglobin. Because of certain amino acid changes, 2,3diphosphoglycerate (2,3DPG) is less firmly bound to fetal haemoglobin (Tyuma and Shimizu, 1969). 2,3DPG interacts with hydrogen ions from amino acid groups in the region of association between α - and non-α chains, and reduces the oxygen affinity of haemoglobin (Benesch and Benesch, 1967). Without the presence of 2,3DPG haemoglobin would have such a high oxygen affinity that, once bound, it would be unable to unload oxygen under normal physiological conditions (Benesch et al., 1968). In adult red cells, the concentration of 2,3DPG is such that oxygen is readily released (Benesch et al., 1971); however, the 'unstable' binding of 2,3DPG in fetal cells increases the oxygen affinity of fetal haemoglobin producing the classical shift in the oxygen dissociation curve seen in Fig. 2. Embryonic haemoglobins have a very similar pattern of oxygen dissociation to fetal haemoglobin. Since this would require the co-operation of pairs of unlike globin chains, the molecular formula of Hb Gower 1 is now thought to be $\zeta_2\varepsilon_2$ rather than ε_4 as originally thought (Huehns and Farooqui, 1975). The relative advantages of having a high oxygen affinity haemoglobin in fetal life have been summarised in several reviews including Huehns and Beaven (1971) and Weatherall et al. (1974) but it essentially means that fetal blood can achieve a fairly high level of oxygen saturation at the relatively low partial pressures of oxygen found in utero.

4. The Red Blood Cell as a Typical Tissue Cell

There is an enormous number of differentiated cell types within the human body, all varying in structure and organelle content according to their function. Numerous cell types are very highly specialised and have greatly modified structures. A sperm cell is almost all nucleus, Golgi material and mitochondria with virtually no cytoplasm; whereas an erythrocyte is the converse, with none of these organelles, but an enlarged haemoglobin-laden granulofibrillar stroma (Lewis et al., 1968; Weinstein, 1974). Mature erythrocytes also lack an endoplasmic reticulum and ribosomes indicating the loss of ability to synthesise protein. This loss of organelles is gradual throughout the maturation process from erythroblasts (Bessis, 1961). The specialisation of both sperm and red blood cell imposes a short, finite life span. In a liver cell, however, a high level of diversified metabolic activity continues and these cells may divide to regenerate damaged tissue. A neurone resembles a liver cell in preserving all of its organelles, but differs in having extra specialised cytoplasmic neurofibrils (Grobstein, 1959). It would therefore appear that there is not really a 'typical' tissue cell-type and that an erythrocyte is not unrepresentative of tissue cells because of its specialisation.

An immunological characteristic of tissue cells is that their surfaces are covered by genetically determined antigens. These are 'histocompatibility' antigens, and in effect this means that if cells or tissues bearing one set of antigens are placed inside the body of someone bearing different antigens, the introduced cells will be destroyed by the immune system of the host. This rejection occurs between species and between allogeneic individuals of the same species.

In man, these histocompatibility or HLA antigens form a complex system of multiple alleles at four different loci (Bright and Munro, 1976). An analogous situation exists with the red blood cell which possesses blood group histocompatibility antigens on its surface. The major blood group antigens in the 'ABO' system were discovered by the presence of anti-A and anti-B antibodies in normal human sera. These major blood group substances may also be found on the surface of certain other tissue cells, e.g. kidney, and also in some secretions, e.g. saliva, sweat and gastric juice (Eisen, 1974).

As with the HLA system, red blood cells of one antigen type

are immunologically destroyed when transfused into a dissimilar host. This has particular significance to the fetus in the Rhesus (Rh) antigen system. In practical terms, if a Rh-ve mother carriers a Rh+ve fetus, immunisation occurs as fetal red cells cross the placenta into the maternal circulation, causing the mother to produce antibodies against her child's cells. This becomes more of a problem in consecutive pregnancies and can cause a haemolytic disease of the newborn known as Erythroblastosis fetalis (Walker et al., 1957). Fortunately, this disease is now controlled by the passive administration of anti-Rh gamma globulins which coat any Rh+ve red cells before they become immunogenic (Bowman, 1975).

Most blood group agglutination takes place at body temperature, the Ii group of antigens are an exception to this and agglutination occurs best at $4^{o}C$. The vast majority of adults are I positive, while neonates are i positive. i appears to be a fetal component of the completely developed I in adults (Marsh et al., 1971). The i condition (but no other 'fetal' red cell characteristics) can be induced in normal adults under conditions of marrow stress which cause red cells to be released prematurely. If the stressing factor is removed, newly developing cells are not i positive indicating that this antigen is characteristic of cell age rather than fetal specific (Hillman and Giblett, 1965).

The association of fetal-type protein recurrence in tissue carcinoma is now fairly well established (Alexander, 1972; Maugh, 1974). The search for fetal proteins has consequently become more than just of interest to developmental biologists - the hope that a new fetal protein will be of some diagnostic use in cancer therapy seems very real. In man, the two best known onco-fetal antigens are carcinoembryonic antigen (CEA) of colonic cancer identified by Gold and Freedman (1965); and α-fetoprotein (AFP) described by Abelev (1968) which is associated with hepatoma. These proteins are currently in use as diagnostic 'markers' both for the initial detection of cancer and to follow the regression of the disease following treatment.

In common with other tissues, certain fetal red cell proteins re-occur in malignant diseases of blood cells. In juvenile chronic myeloid leukaemia (Shapira et al., 1972; Maurer et al., 1972) and erythroleukaemia (Weatherall and Walker, 1965; Horton

et al., 1970) there is a general pattern of regression to fetal erythropoiesis which includes elevated levels of fetal haemoglobin, changes to fetal enzyme levels and quantitative antigenic modifications (Sheridan et al., 1976). In the majority of less severe leukaemias only some 'fetal' characteristics re-appear: increased levels of fetal haemoglobin being the commonest symptom. In these cases it has sometimes been possible to localise the abnormality to distinct erythrocyte populations. This is not consistent with a genuine reversion to fetal erythropoiesis and it is possible that a polychromosomal lesion of either quiescent F cells or adult stem cells may be involved (Pagnier et al., 1973, 1977; Dover et al., 1977). A loss or weakening of blood group antigens, such as occurs with fetal cells may also occur in malignant blood disorders, and 'mosaicism' of ABO antigens with two or more distinct erythrocyte populations has been reported (Kahn et al., 1972; Bird et al., 1976). It has also been reported that tumour cells may acquire antigens identical with, or closely resembling certain erythrocyte antigens - and these may be different blood group antigens to those on the host's own erythrocytes (see Bird, 1977).

It appears that the unexpected appearance of one or more fetal characteristics is a well-known but poorly understood feature of many malignant diseases. A precedent has already been set for fetal proteins to be of some diagnostic use in the treatment and monitoring of progress in many cancers of blood and tissue cells. The search for new fetal proteins is continuing in many research centres and it was hoped that this immunological investigation of fetal red blood cells might 'uncover' a novel fetal antigen which might have some use as a 'marker' in the further understanding and detection of blood cell disorders.

B. IMMUNOLOGICAL ASPECTS

1. Immunology of Red Blood Cells

Immunologically, the red blood cell is best known for its major membrane antigens: the blood group substances, and generally, these seem to be weaker on fetal cells (see Race and Sanger, 1975). As far as the internal, or red cell lysate proteins are concerned, immunological interest seems to have centred almost exclusively on haemoglobin.

Initially, adult haemoglobin was thought to be only weakly antigenic and the corresponding antisera non-specific (Chernoff, 1953a). In contrast, fetal haemoglobin was considered to be much more antigenic and distinct immunological differences were demonstrated among fetal haemoglobins from normal infants (McCormick and Walker, 1960). Specific antisera to fetal haemoglobin (HbF) was used to devise a semi-quantitative precipitin test (Chernoff, 1953b) and more recently Mauran-Sendrail et al. (1973) reported an immunological technique for determination of HbF derived from the Ouchterlony immunodiffusion method, which was highly comparable with the standard alkali denaturation of the Singer test.

The major component of adult haemoglobin (HbA) together with common haemoglobin mutants HbS and HbC were originally reported as being non-antigenic (Heller et al., 1962). Cross-reactive and non-specific antisera were later produced, although the α chain was still considered to be non-antigenic (Yakulis and Heller, 1964). Many authors (Boerma and Huisman, 1964; Schneider and Arat, 1964; Kraus and Sassano, 1965; Maggioni et al., 1966) have since found that haemoglobins A, A_2, F, and Barts are all immunologically heterogeneous in the rabbit, all sharing some antigenic groups and each having some that are distinctive. The data also suggest that the shared groups are on the β, γ or δ polypeptide chains and that these chains are the most reactive immunogenic sites of haemoglobin, although the α chain is immunogenic. Kimura and Suzuki (1969a and b) concluded that a more extensive immunisation schedule is required for the production of anti-α chain antisera. Guinea pig antisera to α and β chains shows that the α chain has antigenic determinants of its own, as well as those shared with other chains (Ovary, 1964).

The adsorption studies of Boerma and Huisman (1964) revealed that antisera to Hb Barts consisted of two types of antibodies: one reacting with HbF ($\alpha_2 \gamma_2$) and Hb Barts (γ_4) and the other reacting with Hb Barts specifically. Specific antibodies for the haemoglobin mutants HbS and HbC were also demonstrated, indicating that the replacement of just one amino acid by another (as is the case with these mutants) can be sufficient to convey specific antigenicity to a haemoglobin mutant, provided a pure sample is used as an immunogen. Antisera sufficiently specific for diagnostic radioimmunoassay have now been produced to a large number of haemoglobin mutants with single amino acid substitutions (Reichlin, 1972; Garver et al., 1977).

Electrophoretically isolated minor fraction of adult haemoglobin (HbA_2) and an unidentified non-haemoglobin component were shown to have specific antigenicity in rabbits (Heller et al., 1962) while another non-haemoglobin protein and an enzyme protein with catalase activity were detected as antigenic components of whole haemolysates. Specific antibodies to red cell carbonic anhydrase B have also been produced by immunisation of rabbits with purified enzyme (Gitlin et al., 1968).

The haemoglobins from many different mammalian species are cross-reactive to varying extents when tested with anti-human haemoglobin antisera (Rachmilewitz et al., 1963; Reichlin et al., 1963). Since the production of antisera depends on an immunological awareness of a 'non-self' determinant, it is perhaps not surprising that the introduction of a protein with great similarity to the hosts produces little response. Human α-chains have a very similar amino acid sequence to those of rabbit haemoglobin chains. β, γ and δ chains are less like the rabbits chains and consequently are more immunogenic in that animal (Reichlin, 1972). Antibodies with specificities for the human α chain can be more readily produced in guinea-pigs because there are greater differences between the human and guinea-pig.

Reichlin (1970) has attempted to correlate the immunogenicity and distribution of rabbit antibody specificity for haemoglobin chains with the similarity between rabbit and haemoglobin chains. The number of radical primary amino acid sequence differences (i.e. those involving changes in charge, bulk or chemical nature of the residues) between human and rabbit

α chains are 7, between human and rabbit β chains are 11, and the number of differences between human γ chains and rabbit β chains are 21. Thus there is a good correlation between the immunogenicity of human γ chains and the differences between individual homologous polypeptide chains of the two species; and it appears that rabbits do not produce antibodies to portions of the primary sequence of the human haemoglobin molecule which are identical to the primary sequence of the rabbit's own haemoglobin molecule (Reichlin, 1972).

Haemoglobin mutants have been used to localise and enumerate antigenic determinants by studying the comparative reactivity of mutants with one amino acid substituted from the normal sequence and normal adult haemoglobin (HbA) with anti-HbA antisera. The results indicate that the substitutions in the mutants distinguished by anti-HbA serum from HbA were located in regions of the primary sequence where rabbit and human haemoglobin chains have many differences. Mutants with substitutions in regions of identical sequence were not distinguishable (Reichlin, 1972; 1974). The reverse situation was also investigated: examinations were made of the ability of antisera to twelve haemoglobin mutants to distinguish between mutant and normal adult haemoglobin. The results indicate that if the single amino acid substitution was in a region of similarity between rabbit and human haemoglobin chains, antibodies were produced which readily distinguished mutant from normal haemoglobin. Conversely antibodies which demonstrated no such distinction were produced if the substitution was in a region of dissimilar chain composition. Reichlin concluded that, in the first instance, antibodies are being directed against a single highly specific determinant, while in the second, the antibodies are being directed against many determinants on a large variable region. It is for this same reason that anti-mutant haemoglobin antibodies more readily distinguish mutants from normal haemoglobin, than do anti-normal haemoglobin antibodies, which only recognise broad differences between rabbit and human haemoglobins while overlooking more subtle distinctions (Reichlin, 1974).

It appears that red cell internal proteins are not very immunogenic and that injection of single, purified antigens has been necessary to elicit an antibody response to minor red cell proteins. In planning the immunological investigation of fetal red cell lysate proteins, it is therefore envisaged that a very

limited antibody response to the immunogen might occur but that this challenge might be met by partial purification of interesting antigens or 'blocking' of the response to unwanted antigens.

2. Methods for the Identification of Specific Immunological Reactions with Particular Reference to Tissue Proteins

Antibodies and antigens work on a 'lock and key' principle with the complex shape of an antigenic determinant fitting the corresponding shape in the antibody combining region and being held there by various forces including coulombic, Van der Waals, hydrogen and hydrophobic bonding (Roitt, 1977). There are basically two groups of methods for determining that an antibody has bound to an antigen: primary binding tests which simply require that an antibody-antigen complex is formed (whether soluble and insoluble) while tests for secondary interactions require that the single antibody-antigen complex cross-link with other complexes or react with a third component (e.g. complement) to visibly demonstrate that the antibody-antigen reaction has taken place. This may be by precipitation (in solution or in gels), agglutination or lysis of cells. There are also tertiary manifestations of antibody-antigen reactions and these principally involve skin tests for hypersensitivity or anaphylaxis. An excellent review of all these immunological techniques together with their relative advantages and limitations may be found in Weir (1978).

a) Gel Precipitation Reactions

In the preliminary study of tissue antigens, gel precipitation is the most commonly used technique. Essentially, the antigen moves through the antibody-containing gel (either by diffusion or electrophoresis) forming soluble isolated complexes until these reach an 'equivalence point' where neither antigen nor antibody is in excess; the complexes can then form a cross-linked lattice and precipitation occurs (see Ouchterlony and Nilsson, 1978). The requirements and limitations of this precipitation are: 1) the antigen is multivalent, 2) sufficient antibodies are present for the antigen to reach the equivalence zone (and vice versa), 3) having reached this point, there are sufficient complexes to form a lattice that can be stained to show a visible precipitin line, band or arc. Despite these limitations, gel precipitation is the principal technique for screening antisera for multiple antigenic

specificities as are likely to be found in tissue extracts.

b) Haemagglutination

Red blood cells will agglutinate in the presence of antibodies to their membrane antigens (e.g. blood group substances). Alternatively, antigens may be artificially coated onto their surface and the indirect agglutination of the red cells used to indicate the antibody-antigen reaction (see Herbert, 1978).

Direct agglutination of red blood cells has been used in the study of antibodies to transformed tissue culture cell lines (Kornstad and Rose, 1962) and brain tissue (Golub, 1973). In both cases the antisera showed extensive cross-reactivity and agglutinated erythrocytes from several species of animal. Bjorklund and Paulsson (1962) used the tanned cell technique to assay normal and malignant tissue extracts with antisera raised against the insoluble residue from transformed HeLa cells. Haemagglutination and inhibition of agglutination with different tissue extracts was performed and demonstrated that the anti-HeLa antibodies agglutinated cells coated with extracts from cancerous tissue significantly more frequently and with a higher titre than cells coated with extracts from comparable normal tissues (Bjorklund, 1969). The significance of this kind of assay for tissue antigens is difficult to assess, due to the lack of information about the number and nature of antigens involved. This is a typical problem when dealing with an unknown mixture of antigens in a tissue extract. Inhibition tests are really only applicable to single highly purified antigens.

c) Cytolytic Tests

Antibody-antigen complexes may activate a complex cascade reaction involving nine protein components (C_1-C_9) which result in complement formation. Antibodies linked to antigens in the presence of complement can produce cell lysis and if the antigen were coated onto a red blood cell, lysis would produce a pool of indicator pigmentation-haemoglobin. This complement fixation is the basis of the plaque assay which uses the lysis of sheep red blood cells to indicate specific antibody production by spleen cells (see Dresser, 1978). The localised haemolysis in gels (LHG) technique as originally described by Jerne et al. (1963) detected only those cells which produced antibody capable of

fixing complement and lysing erythrocytes directly. With certain minor exceptions this antibody is IgM; however the majority of humoral antibodies belong to other classes (IgG; IgA) which are less efficient at fixing complement by themselves but the addition of antiglobulin antibody enhances the reaction (Dresser and Wortis, 1965).

It has been observed that sera from patients with various diseases will fix complement with extracts of normal human tissues (Gajdusek, 1957). Beall (1963) fractionated extracts of normal liver to investigate the significance of this phenomenon. He found that antigenic activity was found in fractions containing gamma globulins and suggested that the complement fixation observed might be due to aggregation of the normal tissue protein with IgG rather than an autoimmune antibody-antigen reaction. Thorpe et al. (1977) reported that many normal human sera were found to be capable of causing complement-mediated lysis of normal human skin cells grown in tissue culture. This lytic activity could be removed by adsorption with first trimester fetal tissue whereas adsorption with a variety of normal adult tissues could not prevent lysis. This apparently indicates that normal human cells in tissue culture express 'neoantigens' present in fetal tissues but not normal adult tissues.

d) Methods for the Localisation of Tissue Antigens

The technique of immunofluorescence is particularly suitable for the examination of antigens within tissue sections or cultured cells. Test antibodies may be conjugated directly to the fluorochrome or an intermediate antiglobulin/fluorochrome bridge may be used. Inhibition of fluorescence with a single purified antigen is the best test of specific activity and this localisation method is frequently used to extend the results obtained from the examination of tissue antigens by gel precipitation techniques. Many examples of the application of this method can be found in Weir (1978).

Immunofluorescence has a higher resolution than the peroxidase method, but the latter is permanent and counterstains may be used to identify specific antigens, e.g. lactate dehydrogenase in muscle (see Johnson et al., 1978). Ferritin conjugated antibody has also been used for ultrastructural localisation of antigens; its distribution can readily be seen

from its density when observed under an electron microscope. This method has been used for specialised ultrastructural localisation of tissue antigens (e.g. Andres et al., 1962; Vogt et al, 1968; Kraehenbuhl and Jamieson, 1974) and was responsible for the localisation of blood group antigens on erythrocyte membranes (Howe and Lee, 1969; Howe et al., 1970; Nicolson et al., 1971).

(2) Tissue Antigens and the Removal or Prevention of Antibodies with 'Unwanted' Specificities

The study of tissue antigens involves several levels of specificity. Many antigens occur in tissues which are specific for a single species. Conversely, some antigens are specific to a single organ, but also occur in many different species. Generally, the farther apart two animals are in evolutionary terms, the fewer tissue antigens will be shared (Weir, 1973).

A further complication in the identification of tissue antibody-antigen reactions is that tissue cells share antigenic components with serum proteins and these are constituent parts of the cell and not contaminants. These serum protein components are also present in human tissue cell cultures grown for many generations free of human serum (Kornstad and Rose, 1962; Bonstein and Rose, 1971) and are generally potent immunogens whereas antigens found only inside tissue cells often have limited immunogenicity (Weir, 1973). Adsorption studies seem to have resolved tissue species-specific antigens into three groups: a) serum cross-reactive; b) red cell cross-reactive; c) residual specific tissue antigens (Kite et al., 1967; Silberman et al., 1967; Shulman and Wypych, 1969; Golub, 1973).

An alternative to removing the unwanted antibody specificities by adsorption is to try and prevent them from occurring in the first instance. One solution is to immunise with highly purified antigen. This is frequently impractical, particularly when using immunological methods to try to detect novel antigens. Another possibility is to first tolerise the animal to be immunised with the 'unwanted' antigens. This may be done by injecting 'unwanted' antigens while the host animal's immune system is underdeveloped: neonatal tolerance (DeCarvalho and Rand, 1963) or impaired by physical : X radiation (Nachtigal and Feldman, 1963) or chemical means: 6-mercaptopurine (LaPlane et al., 1962);

The 'poor immunogenicity' of tissue antigens may be due to quantitative differences since some tissue components are immunogenic when purified and injected at a high enough concentration. The structural characteristics and similarity of antigens to the immunised animal's own accessible components are also relevant and this whole question is considered in more detail in Section A of the Discussion, pages 58 - 63.

cyclophosphamide (Zan Bar et al., 1975). X-irradiation and anti-mitotic drugs like mercaptopurine act by destroying rapidly dividing cells like activated lymphocytes.

C. CONCLUSION

The immunological investigation of intracellular tissue proteins presents a number of interesting problems: how to raise antibodies against antigens with limited immunogenicity, how to detect a specific response and what that response indicates about the number, nature and location of the antigens involved.

Red blood cells may be used as a model for tissue proteins in this context. They have the advantages of being commonly available and having a high concentration of soluble intracellular protein that can be very simply extracted.

The specialised in utero environment has resulted in structural and metabolic differences characteristic of fetal red blood cells. The factors regulating these properties are not wholly understood. For example, little is known about the possible regulatory role of non-haemoglobin red cell proteins in the maintenance of fetal characteristics or the control of fetal to adult gene switches. Since very little immunological work has been done on red cell intracellular proteins besides haemoglobin, it is possible that an immunological investigation of red cell proteins might produce some data on developmental markers which would help elucidate these problems. Fetal specific proteins are also of interest because of their association with malignant disease. Fetal haemoglobin has been associated with the occurrence of many types of leukaemia and it is possible that variations in other fetal red cell proteins might have some diagnostic value.

Thus, this research project involves the raising of antibodies to the antigens in fetal red cell lysates with two aims:

1) the investigation of red blood cell soluble proteins as a model for tissue proteins generally, and

2) to look for novel 'fetal' proteins in red cells by immunological methods.

MATERIALS AND METHODS

* Erythropoietic production sites and cell-types are changing during this phase of fetal life (see pages 1 - 6 and Fig. 1) and hence different cell populations are present in fetal and adult blood. Any recurrence of 'fetal' proteins in adults might therefore reflect a developmental or cell maturation defect rather than a return to intrinsic fetal proteins.

Chemicals

Analar chemicals were used wherever possible, the principal source being BDH chemical company. Agarose of low electro-endosmotic grade, purified (twice crystallized) rabbit and adult human haemoglobin, bovine serum albumin, tannic acid and Coomassie Brilliant Blue R stain were obtained from Sigma chemical company.

Commercial Antisera

Monospecific rabbit anti-human haptoglobins antisera were obtained from Nordic and Miles chemical companies. Monospecific rabbit antisera to human adult haemoglobin, fetal haemoglobin, α_2 macroglobulin and immunoglobulin G were obtained from Behringwerke (Hoechst chemical company).

Prior to the commencement of this investigation a rabbit antiserum to human α-fetoprotein and a sheep antiserum to adult human serum proteins had been prepared for use in the laboratory.

Serum

Standard human serum was obtained from Behringwerke (Hoechst chemical company). The concentration of various proteins, e.g. albumin and haptoglobin had been measured and these values were included in the literature accompanying each batch of serum.

The pooled adult serum used in this investigation was kindly donated by the Virology Department, Glasgow University; and consisted of serum pooled from approximately one hundred healthy adult donors.

Blood from a volunteer in the laboratory (Miss B. M. Kukulska) with haptoglobin phenotype 1:1 was obtained by venipuncture. The blood was allowed to clot at $4^{o}C$ and serum was pipetted from around the clot as it shrank. This enabled serum without red cell lysis to be obtained.

Red Blood Cells

Samples of fetal blood were obtained from a local hospital during the examination of fetuses which had been aborted either spontaneously or by induction for medical or social reasons. Over 4 - 5 months, blood from fifteen fetuses from 13 weeks to 28 weeks gestation (mode = 18 weeks) was obtained.* Four of these fetuses were apparently normal, six had spina bifida, two were anencephalic, one was hydrocephalic, one had Down's syndrome

(Trisomy 21) and one was a male fetus from a carrier of haemophilia. Because the blood samples were collected some hours after death, the blood was rather coagulated. The samples were centrifuged and the red cells dispersed in phosphate buffered saline (PBS) as soon as possible after collection. After washing and lysing (see below) aliquots of lysate from each individual fetus were retained and the rest pooled. This pool formed the 'standard fetal red cell lysate' used in the investigation.

Blood from the umbilical cord was obtained at the birth of babies in the maternity wing of the same local hospital. This term cord blood was collected into heparinised tubes and stored at 4°C until collected (usually the following day). Samples from twelve - fifteen individuals at a time were collected and pooled. This provided sufficient 'standard term red cell lysate' for several months' work. Three such standard pools were used during the course of the investigation.

Adult blood was collected by venipuncture from a single volunteer in the laboratory for the 'standard adult lysate'. The blood was mixed with five volumes of PBS and washed immediately after collection. Fifty millilitres of blood was collected at a time and this was sufficient to provide a standard adult red cell lysate for several months' work. Three such pools were used during the course of the investigation. Small samples of adult red cells from fifteen individuals with blood group O were kindly donated by Mrs. Betty O'Hare. These were pooled and used for agglutination experiments.

* All standard lysates were adjusted to 100 mg/ml protein.

Washing and Lysing: Each set of red blood cells (fetal, term cord and adult) were washed with five volumes of PBS by gently inverting for one minute and centrifuging down in an MSE bench centrifuge at speed setting 5 for four minutes. The supernatant, buffy coat and top layer of red cells were discarded. The red blood cells were washed in a similar manner a further three times. After the final supernatant had been removed, two volumes of distilled water were added to lyse the cells. The tubes containing the red cells were vigorously whirlmixed for two minutes before spinning down in a Sorvall refrigerated centrifuge (SS 34 head) at 15,000 revs/min for fifteen minutes. 25,000 g *

Table 4 Buffers for Antibody-Antigen Crossed Electrophoresis

	1 x Concentrated Electrophoresis Tank Buffer	2 x Concentrated Gel Buffer
Barbitone	13.8 g	11.07 g
Sodium Barbitone	87.6 g	70.07 g
Calcium Lactate	3.84 g	10.24 g
Thiomersal	2.0 g	1 Litre of 1/1000 Tincture of Merthiolate (Commercial .1% Thiomersal Soln (w/v) from Lilley Ltd.)
	pH 8.6	pH 8.6
	Water to 10 L	Water to 5 L

1 g Agarose autoclaved with 50 ml distilled water

50 ml 2 x concentrated gel buffer added while still molten and thoroughly mixed

Maintained at 50-60^{o}C to keep molten for immediate use

Stain	Destain
2% Coomassie Brilliant Blue Stain (w/v)	50% Methanol (v/v)
50% Methanol (v/v)	7% Acetic Acid (v/v)
7% Acetic Acid (v/v)	

The red cell lysate was then decanted off. The protein concentration was measured by the method of Lowry et al. (1951) (see below) and adjusted to 100 mg/ml with PBS if necessary. Ten millilitre aliquots of lysate were then stored at -20°C until required.

Because the adult red cells were mixed with a large volume of PBS immediately after collection, the cells did not clot and formed a uniform suspension when washed. The term and fetal red cells, however, were partially clotted and tended to clump together when washed. Although larger clots were discarded, the washing of the remaining cells was not as consistently thorough as with the adult cells. The reason for the clotting of the term cord cells was insufficient mixing in the heparinised tubes at the time of collection. The nurses collecting the blood at the births were primarily engaged in other activities and could not always ensure that the tubes were inverted sufficiently. Since the completion of this investigation the hospital changed to using heparinised tubes which contain small glass beads. The beads efficiently mix the blood with the heparin and so the time spent inverting the tubes is now reduced to a minimum, and the cells can now be obtained in uniform suspension.

Immunological Techniques

Antibody-Antigen Crossed Electrophoresis (AACE) was performed in agarose gel with barbitone buffers pH 8.6 according to the methods of Laurell (1965, 1966) and Clarke and Freeman (1968). Table 4 opposite gives details of the buffers and stain used. Ten millilitres of 1% agarose solution in gel buffer was applied to 8.2 cm x 8.2 cm x 0.12 cm glass plates. The first dimension of electrophoresis was at 20 mV/cm and took approximately 1½ - 2½ hours according to the distance of electrophoresis required. After the antibody bed had been poured, the gel was turned through 90° and electrophoresed at right angles to the first dimension at 6 mV/cm 3 mA/plate for 18 hours (overnight). All electrophoresis was at 4°C. For one dimensional AACEs samples were applied at the cathodal side of the antibody bed and immediately electrophoresed at 3 mA/plate overnight. After electrophoresis, the gels were washed in 1/10 PBS in boxes into which air was bubbled. The bubbling motion caused the gels to free themselves from the glass plate and float in the

1/10 PBS. This enabled efficient washing from both sides of the gel to take place. After 24 hours washing and 3 - 4 changes of 1/10 PBS buffer the gels were relocated on their glass plates and dried out to a thin film in a stream of air from a small electric fan. To prevent the gels curling at the edges as they dried, filter paper squares were placed on top of gels after washing. These filter papers became detached when the gels were completely dry. After drying, the gels were stained with Coomassie Brilliant Blue protein stain and destained in acid/alcohol until the peaks were clearly visible.

Antibody solutions could be added to the agarose gel up to a concentration of 20% by volume. Higher concentrations resulted in the gel not setting or collapsing during electrophoresis (due to the water maintaining the gel matrix undergoing electroendosmosis). The addition of the tincture of Merthiolate (containing an orange dye) to the gel buffer enabled the location of albumin to be observed. Albumin binds to the dye forming a pink spot in the gel. Since albumin has almost the fastest mobility of any serum protein, the progress of the first dimension of electrophoresis could be monitored by the progress across the gel of the pink spot. After the first dimension of electrophoresis, the centre of the pink spot was marked in the gel with the end of a pasteur pipette (i.e. a small circle was marked but not removed). This served as a reference point to mark the extent of electrophoresis so that comparison between plates run on different days was simplified.

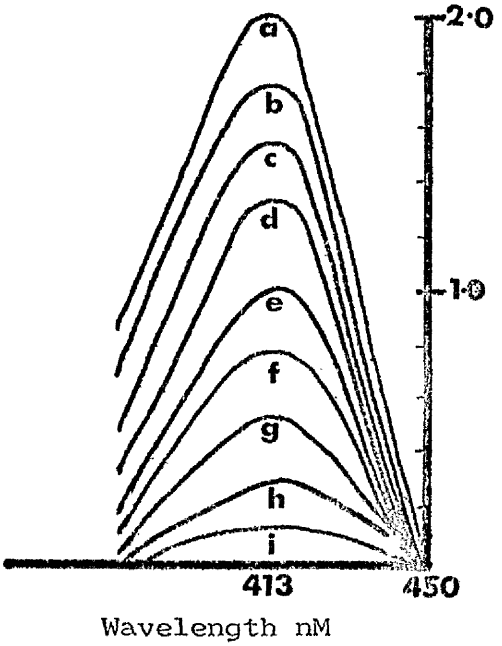
Ouchterlony double immunodiffusion plates were prepared according to Ouchterlony (1958) using the same agarose plates as for AACEs. In this investigation the wells used were 2.0 - 2.5 mm in diameter and the centres of the wells were generally 8 mm apart. The distances used in individual plates varied according to the concentrations of antigens and antibodies employed. The plates were allowed to diffuse for 48 hours in a humidity cabinet before washing and staining as described for AACEs.

Red cell haemagglutination was performed exactly as described by Herbert (1973). After washing, the adult and term

cord red cells were suspended as a 1% solution in PBS containing 1% decomplemented control rabbit serum (to prevent spontaneous agglutination). Using perspex agglutination trays, undiluted rabbit antisera, and antisera in doubling dilutions from 1/10 were made in 100 µl volumes, and 100 µl of the 1% red cell suspension was added. The trays were then covered and left at room temperature overnight before examination and recording the result the following morning.

The formalisation and tanning of sheep red blood cells were also performed exactly as described by Herbert (1973). Twenty five millilitres of washed packed sheep red cells were resuspended in 200 ml PBS in a 500 ml flask. Fifty millilitres commercial formalin (40% formaldehyde from BDH) was pH'd to 5.5 - 6.0 with 1M sodium hydroxide and put into a large dialysis sac so that although air was excluded, the tube was only $\frac{2}{3}$ full. The dialysis sac was then submerged in the red cell suspension and gently agitated at room temperature. After 3 hours the swollen dialysis sac was punctured allowing the formalin to escape, and the gentle mixing was continued overnight. The dark brown cell suspension was then centrifuged and washed several times with PBS. After this procedure the cells pack more closely on centrifugation and are 'sticky' to resuspend. The formalised cells were diluted to a 2.5% suspension in PBS ready for tanning. To 25 ml of the 2.5% formalised cells was added 25 ml freshly prepared 1/10,000 tannic acid solution in PBS. This was agitated gently for two minutes and then spun down in an MSE bench centrifuge speed 6 for 5 minutes. The supernatant was poured off and the cells resuspended in 25 ml PBS 5 ml control cells which were not coated were removed at this point. To 10 ml of the tanned cell suspension was added 10 ml of the protein to be coated at a concentration of 0.1 mg/ml in PBS. The cells were agitated gently for two minutes before spinning down and washing with PBS as before. The tanned cells were finally made up to a 1% solution in PBS (which contained 0.01% sodium azide as a preservative) and stored at $4^{o}C$ until required. The agglutination steps were performed for tanned cells exactly as described for adult and term cord red cells.

