BIOCHEMICAL ASPECTS OF THE IDIOPATHIC RESPIRATORY DISTRESS SYNDROME OF THE NEWBORN

Thesis presented for the degree of
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by

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PART 1

INTRODUCTION AND SURVEY OF THE LITERATURE

1. I. Clinical features of the Idiopathic Respiratory Distress Syndrome of the Newborn.

The features of respiratory distress in a newborn infant are as follows (1).

(i) Grunting expirations commencing within a few hours of birth.

(ii) A respiratory rate of more than 60/min. maintained for more than 3 hours.

(iii) Cyanosis in room air.

(iv) Recession of the ribs and sternum.

(v) Pulmonary crepitations.

Any infant exhibiting two or more of these features, is classified as suffering from respiratory distress.

Respiratory distress may be due to pulmonary or extrapulmonary causes :

Extrapulmonary causes include cerebral and cardiac abnormalities, diaphragmatic hernia, obstruction to the upper respiratory tract and anaemia, due to blood loss (ante-partum haemorrhage) or to haemolysis (Rh-isoimmunization).

Generally-accepted pulmonary causes may be specific or idiopathic.

Specific causes include conditions such as neonatal pneumonia and meconium aspiration.

Idiopathic causes include neonatal disseminated atelectasis (NDA) and hyaline membrane disease (HMD). NDA may be distinguished from HMD on the following grounds :

(i) NDA affects infants of all gestational ages, whereas HMD affects only premature infants.

(ii) The clinical course of NDA lasts about 24 hours, as opposed to 72 hours or more in HMD and the prognosis is good in NDA but poor in HMD.

(iii) The two conditions present different chest X ray patterns: in NDA, centrally-distributed linear atrophy of the lung-fields is present ; in HMD there is "ground glass" opacity of the lung-fields with an air bronchogram.

Additional features of HMD are :

(i) Cyanosis in all cases, unless additional oxygen is given.

(ii) General prostration out of proportion to the respiratory impairment.

(iii) Generalised oedema in all cases, usually obvious at birth.

(iv) Respiratory distress which is not always obvious at birth and which developes progressively during the first 24 hours of postnatal life.

(v) Poor peripheral circulation, low blood pressure, hypovolaemia.

(vi) Low body temperature.

(vii) Paralytic ileus.

(viii) Increased susceptibility to infection.

(ix) Commonly associated complications --- bleeding tendency, pneumothorax, neonatal jaundice (2, 3, 4a). (See figs. I.1, I.2).

The term hyaline membrane disease has now been replaced by the Idiopathic Respiratory Distress Syndrome of the Newborn (IRDS).

Incidence.

The condition affects between 14% and 25% of all live-born premature infants, i.e. infants of gestational age less than 37 weeks, 8% of all infants born by Caesarian Section and 22% of all infants born to diabetic mothers. (2, 3, 5) Male infants are twice as commonly affected as females. The overall mortality rate varies with the standard of clinical care, but is of the order of 50% in most centres. The prognosis is worse in males than in females and it affects infants of all races equally. There is an increased incidence of the disease during the warm months (3). Statistical studies (6, 7) have shown that 80% of mothers of affected infants have a predisposition to give birth repeatedly to affected infants, provided that they are delivered prematurely. Exhaustive familial studies have been made in order to determine whether or not this predisposition is genetic (6). These have revealed no evidence for a genetic factor in its causation.

Clinical course.

The disease runs an average course of 72 hours, during which the hyaline membranes form and, in non-fatal cases, are later removed by phagocytic cells, or aspirated. A diuresis occurs between the second and third days, with disappearance of the oedema. Cases receiving treatment with an intermittent positive pressure respirator (IPPR), sometimes sustain a degree of permanent pulmonary fibrosis (8).

CLINICAL AND HISTOLOGICAL FINDINGS IN IRDS.

FIGURE 1.1 : INFANT WITH IRDS EXHIBITING SEVERE RIB-RECESSION DURING INSPIRATION.

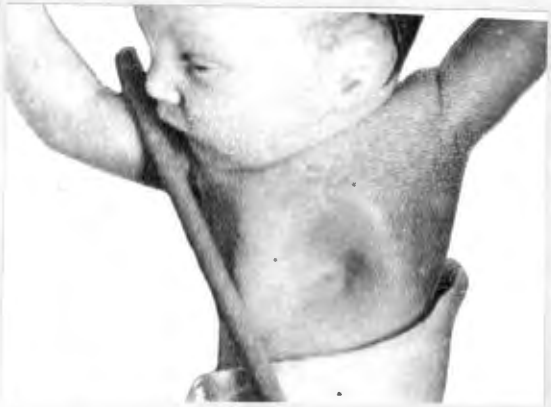


FIGURE 1.2 : TYPICAL CHEST X-RAY PATTERN, SHOWING "GROUND GLASS" OPACITY OF THE LUNG-FIELDS WITH AN AIR BRONCHOGRAM

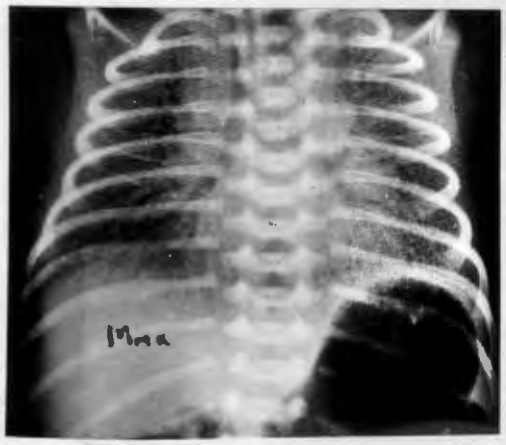
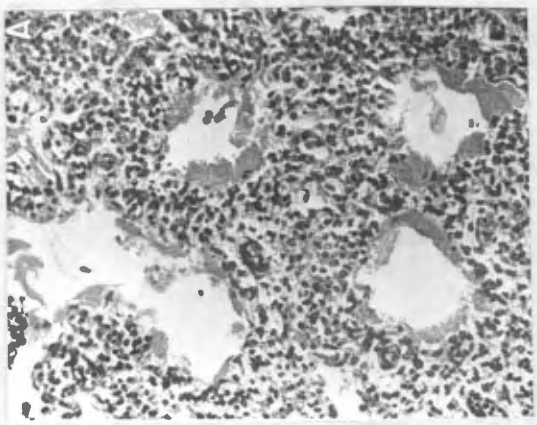


FIGURE 1.3 : MICROPHOTOGRAPH OF THE LUNG PARENCHYMA, SHOWING HYALINE MEMBRANES IN WIDELY-DILATED TERMINAL AIR-SPACES AND COLLAPSED LUNG PARENCHYMA IN OTHER PARTS.



1.II. Pathology and Pathophysiology.

II.1 Lungs.

At autopsy these are "solid", plum-coloured and non-aerated, so that they sink in water. There is intense pulmonary arteriolar constriction, accompanied by engorgement of the pulmonary veins and lymphatics. Many of the terminal air-spaces are collapsed and there is formation of hyaline membranes, i.e. laminated masses of eosinophilic material, containing sloughed cells, attached to the walls of the air-spaces. (See fig. I.3). These form mainly in the alveolar ducts, which are widely dilated (9, 10, 11). The hyaline membranes have been shown by studies with fluorescein-labelled antibodies (12), to contain all the major plasma proteins and fibrin. Quantitation of the protein fractions present was not possible by this method.

It has been suggested (13) that the above changes are explicable on the basis of reflex pulmonary arteriolar constriction, which is the normal response to anoxic insult in utero. Here the reflex has survival value, as it provides a mechanism for diverting blood from the non-essential pulmonary vascular bed to areas where it is more urgently required.(14a) In cases of perinatal asphyxia, this mechanism may persist and result in intense pulmonary arteriolar constriction after birth. As a result, there is inadequate alveolar perfusion and deficient anabolism in the alveolar cells.

This has the following effects :

(i) Inactivation of surfactant (see later).

(ii) Increased permeability of the alveolar wall, with effusion of plasma into the air-spaces.

(iii) Death of the alveolar lining cells and clotting of the exudate to form the hyaline membranes.

It seems unlikely that perinatal asphyxia is operative in all cases, as many appear to be in good condition at birth and to detiorate during the first few hours thereafter(2, 4b), i.e. the triggering mechanism for the vasoconstriction is still obscure. However, once established, arteriolar constriction may well produce a histological picture similar to HMD. It has not been determined why the membranes usually form in the alveolar ducts rather than in the alveoli.

A case has been reported (15) of an infant who died of HMD, and in whom it was found, at autopsy, that four of the five lobes of the lungs had hyaline membranes. The fifth lobe was perfused by aberrant vessels from the aorta and had no such membranes.

A similar histological picture has been produced experimentally in newborn lambs (16), using phenothiazines, or other measures to make the pregnant ewes hypotensive and subsequently delivering the lambs prematurely by Caesarean Section. Here the changes were also attributed to pulmonary vasoconstriction.

In addition to the vasoconstriction, very immature infants may have areas of the lung which are lined with relatively undifferentiated cells and which lack the normal elastin supporting stroma. Pulmonary haemorrhage and pneumothorax are common complictations for infants of all degrees of maturity.

Studies of the surfactant system of the lungs (17, 18, 14b), have shown that lungs of infants with IRDS are deficient in surfactant. It has been found in normal individuals, that the alveoli are lined with a substance which possesses a variable surface tension, the range being 0.5 to 45 dynes/cm., the surface tension increasing with increasing distension of the alveoli. The pleural pressures are low and in order to remain open at these pressures, the alveolar lining must consist of a substance having a surface tension much lower than that of plasma (30 dynes/cm.). As the alveolus becomes distended, the surface tension forces work at a smaller mechanical advantage so that the increasing surface tension tends to prevent overdistension. Hence surfactant acts to stabilize the lung. When it is inactivated, some areas become emmphysematous and others collapse (18), causing markedly increased respiratory efforts.

Surfactant is thought to consist of a monomolecular layer of a phospholipid-protein complex, containing palmitoyl lecithin. It is normally present in adequate amounts in the lungs of foetuses by the 24th week of gestation(17).

In IRDS inactivation of surfactant could be due to ischaemia, as it has been shown in dogs that experimental ligation of the pulmonary vessels on one side causes an increased surface tension of lung extracts and atelectasis on that side.

II.2 Cardiovascular system.

Under normal circumstances, expansion of the lungs at birth causes opening of the pulmonary vascular bed and an increased pulmonary blood flow. This, together with removal of the low-pressure placental vascular bed, reverses the pressure gradient between the right and left atria, which closes the foramen ovale. The ductus arteriosus does not close for a few hours, so that during this period shunts may occur through it in either direction. Closure is governed by the rising arterial oxygen tension accompanying improved pulmonary ventilation (14c).

In IRDS, foetal circulatory patterns tend to persist because of anoxia and poor inflation of the lungs. A common finding is a persistent right-to-left shunt via the ductus, or sometimes also via the foramen ovale, in cases of all grades of severity. In severe cases, there may be large left-to-right shunts via the ductus (16, 20).

Abnormalities of the electrocardiogram have also been found, especially prolongation of the PR and QRS intervals. These appear between the 12th and 60th hours after birth and progress with the disease. These changes have been

attributed to the hyperkalaemia associated with the disease, although it is unusual to find peaked T-waves (2, 3, 21).

Serial radiographic studies have shown progressive cardiac enlargement and eventual cardiac failure in fatal cases (3). A decrease in cardiac size accompanies clinical improvement. The basis for cardiac failure is uncertain : anoxia, shunts, increased pulmonary resistance and biochemical disturbances probably all contribute.

II.3 Other organ-systems.

The main features are :

(i) A generalised capillary lesion.

(ii) Changes secondary to anoxia.

(iii) A general bleeding tendency.

(iv) A defective fibrinolysin system.

(v) Poorly developed lymphoid tissues.

(i) The cappilaries throughout the body are abnormally permeable, resulting in generalised oedema, the oedema fluid having a high protein content (between 1 and 2 gm./100ml.), mostly albumin (2, 3). The capillary lesion is unlikely to be anoxic in origin as studies with the electron microscope (22) have shown that, in the lung, the capillary endothelium is damaged, whereas the alveolar lining cells are frequently normal in appearance.

(ii) Anoxia may aggravate the capilliary lesion and bleeding tendency as well as interfering with the functioning of all metabolically active organs.

(iii) The bleeding tendency gives rise to intracranial haemorrhages, haemorrhage into the adrenals, subcapsular haematomata of the liver, congestion of and haemorrhage into the gut and petechial haemorrhages elsewhere in the body. The haematocrit is low and concomitant neonatal jaundice is common.
(iv) The fibrinolysin system is defective in IRDS. Affected infants lack a plasminogen activator (23, 24, 25).
(v) It has been reported, that in IRDS infants, the lymph nodes are depleted of lymphoid precursors and that there is a mild degree of infiltration with eosinophils (26).

There is no histological evidence that renal function is abnormal, as compared with that of healthy premature infants. However, severely affected infants fail to respond to diuretics (27).

Infants with IRDS tend to have a low birth-weight for their gestational age, particularly after they have lost their oedema fluid (28).

1. III. Biochemical features.

III.1 One of the prominent features is a severe acidosis (4c, 29). Normal infants are mildly acidotic at birth and the blood pH tends to drop still further during the first hour of post-natal life, after which it starts to rise to adult levels. The pH does not normally fall below 7.25. The serum lactate concentration at birth has been found to be proportional to the length of labour and the fall in pH after birth is due to the release of acid metabolites which accumulate in the brief acute asphyxial episode occurring during delivery.

Infants with IRDS, however, suffer from a much more marked acidosis : a blood pH of 7.00 - 7.25 is usual and the pH may fall as low as 6.60 before death, in fatal cases.

(a) At or soon after birth, the acidosis is predominantly respiratory, with a slight matabolic component, the latter due mainly to the presence of lactate. At this stage, the pCO_2 is, on average, 60-70 mm. Hg. and the concentration of HCO_3^- is 20-24 mequ./l.

(b) After about 12 hours, the respiratory acidosis may decrease or remain constant, but the metabolic component of the acidosis becomes increasingly severe, as a result of further accumulation of lactate. The lactate concentration may reach 45 mg./100 ml., or more.

Typical findings at this stage are :

pH 7.1 - 7.25	normal 7.25 - 7.30
pCO_2 55 - 65 mm.Hg.	normal 40 mm.Hg.
HCO_3^- 16 - 18 mequ./l.	normal 19 - 22 mequ./l.

The acidosis is aggravated by the fact that compensatory mechanisms, operative in older individuals, do not function efficiently in the newborn infant.

(i) The kidney is unable to excrete PO_4^{---} and SO_4^{--} adequately during this period. It follows that the phosphate buffering system is absent from the urine (4d).

(ii) NH_3 formation is also not sufficient in the kidney of the neonate. Hence excretion of H^+ is impaired (4d).

(iii) Carbonic anhydrase activity of erithrocytes is low in all premature infants and therefore CO_2 transport and the HCO_3^--forming mechanism are depressed.(199)

(iv) The plasma protein concentration and haematocrit are low and therefore these buffering systems are also defective (30, 31, 32, 33, 34, 35, 36).

If the infant survives, these changes are gradually reversed. Most authorities agree that the following observations are incompatible with survival :

Blood pH less than 7.15

pCO_2 greater than 70 mm.Hg.

HCO_3^- less than 18 mequ./l. (4c)

1.III.2 Plasma electrolytes.

The plasma K^+ is within normal limits (± 4 mequ./l.) for the first few hours after birth and, in the absence of intensive treatment rises to 7 -- 9 mequ./l. at approximately 36 hours after birth (2, 3). In infants that survive, the concentration then falls, but it continues to rise in fatal cases. Normal infants also exhibit transient rise in serum K^+, but never to a concentration in excess of 7 mequ./l.

There is an associated rise in blood urea and inorganic phosphate in the plasma (6 -- 8 mg./100ml., normal 5 mg./100ml.). These follow the same pattern as the plasma K^+. The changes reflect general tissue catabolism, with release of intracellular substances. The kidney fails to excrete the excess K^+, urea and inorganic phosphate. Excretion of Na^+ is increased (4c).

III.3 Blood glucose.

The normal foetus near term has a blood glucose concentration which varies with the corresponding maternal level and which falls rapidly after birth, to reach a minimum concentration between 2 and 3 hours later. The average concentration during the first day postpartum is 35 -- 65 mg./100ml., with a gradual increase over the following few days.

Premature infants have lower concentrations than full-term infants and may develop a marked hypoglycaemia, on starvation or exposure to stress of any kind. However, infants tolerate hypoglycaemia well as compared with adults.

Various explanations have been advanced for this :

(a) The glycogen stores of the lower centres of the brain are higher than normal at birth.

(b) There is evidence (4e) that the brain of the neonate can utilize either protein or amino acids directly.

It is thought that, in normal infants, the hypoglycaemia is due to slower hepatic release of glucose, rather than increased utilization in the newborn. Further, it has been shown that the newborn mammalian liver cannot carry out gluconeogenesis from 3-carbon fragments (39).

Infants with IRDS have extremely low blood-glucose levels (5 -- 30 mg./100 ml.). A number of suggestions have been put forward to explain this :

(i) The stress of laboured respiration may exhaust the glycogen stores of the body.

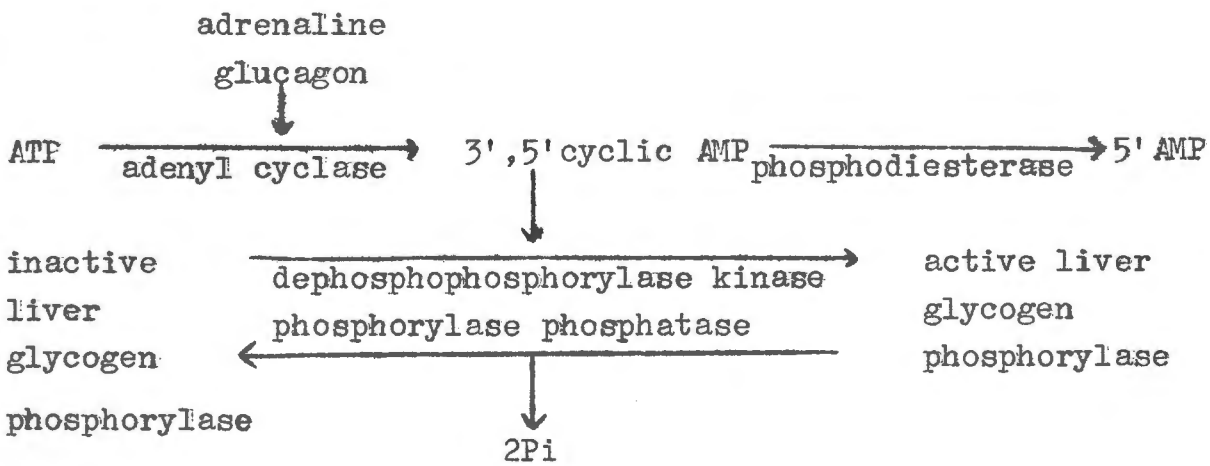
(ii) Adults have two enzymes for the phosphorylation of glucose : one with a low affinity for glucose and a high K_m ; the other a non-specific hexokinase with a high affinity for glucose. (37,38) Neonates have only the latter enzyme. Hence glycolysis proceeds at a slower rate in the newborn.

1.III.4 Hormonal induction of enzymic activity in neonates.

It has been shown, in foetal rabbits (39), that hypophysectomy results in inability of the liver to synthesize adequate amounts of glycogen.

Normally, there is a sharp postnatal rise in the activities of tyrosine-aminotransferase and glucose-6-phosphatase,

which is regarded as being a response to hypoglycaemia. In experimental animals, prenatal administration of glucagon, adrenaline and thyroxine was found to enhance the development of glucose-6-phosphatase activity.(39) According to Greengard (39) administration of insulin, glucocorticoids and somatotropin have no demonstrable effect, in neonates, on induction of the enzymes studied to date. However, cortisol has a marked effect in enhancing the activity of soluble phospho-enol-pyruvate carboxykinase and pyruvate carboxylase, in liver and kidney (40, 200). Cortisol has also been reported to induce tyrosine α-ketoglutarate transaminase activity. Glucagon, adrenaline and thyroxine are all capable of enzyme induction in mature foetuses. In immature foetuses, glucagon is ineffective and 3',5' cyclic AMP is required. The latter is probably a cofactor in enzyme induction and/or activation. For example, the process of activation of liver phosphorylase is as follows :



Most active hormones stimulate adenyl cyclase. Insulin probably acts by stimulating breakdown of cyclic AMP by cyclic 3', 5' nucleotide phosphodiesterase.

III.5.Hormones.

(a) 17-hydroxy-corticosteroids.

During the course of a normal pregnancy, there is a progressive rise in the concentration of these hormones in the maternal serum and a further rise during labour and delivery of the infant. The average serum concentration during the third trimester of pregnancy is 45.9 µg./100ml., of which 39.0 µg./100ml. is protein-bound. Corresponding values in healthy adult males are 17.2 µg/100ml. (16.3 µg. protein-bound) and in Cushing's Syndrome 32.7 µg/100ml. (16.1 µg protein-bound). (41)

Serum concentrations are normally highest at 8 a.m. and reach their lowest levels at 12 midnight. Pregnant women show the usual diurnal variation. Concentrations in cord blood have been observed to parallel those in the maternal blood. After birth, the concentration in the infant's blood falls.

There are conflicting reports on 17-hydroxy-corticosteroid levels in infants with IRDS. Some workers (42) have reported similar concentrations in healthy infants and in those with IRDS, while others(4c) claim that concentrations are high in infants with IRDS born of diabetic mothers and low in other cases.

No definite conclusion can be reached on the evidence available at present: the tissue catabolism would suggest increased steroid secretion, whereas the hypotension, hypoglycaemia, hyperkalaemia and increased urinary sodium excretion are consistent with adrenal hypofunction.

(b) Insulin.

Where infants of diabetic mothers are concerned, it has been observed (43,44) that the plasma insulin-like activity is increased, resulting in a flattened glucose tolerance curve. There is controversy concerning whether or not the fasting blood-glucose concentration is affected.

(c) Catecholamines.

In early life, nor-adrenaline increases oxygen consumption and plays an important part in maintaining the body temperature, by promoting the oxidation of brown fat (45). It is also a potent vasoconstrictor and raises arterial blood pressure by its effect on arteriolar smooth muscle. Adrenaline has a similar effect, but its effect on body temperature and blood pressure is less marked. Adrenaline is released in increased amounts in anoxia. In IRDS, the plasma adrenaline concentration parallels the rise in plasma potassium and lactate concentrations. A causal relationship between these observations has mot been established. Adrenaline increases the heart rate and the pulmonary

arerial blood pressure.

It has been claimed (46) that infants with IRDS have a significantly raised plasma adrenaline concentration.

(d) Progesterone.

Progesterone is known to have relaxant and anaesthetic properties. A proportion of the maternal progesterone is known to cross the placenta and enter the foetal circulation. As the maternal progesterone output falls towards term, it has been suggested that premature infants, being born while the progesterone level is still high, may be depressed by the unusually large amount of circulating hormone (47).

This may be a contributary factor in IRDS.

1. IV. Treatment.

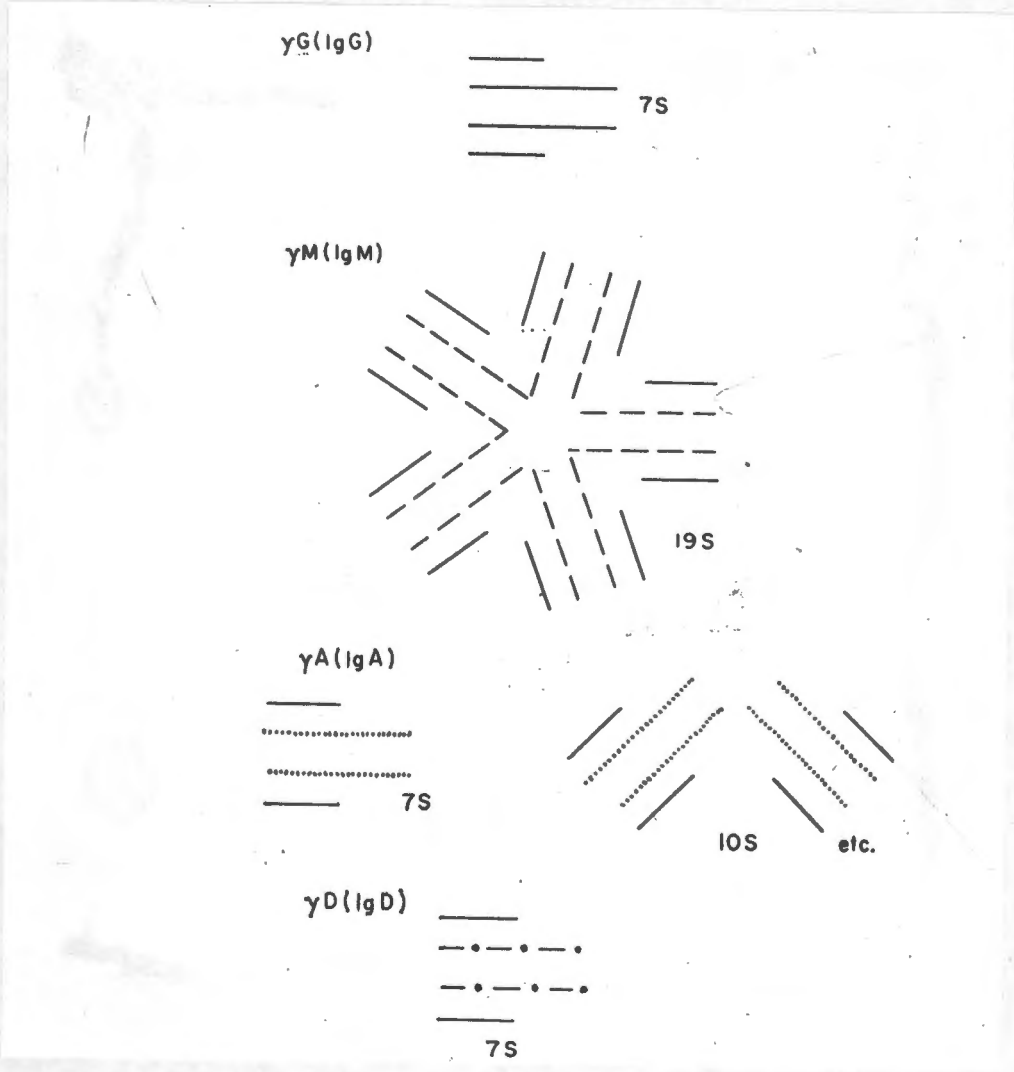
Affected infants are placed in an incubator and additional oxygen is administered. If necessary, the infant is intubated and given artificial respiration by means of an intermittent positive pressure respirator (IPPR). Secretions are aspirated by means of a suction pump at regular intervals. Infants are tube-fed.

The acidosis and hypoglycaemia are corrected, as far as possible, by intravenous infusion of sodium bicarbonate and glucose. Diuretics are given to induce a diuresis and promote the removal of oedema fluid.

Pneumothorax, when present, is treated by aspiration of the air via a needle in the pleural cavity.

Antibiotics are given for intercurrent infection and digitalis for cardiac failure

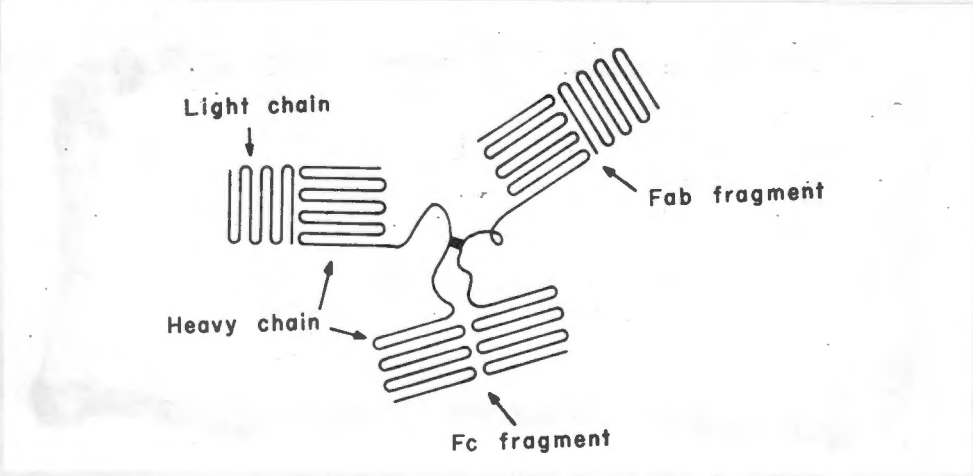
FIGURE 1.4: MOLECULAR CONFIGURATIONS OF THE IMMUNE GLOBULINS.