Fig. 3a Absorbancy of Commercially Purified Haemoglobin Solutions Measured on a Pye Unicam SP 800 Scanning Spectrophotometer



	Folin Protein Concentration (mg/ml Hb)	Absorbance 413 nM
a	0.47	2.0
b	0.43	1.87
c	0.33	1.48
d	0.29	1.25
e	0.24	1.0
f	0.19	0.76
g	0.13	0.5
h	0.068	0.26
i	0.023	0.04

Fig. 3b Standard Curve of Absorbancy of Haemoglobin Solutions

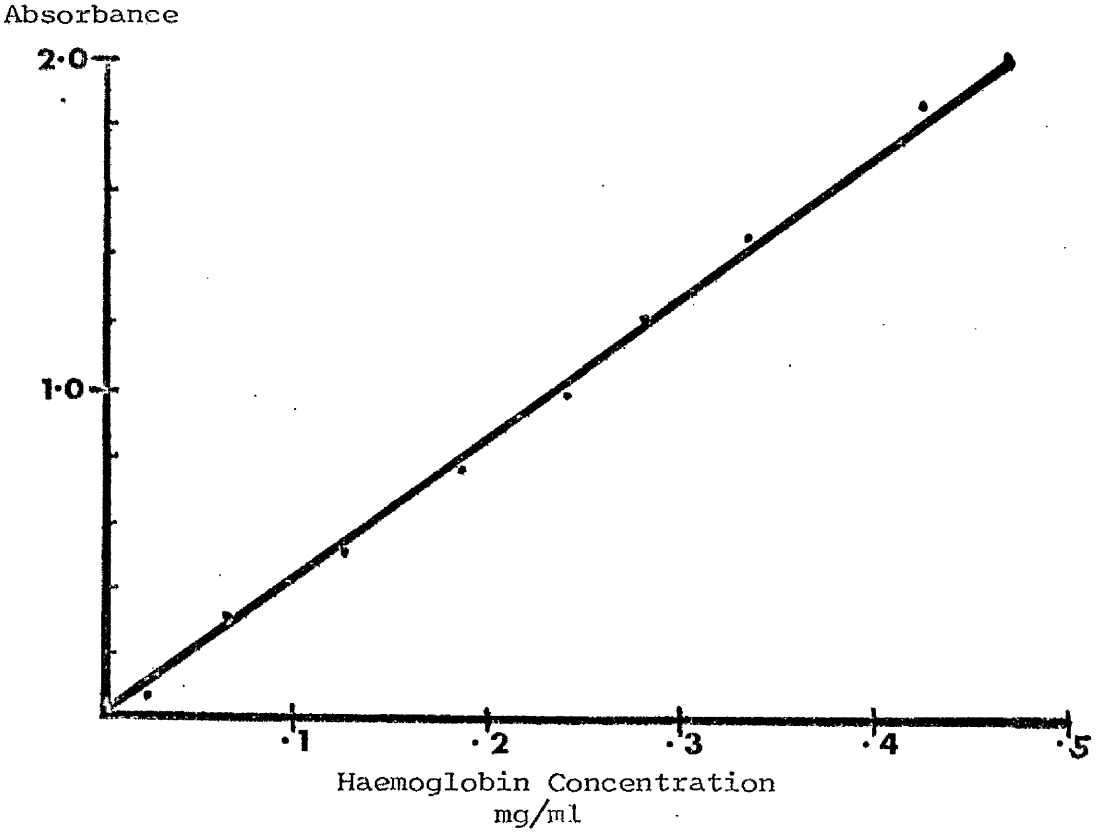
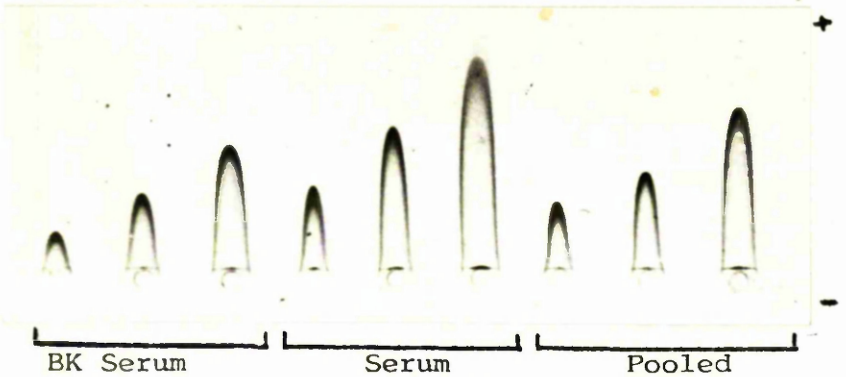
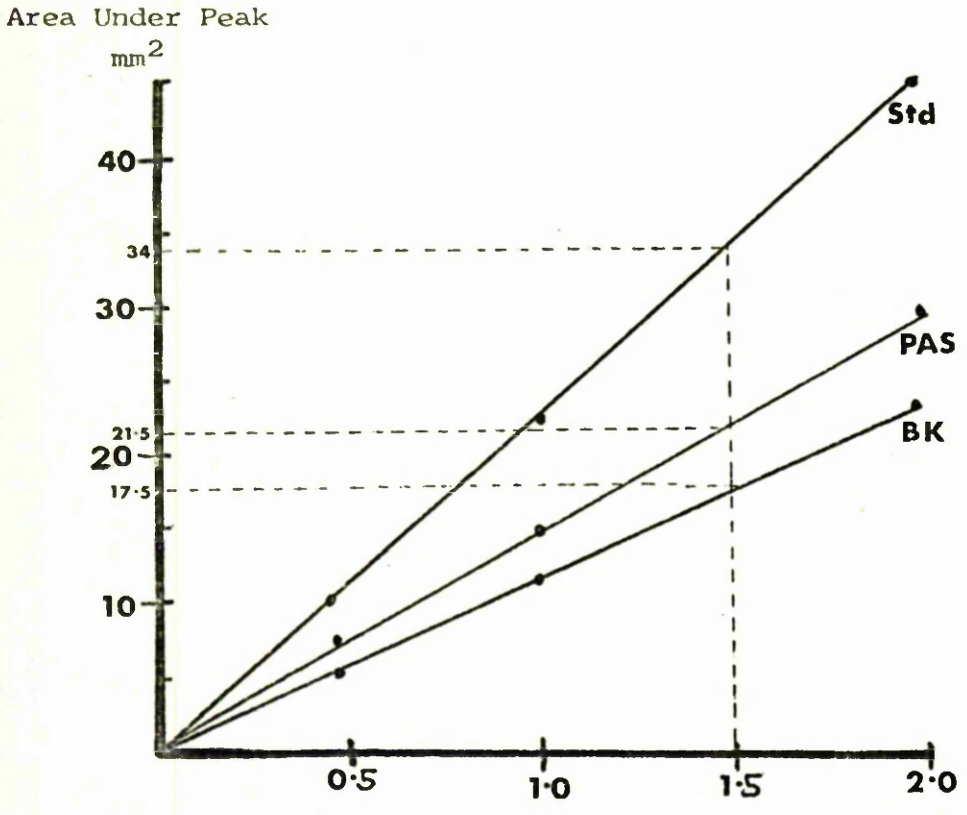


Fig. 4a One Dimensional Antibody-Antigen Crossed Electrophoresis of Serum Samples with Commercial anti-Haptoglobin Antiserum



Sample	BK Serum (Hp 1:1)			Serum Standard (2.08 mg/ml Hp)			Pooled Adult Serum (PAS)		
µl	.5	1	2	.5	1	2	.5	1	2
mm^2 Area Under Peak	5	11	23	10	22	44	7	14	28

Fig. 4b Graph of Haptoglobin Peak Area and µl Serum Applied to AACE Plate



Hp Content of PAS = $2.08 \times \frac{21.5}{34.0} = 1.3$ mg/ml

Hp Content of BK Serum = $2.08 \times \frac{17.5}{34.0} = 1.07$ mg/ml

Quantitation

Protein concentration: Initial estimates of protein concentration were made by optical density readings at 280 nM and 260 nM according to the method of Warburg and Christian (1941) described by Dawson et al. (1969). For more precise measurement, the method of Lowry et al. (1951) utilising Folin-Ciocalteu phenol reagent was used with bovine serum albumin as a standard. A small standard curve of albumin at four different concentrations was produced on each occasion, and the samples were usually duplicated at different protein concentrations.

Haemoglobin: Purified twice crystallized adult human haemoglobin (Hb) was resuspended in PBS at a concentration of 1 mg/ml. A set of dilutions was made and the protein concentration of each measured by the Folin method. While the protein was being measured, the optical density reading of each sample was made at 413 nM on a Unicam SP 800 recording spectrophotometer. Each sample was zero'd against PBS at 450 nM (see Fig. 3a). A standard curve was produced of the peak height at 413 nM against the Folin protein concentration in mg/ml Hb (Fig. 3b). Subsequent haemoglobin concentrations were estimated from diluting the sample until the peak at 413 nM had an adsorbancy of between 0.1 and 2.0 and then reading the haemoglobin concentration equivalent to this peak height from the standard curve.

Haptoglobin, Albumin and α-Fetoprotein: These serum proteins were measured by one dimensional Antibody-Antigen Crossed Electrophoresis (1D AACE) after the 'rocket' method of Laurell (1966, 1972). A series of sample applications (e.g. 2 µl, 4 µl, 6 µl, 8 µl) would be made and the set of peaks produced by suitable antisera on a 1D AACE compared with a set of standard peaks. For haptoglobin and albumin, the standard peaks were produced by Behringwerke adult human standard serum in which these proteins had been measured prior to despatch. The α-fetoprotein standard was a sample of term cord serum in which the α-fetoprotein had been previously quantified in the laboratory.

Fig. 4a shows a typical 1D AACE quantitation plate for

Table 5 Buffers for Polyacrylamide/SDS Gradient Slab Gel Electrophoresis

	Running Gel Buffer	Stacking Gel Buffer
Tris	36.3 g	5.98 g
SDS	0.8 g	0.4 g
	pH to 9.4 with conc. HCl make to 200 ml with H_2O	pH to 6.7 with conc. HCl make to 100 ml with H_2O

Acrylamide Solution

Acrylamide 57.0 g

NN Methylene Bis-Acrylamide 3.0 g

Make up to 100 ml with H_2O

5 x Concentrated Tank Buffers

	Top	Bottom
Tris	31.6 g	60.5 g
SDS	5.0 g	5.0 g
Glycine	20.0 g	
	make to 1 L with H_2O	pH to 8.1 with conc. HCl make to 1 L with H_2O

Boiling Mix

Stacking Gel Buffer	1.0 ml
25% (w/v) SDS	0.8 ml
Mercaptoethanol	0.5 ml
Glycerol	1.0 ml
Bromophenol Blue Soln.	25 ul

Running Gels

	7%	15%
Acrylamide Soln	5.6	12.0
Running Gel Buffer	6.0	6.0
Distilled Water	12.4	3.6
Glycerol	0	2.4
10% (w/v) Ammonium Persulphate	1.50 ul	75 ul
Temed	10 ul	10 ul

Stacking Gel

Acrylamide Soln	2 ml
Stacking Gel Buffer	3 ml
Distilled water	7 ml
10% (w/v) Ammonium Persulphate	200 ul
Temed	10 ul

haptoglobin. The peak heights for the sample and standard are plotted on the same graph and the quantitation measurements made from the gradient of the two lines (see Fig. 4b).

Polyacrylamide Gradient Slab Gel Electrophoresis

Polyacrylamide slab gels (approximately 15 x 20 cm) containing sodium dodecyl sulphate (SDS) at a gradient range of 7 - 15% acrylamide were made according to the method of Marsden et al. (1976). The buffers required are described in Table 5. The samples to be applied were diluted to 200 µg in 50 ul PBS in 1 dram screw top vials. Twenty five microlitres 'Boiling Mix' containing mercaptoethanol and bromophenol blue were added and the vials stood in a boiling water bath for 4 minutes. Five microlitres of the solution was then applied to the sample channels in the stacking gel at the top of the running gel. This resulted in 13.5 µg of the original sample being applied to the gel. On certain occasions, more concentrated samples were required, in which case 25 µl (62.5 µg) of sample were applied. The effect of the stacking gel was to collect the protein into a uniform front as it entered the actual gradient running gel.

Ion Exchange Chromatography

For fractionation of term cord red cell lysate, 250 g CM Sepharose CL 6B (Pharmacea) was equilibriated with four bed volumes of 0.5 M phosphate buffer pH 6.8 on a 30 cm x 2.5 cm column. The pH of the buffer leaving the column was then checked before equilibriation with eight bed volumes of 5 mM phosphate buffer pH 6.8. Three millilitres term lysate at 100 mg/ml (which had been dialysed against 5mM PO_4 pH 6.8) was then applied to the column at 40 ml/hour. The protein which did not bind to the column was collected (in approximately 200 ml), reduced to 10 ml by ultrafiltration over an Amicon PM 10 membrane with an exclusion diameter of 10,000 daltons (this only took 20 - 30 minutes because the protein concentration was so low) and immediately lycphilized prior to storage at $20^{o}C$. The protein bound to the column was eluted off in 10 mM phosphate buffer containing 0.5 M sodium chloride pH 7.2 and the column washed with 0.5 M phosphate buffer pH 6.8 until

* The amount of protein mixed with 1 ml of adjuvant varied with the antigen used for immunisation. Full details are given at the beginning of each antiserum description in the Results section.

the pH of the effluent had returned to pH 6.8. The column was then re-equilibrated with ten bed volumes of 5 mM phosphate buffer pH 6.8. The eluted protein was dialysed against 1/10 PBS before concentration and lycphilization. Haptoglobin was purified on a 25 x 2.5 cm DEAE cellulose column, after the method of Connell and Shaw (1961). 2.5 ml serum with haptoglobin phenotype 1:1 was applied to the column and washed through in 20 bed volumes of 0.01 M Acetate buffer pH 4.7. Haemoglobin was observed passing through the column, and the serum proteins albumin, transferrin and immunoglobin G were detected in the unbound protein by one dimensional AACE. The buffer was then changed to 0.02 M Acetate pH 4.7 and the fractions collected were found to contain haptoglobin as detected with commercial anti-haptoglobin antiserum on one dimensional AACE. Analysis of this protein with multivalent sheep anti-human serum protein by two dimensional AACE showed a major α_2 mobility protein with very little contamination by other serum proteins. No haemoglobin was detected in this material (as determined by optical density readings at 413 nM) and therefore this material was assessed as partially purified 'free' haptoglobin.

Gel Filtration

A 90 cm x 2.5 cm G200 sephadex column was prepared by pre-swelling 10 g sephadex in 600 ml PBS and placing in a boiling water bath for six hours. After cooling, the column was packed at the running speed of 20 ml/hr. After 24 hours a sample of blue dextran (molecular weight 6,000,000) was passed over the column to determine the void volume. Following this, 2 ml serum was applied and 6.5 ml fractions were collected. The column was washed in PBS for a further 24 hours before another sample was applied. The fractions from the column were assayed for the presence of α_2 macroglobulin, immunoglobulin G, haptoglobin and albumin by one dimensional Antibody-Antigen Crossed Electrophoresis, and haemoglobin was assayed by optical density readings at 413 nM.

Production of Antisera

The antigens to be injected were lycphilized and resuspended with vigorous whirlmixing in 1 ml Freunds complete adjuvant.* All injections were given intramuscularly into the haunches of the rabbits. After the preliminary immunisation, the first booster injection was given four weeks later. Subsequent booster injections

were given at four week intervals. All booster injections were given intramuscularly in Freunds complete adjuvant and contained the same amount of protein as the primary injection.

Rabbit bleeding commenced one week after the first booster injection (five weeks from the primary immunisation). Approximately 40 ml blood was taken twice weekly by nicking the outer ear vein with a scalpel blade. The blood was collected in glass beakers and coagulated very quickly. The blood clots were cut into quarters and spun down in a Sorvall refrigerated centrifuge (SS 34 head) at 25,000 g 15,000 revs/min for fifteen minutes. The resultant serum (which was slightly lysed due to the clot being cut) was stored at -20°C until required.

Rabbit antisera were adsorbed as required with pooled adult serum, adult haemoglobin or adult red cell lysate polymerised with gluteraldehyde. Polymerisation was by the method of Avrameas and Ternynck (1969). Haemoglobin and lysate solutions at a concentration of 50 mg/ml were dialysed against 0.1 M phosphate buffer pH 7 prior to polymerisation, but this was found to be unnecessary for pooled adult serum (at approximately 70 mg/ml). For every 5 ml of protein solution, 0.1 ml of a 25% aqueous solution of gluteraldehyde was added dropwise with vigorous stirring. As soon as the gluteraldehyde solution had been added, the stirring was stopped and the protein solution was left to gel (usually took 2 - 5 minutes). It was found that the gel was easier to homogenise into uniformly fine particles if the gel was left overnight at 4°C before homogenising in an MSE blender in PBS. After a brisk homogenisation, the polymer particles were spun gently and briefly either in a bench centrifuge or in the Sorvall centrifuge at 2,000 rev/min (large GSA head). 150 g After decanting the supernatant the polymerised protein was forced through a domestic sieve with a flexible plastic spatula. This had the effect of finely dividing the polymer particles, which were then washed in ten volumes PBS by stirring for two hours, standing for four hours, siphoning off the clear supernatant and washing in ten volumes PBS again. This washing cycle was repeated 3 - 4 times until an even, fine suspension of particles in a totally clear supernatant is achieved. After the final centrifugation, the polymer particles were resuspended in a minimum volume of PBS and stored at 4°C before use.

The adsorption procedure involved decanting a volume of polymer into a screw-topped tube and spinning down to a 'packed'

Table 6 Injection Schedule for Tolerised Rabbits

Days from Birth	mls Adult Serum Injected	mls Adult Red Cell Lysate (1:1 in H_2O) Injected
1	0.3	0.3
2	0.3	0.3
3	0.3	0.3
4	0.3	0.3
7	0.3	0.3
11	0.3	0.3
14	0.3	0.3
20	0.6	0.6
29	0.6	0.6
40	0.6	0.6
54	0.6	0.6
60	0.6	0.6
68	0.6	0.6
80	Bled to determine tolerance	
84	Commence immunisation	
100	Test bleed to determine tolerance after immunisation	
113	First booster injection of immunogen	
120	Commence bleeding for collection of antisera	

volume, in a MSE bench centrifuge and discarding the supernatant. For 5 ml packed polymer 7 ml antiserum would be added, vigorously mixed and inverted overnight on a Matburn cell suspension mixer. The following day, the tubes were vigorously centrifuged and the adsorbed antiserum removed. To test for completeness of adsorption, a two dimensional Antibody-Antigen Crossed Electrophoresis plate was set up with as concentrated a form of antigen and adsorbed antibody as possible (e.g. 10 ul 5 x concentrated pooled adult serum and a 20% antibody bed). If any peaks were still visible, the adsorption step was repeated. In this investigation, a single adsorption step was required on every occasion.

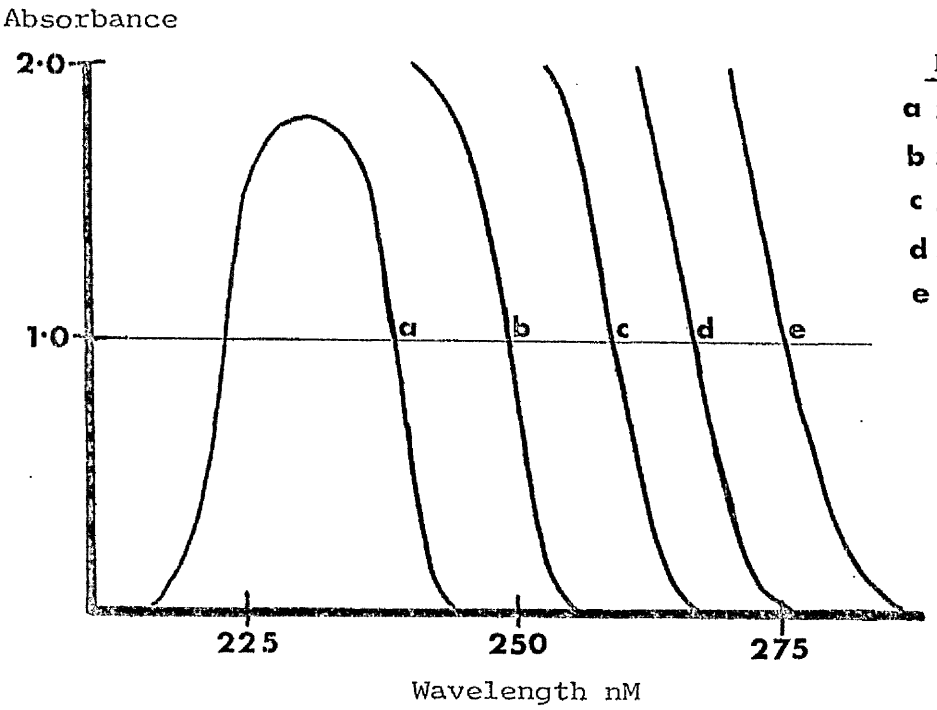
Neonatal Tolerisation: A litter of newborn New Zealand white rabbits were injected subcutaneously with adult serum and red cell lysate according to the schedule in Table 6.

Affinity Chromatography

Antibodies to be immobilised on sepharose were first partially purified (after adsorption with polymerised adult serum if necessary) by precipitation in 45% saturated ammonium sulphate which had been brought to neutral pH with 0.5 M Tris (see Heide and Schwick, 1973).

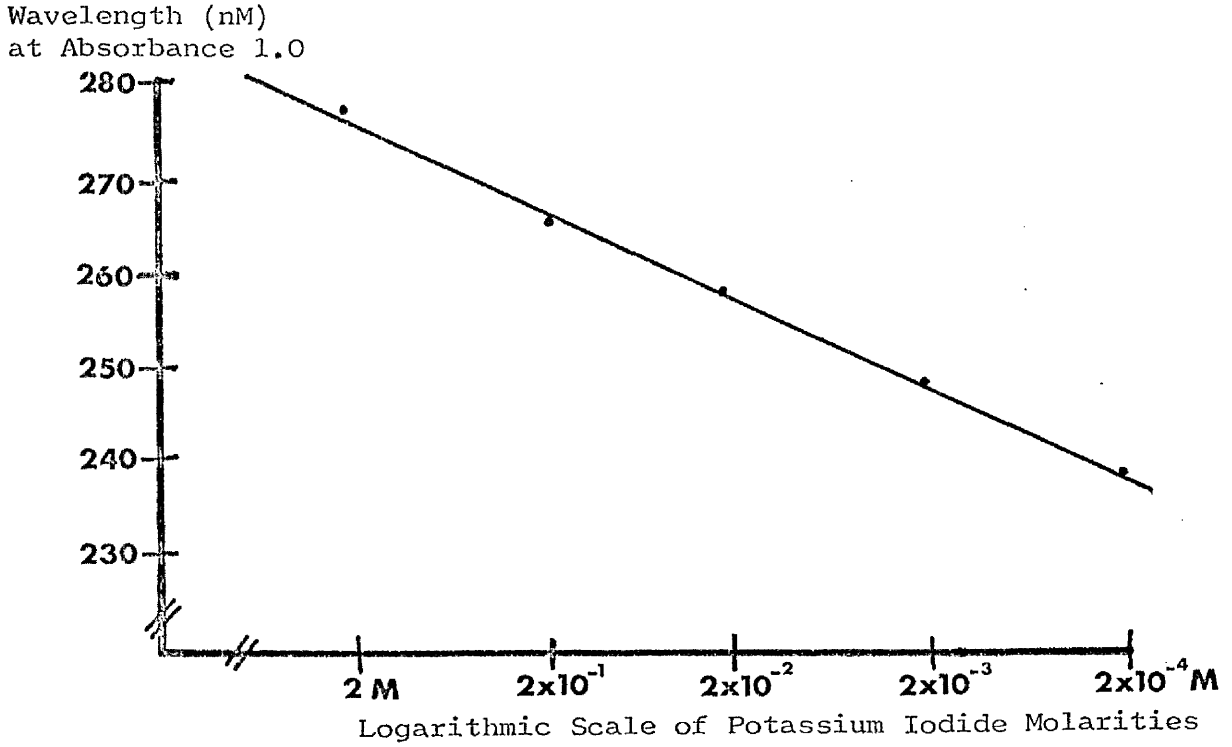
The precipitated immunoglobulins were redissolved and dialysed against 0.1 M sodium bicarbonate. Sepharose 4B (Pharmacea) was activated with cyanogen bromide according to the method described by Porath et al. (1967) and Fuchs and Sela (1973). For every 150 mg of protein to be bound, 10 g drained wet weight of sepharose, 0. 1 g cyanogen bromide and 25 ml water were required. All procedures were carried out in a fume cupboard with a sink, and all equipment was covered with aluminium foil to reduce contamination in the event of an accident. The cyanogen bromide was dissolved in $\frac{2}{3}$ of the reaction volume of water (taking approximately 1 - 2 hours) and then added to the slurry of sepharose containing the remaining water. The slurry was set stirring in the fume cupboard with a pH meter probe in the beaker. Two molar sodium hydroxide was pumped into the beaker to maintain the pH at 11.2 - 11.5 until the pH was maintained without any further addition of sodium hydroxide (approximately 20 - 45 minutes). The activated sepharose was then decanted into a wide diameter column and supernatant pumped off as quickly as possible. All supernatants and washing solutions were pumped into 10 M sodium hydroxide to prevent the formation of cyanide gas which might come

Fig. 5a Absorbancy of Potassium Iodide Solutions Measured on a Pye Unicam SP 800 Scanning Spectrophotometer



	Molarity	Wavelength at Absorbance 1.0
a	2×10^{-4}M	239.5
b	2×10^{-3}M	248.0
c	2×10^{-2}M	258.0
d	2×10^{-1}M	266.5
e	2M	276.5

Fig. 5b Standard Curve of Absorbancy of Potassium Iodide Solutions



from any unreacted cyanogen bromide at low pH. After the supernatant had been removed the activated sepharose was washed with 4 - 5 volumes of distilled water followed by at least 10 volumes of 0.1 M sodium bicarbonate, which terminates the activation procedure. The sepharose was ejected from the column as a 50% slurry in the 0.1 M sodium bicarbonate and the protein to be bound was added to the slurry at a concentration of 10 mg/ml (in 0.1 M sodium bicarbonate). The sepharose and protein were then gently mixed together overnight at room temperature. After this coupling procedure the sepharose slurry was degassed under vacuum and poured into a column. The protein supernatant was drained from the sepharose and the amount of unbound protein estimated from optical density readings at 280 nM and 260 nM. The method of total protein bound to the sepharose by this method was generally 90 - 97%. After any remaining protein had been washed away, the antibody/sepharose column was exposed to fresh 1 M ethanolamine pH 8 for one hour in order to block any remaining reactive groups on the sepharose matrix to which protein had not bound. To remove any non-covalently bound protein, the sepharose was exposed to three washing cycles of 0.1 M acetate buffer 1 M sodium chloride pH 4 and 0.1 M borate buffer 1 M sodium chloride pH 8. After the final washing cycle, several bed volumes of 2 M potassium iodide 50 mM Tris pH 8.6 (which was to be the eluting buffer) were passed through the column before thorough equilibriation with PBS.

The samples were applied to the antibody/sepharose columns at a pump speed of 80 ml/hr which meant that the sample was exposed to the column for about an hour. After washing the sample through with PBS for a further $1\frac{1}{2}$ - $2\frac{1}{2}$ hours, the column was exposed to 2 M potassium iodide 50 mM Tris pH 8.6 for 40 minutes at 160 ml/hr (approximately 100 ml) to elute the protein bound to the column. During re-equilibriation of the column with PBS, the eluted protein was dialysed against 20 volumes of 1/10 PBS (6 changes over the following 2 days) to remove potassium iodide.

To avoid spending too long on the dialysis procedure, a spectrophotometric method of monitoring potassium iodide was devised. Potassium iodide is optically a c tive around 240 - 280 nM, so it was possible to determine when dialysis was complete from scanning the dialysis media after several hours equilibriation and comparing the wavelength at adsorbance 1.0 with a standard curve of dilutions from 2 M potassium iodide to 2×10^{-4} M potassium iodide (Figs. 5a and 5b).

RESULTS

Table 7 Concentration of Haemoglobin and Albumin in Adult, Term and Fetal Red Cell Lysates

Standard Lysate	Total Protein in 1 ml Lysate (mg)	Concentration of Haemoglobin (mg/ml)	Concentration of Albumin (mg/ml)	Concentration of Other Protein (mg/ml)
Adult	100	95	0.024	4.98
Term	100	95	0.31	4.69
Fetal	100	94.5	0.34	5.16

The 'other protein' is the amount of total protein which is not haemoglobin or albumin.

Since the protein concentration was 100 mg/ml in each case, the concentrations of haemoglobin and albumin are also percentages of the total protein present.

Figs. 6a-d AACE of Adult Serum and Adult, Term and Fetal 'Standard' Lysates with Sheep Anti-Human Serum Protein Antiserum

Fig. 6a Pooled Adult Serum

Fig. 6b Adult Lysate

Fig. 6c Term Lysate

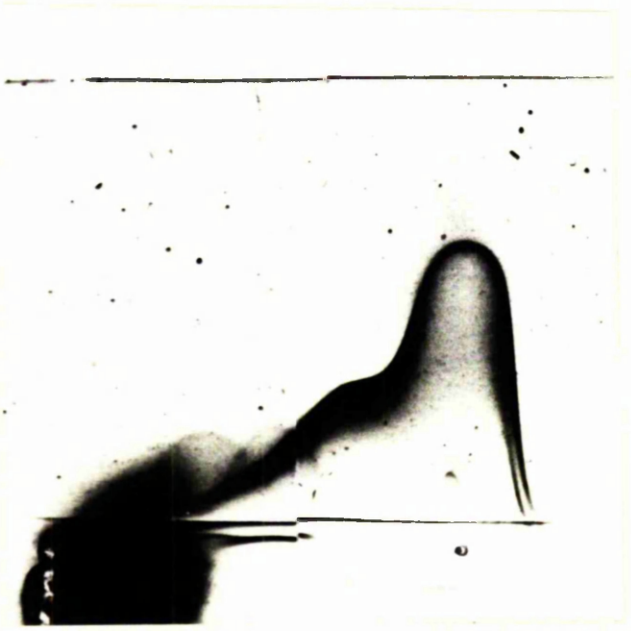


Fig. 6d Fetal Lysate

The major right hand peak has the mobility of serum albumin.

A. COMPOSITION OF RED CELL LYSATES

To quantitate the type of proteins in the lysates, a sample of each of the standard lysates (see page 21) from fetal term and adult red cells was assayed. The total protein concentration was measured by the Folin method, haemoglobin was measured by calibrated optical density readings at 413 nM and albumin was measured by one dimensional Antibody-Antigen Crossed Electrophoresis (AACE) (see Materials and Methods for details).

Albumin is a major serum protein at all stages of development, representing over 70% of the total serum protein in the adult and hence providing an estimate of serum contamination in the lysate protein. A fetal serum protein, α-fetoprotein (AFP) was also measured by one dimensional AACE. No AFP was detectable in the adult and term lysates. A very small peak was observed with the application of 10 mg fetal lysate protein, but this peak was too small to use for accurate quantitation.

Table 7 opposite, shows the concentration of haemoglobin and albumin, and the total protein present in adult, term and fetal lysates.

To see what other serum proteins were present in the lysates, the three types of red cell lysate were then analysed by two dimensional AACE with sheep anti-adult human serum protein and compared with a sample of adult serum electrophoresed into the same antiserum (Fig. 6a - d). The adult serum produced a complex pattern of over thirty peaks. Albumin was the only serum protein detectable in the adult red cell lysate. The term and fetal lysates both showed a similar pattern of a bimodal albumin peak, a fairly strong β mobility peak (higher in term lysate) and three or four other faint peaks.

B. ANTISERA RAISED AGAINST WHOLE RED CELL LYSATES

1. Term Red Cell Lysate

A New Zealand White Rabbit (No. 5895) was immunised with doses of 20 mg term red cell lysate protein.

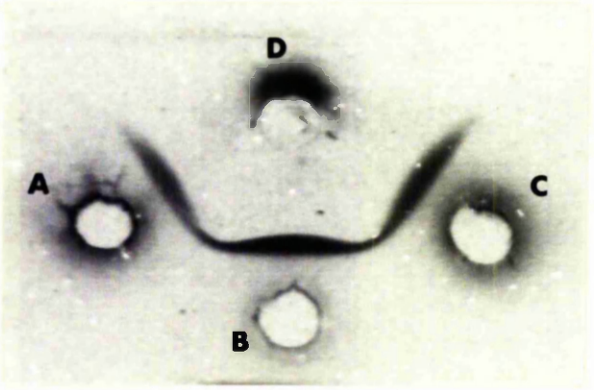
a) Reactions of Unadsorbed Antiserum

When 5895 antiserum was tested with 10 µg adult, term and fetal lysates on a two dimensional AACE, only one precipitin arc

Fig. 7a AACE of Adult, Term and Fetal Lysate with 5895 Antiserum

Adult Lysate Term Lysate Fetal Lysate

Fig. 7b Ouchterlony Plate of Fetal Lysate with 5895 Antiserum and Commercial Anti-Fetal Haemoglobin Antiserum



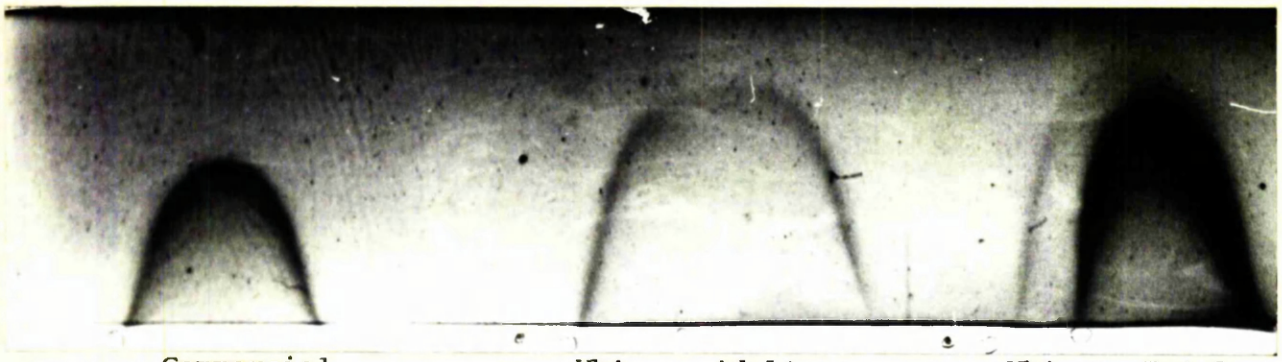
Wells A and C = 5895 Antiserum

Well B = Commercial Anti Fetal Haemoglobin Antiserum

Well D = Fetal Red Cell Lysate

5895 antiserum was raised against unfractionated term cord red cell lysate.