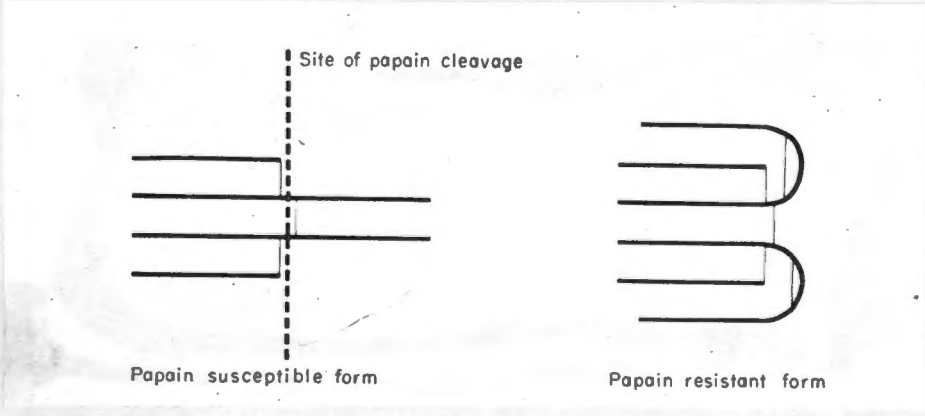


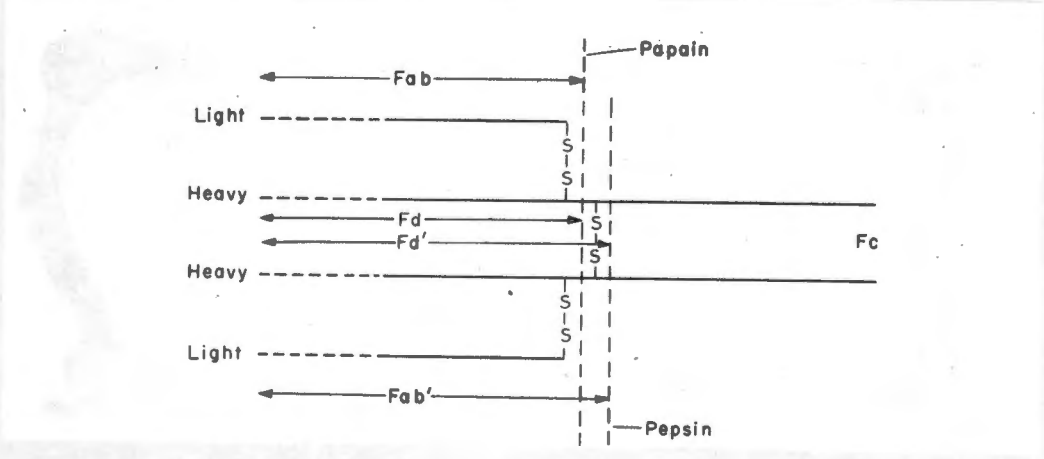
_____ γ-heavy chain; ——— μ-heavy chain;

..... α-heavy chain; -.-.-. δ-heavy chain.

FIGURE 1.5: CONFIGURATIONS OF THE IgG MOLECULE AND SITES OF DISRUPTION BY PROTEOLYTIC ENZYMES.







V.1. Physicochemical properties of the immune globulins.

To date, five major classes of immune globulins have been identified. Some of their established properties are listed in Table 1.1 (48,149,155,156)

Immune globulins consist structurally of heavy and light chains, linked by disulphide bridges. Light chains are common to all classes of immune globulin and are of two types: k and λ. In normal individuals, 65% of the immune globulin molecules have type k light chains and 35% type λ. (See Figs 1 - 4, 1.5).

Light chains have on them the antibody-combining sites of the molecule: each has about 110 amino-acid residues and a molecular weight of 20,000. 30 of these residues provide sites where substitutions may occur, resulting in specific antibody-combining sites. Type k light chain configurations are determined by the Inv genetic locus.

The heavy chains are characteristic of the class of immune globulin. These are termed γ (IgG), μ (IgM), α (IgA), δ (IgD), and ε (IgE). Their approximate molecular weight is 50,000.

IgG, IgA, IgD, and IgE each have two heavy and two light chains joined by disulphide bridges and their structure may be summarized as follows:

IgG : $(\gamma k)_2$ or $(\gamma\lambda)_2$ IgA : $(\alpha^k)_2$ or $(\alpha\lambda)_2$

IgD : $(\delta k)_2$ or $(\delta\lambda)_2$ IgE : $(\varepsilon k)_2$ or $(\varepsilon\lambda)_2$

Table 1.1 : Some properties of the immune globulins.

	IgG	IgM	IgA*	IgD	IgE
Molecular weight (approx)	150,000	900,000	150,000	150,000	200,000
Sedimentation coefficient	7	19	7,11	7	3
No. of antibody combining sites	2	5	? 2	?	?
Serum concentration in adults (mg./100 ml.)	1200	150	300	3	0.025
Percent intravascular	45	80	40	75	50
Biological half-life in days (approx.)	23	5	6	3	?
Placental transfer	+	-	-	-	-
Complement fixation	+	+	-	?	? -
Relative agglutination capacity	low	high	variable	?	low
Electrophoretic mobility	1.2	2.1	2.1	slower than IgA	?
Carbohydrate content %	2.9	11.8	7.5	?	?

* IgA exists in serum mainly in the 7S monomer form but 9 to 15S polymers occur as well. Secretory IgA contains an additional component and exists mainly in the 11S form. The agglutinating capacity of monomers is low ; of polymers high.

The full structure of IgG has recently been elucidated (49). IgM has ten heavy and ten light chains. The structure may be summarized as $(\mu k)_{10}$ or $(\mu\lambda)_{10}$. (see fig. 1.4) The IgM molecule may be disrupted at the disulphide bridges by incubation at 37 C with an equal volume of 0.1 M 2-mercapto-ethanol for one hour (50).

IgG molecules may be split enzymatically with papain, pepsin, or other proteolytic enzymes. Papain digestion of IgG in the presence of cysteine results in the production of fragments termed Fab, Fc' and Fc. Pepsin splits the molecule into fragments Fd, Fc' and light chains. (see figs. 1.6, 1.7) IgG may exist in a papain-resistant form. The Fc fragment of the molecule carries the antigenic determinants characteristic of IgG and is electrophoretically faster than Fab. Normal individuals have four different types of γ-chain, termed γ_{2a}, γ_{2b}, γ_{2c}, γ_{2d}. In addition, there are finer, genetically-determined differences between the IgG's of different individuals (IgG allotypes). The Gm genetic locus governs the configuration of Gm determinants on the Fc fragment of the molecule.

Much of the generally accepted information concerning physicochemical, immunological and biological properties of immune globulins was derived from references 51, 52 and 53

Complex-formation between immune globulins and other plasma constituents.

Immunoelectrophoretic studies (54,55,56,57) have

demonstrated that IgG and IgA, and possibly fragments of the immune globulin molecule may form complexes with albumin and other serum constituents such as lipids, polysaccharides, metals, etc. Complexes of this kind generally have an electrophoretic mobility intermediate between that of the main components.

It has been suggested that complex-formation may be responsible, in part, for the wide range of electrophoretic mobilities encountered, especially in the case of IgG.

Low molecular-weight fragments of immune globulin.

Free k and λ -type polypeptide chains and heavy chain fragments, resembling the Fc fragment of IgG after papain digestion, have been observed to be present in small amounts in normal plasma and urine (150, 151, 152, 153, 154). The heavy chain fragments are presumably the result of catabolism of immune globulins, whereas the light chain fragments are thought to be anabolic in origin (150, 151, 152, 153, 154, 174, 175, 176, 177, 178). Catabolism of light chains is reported to occur mainly in the kidney (173, 179), although the liver plays an important part in IgG catabolism (51).

V.2 Immune globulins.

(a) Site of synthesis.

Immune globulins are produced by plasma cells in the reticulo-endothelial system (RES). These cells are 8--10 μ in diameter, with an eccentric "cartwheel" nucleus and basophilic cytoplasm. They have the well-developed, rough endoplasmic reticulum and polyribosomes characteristic

of protein-secreting cells. Fluorescent-antibody studies (58, 59, 60, 61, 62) have led to the conclusion that any one cell produces only one type of immune globulin and with only one type of light chain.

(b) Functions.

(i) The functions of IgD and IgE are still speculative. The IgE molecule has the property of attaching itself firmly to tissue cells and is believed to participate in atopic allergic reactions. Little or none of these proteins are present in the serum of newborn infants (84, 85, 156, 157, 158, 163).

(ii) IgA is the main immune globulin found in secretions of all kinds (saliva, tears, respiratory tract secretions, colostrum,etc.) (86). It is present in large amounts in the gut and faeces of the newborn,where it has been described as "coproantibody" (63, 64), The concentration in the gut decreases with increasing age. Umbilical cord blood contains, on average, about 1/50 of the adult IgA concentration (157, 158, 159, 163).

(iii) IgM is predominantly intravascular. It has five combining sites and is particularly effective in agglutinating cells and other large circulating particles. In the presence of complement, it may participate in lysis of cells, following agglutination.

The primary antibody response to a foreign antigen is usually of the IgM type and is followed by production of IgG.

Agglutinating antibodies of the IgM type, directed against IgG and/or fragments of the IgG molecule, have been demonstrated in the sera of individuals with auto-immune disease, e.g. Rheumatoid Arthritis, in multiply-transfused individuals and in certain normal individuals (65, 66, 67, 68).

In the case of normal individuals, antibodies to IgG fragments are common (60% of normal adult sera), the incidence rising with increasing age (69, 70, 71, 72). Their presumed function is to remove from the circulation the end-products of IgG catabolism. Antibodies to the intact IgG molecule, when present, are usually directed against an antigenic determinant foreign to that individual and present on the IgG molecules of his or her mother. It is unusual to find antibodies of this type in healthy neonates, although they are occasionally present (68, 160, 161). Such antibodies have been observed to persist for years in the absence of antigenic stimulation (162). Umbilical cord blood has, on average, one tenth of the adult IgM concentration.

(iv) IgG. This is the major immune globulin present in the plasma and interstitial fluid. IgG is the antibody-type usually formed during the secondary response to antigenic stimulation. It participates in precipitin and complement-fixation reactions (51, 52).

The normal foetus has an extremely limited capacity for IgG synthesis (73, 74). Most or all of the IgG present in the infant's body fluids at birth is derived from the

maternal circulation. Whether or not the foetus synthesizes its own IgG insignificant amounts is dependent on the antigenic stimulation in utero (165, 166). As an example, infants who suffer infections in utero are born with well-developed lymphoid tissues and the capacity for producing large amonuts of IgG (75, 76, 165, 166).

In normal foetuses, IgG appears in the circulation in measurable amounts at 11--14 weeks after conception. At term, the concentration in the foetal blood is the same as, or higher than, that in the maternal blood. Virtually all of this is derived from the mother by selective and active placental transfer. Hence at birth, the normal infant has its mother's IgG phenotype (77, 78, 159, 170). For normal infants, a linear relationship between gestational age and serum IgG concentration, up to the gestational age of 40 weeks, has been demonstrated. In postmature infants, the IgG level falls (79, 80).

There is some evidence that foetal IgG differs from maternal IgG : immunoelectrophoretic analyses have shown that the foetal IgG precipitin arc is shorter on the anodic side and sometimes shows longitudinal splitting (51).

After birth, there is an exponential decline in the plasma IgG concentration, until the age of 2--3 months (78, 81, 82, 83). The lowest level reached at this stage is of the order of 200--400mg./100ml. Thereafter the IgG concentration rises, as the infant synthesizes increasing

amounts of its own IgG. Adult levels are reached at about one year of age (15, 7, 78, 81, 82, 83, 158, 163).

It is uncertain whether or not the placenta can synthesize IgG. In vitro attempts to demonstrate synthesis have failed (87), but it has been observed that the placenta contains plasma cells (88). Also there have been cases reported of agammaglobulinaemic women, who acquired the ability to synthesize IgG during the third trimester of pregnancy (89). If the placenta does synthesize IgG, this protein appears to be released into the maternal, rather than the foetal circulation (87).

Amniotic fluid has a smaller proportion of IgG, relative to thetotal protein concentration as compared with serum. The proteins of the amniotic fluid are beleived to be predominantly of maternal origin and to reach the foetal circulation in very limited amounts (51).

In man, the sole site of transmission of appreciable amounts of IgG, has been shown to be the placenta and not the amnion, yolk sac, or colostrum (90, 91, 92, 93).

Mechanism of transmission of IgG.

It has been suggested (94) that the sequence of events is as follows :

(i) Non-selective pinocytosis of a droplet of maternal extracellular fluid, containing a mixture of proteins, by the cells of the syncitial trophoblast.

(ii) Fusion of the pinocytotic vesicle with a lysosome.

(iii) The placenta is rich in acid proteases which are inactive in the pH range above 5--6, but highly active at pH 2--4. These pour into the lysosome and destroy all the proteins except 10--20% of the IgG.
(iv) The intact IgG molecules escape into the interstitial space of the placental villus.

It is likely that the Fc fragment of the molecule has on it a specific inhibitor of the acid proteases. The evidence in support of this is:
(a) Fc fragments are readily transmitted, but Fab fragments only to a small extent (93,77).
(b) IgA, IgM, IgD and IgE have no Fc fragment and are not transmitted.

V.3. Maternal-foetal immunological interactions.

These have been under investigation since 1890 when Heape (172) performed the first foster-pregnancy experiment. The main point under investigation is why the foetal homograft is so much more successful than homografts of different (e.g. paternal) origin.

Immunization of the mother by foetal antigens occurs, but is unusual (for example Rh-isoimmunization). The currently accepted view is that the trophoblast, which is weakly antigenic, forms a physiological barrier between mother and foetus and excludes most foetal antigens from the maternal circulation (167,168). Ancillary measures may include damping of the immune response by the raised plasma cortisol and acquired tolerance to paternal antigens with increasing parity.

1. VI. Foetal plasma proteins.

During foetal life, the total plasma protein concentration rises from about 1.5 Gm./100ml. at between six and eight weeks of age to about 5.5 Gm./100ml. ar birth. There is an overall resemblance between the foetal and maternal plasma protein patterns, with the following exceptions:
(i) There is a "pregnancy zone" in the α_2 -globulin region of the maternal serum, which is not present in the foetus (95).
(ii) The foetal IgG is slightly different from that of the mother (see Part 1:V). Levels of IgA and IgM are very low and IgD and IgE are probably absent in the foetus and newborn. (78,81)
(iii) Foetal serum contains no haptoglobins (51).
(iv) Foetal serum contains a protein or proteins not present in normal adult serum.
Tatarinov (96) and Adinolfi and Gardner (97) have claimed that, during the first half of intrauterine life, there are at least four foetus-specific proteins, migrating electrophoretically with the α - and β -globulins and that in the second half there are two: one α - and one β -globulin. Other investigators (98,99,100) have dected only one foetus-specific protein migrating just behind serum albumin on cellulose acetate at alkaline pH. This protein has been termed α -fetoprotein. (98)

In human foetuses, α -fetoprotein appears early

in embryonic life and maximum concentrations in the plasma are reached at the age of 12 to 13 weeks. The total rate of synthesis increases increases until about 20 weeks, but the plasma concentration falls, because of rapid foetal growth. After this, the total amount in the extracellular fluid remains relatively constant (but the serum concentration continues to fall) until 2--4 weeks before birth, when the amount present falls abruptly, whether the birth is premature or full-term, i.e. curtailment of synthesis appears to be linked with the imminent onset of labour.

After birth, production of α-fetoprotein apparently ceases and it disappears from the plasma during the first few days of post-natal life.

α-fetoprotein has been found in sera of women with spontaneous abortion, where it may have gained access to the maternal serum as a result of placental detachment with rupture of placental vessels. An apparently identical protein is present in the serum of most or all patients with Primary Carcinoma of the Liver.(96,101) It is, however, not present in other types of liver disease, other neoplastic disease, or cases with liver metastases originating elsewhere in the body (96,101,164).

α-fetoprotein has an estimated molecular weight of 79,000 and a sedimentation coefficient of 5.0. It is not a glycoprotein or a lipoprotein. The site of

synthesis is probably the liver or yolk sac (99).

There is some evidence that it may be a growth-promoting factor. Foetal serum promotes the spreading of cells in tissue culture to a greater extent than does adult serum(102)

Similar foetal proteins have been found in species as diverse as the opossum, squirrel monkey, armadillo, mouse, rat, rabbit, seal, dog, cat, cow and sheep. Antisera raised against the foetal protein of one species frequently cross-react with foetal proteins of other species, suggesting that they are immunochemically and functionally related(96, 103).

It has been reported (104) that 11% of pregnant women develop skin reactions on injection of foetal serum and/or extracts of amniotic fluid. The significance of this uncertain.

Another observation (185) is that, whereas sera from women in early pregnancy stimulate the growth of cells in tissue culture, sera from women in the second half of pregnancy have an inhibitory effect. The inhibitory activity is associated with the appearance in the serum of an abnormal α_1-globulin, which disappears shortly after delivery of the infant.

The foetus is believed to synthesize all its own plasma proteins, with the exception of IgG.

1. VII. Properties of Somatotropin (Human Growth Hormone).

Human growth hormone (HGH) is secreted by the acidophil cells of the adenohypophysis. Serum-concentrations are highest in foetuses and young infants (50--60 gm./ml.) and lowest in healthy adults (0.6--0.7gm./ml.). There is a diurnal fluctuation in secretion of the hormone : a rise 4 hours after breakfast and a fall after lunch. During sleep the level rises and is highest at midnight (108).

The known actions of the hormone are to raise the concentrations of glucose and non-esterified fatty acids (NEFA) in the blood and to facilitate the incorporation of amino-acids into cells for protein synthesis (105, 106, 107). The hormone also produces a transient increase in permeability of adipose tissue cell-membranes to sugars (107).

The HGH molecule consists of 188 amino-acid residues with phenylalanine at both the C- and N- terminal ends. It contains no carbohydrate and no lipid. The molecular weight is 21,000 , but the usual methods of extraction of the hormone from human pituitaries readily produce aggregates of a higher molecular weight (109, 110).

The native hormone in serum migrates as an α-globulin on cellulose-acetate electrophoresis at alkaline pH. Commercial preparations are often contaminated with traces of other serum-proteins, particularly albumin and protein(s) migrating in the γ-globulin region. (110, 111, 112, 113)

Immunologically, HGH is related to Lactogenic Hormone and to Human Placental Lactogen (HPL), but not to Human Chorionic Gonadotropin (HCG) or to Luteinizing Hormone. Antisera to HGH cross-react with Simian Growth Hormone and with other mammalian Growth Hormones, if the latter, with the exception of Simian Growth Hormone, are partially digested with pepsin prior to testing, or exposed to sodium dodecyl sulphate (114,115).

Laron and Assa(116) separated HGH, prepared by the method of Raben (109) into three components (C1,C2,C3) by electrophoresis on starch gel and prepared antisera against each.They found that the antiserum against C3 cross-reacted with C1,C2,C3 and also human serum albumin (HSA) when these were coated onto tanned sheep erythrocytes. Antisera to C1 and C2 reacted with C1, C2 and C3 only. There was no demonstrable reaction between HSA and any of these antisera when tested by means of immune diffusion in agar gel. The reactions demonstrated between C1, C2, and C3 and the antisera were reactions of identity.

Recently it has been reported (111,112) that further purification of HGH, supplied by the NIH (see part II:2), can be achieved by the use of ultrafiltration to eliminate aggregated material. Such preparations contain no HSA, as tested by immunoelectrophoresis, but have a component migrating in the γ-region. These investigators could demonstrate no reaction between this component and an

antiserum against human 7S-γ-globulin prepared in rabbits.

HPL has been shown to be produced by the cells of the syncitiotrophoblast (120). While being related to HGH immunologically, it has little growth hormone-like activity, but is strongly lactogenic.

Lactogenic Hormone, like HGH, is produced by the adenohypophysis and also shares certain antigenic determinants with HGH. However, it functions entirely as a lactogenic agent.

1. VIII. Properties of Human Serum Albumin (HSA).

HSA is present in the serum of healthy adults in a concentration of approximately 3.5--4.0 Gm./100ml. It has a molecular weight of 69,000, 4.6S, and an isoelectric point of 4.9. The molecule contains 0.2% lipid and 0.08% carbohydrate by weight. Normally, two-thirds of the albumin present in serum of healthy individuals exists as mercaptalbumin, the remaining one-third lacking an additional cysteine residue. Albumin undergoes pH-dependent structural alterations (N-F transformation) and has been observed to form dimers and higher polymers.

Complex-formation between HSA and immune globulins has already been discussed (Part2 :V.1). In addition, HSA is known to bind other substances, such as bilirubin, dyes and certain hormones. Bound substances tend to alter the electrophoretic mobility of the protein. Many substances with an affinity for HSA are able to inhibit the N - F transformation.

Microheterogeneity of the primary structure occurs: the different cysteine content of one third of normal HSA molecules was noted above. Healthy individuals have also a small and variable amount of low-molecular-weight (30,000, 2.6 S) albumin in their serum and urine. This is indistinguishable in other ways from serum albumin of normal molecular weight (190,191,192)

In cadmium toxicity, low molecular weight albumins,

entirely lacking in tryptophan and deficient in lysine and cystine, are present in the serum and are excreted in the urine. These range in molecular weight between 5,000 and 20,000 and have been termed minialbumins (187, 188,189). Minialbumins are antigenically identical with normal serum albumin and aggregate readily to form molecules of the usual size, or larger, in low-salt media.

Bisalbuminaemia is a rare congenital abnormality, genetically determined, in which there are two main albumin fractions present in the serum. These are immunochemically identical, but differ in their electrophoretic mobilities. As a rule, the albumin components do not differ significantly as regards their biological functions. The slow albumin component in this condition is reported to be unrelated to α -fetoprotein.

1.IX. Objectives of the present study and main conclusions .

This study was undertaken primarily to investigate the plasma protein system in infants with IRDS, as compared with heathy premature infants, as it had previously been reportd that the plasma protein concentration in affected infants was abnormally low. It was attempted further to establish biochemical and/or immunological criteria for diagnosis of the disease and to discover reasons for the low IgG concentrations and raised α-fetoprptein concentrations found in the sera of these infants. Maternal serum proteins were also studied during pregnancy and at and after delivery of the infant. Interrelationships between α-fetoprotein, Human Growth Hormone and other proteins, in immunochemical systems, were investigated.

In summary, the main conclusions reached were as follows:

(i) The total serum-protein concentration in affected infants is much reduced, as compared with healthy premature infants of the same gestational age.

(ii) In IRDS infants, the relative and absolute concentrations of IgG is extremely low, whereas concentrations of other immune globulins, as far as could be determined, are within normal limits.

(iii) Mothers of affected infants have significantly

lower . concentrations , both of serum IgG and of IgM, than mothers of healthy premature infants. These changes in the serum-proteins are present throughout pregnancy. By six weeks post-partum, the IgG level has returned to normal, but the IgM level remains low. Concentrations of IgA and total serum-protein are normal at all times.

(iv) Examination of oedema fluid, urine , faeces and amniotic fluid for γ-globulin content, has excluded the possibility that IgG is being lost from the circulation by these routes.

(v) IRDS infants have, in their serum, agglutinins of the IgM type directed against the intact maternal IgG molecule. Similar agglutinins are present in a minority of healthy premature infants. Both IRDS and healthy infants have agglutinins against IgG fragments, in approximately 50% of cases. Agglutinin titres against these are similar in the two groups, but the incidence of agglutinins against Bence Jones protein type L is raised in IRDS.

(vi) Affected infants have an elevated serum concentration of α-fetoprotein , which disappears from the serum during the first week of post-natal life.

(vii) The majority of pregnant women examined have been observed to have serum agglutinins directed against α-fetoprotein. These cross-react with albumin prepared from sera of healthy adult males. α-fetoprotein has been found in the serum of many pregnant women, especially

during the second trimester.

(viii) Immunological interrelationships between α-fetoprotein human serum albumin, Human Growth Hormone and human IgG have been demonstrated.

(ix) Infants suffering from Rh-isoimmunization exhibit a serum- protein pattern similar to that seen in IRDS.

Biochemical and immunological criteria for the dianosis of IRDS have thus been established. The data to be presented indicate the presence of an immunological factor in the aetiology of the disease.

Some of the findings recorded in this thesis have been published. Reprints of these publications form an addendum.

PART 2

MATERIALS AND METHODS

2.I. Cases selected for the study on clinical and autopsy findings

Clinical diagnoses were made, in most cases, by Dr V.C.Harrison and Dr M. Klein of the Neonatal Respiratory Unit, Groote Schuur Hospital, or by Registrars in Paediatrics at other teaching hospitals attached to the University of Cape Town.

The infants manifested the signs of idiopathic respiratory distress as described in Part 1.I. and the diagnosis was confirmed radiologically and/or at autopsy. The group labelled IRDS ? in Table 3.9 manifested clinical signs of IRDS, but radiological or histological confirmation of the diagnosis was not possible, because of lack of facilities or severity of illness. The control groups consisted of healthy premature infants of a similar gestational age and full-term infants, born at any of the teaching hospitals.

The mothers studied were selected on the basis of (i) their previous obstetrical history and (ii) the condition of the infant at birth. The antenatal cases were patients attending the antenatal clinic at Groote Schuur Hospital, or cases admitted to the Obstetrical wards.

Data concerning blood gas tensions, blood pH, base excess, buffer base, etc., were supplied by the Paediatrician in charge.

Chest X-rays were taken by the Department of Radio-diagnosis, Groote Schuur Hospital. Autopsy findings were supplied by the Department of Pathology, University of Cape Town.

2.II. Biological materials and preparation for analysis

1. Blood

In infants this was obtained from:

(i) The umbilical cord at birth and/or (ii) A catheterized umbilical vein, during the first 72 hours of life and/or (iii) A peripheral vein, at varying periods after this.

Maternal blood was obtained from a peripheral vein at intervals during pregnancy, and/or at delivery. In some instances, additional specimens were obtained 6 weeks or more post-partum.

Foetal blood was aspirated, by cardiac puncture at hysterotomy, or after a non-infected abortion, from foetuses ranging in maturity from 12 - 24 weeks.

The blood specimens were either:

(a) Allowed to clot, after which the serum was separated by centrifugation. Sera were stored at 4°C with 0.1% sodium azide as a preservative, except in cases where enzyme studies were to be performed, where the addition of preservative was omitted;

or (b) Collected in a tube containing sodium citrate as an anticoagulant. The red blood cells were centrifuged, washed with 0.15M sodium chloride and a haemolysate was prepared, by alternate freezing and thawing, followed by removal of the red cell membranes by ultracentrifugation. This preparation,or

serum without preservative, was employed for enzyme studies.

Serum of patients with Primary Carcinoma of the Liver (Hepatoma) was kindly supplied by Dr L.S. Purves, Department of Chemical Pathology, University of the Witwatersrand, Johannesburg. (The incidence of Hepatoma is high in the vicinity of Johannesburg).

2. Urine

24-Hourly specimens were collected from (i) healthy premature infants (ii) from infants with IRDS (iii) ante-partum from six mothers, of whom four subsequently gave birth to healthy premature infants and two to infants with IRDS.

These specimens were dialyzed for 24 hours against distilled water at 4°C, using Visking membranes previously boiled for 30 minutes in distilled water, to decrease the pore-size. Care was taken to prevent the membranes from becoming tense during dialysis. The sac contents were lyophilized and the diffusate concentrated by means of a rotary evaporator.

3. Meconium and Faeces were collected from (i) normal and affected infants and (ii) ante-partum from the six mothers mentioned in (2). These were homogenized in an equal volume of sodium chloride, 0.15M, centrifuged and the deposit discarded.