Fig. 8 AACE of 5895 Antiserum with Commercially Purified Adult Haemoglobin, Adult Lysate and Fetal Lysate



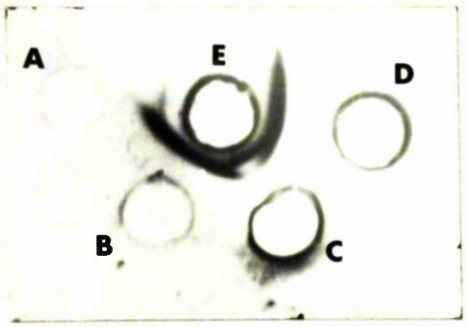
Commercial HbA Alone

HbA - Adult Lysate Applied in Tandem Wells 5 mm Apart

HbA - Fetal Lysate Applied in Tandem Wells 5 mm Apart

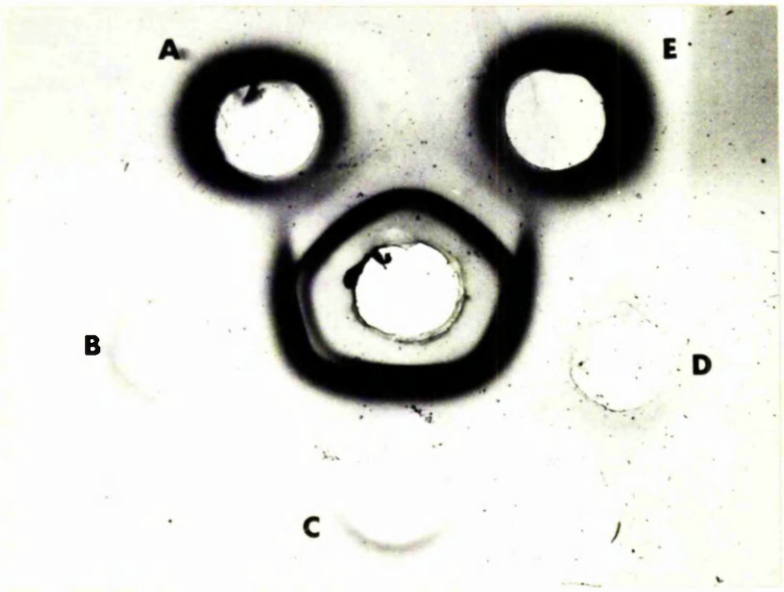
5895 antiserum was raised against unfractionated term cord red cell lysate.

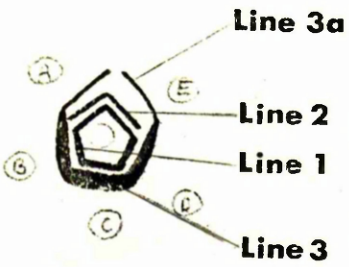
Fig. 9a Ouchterlony Plate of 5895 Antiserum With Fetal Lysate and Commercially Purified Adult Haemoglobin



Wells A and C = HbA
B and D = Fetal Lysate
E = 5895 Antiserum

Fig. 9b Ouchterlony Plate of 5895 Antiserum With Adult, Term and Fetal Lysates, Adult and Fetal Haemoglobin





Well A = HbA
B = HbF
C = Term Lysate
D = Fetal Lysate
E = Adult Lysate
Centre Well = 5895 Antiserum

5895 antiserum was raised against unfractionated term cord red cell lysate

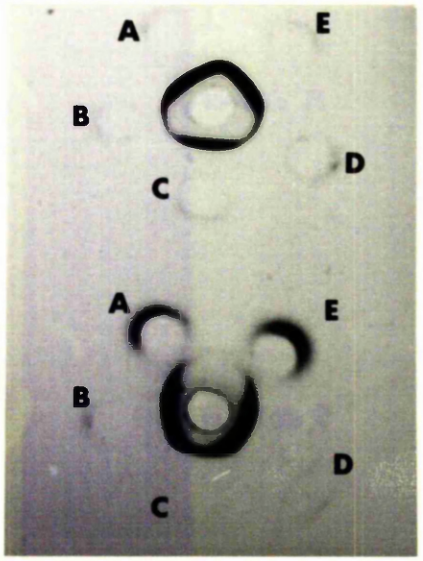
was observed (Fig. 7a). This peak was particularly intense with term and fetal lysates and had the slow mobility and broad shape characteristic of haemoglobin. An Ouchterlony double diffusion plate was set up to compare the reaction to fetal lysate of 5895 and commercial anti-fetal haemoglobin and a fused line of identity was observed (Fig. 7b). Commercially purified adult haemoglobin (HbA) was examined by AACE with 5895 antiserum and produced the same shaped peak as had been observed with the lysates (on left of Fig. 8). Pure HbA was added to adult and fetal lysate in tandem sets of wells spaced apart (Fig. 8). HbA combined with adult lysate to give a fused elongated peak indicating a reaction of identity. However, the tandem addition of HbA to fetal lysate (Fetal RCL) gave a clear fetal haemoglobin peak joined to a fainter half peak of HbA on the left hand side. This indicated partial identity. To exclude the possibility of the two peaks overlapping and the right hand side of the HbA peak being obscured by the intense fetal RCL peak, the distance between the application wells was increased until the HbA and adult lysate peaks just met. The HbA and fetal lysate peaks still showed partial identity as before.

It therefore appeared that HbA and haemoglobin from fetal lysates had some antigenic determinants in common and that fetal haemoglobin had some further determinants not detected on HbA by this antiserum. This reaction of partial identity was initially confirmed with an Ouchterlony plate showing spurs of a fetal lysate precipitin line (Fig. 9a). However, a further Ouchterlony plate comparing the reaction of 5895 antisera to different haemoglobin sources revealed a more complex pattern of identities (Fig. 9b). Adult, term and fetal lysates were tested, together with commercially purified HbA and HbF purified on a CM sepharose ion exchange column (see Materials and Methods and Section C of Results for details). There appeared to be a precipitate line fused for all the antigens tested.[1*] Just outside this was a precipitate line only occurring with adult lysate and HbA.[2] On the outside of this was a very intensely staining line fused for fetal and term RCL and HbF.[3] This line appeared to join very much fainter lines from adult RCL and HbA.[3a]

To try to elucidate these patterns, this Ouchterlony plate was repeated using commercial anti-adult haemoglobin and anti-fetal

* These superscript numbers refer to the numbered lines in the drawing of Fig. 9b.

Fig. 9c Ouchterlony Plates of Commercial Anti-Adult Haemoglobin (Top) and Commercial Anti-Fetal Haemoglobin (Bottom) With Adult, Term and Fetal Lysates, Adult and Fetal Haemoglobin



α–Hb A

α – Hb F

Wells A = HbA
B = HbF
C = Term Lysate
D = Fetal Lysate
E = Adult Lysate
Upper Centre Well = Commercial anti-HbA antiserum
Bottom Centre Well = Commercial anti-HbF antiserum

5895 antiserum was raised against unfractionated term cord red cell lysate.

Fig. 10a AACE of 5895 Antiserum with Pooled Adult Serum

Fig. 10b AACE of 5895 Antiserum with Haemolysed Adult Serum

5895 antiserum was raised against unfractionated term cord red cell lysate

The extent of electrophoresis is indicated by a spot on the right hand side of the plate marking the progression of a serum albumin marker.

Fig. 11a AACE of Pooled Adult Serum With 5% Commercial Anti-Haptoglobin (Bottom) and 5895 Antiserum (Top)

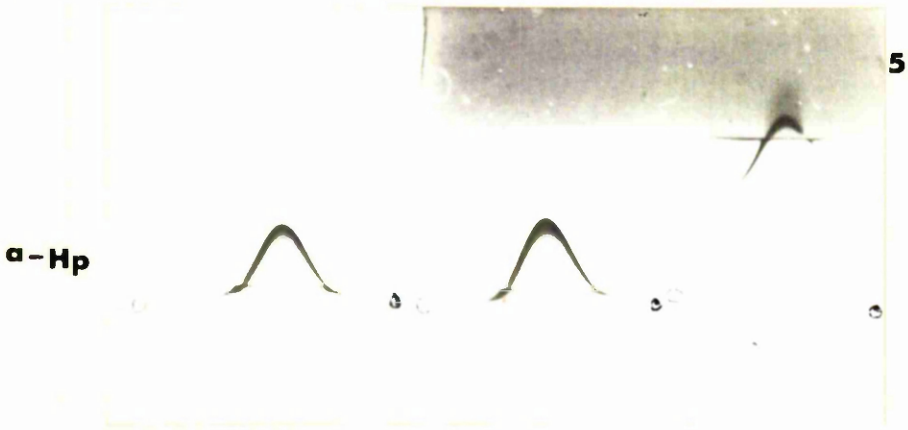


Fig. 11b AACE of Pooled Adult Serum With 1% Commercial Anti-Haptoglobin (Bottom) and 5895 Antiserum (Top)

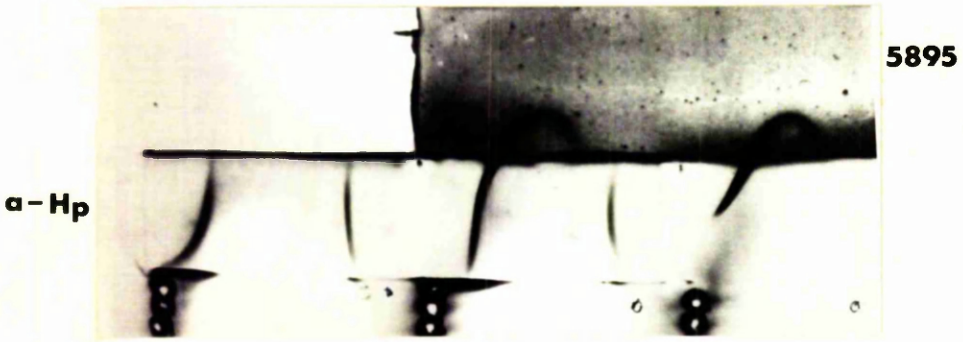


Fig. 11c AACE of Pooled Adult Serum With Commercial Anti-Adult Haemoglobin, Commercial Anti-Haptoglobin and 5895 Antisera

a - Hp a - Hp + 5895 5895 5895 + a - HbA a - HbA

5895 antiserum was raised against unfractionated term cord red cell lysate.

haemoglobin antisera (Fig. 9c).

A single line fused for all the antigens tested formed around anti-HbA; the lines for adult RCL and HbA being slightly more intense than the other haemoglobin sources. An intense line fused for fetal and term RCL and HbF formed around anti-HbF. This line appeared to show small spurs past a very faint line fused for adult RCL and HbA.

These commercial antisera were also analysed by two dimensional AACEs using 10 µg adult, term and fetal lysates. Commercial anti-HbA antiserum produced a clear peak with adult RCL, a fainter peak with term RCL and a smaller faint peak with fetal RCL. Commercial anti-HbF antiserum produced intense but not completely distinct peaks with term and fetal RCL and no reaction with adult RCL.

When 5895 antiserum was tested with pooled adult serum (PAS) on a two dimensional AACE, only one precipitin arc was observed (Fig. 10a). This peak appeared incomplete and looked like only half a normal peak. A completed peak was produced, however, when serum in which some red cell lysis had occurred was tested (Fig. 10b). This suggested that the form of the peak might be reflecting the concentration of haemoglobin in the serum. Haptoglobin is a serum protein which binds extracellular haemoglobin <u>in vivo</u> and it seemed possible that this peak was haptoglobin or a haptoglobin complex.

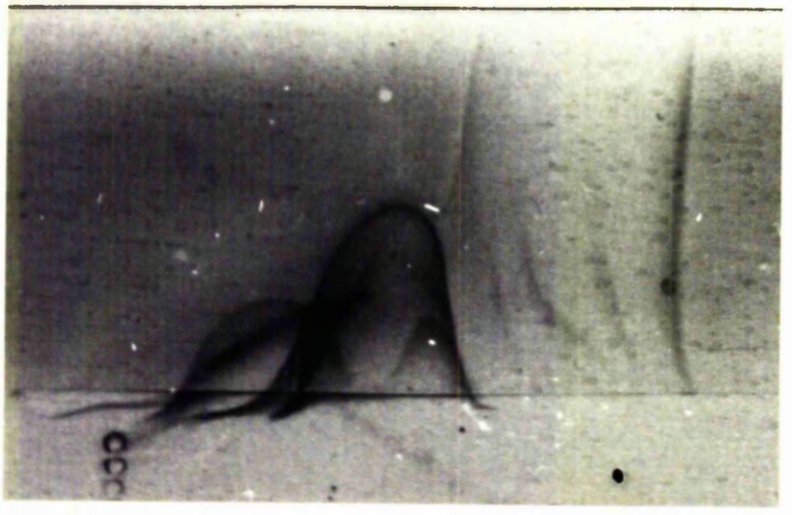
To test this possibility an antibody interaction between 5895 antiserum and commercial anti-haptoglobin antiserum was made on an AACE with the 5895 antibody bed set in above the anti-haptoglobin bed as shown in Fig. 11a. The principle of this test is that if the two antisera are recognising the same antigen, the first antibody bed precipitates the antigen so that it does not reach the second antibody bed on top. A control, without a bottom bed, is also included to demonstrate that the second antibody bed can precipitate the antigen despite the distance travelled. Fig. 11a demonstrates this blocking effect of anti-haptoglobin antiserum on 5895. In Fig. 11b, the percentage of anti-haptoglobin antiserum in the bottom bed has been reduced so that the haptoglobin peak is not completed in the first antibody bed. This results in a precipitate line from the 5895 bed fusing with the peak in the anti-haptoglobin bed.

In an attempt to define whether this peak with adult serum was haptoglobin or a haemoglobin-haptoglobin complex, 'addition interactions' were set up between 5895 antiserum and commercial anti-haptoglobin and anti-adult haemoglobin. To do this, five identical samples of adult serum were electrophoresed in the first dimension of an AACE plate. In the second dimension, the reactions of 5895 and commercial antisera were compared individually and then added together in the same antibody bed (Fig. 11c). Anti-haptoglobin antiserum alone produced a clear complete peak. This became smaller and more intense when 5895 antiserum was added. This suggests that the antisera are acting together in recognising the same antigen. An indistinct half peak occurred with 5895 antiserum alone, anti-adult haemoglobin antiserum alone and with the two antisera together. This suggests that the serum antigen has determinants recognised by 5895 and anti-haemoglobin antisera. These reactions of apparent identity with both anti-haptoglobin and anti-haemoglobin antisera suggest that 5895 antiserum may be recognising haemoglobin-haptoglobin complex determinants. The nature of the antibodies involved in these reactions were subsequently investigated further and the results may be found in Section G.

b) Reactions of Antiserum Adsorbed with Adult Human Serum

Adsorption of 5895 antibodies with polymerised adult serum resulted in a total loss of reactivity with adult or fetal serum when tested on a two dimensional AACE. Examination by AACE of 10 µg adult, term and fetal lysate with adsorbed 5895 antiserum showed haemoglobin peaks only for term and fetal lysates. This appearent adsorption of a red cell protein by serum was puzzling until the optical density analysis of the pooled adult serum showed that it contained approximately 0.04 mg/ml haemoglobin. This was estimated from the calibrated standard curve for haemoglobin readings at 413 nM. It therefore appeared that it was the very slight contamination of haemoglobin from red cell lysis that had adsorbed out the adult haemoglobin peak on AACE. To try to detect minor antigenic components, the amount of fetal lysate applied to the AACE plate was increased one hundred fold to 1 mg. No further peaks were observed and the haemoglobin peak was greatly enlarged.

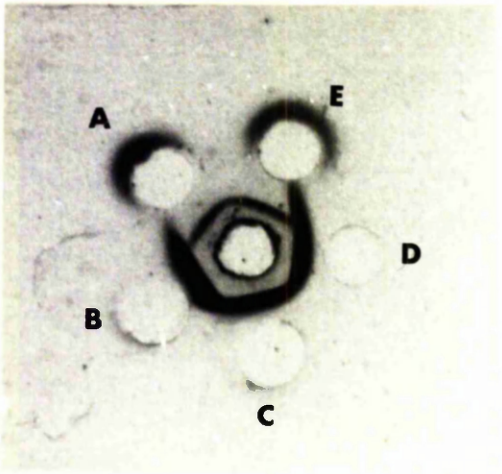
Fig. 12 AACE of Pooled Adult Serum with Unadsorbed FRC Antiserum



FRC antiserum was raised against pooled fetal red cell lysate.

The major right hand peak has the mobility of serum albumin.

Fig. 13 Ouchterlony Plate of Unadsorbed FRC Antiserum with Adult, Term and Fetal Lysates, Adult and Fetal Haemoglobin



Well	A	=	HbA	Well	D	=	Fetal Lysate
	B	=	HbF		E	=	Adult Lysate
	C	=	Term Lysate		Centre Well	=	FRC antiserum

FRC antiserum was raised against pooled fetal red cell lysate.

Δ

After adsorption with adult serum, 5895 antiserum was further adsorbed with either adult lysate or HbA (both polymerised with gluteraldehyde) to test for fetal specificities. In either case haemoglobin peaks were no longer visible when adult, term or fetal lysates were tested on AACE.

2. Fetal Red Cell Lysate

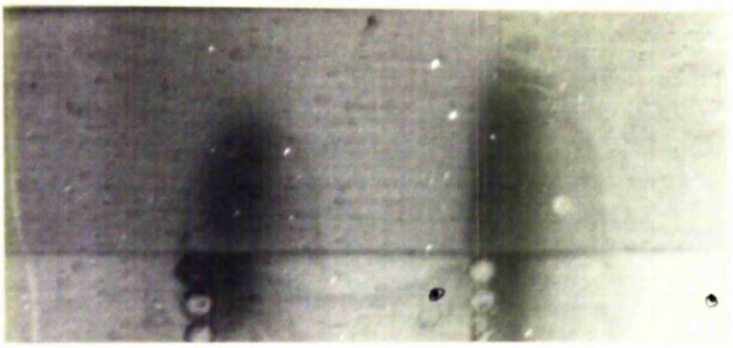
Two New Zealand White rabbits at the Radiochemical Centre, Amersham, were injected with fetal red cell lysate protein. The first (FRC1) was immunised with 100 mg doses, the second (FRC2) with 300 mg doses. The initial reactions of these antisera appeared to be indistinguishable and since only small volumes of blood were obtained, antisera from similar dates were pooled.

a) Reactions of Unadsorbed Antisera

When tested by Antibody-Antigen Crossed Electrophoresis (AACE) the specificities of the unadsorbed FRC antisera for adult serum were more extensive than had been observed with 5895 antiserum. Four peaks were visible after the first booster injection and ten or more after the second (Fig. 12). The half-peak observed with 5895 antiserum was present. The response to 10 µg of adult, term and fetal red cell lysates as tested on AACE with unadsorbed FRC antisera was similar to that seen with 5895. A strong haemoglobin-type peak was noted with term and fetal lysates, but the adult lysate peak was only a very weak shadow. FRC antisera had such a low titre of antibodies to haemoglobin in adult lysate that a peak with commercially purified adult haemoglobin (HbA) was not visible. An Ouchterlony plate comparing the response of commercial anti-fetal haemoglobin and FRC antisera to fetal lysate showed a continuous fused line of identity. A further Ouchterlony plate (Fig. 13) was set up to compare the reaction of FRC antiserum with adult, term and fetal red cell lysates (RCLs), HbA and ion exchange purified fetal haemoglobin (HbF). An intense, rather blurred fused line of identity was observed for fetal and term RCLs and HbF, which showed spurs past fainter fused lines of identity for adult RCL and HbA. This indicates partial identity between adult and fetal haemoglobin, there being certain determinants in common and others which were specific for fetal haemoglobin when tested with this antiserum.

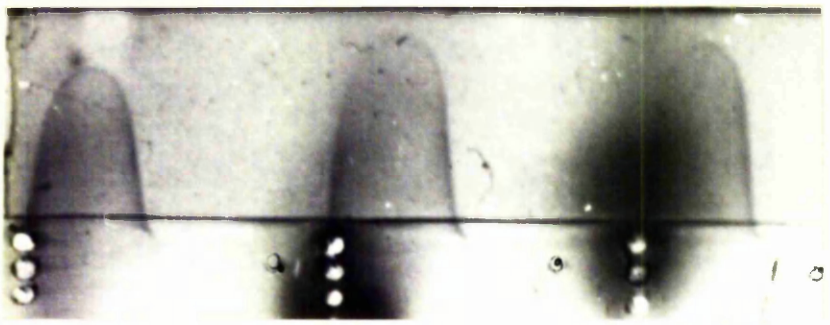
Figs. 14a-c AACE of Adult and Fetal Lysate with FRC Antiserum Adsorbed with Adult Serum

Fig. 14a



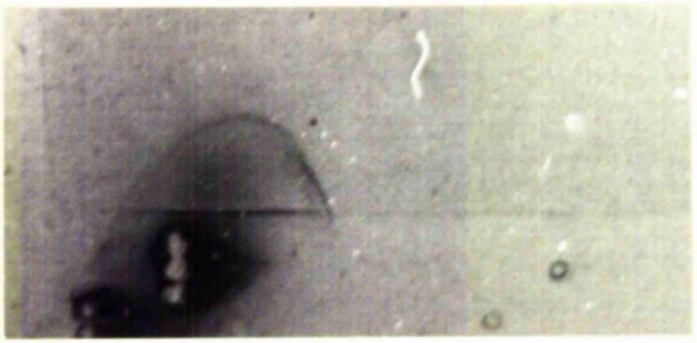
Adult RCL | Fetal RCL

Fig. 14b



Adult RCL | Adult RCL plus Fetal RCL | Fetal RCL

Fig. 14c

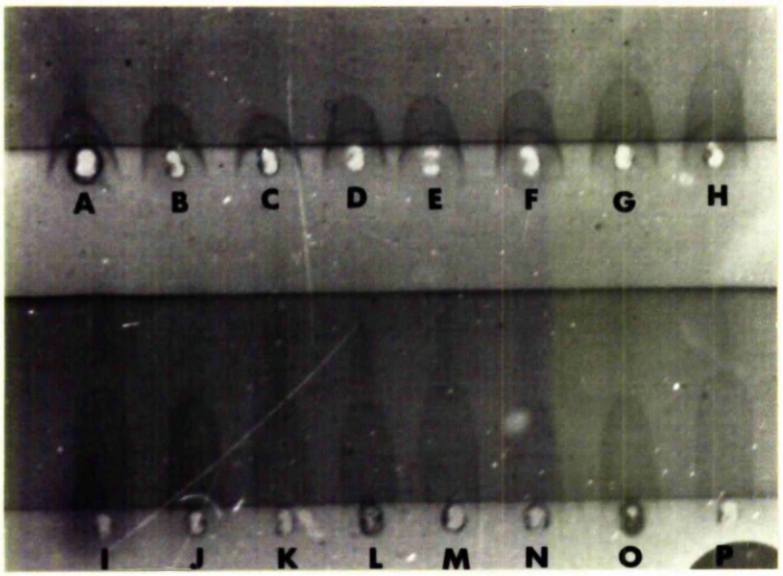


Adult RCL - Fetal RCL

FRC antiserum was raised against pooled fetal red cell lysate.

The extent of electrophoresis is indicated by a spot on the right hand side of the plate marking the progression of a serum albumin marker.

Fig. 15 One Dimensional AACE of Fetal, Term and Adult Lysates with FRC Antisera Adsorbed with Adult Serum



Sample A 13 Weeks Gestation Fetal Lysate
B 15 " " " "
C 18 " " " "
D 22 " " " "
E 25 " " " "
F 28 " " " "
G Term Cord Lysate)
H " " ")
I " " ") From Different Individuals
J " " ")

K Adult Lysate)
L " ")
M " ")
N " ") From Different Individuals
O " ")
P " ")

b) Reactions of Antisera Adsorbed with Adult Human Serum

Adsorption of FRC antibodies with polymerised adult serum resulted in a loss of reactivity with adult or fetal serum when tested on a two dimensional AACE. Examination by AACE of 10 µg adult, term and fetal lysates with adsorbed FRC antisera showed faint haemoglobin peaks only for term and fetal lysates. To try to detect minor antigenic components, the amount of lysate applied to the AACE plate was increased one hundred fold to 1 mg. A weak β mobility peak was visible with adult and fetal lysate (Fig. 14a). To determine if these peaks were the same for both adult and fetal lysate, both antigens were added together in the same wells and in a tandem set of wells spaced apart. The straight-forward addition produced a single larger peak (Fig. 14b) and the tandem addition produced a fused elongated peak (Fig. 14c). These results indicate that the antigen being recognised is the same in adult and fetal lysate. To see if there was any change in antigen concentration during fetal development, 250 µg adult, term and fetal lysates of various gestational ages were tested on one dimensional AACE with serum adsorbed FRC antisera (Fig. 15). The peak height seemed to increase from the earliest fetal sample through to term, at which time the height was similar to that found in adult samples. This indicates that the concentration of the β mobility antigen present in lysates increases from approximately one quarter adult values at 13 weeks gestation to near-adult values at term.

Further adsorption of serum-adsorbed FRC antisera with polymerised adult haemoglobin was carried out to see if β mobility antigen detection was resistant to adsorption with haemoglobin chains. The β mobility peak remained unchanged indicating that this antigen was not a haemoglobin chain.

To detect any antibodies specific for antigens in fetal red cell lysates, FRC antisera were further adsorbed with adult serum and adult red cell lysate, both of which had been polymerised with gluteraldehyde. No peaks were detected on AACEs with 1 mg lysate for β mobility antigen detection or with 10 µg lysate for haemoglobin detection.

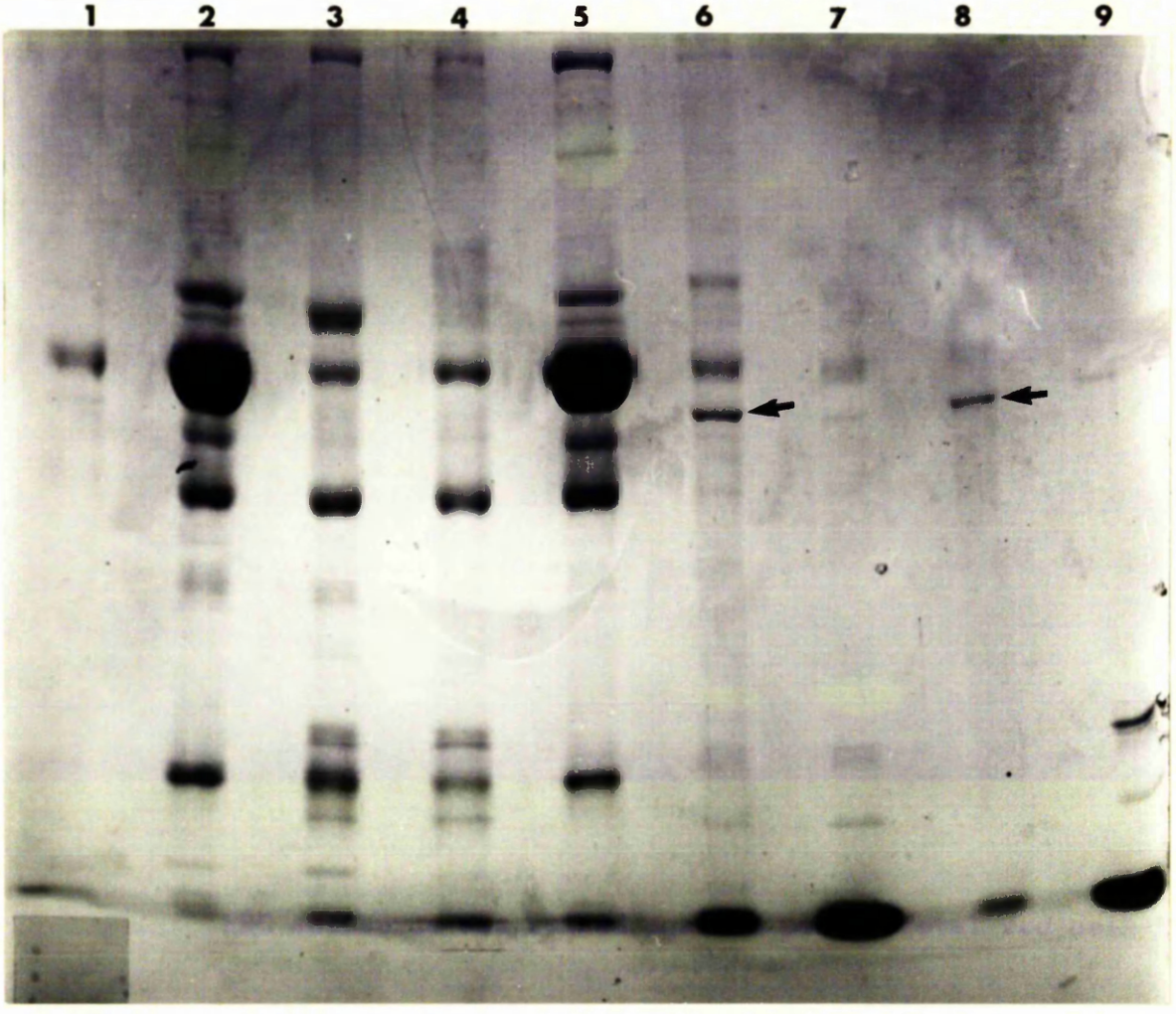
c) Affinity Chromatography

Although there are many non-haemoglobin proteins in red

Table 8 Total Protein Applied and Recovered in FRC Affinity Chromatography Experiments

Sample	mg Protein Applied	mg Protein Recovered
Blank Eluate	-	0.14
Adult Serum	700	3.8
Term Cord Serum	700	4.3
Adult Red Cell Lysate	700	3.1
Term Cord Red Cell Lysate	700	3.5

Fig. 16 Analysis of Protein Eluted from FRC Affinity Column by Polyacrylamide/SDS Slab Gel Electrophoresis



Track 1 = 'Blank' Eluate
2 = Adult Serum
3 = Adult Serum Eluate
4 = Term Cord Serum Eluate
5 = Term Cord Serum
6 = Term Cord Lysate Eluate
7 = Term Cord Lysate
8 = Adult Lysate Eluate
9 = Adult Lysate

FRC antiserum was raised against pooled fetal red cell lysate

cells only one had been detected so far. It was therefore concluded that either antibodies had not been raised against those proteins, or the principal detection system (AACE) was not sensitive enough to detect antigens at low concentration. It was decided to try affinity chromatography in order to increase the concentration of antigens for which this antiserum had specificities. By this method, a mixture of lysate antigens could be passed over immobilised FRC antibodies and antigens which bind to the antibodies could be retained while the rest pass through. These antigens could be desorbed (e.g. by 2M KI), dialysed to restore normal salt strength and analysed for further lysate specificities.

Antibodies from 150 ml FRC antisera were partially purified by 45% ammonium sulphate precipitation and were immobilised on 150 g sepharose. Unadsorbed antibodies were used since serum adsorption had been shown to remove haemoglobin specificities and it was possible that specificities for minor red cell proteins might likewise be adsorbed.

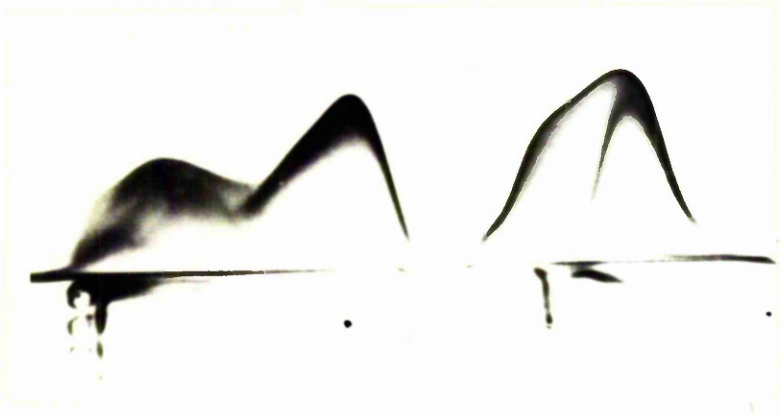
The FRC/sepharose column was eluted with 2M KI before any samples were passed over. This 'blank' eluate was to act as a control for any protein which might become detached from the affinity column during subsequent desorption of antigens with 2M KI. The sepharose was then divided into two parts. Term and adult lysates were passed over one half, while term cord and adult serum were passed over the other. Serum eluates were collected to control for the serum contamination in the lysates. The serum and lysate columns were separated so that the serum eluates would not become contaminated by lysate antigens from previous runs and vice versa. This might happen because antigens are sometimes not desorbed fully from a column by a single elution and may appear in subsequent elution cycles.

Table 8 opposite shows the amount of protein (as measured in a Folin protein determination) in the eluates from the various samples applied to the FRC columns.

The antigens eluted from this column were compared on a polyacrylamide/SDS gradient slab gel (Fig. 16). Two bands were visible with the blank eluate in Track 1. The eluates obtained

Figs. 17a + b Analysis of Protein Eluted from FRC Affinity Column by AACE with Sheep-Anti Human Serum Protein

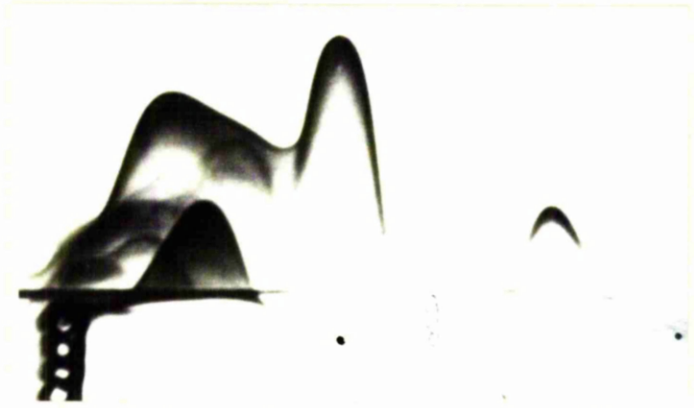
Fig. 17a



Adult Serum Eluate

Adult RCL Eluate

Fig. 17b



Term Cord Serum Eluate

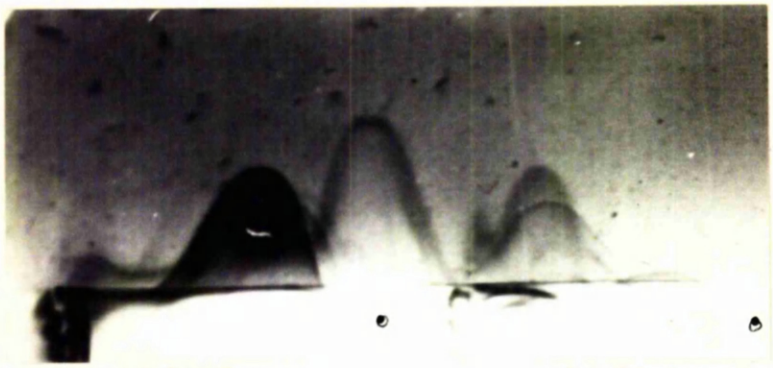
Term RCL Eluate

FRC antiserum was raised against pooled fetal red cell lysate

The extent of electrophoresis is indicated by a spot on the right hand side of the plate marking the progression of a serum albumin marker.

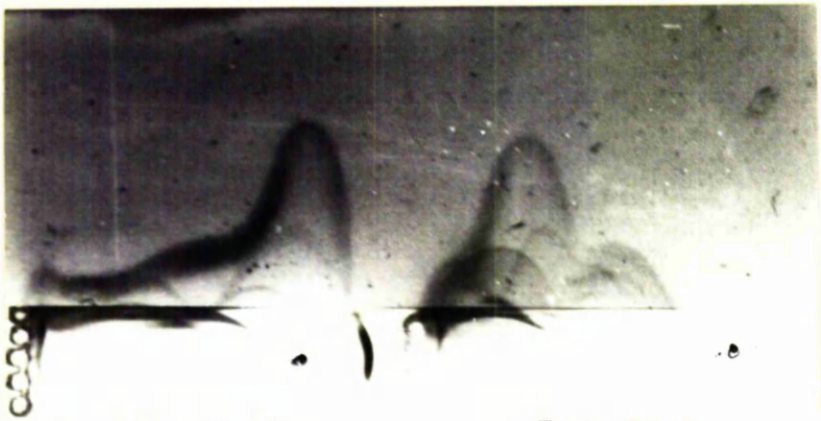
Figs. 17c + d Analysis of Protein Eluted from FRC Affinity Column by AACE with Unadsorbed FRC Antisera

Fig. 17c



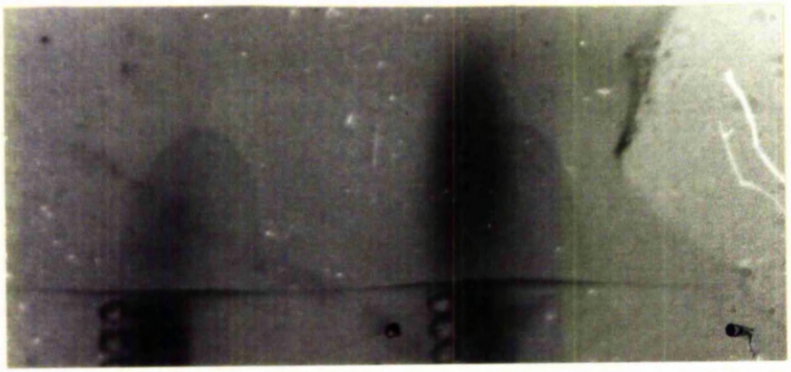
Adult Serum Eluate | Adult Lysate Eluate

Fig. 17d



Term Cord Serum Eluate | Term Cord Lysate Eluate

Fig. 17e Analysis of Adult and Term Cord Lysate Protein Eluted from FRC Affinity Column by AACE with FRC Antisera Adsorbed with Adult Serum



Adult Lysate Eluate | Term Cord Lysate Eluate

FRC antiserum was raised against pooled fetal red cell lysate.

from adult and term cord serum (Tracks 3 and 4) appeared to contain many of the bands visible in the unfractionated starting material (Tracks 2 and 5). Both the lysates and lysate eluates have far fewer bands than serum. The eluate from term lysate (Track 6) contains more bands than unfractionated term lysate (Track 7) but most of these bands also appear to be in term cord serum. The eluate from adult lysate (Track 8) appears to contain less bands than unfractionated adult lysate (Track 9). Approximately half the way down the gel in the lysate eluate tracks there is a clear band (marked with an arrow) which does not seem to be present in serum. This might be the β mobility antigen: The thick band at the bottom of the gel (particularly prominent in the lysate tracks) is a double band corresponding to the haemoglobin α and non-α chains. The very large and heavily stained bands which are particularly prominent in the serum tracks correspond to albumin.

Examination of the eluates was also made by Antibody-Antigen Crossed Electrophoresis (AACE). When tested with sheep anti-adult human serum, the eluates show a number of indistinct peaks with adult and term cord serum, a few (predominantly slower) mobility peaks with adult lysate and a slow mobility peak underlined by another with term lysate (Figs. 17a and b). A larger number of peaks were observed when the eluates were tested with unadsorbed FRC antiserum, but in general they were faint and indistinct, with some peaks either not completed or merged with others (Figs. 17 c and d). Single very faint β mobility peaks were visible when the eluates from adult and term lysates were examined by AACE with the adsorbed FRC antiserum which had previously been used to detect this antigen (Fig. 17e). These peaks were shown in one dimensional addition reactions to be the same as those originally observed on AACE with untreated lysates.

From these results it appears that affinity chromatography had failed to concentrate any further lysate antigens detectable on AACE with this antiserum. It was therefore concluded that a different method of presenting the red cell protein as an immunogen was required and it was decided to fractionate the lysate proteins so as to maximise the non-haemoglobin protein concentration.

C. ION EXCHANGE FRACTIONATION OF RED CELL LYSATES

1. Background

Several workers (e.g. Hennessey et al., 1962; Haut et al., 1964) had successfully used ion exchange methods to concentrate erythrocyte enzymes and remove haemoglobin which was obscuring starch gel bands or interfering with colourometric enzyme assays. Attempts had also been made to fractionate lysates by gel filtration. This is a more gentle fractionation method and is less prone to denature proteins since abrupt changes in salt concentration are not required. However, its disadvantages are that a large scale preparative column can take a long time to complete a cycle, and the quality of fractionation is not great. Aebi et al. (1964) found that gel filtration gave a very low recovery of general haemoglobin-free red cell proteins although in a few specific cases, e.g. catalase, a good separation could be achieved.

Two alternative methods of ion exchange had been used, these either used diethylaminoethyl (DEAE) cellulose in 3 mM phosphate buffer pH 7.0, or carboxymethyl (CM) cellulose ion exchange media with 5 mM phosphate buffer pH 6.8. In a comparison of the two methods Haut et al. (1962) achieved slightly better results with the CM cellulose method, despite the non-haemoglobin proteins being diluted in the process. This is because these proteins pass through the column in the low strength buffer, while haemoglobin is retained on the column. Because the non-haemoglobin proteins are in such a low strength buffer, however, they may be directly concentrated by lycphilisation.

2. Results

Three hundred milligrams term lysate was passed over a 2.5 cm x 29 cm CM sepharose column in 5 mM phosphate buffer pH 6.8 at 40 ml/hour. Haemoglobin could be observed binding to the column and the protein which passed through had a greatly reduced haemoglobin concentration. Because of the reduction in haemoglobin content, this fractionated product was termed 'reduced-haemoglobin red cell protein' (rHbRCP). The amount of rHbRCP prepared from 300 mg term lysate in a single ion exchange cycle was 6.8 mg, as measured by a Folin protein determination.

Fig. 18 Optical Density Profile of Term Red Cell Lysate Before and After Fractionation on a CM Sepharose Ion Exchange Column (Pye Unicam SP 800 Scanning Spectrophotometer)

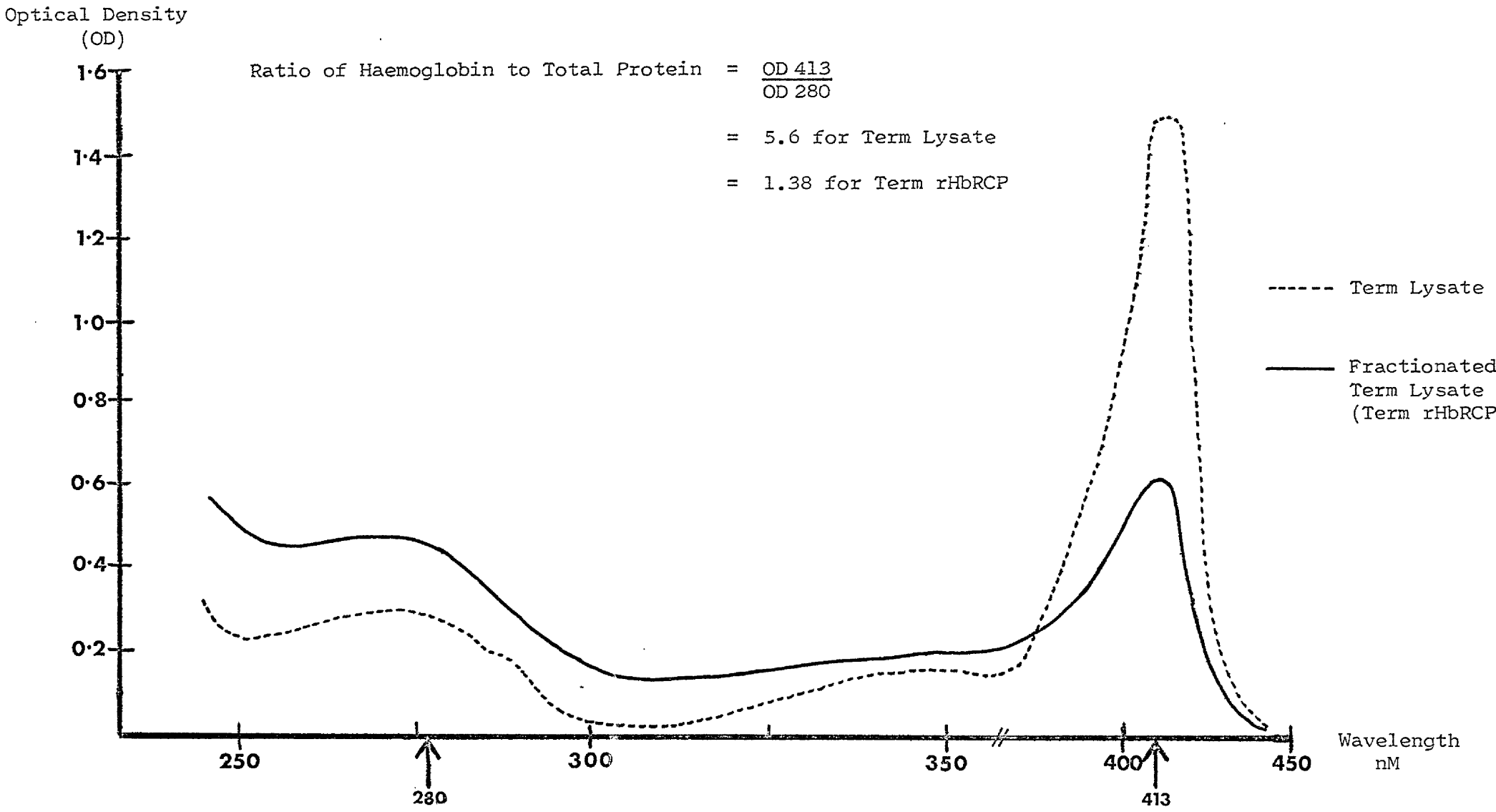


Table 9 Haemoglobin and Total Protein Content of Reduced Haemoglobin Red Cell Protein from Term Lysate Following Serial Passages Over a CM Sepharose Ion Exchange Column

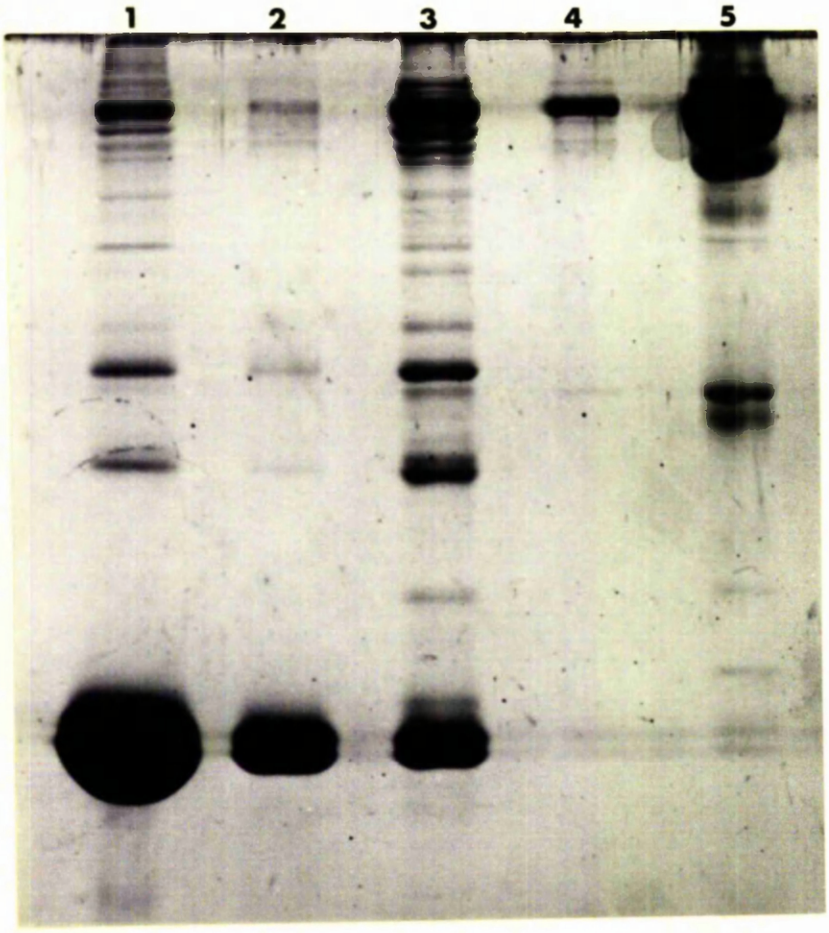
Sample Applied	Total Protein Recovered	Haemoglobin to Total Protein Ratio (OD 413/ OD 280)
300 mg Term Lysate	6.8 mg	1.59
4 mg Term rHbRCP (already passed once over column)	2.5 mg	1.6

Table 10 Comparison of Fractionated and Unfractionated Term Red Cell Lysate

Sample	Protein Concentration mg/ml	Haemoglobin Concentration mg/ml	Albumin Concentration mg/ml	'Other Protein' Concentration mg/ml	Ratio of 'Other' to Total Protein	Ratio of 'Other' to Haemoglobin Protein
Unfractionated Term Lysate	100	95	0.5	4.5	$\frac{4.5}{100} = 0.045$ ie 1	$\frac{4.5}{95} = 0.047$ ie 1
Reduced Haemoglobin Red Cell Protein Preparation from Term Lysate	50	20	8.5	21.5	$\frac{21.5}{50} = 0.43$ ie 9.7	$\frac{21.5}{20} = 1.075$ ie 29

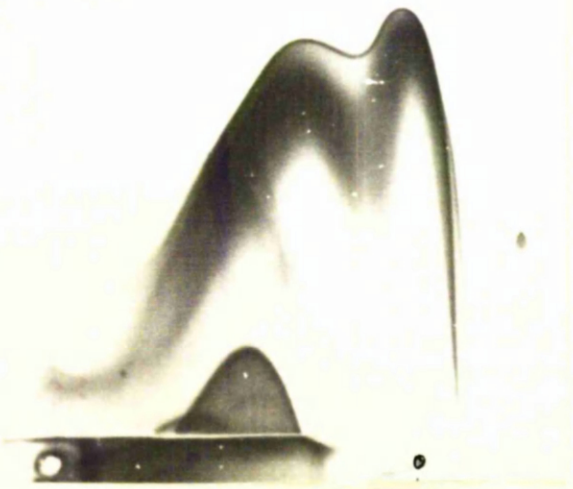
The 'other' protein is the amount of total protein which is not haemoglobin or albumin (i.e. non-haemoglobin red cell protein plus unknown serum contaminants).

Fig. 19 Analysis by Polyacrylamide/SDS Slab Gel Electrophoresis of Term Cord Lysate Protein Fractionated by CM Sepharose Ion Exchange Chromatography



Track 1 = 65 ug (5x concentrated) Term Cord Lysate
2 = 13.5 ug Term Cord Lysate
3 = 13.5 ug 'Reduced Haemoglobin Red Cell Protein'
(4 = Contaminated Track)
5 = Term Cord Serum

Fig. 20 AACE of 'Reduced Haemoglobin Red Cell Protein' From Term Cord Lysate with Sheep-Antihuman Serum Protein



The extent of electrophor
hand side of the plate ma
marker.

The optical density profile of this material was compared with unfractionated term lysate at the same Folin protein concentration (Fig. 18).

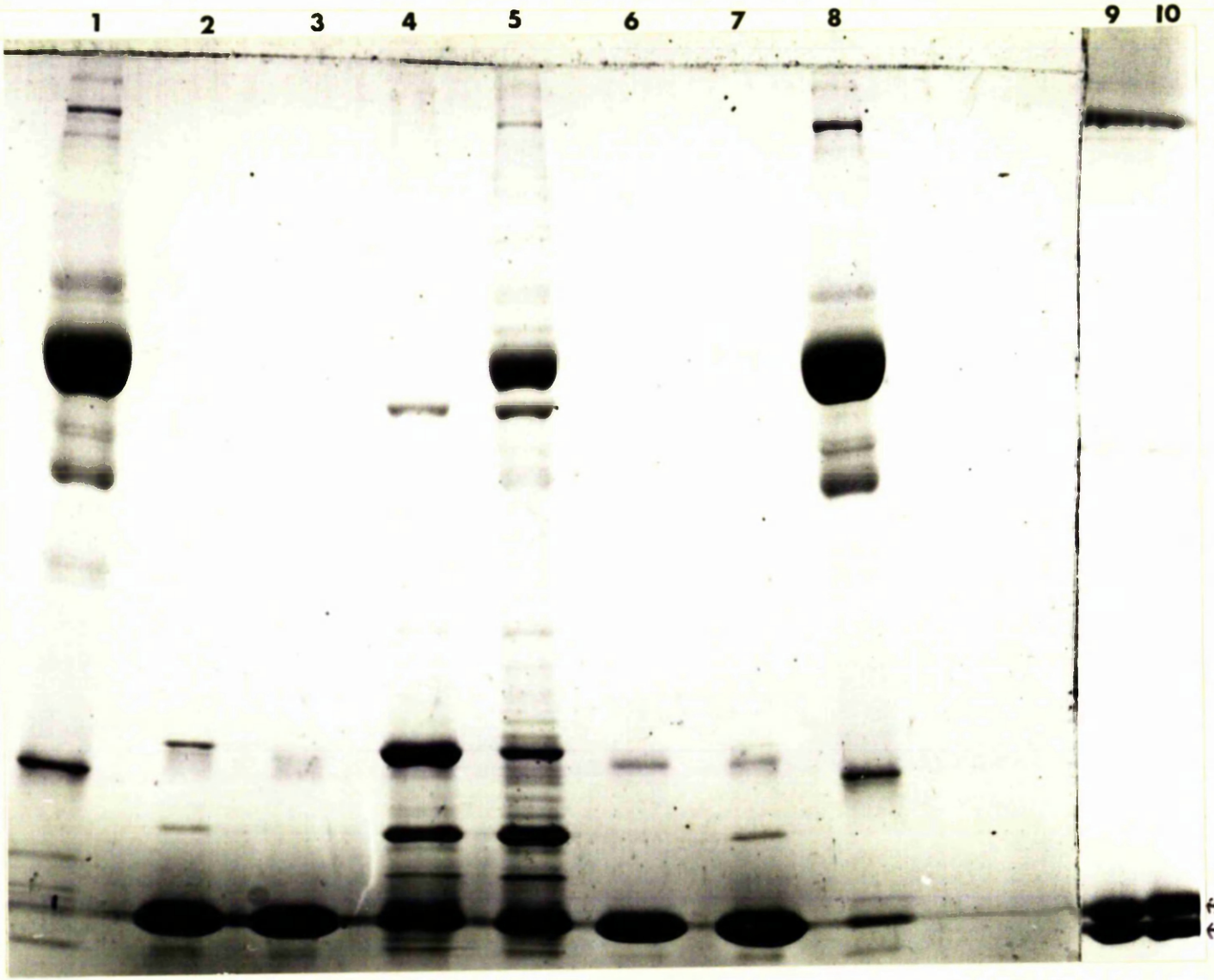
To see if the haemoglobin to total protein ratio could be reduced further, a term rHbRCP preparation from a single column passage was passed over the ion exchange column a second time. Optical density readings were made at 413 nM for haemoglobin and 280 nM for total protein. The total protein recovered was measured by the Folin method. These results are summarised in Table9 and indicate that less than two-thirds of the protein had been recovered and the haemoglobin to total protein ratio had not been improved at all by a second passage over the column.

Haemoglobin and albumin were measured in a sample of rHbRCP from a single column passage and in the term lysate from which the rHbRCP was derived. The concentration of haemoglobin was estimated from the optical density readings and albumin was measured by one dimensional AACE . The protein concentration was also measured by the Folin method. These results are summarised in Table 10, and indicate that a considerable increase in non-haemoglobin protein had been achieved. The proportion of total protein which was not haemoglobin or albumin had increased ten fold and the ratio of non-haemoglobin to haemoglobin protein had increased thirty fold. However, non-haemoglobin red cell proteins were not the only class of proteins to have increased in concentration. The albumin concentration had increased in parallel and it was possible that contamination by other serum proteins might likewise be increased.

To investigate whether any worthwhile concentration of non-haemoglobin red cell protein had been achieved, the ion exchange preparation was compared with term red cell lysate and term cord serum on a polyacrylamide / SDS gradient slab gel (Fig. 19). Many bands were visible with application of 13 µg reduced haemoglobin red cell protein (rHbRCP) from term lysate (Track 3) which were not visible with 65 µg unfractionated term lysate (Track 1) and which did not appear to correspond to serum protein bands (Track 4). Five hundred micrograms of term rHbRCP was analysed by AACE using sheep anti-adult human serum. The result (Fig. 20) shows a limited number of peaks with fairly

ndicated by a spot on the right
progression of a serum albumin

Fig. 21 Analysis by Polyacrylamide/SDS Slab Gel Electrophoresis of Adult and Term Cord Lysate Protein Fractionated by CM Sepharose Ion Exchange Chromatography



Track 1 = Adult Serum

2 = Adult Lysate

3 = Adult Lysate Protein Bound to Column

4 = Unbound Adult Lysate Protein (Adult rHbRCP)

5 = Unbound Term Lysate Protein (Term rHbRCP)

6 = Term Lysate Protein Bound to Column

7 = Term Lysate

8 = Term Cord Serum

9 = Fetal Lysate Protein Bound to Column (HbF)

10 = Commercially Purified Adult Haemoglobin (HbA)

intense α_2 mobility peak and indistinct bimodal albumin peaks.

It was felt, however, that the ion exchange fractionation of the lysate protein had achieved a sufficient degree of non-haemoglobin red cell protein concentration to make immunisation with this material worthwhile. Although the albumin concentration had been increased, it was probable that any further processing of the rHbRCP preparation to remove albumin (possibly on a blue dextran/sepharose column) might reduce the yield of red cell enzymatic proteins still further due to the deleterious effect of increased time in a dilute state. It might have been possible to remove the bulk of the contaminating serum proteins by affinity chromatography using immobilised sheep anti-adult human serum protein antibodies. However, the immunogen used to produce this antiserum was adult serum in which some red cell lysis had occurred, and therefore the antibodies probably had red cell protein specificities as well, which would be unwelcome in this instance.

Adult red cell lysate was also fractionated by CM sepharose ion exchange and compared with an equal amount of the fractionated term lysate on a polyacrylamide/SDS gradient slab gel (Fig. 21). There are differences between the term and adult reduced haemoglobin red cell protein tracks, but these appear to be qualitative rather than quantitative differences. The albumin band in particular is larger and more darkly stained in the fractionated term lysate track.

Ten milligrams pooled fetal lysate was passed over a smaller 1.5 cm x 15 cm CM sepharose column run at 8 ml/hour. The haemoglobin eluted from the column was compared with an equal amount of purified twice crystallised human adult haemoglobin (Sigma Chemical Co.). Both haemoglobin preparations gave strong double bands on polyacrylamide/SDS gradient slab gels (Fig. 21 right hand side) because of the presence of α and non-α polypeptide chains. The α chains, having slightly lower molecular weight, are nearer the bottom of the gel.

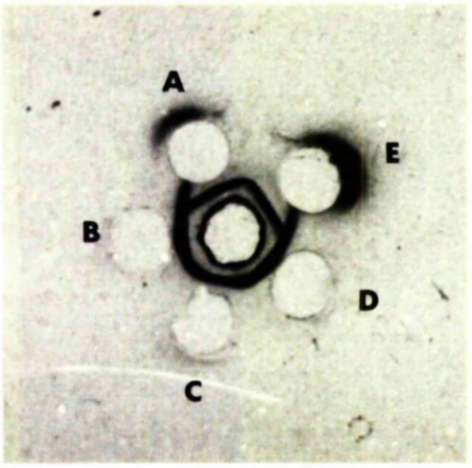
The fetal haemoglobin eluted from the ion exchange column appeared to be as pure as the haemoglobin prepared commercially and was therefore used as a source of purified fetal haemoglobin in other experiments.

One hundred and eighty micrograms reduced haemoglobin red cell

Fig. 22 AACE of Pooled Adult Serum With 6791/2 Antisera

The major right hand peak has the mobility of serum albumin.

Fig. 23 Ouchterlony Plate of 6791/2 Antisera with Adult, Term and Fetal Lysates, Adult and Fetal Haemoglobin



Well A = HbA
B = HbF
C = Term Cord Lysate
D = Fetal Lysate
E = Adult Lysate
Centre Well = 6791/2 Antisera

6791/2 antisera were raised against term cord lysate which had been fractionated by ion exchange chromatography to concentrate non-haemoglobin red cell proteins.

protein (rHbRCP) was produced in this experiment. This was just sufficient to establish the similarity to term rHbRCP on Antibody-Antigen Crossed Electrophoresis. (See next section of Results).

D. ANTISERA RAISED AGAINST CM SEPHAROSE ION EXCHANGE FRACTIONATED TERM CORD RED CELL LYSATE

Two New Zealand White rabbits (Nos. 6791 and 6792) were injected with 16 mg each of CM sepharose ion exchange reduced-haemoglobin red cell protein (rHbRCP) from term lysate. From Table 10 in the previous Section, it can be seen that 43% of the total protein in the term rHbRCP immunogen was not haemoglobin or albumin. It therefore follows that the maximum amount of true 'non-haemoglobin red cell protein' injected into each of these rabbits was 6.8 mg. The work of Hennessey et al. (1962) indicates that the total amount of non-haemoglobin red cell protein extractable from a red cell lysate is 4 - 5%. The amount of non-haemoglobin red cell protein in 150 mg of lysate (as was injected in rabbit FRC 1; see Section B ii of Results) would be 6 - 7.5 mg which is comparable to the amount injected into rabbits 6791 and 6792 as described in the present Section.

Preliminary results showed these two antisera to be indistinguishable and serum from both rabbits was subsequently pooled.

a) Reactions of Unadsorbed Antisera

When tested by Antibody-Antigen Crossed Electrophoresis (AACE) the specificities of the unadsorbed 6791/2 antisera for adult serum produced a more complex pattern of peaks than had been observed with antisera from rabbits 5895 and FRC 1 and 2 (Fig. 22). Most of the peaks were quite faint except for those in the α_2 mobility region. The half peak observed with 5895 antiserum was present but appears obscured behind other intensely staining peaks in the photograph of the AACE plate (Fig. 22).

The response to 10 µg adult, term and fetal red cell lysates as tested on AACE with unadsorbed 6791/2 antisera was a

Fig. 24a AACE of Adult and Term Cord Lysate with 6791/2 Antisera

Fig. 24b AACE of Adult and Term Cord 'Reduced Haemoglobin Red Cell Protein' with 6791/2 Antisera

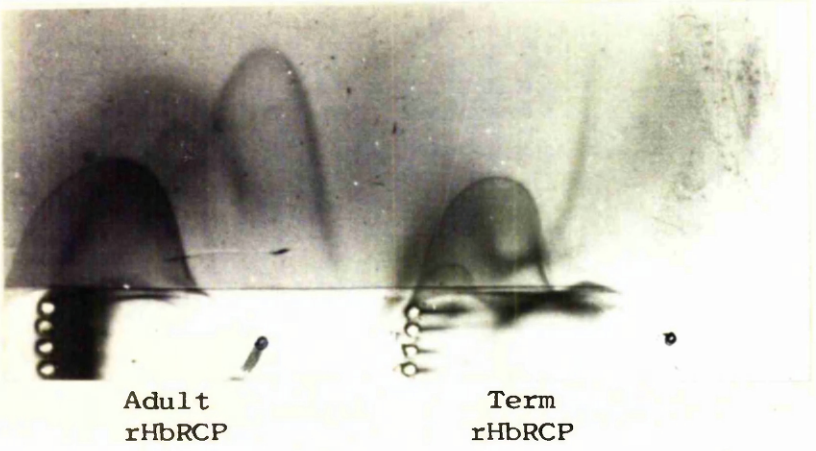
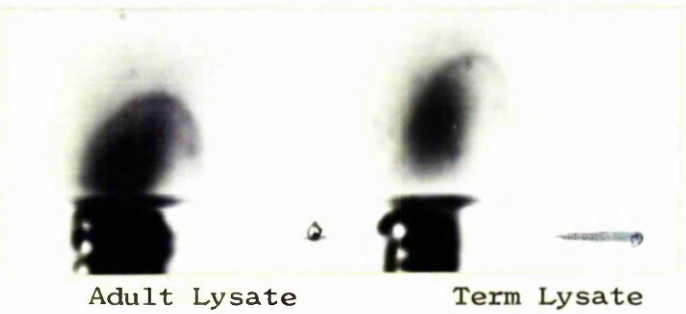


Fig. 25 AACE of Adult and Term Lysate with 6791/2 Antisera Adsorbed with Adult Serum



6791/2 antisera were raised against term cord lysate which had been fractionated by ion exchange chromatography to concentrate non-haemoglobin red cell proteins. This material was called 'reduced haemoglobin red cell protein' or 'Term rHbRCP'.

The extent of electrophoresis is indicated by a spot on the right hand side of the plate marking the progression of a serum albumin marker.

broad haemoglobin-type peak. The peaks with term and fetal lysates were considerably more intense than with adult lysate. To look for identity between various sources of adult and fetal haemoglobin, an Ouchterlony plate was set up with adult, term and fetal lysates, (RCLs) commercially purified adult haemoglobin (HbA) and fetal haemoglobin (HbF) purified by ion exchange. The result (Fig. 23) showed fused lines of identity with term RCL, fetal RCL and HbF, which showed spurs past fainter fused lines of identity for adult RCL and HbA. This indicates partial identity between adult and fetal haemoglobin, with some extra determinants being recognised on fetal haemoglobin by these antisera. When 1 mg adult, term and fetal lysates were tested by AACE with unadsorbed 6791/2 antisera a small number of large, very faint peaks with a more intense β mobility peak was observed (Fig. 24a). A similar result was recorded when 0.5 mg adult and term reduced haemoglobin red cell protein (rHbRCP) was examined (Fig. 24b). Two extra peaks were observed with the term lysate which did not appear to be present in the other samples. Examination of these antisera during two further boosts at four week intervals showed these results to remain fairly constant.

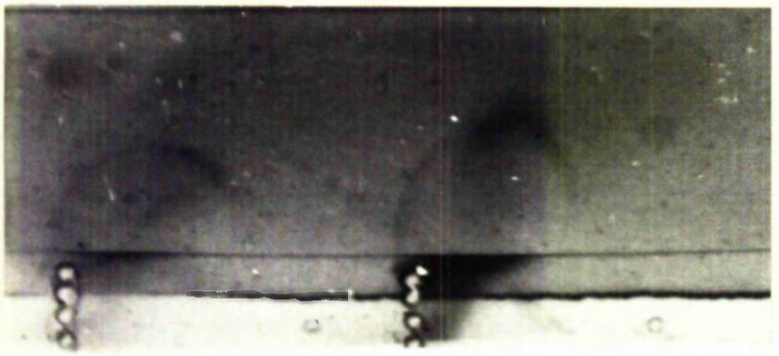
b) Reactions of Antisera Adsorbed with Adult Human Serum

Adsorption of 6791/2 antibodies with polymerised adult serum resulted in a total loss of reactivity with adult or fetal serum when tested on a two dimensional AACE. Examination by AACE of 10 µg adult, term and fetal lysates with adsorbed 6791/2 antisera showed very faint haemoglobin peaks only for term and fetal lysates; antibody specificities for adult haemoglobin probably being adsorbed by the haemoglobin in the adult serum polymer.

When 1 mg adult and term lysates were examined on AACE with adsorbed 6791/2 antisera, a very faint, broad β mobility peak was observed in both cases (Fig. 25). To determine if these peaks were the same, tandem and direct additions of adult and term lysates were made and resulted in single fused peaks. This indicates that the antisera were recognising the same antigen in both adult and term lysate.