4. Amniotic fluid was obtained by needle puncture and aspiration from the amniotic cavity at hysterotomy, or by amniocentesis at intervals during pregnancy. The fluid was

centrifuged to remove debris, lyophilyzed and redissolved in 1/10 its original volume of distilled water.

5. Fresh Placentas were obtained from one infant with IRDS, two healthy premature infants and two normal full-term infants. These were cut in strips, about 0.5 cm thick, rinsed thoroughly in cold 0.15 M sodium chloride to free them of residual blood and homogenized with the smallest possible volume of cold 0.15 M sodium chloride, in a Waring Blendor. The homogenate was centrifuged and the deposit discarded.

6. Hyaline membranes were obtained by aspiration from some of the affected infants. Lipid was extracted by homogenizing these in a mixture of ethanol and ether, 3:1 v/v, followed by centrifugation. The supernate was examined for lipid content and the deposit kept for amino-acid analysis.

7. Bence Jones Proteins, prepared from the urine of patients with Multiple Myeloma, were kindly supplied by Dr G.M. Potgieter, Department of Chemical Pathology, University of Cape Town (UCT). Prior to use they were dissolved in 0.15 M sodium chloride.

8. Bovine serum albumin (Cohn Fr. V) was supplied by Nutritional Biochemicals, inc., (Cleveland, Ohio).

9. Goose serum albumin was kindly supplied by Mr P. Gubb, Department of Chemical Pathology, U.C.T.

10. Amandin, purified from almonds, was kindly supplied by Dr L. Kaminsky, Department of Physiology and Medical Biochemistry, U.C.T.

Preparations 8 - 10 were dissolved in 0.15 M sodium chloride prior to use.

11. Somatotropin and an antiserum to it, were supplied by the Endocrinology study section of the National Institutes of Health, Bethesda, Maryland, U.S.A. The Somatotropin was dissolved, at a concentration of 1 mg/ml, in barbitone buffer, pH 8.6, ionic strength = 0.075.

12. Sheep erythrocytes were obtained by jugular puncture, from sheep at the Cape Town Municipal Abattoirs, preserved in Alsever's solution and formalinized as described in Part 2.V. (11). The cells were then stored at $4^{o}C$ in 0.15 M sodium chloride, with 0.02% merthiolate as a preservative.

13. Human chorionic gonadotiopin was supplied by Organon laboratories, Amsterdam.

2.III. Immunological reagents used

1. Monospecific antisera against human serum albumin, human IgG, IgA, IgM, Bence Jones proteins types K and L, human chorionic gonadotropin (HCG) and a polyvalent antiserum against adult human serum were supplied by Hyland laboratories. (See Fig. 2.1).

These antisera were produced by immunization of rabbits or goats with the intact protein(s) in the case of all the proteins except IgG, IgA and IgM. The antisera to the latter were produced by immunizing animals with the appropriate heavy chain fragments (122).

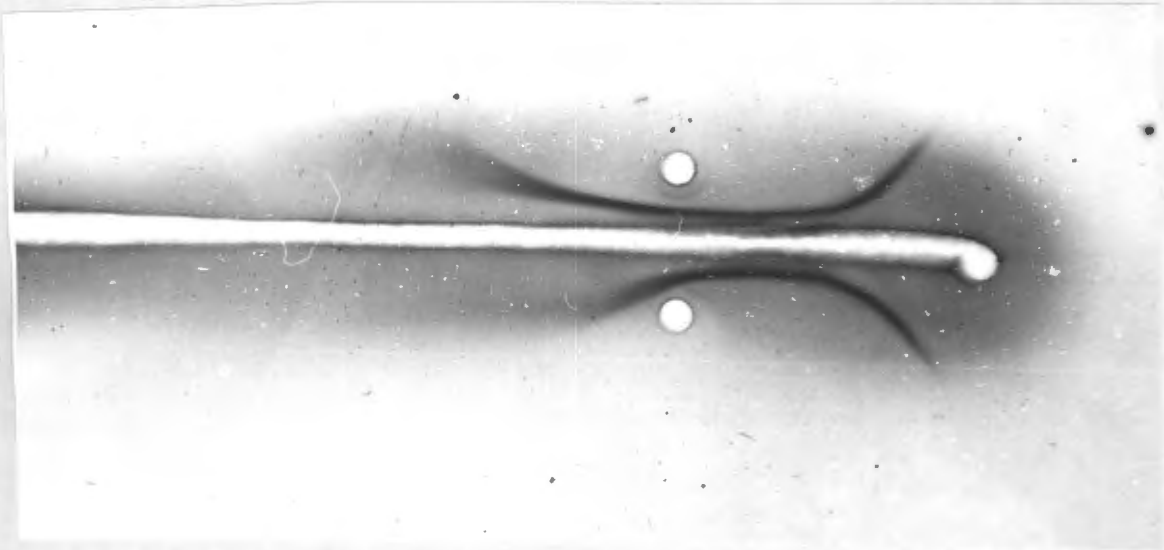
A monospecific antiserum to α- fetoprotein was originally supplied by Dr L.S. Purves. Subsequently a similar antiserum was prepared by the candidate. Rabbits were immunized with foetal serum, as described in Part 2. VII, and antibodies to serum proteins, other than α-fetoprotein, were absorbed out by addition of excess adult male serum.

2. Immunoplates for the immunochemical quantitation of human IgG, IgA and IgM, were supplied by Hyland laboratories. (Fig. 2.2).

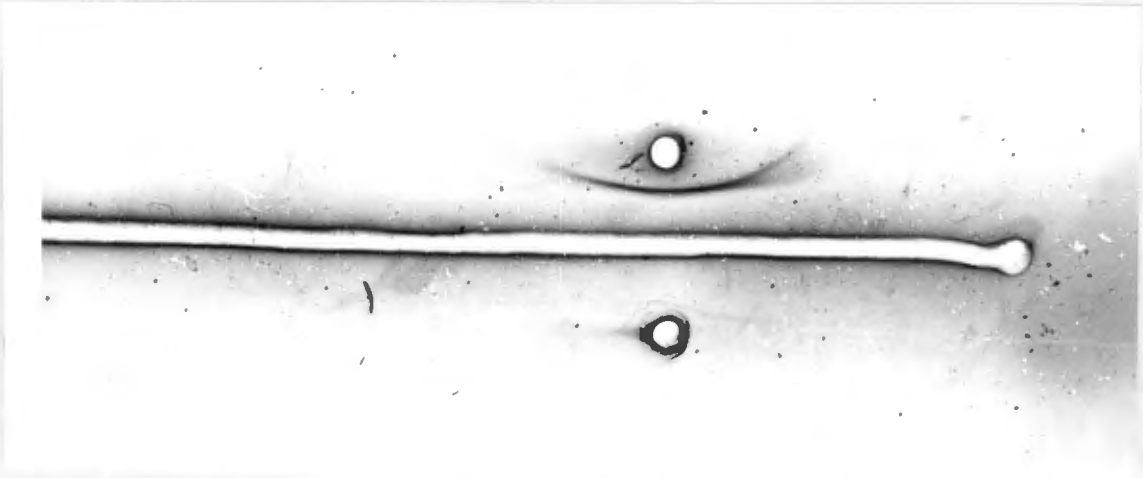
3. Fetoprotein immunoplates were prepared as described by Russchen (123). (See Fig. 2.3).

FIGURE 2.1

IMMUNOELECTROPHORETIC DEMONSTRATION OF THE MONOSPECIFICITY OF THE ANTISERA TO IgG AND IgM USED.



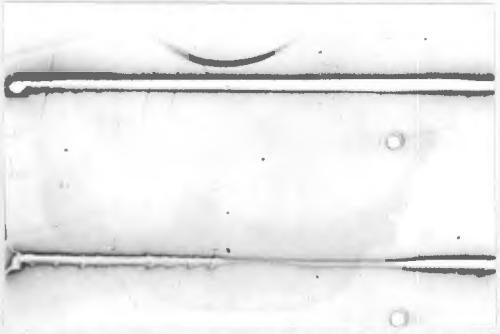
UPPER WELL: WHOLE SERUM; LOWER WELL: IgG PREPARATION ANTISERUM TO HUMAN IgG IN SLOT.



UPPER WELL: WHOLE ADULT SERUM; LOWER WELL: IgG ANTISERUM TO IgM IN SLOT.

FIGURE 2.1 (CONTINUED)

IMMUNOELECTROPHORETIC DEMONSTRATION OF THE MONOSPECIFICITY OF THE ANTISERA TO HUMAN SERUM ALBUMIN, α-FETOPROTEIN AND BENCE JONES PROTEINS OF TYPES K AND L.



1. UPPER WELL: FOETAL SERUM. LOWER WELLS: VARIOUS ADULT SERA. ANTI α-FETOPROTEIN IN SLOTS.

2. UPPER WELL: WHOLE SERUM. LOWER WELL: SERUM ALBUMIN PREPARATION. ANTISERUM TO HUMAN SERUM ALBUMIN IN SLOT.

3. ADULT MALE SERUM IN LOWER WELL. ANTISERUM TO BENCE JONES PROTEIN TYPE K IN SLOT.

4. ADULT MALE SERUM IN LOWER WELL. ANTISERUM TO BENCE JONES PROTEIN TYPE L IN SLOT.

2.IV. Methods of protein fractionation

1. Column chromatography on Sephadex G200 (124)

The column used was 38 cm long, with an internal diameter of 2 cm and a coarse-grade, sintered-glass disc at the base. This was packed with Sephadex G200 (Pharmacia, Uppsala) which had previously been equilibrated with 0.1 M Tris-HCl buffer, pH 8.0, containing 0.2 M sodium chloride and 0.05 M ethylene-diamine-tetra-acetic acid (EDTA). 0.5 ml of protein-containing sample was applied to the upper surface of the gel, under a layer of buffer, and eluted downwards. The buffer flow-rate was maintained at 20 ml/hour. Emergence of the protein peaks was monitored by means of a Uvicord absorptiometer (L.K.B.) attached to a Beckman 5" recorder. Sera from IRDS and normal infants and their mothers were examined by this method.

2. Preparative electrophoresis on polyacrylamide Gel

Initially, the Shandon preparative apparatus was used: this employs a 7.5% (w/v) annular gel, 6 mm thick and not more than 12 cm in height, with a circulating tap-water cooling system (125a). The gel was prepared by mixing in the following order, equal volumes of (i) 30.0 gm acrylamide 0.8 gm N-N-methylene-bis-acrylamide in 100 ml bidistilled water.

(ii) 1.6% v/v dimethylamino propionitrile in Tris-glycine

buffer * (initiator).

(iii) 0.03% potassium ferricyanide in bidistilled water (inhibitor).

(iv) 0.48% ammonium persulphate in bidistilled water (catalyst).

The mixture was degassed before pouring the gel.

3. Preparation of IgG

Most of the serum proteins were precipitated by (i) The dropwise addition of 0.1 N NaOH to the serum, with continuous shaking, to adjust the pH to 9.4, followed by

---

* The buffer used in both the gel and the electrode vessels was:
3 gm Tris-hydronymethylamino-methane
14.4 gm glycine
in 1l bidistilled water.
The capacity of the gel was not in excess of 60 mg protein. The sample was applied to the upper surface of the gel, under a layer of buffer and the upper electrode vessel fitted and filled, taking care not to disturb the sample.

A current of 80 milliamps was passed through the system and the protein fractions were eluted as they emerged from the lower surface of the gel, using Tris acetate buffer, pH 8.3. These were monitored with a Uvicord-absorptiometer and recorded as in (1).

This technique was applied to sera from IRDS infants, healthy premature infants, foetuses, pregnant women, normal adult males, to serum protein fractions obtained by methods described in (Part 2.IV.3,4,5) and to somatotropin. (NIH).

Subsequently the Pleuger preparative apparatus was used: the composition of the gels and buffer were the same as described above. The gels were 1.5 cm in diameter and 10.5 cm long. The electrophoretic run was carried out in the cold laboratory (4°C); the gels were sliced and corresponding slices pooled. Protein was eluted from these, using the Tris-glycine buffer described above.

(ii) Mixing the serum with 0.4% Rivanol (w/v) in distilled water in the proportions 1 ml serum : 3.5 ml Rivanol (126, 127). (Rivanol is 6,9, diamino-2-ethoxyacridine-lactate). The Rivanol was then precipitated by dropwise addition of saturated KBr, or removed with activated charcoal. After centrifugation, the supernate contained IgG and traces of IgA, α_2- and β-globulins.

IgA and α_2- and β- globulins were eliminated in one of two ways:

(iii) IgG was precipitated by adjusting the pH to 6.8 and adding ethanol, at -5°C, to a final concentration of 25% by volume (128).

(iv) IgG was precipitated, by adding to the supernate an equal volume of saturated ammonium sulphate at room temperature.

Both procedures yielded preparations that were pure on immunoelectrophoresis (Fig. 2.4). Some of the faster moving IgG was lost, but the preparation retained its immunological and biological properties. This is in agreement with the findings of other workers. (126,129).

In more recent experiments (iv) was used, as this procedure was more convenient. This method was used to prepare IgG from the serum of mothers and of some of the infants.

4. Preparation of Albumin

Two methods were followed:

(i) The method of Vallance-Owen and Campbell (130)

The procedure was carried out at 4°C.

(a) To 1 vol of serum was added 1 vol of 10% trichloracetic acid (w/v), with continuous stirring.

(b) The preparation was then centrifuged and the supernatant discarded.

(c) The precipitate was washed twice with 5% trichloracetic acid (w/v).

(d) The precipitate was then resuspended in 3 vols of 1% trichloracetic acid (w/v) in 96% aqueous ethanol (v/v) and stirred thoroughly to redissolve the albumin.

(e) The preparation was centrifuged, the precipitate discarded and the supernatant solution dialyzed at 4°C, against repeated changes of distilled water until it was free from ethanol.

(f) The dialyzate was lyophilyzed.

This method was used to prepare albumin from sera of mothers, new-born infants, foetuses and adult males. Pure albumin was prepared by this method, irrespective of the origin of the serum.

(ii) The method of Fernandez et al (131)

This procedure may be performed at room temperature. Recovery of serum albumin was found to be 80 - 90%.

(a) 0.5 ml of serum was diluted to 2.5 ml with 0.15 M sodium chloride in a 50 ml centrifuge tube.

(b) Using a burette, 22.5 ml of HCl-ethanol mixture (1:600 v/v) was added dropwise with continuous shaking and this was left to stand for 30 mins at room temperature.

(c) The preparation was centrifuged and the precipitate discarded. The supernate was mixed with 2.5 - 3.0 ml of 0.2M sodium acetate in absolute ethanol and left to stand for 10 - 20 mins at room temperature.

(d) After centrifugation, the supernate was discarded and the precipitate washed with ethanol/ ether 3:1 (v/v) to remove traces of lipid.

(e) The precipitate was redissolved in buffer or distilled water.

This method yielded pure albumin when applied to normal adult serum. However, when applied to foetal serum, the resulting product contained albumin and α- fetoprotein.

5. Preparation of α- fetoprotein + albumin

As it was not possible to separate - fetoprotein from albumin satisfactorily by any of the methods described, a preparation containing both was made, and where necessary, pure albumin, prepared from foetal serum by the method of Vallance-Owen (130) or from adult male serum by either method described in (4) was used as a control.

Two methods were employed for the preparation of α-fetoprotein + albumin:

(i) The method of Fernandez et al (Part 2 IV.2).

(ii) If the serum was from a foetus of gestational age less than 16 weeks, the following method could also be used. However, this method did not give consistent results, if applied to hepatoma serum, or to serum of more mature foetuses.

(a) The serum was diluted to 3 times its original volume with 0.15M sodium chloride.

(b) The diluted serum was saturated with methylene dichloride with continuous shaking, to remove lipoproteins and the mixture centrifuged at 10,000g for 30 mins. The precipitate was discarded.

(c) To the supernate was added an equal volume of saturated ammonium sulphate, with continuous stirring (i.e. the preparation was 50% saturated with ammonium sulphate.

(d) After centrifugation, the precipitate was discarded, and to one volume of supernate was added a half-volume of saturated ammonium sulphate, thus raising the ammonium sulphate saturation to 60%.

(e) This mixture was centrifuged: supernate and precipitate both contained a mixture of albumin and α- fetoprotein but the relative proportion of α- fetoprotein in the precipitate was higher than that of albumin. Therefore:

(f) The supernate was discarded, the precipitate was washed with 60% saturated ammonium sulphate, previously adjusted to pH 6.8, redissolved in distilled water and

dialyzed against distilled water at 4°C to remove excess ammonium sulphate.

(g) The preparation was lyophilyzed and redissolved in 0.15M sodium chloride, or buffer.

6. Preparation of Bence Jones proteins

Bence Jones proteins of types K and L were prepared from the urine of patients suffering from paraprotein-aemias as follows:

After preliminary filtration and dialysis against distilled water at 4°C, proteins were precipitated with 50% saturated ammonium sulphate, redissolved, dialyzed against distilled water at 4°C and lyophilyzed.

DEAE cellulose (Whatman) was pre-cycled in the following way:

(i) Treated with 5% HCl in 96% ethanol (v/v) for 45 mins.

(ii) Washed to pH 4 with distilled water on a Buchner funnel with suction from below.

(iii) Treated with 0.5M sodium hydroxide in distilled water (w/v) for 30 mins.

(iv) Washed and re-treated with 0.5M sodium hydroxide.

(v) Washed, as before, on a Buchner funnel with water until the pH was neutral.

(vi) Equilibrated with 0.01M Tris buffer pH 8.0.

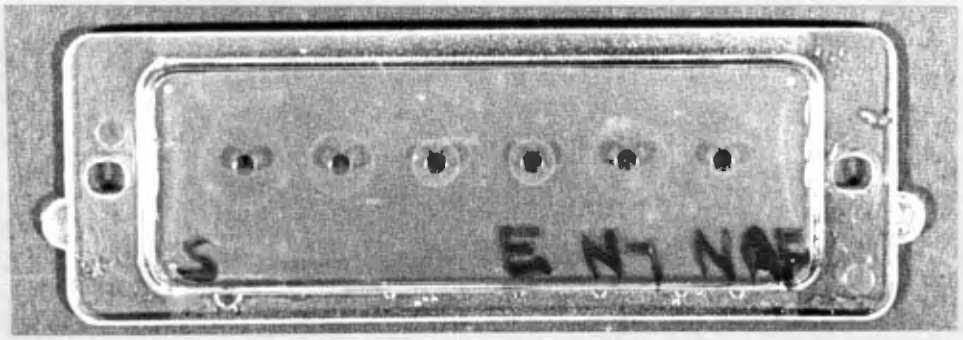
The cation-exchanger was poured into a column (2 x 22 cm)

with a coarse-grade sintered-glass disc at the base.

300 mg Of protein was applied and a gradient elution performed, increasing the molarity of the Tris buffer from 0.01 to 0.30 and employing a gradient-elution pump with a linear cam. The flow-rate was maintained at 37 ml/hr and the emergence of protein fractions detected with a spectro-monitor. The first fraction to emerge contained the Bence Jones proteins. This fraction was collected, dialyzed as before, and lyophilyzed.

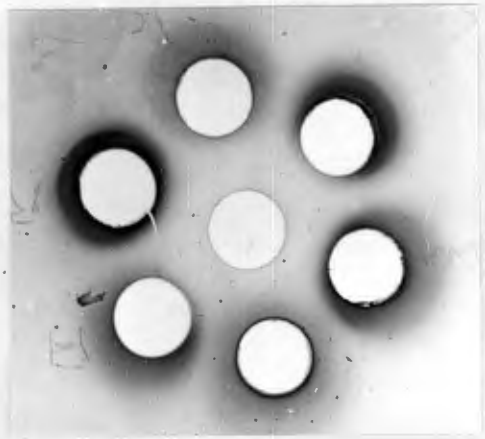
To achieve further purification, the resulting preparation was subjected to Chromatography on Sephadex G100 in a column of dimensions 3.0 x 70 cm. The buffer used was 0.1M Tris, pH 7.4,with 0.2M NaCl and the flow rate 20 ml/hour. Preliminary calibration of the column was performed by prior passage of IgG, haemoglobin and myoglobin. 5 ml Fractions were collected: IgG appeared at about fraction 30, haemoglobin at fraction 40 and myoglobin at fraction 60. The Bence Jones proteins were present, in pure form, in fractions 50 - 55. These fractions were collected, dialyzed and lyophilyzed as described previously. (132, 133).

FIGURE 2.2 : IMMUNOPLATES (HYLAND) FOR QUANTITATION OF IMMUNE GLOBULINS.



STANDARDS ARE PLACED IN THE THREE LEFT-HAND WELLS AND UNKNOWNS IN THE RIGHT-HAND WELLS.

FIGURE 2.3 : FETOPROTEIN IMMUNOPLATES.

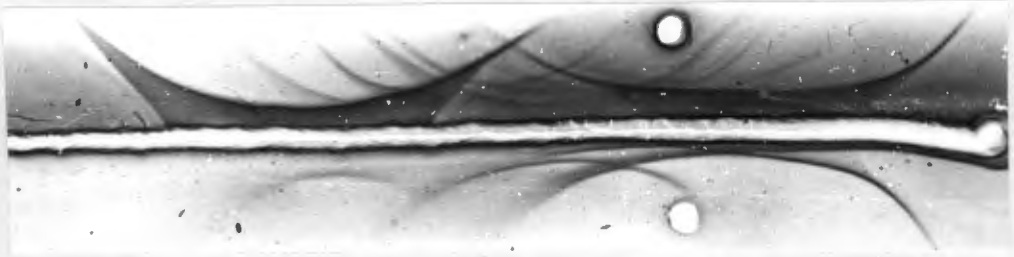


NOTE DARKLY-STAINING, ASYMMETRICAL PRECIPITIN DISCS SURROUNDING WELLS CONTAINING CERTAIN MATERNAL SERA.

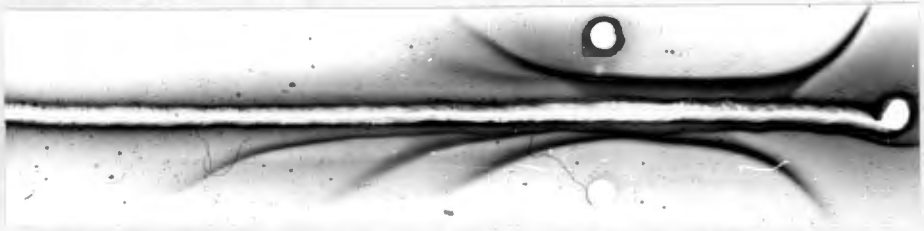
FIGURE 2.4

IMMUNOELECTRIPHORETIC ANALYSIS OF IgG AND PAPAIN DIGESTS OF THE IgG MOLECULE.

1. IgG IN UPPER WELL; WHOLE SERUM IN LOWER WELL. POLYVALENT ANTISERUM TO ADULT HUMAN SERUM IN SLOT.

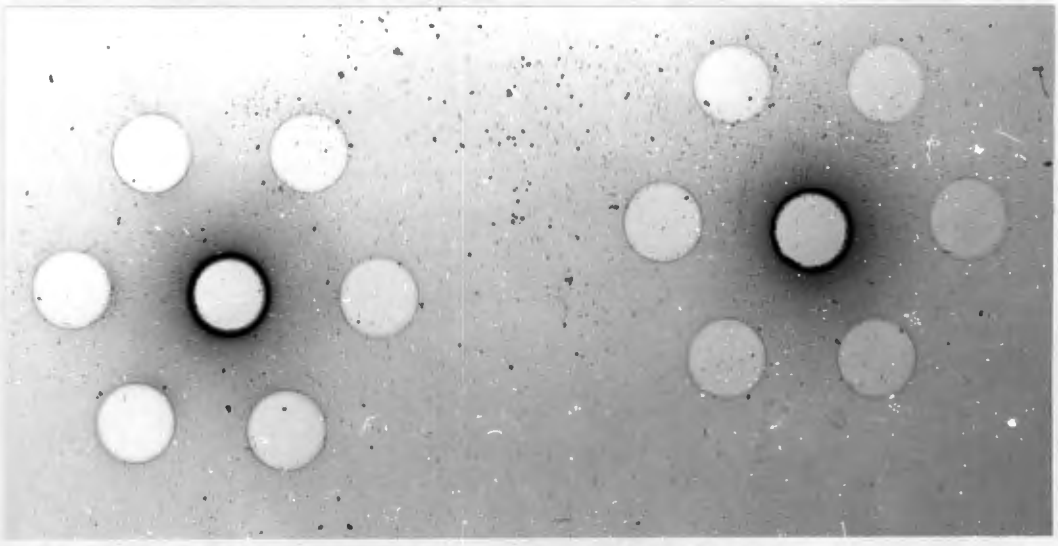


2. WHOLE SERUM IN UPPER WELL; PAPAIN DIGEST OF IgG IN LOWER WELL. POLYVALENT ANTISERUM TO, ADULT HUMAN SERUM IN SLOT.

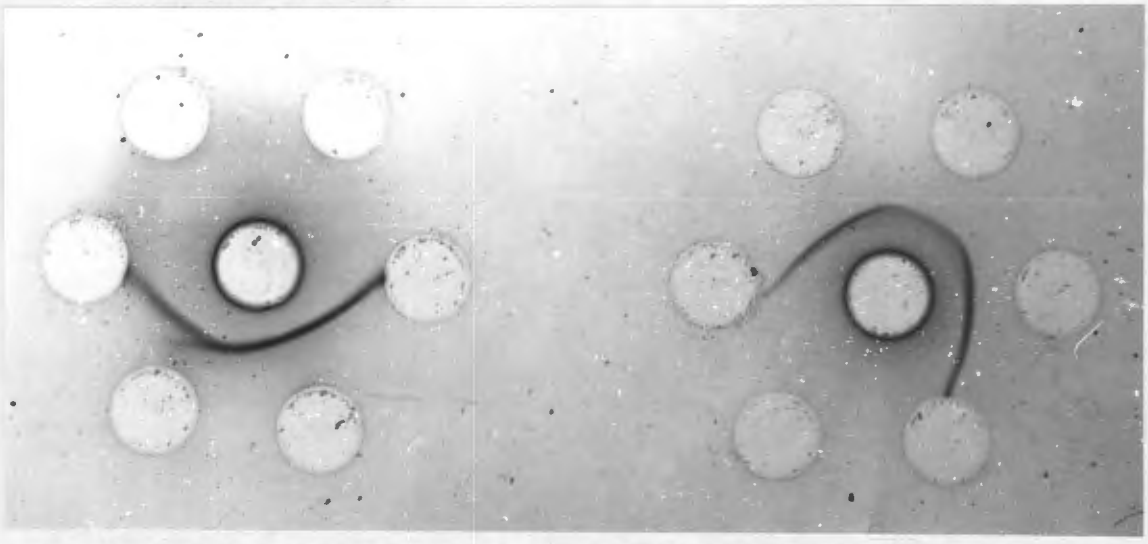


3.IgG IN UPPER WELL; PAPAIN DIGEST OF IgG IN LOWER WELL. MONOSPECIFIC ANTISERUM TO IgG IN SLOT.

FIGURE 2.4 (CONTINUED) : DEMONSTRATION OF PURITY OF BENCE-JONES PROTEINS BY MEANS OF OUCHTERLONY PLATES.

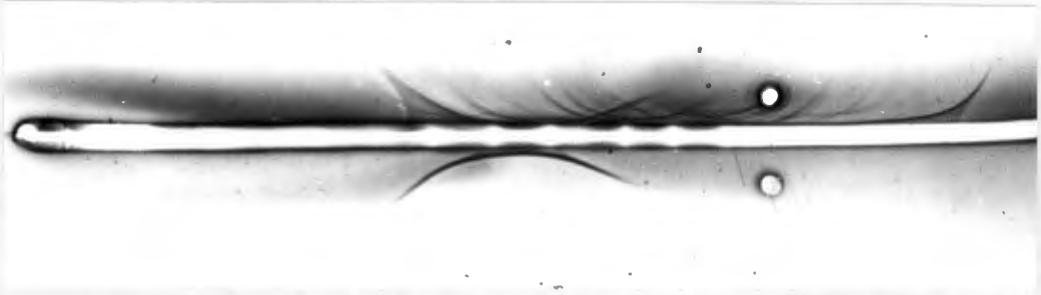


1. ANTISERA TO HUMAN IgG AND IgA IN LEFT AND RIGHT-HAND CENTRE WELLS RESPECTIVELY. BENCE-JONES PROTEINS IN PERIPHERAL WELLS.