When 0.5 mg adult and term reduced haemoglobin red cell

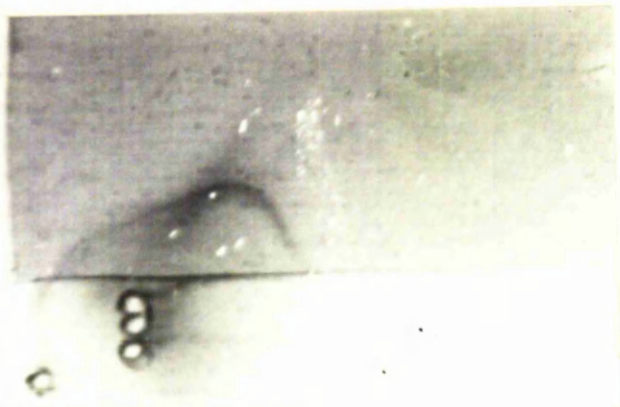
Fig. 26a AACE of Adult and Term 'Reduced Haemoglobin Red Cell Protein' with 6791/2 Antisera Adsorbed with Adult Serum



Adult rHbRCP | Term rHbRCP

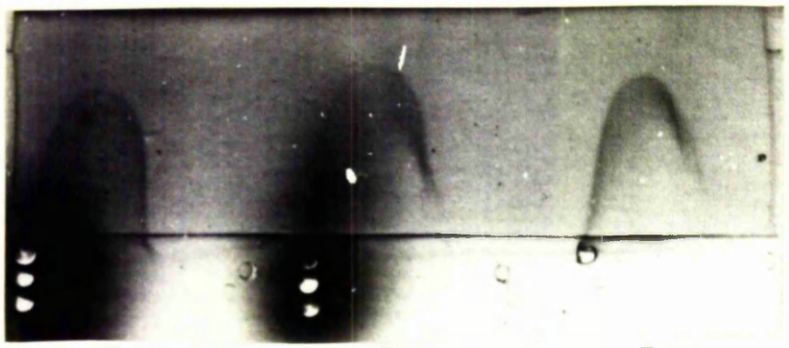
Figs. 26b + c AACE of Term Cord Serum and Term 'Reduced Haemoglobin Red Cell Protein' with 6791/2 Antisera Adsorbed with Adult Serum

Fig. 26b



Term rHbRCP _ Term Lysate

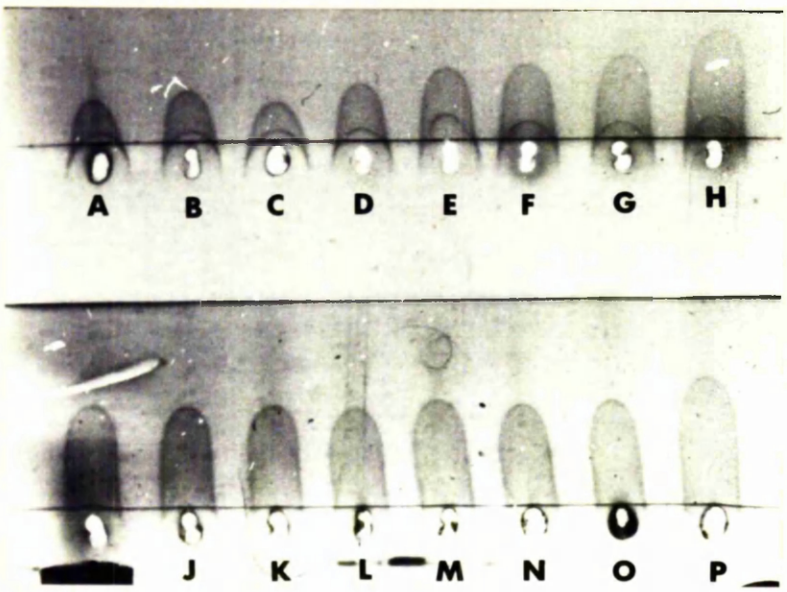
Fig. 26c



Term Lysate 500 ug | Term Lysate plus Term rHbRCP | Term rHbRCP 100 ug

6791/2 antisera were raised against term cord lysate which had been fractionated by ion exchange chromatography to concentrate non-haemoglobin red cell proteins. This material was called 'reduced haemoglobin red cell protein' or 'Term rHbRCP'.

Fig. 27 One Dimensional AACE of Fetal, Term and Adult Lysates with 6791/2 Antisera Adsorbed with Adult Serum



Sample A 13 Weeks Gestation Fetal Lysate
B 15 " " " "
C 18 " " " "
D 22 " " " "
E 25 " " " "
F 28 " " " "
G Term Cord Lysate)
H " " ") From Different Individuals
I " " ")
J " " ")

K Adult Lysate)
L " ")
M " ") From Different Individuals
N " ")
O " ")
P " ")

N.B. The arched bases of the upper row of peaks are artifacts which sometimes occur when two separate one-dimensional antibody beds are used on the same plate. Fetal samples which had been individually examined previously did not exhibit this type of base and unfortunately there was insufficient 6791/2 antisera remaining to repeat this plate.

6791/2 antisera were raised against term cord lysate which had been fractionated by ion exchange chromatography to concentrate non-haemoglobin red cell proteins.

Fig. 28a AACE of Fetal Lysate with Serum Adsorbed 6791/2 (Top) and FRC (Bottom) Antisera

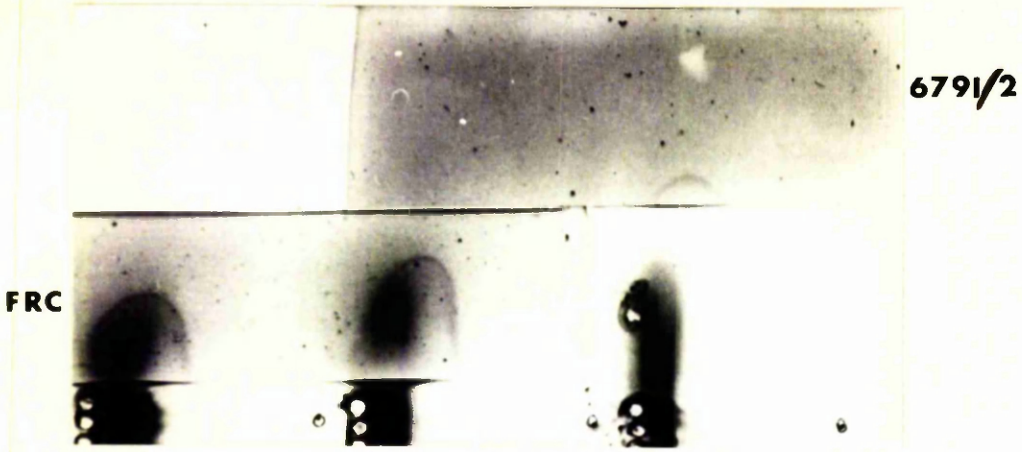
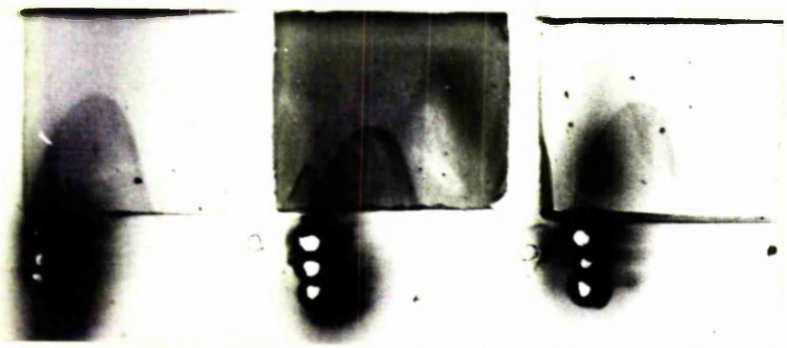


Fig. 28b AACE of Fetal Lysate with Serum Adsorbed 6791/2 and FRC Antisera



6791/2 antisera were raised against term cord lysate which had been fractionated by ion exchange chromatography to concentrate non-haemoglobin red cell proteins.

FRC antiserum was raised against pooled fetal red cell lysate.

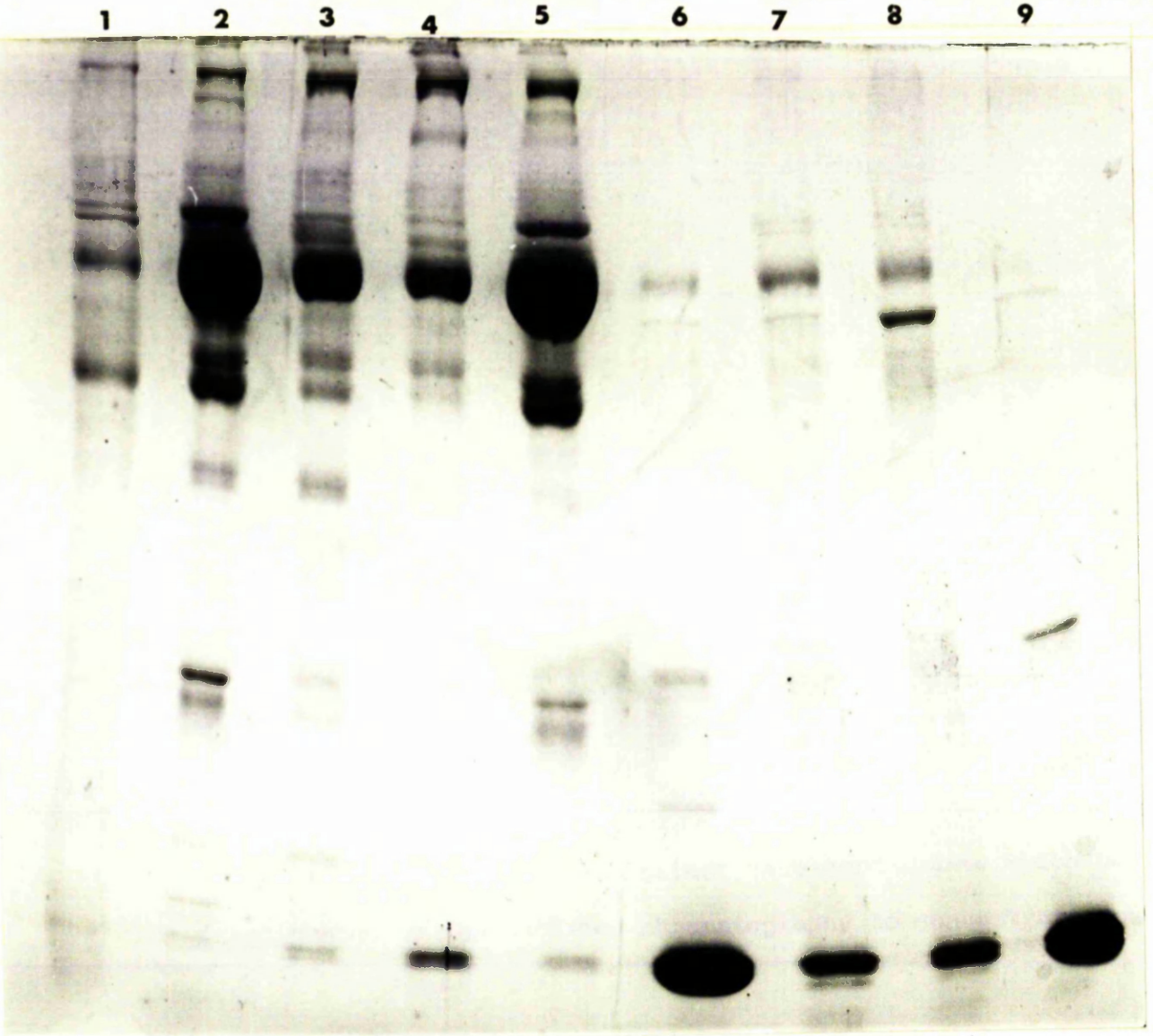
protein (rHbRCP) were examined on AACE with adsorbed 6791/2 antisera, rather faint indistinct peaks resulted, (Fig. 26a). Adult rHbRCP produced two incomplete peaks while term rHbRCP showed an incomplete peak which merged into a more intensely staining line. To test for identity between the peaks with term lysate and term rHbRCP, a tandem interaction was made on AACE (Fig. 26b) and resulted in an elongated continuous precipitin arc. This indicated that the same antigen was being recognised in both fractionated and unfractionated lysate. This was further supported by the direct addition of term lysate and term rHbRCP on an AACE (Fig. 26c). On this plate 100 µg term rHbRCP produced approximately the same size peak as 500 µg term lysate, indicating that the concentration of the mobility antigen is about five times higher in the reduced haemoglobin red cell protein preparation.

The fetal rHbRCP produced on the ion exchange column when purifying fetal haemoglobin was analysed on AACEs with adsorbed 6791/2 antisera in a similar manner. The results were indistinguishable from those with fractionated term red cell lysate. A single peak was produced which showed identity with the β mobility antigen in term lysate. To see if adsorbed 6791/2 antisera could detect any change in the β mobility antigen strength during fetal development, 250 µg fetal lysates of various gestational ages were tested by one dimensional AACE with similar samples of term and adult lysate (Fig. 27). The peak height increased from approximately one fifth adult values at 13 weeks gestation to near-adult values at term. This was a very similar pattern to that observed with FRC antisera (see Section B ii of Results). To test the possibility that 6791/2 and FRC antisera were recognising the same antigen in fetal lysates, an interaction between the two antisera was made on an AACE with the 6791/2 antibody bed set in above the FRC antibody bed as in Fig. 28a. The presence of a bed containing FRC antibodies prevented a peak forming in the upper 6791/2 antibody bed and this indicates that the two antisera are recognising the same antigen. The two antisera were also mixed together in an antibody bed (Fig. 28b). The occurrence of a single peak, which is smaller and more intense than with the individual antisera alone indicates an additional effect of the antisera acting

Table 11 Total Protein Applied and Recovered in 6791/2 Affinity Chromatography Experiments.

Sample	Unadsorbed 6791/2		Serum Adsorbed 6791/2	
	Protein Applied mg	Protein Recovered mg	Protein Applied mg	Protein Recovered mg
Blank Eluate	-	0.08	-	0.09
Adult Serum	300	4.6	300	1.1
Term Cord Serum	300	5.7	300	1.0
Adult Red Cell Lysate	300	6.7	300	1.4
Term Cord Red Cell Lysate	300	8.5	300	2.1

Fig. 29 Analysis by Polyacrylamide/SDS Slab Gel Electrophoresis of the Protein Eluted from the Unadsorbed 6791/2 Affinity Column



Track 1 'Blank Eluate'
2 Adult Serum
3 Adult Serum Eluate
4 Term Cord Serum Eluate
5 Term Cord Serum
6 Term Cord Lysate
7 Term Cord Lysate Eluate
8 Adult Lysate Eluate
9 Adult Lysate

6791/2 antisera were raised against term cord lysate which had been fractionated by ion exchange chromatography to concentrate non-

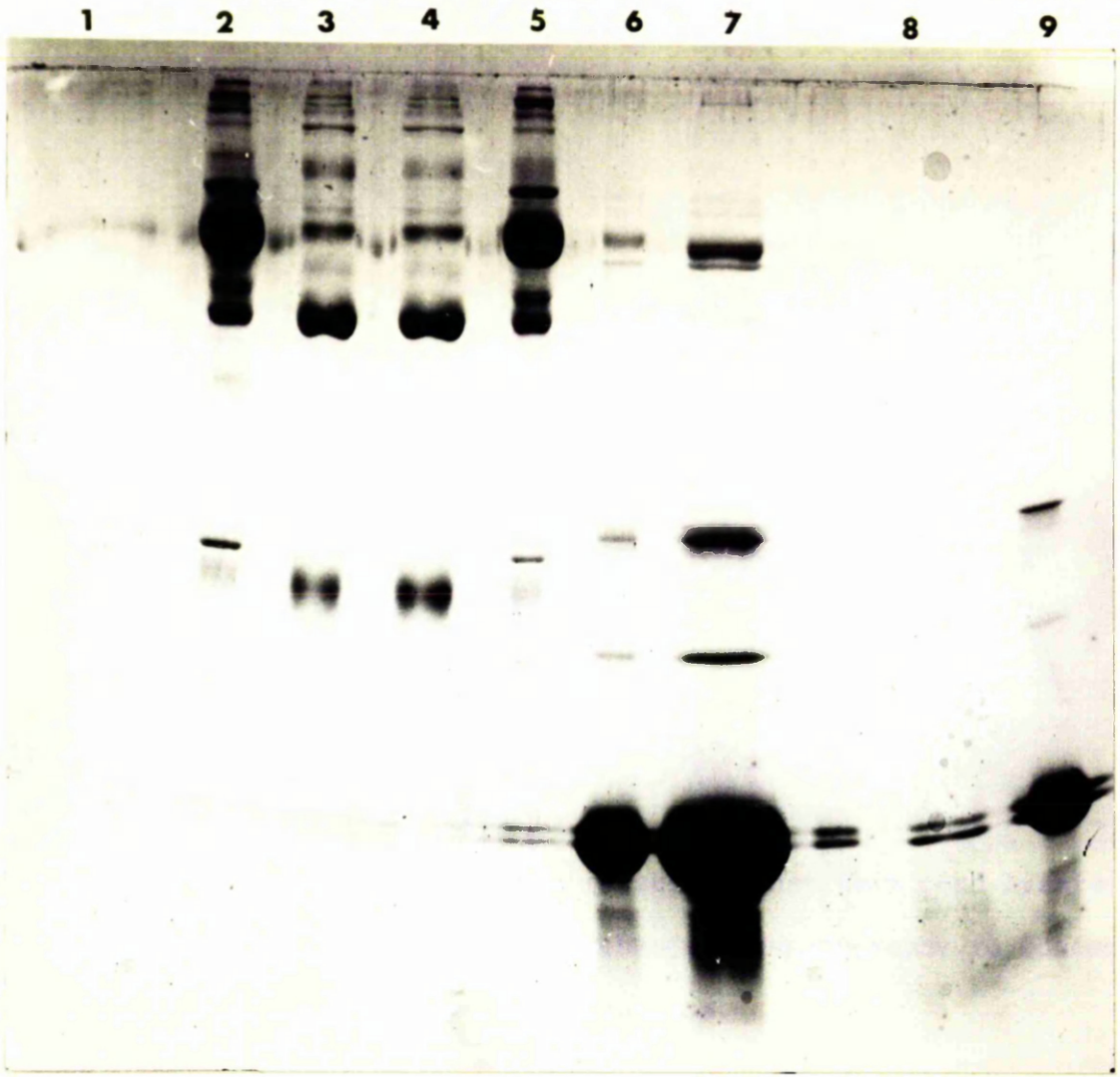
together on the same antigen.

To try to detect any antibodies specific for antigens in fetal red cell lysates, 6791/2 antiserum adsorbed with adult serum was further adsorbed with adult red cell lysate polymerised with gluteraldehyde. This antiserum was then tested on AACEs with 0.5 mg fetal reduced haemoglobin red cell protein or 1 mg fetal lysate or 10 µg fetal lysate. No peaks could be observed with any samples applied, indicating a lack of antibody specificities resistant to adsorption with adult red cell lysate.

c) Affinity Chromatography

It was decided to investigate the possibility of detecting further red cell proteins by using affinity chromatography to increase the concentration of antigens for which 6791/2 antibodies had specificities. One hundred millilitres each of native and serum adsorbed antisera were fractionated by 45% ammonium sulphate precipitation and immobilised on two 100 g aliquots of sepharose. Both the columns were eluted with 2 M KI before any samples were applied. These 'blank' eluates were to control for any protein which might become detached from the affinity columns during subsequent elution cycles. To limit contamination from previous samples, each aliquot of sepharose was divided into two equal parts as with the FRC affinity columns (see page 37). Adult and term lysate were passed over one half, while adult and term cord serum were passed over the other. Table 11 shows the amount of protein (as measured in a Folin protein determination) in the eluates from the various samples applied to the adsorbed and unadsorbed 6791/2 columns. Samples of the protein eluted from each experiment was examined on polyacrylamide/SDS gradient slab gels. Fig. 29 shows the result for the unadsorbed 6791/2 column. The 'blank' eluate (Track 1) shows many bands but this is probably due to contamination from the serum in the next track. There appears to be little difference between the eluted protein obtained from the adult and term cord serum (Tracks 3 and 4) and these tracks have many of the bands found in the corresponding unfractionated serum tracks (2 and 5). The tracks for both the lysates and lysate eluates have far fewer bands than the serum tracks. The eluted protein from term and adult lysates (Tracks 7 and 8) contain a

Fig. 30 Analysis by Polyacrylamide/SDS Slab Gel Electrophoresis of the Protein Eluted from the Serum Adsorbed 6791/2 Affinity Column



Track 1 'Blank Eluate'
2 Adult Serum
3 Adult Serum Eluate
4 Term Cord Serum Eluate
5 Term Cord Serum
6 Term Cord Lysate
7 Term Cord Lysate Eluate
8 Adult Lysate Eluate
9 Adult Lysate

6791/2 antisera were raised against term cord lysate which had been fractionated by ion exchange chromatography to concentrate non-haemoglobin red cell proteins.

Table 12 Summary of the Antibody-Antigen Crossed Electrophoresis Analysis of the Protein Eluted from the Adsorbed and Unadsorbed 6791/2 Columns

Antisera Used for Analysis	Unadsorbed 6791/2		Serum Adsorbed 6791/2	
	Adult Serum / Term Cord Serum	Adult Lysate / Term Cord Lysate	Adult Serum / Term Cord Serum	Adult Lysate / Term Cord Lysate
Sheep anti-adult human serum protein	15+ peaks including albumin. Several intensely staining slow mobility peaks.	8+ very small peaks. No albumin peak. Several peaks bimodal.	5+ small peaks. Several peaks linked together, especially near origin.	2 peaks and 2 lines / 1 line and 1 large very faint peak.
Unadsorbed 6791/2 antiserum	5? mostly faint or incomplete peaks. Term cord serum also has 2 intense peaks	5? faint peaks. 1 intense near origin. Term lysate also has 1 large bimodal peak.	Intense α_2 mobility peak and 2 very faint slower mobility peaks.	Possibility of a few peaks but too faint to see clearly.
Serum adsorbed 6791/2 antiserum	Possibility of 1? large incomplete peak but too faint to see clearly.	Possibility of a β mobility peak.	Possibility of an α_2 mobility peak but too faint to see clearly.	No detectable reactions.

Most of the AACE plates ummarised here showed peaks which were extremely faint and either blurred or incomplete. It was therefore very difficult to compare plates. Most peaks are reported as being present in both adult and term material because the peaks appeared to have the same mobility and general appearance in both cases. The only differences between adult and term material occurred with unadsorbed 6791/2 antiserum, when two extra peaks were observed with term cord serum and one extra bimodal peak with term lysate. These extra specificities all disappeared when the antiserum was adsorbed with adult serum.

few more bands than the unfractionated lysate tracks (6 and 9) and these extra bands appear to correspond to ones in the serum tracks. The thick bands at the bottom of the gel (particularly prominent in the lysate tracks) are caused by haemoglobin chains and the very large, heavily stained bands which are prominent in the serum tracks correspond to albumin.

The slab gel analysis of the eluates from the serum adsorbed 6791/2 column (Fig. 30) follows a similar pattern. There are no obvious differences between the eluted protein from adult and term cord serum (Tracks 3 and 4) although there are many bands present. The term and adult lysate eluates (Tracks 8 and 9) were very similar and only appear to have the two bands corresponding to haemoglobin chains. The 'blank' eluate in Track 1 shows no clear bands.

Examination of the eluates from both native and adsorbed 6791/2 columns was also made on Antibody-Antigen Crossed Electrophoresis. These results are summarised in Table 12.

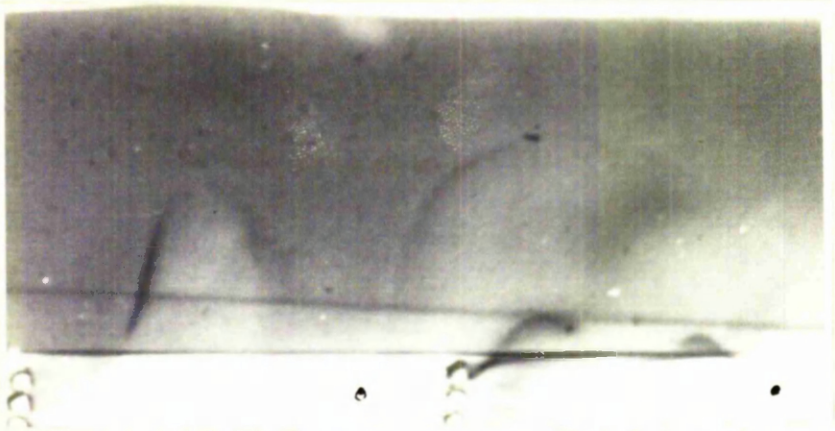
Conclusion

The ion exchange fractionation of the lysates achieved an approximately five-fold concentration of the β mobility antigen recognised in red cell lysates by both FRC and 6791/2 antisera. (Raised against unfractionated fetal lysate and ion exchange fractionated reduced haemoglobin red cell protein from term lysate respectively). No new antigens had been discovered, however, and therefore there was apparently no advantage in immunising with fractionated term lysate which contained the same amount of non-haemoglobin red cell protein. It was therefore decided to take advantage of a different method for blocking the response to unwanted antigens - neonatal tolerance, which was currently available in the laboratory.

E. ANTISERA RAISED IN NEONATALLY TOLERISED RABBITS

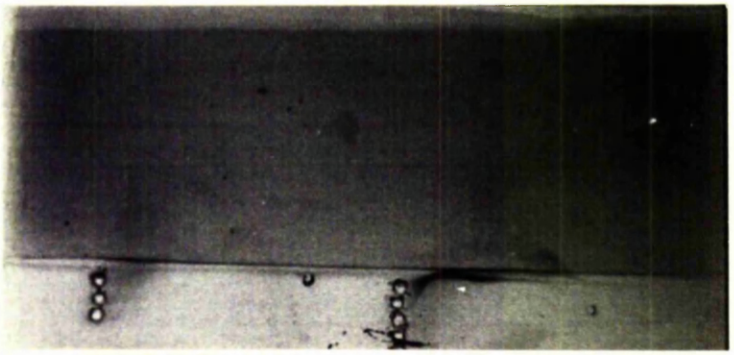
Two young rabbits, made tolerant to adult serum and adult red cell lysate were immunised with 100 mg and 300 mg doses of pooled fetal red cell lysate. These rabbits were numbered 7041 and 7043 respectively.

Fig. 31a AACE of Adult Serum and Fetal Lysate with 7041 Antiserum (After Two Injections of Immunogen)



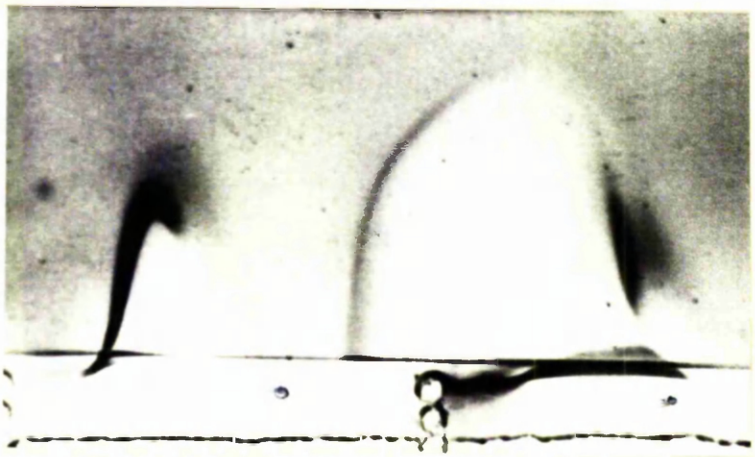
Adult Serum Fetal Lysate

Fig. 31b AACE of Adult Serum and Fetal Lysate with 7043 Antiserum (After Two Injections of Immunogen)



Adult Serum Fetal Lysate

Fig. 31c AACE of Adult Serum and Fetal Lysate with 7041 Antiserum (After Three Injections of Immunogen)

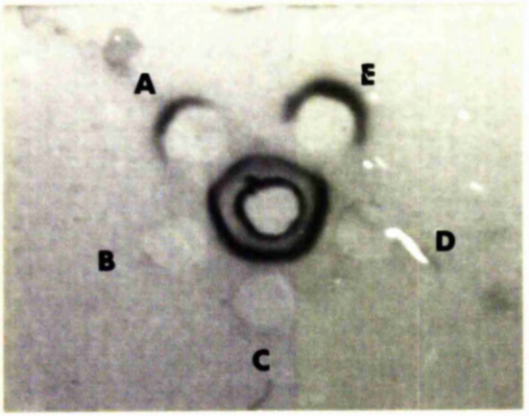


Adult Serum Fetal Lysate

7041 and 7043 antisera were raised against pooled fetal lysate following neonatal tolerisation to adult serum and red cell protein.

The extent of electrophoresis is indicated by a spot on the right hand side of the plate marking the progression of a serum albumin marker.

Fig. 32 Ouchterlony Plate of 7041 Antiserum with Adult, Term and Fetal Lysate, Adult and Fetal Haemoglobin



Well A = HbA
B = HbF
C = Term Cord Lysate
D = Fetal Lysate
E = Adult Lysate
Centre Well = 7041 Antiserum

7041 antiserum was raised against pooled fetal lysate following neonatal tolerisation to adult serum and red cell protein.

a) Reactions of Unadsorbed Antisera

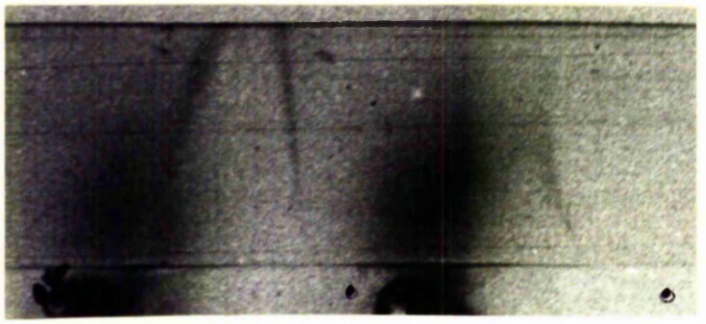
The rabbits were bled before and after the initial injection of immunogen. The antisera were tested with pooled adult serum on Antibody-Antigen Crossed Electrophoresis (AACE). At both times no peaks were observed with 7043 antiserum. Three extremely faint peaks were initially noted with 7041 antiserum, but these disappeared after immunisation.

These antisera were examined again after the first booster injection (four weeks after the initial immunisation). When 7041 antiserum was tested on AACE with pooled adult serum a weak half-peak was observed. There was no reaction with 10 µg adult, term and fetal red cell lysates but examination of 1 mg fetal lysate showed two small peaks and half of a weak slow mobility peak (Fig. 31a). Examination of 7043 antiserum on AACE with pooled adult serum showed two or three very faint peaks. There was no reaction observed with 10 µg adult, term and fetal lysates, but examination of 1 mg fetal lysate showed a very slow mobility peak and another very faint faster mobility peak (Fig. 31b).

After the second booster injection, examination of 7041 antiserum on AACE showed that the half peak observed with adult serum had intensified and the response to 1 mg fetal lysate now included a large faint haemoglobin-type peak with the small peaks originally observed (Fig. 31c). This was also the response observed after the third boost. The responses of 7043 antibodies as tested on AACE never improved beyond two extremely faint peaks with adult serum and one faint incomplete peak with fetal lysate. This rabbit was therefore discarded after the second boost.

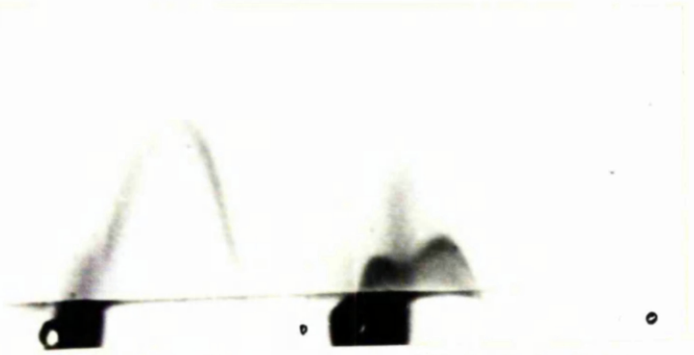
After the second boost, examination of 7041 antiserum on AACE with 10 µg adult, term and fetal lysates showed a haemoglobin pattern very similar to that observed with FRC and 6791/2 antisera. Term and fetal lysates gave intense peaks while adult lysate produced a weak hazy peak. An Ouchterlony plate was set up to compare the reactions of unadsorbed 7041 antiserum with adult, term and fetal red cell lysates (RCLs), commercial adult haemoglobin (HbA) and ion exchange purified fetal haemoglobin (HbF). The result (Fig. 32) showed fused lines of identity between term RCL, fetal RCL and HbF which had very faint

Fig. 33a AACE of Adult and Fetal Lysate with 7041 Antiserum Adsorbed with Adult Serum (After Two Injections of Immunogen)



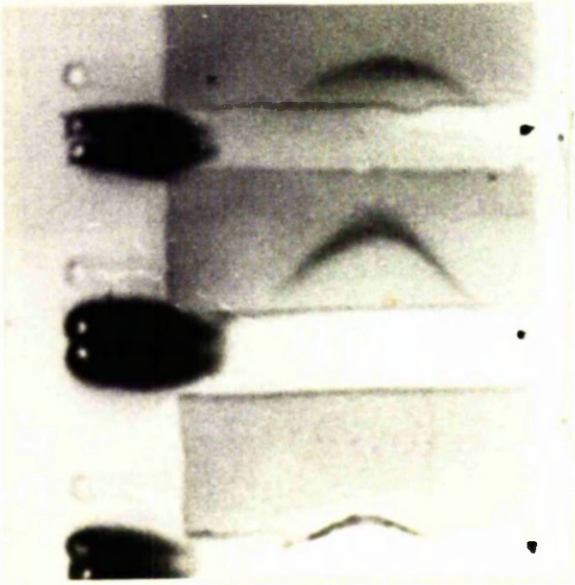
Adult Lysate Fetal Lysate

Fig. 33b AACE of Adult and Fetal Lysate with 7041 Antiserum Adsorbed with Adult Serum (After Three Injections of Immunogen)



Adult Lysate Fetal Lysate

Fig. 34 AACE of Adult and Fetal Lysate with Serum Adsorbed 7041 Antiserum



Adult Lysate

Adult plus Fetal Lysate

Fetal Lysate

7041 antiserum was raised against pooled fetal lysate following neonatal tolerisation to adult serum and red cell protein.