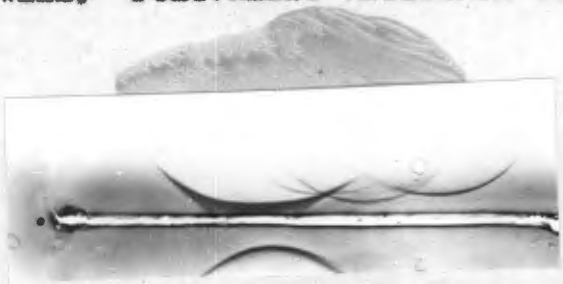


2. ANTISERA TO BENCE-JONES PROTEINS TYPES K AND L IN LEFT AND RIGHT-HAND CENTRE WELLS RESPECTIVELY. BENCE-JONES PROTEINS IN PERIPHERAL WELLS.

FIGURE 2.4 (CONTINUED) : PROPERTIES OF ALBUMIN AND ALBUMIN + α -FETOPROTEIN PREPARATIONS.



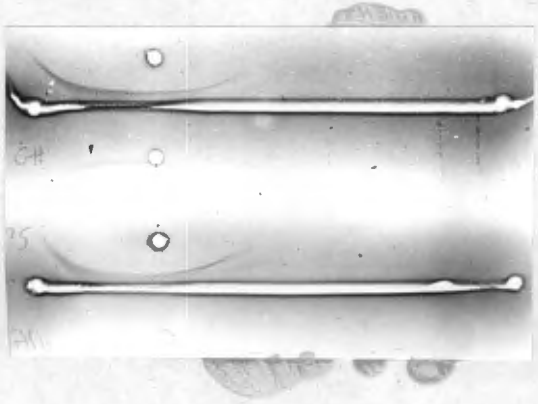
1. ADULT HUMAN SERUM IN UPPER WELL, ALBUMIN PREPARATION IN LOWER WELL. POLYVALENT ANTISERUM TO HUMAN SERUM IN SLOT.



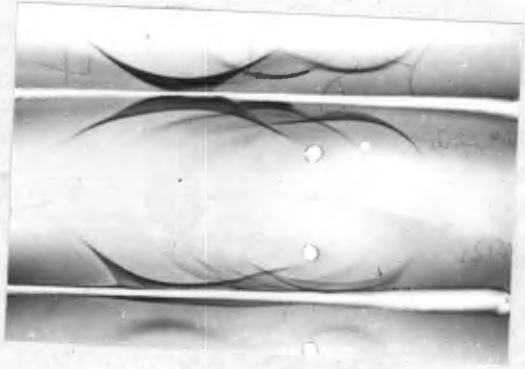
2. FOETAL SERUM IN UPPER WELL, α -FETOPROTEIN + ALBUMIN IN LOWER WELL. POLYVALENT ANTISERUM TO ADULT HUMAN SERUM IN SLOT.

3. ADULT SERUM IN UPPER WELL, α-FETOPROTEIN + ALBUMIN IN LOWER WELL. ANTISERUM TO α -FETOPROTEIN IN SLOT.

FIGURE 2.4 (CONTINUED) : IMMUNOELECTROPHORETIC ANALYSIS OF THE GROWTH HORMONE PREPARATION STUDIED.



1. SERA IN 1st AND 3rd WELLS FROM TOP, GROWTH HORMONE IN 2nd WELL. ANTISERUM TO HUMAN IgG IN BOTH SLOTS.



2. FOETAL SERUM IN UPPERMOST WELL, ADULT SERA IN 2nd AND 3rd WELLS, GROWTH HORMONE IN 4th WELL.
POLYVALENT ANTISERUM TO ADULT HUMAN SERUM IN BOTH SLOTS.

2.V. Methods of protein quantitation and identification

1. Estimation of total protein (provided that the solution was not highly coloured) was performed by the buiret method of Gornall et al (134). This was routinely applied to sera, amniotic fluid and urine concentrates. (Estimation of individual protein fractions is described in V.2,3).

2. Paper electrophoresis

0.01 ml Of sample was applied, using a 0.1 ml graduated pipette, to a Whatman No.1 filter paper electrophoresis strip. This was placed in a Durrum-type tank containing barbitone buffer, pH 8.6, ionic strength 0.075, and a potential difference of 150 V was applied for 15 - 18 hrs. The strips were then heated and stained with lissamine green, according to the method of Gorringe (135). Dye was eluted from the bands corresponding to the protein fractions with phthalate buffer, pH 6.0. Eluates were filtered and the optical density corresponding to each was read in a Klett-Summerson photometer, using a red filter (λ = 660 mμ). Optical densities were assumed to be proportional to the concentrations of the protein fractions. The latter were calculated by simple proportion from the total protein concentration as determined by the biuret method. (V.1).

The resulting values were checked against those obtained by precipitating the protein fractions with a 23% w/v solution

of sodium sulphate. The corresponding values from the two methods did not differ by more than 6% of the mean, over a wide range of albumin and globulin concentrations.

In order to determine experimental error, 10 serial measurements were made on the same serum. The results are in Table 2.1. (All fractions are expressed as percentages of the total protein).

TABLE 2.1

No.	Albumin	Globulins			
		$\alpha 1$	$\alpha 2$	β	γ
1	64.1	5.2	4.6	14.4	11.8
2	63.9	5.1	5.1	14.6	11.4
3	66.4	2.9	4.3	15.0	11.4
4	64.7	5.0	4.3	14.5	11.5
5	64.8	5.5	5.5	14.8	9.4
6	68.3	4.0	4.0	11.3	12.4
7	65.0	5.4	5.0	13.0	11.6
8	64.9	4.6	4.4	14.1	12.0
9	65.3	4.0	5.4	14.4	10.9
10	65.3	5.6	4.0	14.6	10.5
Mean ± SD	65.3 ± 1.0				11.3 ± 0.6
Range	5.4				3.0
Standard of error of the mean	0.4				0.3

Experimental error was less than 5% of the mean for each fraction.

This method was used for quantitation of protein fractions in all the sera studied in the first series.

3. Large-scale electrophoresis on cellulose acetate

After experience with paper electrophoresis, it was decided to utilize cellulose acetate (Oxoid) as a supporting medium instead, as this medium gave better resolution and the technique was less laborious. The procedure of Kohn (125(b)) was followed:

Oxoid strips, 5 cm wide, were impregnated with barbitone buffer (pH 8.6, ionic strength = 0.075), blotted dry and 0.01 ml of sample was applied with a Beckman sample applicator. Strips were placed in a horizontal electrophoresis tank containing the same buffer. Electrical contact was effected by means of paper wicks with their ends in the outer electrode vessel, the distance between the wicks being 10 cm. (See Fig. 2.7).

It was found that, if the line of application of the sample was placed 1 cm from the cathodal wick, electroendosmosis could be minimized so that all the protein fractions migrated towards the anode. Hence, denatured material deposited at the origin did not interfere with quantitation by the Densitometer.

A potential difference of 150 V was applied for 1½ to 2 hrs. The wet strips were then stained with lissamine green (1% w/v, containing salicylsulphonic acid 1.5% w/v as protein

precipitant), washed in 3 changes of 1% acetic acid (v/v) and dried in room air, or under pressure, between sheets of blotting paper. The latter were preserved as records of the serum-protein patterns. (See Figs. 3.3 - 3.7). In the former, bands corresponding to the main protein fractions were dissolved in chloroform/ethanol 9:1 (v/v), and the corresponding optical densities read as before.

A determination of experimental error, similar to that for paper electrophoresis, was performed. The error was found to be less than 5% of the mean.

In addition, experiments were performed to determine whether the quantitative results obtained on paper and on cellulose acetate were comparable. Accordingly, albumin and γ- globulins were estimated by paper electrophoresis and by cellulose acetate electrophoresis in 10 different sera, with the following results. (Table 2.2).

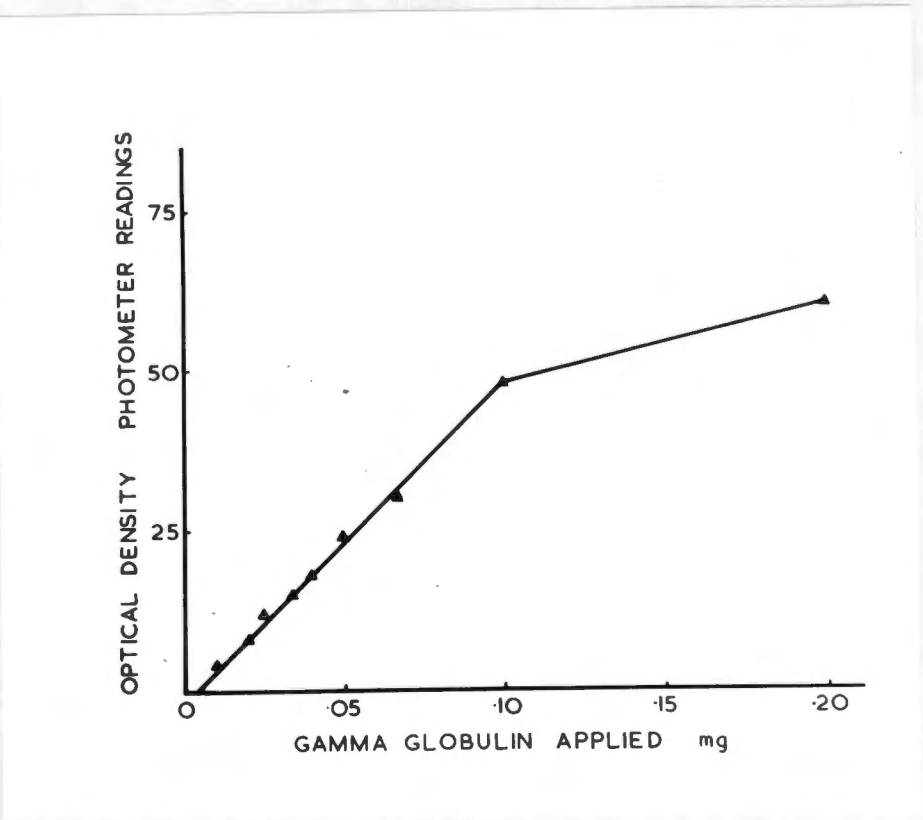
There was no significant difference between mean values obtained for albumin and γ- globulins by the two methods. It was therefore considered justified to pool data obtained by the two methods, for certain purposes.

A standard curve for the recovery of known quantities of IgG applied to a cellulose acetate strip was constructed. After impregnation with buffer, the strip was blotted dry and held taut between the electrode wicks. A known quantity of IgG was then applied directly, by means of a 0.1 ml

TABLE 2.2

No.	Cellulose acetate		Paper	
	Albumin (% of total)	γ - globulins (% of total)	Albumin (% of total)	γ - globulins (% of total)
1	73.1	9.9	72.1	10.0
2	70.0	11.8	65.5	13.1
3	71.2	10.0	70.0	8.9
4	65.9	11.5	69.8	10.0
5	63.8	10.5	62.1	13.7
6	62.4	10.2	66.6	8.4
7	70.5	11.0	71.4	12.7
8	62.1	14.5	64.7	13.0
9	66.7	15.0	63.3	15.0
10	78.0	7.0	75.1	8.2
Total	683.7	111.4	680.6	113.0
Mean	68.4	11.1	68.1	11.3

FIGURE 2.5 : RECOVERY CURVE FOR γ-GLOBULINS APPLIED TO A CELLULOSE ACETATE STRIP.



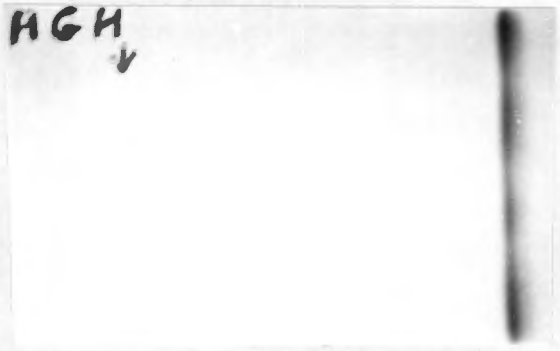
THE INTERCEPT ON THE ABSCISSA IS DUE TO BACKGROUND STAINING OF THE STRIP.

FIGURE 2.6 : CELLULOSE ACETATE ELECTROPHORESIS OF PROTEIN PREPARATIONS.

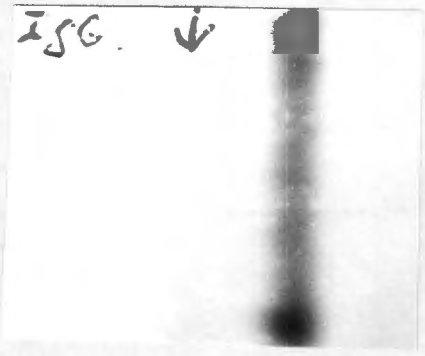
1. ALBUMIN. ORIGIN IS AT THE ARROW.

2. α-FETOPROTEIN + ALBUMIN. ORIGIN IS AT THE ARROW.

FIGURE 2.6 (CONTINUED). CELLULOSE ACETATE ELECTROPHORESIS OF PROTEIN PREPARATIONS.



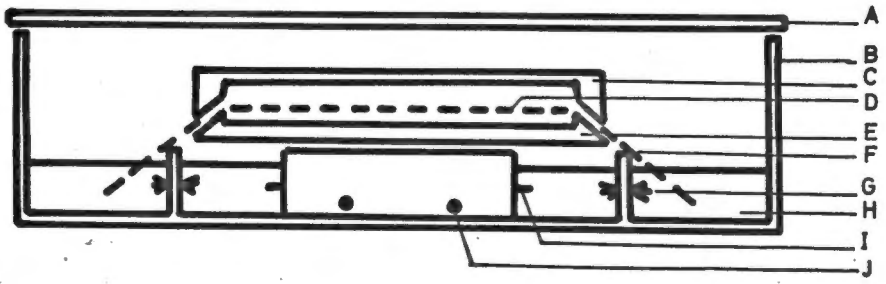
3. HUMAN GROWTH HORMONE. ORIGIN IS AT THE ARROW.



4. HUMAN IgG. ORIGIN IS AT THE ARROW.

FIGURE 2.7A: APPARATUS FOR LARGE-SCALE CELLULOSE ACETATE ELECTROPHORESIS.

CELLULOSE ACETATE ELECTROPHORESIS. APPARATUS.



A. AIRTIGHT COVER
B. PERSPEX TANK
C. BAR FOR KEEPING STRIPS TAUT
D. CELLULOSE ACETATE STRIP
E. SUPPORT FOR STRIPS
F. WHATMANS NO.1 WICK
G. COTTON WICK
H. BUFFER
I. TERMINAL
J. ELECTRODE

graduated pipette. IgG concentration was plotted against photometer readings (Fig.2.5). The range of concentrations of IgG encountered in the studies to be outlined, fell within the linear portion of the curve. The intercept on the abscissa was ascribed to background-staining of the strip. (IgG was prepared as in Part 2.IV.3).

4. Electrophoresis and staining for lactic-dehydrogenase (LDH) isozymes (136).

Cellulose acetate strips were prepared in duplicate and one set impregnated with 0.07M barbitone buffer, pH 8.6. Electrophoresis was performed as in IV.3, using 0.01 ml of serum or of a red blood cell haemolysate. (Part 2.II.1). The sample was applied in the centre of the strip. 5 mins Before the completion of the electrophoretic run an incubation medium was prepared as follows:

0.1M sodium lactate: 1 vol

1% (Nicotine adenine dinucleotide (NAD)) in distilled water (w/v): 1 vol

0.1% 3(4,5 - dimethylthiazolyl - 2)-2,5 diphenyltetrazolium bromide in distilled water (w/v): 3 vol

0.1% phenazine methosulphate in distilled water: 0.3 vol

The second set of cellulose acetate strips was impregnated with this medium, contained in separate petri dishes for individual strips. Care was taken to achieve adequate impregnation without overloading any strip. On completion

of the electrophoretic run, 1 cm was cut from each edge of the first set of strips, which were then layered over the second in pairs without trapping air bubbles between them. The pairs of strips were incubated at 37°C for 30 minutes. Isozyme bands were fixed in a solution of 4% formaldehyde (v/v) in 0.15M sodium chloride. Intensity of the zones was recorded by means of a photoelectric scanner. (See Fig.2.1(c)). Sera of healthy and IRDS infants and adult controls were examined in this way.

5. Electrophoresis and staining for glycoproteins (125c).

Electrophoresis was performed on 0.01 ml of serum as in (3). Strips were washed in industrial alcohol for 10 mins and then transferred to:

Periodic acid	2.5 ml
M/5 sodium acetate	25.0 ml
Distilled water	250.0 ml

for 10 mins, followed by rinsing in N/1,000 hydrochloric acid. They were then transferred to

Potassium iodide	40.0 gm
Distilled water	400.0 ml
N/10 hydrochloric acid	10 ml added just before use.

Saturated ammonium thiosulphate was then added dropwise, until the brown colour disappeared, and the strips were allowed to remain until the stain had just cleared from the iodine-stained bands. They were then rinsed in N/1,000 hydrochloric

acid and transferred to Schiff's stain (G.T.Gurr), until the bands had acquired a purple colour (15 - 30 mins). Thereafter the strips were washed in 3 changes of 0.1N nitric acid, finally rinsed in N/1,000 hydrochloric acid and dried at room temperature under pressure between sheets of blotting paper. (See Fig. 2.10).

6. Analytical electrophoresis on polyacrylamide gel (137)

Analytical electrophoresis was performed on 7.5% cylindrical polyacrylamide gels, 5 to 6 cm long and 7 mm in diameter. The gel reagents and buffer were prepared as in Part 2.IV.2. The Pleuger analytical apparatus was used and bromophenol blue added as indicator to the upper electrode vessel. 5 µl Of sample was introduced on the upper surface of each gel. A potential difference of 80 volts was applied for 45 minutes and then increased to 160 volts. Electrophoresis was allowed to proceed, until the indicator band reached the lower end of the gel.

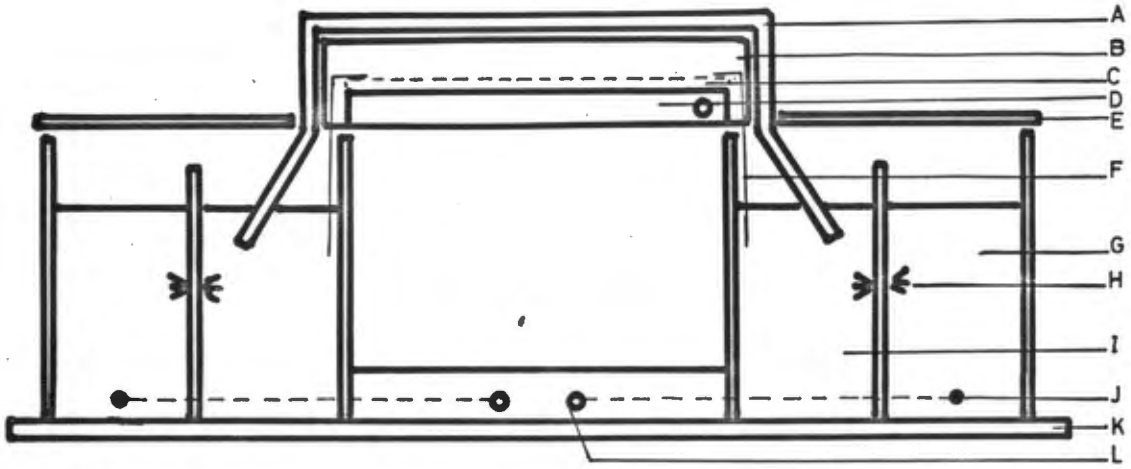
The gels were stained for 1 hour with 0.6% amidoschwartz (w/v) in 7% acetic acid (v/v), followed by electrophoretic de-staining in 7% acetic acid.

7. Immunoelectrophoresis (138)

Glass microscope slides 3" long and 1 or 2" wide were employed. These were covered to a thickness of 1 mm with 1% (w/v) agarose gel prepared in 0.05M Tris buffer, pH 8.2

FIGURE 2.8

IMMUNO-ELECTROPHORESIS. APPARATUS.



A. CHAMBER COVER.
B. COVER SUPPORT.
C. AGAR PLATE.
D. COOLING CHAMBER.
E. TANK COVER PLATE.
F. WHATMANS NO 1 WICK.
G. ELECTRODE CHAMBER.
H. COTTON WICK.
I. BUFFER TANK.
J. ELECTRODE.
K. PERSPEX TANK.
L. ELECTRODE TERMINAL.

FIGURE 2.9

POLYACRYLAMIDE ELECTROPHORESIS. APPARATUS.

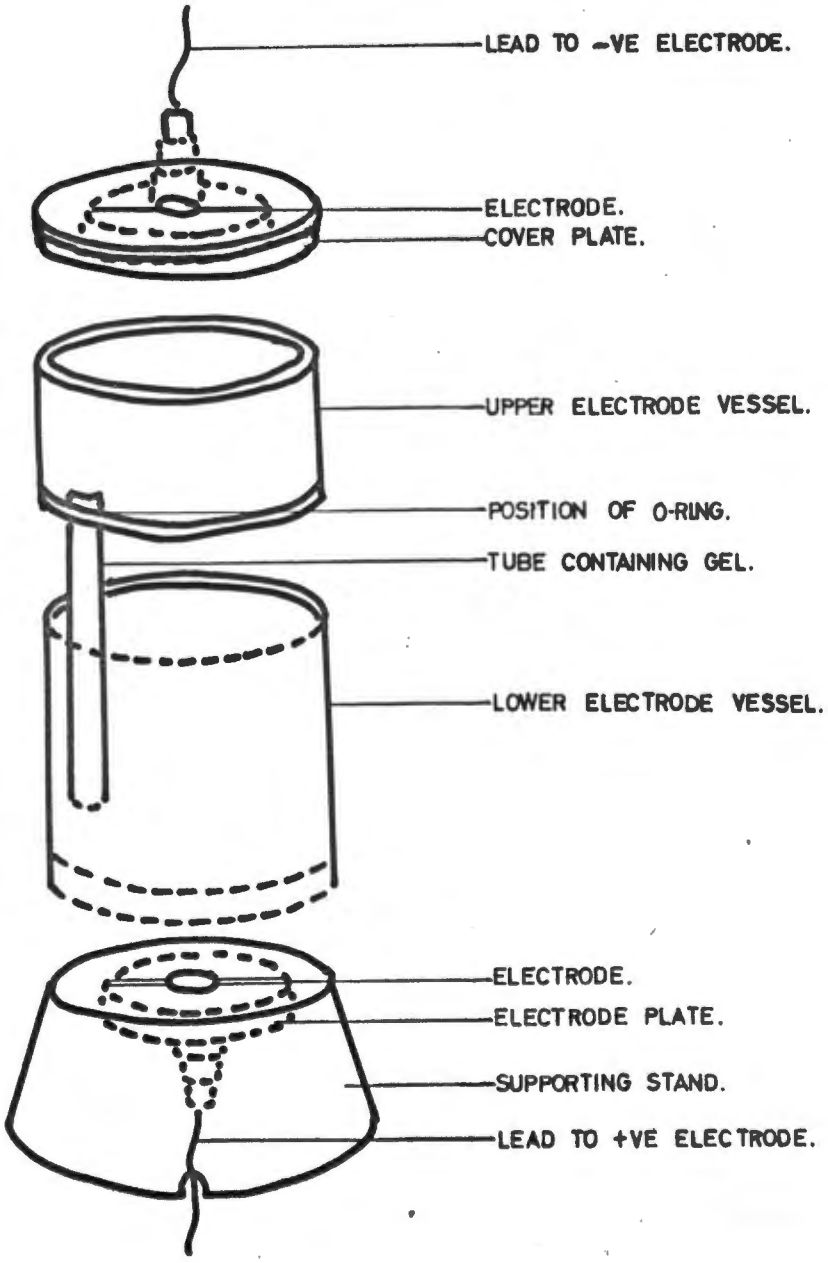
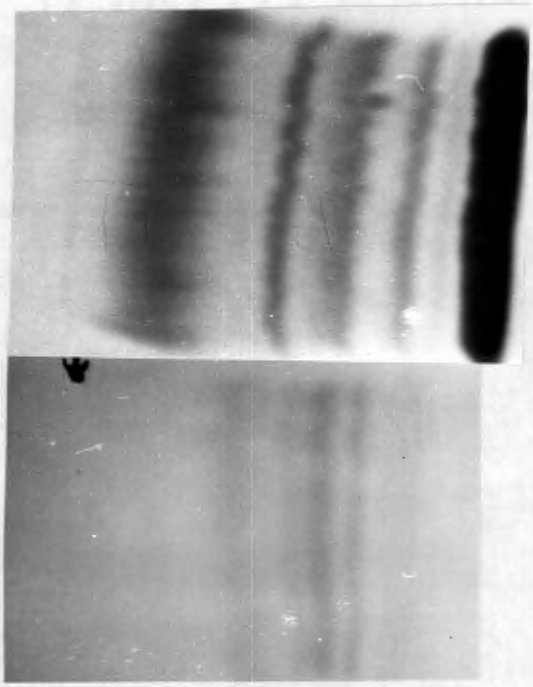
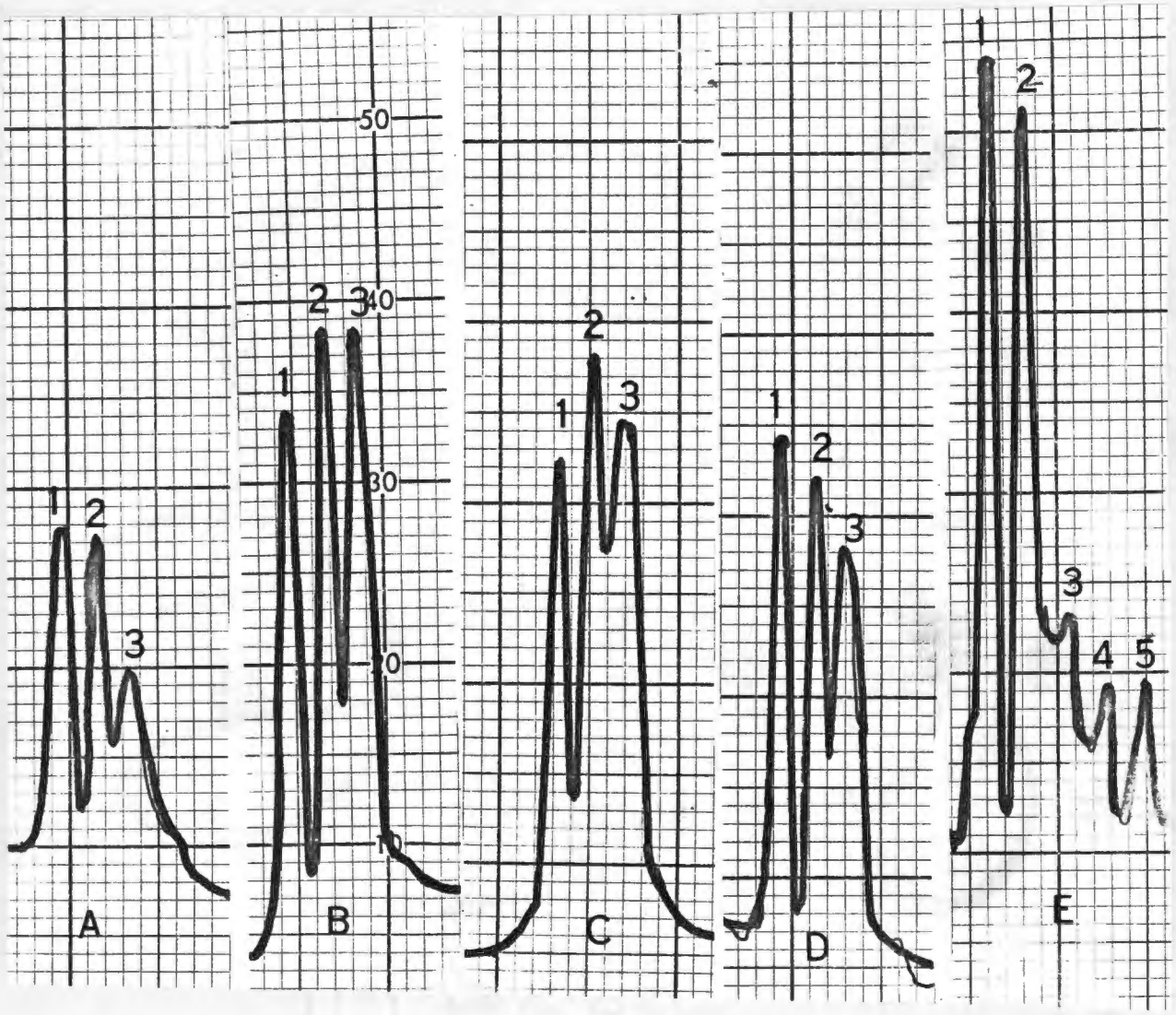


FIGURE 2.10. STAINING OF SERA OF IRDS INFANTS FOR GLYCOPROTEINS.