The extent of electrophoresis is indicated by a spot on the right hand side of the plate marking the progression of a serum albumin marker.

Table 13 Summary of Reactions of Adsorbed 7041 Antiserum Observed on Antibody-Antigen Crossed Electrophoresis

	Adult Lysate		Fetal Lysate	
	Grade of Peak Intensity	Type of Peak	Grade of Peak Intensity	Type of Peak
First Boost 14.6.77				
7 days after 1st boost	1	single	2	single
10 "	1	single	2	single
14 "	2	single	3	bimodal
17 "	No visible reaction		1	bimodal
24 "	"		3	bimodal
31 "	"		1	single
Second Boost 19.7.77				
7 days after 2nd boost	2	single	3	bimodal
11 "	4	single	4	bimodal
15 "	4	single	3	single?
Third Boost 25.8.77				
7 days after 3rd boost	1	single	2	single

Key 1 = very faint indistinct peak
2 = indistinct peak
3 = clear peak
4 = very intense clear peak

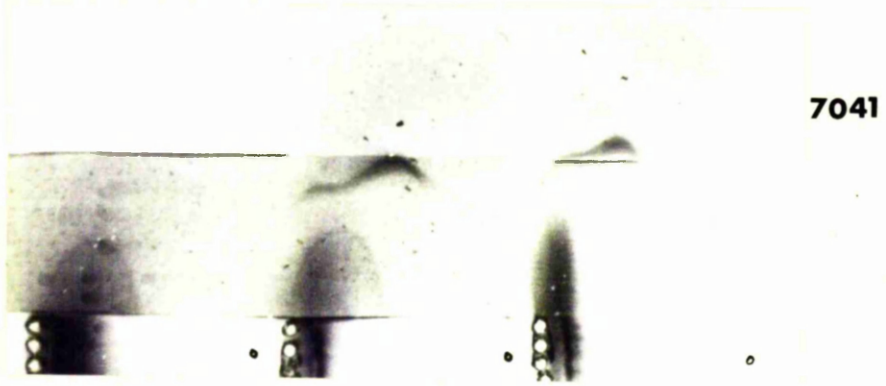
spurs which extended past fainter fused lines of identity for adult RCL and HbA. This indicates partial identity between adult and fetal haemoglobin, with some extra determinants being recognised on fetal haemoglobin by this antiserum. This result was also recorded for FRC and 6791/2 antisera.

b) Reactions of Antisera Adsorbed with Adult Human Serum

Adsorption of 7041 antibodies from after the first boost, with polymerised adult serum resulted in a loss of reactivity with adult or fetal serum when tested on AACE. Examination by AACE of 10 µg adult, term and fetal lysate with adsorbed 7041 antiserum showed extremely faint haemoglobin peaks only for term and fetal lysates. When 1 mg adult and fetal lysate were examined by AACE, one indistinct peak was observed, which was much larger with adult lysate (Fig. 33a). Addition of these two lysates together produced a single peak indicating that the antigens share identity. Examination by AACE of adsorbed 7041 antiserum after the second boost showed a distinct bimodal peak with 1 mg fetal lysate and a larger monomodal peak with adult lysate (Fig. 33b). The bimodal fetal peak suggests two electrophoretic variants of a single protein and to see which of the fetal peaks the adult peak corresponded to, the fetal and adult lysates were run separately and together in vertical parallel positions so that the electrophoretic charge across the plate was the same for all the samples. Unfortunately, two fetal peaks were never produced whenever this experiment was attempted (see Fig. 34). Adsorbed 7041 antiserum produced extremely variable results. There was considerable difference between the number, height and intensity of peaks observed on AACE from bleeds only a few days apart (Table 13). This variability even occurred with adsorbed antiserum from the same date used on different occasions. These erratic results made interpretation or manipulation of antigens to show identity extremely difficult. Individual fetal lysates of varying gestational ages gave different numbers and sizes of peaks every time they were examined by AACE with adsorbed 7041 antiserum. However, peaks formed with adult lysates were never bimodal, and tended to be larger than peaks which were formed when fetal lysates were examined.

In general, the AACE peaks observed when 1 mg adult and fetal

Fig. 35 AACE of Fetal Lysate with Serum Adsorbed 7041 Antiserum (Top) and Serum Adsorbed 6791/2 Antisera (Bottom)



7041 antiserum was raised against pooled fetal lysate following neonatal tolerisation to adult serum and red cell protein.

6791/2 antisera were raised against term cord lysate which had been fractionated by ion exchange chromatography to concentrate non-haemoglobin red cell proteins.

lysates were tested with adsorbed 7041 antiserum were of α_2 mobility and were pointed at the apex. The peaks observed when adsorbed FRC and 6791/2 antisera were tested on AACE with 1 mg adult and fetal lysate were of β mobility and were much more rounded in outline. To test for antibody interactions between 7041, FRC and 6791/2 antisera, 1 mg fetal lysate was applied to AACEs with two antibody beds. Adsorbed FRC or 6791/2 antibodies were placed in the lower bed nearest the track of first dimension electrophoresis with adsorbed 7041 antibodies in the upper bed (see Fig. 35). This resulted in a rounded β mobility peak forming in the lower bed and a faster mobility, pointed bimodal peak appearing nearer the upper 7041 antibody bed. The bimodal peak did not merge or fuse with the β mobility peak and did not form from the base of the FRC or 6791/2 antibody beds. This suggests that the bimodal antigen does not have any identity with the mobility antigen and is probably not recognised by FRC and 6791/2 antibodies. The lowering of the bimodal peaks from the 7041 antibody bed could be due to the backward diffusion of antibodies towards the cathode during second dimension electrophoresis. Experimentation with the design of AACEs made during the course of other laboratory investigations showed that it was possible to get a precipitate peak to appear some distance below an antibody bed provided the bed contained concentrated antibodies and was set far enough back from the first dimension electrophoresis track.

To try to elucidate the antibody identities, 1 mg fetal lysate was applied to AACEs in which adsorbed 7041 antibodies were mixed with adsorbed FRC or 6791/2 antibodies in a single bed, and compared with the results from antisera in individual beds. Unfortunately, the results were inconclusive again since the 7041 antibodies did not produce any precipitate peaks when this experiment was attempted.

To try to detect any antibodies specific for antigens in fetal red cell lysates, adsorbed 7041 antiserum was further adsorbed with adult red cell lysate polymerised with gluteraldehyde. This antiserum was then tested on AACEs with 10 µg and 1 mg adult, term and fetal lysates. No peaks could be observed with any samples applied indicating a lack of antibody specificities resistant to adsorption with adult red cell lysate.

Table 14 Summary of Results Obtained from Examination of Unadsorbed Antisera by Antibody-Antigen Crossed Electrophoresis

	5895	FRC 1/2	6791/2	7041/3
Type of Immunogen	Term Cord Red Cell Lysate	Fetal Lysate	Reduced haemoglobin red cell protein (ion exchange fractionated) Term Lysate	Fetal Lysate after tolerisation with adult serum and red cells
RESPONSES OF UNADSORBED ANTISERA to: Adult Serum	Half peak	4 peaks initially - increasing to 10+ peaks after second boost including half peak	15+ peaks including half peak	No clear response initially - intensifying to clear half peak after second boost
10 ug Lysates	Very intense haemoglobin peaks with term and fetal lysates. Distinct peak with adult lysate.	Distinct haemoglobin peaks with term and fetal lysates. Much fainter haemoglobin peak with adult lysate.		
1 mg Lysates	Greatly enlarged haemoglobin peak	4 - 5 indistinct peaks including haemoglobin peak with fetal lysate	Adult - 1 clear peak, haemoglobin outline, albumin, + 1 other? Term - 3 clear peaks, haemoglobin outline, + 1 other?	1 peak + weak fetal haemoglobin peak after second boost
0.5 mg Reduced Haemoglobin Red Cell Protein			Adult and term - 1 clear peak, 6 + large, indistinct peaks	

There are no major differences detected by AACE in the response to adult and term lysate by any of the antisera. The peaks reported were of the same mobility and appearance in both adult and term lysate samples. However, the response of unadsorbed 6791/2 antisera to term lysate did contain one clear peak not present in adult lysate (see Fig. 24A) but this disappeared completely when the antiserum was absorbed with serum protein.

Table 15. Summary of Results Obtained from Examination of Adsorbed Antisera by Antibody-Antigen Crossed Electrophoresis

	5895	FRC 1/2	6791/2	7041/3
Type of Immunogen	Term Cord Red Cell Lysate	Fetal Lysate	Reduced Haemoglobin Red Cell Protein (ion exchange fractionated) Term Lysate	Fetal Lysate after tolerisation with adult serum and red cells
RESPONSES OF ANTISERA ADSORBED WITH ADULT SERUM to: Adult Serum	No peaks observed with adult or fetal serum			
10 µg Lysates	Clear haemoglobin peaks with term and fetal lysates. No peak with adult lysate.		Faint haemoglobin peak with term and fetal lysate. No peak with adult lysate.	Very faint, indistinct haemoglobin peaks with term and fetal lysate. No peak with adult lysate.
1 mg Lysates	No reaction	1/β mobility rounded peak with same determinants recognised by FRC and 6791/2 antisera. Concentration in early fetal lysates approximately fifth adult values.		Generally one α_2 mobility peak with adult lysate. Smaller, sometimes bi-modal peak with fetal lysate. Very erratic results.
0.5 mg Reduced Haemoglobin Red Cell Protein			Same β mobility antigen as detected in unfractionated lysates plus further weak peak with adult rHbRCP	
RESPONSES OF ANTISERA ADSORBED WITH ADULT SERUM AND ADULT RED CELL LYSATE	No Reaction Observed			

Conclusion

It is very difficult to draw any conclusions about the reactions of this antiserum. It would appear that the cause of the variability does lie with the antiserum and not with the red cell lysates, since these continued to give reproducible results with other antisera. A gel precipitation system does have many advantages for screening antibodies for unknown specificities. The techniques are fairly simple and not time consuming. The results are permanently recorded and a technique such as Antibody-Antigen Crossed Electrophoresis (AACE) is highly adaptable and multiple specificities of several antisera can be demonstrated by varying the design of the plate. Tables 14 and 15 summarise the results obtained from examination by AACE of the antisera reported in this thesis.

Gel precipitation techniques are limited, however, to antigens which can form a lattice at equivalence with the particular concentration of specific antibodies present. In view of the inconsistent results obtained by examination of 7041 and 7043 antisera by AACE it was decided to investigate other methods of detecting antibody/antigen reactions. Agglutination of red blood cells, either directly or with a coating of antigen is a commonly used detection method, and it was felt that for examination of antisera raised against red cell proteins, this would be a good technique to try in the time available.

F. HAEMAGGLUTINATION EXPERIMENTS

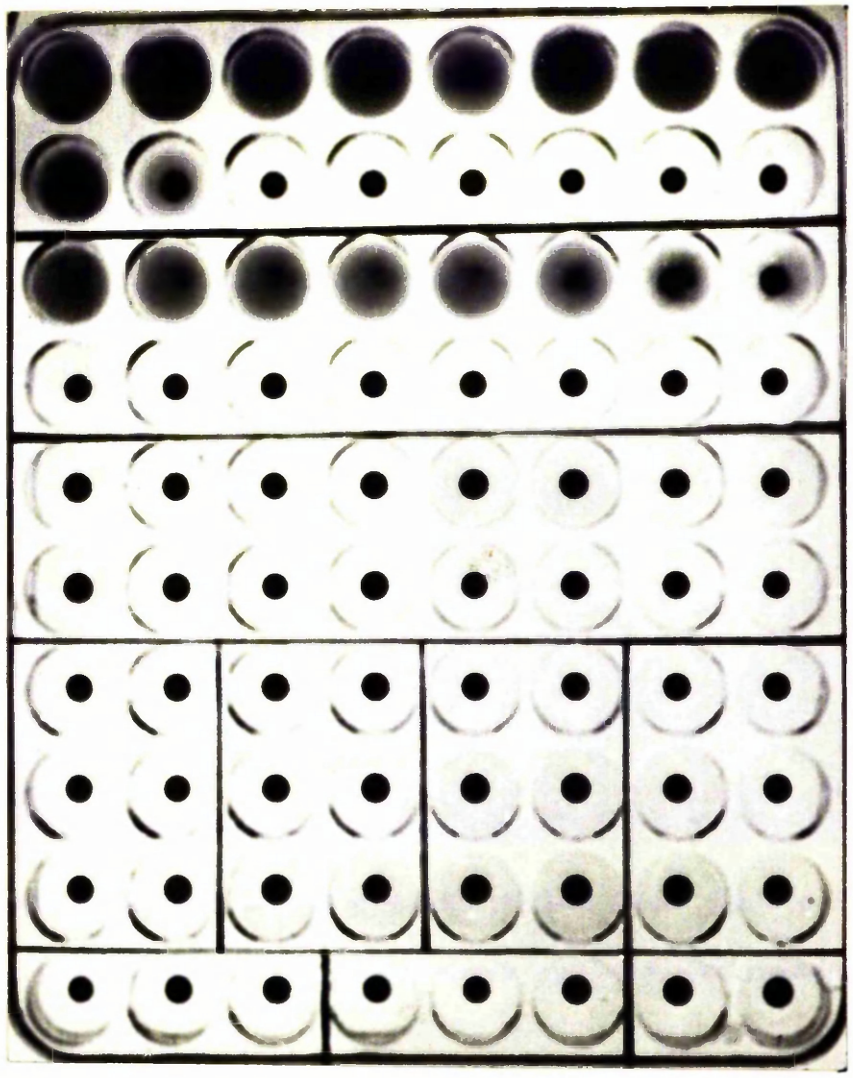
1. Direct Agglutination

Freshly washed samples of red cells from adult and term cord blood were tested for ABO blood group antigens by Mrs. Betty O'Hare. Only Group O blood cells were used for agglutination experiments. The cells were diluted to a 1% solution with a diluent consisting of phosphate buffered saline with 1% decomplemented control rabbit serum (to prevent spontaneous agglutination). Antisera to be tested were adsorbed with rabbit red cells (to remove any non-specific agglutinins) before decomplementing by heat treatment at 56^{o}C for thirty minutes.

Table 16 Summary of Results from Direct Agglutination Experiments

Antiserum	Dilution End Point for Agglutination of Term Cord Red Cells	Dilution End Point for Agglutination of Adult Red Cells
5895	No agglutination	No agglutination
FRC 1/2 after 1st boost	1/10	No agglutination
2nd	1/160	1/160
6791	No agglutination	No agglutination
6792	No agglutination	No agglutination
7041 after 1st boost	1/160	1/160
2nd	1/1280	1/320
3rd	1/320	1/160
7043 after 1st boost	1/320	1/320
2nd	1/1280	1/640

Fig. 36 Direct Agglutination of Adult, Term Cord and Control Rabbit Red Blood Cells with 7041 Antiserum Adsorbed with Adult Serum



Term Cord Cells — Dilutions of 7041 Antiserum — End Point = 1/1280

Adult Cells — Dilutions of 7041 Antiserum — End Point = 1/320

Control Rabbit Cells — Dilutions of 7041 Antiserum

Term, Adult and Rabbit Cells — Undiluted Serum from four Unimmunised Rabbits

Term, Adult and Rabbit Cells — Diluent alone (No Rabbit Serum)

7041 antiserum was raised against pooled fetal lysate following

neonatal tolerisation to adult serum and red cell protein

0.1 ml dilutions of antiserum in diluent were made from 0 to 1/163840 (doubling dilutions from 1/10) in perspex agglutination trays. 0.1 ml of the 1% adult and term cells were added and the trays were covered before leaving at room temperature overnight. One per cent rabbit red cell solution was also tested at 1/10 antiserum dilution to control for non-specific agglutination by the antiserum. Serum at 1/10 dilution from four control rabbits (A, B, C and D) was tested with adult, term and rabbit red cells to control for spontaneous agglutination of the cells. Cells were also tested with diluent alone. The results of the direct agglutination experiments are summarised in Table 16. Figure 36 shows the result of the agglutination experiment testing 7041 antiserum after the second boost. The FRC, 7041 and 7043 antisera which had agglutinated term and adult cells were adsorbed with adult red cells and retested to try to detect any specific agglutinins for term red blood cells. No agglutination for either adult or term cells was observed in all cases.

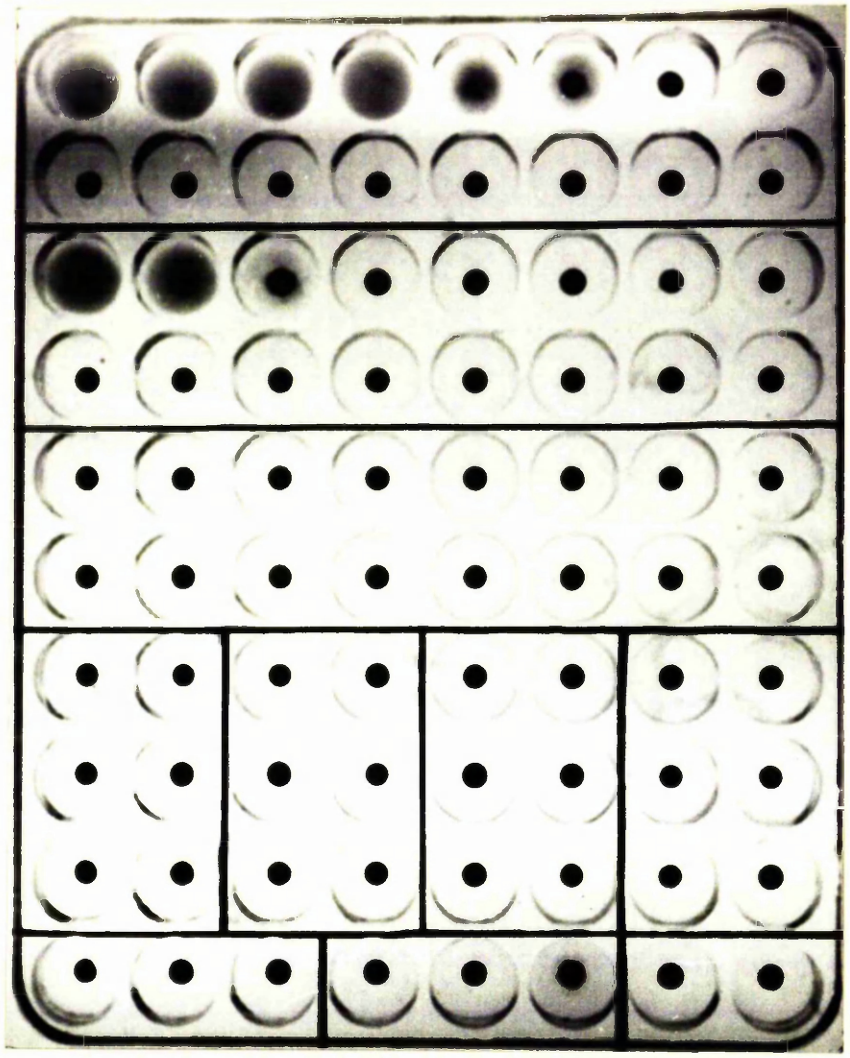
2. Agglutination of Tanned Cells

Fresh sheep red blood cells were formalised and tanned according to the methods of Herbert (1973) (see Materials and Methods for details). A 20 ml aliquot of these cells was then coated with fetal haemoglobin eluted during the CM sepharose ion exchange fractionation of fetal red cell lysate (see Section C of Results) at a concentration of 0.1 mg/ml. A further aliquot of tanned formalised cells was coated with commercially purified adult haemoglobin at a similar concentration. These cells were diluted with phosphate buffered saline containing 1% decomplemented control rabbit serum. With the addition of 0.01% sodium azide as a preservative these cells were kept at $4^{o}C$ for several months without any loss of reactivity. Antisera which had been used for direct agglutination experiments were further adsorbed with uncoated tanned formalised sheep cells and decomplemented again. The agglutination trays were set up with 0.1 ml dilutions of antiserum as before and 0.1 ml tanned cells coated with either adult or fetal haemoglobin were added. Uncoated tanned cells were used instead of rabbit red cells to control for non-specific agglutination.

Table 17 Summary of Results of Agglutination Experiments with Tanned Cells

Antiserum	Dilution End Point for Agglutination of Tanned Cells Coated with Fetal Haemoglobin	Dilution End Point for Agglutination of Tanned Cells Coated with Adult Haemoglobin
5895	1/80	0 - 1/10
FRC	0 - 1/10	0
6791/2	No agglutination	No agglutination
7041/3	No agglutination	No agglutination

Fig. 37 Tanned Cell Agglutination with 5895 Antiserum



Cells Coated with HbF

Dilutions of 5895 Antiserum — End Point = 1/80

Cells Coated with HbA

Dilutions of 5895 Antiserum — End Point = 0-1/10

Control Tanned Cells without Coating

Dilutions of 5895 Antiserum

Tanned Cells Alone, With HbA, and HbF

Undiluted Serum from four Unimmunised Rabbits

Tanned Cells Alone, With HbA, and HbF

Diluent Alone (No Rabbit Serum)

5895 antiserum was raised against unfractionated term cord red cell lysate.

The results of the experiments with tanned cells are summarised in Table 17. Figure 37 shows the results of the agglutination of tanned cells with 5895 antiserum. The 5895 and FRC antisera which had shown some agglutination ability were adsorbed with tanned red cells which had been coated with adult haemoglobin and retested to try to detect any specificities for fetal haemoglobin. No agglutination was observed.

Conclusion

The antisera which had been raised by injecting large amounts (100 mg and 300 mg) of fetal lysate contained antibodies= with specificities for red cell membranes. This was possibly due to incomplete spinning down of the membrane 'ghosts' when preparing the lysates from semi-clotted blood. This could result in the injection of membrane particles with the fetal lysate protein. 7043 antiserum, which produced very weak and indistinct precipitate arcs on Antibody Antigen Crossed Electrophoresis, had quite a high agglutination titre for adult and term red cells.

Tanned, formalised cells were much easier to handle than fresh red cells and produced more precise agglutination end-points. Two antisera (6791/2 and 7041) did not show any agglutination ability with tanned cells coated with adult or fetal haemoglobin although distinct haemoglobin specificities had been observed by gel precipitation techniques. This may well have been due to the lack of experience with the coating of tanned cells resulting in low or uneven distribution of antigen on the cells and causing low agglutination titres.

Having gained some degree of familiarity with this technique it would have been possible to continue with this line of approach and investigate the importance of various factors like decomplementation, non-specific agglutination and the concentration of antigen used to coat cells. It would also have been possible to coat the tanned cells with other purified red cell antigens or investigated agglutination inhibition by the addition of monospecific antisera. However, it was felt that the time required to succeed with such a line of research was greater than that available and so it was decided to return to familiar gel precipitation techniques to investigate further a particular result which had been noted with all of the

Table 18 Summary of Haemoglobin Additions to Pooled Adult Serum

Volume of Pooled Adult Serum (Containing 0.04 mg/ml Hb) ml	Hb Added at 100 mg/ml ul	mg	% By Volume Hb Added	Total Hb Present mg/ml
1.0	1	0.1	0.1	0.14
1.0	5	0.5	0.5	0.54
0.99	10	1.0	1.0	1.04
0.95	50	5.0	5.0	5.04
0.9	100	10.0	10.0	10.04

Fig. 38a AACE of 5895 Antiserum with Adult Serum Containing Increasing Amounts of Adult Haemoglobin

Sample
A = PAS
B = PAS + 0.1% HbA
C = PAS + 0.5% HbA
D = PAS + 1% HbA
E = PAS + 5% HbA
F = PAS + 10% HbA

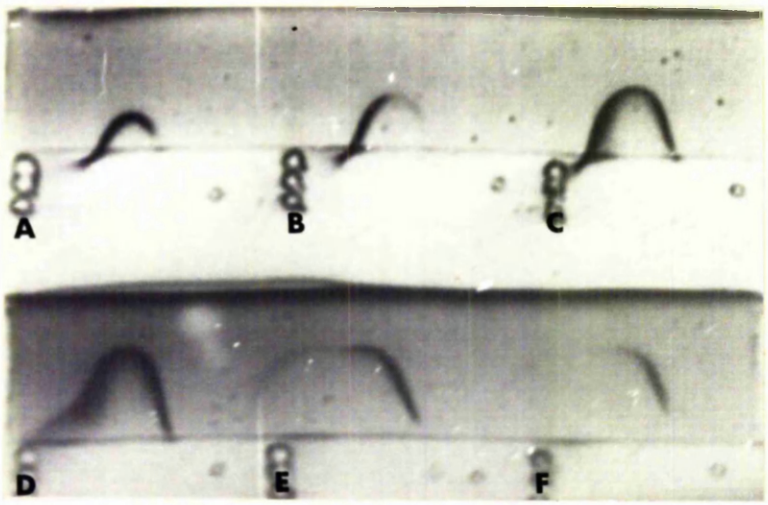


Fig. 38b AACE of 5895 Antiserum with Adult Serum Containing Increasing Amounts of Term Lysate (TRCL)

Sample
A = PAS
B = PAS + 0.1% TRCL
C = PAS + 0.5% TRCL
D = PAS + 1% TRCL
E = PAS + 5% TRCL
F = PAS + 10% TRCL

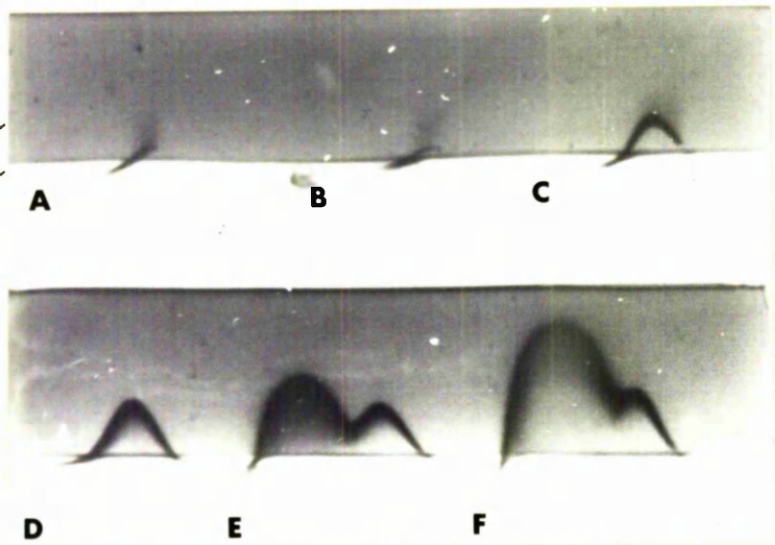
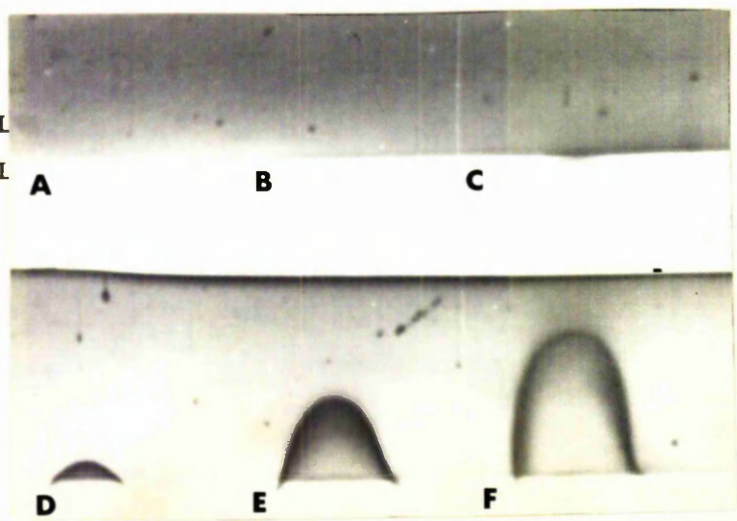


Fig. 38c AACE of 5895 Antiserum with Saline Containing Increasing Amounts of Term Lysate (TRCL)

Sample
A = Saline
B = Saline + 0.1% TRCL
C = Saline + 0.5% TRCL
D = Saline + 1% TRCL
E = Saline + 5% TRCL
F = Saline + 10% TRCL



5895 antiserum was raised against unfractionated term cord red cell lysate.

antisera raised in the course of this investigation - the peculiar half peak formed when adult serum was examined by Antibody Antigen Crossed Electrophoresis.

G. FURTHER INVESTIGATION OF THE IMMUNOLOGICAL NATURE OF THE HAEMOGLOBIN-HAPTOGLOBIN COMPLEX

All of the antisera raised against whole or fractionated lysates produced an intense half peak when tested with pooled adult serum on AACE (Figs. 10a, 12 and 31c). As described previously (page 33 , Figs. 11a and b) the half peak gave reactions of identity with commercial anti-haptoglobin antiserum. Further, the same half peak gave reactions of apparent identity with commercial anti-adult haemoglobin (Fig. 11c). This raised the question as to what properties of the antigen, and what specificities of antibody could account for this very unusual incomplete peak.

The antiserum used in these investigations was from rabbit 5895, which was immunised with 20 mg doses of term red cell lysate. This antiserum was chosen because of the simplicity of its reactions when examined on AACE. Only an intense half peak was formed when tested with pooled adult serum (PAS), and a clear haemoglobin peak when tested with adult lysate.

Since the presence of haemoglobin affected the shape of this half peak, controlled amounts of adult red cell lysate (RCL) or commercially purified adult haemoglobin (HbA) were added to aliquots of PAS according to Table 18.

These PAS samples were subjected to a short period of electrophoresis for a two dimensional AACE. Addition of adult lysate or HbA gave identical results (Fig. 38a). The half peak initially increases in height (+ .1% Hb), becomes a complete peak (+ .5% Hb), gains a small cathodal spur (+ 1% Hb), becomes fused to a much fainter peak on the cathodal side (+ 5% Hb) and becomes increasingly smaller and less complete on the cathodal side (+ 10% Hb). The very faint fused peak on the cathodal side had the mobility and shape of a haemoglobin peak.

Since this antiserum had a fairly weak response to adult haemoglobin, but a much stronger response to haemoglobin in term

Fig. 39 AACE of Commercial Anti-Adult Haemoglobin Antiserum with Adult Serum Containing Increasing Amounts of Adult Haemoglobin

Sample

A = PAS

B = PAS + 0.1% HbA

C = PAS + 0.5% HbA

D = PAS + 1% HbA

E = PAS + 5% HbA

F = PAS + 10% HbA

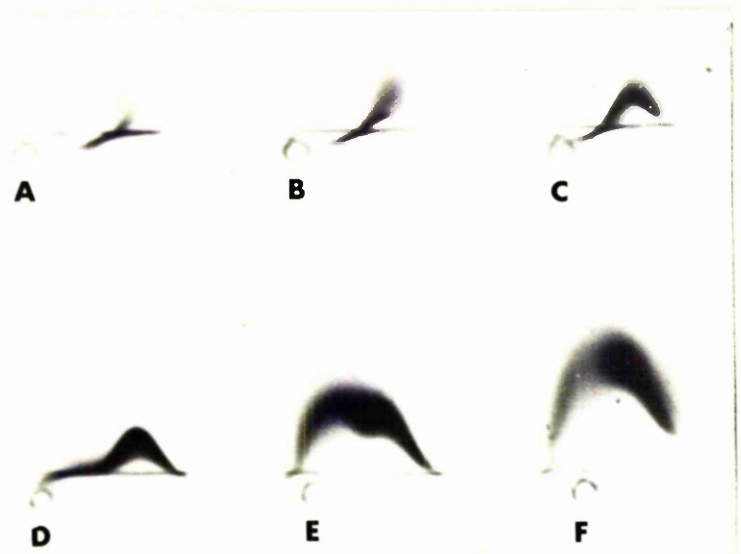


Fig. 40 AACE of Commercial Anti-Haptoglobin Antiserum with Adult Serum Containing Increasing Amounts of Adult Haemoglobin

Sample

A = PAS

B = PAS + 0.1% HbA

C = PAS + 0.5% HbA

D = PAS + 1% HbA

E = PAS + 5% HbA

F = PAS + 10% HbA

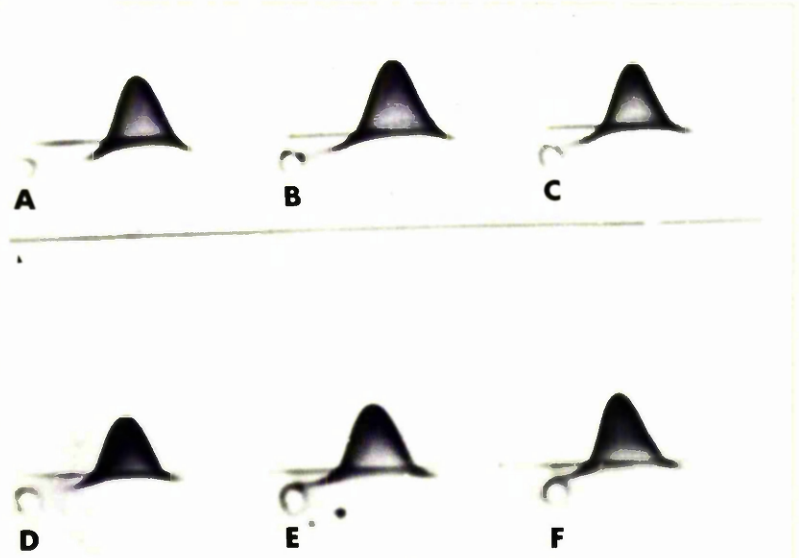
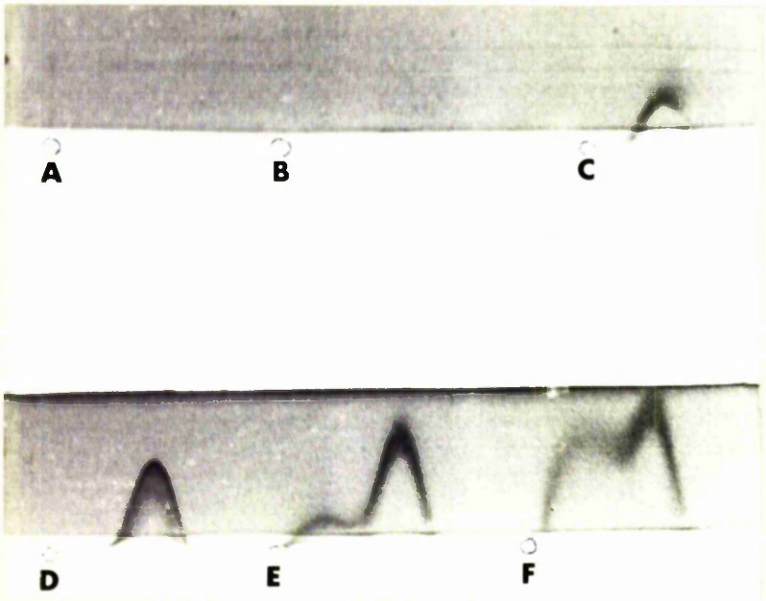


Table 19 Summary of Haemoglobin Additions to Partially-Purified Haptoglobin

Volume of Haptoglobin Containing 1.25 mg/ml protein (ml)	Term Lysate Added at 100 mg/ml µl	µg	% By Volume Term Lysate Added
1.0	.1	10	0.01
1.0	.5	50	0.05
1.0	1	100	0.1
1.0	5	500	0.5
0.99	10	1000	1.0

Fig. 41 AACE of 5895 Antiserum with Partially Purified Haptoglobin Containing Increasing Amounts of Term Cord Lysate (TRCL)

Sample A = Hp
B = Hp + 0.01% TRCL
C = Hp + 0.05% TRCL
D = Hp + 0.1% TRCL
E = Hp + 0.5% TRCL
F = Hp + 1% TRCL



5895 antiserum was raised against unfractionated term cord red cell lysate

lysate, this experiment was repeated with additions of term lysate to PAS and with equivalent dilutions of term lysate in phosphate buffered saline (Figs. 38b and c). The addition of term lysate to PAS intensified the cathodal peak visible with additions of 5% and 10% term lysate. The equivalent dilutions of term lysate without PAS produced peaks of the characteristic haemoglobin type in the same position and of the same size as the cathodal peaks.

Pooled adult serum samples with additions of adult haemoglobin were also analysed on AACE with commercial anti-adult haemoglobin antisera (Fig. 39). This resulted in a very similar pattern of peak changes to that observed with 5895 antiserum. Similar analysis with anti-haptoglobin antiserum showed uniform completed peaks regardless of the amount of haemoglobin added (Fig. 40).

To test that haptoglobin is involved in these reactions a sample of haptoglobin was partially purified on a DEAE sepharose ion exchange column using 0.01 M - 0.08 M acetate buffers pH 4.7, (see Materials and Methods for details). This preparation of haptoglobin was then tested against 5895 antiserum by AACE, in the presence of increasing amounts of term lysate as in Table 19. The effect of adding term lysate to the partially purified haptoglobin (Fig. 41) was very similar to that observed when term lysate was added to pooled adult serum (Fig. 38b) although the haptoglobin alone did not form a precipitate arc.

At this stage it was tentatively concluded that the precipitate arc observed was caused by haemoglobin-haptoglobin complexes and that the half peak became complete when haptoglobin was saturated with haemoglobin. To test this hypothesis, the isolation of haemoglobin-haptoglobin complexes was required. This could be achieved by gel filtration if haptoglobin of a known molecular weight was used. Haptoglobin occurs in three major phenotypes, 1.1, 2.1 and 2.2 which are distinguishable by banding patterns on starch gels. Haptoglobin 2.1 and 2.2 form chains of polymers up to 700,000 daltons M.Wt. However, haptoglobin 1.1 exists as discrete molecules of approximately 100,000 daltons M.Wt. (Putnam, 1975).

Because serum from a very large number of individuals was pooled to form the PAS stock serum, all the 3 major haptoglobin types were present. Serum samples were therefore taken from volunteers in the laboratory for haptoglobin typing, which was

Fig. 42 Elution Profile from a 2.6 x 90 cm Ultrogel AcA 34 Column to which 2 ml Serum (Haptoglobin 1:1 phenotype) plus 20 mg Haemoglobin had been Applied

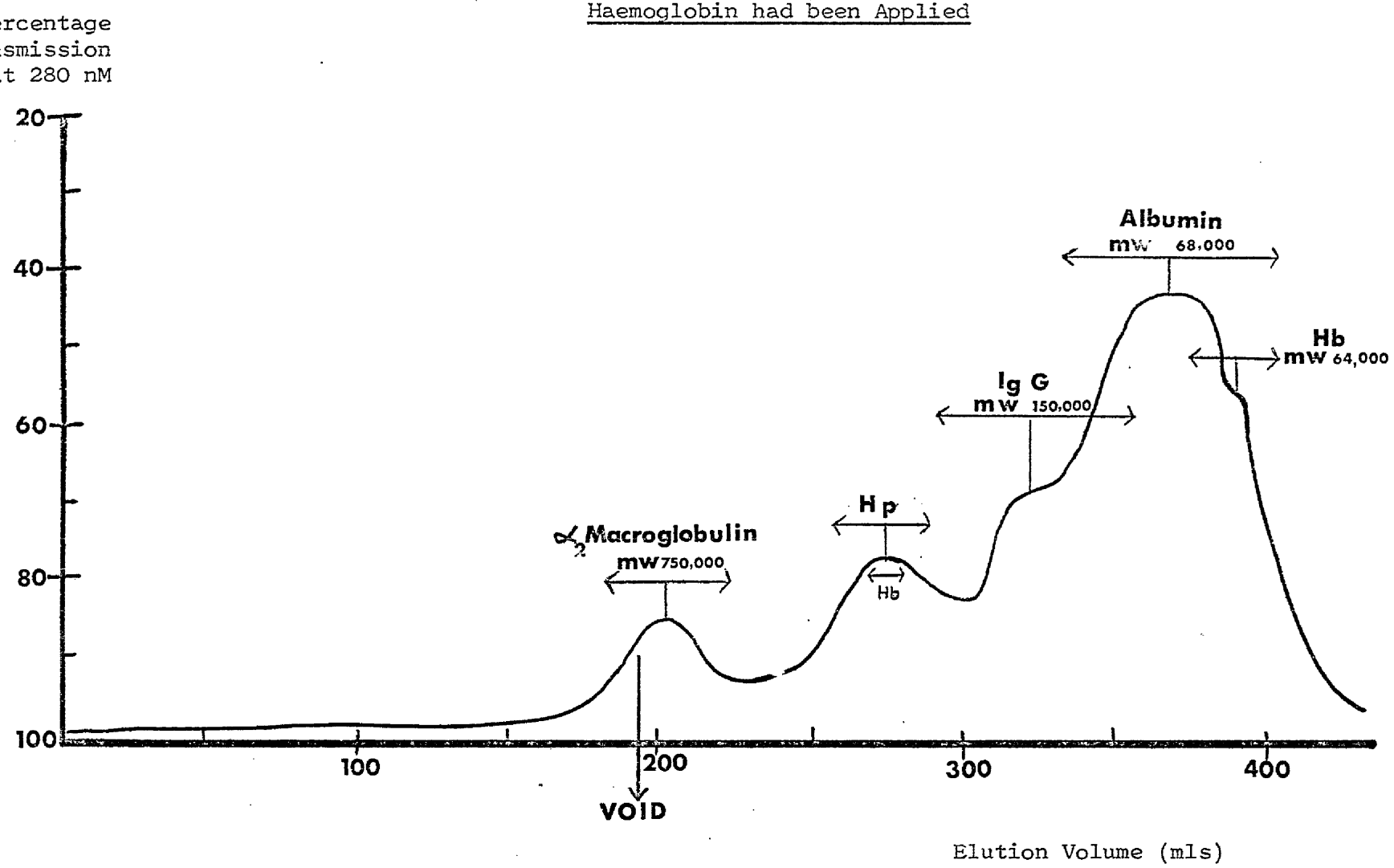
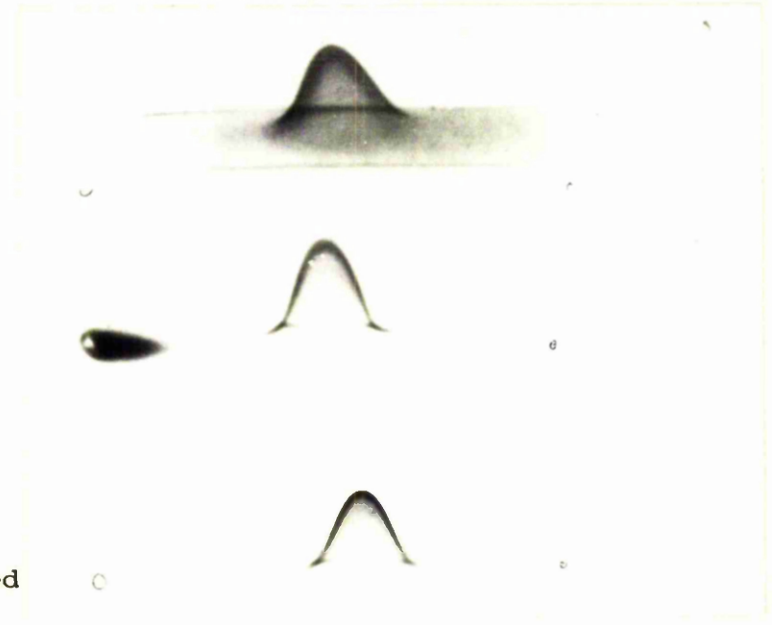


Fig. 43 AACE of Commercial Anti-Haptoglobin Antiserum with Unfractionated Serum of Type 1:1, Isolated Haemoglobin-Haptoglobin 1:1 Complexes and Partially Purified Haptoglobin 1:1. (Samples run in Parallel)



kindly performed by Mrs. Marion Stone. One of the volunteers (BK) had the Hp 1.1 phenotype. BK serum produced the same pattern of peaks (when tested on AACE with 5895 antiserum) as that observed with samples of pooled adult serum to which adult haemoglobin had been added (see Fig. 38a).

To isolate haemoglobin-haptoglobin complexes, a solution of BK serum containing 10.04 mg/ml haemoglobin was passed over an Ultrogel AcA 34 column. (Fractionation range 20,000 - 350,000 daltons). Fractions containing albumin, immunoglobin (IgG), haptoglobin and α_2 macroglobulin were located by one dimensional AACE using commercial antisera. Fractions containing haemoglobin were located visually and the peak fractions confirmed by optical density readings at 413 nM. The void volume was determined by passing a sample of blue dextran solution over the column. Figure 42 shows the elution profile from this experiment. Haptoglobin was detectable as a single peak before the IgG peak fractions. This indicates that the haptoglobin had a molecular weight of higher than 150,000 daltons. Haemoglobin was also detectable in these fractions although the majority of haemoglobin eluted off the column just after the albumin peak. The fractions containing haptoglobin 1:1 (M. Wt. 100,000 daltons) and haemoglobin (M.Wt. 64,000) eluted from the column in a position indicative of a molecular weight higher than either of the component proteins and approximately equal to their sum. It was therefore concluded that these fractions contained isolated haemoglobin/haptoglobin complexes. When these fractions were pooled and tested on AACE with 5895 antiserum, a uniform complete peak was observed (see Fig. 43, centre track).

The concentration of haptoglobin in BK serum was estimated by one dimensional AACE using anti-haptoglobin antiserum and a commercial human serum standard which contained 2.08 mg/ml haptoglobin (see Figs. 4a and b in Materials and Methods). Using this method, BK serum was calculated to contain 1.03 mg/ml haptoglobin. The amount of addition haemoglobin required to produce a complete peak (when tested on AACE with 5895 antiserum) with BK serum containing 0.04 mg/ml haemoglobin was approximately 0.5 mg. Since haptoglobin and haemoglobin bind together in a 1 to 1 molar ratio (Laurell, 1960) it would be expected that 1.03 mg/ml haptoglobin would require $1.03 \times \frac{64,000}{100,000}$ mg/ml

haemoglobin for complete saturation. From this calculation it appears that 0.66 mg haemoglobin would be required by 1 ml BK serum for complete saturation - a similar saturation to that observed on AACE. This supports the theory that at equivalent molar concentrations a complete peak is formed and that the half peak observed with PAS was attributable to haemoglobin-haptoglobin complexes.

However, the reason for the incompleteness of the peak seen on AACE with adult serum alone was still perplexing. Because 5895 antiserum was raised against a mixture of proteins in term lysate, it was possible that non-precipitating antibodies were present which were reacting with a slightly faster mobility protein than the haemoglobin-haptoglobin complex. This hypothetical protein would also move faster in the second dimension and might bind antibodies before the haemoglobin-haptoglobin complex. If these antibodies reacted with the faster mobility protein and the complex, this faster removal of antibodies from the anodal side of the complex peak might account for the peak's unique shape. A possible protein which might react in this manner was uncomplexed haptoglobin. To test for a mobility difference between free and complexed haptoglobin, samples of BK serum, haptoglobin complex (isolated by gel filtration) and free haptoglobin (isolated by ion exchange) were examined with anti-haptoglobin antiserum on a two dimensional AACE with the first dimension tracks in parallel, (Fig. 43). A small but distinct difference in mobility was noted, with the free haptoglobin moving slightly faster than complexed haptoglobin. BK serum, which contained a mixture of free and complexed haptoglobin produced a broader peak spanning the mobilities marked by the two separate components. This result supported the idea that antibodies against haptoglobin determinants could be involved in the formation of the half peak observed on AACE. To test the possibility of haptoglobin 'interfering' in some way with the formation of the serum half peak, tandem and straightforward additions of partially purified haptoglobin and BK serum were made on a two dimensional AACE with 5895 antiserum. This experiment was attempted with additions of 12.5 µg, 10 µg, 3.75 µg, 1.25 µg and 0.125 µg haptoglobin. Addition of 12.5 µg haptoglobin in any position (in front, together

Fig. 44a AACE of 5895 Antiserum with Serum of Hp Type 1:1(S) and 12.5 µg Partially Purified Haptoglobin

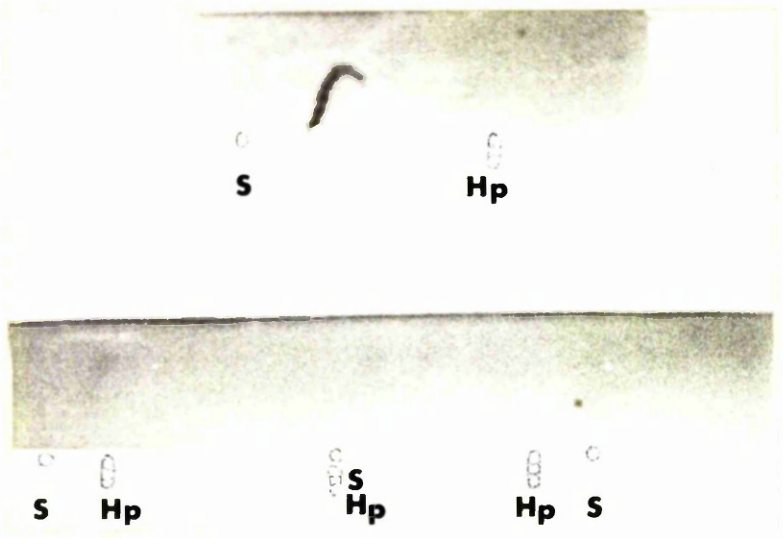
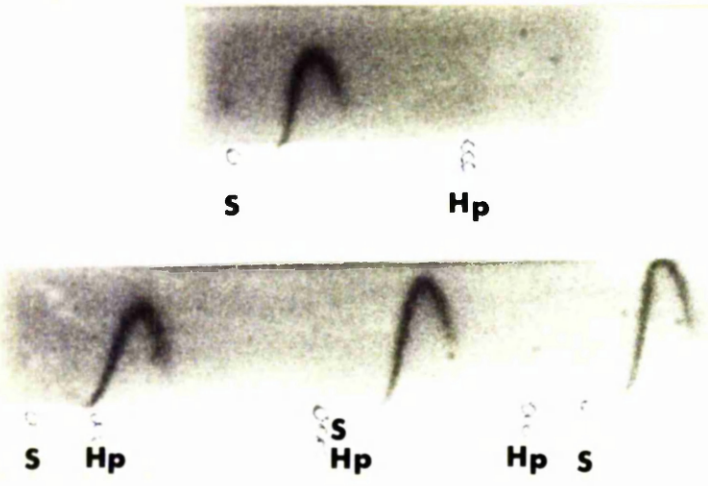


Fig. 44b AACE of 5895 Antiserum with Serum of Type Hp 1:1 and 10 µg Partially Purified Haptoglobin



5895 antiserum was raised against unfractionated term cord red cell lysate.

with or behind the serum) removed all precipitin arcs, although serum alone maintained its half peak (Fig. 44a). At all dilutions of haptoglobin below this, the half peak was unaffected (Fig. 44b).

Although these results are inconclusive, it was not possible to repeat or enlarge on these experiments, due to the termination of the research project.

DISCUSSION

Table 20 A Summary of Results from Antisera Obtained from Immunisation with Pooled Fetal and Fractionated Term Cord Red Cell Lysates

FRC 1 and 2	6791 and 6792
Immunised with 150 and 300 mg doses of pooled fetal red cell lysate. Both rabbits injected gave similar results.	Immunised with 16 mg ion-exchange fractionated term red cell lysate (reduced haemoglobin red cell protein). Both rabbits injected gave similar results.
Reactions of Unadsorbed Antisera	
5 peak reaction to adult serum on AACE.	15 peak reaction to adult serum on AACE.
Strong reaction to haemoglobin in fetal and term lysate. Faint adult haemoglobin peak on AACE. Fetal haemoglobin spurs past adult haemoglobin lines on ouchterlony plate.	
Reactions of Antiserum Adsorbed with Adult Serum	
(Both sets of antisera were adsorbed in parallel so dilution effect was same in both cases)	
β mobility antigen detected on AACE in adult, term and fetal lysate. Peaks approximately the same height with both sets of antisera so antibody titre for this antigen approximately the same.	
β mobility antigen detected in protein eluted from affinity columns.	β mobility antigen not detected in protein eluted from affinity columns.

A. THE RAISING OF ANTISERA

1. Comparison of Immunisation Regimes

In general, the further removed in evolutionary terms, the source of antigen is from the animal being immunised, the more extensive the response becomes. However, practical considerations usually result in rodents or other small mammals being used. Rabbits, goats and sheep are believed to be the best species for the production of precipitating antibodies. Horses have precipitating antibodies with a very narrow equivalence zone which means that a distinct line or precipitate arc is produced just in the equivalence zone. This is highly desirable for immunoelectrophoresis because multiple overlapping arcs become more distinct; but is not suitable for Antibody-Antigen Crossed Electrophoresis (AACE) where precipitation occurs in antibody excess (Herbert, 1978).

In random bred rabbits, the peak antibody titres measured following a simple immunisation procedure may vary by as much as five hundred times amongst individuals. This difference between individuals is probably increased by slight differences in the injection site of immunogen in relation to lymph nodes, and other external factors which make differences between even pure inbred stock considerable (Ganrot, 1972; Herbert, 1978).

It is therefore very difficult to draw any comparisons between the responses produced by the pairs of rabbits used to raise antisera for the experiments described in this thesis. The rabbits injected with 150 mg and 300 mg pooled fetal red cell lysate (FRC 1 and 2) produced antisera which were very similar (when examined by AACE) to the antisera from rabbits (6791 and 6792) injected with 16 mg ion exchange fractionated term red cell lysate. These results are summarised in Table 20, which shows that the only major difference between FRC and 6791/2 antisera was the detection of the β mobility antigen in the protein eluted from the FRC affinity column. Aside from small unintentional technical variations between the two experiments (e.g. in the immobilisation on sepharose or during the treatment of the eluted protein) this might indicate a difference in the association constants of the antibodies binding the β mobility antigen.

Table 21 Summary of Results from Antisera Obtained From Immunisation with Pooled Fetal Red Cell Lysate With and Without Prior Tolerisation to Adult Blood Proteins

FRC 1 and 2	7041	7043
Immunised with 150 mg and 300 mg doses of pooled fetal red cell lysate.	Neonatally tolerised to whole adult blood prior to immunisation with:	
	150 mg pooled fetal red cell lysate	300 mg pooled fetal red cell lysate
Reactions of Unadsorbed Antisera		
5 peak reaction to adult serum on AACE Strong reaction to haemoglobin as observed on AACE and ouchterlony plates.	No reaction to adult serum after first boost. Peculiar ½ peak observed with adult serum on AACE after second boost. Very faint with 7043. No reaction to haemoglobin after first boost.	
	Weak reaction to haemoglobin after second boost.	No further reactions.
Reactions of Antisera Adsorbed with Adult Serum		
Rounded β mobility antigen detected by AACE in adult, term and fetal lysate.	Pointed α_2 mobility antigen detected on AACE. Sometimes bimodal with fetal lysate. Very erratic results.	No reaction.

Haemagglutination Experiments

All rabbits in both experimental regimes agglutinated adult and term cord red cells to approximately the same dilution end points.

It is probably rather simplistic to comment that 6791/2 and FRC antisera, despite the use of different immunogens, had a similar response to red cell lysate proteins, since only one detection system (AACE) was utilised, it is possible that the superficial similarities in gel precipitation reactions of the two sets of antisera disguised more distinct differences in antibody specificity which tanned cell agglutination, immunofluorescence or radioimmunoassay might have shown up. However, these techniques were not really applicable at this stage of the investigation because they require purified antigen for maximum sensitivity.

The reactions of antisera raised against pooled fetal lysate in adult rabbits (FRC 1 and 2) and in young adult rabbits neonatally tolerised to whole adult human blood (7041 and 7043) were apparently very different (see Table 21). It is possible that variation between individual rabbits might be responsible for the differences observed in the two types of antisera. However, it seems more likely that the tolerisation to adult protein, prevented antibodies to the β mobility antigen being produced in 7041/3 antisera since there is no apparent immunological difference in the β mobility antigen from adult and fetal lysates. Although a response was produced to an adult form of the α_2 mobility antigen, the fetal form did appear to possess some electrophoretic variability and hence might be more immunologically distinctive. The lack of response to the α_2 mobility antigen by FRC (and 6791/2) antibodies might be due to a very low concentration of antigen in the immunogen which required tolerisation to other more abundant proteins, before a response was produced.

There was one experiment which showed a similarity between FRC, 7041 and 7043 antisera: all demonstrated similar ability to agglutinate adult and term cord red blood cells. No other antisera directly agglutinated cells, but it is not known whether the agglutination was due to recognition of the same membrane antigens by FRC, 7041 and 7043 antibodies.

2. Presentation of Immunogen

a) Composition

The presentation and composition of tissue immunogens is of great importance. Serum proteins, either as contaminants or as soluble intracellular components in tissue homogenates are

highly immunogenic and may form the main focus of the immune response (Bonstein and Rose, 1971; Weir, 1973). The amount of serum contamination in the adult, term and fetal red cell lysate pools varied according to the thoroughness of the washing procedure (see page 21). Fetal and term blood samples were partially coagulated when collected which restricted the washing technique, and as a consequence lysates from fetal and term red blood cells (which were used for immunisation) contained a higher concentration of albumin and other serum proteins than did adult lysate (see Figs. 6a-c and Table 7).

The immunisations with ion exchange fractionated term lysate protein (rabbits 6791 and 6792) represented an attempt to extend the range of antibody specificities produced by increasing the concentration of non-haemoglobin red cell proteins relative to haemoglobin. The extent to which the fractionation was successful is difficult to assess. Some non-haemoglobin red cell proteins were apparently increased in concentration: the β mobility antigen was approximately five times more concentrated in the reduced-haemoglobin red cell protein (rHbRCP) than in term red cell lysate (see Fig. 26c; page 44).

b) Stability

The lysates and reduced-haemoglobin red cell protein were lyophilised and resuspended in adjuvant for immunisation. This meant that the antigens were presented in particulate form which usually stimulate good antibody production due to their stability and insolubility in rabbit body fluids (Herbert, 1978). Particulate antigens may elicit an increased IgM response and since gel precipitation reactions generally involve IgG antibodies, some of the antibody specificities produced may not have been detected. (IgM antibodies are particularly effective at agglutination or cytolysis).

Non-persistence in the injected animal is a major reason for failing to produce an extensive antibody response (Herbert, 1978). Although the particulate form of the lysate immunogen would encourage persistence, the majority of the non-haemoglobin red cell proteins are enzymes (Hennessey et al., 1962) and many of these, while not unstable at body temperature, might be metabolised by the rabbit or degraded by rabbit proteases before antibody production was stimulated.

Tsunoo et al. (1974) compared the relative effectiveness of free adult human haemoglobin and adult haemoglobin bound to rabbit haptoglobin in producing an antibody response in rabbits. They found that the complexed haemoglobin was far more immunogenic although it appeared from Ouchterlony double immunodiffusion data that the antigenic determinants of haemoglobin had not been masked or modified, and that new antigenic determinants had not been produced. To explore the reasons for this increased immunogenicity, the resistance of the haemoglobin-haptoglobin complex to cathepsins and other proteases was compared with that of free haemoglobin; and found to be 50-80% higher with the complex. The in vivo significance of human haemoglobin/rabbit haptoglobin complex formation is difficult to assess since it is not clear that rabbit haptoglobin would bind haemoglobin after it had been injected as a suspension in adjuvant, but these data do suggest that antigen susceptibility to proteases in the injected animal may have a role in modifying the antibody response to that antigen.

c) Denaturation of Immunogens

When ion exchange fractionated term lysate (rHbRCP) was examined by AACE with adsorbed 6791/2 antisera (raised against this material) a rather indistinct peak was observed (Fig. 26a). Furthermore, when this rHbRCP material was analysed with sheep anti-adult human serum protein antiserum, several of the peaks observed were hazy and linked together (Fig. 20). Indistinct multiple peaks are frequently associated with denatured or antigenically altered proteins (Bjerrum and Bog-Hansen, 1975; Bjerrum et al., 1975; Kukulska and Sutcliffe, 1978) so it is probable that the fractionation had partially denatured some of the proteins present. Albumin, and possibly other serum proteins, had also increased in concentration during this fractionation, but further processing to remove the serum protein contaminants would almost certainly have caused a further decrease in the recovery of unaltered non-haemoglobin red cell proteins.

d) Complexity and Number of Antigens Injected

Injection of a single antigen may induce an antibody response to that antigen which does not occur when the same

produced. It is thought, for example, that in some instances antibody produced as a result of primary immunisation may act through an inhibitory feed-back mechanism in specifically limiting the magnitude of the secondary response to a subsequent antigenic challenge (Uhr and Moller, 1968; Diener and Feldmann, 1972; Grantham and Fitch, 1975).

The molecular factors involved in tolerance induction and the co-operation between cells in the immune system is very complex and much attention has been focused on their elucidation (Feldmann and Nossal, 1972; Nossal, 1973, 1974; Klaus et al., 1976; Taylor and Basten, 1976; Waksman, 1977). In the particular case of immunisation with tissue homogenates, it seems likely that non-specific suppression of an immune response may occur as a result of the injected animal being bombarded by a large number of different antigens all of which probably bear a basic resemblance to the injected animals' own tissues (Weir, 1973). Neonatal tolerance is based on the introduction of 'foreign' material at a time when the developing immune system is unable to stimulate antibody producing cells. The 'foreign' material therefore effectively becomes 'self' and subsequent challenges when the immune system has matured, results in an extremely limited response to these antigens. In these circumstances antibody specificities may be directed against minor antigenic variants to the antigens used for tolerisation. In the present investigation, neonatal tolerance to adult serum and red cells (as with rabbits 7041 and 7043, see Materials and Methods, page 29 for details) was attempted as a method to detect fetal specific proteins in fetal red cell lysates. The extent to which the prior state of neonatal tolerance to some of the components in the immunogen is responsible for the variability of the gel precipitation responses of 7041 antiserum is unknown. A similarly tolerised littermate (which was later immunised with fetal liver homogenate) also produced somewhat variable responses to an antigen in term cord serum (Sutcliffe and Nicholson, unpublished data). It is possible that marginal variation in ionic strength of gel or electrophoresis buffers might cause slight variation in antigen conformation (especially since the bimodality of the α_2 mobility antigen suggested

electrophoretic variation). Conformational changes might alter the affinity of 7041 antibodies which only weakly reacted with the antigen and caused the variability observed. Reports of such variability were not found in the literature, but it is possible that such a result would not be described in favour of other more successful results.

3. Conclusion

The effects of antigen dose and its timing, antibody suppression and tolerance have great theoretical significance, but are of limited application in a practical situation where a number of unspecified antigens of mixed immunogenicity are involved (Williams and Chase, 1973). There is a great range in concentration of red cell antigens, with haemoglobin accounting for approximately 95% of the total soluble protein in red cells and the rest of the intracellular proteins together only amounting to 5% (Hennessey et al., 1962). The concentration of particular red cell enzymes varies with cell age and between individuals, and although carbonic anhydrase and catalase are generally present in a much higher concentration than most other red cell enzymes together, while certain other enzymes (e.g. glycerol-1-phosphate dehydrogenase) are only present in trace amounts (Brewer, 1974). This means that it is impossible to consider 'antigen dose' in a complex red cell lysate in any meaningful way. The exact amounts of antigen required to give high and low zone-tolerance need to be calculated from trials for individual antigen test-systems and so it was not known beforehand whether, for example, injecting 300 mg pooled fetal lysate would produce tolerance to haemoglobin. The pooled fetal lysate also contained a very small amount of serum α-fetoprotein as a contaminant and it has been suggested that this protein might have immuno-suppressive properties in utero, as demonstrated in mice by Murgita and Tomasi (1975a and b), but this has been discounted at any significant level for human α-fetoprotein (Littman et al., 1977).

With insufficient animals and antigen available, it was not practically possible to investigate the optimum conditions of timing or the amount of complex antigen to inject to achieve

a detectable antibody response to the maximum number of components present. The immunisation schedules followed were therefore based on generally accepted guidelines (Herbert, 1978) and it is not known whether the general lack of diversity in the antisera produced was a result of this, although it is probably more likely to be the result of a number of factors, of which the manner of antigen presentation could be one.

B. METHODS FOR DETECTING IMMUNOLOGICAL RESPONSES

The principal detection system used in this investigation was precipitation in gels. This has the advantages of being simple, reproducible and adaptable (see Ouchterlony and Nilsson, 1978) although there are also a number of limitations. Gel precipitation techniques require precipitating or 'complete' antibodies (usually IgG) to be present, and in suitable specific concentrations for the equivalence point of the antigens under examination to be reached. The sensitivity of Ouchterlony plates also depends on there being a difference in the relative concentration of antibodies for different antigens so that precipitin lines are not superimposed. Antibody-Antigen Crossed Electrophoresis (AACE) has superior resolution of multiple precipitate arcs due to the electrophoretic separation of antigens in the first dimension.

The AACE technique is widely used in the study of tissue, tumour and membrane antigens (see Axelsen, 1973 , 1975) and the peak morphology is sensitive enough to distinguish between slightly denatured or immunologically altered antigens (Bjerrum et al., 1975; Kukulska and Sutcliffe, 1978). The shape of a one-dimensional AACE peak also varies between proteins. Albumin and haptoglobulin, for example, produce narrow parallel sided peaks (see Fig. 4a), whereas IgG, haemoglobin and the β mobility antigen detected in red cell lysates by certain antisera produced extremely rounded peaks (see Fig. 27). The precise reason for this is unknown but it is possible that a slower mobility allows a certain amount of sideways diffusion to occur before precipitation. Backward diffusion of antibodies may be responsible for the occasional appearance of peaks below the antibody bed (see Fig. 35).

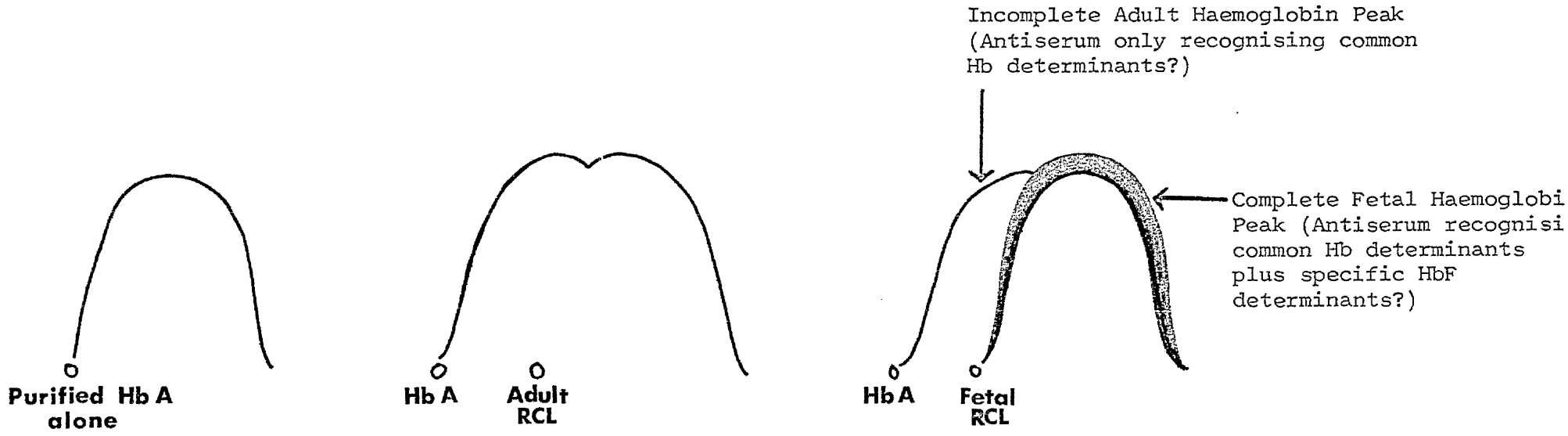
Although gel precipitation techniques were mainly used in this investigation, consideration was given to other methods for determining antibody-antigen reactions, and although the limited time available prevented the investigation of immunofluorescence or assays with radioactively labelled antigens, some haemagglutination experiments were attempted.