NOTE THAT α-FETOPROTEIN DOES NOT COINCIDE WITH A GLYCOPROTEIN BAND.

FIGURE 2.10A: SCANS OF LACTIC-DEHYDROGENASE ISOZYME PATTERNS OF HEALTHY PREMATURE AND IRDS INFANTS AND OF NORMAL ADULTS.



A,B. HEALTHY PREMATURES. C,D. IRDS INFANTS.

E. HEALTHY ADULT.

Sodium azide, 0.5 gm/l, was added to all buffers as a preservative. Generally, the wells were placed 3 mm on either side of the central trough and about 1/3 of the length of the slide from the cathode. Filter paper wicks made contact between the edges of the agar and the buffer (0.1 Tris, pH 8.2) in the outer electrode vessels. Phenol red was applied level with the wells as a marker and tap water, circulating underneath the slides, provided a cooling system. A potential difference of 300V was applied until the marker reached the anodic wick, after which antiserum was added to the trough. The slides were incubated at 4°C in a humid chamber for 24 - 120 hrs, the duration depending on the antibody-antigen system under examination. Thereafter excess (unreacted) protein was removed by dialysis at room temperature against 0.15M sodium chloride for 24 - 48 hrs, with 3 changes of saline during this period. The slides were then fixed in a mixture of ethanol 70%, acetic acid 5% and H_2O 25% by volume for 30 - 60 mins, stained with 0.02% aqueous nigrosine w/v for 60 mins and washed for 24 - 48 hrs in 2 - 3 changes of 5% acetic acid (v/v).

8. Immune diffusion

The procedure followed was that of Ouchterlony (139), using the same gel as described for immunoelectrophoresis. A central well and six equidistant peripheral wells, all 5 mm in diameter and with midpoints 12.5 mm from the central

well, were punched out with a plate-cutter. Reagents were placed in these and allowed to diffuse at 4°C, with resultant precipitin arcs or rings. Subsequent dialysis, washing and staining were as described in V.7.

9. Immunoplates (Hyland)

These are agar plates, uniformly impregnated with an antiserum monospecific for the protein to be quantitated. 3 Standards are provided to enable the investigator to construct a calibration curve: these were placed in 3 of the 6 wells in the plate. Protein mixtures containing an unknown concentration of the protein to be measured were placed in the other 3. The plate was incubated for 4 hrs at 37°C for determination of IgG and for 16 hours at room temperature for determination of IgM and IgA. Precipitin discs appeared, the diameter of the disc being proportional to the logarithm of the protein concentration. The diameters of the discs were read by means of a dissecting microscope and, after plotting the diameters of the standards on semilogarithmic graph paper (thus obtaining a linear calibration curve), the concentrations of protein in the unknown mixtures were read directly from the graph.

10. Fetoprotein immunoplates

These were not available commercially. Therefore one attempted to make them by cutting a number of smaller wells at intervals between the main wells of an Ouchterlony plate

introducing the fetoprotein-albumin mixture into the former and allowing the proteins to diffuse for 3 days. The agarose gels were kept moist by storing them sealed under a glass plate with silicone grease around the edges. Maternal sera were then introduced into the main wells and the plates were incubated for a further 2 - 3 days. Dialysis and staining were performed as described in Part 2.V.7.

11. Passive haemagglutination

(a) Sheep erythrocytes preserved as in Part 2.II.9, were formalinized by the Czismas method (140).

The cells were separated from the preservative and serum by centrifugation and washed 5 - 7 times in 10 volumes of cold 0.15M sodium chloride. Care was taken at all times to avoid frothing of the cell suspension. The washed, packed cells were resuspended in 8 volumes of cold 0.15 sodium chloride and ¼ volume of 40% formaldehyde solution, encased in dialysis tubing, 1/3 empty, with the excess air expelled was introduced. The suspension was agitated gently by means of a mechanical shaker for 2 hours at room temperature. After this manoeuvre, the dialysis tubing was removed, punctured and its contents poured into the cell suspension. Shaking at room temperature was continued for 12 - 18 hrs. The resulting suspension was filtered through gauze to remove debris and washed 6 times in 15 vol of cold 0.15M sodium chloride. Finally, the packed cells were resuspended

in an equal volume of 0.15M sodium chloride with 0.02% thiomersal w/v as a preservative.

(b) Tanning and coating of formalinized cells with antigens - For IgG

The method described by Daniel and Stavitsky (141,142) was followed. However, as human IgG and a commercial antiserum to it (Hyland) were being investigated, preliminary experiments were undertaken to determine optimal conditions with respect to the following variables:

(i) Concentration of tannic acid
(ii) Time of exposure of cells to tannic acid
(iii) pH At which the tanning procedure was performed
(iv) Temperature for the tanning procedure
(v) Concentration of IgG for sensitizing tanned cells
(vi) Time of incubation of tanned cells with IgG
(vii) Temperature of incubation of tanned cells with IgG
(viii) pH Of incubation of tanned cells with IgG

Results of these were as follows: (See Table 2.3)

Hence a tannic acid concentration of 0.5 mg/100 ml was selected as being the most reliable. (See Table 2.4)

Although higher titres could be achieved by incubating the cells with tannic acid (0.5 mg/100 ml) for 15 - 120 minutes, specific inhibition of agglutination by prior addition of IgG was not observed if the incubation time exceeded 10 mins. For this reason, a standard incubation

TABLE 2.3

Concentration of tannic acid solution in 0.15M NaCl for tanning formalinized cells

Tannic acid concentration (mg/100 ml)	Titre (Commercial antiserum)
10.000	(Spontaneous agglutination of all cells variable
2.000	
1.000	
0.500	1:320
0.200	1:320
0.100	1:320
0.050	(Atypical settling patterns
0.025	

TABLE 2.4

Time of exposure of formalinized cells to tannic acid (0.5 mg/100 ml in 0.15M NaCl)

Time (mins)	Titre
5	1:80
10	1:320
15	1:640
20	1:640
30	1:640
60	1:640
120	1:640

time of 10 minutes with tannic acid (0.5 mg/100 ml 0.15 M NaCl) was adopted in subsequent experiments.

TABLE 2.5

Temperature of incubation of formalinized sheep cells with tannic acid (0.5 mg/100 ml 0.15M NaCl)

Temperature (°C)	Titre
4	1:20
20	1:80
37	1:320
56	1:320

Maximal titres were obtained by incubation with tannic acid at 37°C and 56°C. As the lower temperature involved less risk of denaturation of proteins in the system, incubation was routinely performed at 37°C.

pH Of tannic acid solution

Solutions of tannic acid (0.5 mg/100 ml) were prepared in phosphate-buffered saline (0.15M) with final pH's ranging from 3.8 - 7.5. The tanning procedure was performed as described above, at each pH of the tannic acid solution, but variation of pH in this range appeared to have no significant influence on the uptake of IgG by the tanned cells. Consequently, tannic acid (0.5 mg/100 ml) in 0.15M unbuffered sodium chloride was used. An average value for the pH of

this solution was 4.2.

TABLE 2.6

Optimal concentration of IgG for sensitizing sheep red blood cells, tanned with tannic acid (0.5 mg/100 ml 0.15M sodium chloride) for 10 mins at pH 4.2 and 37°C

Concentration of IgG (gm/ml)	Titre (Commercial antiserum
25	1:305
50	1:310
100	1:320
150	1:335
200	1:340
250	1:360
300	1:380
350	1:380
400	1:400

Concentration of IgG was estimated (1) by the biuret method and (2) by immunoplates. The mean of the two values was calculated and an appropriate dilution made in each case. If the IgG concentration was in excess of 100 ~~mg~~ /ml, specific inhibition of haemagglutination by prior addition of IgG was not consistently observed. A sensitizing concentration of 100 gm IgG/ml was therefore chosen as a standard procedure.

TABLE 2.7

pH Of incubation of 100 µgm IgG/ml with sheep erythrocytes, formalinized and tanned as described above

pH Of incubation	Titre (Commercial antiserum)
4.5	1:20
5.0	1:100
5.5	1:290
6.0	1:310
6.5	1:320
7.0	1:240
7.5	1:200
8.0	1:160

Uptake of IgG by erythrocytes, tanned as previously described was tested in the pH range 4.5 - 8.0, pH being adjusted by means of 0.15M phosphate-buffered saline.

Optimal uptake of IgG occurred at approximately pH 6.5. Hence cells were routinely incubated with IgG in phosphate-buffered 0.15M saline at pH 6.5. (See Table 2.8)

The titre did not vary with temperature ranging between 4°C and 25°C, but above this temperature, the value rose. For fear of denaturation of the proteins at the higher temperature, this incubation was performed at room temperature. (18 - 25°C).

TABLE 2.8

Temperature of incubation of tanned sheep erythrocytes with 100 µgm IgG/ml

Temperature (°C)	Titre (Commercial antiserum)
4	1:320
20	1:320
37	1:640
56	1:1280

TABLE 2.9

Time of incubation of tanned sheep erythrocytes with 100 µgm IgG/ml at room temperature

Time of incubation (hours)	Titre (Commercial antiserum)
0.25	1:240
0.50	1:320
0.75	1:320
1.0	1:320
3.0	1:400
6.0	1:400
12.0	1:480
18.0	1:480
24.0	1:480

Although increasing time of incubation yielded higher titres, it was found that specific inhibition of agglutination could not be demonstrated consistently, if the incubation time exceeded 30 mins. This was selected as the standard incubation time.

Accordingly, the final procedure was as follows:

Formalinized sheep erythrocytes preserved in 0.15M sodium chloride with 0.02% methiolate (w/v) were washed 3 times in 0.15M sodium chloride and diluted to make a 2.5% suspension by volume in 0.15M NaCl. An equal volume of tannic acid (0.5mg./100ml 0.15M sodium chloride) was added and the mixture incubated for 30 mins with IgG (100 μg/ml) at pH 6.5. The erythrocytes were now washed 3 times with normal rabbit serum (NRS), previously heat-inactivated at 56°C for 30 minutes and diluted 1:200 v/v in 0.15M sodium chloride.

Finally, the sensitized cells were resuspended in an equal volume of NRS. NRS 1:200 was chosen as the diluent in these titrations, as it gave the most satisfactory settling patterns, i.e. clear end-points. NRS 1:100 gave lower titres without any increased reproducibility. With NRS solutions more dilute than 1:200, some of the cell preparations exhibited non-specific pan-agglutination.

<u>For human serum albumin, bovine serum albumin, goose serum albumin and albumin + α-fetoprotein</u>, the method

described for albumin by Daniel et al (141,142) was chosen, as these workers had made a detailed study of the variables involved for human serum albumin and the other proteins studied had similar isoelectric points. The procedure was similar to that for coating cells with IgG, except that incubation with antigen was performed at pH 5.6. The same antigen concentration as for IgG was used. Quantitation of the antigen was by the biuret method.

It was assumed that in the albumin + α-fetoprotein preparation, both proteins were taken up by the tanned cell surface. This was confirmed by the observation that a monospecific antiserum against α-fetoprotein and a similar antiserum against human serum albumin, agglutinated these cells to a titre of 1:1010 and 1:1280 respectively.

For papain-digested IgG, Bence Jones proteins and γ-globulin fragments extracted from urine, the method described for coating cells with IgG was used. (141,142). Controls were monospecific commercial antisera against Bence Jones proteins, types K and L and against IgG.

For amandin, tanning and coating of the cells was as before, except that the coating procedure was performed at pH 5.8, the isoelectric point of this protein.

(c) Titration of coated cells against sera.

Serial doubling dilutions of the sera to be tested were made with NRS in a perspex haemagglutination tray, starting

at a dilution of 1:10. 0.05 ml Of erythrocytes coated with the appropriate antigen were added to each well and stirred to make an even suspension. Care was taken to avoid cross-contamination. Sera were not heat-inactivated as this is unnecessary when using formalinized erythrocytes: these were found to be extremely resistant to lysis, in accordance with the observations of other workers. (143,144).

Controls were as follows:

(i) Coated cells in plain diluent

(ii) Coated cells titrated against a commercial antiserum (monospecific and free of soluble complexes) to the antigen.

(iii) An inhibition titration duplicating the assay titration, but with 0.2mg of antigen added to each well before the introduction of the cells.

(iv) An attempt was made to set up an additional control with formalinized, tanned cells not coated with antigen. This was unsatisfactory, as these cells agglutinated with every serum against which they were titrated. Presumably the mechanism is that such cells take up proteins from the serum, the protein molecules then forming bridges between the cells and resulting in agglutination.

Instead, cells coated with human serum albumin in some experiments and with other albumins or

amandin in others, provided a satisfactory control.

Titrations were allowed to settle overnight at room temperature and agglutination was graded -, ±, +, ++, +++, according to the diameter of the resulting agglutinate.

2.VI. Methods of protein digestion

1. Papain digestion of IgG

This was according to the method of Porter (145). 150mg IgG, prepared by the method described in IV.3(b) and 1.5mg Hg papain (twice recrystallized) were dissolved in 10ml buffer (sodium phosphate pH 7.0, 0.01M cysteine, 1mM EDTA). The mixture was incubated for 16 hrs at 37°C in the presence of toluene, after which it was dialyzed against distilled water at 4°C for 48 hours, with stirring and several changes of the outer liquid during this period. This removes cysteine and EDTA, facilitates oxidation and inactivates the papain. The digestion products (Fab, Fc ', Fc) were checked for purity by immunoelectrophoresis and finally lyophilysed. No attempt was made to separate the fragments.

2. Treatment of serum with 2-mercapto-ethanol.(146)

1 Volume of serum was mixed with 1 volume of 0.1M 2-mercapto-ethanol in 0.15M sodium chloride and incubated for 2 hours at 37°C. The preparation was then dialyzed at 4°C against repeated changes of distilled water, until it was free of 2-mercapto-ethanol.

3. Preparation of deposit from hyaline membranes for amino-acid analysis

The deposit was redissolved in 0.3M sodium hydroxide. 5mg Protein from this was placed in a hydrolysis tube with

1ml 6N hydrochloric acid and the tube was sealed. Hydrolysis was carried out in an oil bath at 110°C for 12 hours, after which the amino-acid composition of the hydrolysate was determined, using the Beckman amino-acid analyzer, Model 120B.

2.VII. Immunization of rabbits with foetal serum

6 Young adult rabbits were selected. Serum from a 14-week foetus was sterilized by passing it through a millipore filter (GSWP 025.00; GS 0.22). 0.03ml Serum was diluted to a total volume of 1.0ml with sterile 0.15M NaCl and emulsified with 1ml complete Freund's adjuvant in an autoclaved container. 2ml Of this emulsion was given intramuscularly to each rabbit at 2-weekly intervals for a total of 12 injections. Blood was taken every 4 - 6 weeks in order to examine for antibody content. The rabbits were bled as follows: the dorsum of the ear was shaved and made hyperaemic by warming and the application of Xylol. A dorsal ear artery was then incised with a scalpel and 5 - 8ml of blood collected from each rabbit.

Immunoelectrophoresis was performed on each rabbit antiserum, against foetal and healthy adult male serum, to detect the presence of anti-α- fetoprotein antibodies. (Fig 2.1). When these appeared, antibodies against the adult serum proteins could be absorbed out by the addition of excess adult male serum. (0.2 - 0.3ml per 1ml rabbit serum). It was occasionally necessary to concentrate the antiserum (by dialysis, lyophilyzation and reconstitution in a smaller volume of 0.15M sodium chloride) in order to demonstrate antibody activity.

2.VIII. Miscellaneous other methods used

1. Free plasma 11 - hydroxycorticosteroids

These were measured by the method of Mattingly (147). The fluorescence reagent was 7 volumes concentrated sulphuric acid + 3 volumes ethanol.

Cortisol standards were prepared by diluting aliquots of a stock solution of 50mg cortisol in 50ml purified ethanol with distilled water, to a final concentration of 1 µg/ml. This was stored not longer than 1 month at 4°C.

Steroids were extracted from plasma by mixing 2.0ml plasma (from heparinized blood) and 15.0ml methylene chloride, pre-treated as follows:

(i) Stood over concentrated sulphuric acid for 3 - 4 days, shaking occasionally.

(ii) 11 Then washed by shaking (a) with 100ml concentrated sulphuric acid, (b) 100ml 1.0N sodium hydroxide, (c) 2 washes with 200ml distilled water.

(iii) Dried over anhydrous sodium sulphate for 24 hours.

(iv) Distilled, and fractions coming over between 39 and 40°C collected, after which:

(v) kept in dark.

Plasma and methylene chlorine mixture(s) were then centrifuged for 30 min at 33 r.p.m. and excess plasma removed. The reagent blank (2ml water) and cortisol standard (0.2µg) were subjected to the same procedure.

5.0ml Of the fluorescence reagent was added to 10.0ml of each extract, shaken for 20 seconds and the fluorescence read serially at 13 minutes after mixing. The blank was set at zero and the cortisol standard at 100. The timing of the reading was arranged so as to ensure a relatively constant level of non-specific fluorescence during reading. Care was taken to use glassware cleaned only with chromic acid and distilled water. Readings were made, using a mercury lamp light source and primary and secondary filters: Chance OB 10 blue (λ = 430mμ) and Chance OGrI + OY3, giving peak transmission at λ = 540m . Fluorimetry was processed on batches of 6 plasma extracts, a blank and cortisol standard, using 5ml cells. This standard gave fluorimeter readings in μg/100ml, SD 0.1 μg/100ml. There was a linear relationship between fluorescence intensity and concentration of cortisol and corticosterone standards, in the concentration range 0.1 - 2.0 μg. Mean recovery of these steroids from plasma was 98%, and they together accounted for more than 95% of the total fluorescence due to steroids.

There was a good correlation between results obtained by this method and by the Porter-Silber chromogen method. Synthetic steroids did not fluoresce significantly.

2. Serum electrolyte concentrations, blood urea levels and lipid estimations were determined by standard methods in the laboratories of the Department of Chemical

Pathology, handling diagnostic investigations for Groote Schuur and other teaching hospitals in Cape Town.

3. Spectrophotometry of serum

Serum diluted 1:10 with 0.15M sodium chloride was placed in a 3ml cuvette with a 1cm light-path and scanned for absorption peaks over the wavelength range 750 - 220mμ with a Beckman DU spectrophotometer and recorder.

PART 3

RESULTS

3.I. Quantitation of serum proteins in infants

Measurements were made on three separate series of infants with IRDS and on normal controls.

1. In the first series, infants were grouped according to birth-weight. Estimation of total serum proteins and the various serum-protein fractions, was by the biuret method and by paper electrophoresis. (See Part 2.V.1,2.)

Data were grouped in two ways:

(a) Mean protein concentrations (± standard error) were calculated for each birth-weight range over the first 72 hours of life in both the normal and in the IRDS groups.

Results were as follows: (See Table 3.1)

In all groups

As can be seen from the table, there was a progressive and statistically significant increase in concentrations of total serum-protein, serum albumin and serum γ-globulins with increasing birth-weight.

In the normal group

(i) The concentrations of total protein, albumin and γ-globulins were significantly higher ($P < .01$) in infants weighing more than 4.5 Kg at birth than in those weighing less than 2.0 Kg.

(ii) However, there was no significant difference between the proportions of albumin and γ-globulins (expressed as a percentage of the total) in the various weight groups.

TABLE 3.1

Group	Weight (kg)	No. in group	Total serum protein (gm/ 100ml)	Albumin		γ-globulins	
				gm/ 100ml	% of total	gm/ 100ml	% of total
Normal infants	2.0	30	4.9 ±0.2	3.19 ±.05	64.5 ±1.1	0.71 ±.05	14.2 ±0.5
	2.0-2.5	16	5.5 ±0.2	3.74 ±.06	67.9 ±1.1	0.83 ±.03	15.0 ±0.6
	2.5-3.5	38	5.4 ±0.1	3.62 ±.04	67.6 ±0.6	0.77 ±.02	14.4 ±0.4
	3.5-4.5	30	5.5 ±0.1	3.72 ±.03	68.2 ±0.6	0.77 ±.03	14.0 ±0.5
	4.5	16	5.7 ±0.2	3.75 ±.11	65.9 ±1.4	0.88 ±.04	15.5 ±0.6
Means for all weight groups:			5.3 ±0.2	3.54 ±.06	66.8 ±0.7	0.77 ±.03	14.5 ±0.5
IRDS	2.0	9	3.8 ±0.2	2.75 ±.06	72.3 ±1.6	0.33 ±.02	8.4 ±0.6
	2.0-2.5	5	4.6 ±0.4	3.32 ±.01	72.3 ±0.3	0.38 ±.07	8.3 ±1.5
	2.5-3.5	5	5.4 ±0.3	3.97 ±.01	76.2 ±0.2	0.45 ±.01	8.6 ±1.8
Means for all weight groups:			4.4 ±0.3	3.20 ±.03	73.4 ±0.7	0.37 ±.09	8.4 ±1.3

TABLE 3.2

Mean values for electrophoretic quantitation of serum-protein fractions (± standard error)

Group	Age (days)	No. in group	Total serum-protein (gm/100ml)	Albumin gm/100ml	Albumin % of total	γ-globulins gm/100ml	γ-globulins % of total
IRDS (Mean gestational age = 34.4 wks)	Cord	54	3.7 ±0.1	2.93 ±0.03	74.5 ±1.0	0.38 ±0.02	10.3 ±0.5
	1st day	33	3.7 ±0.1	2.73 ±0.05	75.0 ±1.5	0.38 ±0.02	10.3 ±0.4
	2nd day	18	3.6 ±0.2	2.50 ±0.06	75.2 ±1.7	0.31 ±0.04	8.9 ±0.5
	3rd day	14	3.7 ±0.2	2.68 ±0.06	75.2 ±2.0	0.35 ±0.03	9.5 ±0.5
	4th-10th day	14	4.0 ±0.3	2.95 ±0.06	76.0 ±1.4	0.40 ±0.03	10.0 ±0.5
	Late (3wks-2mths)	10	4.2 ±0.3	3.13 ±0.10	76.3 ±1.8	0.45 ±0.03	10.2 ±0.9
Healthy prematures (Mean gestational age = 33.8 wks)	Cord	109	5.3 ±0.1	3.51 ±0.30	70.0 ±1.3	0.81 ±0.02	14.4 ±0.3
	1st day	19	5.4 ±0.2	3.56 ±0.07	70.5 ±2.0	0.78 ±0.04	14.4 ±0.7
	2nd day	16	5.3 ±0.2	3.55 ±0.06	69.8 ±1.7	0.76 ±0.04	14.4 ±0.6
	3rd day	17	5.1 ±0.2	3.49 ±0.06	70.3 ±1.2	0.73 ±0.03	14.4 ±0.6
	4th-10th day	15	5.1 ±0.2	3.49 ±0.05	71.1 ±2.1	0.72 ±0.04	14.3 ±0.4

In the IRDS group the pattern was markedly different:

(i) The total serum protein concentration was markedly lowered, except in the weight-group 2.5 - 3.5 Kg.

(ii) In this last group, the albumin concentration was significantly higher than normal ($P < .01$).

(iii) In all IRDS infants, the albumin accounted for a significantly higher proportion of the total protein than normal ($P < .001$).

(iv) In all cases, the relative and absolute concentrations of γ-globulins were much below normal ($P < .001$).

(b) Attempts were made to follow changes in the serum protein pattern during the first 72 hours of life and again between the 4th and 10th days after birth. Where possible, follow-up specimens were collected, at ages ranging between 3 weeks and 2 months.

For this purpose, healthy premature infants, of a similar mean gestational age, served as controls. Data obtained from cellulose acetate electrophoresis and paper electrophoresis (Part 2.V.3,2) were pooled. IgG and IgM were determined in some cases, using immunoplates (Part 2.V.9). IgA was not quantitated as the concentration in the serum was too low to produce a measurable precipitin ring. It was not possible to obtain complete sets of observations on each infant, as some died, or were discharged from hospital during the period of observation. Results of the electropho-

retic measurements are given in Table 3.2, and immunoplate readings in Table 3.3.

The results in Tables 3.1 and 3.2 are illustrated graphically in Figs 3.1 and 3.2.

TABLE 3.3

Mean values for quantitation of IgG and IgM by immunoplates (± standard error)

Group	Age (days)	No.in -group	IgG(gm/100ml)	IgM(mg/100ml)
IRDS (Mean gesta-tional age =34.1 wks)	Cord	15	0.51 ± 0.04	11 ± 2
	1st day	11	0.54 ± 0.05	16 ± 2
	2nd day	6	0.46 ± 0.03	15 ± 2
	3rd day	6	0.44 ± 0.05	10 ± 3
	4th - 10th day	10	0.57 ± 0.06	22 ± 3
	Late (3wks-2mths)	7	0.48 ± 0.03	38 ± 3
Healthy prema-tures (mean gest. age=33.9 wks)	Cord	18	0.96 ± 0.05	11 ± 2
	Late (3wks-2mths)	5	0.66 ± 0.10	20 ± 3

As can be seen from the tables, the IRDS group had lowest absolute concentrations of all serum-protein fractions at the 2nd to 3rd day, after which concentrations rose. The differences between absolute concentrations of albumin and of γ-globulins (Table 3.2) in the cord blood and on the 2nd day of life; and between the 2nd day and the 4th - 10th

day are statistically significant. ($P < 0.05$).

Unlike the present control group (see below), or those studied by other workers (78,79,81,82,83), the concentrations of total γ-globulins and of IgG, in the follow-up specimens obtained at 3 wks - 2 mths, did not differ significantly from those observed in the cord blood*

In the control group there was a slight fall in the concentrations of all protein fractions between birth and the 10th day of life. However, this was not statistically significant.

Immunoplate readings gave, on average, higher values for immune globulins than did the electrophoretic technique, but the observations followed the same general pattern during the first 10 days of life. In accordance with the findings of other workers (81, 82) the concentrations of IgM rose steadily after birth. IgM was not invariably present, in measurable amounts, in the cord blood of either group and there was a wide scatter in the observations. As before, there was no significant further fall in relative, not absolute concentration of IgG in the IRDS group*, whereas the control group exhibited a significantly lower concentration than was present in the cord blood.

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* This may indicate independent synthesis(or slower rate of catabolism) of IgG by IRDS infants. See Discussion.

Gel filtration on Sephadex G200 (Part 2.IV.1) showed a smaller area under the 7S peak in infants with IRDS, as compared with normal controls*.

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* Taken in conjunction with a lessened concentration of IgG, the implication of this observation is that the IgG present in IRDS babies is probably of normal molecular weight.