The antisera which were raised against 150 mg and 300 mg doses of pooled fetal lysate, either in normal adult rabbits (FRC antisera) or in neonatally tolerised rabbits (7041 and 7043 antisera) agglutinated adult and term red cells directly. To try to avoid reactions with the major AB blood group antigens, only cells of blood group O were used (although H substance, a precursor of A and B antigens, is still present on group O cells). There was apparently no fetal specificity involved since adsorption with adult red cells inhibited agglutination with all cells. The nature of the antigen or antigens recognised by these antisera is unknown. It may be a minor blood group antigen or a structural membrane antigen.

If the haemagglutination studies had been continued, it would have been possible to screen cells of known minor blood groups or inhibit agglutination by prior incubation with monospecific blood group antisera. To test the possibility of a structural antigen, it is probable that solubilisation and fractionation of erythrocyte membrane components would be required, and assayed by agglutination inhibition with controls for blood group antigens included. From the practical point of view, however, it was found that direct agglutination was an extremely time-consuming technique. The requirement for fresh blood cells meant that continuous visits to a local hospital were required, and the centrifugation and washing of the individual samples (which could only be pooled after ABO blood grouping analysis) was prohibitive in the 'part-time' circumstances of this investigation.

The use of formalised tanned cells was much more satisfactory. Once prepared, the cells were kept for a year at $4^{o}C$ with no apparent loss in reactivity. The agglutination results were also more precise and less prone to spurious positive results.

Fig. 45 Drawing of AACE Fig. 8 in Results, Showing Reaction of 5895 Antiserum to Haemoglobin in Adult and Fetal Lysates



The failure of some adsorbed antisera to give a positive result with cells coated with fetal haemoglobin when reactions were observed by gel precipitation (although very faintly) is almost certainly due to insufficient or irregular coating. If the agglutination experiments had continued, it is probable that varying the coating procedure would increase the sensitivity of the assay. The applicability of this method to red cell components generally is limited unless purified antigens could be prepared. This is the major problem with the majority of the non-gel precipitation techniques used for the investigation of antibody-antigen reactions: the sensitivity of immunofluorescence inhibition, radioimmunoassay, complement fixation as well as agglutination inhibition, depend on the certainty of a single known antigen being involved in the reactions observed. If this is not the case, the interpretations of the results and suitability of controls becomes a major source of concern.

C. SPECIFICITIES OF THE RESPONSES OBSERVED

1. Haemoglobin

When comparing the identities of purified adult haemoglobin and haemoglobin in fetal red cell lysates with unadsorbed 5895 antiserum (raised against 20 mg doses of pooled term lysate) on AACE the result was a reaction of partial identity involving a single precipitate arc for each source of haemoglobin (see Fig. 8 in Results and Fig. 45 overleaf). The more intense, completed fetal haemoglobin peak is probably formed by antibodies with specificities for common haemoglobin determinants (possibly located on the α chain) plus those which bind the special determinants peculiar to fetal haemoglobin (probably located on the γ chain). The fainter adult haemoglobin peak, on the other hand, may be formed by the precipitation of antibodies which are only recognising the common determinants of fetal and adult haemoglobin. The removal of the anodal half of the adult haemoglobin peak by the complete fetal haemoglobin peak might therefore be due to 'in situ' adsorption in the gel bed of all anti-haemoglobin antibodies present by fetal haemoglobin. This

Drawing of Ouchterlony Plate Shown in Fig. 9b

Well A = HbA

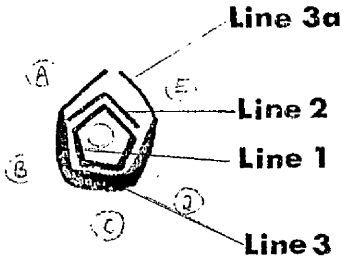
B = HbF

C = Term Lysate

D = Fetal Lysate

E = Adult Lysate

Centre Well = Antiserum raised against term lysate



*These superscript numbers refer to the numbered lines in the drawing of Fig. 9b.

deduction is supported by reports of the 'superior immunogenicity' of fetal haemoglobin which more readily induces precipitating antibodies (Chernoff, 1958; Heller et al., 1962) and by the observations of Reichlin (1970) who reported that in rabbit anti-HbF sera, there is always a preponderance ($\frac{2}{3}$ to $\frac{3}{4}$) of antibodies to γ chains compared to α chains.

Although Antibody-Antigen Crossed Electrophoresis (AACE) can detect subtle changes in antigens, an occasion was found in this investigation when the results were not as informative as with the Ouchterlony Double Immunodiffusion technique. Thus this reaction of partial identity between adult and fetal haemoglobin was supported by initial Ouchterlony plate results (Fig. 9a) but increasing the sensitivity of the Ouchterlony plate by increasing the concentration of both antigen and antibody and increasing the distance between wells showed that more than one antigenic specificity was involved. Adult, term and fetal red cell lysates, commercially purified adult haemoglobin (HbA) and ion exchange purified fetal haemoglobin (HbF) were compared (Fig. 9b) and four different precipitate lines were formed. The innermost line was fused for all the samples applied and the staining was of equal intensity.[1*] This line could represent a determinant on the α chain or in common regions of the β, γ or δ chains. It is unlikely to be a serum protein or a non-haemoglobin red cell protein because it occurs at approximately the same relative concentration (as indicated by the position and intensity of the precipitate arc) in the unfractionated lysates and in the purified haemoglobin samples, which were shown on an SDS/polyacrylamide slab gel to contain virtually no contaminants (see right hand side Fig. 21). Outside this fused line was a line common to adult lysate and HbA.[2] This suggests a β chain specificity. Just outside this precipitate line was a broad, very intensely stained line which fused for fetal and term lysates and HbF.[3] The intensity of the staining suggests that this is the major specificity recognised by 5895 antibodies and is therefore likely to be on the γ chain. Apparently fused to this very intense precipitate line are two very faint lines from HbA and adult lysate.[3a] The position and faintness of these lines suggests that a small number of both antigens and antibodies is involved. A possible explanation for this is that there are some antibodies present which bind the γ chain determinant

and also cross-react with a determinant on one of the adult chains. This could possibly be a determinant on the δ chain since Boerma and Huisman (1964) defined a population of antibodies reacting with isolated γ and δ chains but not α or β chains.

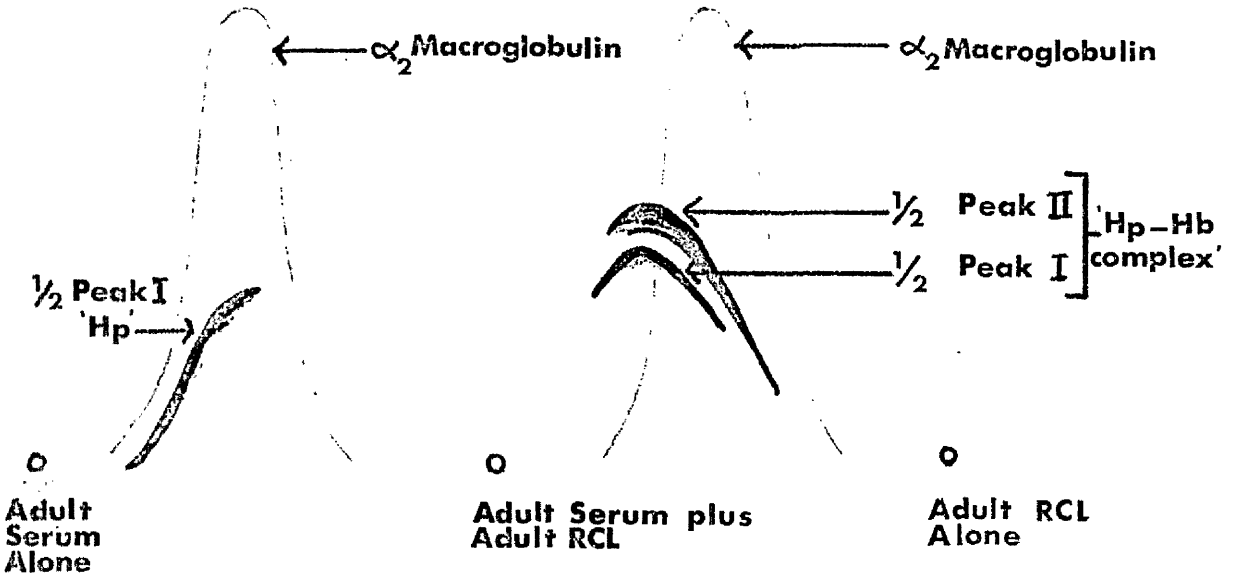
Examination of the reactions of the other antisera (FRC 6791/2 and 7041) to haemoglobin by the Ouchterlony technique all displayed a similar pattern: a single line fused for all haemoglobin and lysate samples applied with the fetal samples showing spurs of partial identity past the foreshortened adult precipitate lines. At first this suggested that there was a single common specificity with extra 'fetal specific' determinants recognised by these antisera. However, 5895 antiserum also produced this pattern of lines at certain relative concentrations of antibody to antigen, but then revealed that the fetal specific' spur fused with very faint lines from adult lysate and haemoglobin. This could have been the case with the other antisera (FRC, 6791/2, 7041/3) except that the adult precipitate lines were not visible. This might be supported by the fact that adsorption experiments also failed to provide evidence of any fetal specificity. Adsorption of every antiserum with adult lysate abolished all precipitate lines with fetal lysate, although the possibility of fetal specificities being too dilute to precipitate or that adsorption removed 'fetal type' specificities by cross-reactivity cannot be excluded.

2. Haemoglobin-Haptoglobin Complex

It would appear that 5895 antiserum (raised against 20 mg doses of term lysate) does have specificities for haemoglobin. Whether these same specificities are involved in the formation of the peculiar half peak (e.g. Fig. 10a) observed when adult serum was examined by AACE with 5895 antiserum is not completely certain.

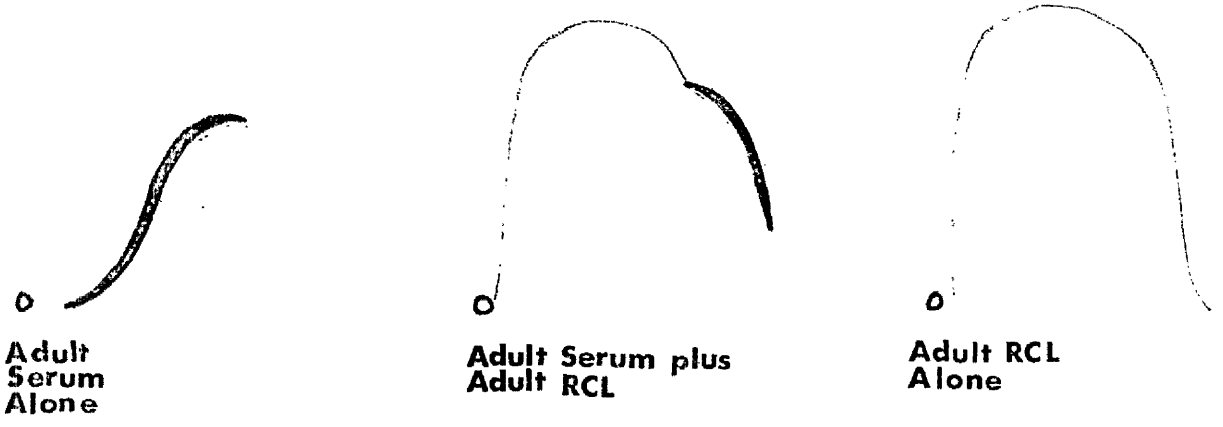
The reaction of unadsorbed 5895 antiserum to pooled adult serum was compared on AACE with the reactions of commercial anti-haptoglobin and anti-adult haemoglobin, and apparently showed identity with both, (see Fig. 11c). A single smaller peak was observed when anti-haptoglobin and 5895 antisera were added together in the same bed. This indicated that both antisera

Fig. 46a Drawing of the AACE Peaks from the Report of Hegelund et al. (1976)



Antiserum raised against an extract of fetal pancreas and partially adsorbed with adult serum, adult red cell lysate and extract of normal adult pancreas.

Fig. 46b Drawing of AACE Peaks from Figs. 7a and 38a in Results Section



Antiserum raised against term cord red cell lysate (unadsorbed).

had antibodies against determinants on the same molecular entity. However, the pooled adult serum has a haemoglobin content of approximately 0.04 mg/ml (see page 34 of Results) and at this concentration all the haemoglobin would be bound to haptoglobin, which would be in excess since it was calculated that a haemoglobin concentration of approximately 0.66 mg/ml would saturate the haptoglobin present in the serum (see page 55-6 of Results). It was therefore possible that this apparent reaction of identity was caused by 5895 antibodies reacting with haemoglobin and anti-haptoglobin antibodies reacting with haptoglobin but both precipitating to produce a single peak because haemoglobin and haptoglobin were complexed together. This could also account for the 'blocking' of a peak in the upper 5895 antibody bed by an intermediate anti-haptoglobin bed, or the merging of the peak from lower into the upper 5895 bed (Figs. 11a and b).

Hegelund et al. (1976) noted a very similar half peak during the examination by AACE of an antiserum raised against fetal pancreas. Their antiserum had been partially adsorbed with adult serum, adult red cell lysate and an extract of normal human pancreas. No reaction was observed when this adsorbed antiserum was tested against adult lysate but examination of adult serum demonstrated a peak identified as α_2 macroglobulin and the peculiar half peak (½ Peak I). Addition of adult lysate to the serum produced a second hazy incomplete precipitate line (½ Peak II) which curved over the original half peak (see Fig. 46a). This second peak did not have the slower mobility and characteristic rounded appearance of haemoglobin. In this brief report, experimental details were excluded, but Hegelund et al. reported that both the half-peaks cross-reacted with both haemoglobin and haptoglobin and concluded that these peaks were therefore haemoglobin-haptoglobin complexes.

Although this conclusion might be applied to the present results, there are some differences between the two sets of data. In the present study no AACE peaks were visible if the antiserum was adsorbed with adult serum or adult red cell lysate. With 5895 antiserum, the addition of adult lysate to adult serum showed a second haemoglobin-type peak of slower mobility which

fused with the cathodal side of the half-peak (see Fig. 46b). In contrast, Hegelund's second peak (II) was of approximately the same mobility, and essentially pointed in outline, and was not clearly fused to the half peak (I) (Fig. 46a).

Despite these differences between the results, it would appear that the unique half peak is associated with haemoglobin-haptoglobin complexes and that the differences observed are probably attributable to variations in antibody specificity. Nevertheless, it was difficult to account for the creation of the half peak and therefore an attempt was made to try and find out why haemoglobin-haptoglobin complexes did not always form a conventionally-shaped arc.

a) Evidence for the Involvement of Haemoglobin Specificities

When increasing amounts of haemoglobin were added to adult serum, a point was reached when the half peak (which was anodally incomplete) became a conventional symmetrical peak. If further additions of haemoglobin were made, the peak became incomplete cathodally and appeared to fuse into a typical haemoglobin-type peak (Figs. 38a and b). This occurred when the serum samples were analysed by AACE with 5895 and commercial anti-haemoglobin antisera. Examination of the serum samples with commercial anti-haptoglobin antiserum showed a uniform symmetrical peak in all cases. These results suggest that only haemoglobin specificities may be involved. However, the peaks with anti-adult haemoglobin were noticeably thicker and more blurred. This may indicate that the antibodies present were reacting with haemoglobin which had been modified by complexing to haptoglobin. It has been claimed that haptoglobin does not modify or block antigenic determinants on haemoglobin and that antibodies bind to haemoglobin in different regions to haptoglobin (Cohen-Dix et al., 1973; Tsunoo et al., 1974; Sasazuki et al., 1974). This is based on the non-alteration of free haemoglobin precipitate patterns observed on Ouchterlony plates when complexed with haptoglobin, and on radioimmunoassay competition and saturation analysis with anti-haemoglobin antibodies and antibody combining region (Fab) fragments. This view is also maintained by Putnam (1975). However, all agree

that haemoglobin bound to haptoglobin of all three phenotypes (1:1, 2:1 and 2:2) is more immunogenic and does show increased precipitability with anti-haemoglobin antibodies as indicated by end-point titration of precipitation in solution and with precipitation of labelled anti-haemoglobin antibodies fractionated by 20% and 38% saturated ammonium sulphate (e.g. Tsunoo et al., 1974).

Haemoglobin splits into α/β chain dimers in order to bind with haptoglobin (Nagel and Gibson, 1971; Malchy et al., 1973) and Javid and Pettis (1975) considered that this change in the conformation of haemoglobin might expose 'neoantigenic' determinants previously concealed. It has been argued, however, that this is unlikely since antibodies and haptoglobin do not bind to haemoglobin in the same regions. The alternative hypothesis is that the increased precipitability of haemoglobin bound to haptoglobin is due to enhanced lattice formation due to the increased size and molecular weight of the haemoglobin-haptoglobin complex (Tsunoo et al., 1974; Putnam, 1975). This idea is supported to some extent by the work of Javid and Pettis (1975) in that they showed that the increased precipitability only occurs when complexes of the polymeric forms of haptoglobin (i.e. Hp 2:1 and 2:2) with high molecular weights are involved.

It seems to me that a third possibility exists. In all of the above papers (Sasuzuki, 1971; Sasuzuki et al., 1974; Tsunoo et al., 1974; Cohen-Dix et al., 1974; Javid and Pettis, 1975) the antisera used in the investigation were raised against haemoglobin purified by DEAE ion exchange methods, yet no consideration was apparently given to the slight possibility of haptoglobin being present in the immunogen and antibodies with specificities for haptoglobin or haemoglobin-haptoglobin complexes being present in the anti-haemoglobin antisera. Haptoglobin is very immunogenic and only trace amounts (possibly the result of some haemoglobin-haptoglobin complexes co-purifying with free haemoglobin) might be required to elicit an antibody response. If haptoglobin was present in the haemoglobin immunogen it would almost certainly be complexed, and the observations of Korngold (1965) that the polymeric haptoglobins have more determinants

could be in accordance with the observation that the increased precipitability of haemoglobin occurs most markedly when it is complexed to polymeric haptoglobin. The increased precipitation and expression of 'neoantigenic determinants' might therefore be due to detection of antibody specificities for the haptoglobin component of a haemoglobin-haptoglobin complex.

The significance of these findings is uncertain. It has been established that haptoglobin does alter the conformation of haemoglobin so that certain characteristics are altered, but it is not clear whether the immunological differences that have been observed between free and complexed haemoglobin are due to determinants of haemoglobin being modified by haptoglobin or whether haptoglobin (either by itself or as part of the haemoglobin-haptoglobin complex) is an integral requirement.

b) The Possible Involvement of Antibody Specificities for Haptoglobin Complexes

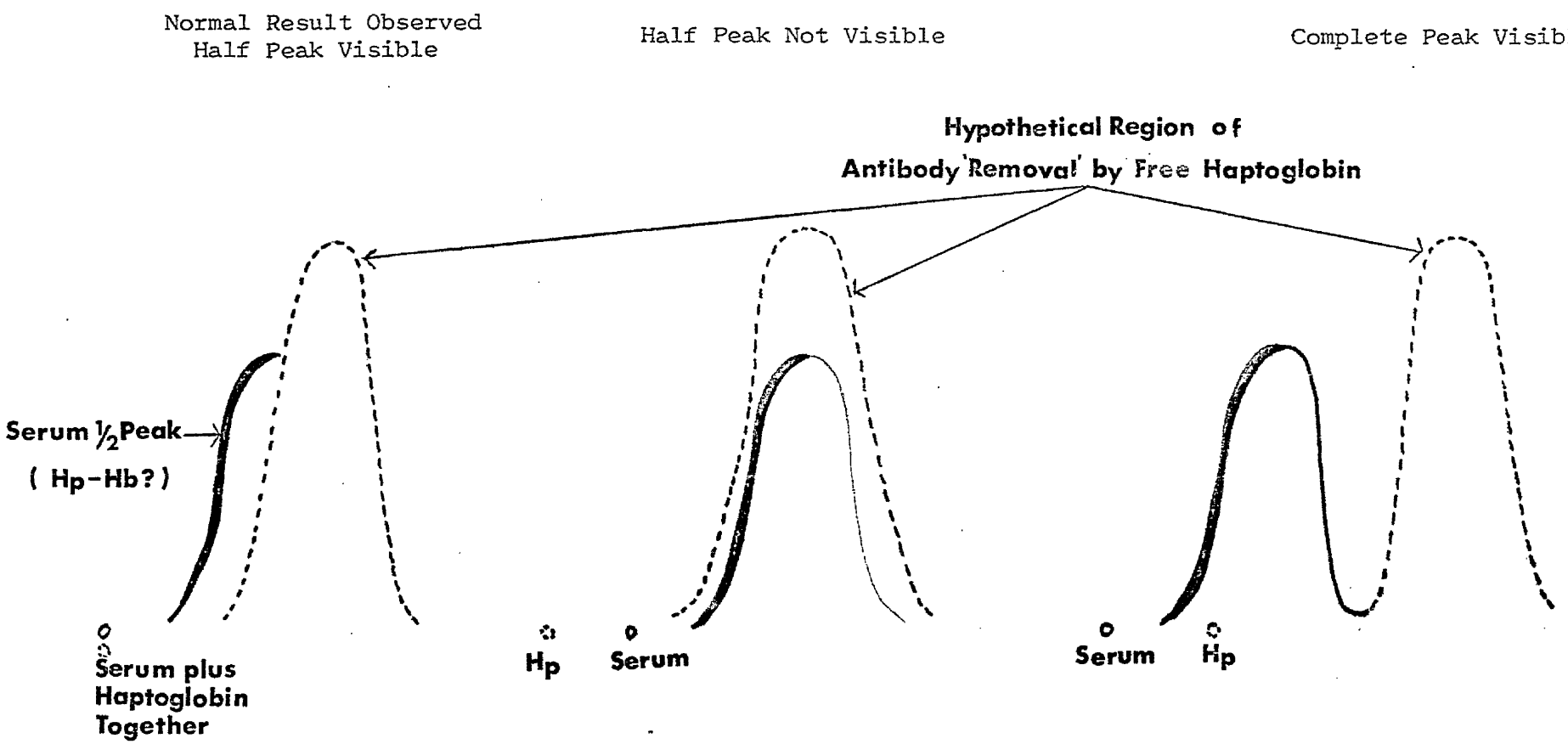
While not forming a precipitin arc by itself, partially purified haptoglobin does modify the pattern of haemoglobin peaks observed with 5895 antiserum (see Fig. 41). The presence of haptoglobin and haemoglobin in the precipitin arcs is indicated by the reactions of apparent identity with antisera to both antigens (see Figs. 11a-c). The possible involvement of antibody specificities for haemoglobin-haptoglobin complexes is also suggested by the formation of a normal complete peak when complexes isolated by gel filtration were examined (see centre track of Fig. 43).

However, these experiments cannot really distinguish between antibody specificities for haemoglobin modified by haptoglobin or specificities for the complex as a whole. In order to make this kind of distinction, radioactively labelled isolated haemoglobin-haptoglobin complexes and components would be required.

c) The Cause of the Incomplete Precipitin Arc

Although partially purified haptoglobin alone did not produce a precipitate peak when tested with 5895 antiserum, it did modify the response of this antiserum to haemoglobin. It is

Fig. 47 Diagrammatic Representation of Experimental AACE Results Expected if Antibodies with Specificities for Haptoglobin are Involved in the Formation of the 'Half Peak'



Antiserum is anti-term cord red cell lysate (5895)

therefore possible that haptoglobin was involved in the disappearance of the anodal half of the serum peak. It is possible that complex formation with haptoglobin would have a two-fold effect on haemoglobin: 1) the electrophoretic charge of the complexed haemoglobin molecules would increase, and 2) such complex formation might block antigenic determinants on haemoglobin. In theory, these two effects would result in an incomplete peak due to a population of slightly faster mobility haemoglobin molecules which would not form an immune precipitate. However, since Cohen-Dix et al. (1973), Tsunoo et al. (1974) and Sasazuki et al. (1974) all consider that haptoglobin does not 'mask' any antigenic determinants on haemoglobin, a further possible explanation was sought.

In principle, a possible cause of the incomplete peak is the removal by another antigen of antibodies which would otherwise complete the precipitin arc. Since it is the anodal part of the peak that is missing, a protein of faster mobility is invoked. This might well travel into the antibody bed faster in the second dimension and adsorb antibodies before they could complete the precipitate peak of the haemoglobin-haptoglobin complex. For this to happen the hypothetical protein would have to react or cross-react with antibodies (which would otherwise react with the complex) but not precipitate in the gel. Uncomplexed haptoglobin is a possible candidate for such a protein since it has a marginally faster mobility than the haemoglobin-haptoglobin complex (see Fig. 43).

To test whether non-precipitating antibodies for haptoglobin might have such an effect, direct and tandem additions of partially purified haptoglobin were made to serum to try to interfere with the formation of the remaining half of the peak (see diagram in Fig. 47 opposite). If this hypothesis was correct, the interference with the whole peak would happen if the haptoglobin were applied slightly behind the serum, so that at the end of the first dimension of electrophoresis, the location of the haptoglobin and haemoglobin-haptoglobin would coincide and no peak would appear. Such a result would indicate that some antibodies were present which had specificities for haptoglobin (or for a protein which co-purified with haptoglobin) and the

haemoglobin-haptoglobin complex. This experiment was attempted with additions of 12.5 µg, 10 µg, 3.75 µg, 1.25 µg and 0.125 µg haptoglobin. Addition of 12.5 µg haptoglobin in any position (in front, together with, or behind the serum) removed all precipitin arcs, although serum alone maintained its half peak. At all dilutions below this, the half peak was unaffected. These results are inconclusive and puzzling because such a major difference between the application of 12.5 µg and 10 µg haptoglobin would not be expected in view of the positions the additions were made in. For example, application of the haptoglobin in front of the serum would carry the haptoglobin even further away from the serum and hence a complete serum peak should be visible (see diagram in Fig. 47). The lack of any kind of influence on peak formation of the 10 µg haptoglobin might be due to the denaturing effects of storage, since this final experiment with haptoglobin was performed several weeks after the initial observation, and the protein concentration of the partially purified haptoglobin was moderately low (1.25 mg/ml). A further possibility is that the original observation when the peaks were removed, was a mistaken one due to some artifact. Unfortunately, it was not possible to complete these experiments. Had time been available, however, further purification and radiolabelling of the haptoglobin and haemoglobin would have been attempted in order to follow the reactions of these antigens in the formation of the peculiar half peak.

Experiments with labelled antigens would also help to elucidate the nature and specificities of the antibodies present in 5895 antiserum. Antibodies with any sort of specificity for haptoglobin have not currently been demonstrated and although precipitating specificities have been observed with free and complexed haemoglobin, it is not known whether these antibodies distinguish free from complexed haemoglobin and whether there are any antibodies specific for just bound or unbound haemoglobin.

The finding that addition of haemoglobin to pooled adult serum did not alter the precipitation pattern of haptoglobin with anti-haptoglobin antisera (Fig. 40) contrasts with the results of Korngold (1965) who observed that haemoglobin addition blocked an increasing number of antigenic determinants on haptoglobin, as judged by diminishing spur formation on Ouchterlony plates. This effect was most conspicuous with haptoglobin 1:1 phenotype and

it is possible that the mixture of haptoglobin types in pooled serum minimises this effect, or it is not detected by AACE.

3. The β Mobility Antigen

A β mobility antigen was recognised in red cell lysates by antisera raised against 150 mg and 300 mg pooled fetal lysate (FRC antisera) and 16 mg ion-exchange fractionated term cord lysate (6791/2) antisera (Figs. 14a and 25a). Both sets of antisera were shown to be recognising the same antigen (Figs. 28a and b) and there was a four-five fold increase in the concentration of this antigen from 13 wks gestation to adult (Figs. 15 and 27). Whether this antigen is red cell specific is unknown. To investigate this, protein extracted from cultured human tissue cells would be required, since it is impossible to obtain a tissue homogenate which is totally lacking in red cell lysis. This aspect of the investigation was unfortunately not completed.

If the cultured cells did contain the same β mobility antigen, this would indicate a ubiquitous tissue enzyme or structural protein. If the β mobility antigen was absent from non-haematopoietic cultured cells, red cell specificity would be indicated. Further identification might be achieved by using the antibodies to inhibit specific red cell enzyme assays, although careful correlation between these results and the results from gel precipitation experiments would be needed.

4. The α_2 Mobility Antigen

The tissue specificity of the erratic 7041 α_2 mobility antigen (see Fig. 33b and Table 13) is also unknown. The occasional bimodality of the fetal peak suggests two electrophoretic forms of the same protein. The antigen in adult lysate appeared to have determinants in common with both fetal lysate peaks since addition reactions between both fetal and single adult peaks resulted in a single large peak. The lack of reproducible results is puzzling and the exact cause is unknown, however it is possible that slight unintentional variation in the conditions of electrophoresis might have affected this antigen and caused the occasional separation of two fetal forms in the first dimension of electrophoresis.

5. Fetal Specificities

The total lack of a precipitating response when all the antisera (5895, FRC, 6791/2 and 7041/3) adsorbed with adult red cell lysate were examined by AACE indicates that no fetal specific red cell protein antigens were detected by the antisera in this investigation. It is possible, however, that such specificities were present but could not be detected by gel precipitation methods either because 'incomplete' antibodies were formed or because the concentration of antibody-antigen complexes in the gel was too low to form a cross-linked lattice. A further possible source of misinterpretation is the purity or specificity of the adult red cell lysate adsorbent. Although fetal haemoglobin, for example, is not detectable in normal adult red cells, it is possible that trace amounts of fetal proteins might be present in sufficient quantity to adsort out any fetal specificities present. If a 'fetal-type' protein had some homology with the structure of its adult counterpart (e.g. haemoglobin) the fetal and adult determinants might be very similar and hence adsorption of antisera with the adult protein might also remove antibodies with fetal specificities. Alternatively, the remaining antibodies might be at such a low concentration that they could not be detected by gel precipitation.

Unfortunately there was not time to investigate the distribution and concentration of the β mobility antigen and the variable bimodal α_2 mobility antigen in pathological states. Moreover, the preliminary results were not very encouraging to this line of research since there was not a very distinctive difference in the concentration of the β mobility antigen in the adult and fetal lysates tested; and a reproducible assay for the more interesting α_2 mobility antigen (which possibly had different adult and fetal forms) was not achieved.

6. Affinity Chromatography

The use of affinity chromatography did not reveal any previously undetected fetal specificities in red cell lysates with serum adsorbed FRC or 6791/2 antisera (raised against pooled fetal lysate and ion exchange fractionated term cord lysate respectively). However, the protein eluted from the columns after adult and term cord lysates had been applied showed a different

pattern of peaks when examined on AACE with unadsorbed FRC and 6791/2 antisera and sheep anti-adult human serum protein (compare Figs. 17a, b, c and d with the Results summarised in Table 12). It appeared that there was a higher concentration of certain serum proteins in the eluted protein compared with the original red cell lysates.

In the experiments using unadsorbed-antibody columns, this increase in serum proteins could have been due to non-specific binding of serum antigens to the immobilised antibody/sepharose matrix; or to specific binding, causing a concentration of antigens for which the antibodies had specificity. Although it was not possible to distinguish between specific and non-specific binding of antigens to the affinity columns, the possibility of contamination between serum and lysate samples applied to the columns could be excluded because of the use of separate columns for these two groups of samples. In the experiment in which serum-adsorbed antibodies were immobilised, a further possible source of serum protein was the polymerised serum used to adsorb the antiserum. However, examination on a polyacrylamide/SDS gradient slab gel of the material obtained from subjecting the antibody column to the dissociating agent prior to any samples being applied (the 'blank' eluate in Track 1 of Fig. 30) revealed virtually no material had been lost from the column itself under dissociating conditions.

The protein eluted from the columns was dialysed, concentrated and lycphilised before being examined and the delay caused by this processing may have partially denatured certain proteins. The faint, blurred precipitate lines observed running together (Figs. 17a, b, c, and d) are typical of the type produced by denatured proteins (Bjerrum et al., 1975). A modification of the affinity chromatography procedure described which might minimise the problem of denaturation would be the immediate desalting of the eluted protein by G25 sephadex gel filtration. This modification was used with success in the partial purification by affinity chromatography of the pregnancy associated placental protein (PAPP-A) by Kukulska and Sutcliffe (1978).

D. CONCLUSION

The results obtained during this immunological investigation indicate a very limited antibody response to the many proteins present in red blood cells. This could have been due to inherent non-immunogenicity of the red cell proteins, to the manner in which the proteins were presented to the rabbits (antigen dose, complexity, frequencing of boosting, etc.) or to the insensitivity of the methods used to detect the immune response.

Although no firm conclusions can be drawn from these results, they are not entirely unexpected or inconsistent with the principal aim of this investigation, which was to make an immunological study of red blood cell protein as a model for other tissue cells. Weir (1973) comments on the apparent lack of immunogenicity of whole tissue homogenates compared with the antibody response to isolated antigens from the same homogenate. In this respect, this study did find red cell lysate proteins to be typical of the soluble proteins from other tissue cells.

The second aim of this investigation, to try to detect novel fetal proteins in red cells, was unsuccessful in that no fetal specificities were detected in the antisera produced. Whether this was because they were undetected by the methods employed or because immunological differences between fetal and adult red cell proteins do not exist (with the exception of haemoglobin) is not known. Many of the apparent differences between fetal and adult red cells are directly attributable to qualitative rather than quantitative changes. For example, a combination or subtle differences in the activities of several enzymes is apparently responsible for modifying the metabolic characteristics of fetal erythrocytes so that they appear quite different to their adult counterparts. While 'fetal specific' enzymes have been postulated, e.g. 2,3-diphosphoglycerate phosphorylase (Oski and Komazawa, 1975) no conclusive proof of the existence of new fetal proteins has yet been produced, despite the intense research activity in this field.

Despite the limited number of antibody specificities detected in this investigation, it was felt that a great deal had been personally gained, and it was finally concluded that the human red blood cell remains a challenge to the immunologist studying tissue proteins.

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