FIGURE 3.1

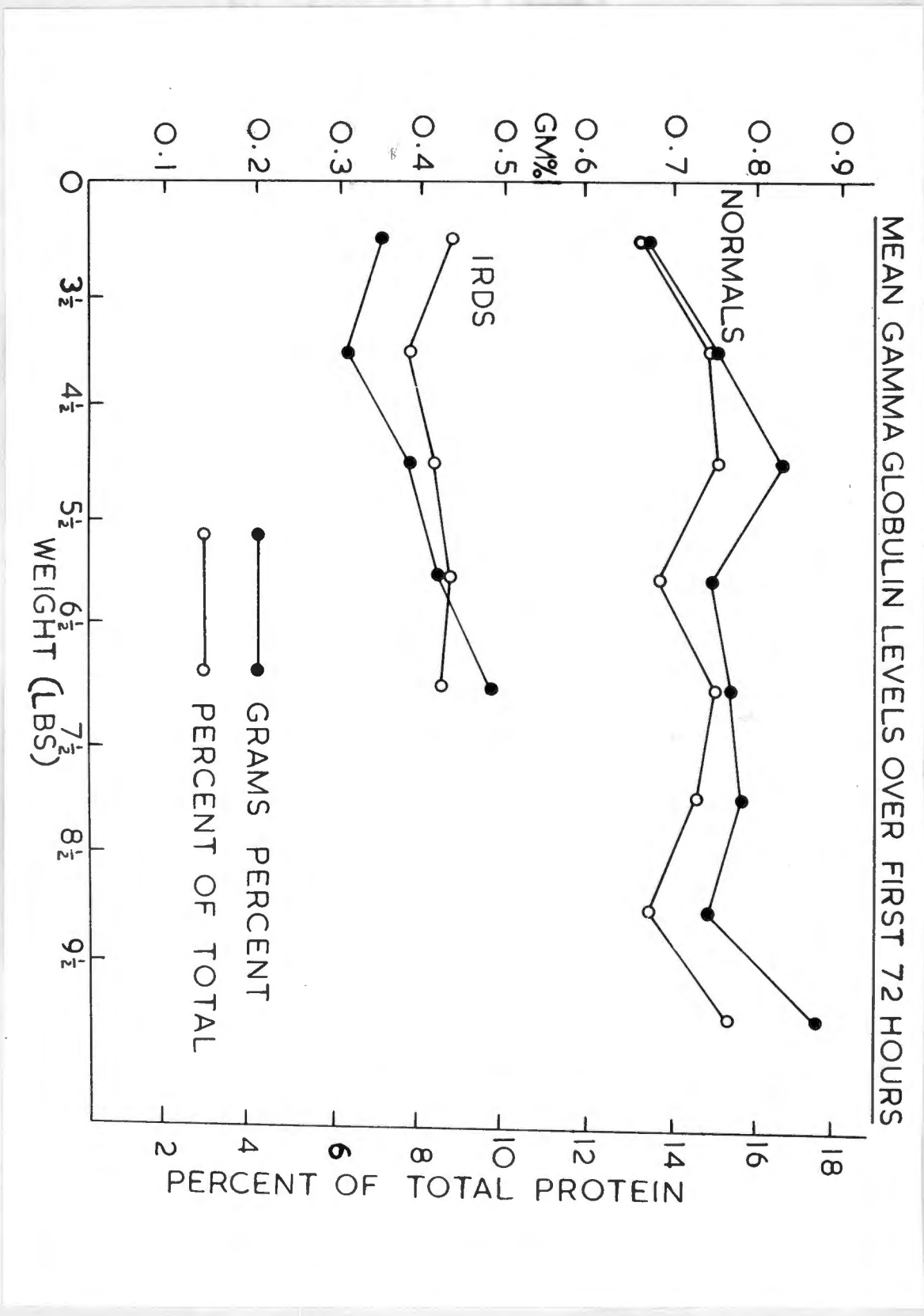

MEAN GAMMA GLOBULIN LEVELS OVER FIRST 72 HOURS
NORMALS
IRDS
GM%
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
3½
4½
5½
6½
7½
8½
9½
WEIGHT (LBS)
GRAMS PERCENT
PERCENT OF TOTAL
18
16
14
12
10
8
6
4
2
PERCENT OF TOTAL PROTEIN

FIGURE 3.2

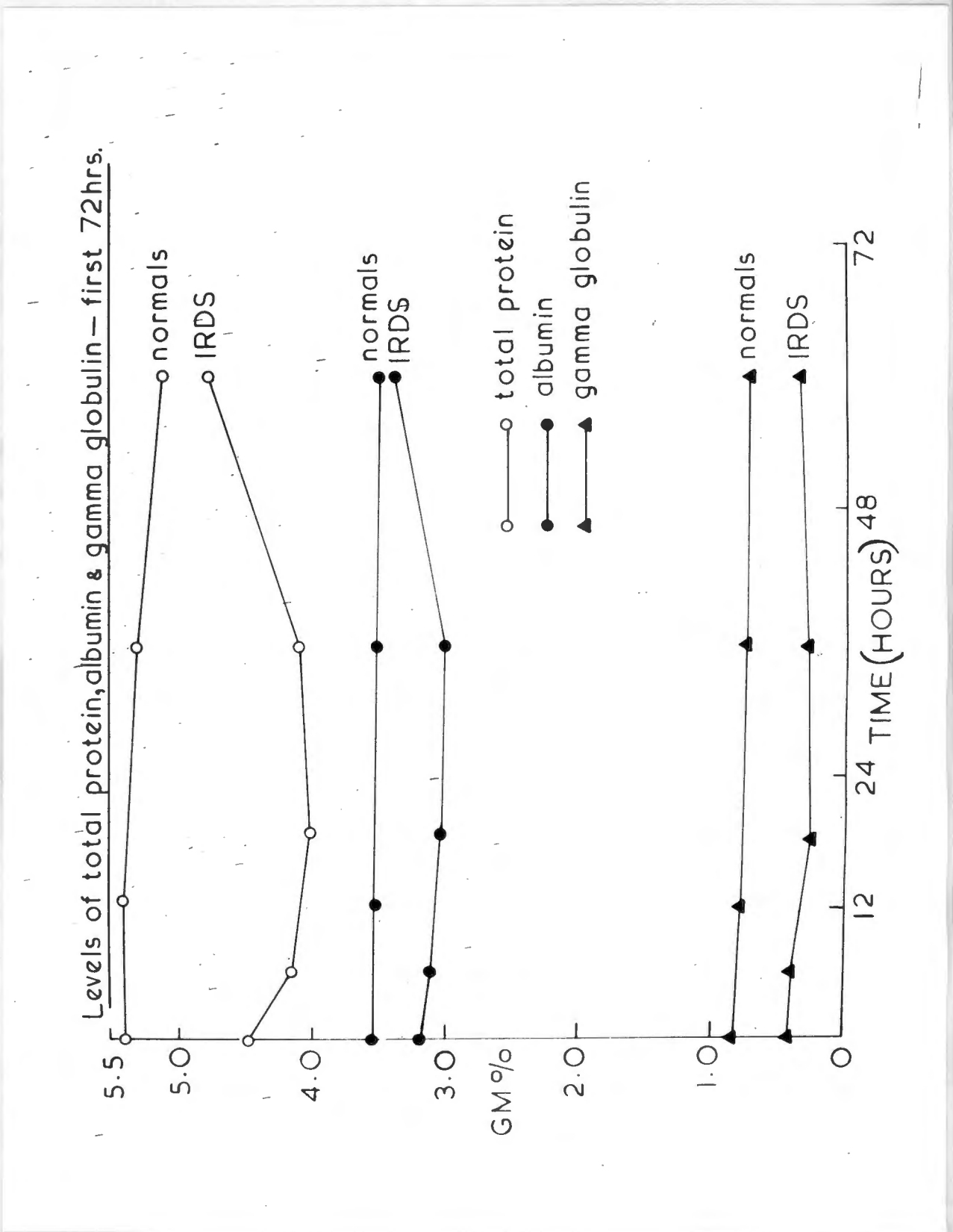
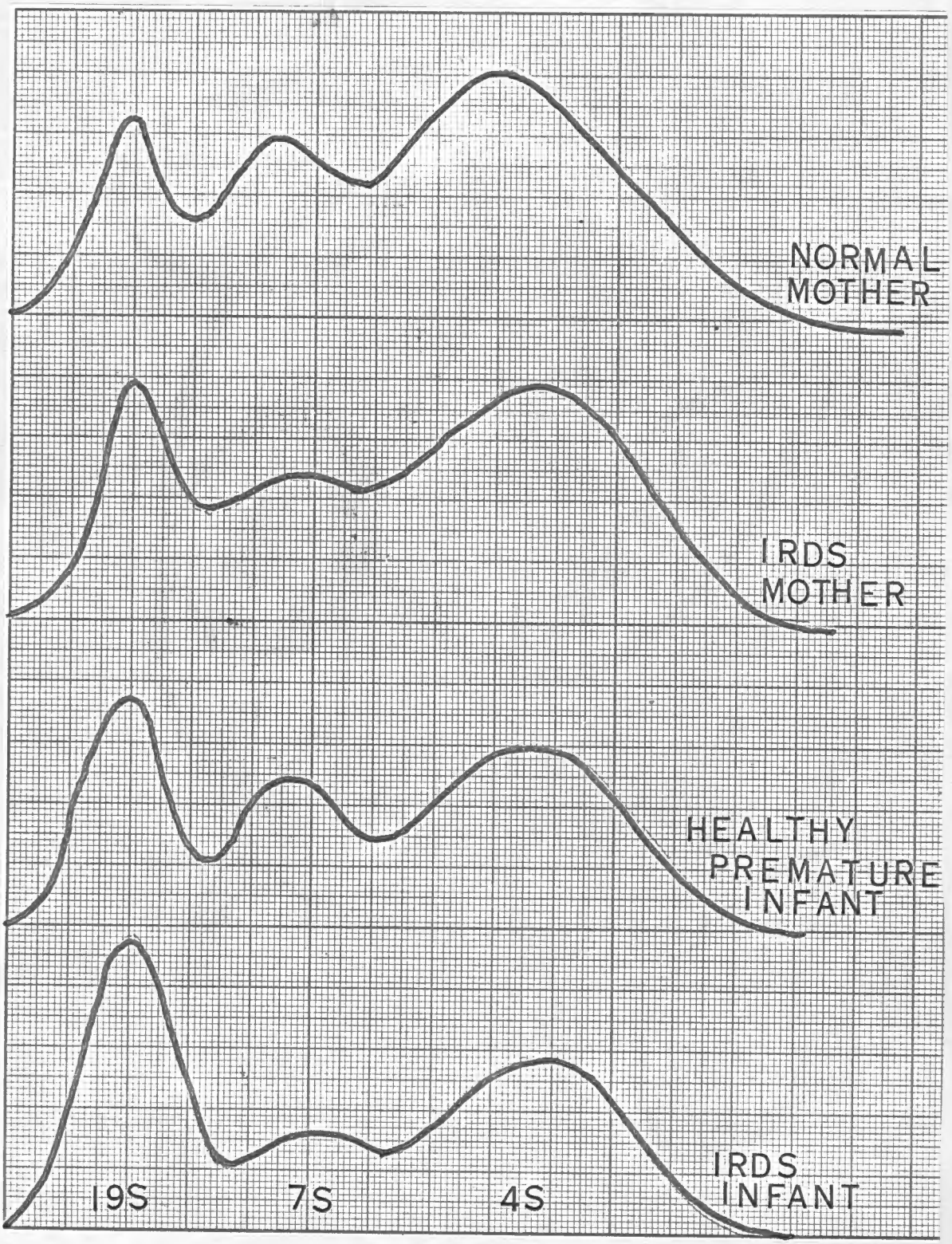

Levels of total protein, albumin & gamma globulin – first 72hrs.
normals
IRDS
normals
IRDS
normals
IRDS
total protein
albumin
gamma globulin
GM %
5.5
5.0
4.0
3.0
2.0
1.0
0
12
24
48
72
TIME (HOURS)

FIGURE 3.2A: COLUMN CHROMATOGRAPHS OF MATERNAL AND INFANTS' SERA.



3.II. Observations on α-fetoprotein

On inspection of the cellulose acetate strips, it was observed that sera of infants with IRDS contained a protein, generally in low concentration, and migrating between albumin and the α_1-globulins. (See Fig 3.3). This protein was described in 1966 by Gitlin (98) who named it α-fetoprotein. The protein was not usually obvious in the sera of healthy premature infants. Thus, in 70 sera from infants with IRDS, the band was visible to the naked eye in 60 instances, while in 90 sera from healthy premature infants, it was visible in only 20. Further, of 5 sets of twins, in each of which only one twin suffered from IRDS, the other being healthy, in 4 pairs of twins the band was visible only in the serum of the affected one. (Fig 3.4). In the 5th set of twins it was present in both individuals. Quantitation of the protein by the method of Part 2.V.3 was not possible because of its low concentration (generally about 10 - 20 mg/100ml serum).

The band was observed to become fainter and to disappear during the first 7 - 8 days of postnatal life. One severely ill infant was kept alive with the intermittent positive pressure respirator for 8 months. The post-albumin band was present in his cord serum and followed the usual pattern of progressive disappearance. At the age of 5 months, he was given a slow intravenous infusion of 1 gm of sterile IgG,

prepared from pooled human serum. He became severely distressed and the post-albumin band reappeared in his serum, disappearing again over the next 4 days. Infants suffering from Rh-isoimmunization had the same protein in their sera.

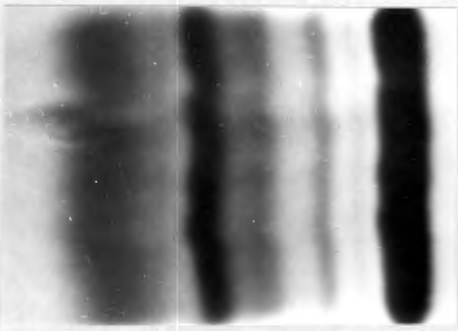
Serum was also obtained as described (Part 2.II.1) from a number of foetuses ranging in maturity from 12 - 24 weeks, and from patients suffering from primary carcinoma of the liver. These were observed to have a band migrating in the same position, the intensity being greatest in the least mature foetuses.

Sera containing the post-albumin band were stained for glycoprotein, after electrophoresis on cellulose acetate. This fraction was found not to be a glycoprotein. (Fig 2.8).

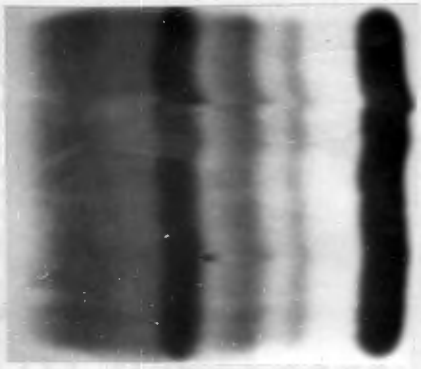
On obtaining a specific antiserum to the protein, as described in Part 2.VII, immunoelectrophoresis and immune diffusion experiments were performed. (Part 2.V.7,8). These established immunochemical identity between the post-albumin band seen in IRDS and the α-fetoprotein present in the serum of foetuses and patients, with primary carcinoma of the liver. (Fig 3.8). Immunoelectrophoresis demonstrated the presence of α-fetoprotein in the serum of normal infants, but in far lower concentration.

The sera were also electrophoresed on analytical and preparative polyacrylamide gels. (See later). (Part 2.V.6 and

FIGURE 3.3 : SERUM OF AN INFANT WITH IRDS COMPARED WITH THAT OF A HEALTHY PREMATURE INFANT.

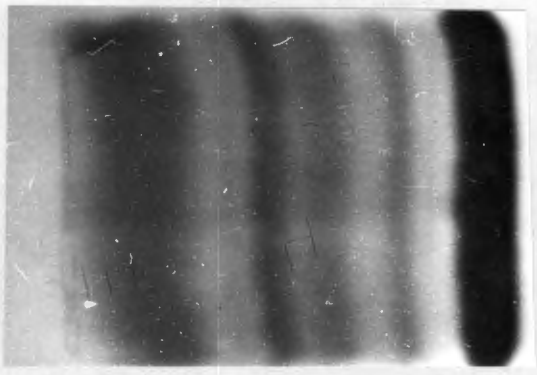


1. IRDS INFANT. NOTE PRESENCE OF A PROMINENT α-FETOPROTEIN BAND.

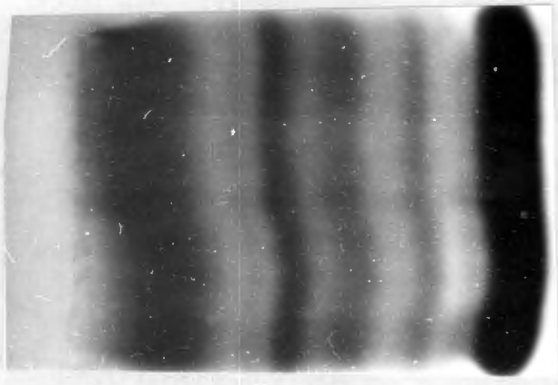


2. HEALTHY PREMATURE INFANT. NOTE ABSENCE OF THE α-FETOPROTEIN BAND.

FIGURE 3.4 : COMPARISON OF SERA OF A PAIR OF TWINS OF WHOM ONE HAD IRDS.

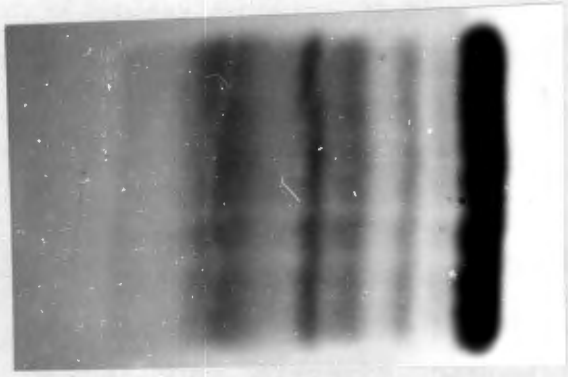


1. SERUM OF AFFECTED TWIN. NOTE PRESENCE OF α-FETOPROTEIN BAND.

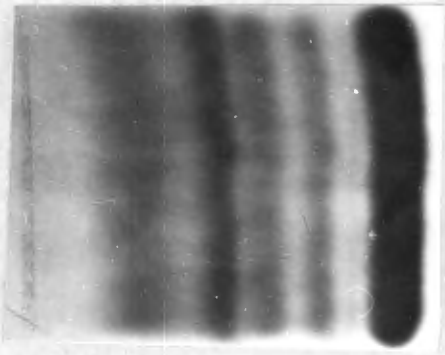


2. SERUM OF HEALTHY TWIN. NOTE ABSENCE OF α-FETOPROTEIN BAND.

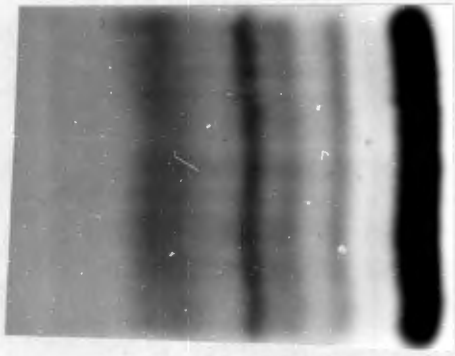
FIGURE 3.5 : SERA OF AN INFANT WITH IRDS ; SPECIMENS TAKEN ON SUCCESSIVE DAYS.



1. CORD SERUM.



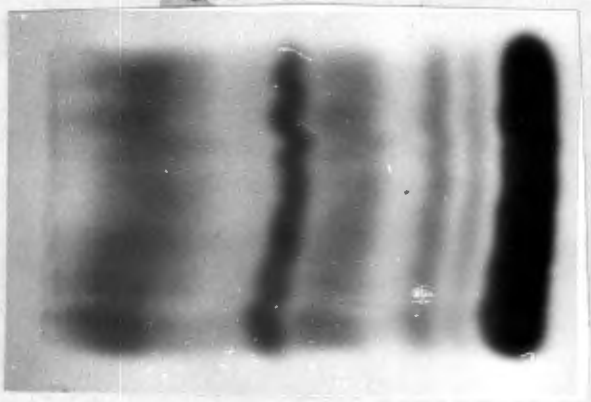
2. SECOND DAY OF POSTNATAL LIFE.



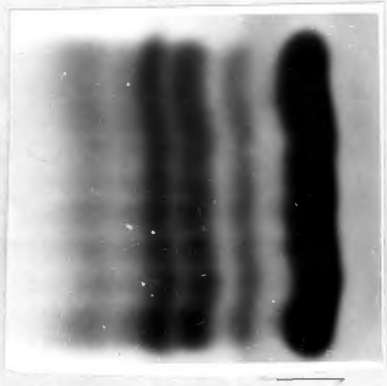
3. THIRD DAY OF POSTNATAL LIFE. NOTE THE GRADUAL FADING OF THE α-FETOPROTEIN BAND.

FIGURE 3.6

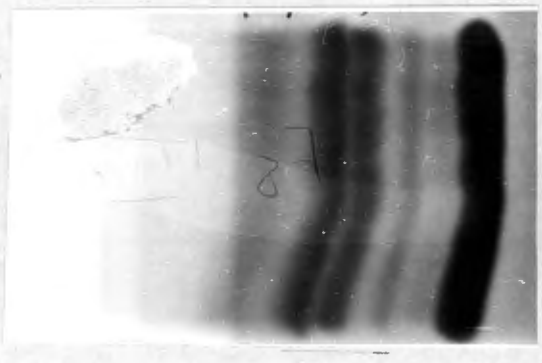
SERA OF BABY ENGELBRECHT: NOTE PRESENCE OF AN α-FETOPROTEIN BAND IN THE CORD SERUM, WITH SUBSEQUENT DISAPPEARANCE AND REAPPEARANCE OF THIS FRACTION FOLLOWING AN INFUSION OF IgG.



1. CORD SERUM



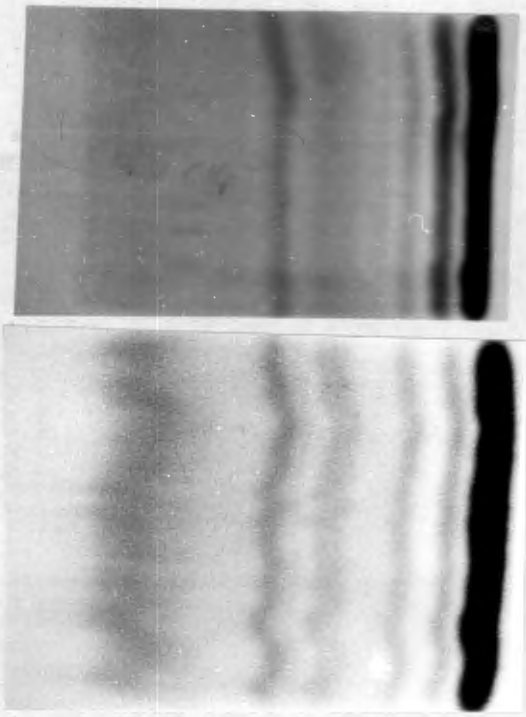
2. SERUM AT THREE MONTHS OF AGE



3. SERUM ON DAY FOLLOWING INFUSION OF IgG

FIGURE 3.7: SERA OF FOETUSES, GESTATIONAL AGES 14 AND 16 WEEKS, COMPARED WITH SERUM OF A PATIENT WITH PRIMARY CARCINOMA OF THE LIVER.

1. FOETAL SERA.



2. HEPATOMA SERUM.

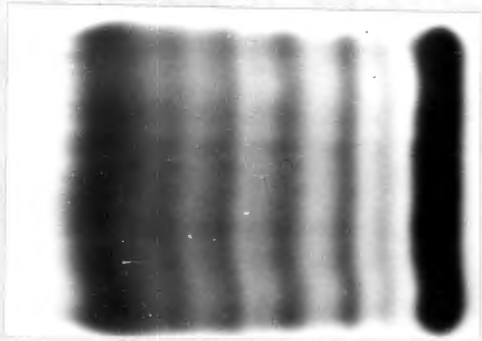
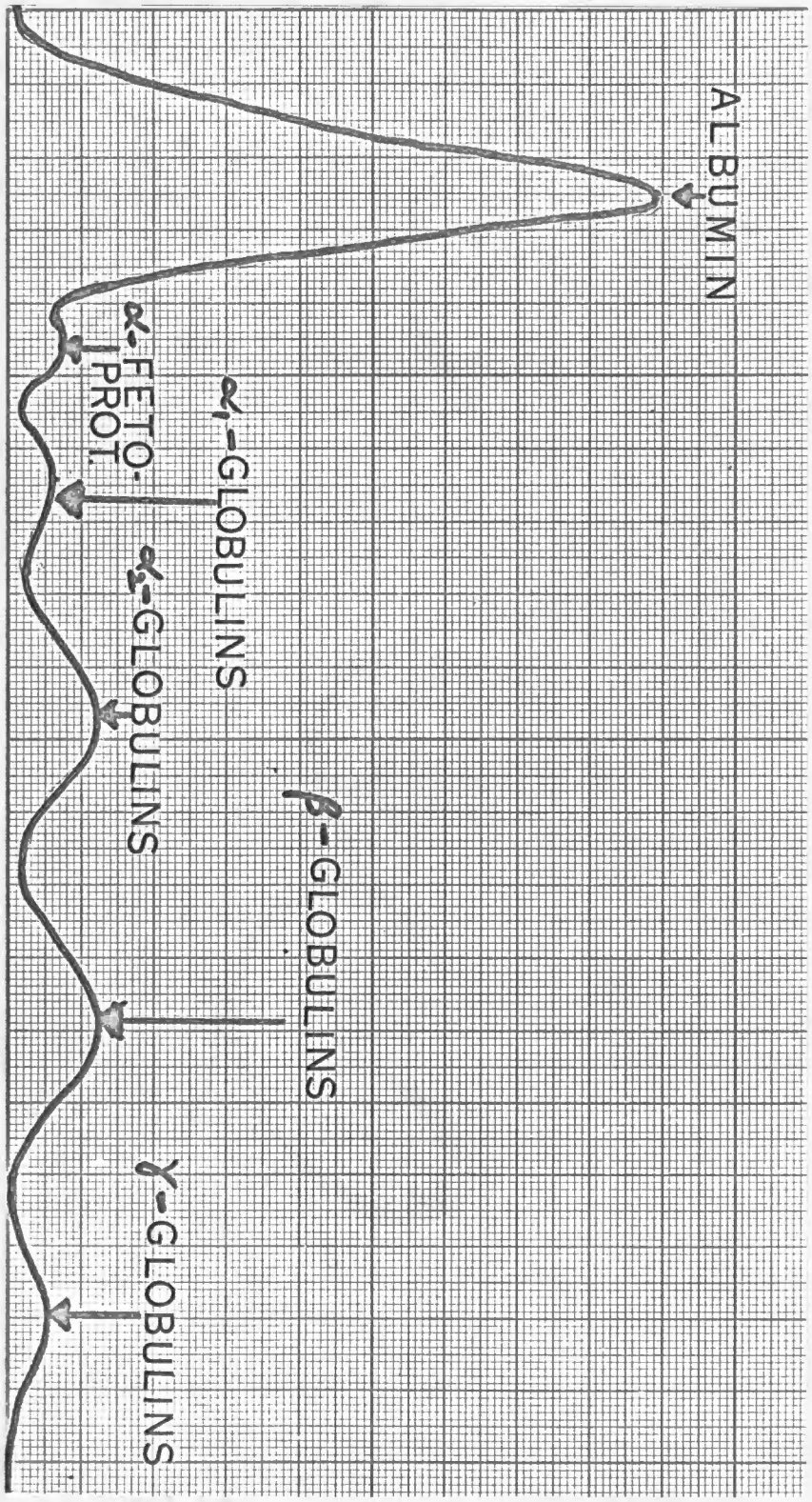


FIGURE 3.7A: DENSITROMETRY OF SERUM OF IRDS INFANT TO DEMONSTRATE RELATIVE DEFICIENCY OF IgG.



IV.2). The ∝-fetoprotein fraction was observed to migrate just behind serum albumin, as on cellulose acetate (Fig 3.9). Attempts to separate ∝-fetoprotein from albumin, by preparative electrophoresis and fractional precipitation with ammonium sulphate (Part 2.IV.2,4), were unsuccessful. Hence a preparation containing ∝-fetoprotein + albumin was used in subsequent experiments.

As it had been reported (102) that foetal serum was superior to adult serum in promoting growth of cells in tissue culture, it was decided to investigate the immunochemical interrelationships between ∝-fetoprotein and somatotropin, placental hormones (human placental lactogen (HPL), human chorionic gonadotropin (HCG)), and lactogenic hormone.

Possible interrelationships of ∝-fetoprotein and somatotropin with IgG were also investigated, in view of the low serum IgG levels in cases of IRDS and of reports of complex-formation between IgG and other serum-proteins. (54,55,56,57).

1. Analytical and preparative electrophoresis was performed on polyacrylamide gel as described in Part 2.IV.2 and V.6.

The preparations examined on the analytical gels were: normal adult male serum, normal non-pregnant adult female serum, pregnant female serum, serum from foetuses of varying gestational ages, serum from healthy infants and infants with

IRDS, preparations of serum albumin and of serum albumin + α-fetoprotein, both of which were shown by immuno-electrophoresis to contain no other proteins, serum from a patient with primary carcinoma of the liver, a preparation of IgG from pooled serum, a preparation of somatotropin supplied by the NIH and pure serum albumin mixed with pure IgG and incubated at 37° for 1 hour.

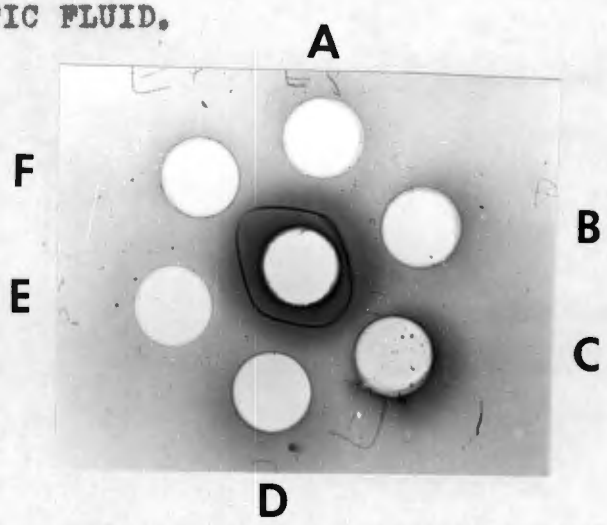
(a) The sera exhibited a large number of protein bands, identification of all of which was not possible. In foetal sera the most prominent zones were those ascribable to serum albumin, a band of varying intensity directly behind albumin, a moderately intense band in the β-globulin region and sharply-defined band in the γ-globulin position.

(b) The pure albumin preparation exhibited 3 bands: an intense one corresponding to the expected position for the main albumin fraction, a moderately intense band in the β-globulin region and a series of faint bands in the normal "belt" region, i.e. near the cathodal end of the gel. (137).

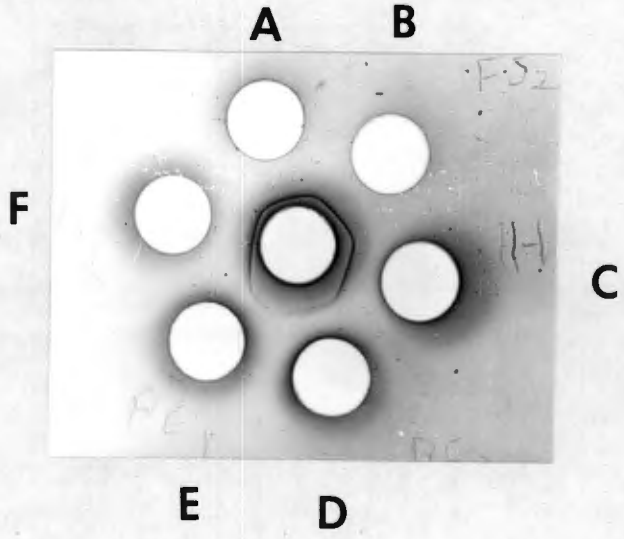
(c) The α-fetoprotein + albumin preparation exhibited an albumin band with one other faint component immediately behind it, two moderately intense bands in the β-globulin region and a series of faint bands, similar to those in the albumin gel, in the normal "belt".

(d) The growth hormone preparation showed two moderately

FIGURE 3.8 : DEMONSTRATION BY MEANS OF OUCHTERLONY PLATES OF THE IMMUNOCHEMICAL IDENTITY BETWEEN α-FETOPROTEIN FOUND IN THE SERA ENUMERATED BELOW AND IN PLACENTAL EXTRACTS AND AMNIOTIC FLUID.

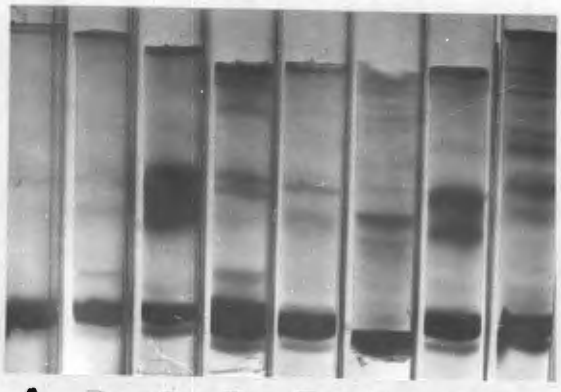


1. A. IRDS INFANT SERUM, B. FOETAL SERUM,
C, D. HEALTHY PREMATURE SERA, E. HEPATOMA SERUM,
F. PREGNANT FEMALE SERUM. ANTI-α-FETOPROTEIN IN CENTRE WELL.

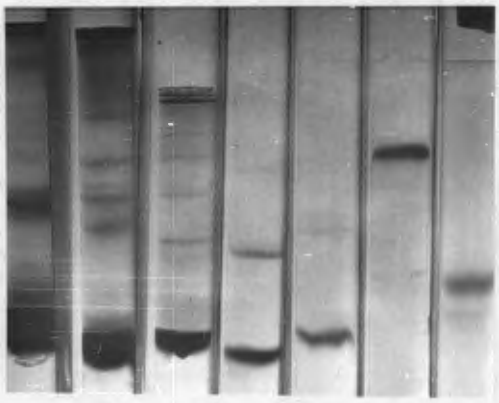


2. A, B, F. FOETAL SERA, C. HEPATOMA SERUM,
D. AMNIOTIC FLUID CONCENTRATE, E. PLACENTAL EXTRACT.
ANTI-α-FETOPROTEIN IN CENTRE WELL.

FIGURE 3.9 : ANALYTICAL POLYACRYLAMIDE GEL ELECTROPHORESIS OF SERA AND VARIOUS PROTEIN PREPARATIONS.



1. A, B, C, D, E. SERA OF FOETUSES RANGING IN GESTATIONAL AGE BETWEEN 14 AND 22 WEEKS, F. SERUM OF HEALTHY PREMATURE INFANT, G. SERUM OF IRDS INFANT, H. SERUM OF A PREGNANT WOMAN.



2. A, B, C. SERA OF A NON-PREGNANT FEMALE, A PATIENT WITH PRIMARY CARCINOMA OF THE LIVER AND AN ADULT MALE,
D. SERUM ALBUMIN, E. α-FETOPROTEIN + ALBUMIN,
F. IgG , G. HUMAN GROWTH HORMONE.

intense bands in the α-globulin region and a very faint band in the γ_1-globulin region.

(e) The IgG preparation exhibited one well-defined band near the cathodal end of the gel and two faint bands nearer the anode. In this system, much of the IgG preparation does not enter the gel because of electroendosmosis.

(f) The IgG + albumin preparation exhibited only the bands seen with pure albumin and with pure IgG, i.e. there was no evidence of complex-formation, as evidenced by the appearance of additional bands.

Photographs of the gels described are shown in Fig 3.9.

2. Immune diffusion and immunoelectrophoresis were carried out as described in Part 2.V.7,8,10.

(a) Immune diffusion

Sera examined were from: adult males, pregnant and lactating females, foetuses, healthy premature infants and infants with IRDS and from cases of primary carcinoma of the liver. In addition, placental extracts, preparations of pure albumin (from adult male serum), albumin + α-fetoprotein, IgG (from pooled adult serum), somatotropin (NIH), human chorionic gonadotropin (Organon) and concentrates of amniotic fluid were studied. These were allowed to react with monospecific antisera against IgG, IgM, IgA Bence Jones proteins types K and L, α-fetoprotein, somatotropin and human

chorionic gonadotropin, on Ouchterlony plates.

(i) When anti-IgG was used, precipitin lines formed against the wells containing all the sera tested and also the placental and amniotic fluid preparations; against the somatotropin well and against the well containing α-feto-protein + albumin. There was no visible precipitin arc opposite the albumin well. The reaction demonstrated was one of complete identity between the proteins tested. (Fig 3.10).

(ii) Anti-IgM formed precipitin lines with all the sera tested, with the exception of foetal serum, but not against any of the other protein preparations. (Fig 3.11)

(iii) Anti-IgA reacted with the adult sera, but not with the other sera or protein preparations.

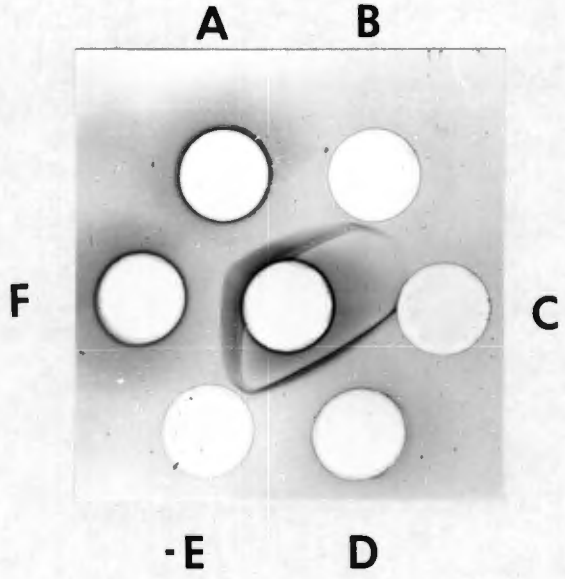
(iv) Antisera to Bence Jones proteins

These were weak antisera and frequently elicited no precipitin line on immunoelectrophoresis with sera containing low concentrations of immune globulins. However, they consistently produced precipitin lines with preparations known to contain immune globulins in significant amounts, when placed on Ouchterlony plates.

(a) The antiserum to Bence Jones protein, type K

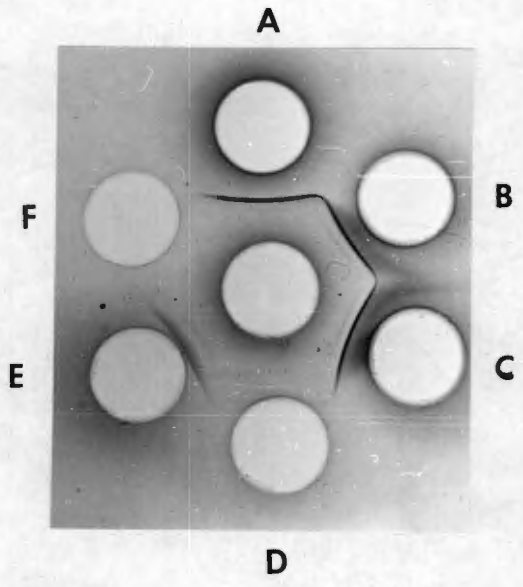
This gave positive cross-reactions with IgG, hepatoma and adult male serum pregnant and lactating female serum,

FIGURE 3.10 : OUCHTERLONY PLATE SHOWING CROSS-REACTION BETWEEN HUMAN GROWTH HORMONE AND AN ANTISERUM TO HUMAN IgG.



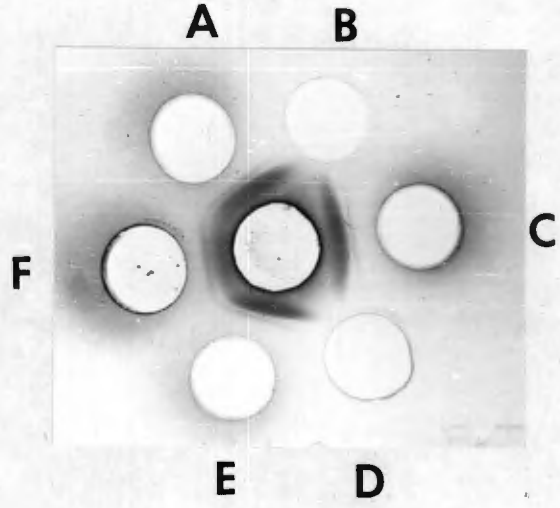
A. HUMAN IgG , B. HUMAN GROWTH HORMONE,
C. HUMAN SERUM ALBUMIN, D, E, F. SERA OF AN ADULT MALE,
A 14-WEEK FOETUS AND A PREGNANT FEMALE.
CENTRE WELL : ANTISERUM TO HUMAN IgG.

FIGURE 3.11 : OUCHTERLONY PLATE SHOWING REACTIONS BETWEEN AN ANTISERUM TO HUMAN IgM, VARIOUS SERA AND HUMAN GROWTH HORMONE.



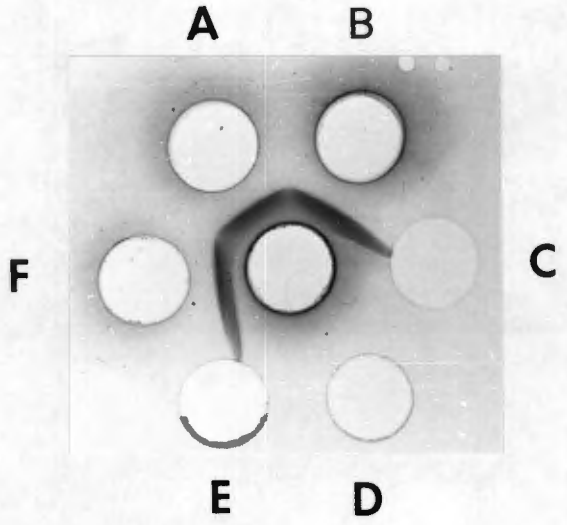
A, B, C, ADULT SERA : MALE, PREGNANT AND NON-PREGNANT FEMALE. D. HUMAN GROWTH HORMONE, E. HEALTHY PREMATURE INFANT SERUM, F. FOETAL SERUM. CENTRE WELL : ANTISERUM TO HUMAN IgM.

FIGURE 3.12 : OUCHTERLONY PLATE SHOWING CROSS-REACTIONS BETWEEN AN ANTISERUM TO BENCE JONES PROTEIN TYPE K AND VARIOUS SERA AND PROTEIN PREPARATIONS.



A. HUMAN IgG , B. HUMAN GROWTH HORMONE , C. ADULT MALE SERUM, D. α-FETOPROTEIN + ALBUMIN , E. ALBUMIN + IgG, F. HEPATOMA SERUM. ANTISERUM TO BENCE JONES PROTEIN TYPE K IN CENTRE WELL.

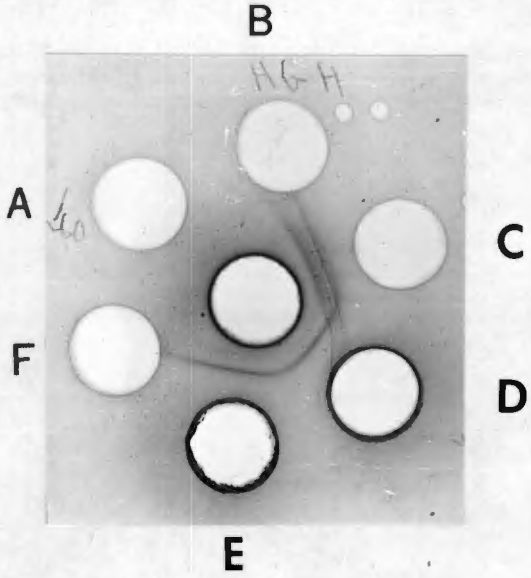
FIGURE 3.12 (CONTINUED) : OUCHTERLONY PLATE SHOWING CROSS-REACTIONS BETWEEN AN ANTISERUM TO BENCE JONES PROTEIN TYPE L AND VARIOUS SERA AND PROTEIN PREPARATIONS.



A. HUMAN GROWTH HORMONE, B. HUMAN IgG , C. SERUM ALBUMIN, D. α -FETOPROTEIN + ALBUMIN , E. BOVINE SERUM ALBUMIN, F. ADULT MALE SERUM.

ANTISERUM TO BENCE JONES PROTEIN TYPE L IN CENTRE WELL.

FIGURE 3.13 : OUCHTERLONY PLATE SHOWING CROSS-REACTIONS BETWEEN AN ANTISERUM TO HUMAN CHORIONIC GONADOTROPIN AND VARIOUS SERA AND PROTEIN PREPARATIONS.



A. FOETAL SERUM, DILUTED 1 : 40 , B. HUMAN GROWTH HORMONE,

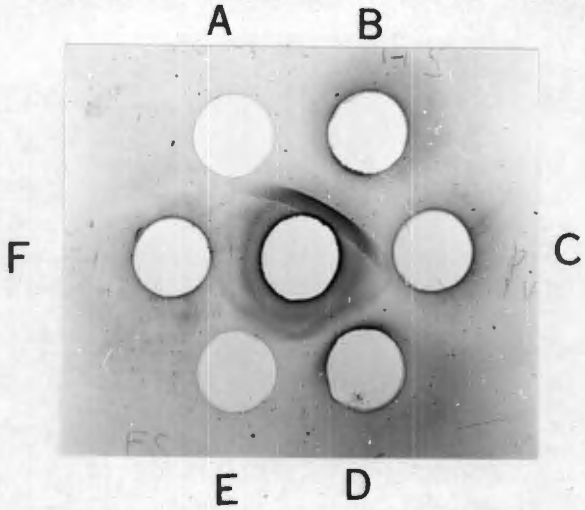
C. HUMAN CHORIONIC GONADOTROPIN,

D, E. SERA OF LACTATING AND PREGNANT FEMALES,

F. α-FETOPROTEIN + ALBUMIN.

ANTISERUM TO HUMAN CHORIONIC GONADOTROPIN IN CENTRE WELL.

FIGURE 3.14 : OUCHTERLONY PLATE TO DEMONSTRATE CROSS-REACTIONS BETWEEN VARIOUS SERA, HUMAN GROWTH HORMONE, PLACENTAL EXTRACTS AND AN ANTISERUM TO α-FETOPROTEIN.



A. HUMAN GROWTH HORMONE, B. HEPATOMA SERUM, C. EXTRACT OF A FULL-TERM PLACENTA, D. PREGNANT FEMALE SERUM, E. FOETAL SERUM, F. EXTRACT OF PLACENTA FROM A PREMATURE INFANT.

and with the serum albumin + IgG preparation. In each instance, 2 lines were visible on the Ouchterlony plate. There was no reaction against any other serum or protein preparation. (Fig 3.12).

(b) The antiserum to Bence Jones protein,type L reacted differently:

There were positive cross-reactions with all the proteins giving positive reactions with anti-Bence Jones protein, type K. In addition, this antiserum cross-reacted with albumin +∝fetoprotein and with human growth hormones, but not with albumin or any other protein mixtures. (Fig 3.13).

(v) Anti-HCG invoked precipitin lines against HCG and the sera of pregnant and lactating women, but against no other sera or protein preparations.

(vi) Anti-∝-fetoprotein formed precipitin lines against sera from foetuses, premature infants, cases of primary carcinoma of the liver, the ∝-fetoprotein + albumin preparation, the IgG preparation, with somatotropin, and with placental extracts. There was partial identity between somatotropin and ∝-fetoprotein with this antiserum.(See Fig 3.14).

(vii) Anti-somatotropin. (Anti-HGH)

This was a weak antiserum, intended for use in radioimmunoassay. (Produced in guinea pigs and diluted 1:2,000

with 0.15M sodium chloride). The antiserum was concentrated by lyophilyzation (after dialysis to remove electrolytes) and reconstituted to 1/10 the original volume with 0.15M sodium chloride. No precipitin arc could be demonstrated between this anti-HGH and the HGH preparation. However, it did give weak cross-reactions with IgG, α-fetoprotein + albumin, a number of sera and with the placental extract.

(viii) Anti-albumin

This produced precipitin lines with all the sera studied, the placental extract, the HGH preparations listed before, with the exception of IgG.

*

(b) Immunoelectrophoresis

The sera and protein preparations examined were the same as in (a) and the same antisera were employed. In addition, a polyvalent antiserum to adult human serum was utilized for certain experiments.

(i) With the polyvalent antiserum the various sera showed the usual precipitin arcs; foetal and cord sera exhibited fewer arcs than did adult serum: in 12 - 14 week foetuses there were arcs corresponding only to serum albumin, β-globulin and γ-globulin. (Fig 4.1).

Albumin and albumin + α-fetoprotein produced arcs corresponding only to albumin and IgG gave rise to a single

*(ix) An interesting recent observation was that whenever placental extracts were placed in a well adjacent to a preparation containing -fetoprotein a strong precipitin line formed between the wells.

arc in the γ-globulin region.

Placental extracts had arcs corresponding to the principal serum protein components. (Fig 3.15a). Somatotropin appeared as three arcs: two in the albumin-α_1-globulin region and one in the γ-globulin region.

(ii) With the monospecific antiserum to IgG, sera, placental extracts and IgG all gave single precipitin arcs in the γ-globulin region. There was no demonstrable reaction with albumin or with albumin + α-fetoprotein, although the latter preparations had consistently formed a precipitin line with this antiserum on Ouchterlony plates. Possibly the quantity of protein which could be placed in the wells of an immunoelectrophoresis plate was too small to produce a visible precipitin arc.

Somatotropin gave a single arc in the γ-globulin region with this antiserum.

(iii) The antiserum to IgM generated single precipitin arcs at or near the origin with adult sera but not with any of the other preparations, despite the fact that serum of neonates gave precipitin lines with this antiserum on Ouchterlony plates. Presumably this was a concentration effect, similar to that suggested in connection with albumin + α-fetoprotein in (ii).

(iv) The antiserum to IgA gave rise to single precipitin arcs with adult sera. None of the other sera or protein preparations reacted visibly with this antiserum.

(v) Similarly, the antisera to Bence Jones proteins, types K and L, gave a visible reaction only with adult sera and the IgG and IgG + albumin preparations, in the γ-globulin region. This result was unexpected, as foetal and neonatal sera obviously contained immune globulins. However, the antibody titre of these antisera was low. As mentioned previously, these antisera gave rise to a greater number of visible cross-reactions on Ouchterlony plates.

(vi) The antiserum to α_1-fetoprotein gave a single precipitin arc in the α_1-globulin region, with serum from foetuses, premature and full-term infants, patients with primary carcinoma of the liver and with the albumin + fetoprotein preparation. There was no visible reaction with normal adult male serum, other adult sera, albumin or somatotropin, although somatotropin had reacted with this antiserum on Ouchterlony plates. The explanation for this may be similar to that given in (ii) and (v).

Results of these experiments are summarized in Table 3.4.

(vii) The antiserum to HGH was too weak to give visible bands with any of the sera or protein preparations by this method.

TABLE 3.4

Reactions between various antisera and protein preparations studied*

Proteins ↓	Anti-IgG	Anti-IgA	Anti-IgM	Anti-BJ(K)	Anti-BJ(L)	Anti-FP	Anti-HSA	Anti-HGH	Anti-HCG	Anti-adult human serum
← Antisera →	O ie	O ie	O ie	O ie	O ie	O ie	O ie	O ie	O ie	ie pattern
IgG	+ +	− −	− −	+ +	+ +	+ −	− −	± −	− −	Single arc in the γ-region
HSA	− −	− −	− −	− −	− −	− −	+ +	− −	− −	Single arc in albumin region
FP+HSA	+ −	− −	− −	− −	± −	+ +	+ +	± −	− −	Single arc in albumin-region
HGH	+ +	− −	− −	− −	+ −	+ −	+ +	− −	− −	2 arcs: 1 in α; 1 in γ-region
Foetal serum	+ +	− −	− −	− −	− −	+ +	+ +	± −	+ −	3 main bands: albumin, β-γ-globulins
Hepatoma serum	+ +	+ +	+ +	+ +	+ +	+ +	+ +	+ −	− −	Usual serum pattern
Pregnant female serum	+ +	+ +	+ +	+ +	+ +	+ −	+ +	− −	+ +	Usual serum pattern
Lactating female serum	+ +	+ +	+ +	+ +	+ +	− −	+ +	− −	+ ±	Usual serum pattern
Adult male serum	+ +	+ +	+ +	+ +	+ +	− −	+ +	± −	− −	Usual serum pattern
Placental extract	+ +	± −	− −	+ +	+ +	+ +	+ +	− −	+ −	Main serum protein fractions
Healthy premature serum	+ +	− −	+ ±	+ −	+ −	+ +	+ +	± −	+ −	Usual serum pattern
IRDS infant serum	+ +	− −	+ ±	+ −	+ −	+ +	+ +	± −	+ −	Usual serum pattern

* O = Ouchterlony plate
ie = immunoelectrophoresis
BJ(K,L) = Bence Jones proteins types K or L
HSA = Serum albumin from a healthy adult male
FP = α-fetoprotein
HCG = Human chorionic gonadotropin
HGH = Human growth hormone
\+ = positive cross-reaction
± = doubtful
− = no reaction

(viii) The antiserum to albumin gave single precipitin arcs in the expected position with the sera, placental extract, albumin, ∝-fetoprotein + albumin and with the HGH preparation.

Results of the immune diffusion and immunoelectrophoresis experiments are summarized in Table 3.4

11% Of pregnant women have been observed to develop skin reactions following subcutaneous injection of foetal serum and/or amniotic fluid extracts (104). In order to establish whether mothers of infants with IRDS had antibodies against ∝-fetoprotein, the following experiments were performed:

(a) Sera from normal and affected infants and from foetuses 14 - 22 weeks of age were electrophoresed in agarose gel as in Part 2.V.7 and incubated with sera of mothers of normal and IRDS infants. No precipitin arcs could be demonstrated by this method.

(b) Ouchterlony plates were prepared with maternal serum in the central well and infants' and foetal sera in the peripheral wells. It was found that, as a rule, asymmetrical precipitin discs formed around the wells containing infants' sera. Occasionally a precipitin line was observed, but this method did not give results which could be interpreted quantitatively. Hence a difference between

the normal and IRDS groups was not established.

(c) Fetoprotein immunoplates were prepared as in Part 2.V.10 and maternal sera placed in the main wells. As before, (b), asymmetrical precipitin discs were discernable around some of the wells. These were produced by sera from mothers of normal premature as well as affected infants. As it was not possible to discover whether or not there was a significant difference between the two groups, it was decided to make use of a passive haemagglutination technique instead. These experiments are described in Part 3.V.3.

3.III. Quantitation of maternal serum-protein

(a) Maternal blood was obtained at delivery from women giving birth to healthy or to affected premature infants. (b) In addition, blood-specimens were taken at intervals of 2 - 3 weeks from women attending the antenatal clinics attached to Groote Schuur Hospital. The investigation was started at the earliest time that the pregnant women came under observation (generally 16 - 20 weeks after the last menstrual period) and continued until delivery. (c) Where possible, follow-up blood specimens were taken, 6 weeks or more post-partum, from the same women, for comparison with the findings during pregnancy.

Serum-protein fractions were quantitated by cellulose acetate electrophoresis and by immunoplates, as described in Part 2.V.1,3,9.

(a) Blood-specimens taken at delivery

Here, 30 mothers of infants with IRDS were compared with 25 mothers of healthy premature infants of a similar mean gestational age. Results are set out in Table 3.6.

The concentrations of total protein, IgM and IgA did not differ significantly in the two groups. However, the IgG concentration, both as estimated electrophoretically and by immunoplates, was lower in the mothers of IRDS infants, the difference being highly significant ($P < 0.001$).

TABLE 3.6

Mean serum-protein concentrations (± standard error) in specimens taken at delivery from IRDS mothers and from mothers of healthy premature infants

Group	No. in group	Mean gest. age (wks)	Total proteins (gm/100ml)	Serum albumin (gm/100ml)	γ-globulins (electrophoretic)	Immunoplates		
						IgG gm%	IgM mg%	IgA mg%
Mothers of healthy premature infants	25	33.9	5.3 ±0.1	3.12 ±0.05	0.90 ± 0.04	0.92 ±0.09	134 ±2	268 ±2
Mothers of IRDS infants	30	34.1	5.2 ±0.8	2.90 ±0.04	0.70 ±0.04	0.67 ±0.05	134 ±3	271 ±2

There was nevertheless a considerable overlap between the groups in the values obtained.

(b) Patients observed from early pregnancy to delivery

(i) Mothers giving birth to healthy infants

There were 40 women in this series, who subsequently delivered healthy premature or full-term infants. The total serum-protein concentration as well as the concentrations of serum albumin and of γ-globulins, underwent minor fluctuationa throughout pregnancy, with lowest levels within the period 32 - 37 weeks, but the variations were not statistically significant. There was good agreement between γ-globulin levels as measured electrophoretically and by immunoplates. The observations were similar to those of other workers (169, 170, 180).

(ii) Mothers giving birth to infants with IRDS

There were 12 women in this series including 3 diabetic patients. In these cases, the concentrations of total serum-protein and of albumin did not differ significantly from those found in healthy mothers, at the same stage of pregnancy. The lower values found at delivery were due to the fact that these women were all delivered before 37 weeks, i.e. at a stage when healthy women have lower values than at term. The γ-globulin level, however, was significantly lower than that observed in healthy women and remained consistently so throughout pregnancy. Lowest levels were

reached at 29 - 31 weeks gestation and remained low thereafter. These results are presented in Table 3.6. Comparison with Table 3.5 shows that mothers of healthy premature infants have, at delivery (i.e. 30 - 37 weeks) a higher concentration of γ-globulins than have mothers of healthy full-term infants at the same stage of pregnancy. This difference is statistically highly significant ($P < 0.001$) and suggests that mothers of healthy premature infants constitute a different population. (See Table 3.7).

The IgG and IgM levels, as measured by immunoplates were significantly lower than normal in mothers of IRDS infants ($P < .001$). There was no significant difference in the IgA concentrations of the two groups.

The observations on IgM levels are not in agreement with findings on the larger group at delivery (III.(a)). This requires further investigation.

(c) Follow-up specimens obtained 6 weeks or more post-partum

Blood-specimens were obtained from twenty of the mothers of healthy infants and from fifteen who had delivered infants with IRDS. In both groups, the rise in the concentrations of total serum-protein and of serum albumin were of the same order. The control mothers showed a slight but non-significant rise in the total γ-globulins and in IgG, whereas in

TABLE 3.7

Mean values for total proteins, serum albumin and γ-globulins (electrophoretically estimated); IgG, IgM and IgA (estimated by immunoplates) during pregnancy

Group			Duration of pregnancy (weeks)						Delivery
			0-20	21-28	29-31	32-34	35-37	38-40	
Healthy mothers (40)	Total serum-proteins (gm/100ml)		5.4 ±0.1	5.4 ±0.1	5.5 ±0.1	5.4 ±0.1	5.3 ±0.2	5.5 ±0.2	5.7 ±0.2
	Serum albumin (gm/100ml)		3.24 ±0.05	3.19 ±0.05	3.14 ±0.04	3.08 ±0.04	2.96 ±0.03	3.08 ±0.05	3.20 ±0.09
	γ-globulins (electrophoretic) (gm/100ml)		0.77 ±0.03	0.72 ±0.03	0.70 ±0.03	0.68 ±0.02	0.71 ±0.03	0.73 ±0.03	0.83 ±0.02
Healthy mothers (12)	Immuno-plates	IgG (gm/100ml)	0.76 ±0.10	0.82 ±0.09	0.71 ±0.11	0.73 ±0.08	0.66 ±0.12	0.70 ±0.10	0.81 ±0.07
		IgM (mg/100ml)	107 ±4	214 ±3	73 ±5	134 ±2	80 ±6	76 ±4	71 ±4
		IgA (mg/100ml)	171 ±2	171 ±6	198 ±8	226 ±7	194 ±4	192 ±5	219 ±5
IRDS mothers (12)	Total serum-proteins (gm/100ml)		5.8 ±0.3	5.3 ±0.2	5.2 ±0.2	5.2 ±0.2	5.2 ±0.2	--	5.2 ±0.3
	Serum albumin (gm/100ml)		3.36 ±0.14	3.08 ±0.06	3.12 ±0.07	2.92 ±0.10	2.76 ±0.13	--	3.17 ±0.07
	γ-globulins (electrophoretic) (gm/100ml)		0.68 ±0.02	0.65 ±0.02	0.59 ±0.01	0.60 ±0.04	0.61 ±0.05	--	0.60 ±0.03
IRDS mothers (4)	Immuno-plates	IgG (gm/100ml)	0.68 ±1.20	0.66 ±1.30	0.70 ±1.00	0.62 ±1.10	0.47 ±2.00	--	0.60 ±0.09
		IgM (mg/100ml)	68 ±6	53 ±4	60 ±4	56 ±6	57 ±5	--	40 ±5
		IgA (mg/100ml)	215 ±6	238 ±5	201 ±9	223 ±4	139 ±5	--	113 ±8

mothers of infants with IRDS the rise between the time of delivery and the time when the follow-up specimen was taken was pronounced and significant. (See Table 3.8).

TABLE 3.8

Mean values 6 weeks or more post-partum (± standard error)

Group	No.	Total protein (gm/100ml)	Serum albumin (gm/100ml)	γ-globulins (electrophoretic (gm/100ml)	Immunoplates		
					IgG (gm/100ml)	IgM (mg/100ml)	IgA (mg/100ml)
Mothers of healthy prematures	20	5.7 ± 0.1	3.82 ± 0.07	0.93 ± 0.03	1.00 ± 0.10	136 ± 5	275 ± 3
Mothers of IRDS infants	15	5.8 ± 0.1	3.83 ± 0.06	1.01 ± 0.04	1.00 ± 0.07	85 ± 4	195 ± 4

There was no significant change in IgA in either group and in the IRDS mothers the IgM remained significantly lower than in the control group.

Gel filtration of the sera on Sephadex G.200 confirmed that the 7S peak in serum taken at delivery from IRDS mothers was smaller than the corresponding 7S peak in serum of normal mothers.

3.IV. Examination of faeces and body fluids other than blood, for protein content

1. Daily specimens of urine were collected from 10 infants with IRDS and from 10 newborn premature infants suffering from respiratory distress, caused by meconium aspiration or pneumonia. These were treated as described in Part 2.II.2 and examined for protein content by electrophoresis. Exact measurements of the total protein content were not feasible as the collections were not complete/24 hrs.

The infants with IRDS had approximately twice as much protein in their urine (w/v) as compared with the control group during the first 3 days of life: this was mainly serum albumin, only traces of γ-globulins being present.

After the third day there was no significant difference between the IRDS and control groups. In view of the method of collection, it was difficult to interpret these findings.

2. Meconium and faeces were collected from 10 infants with IRDS and from 15 healthy premature infants. All of the homogenates from both groups contained appreciable quantities of γ-globulins, average values being 50mg/100ml for IgG and 20mg/100ml for IgA. IgM was not present in detectable amounts. There was no significant difference between the two groups.

Faeces were collected antepartum from 4 mothers who gave birth to healthy premature infants and from 2 mothers of infants with IRDS. There was no detectable -globulin in any of the homogenates from these individuals.

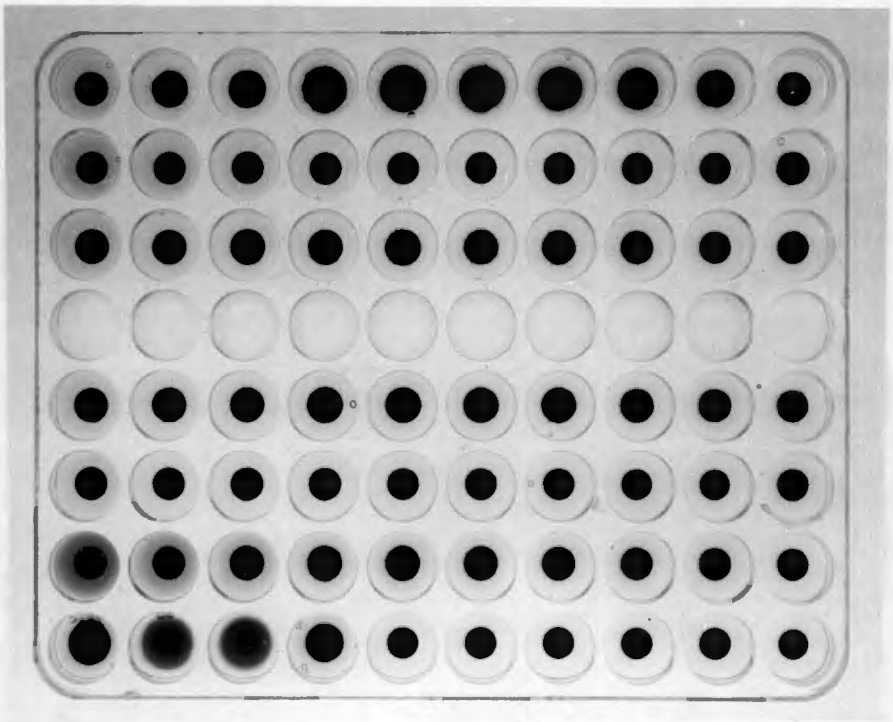
3. Oedema fluid was withdrawn by subcutaneous needle puncture from 5 of the most severely oedematous infants with IRDS. The mean total protein content was 2.1gm/100ml, of which more than 90% was albumin. γ-Globulins were not present in significant concentrations.

4. Amniotic fluid specimens

These were obtained by needle puncture at 33 - 36 weeks from 6 women who gave birth to normal infants and from two who gave birth to infants with IRDS. There was no significant difference between the two groups as regards the protein content. Mean values for total protein were 0.050gm/100ml, serum albumin 0.031gm/100ml, I_gG 0.008gm/100ml, IgA 0.002gm/100ml. No IgM was detected.

FIGURE 3.15 : HAEMAGGLUTINATION TITRATIONS.

1. INFANTS' SERA TITRATED AGAINST TANNED SHEEP ERYTHROCYTES COATED WITH MATERNAL IgG.



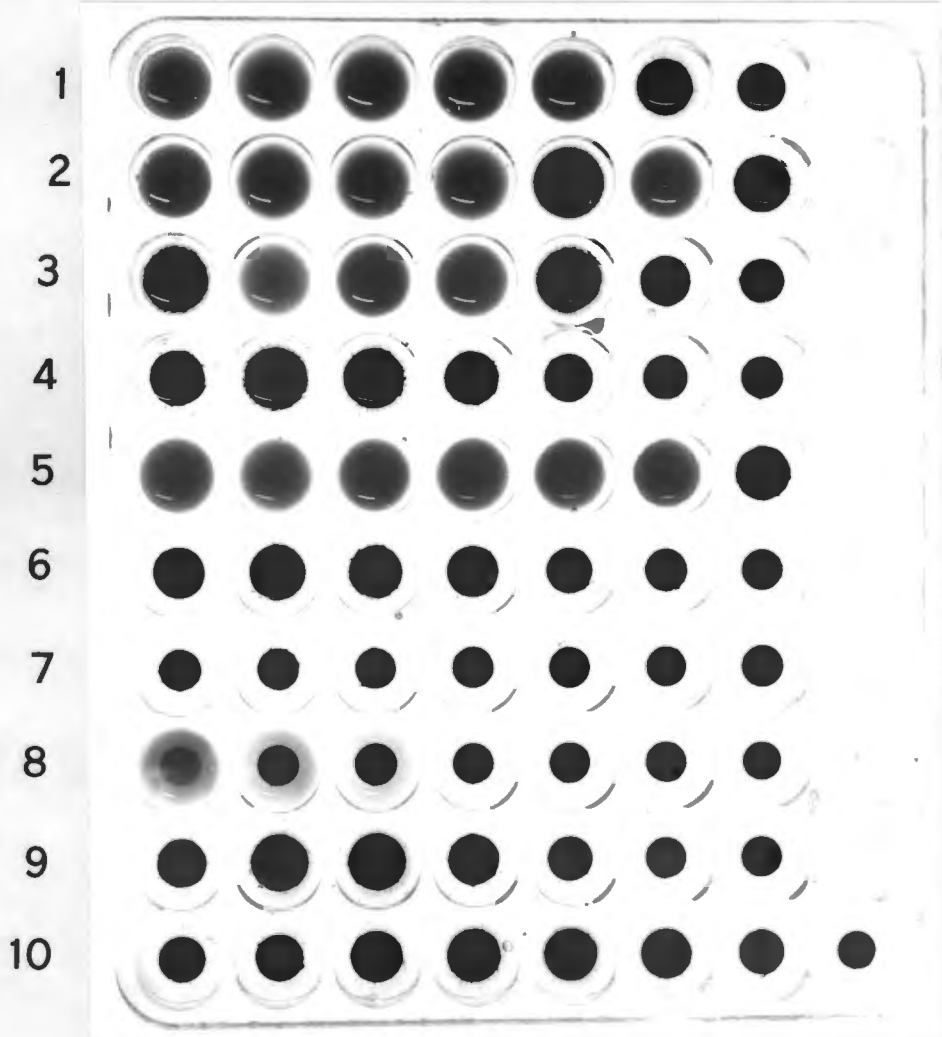
ROP ROW : SERUM OF IRDS INFANT.

BOTTOM ROW : CONTROL TITRATION WITH A COMMERCIAL ANTISERUM TO HUMAN IgG.

INTERMEDIATE ROWS : SERA OF HEALTHY INFANTS.

FIGURE 3.15 (CONTINUED) :

2. TITRATION OF INFANTS' SERA AGAINST IgG FRAGMENTS.



ROWS 1 - 3 : SERA OF HEALTHY INFANTS.

ROWS 4 - 6 : SERA OF IRDS INFANTS.

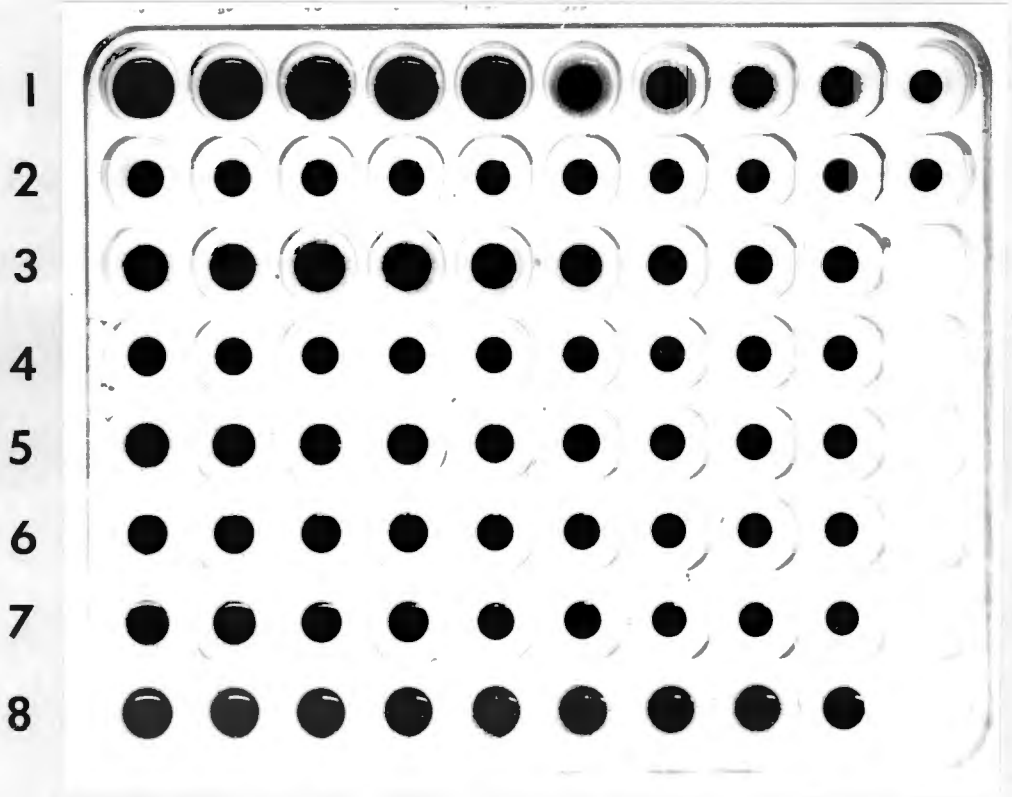
ROWS 7 , 8 : SERA OF HEALTHY INFANTS.

ROW 9 : SERUM OF IRDS INFANT.

ROW 10 : CONTROL TITRATION WITH A COMMERCIAL ANTISERUM TO BENCE JONES PROTEIN TYPE K.

FIGURE 3.15 (CONTINUED) :

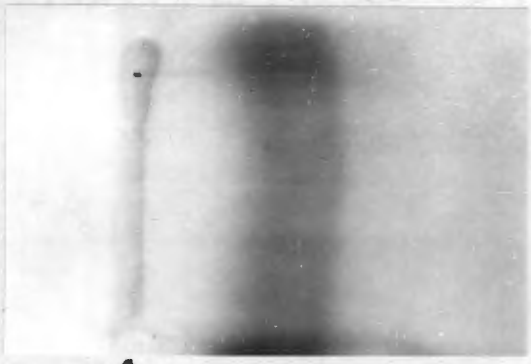
3. TITRATION OF MATERNAL SERA AGAINST TANNED SHEEP ERYTHROCYTES COATED WITH α-FETOPROTEIN + ALBUMIN.



ROWS 1 - 7 : TITRATIONS OF SERA FROM MOTHERS OF HEALTHY AND IRDS INFANTS.

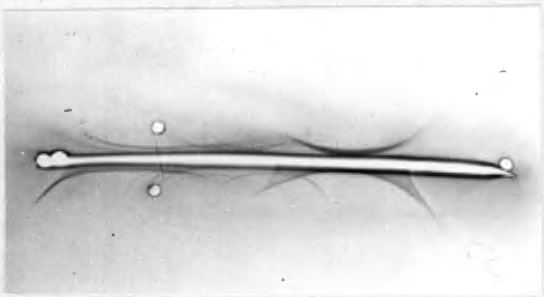
ROW 8 : CONTROL TITRATION WITH A MONOSPECIFIC ANTISERUM TO α-FETOPROTEIN.

FIGURE 3.16 : CELLULOSE ACETATE ELECTROPHORESIS OF AN EXTRACT OF FAECES FROM A PREMATURE INFANT TO DEMONSTRATE γ-GLOBULIN CONTENT.



ORIGIN IS AT THE ARROW.

FIGURE 3.17 : IMMUNOELECTROPHORETIC ANALYSIS OF AMNIOTIC FLUID PROTEINS.



UPPER WELL : AMNIOTIC FLUID CONCENTRATE.

LOWER WELL : ADULT HUMAN SERUM.

POLYVALENT ANTISERUM TO ADULT HUMAN SERUM IN SLOT.

3.V. Haemagglutination experiments

The technique of passive haemagglutination was employed to demonstrate agglutinins in the sera of infants and of their mothers, as it is capable of detecting 0.005 µg of antibody N/ml. 3-20 µg Antibody N/ml is required to produce a precipitin band in agar gel.

1. Titration of infants' sera against cells coated with maternal IgG

Sheep red blood cells were formalinized, tanned as described in Part 2.V.11 and coated with IgG prepared from each of 70 maternal sera, using the methods described in Part 2.IV.3. The sera were collected from mothers of 40 healthy premature infants, from the mothers of 13 patients with IRDS confirmed by chest X-ray and from the mothers of 17 babies diagnosed as having respiratory distress on clinical grounds, but without radiological confirmation: chest X-ray in these cases was precluded by the severity of the illness, or lack of facilities. This group included 8 very immature infants (gestational age less than 32 weeks), some of whom may not have been true cases of IRDS. These are labelled IRDS? in Table 3.10. Table 3.9 gives clinical and blood-gas data on ten of the infants with confirmed IRDS. Similar data were not available for the other 3 in this group, but the diagnosis was confirmed at autopsy. Blood was taken from the umbilical cord of all infants at birth

TABLE 3.9

Clinical, biochemical and autopsy findings on infants with confirmed IRDS

Infant	Sex	Weight (kg)	Gestational age (weeks)	Lowest arterial pO_2 of infant on 100% oxygen **(mm.Hg)	Lowest arterial blood pH *	Arterial pCO_2 (mm.Hg) on admission	Base Excess (meq/l) on admission	Outcome	Other relevant data
Hof	male	2.67	33	34	7.27	48	-6	died	Infant of diabetic mother, neonatal jaundice, hyaline membranes at autopsy. Required IPPR x
Fa	male	2.35	36	120	7.31	61	+1	survived	Neonatal jaundice: no cause found. No IPPR.
Bu	male	1.59	32	46	7.20	72	-5	died	Cerebral haemorrhage and hyaline membranes found at autopsy.Required IPPR.
Da	male	2.50	36	45	7.11	59	-12	survived	Required IPPR.
Pr	female	1.88	36	71	7.29	66	+5	survived	Responded well to treatment. No IPPR.
Po	male	3.90	38	290	7.37	53	+2	survived	Pneumothorax as well as underlying IRDS No IPPR.
Ho	female	2.40	36	60	7.28	49	-5	survived	Glucose-6-phosphate dehydrogenase deficiency, jaundice. No IPPR.
dk	male	2.34	32	54	7.14	68	-9	died	Hyaline membranes at autopsy. Required IPPR.
vA	male	2.50	35	42	7.10	75	-7	died	Hyaline membranes and pulmonary haemorrhage at autopsy. Required IPPR.
Bo	male	2.58	34	110	7.28	56	-1	survived	Responded well to treatment. No IPPR.

x IPPR - treatment with intermittent positive pressure respirator.

* Blood pH, arterial pCO_2 and base excess were measured after intravenous therapy with 2.0 m.eq. $NaHCO_3$/kg body weight had commenced.

** Blood samples for these data were obtained through an indwelling catheter, passed via umbilical artery into the abdominal aorta. Lowest arterial pO_2 of infants given 100% O_2 occurred at variable times after birth, usually at about 36 hours of age.

and follow-up specimens were obtained from a peripheral vein at the age of a few weeks from 4 cases of IRDS and 2 healthy premature infants.

Serial doubling dilutions of the infants' sera were titrated against cells coated with the infants' own mothers' IgG. (See Part 2.V.11). The mean anti-IgG titre was calculated for each group.

It was found that (i) All the infants with IRDS had agglutinins against their maternal IgG, with titres ranging from 1:32 to 1:5120.

(ii) Of the 17 doubtful cases, 7 had agglutinins in the serum, the titres ranging from 1:20 to 1:512.

(iii) Amongst the 40 healthy infants there were 9 that had agglutinins against their own maternal IgG, the titres ranging from 1:10 to 1:160.

No agglutinins could be demonstrated in the sera of the remainder.

The results are summarized in Table 3.10.

As can be seen, the mean anti-IgG titre was markedly increased in the IRDS group as compared with normal controls. The IRDS? group had an intermediate mean titre. Other serum-protein findings are in agreement with earlier results. (Part 3.I).

TABLE 3.10

Mean gestational age, protein concentrations and mean anti-IgG titres (± standard error) in the three groups of infants previously described

Group	No.of cases	Mean gestational age (weeks)	Total serum-protein concentration (gm/100ml)	IgG (gm/100ml)	IgM (mg/100ml)	Mean anti-IgG titre (dilution)
Normal prematures	40	34.1 ± 0.4	4.70 ± 0.02	1.04 ± .06	11 ± 1.1*	1:20
IRDS?	17	32.1 ± 0.6	4.10 ± 0.02	0.64 ± .08	8 ± 1.4	1:82
Confirmed IRDS	13	34.4 ± 1.1	3.60 ± 0.35	0.56 ± .06	8 ± 0.4	1:550

* The normal premature group included one infant who had, atypically, an IgM concentration of 90mg/100ml. This inflated the mean and standard error for this group.

In an attempt to assess the specificity of the reaction, 10 infants' sera containing agglutinins against their own mothers' IgG were titrated against cells coated with IgG prepared from other maternal sera.

The results are recorded in Table 3.11.

TABLE 3.11

Titrations of the sera of infants who possessed agglutinins against their own mothers' IgG, with erythrocytes sensitized with IgG prepared from other maternal sera

Infant No.	No.of IgG-coated preparations titrated	No. of positive reactions	No. of negative reactions
1	11	8	3
2	11	0	11
3	19	4	15
4	18	6	12
5	10	6	4
6	10	0	10
7	10	0	10
8	10	7	3
9	8	3	5
10	9	9	0
Mean	11.6	4.3	7.3
%	100	37	63

3 Of the infants' sera did not agglutinate any of the maternal IgG-coated preparations, one serum agglutinated all of them and the remaining 6 reacted with some but not all. On average there were more negative than positive reactions.

To 10 different agglutinating sera was added a monospecific antiserum against IgM and the titration procedure was repeated with each serum, after centrifuging and discarding any precipitate that formed. In each instance. the agglutinating activity of the serum was abolished or much diminished.

The agglutinating activity of these sera was abolished by incubation with 0.1M 2-mercapto-ethanol for 2 hrs at 37°C. (See Part 2.VI.2).

From these observations it was concluded that the agglutinin was in the infant's rather than the maternal serum and was probably of the IgM type.

The results obtained on follow-up specimens are set out in Table 3.12.

As can be seen, the titre had risen markedly in 3 out of the 4 cases of IRDS. The fourth infant died at 2 days of age.

Healthy infants had no agglutinins in the follow-up specimens.

TABLE 3.12

Anti-IgG titres in follow-up specimens as compared with titres in cord serum

Group	Infant No.	Titre of Anti-IgG in cord serum	Age at which follow-up specimen obtained	Anti-IgG titre of follow-up specimen
IRDS	1	1:64	14 days	1:640
	2	1:32	15 days	1:256
	3	1:64	40 days	1:1024
	4	1:80	2 days	1:80
Normal	1	0	14 days	0
	2	0	30 days	0

2. Titration of infants' sera against fragments of the IgG molecule

Sera of 7 infants with IRDS and of 12 healthy infants, were titrated against formalinized sheep cells coated with Bence Jones proteins of types K or L, prepared as described in Part 2.IV.6, or with a papain digest of pooled maternal IgG, made as in Part 2.VI.1.

The tanning and coating procedure was the same as described for intact IgG. The results of these titrations are in Tables 3.13 and 3.14.

As can be seen, the IRDS group had a higher mean titre of anti-Bence Jones protein, type L than the normal group. However, the scatter observed was too large for the difference to be statistically significant. The incidence of anti-Bence Jones type L was significantly higher in IRDS. The differences in mean titres against Bence Jones protein, type K and the papain digest of IgG were also not significant.

The results indicate that the two groups differ markedly in their reactions to the intact IgG molecule, rather than to fragments of the molecule.

3. Investigation of maternal sera for anti-α-fetoprotein antibodies

Experiments on agar gel (Part 3.II.2) were followed by haemagglutination experiments, as the latter technique

TABLE 3.13

Agglutinin titres of infants' sera against cells coated with a papain digest of IgG

		Dilution of infants' sera							
Normal infants	Infant No.	1:10	1:20	1:40	1:80	1:160	1:320	1:640	Titre
	1	-	-	-	-	-	-	-	0
	2	+++	+++	+++	+++	++	+	-	1:160
	3	±	±	-	-	-	-	-	1:20
	4	++	++	+	±	-	-	-	1:80
	5	-	-	-	-	-	-	-	0
	6	-	±	±	±	-	-	-	1:80
	7	++	+++	+++	+++	+++	+	-	1:320
	8	+++	+++	+++	+++	+++	+++	+	1:640
	9	++	+++	+++	+++	+++	+++	+	1:640
	10	+	++	++	±	-	-	-	1:80
	11	+++	+++	+++	+++	+++	++	±	1:640
	12	±	+	+	±	-	-	-	1:80
								Mean	1:228
Infants with IRDS	1	+++	+++	+++	+++	+++	++	+	1:640
	2	+++	+++	+++	+++	++	±	-	1:320
	3	-	-	-	-	-	-	-	0
	4	±	-	-	-	-	-	-	1:10
	5	±	±	+	±	-	-	-	1:80
	6	-	-	-	-	-	-	-	0
	7	-	-	-	-	-	-	-	0
								Mean	1:150
Control: Commercial antiserum		-	-	±	+	+	±	-	

TABLE 3.14

Agglutinating activity of infants' sera against maternal IgG as compared with activity against Bence Jones proteins of types K and L

Normal infants							Infants with IRDS						
Infant No.	vs Maternal IgG		vs type K		vs type L		Infant No	vs Maternal IgG		vs type K		vs type L	
	Max. intensity observed	titre	Max. intensity observed	titre	Max. intensity observed	titre		Max. intensity observed	titre	Max. intensity observed	titre	Max. intensity observed	titre
1	-	0	-	0	-	0	1	++	1:320	-	0	-	0
2	-	0	-	0	±	1:40	2	+	1:160	-	0	+	1:64
3	+++	1:160	++	1:320	++	1:320	3	±	1:1024	-	0	±	1:20
4	-	0	+	1:16	-	0	4	±	1:64	-	0	+	1:10
5	-	0	±	1:40	-	0	5	+	1:32	++	1:160	+++	1:160
6	-	0	-	0	±	1:40	6	+++	1:80	+++	1:20	+++	1:80
7	-	0	-	0	-	0	7	+++	1:80	+++	1:20	+++	1:80
8	-	0	-	0	-	0							
9	-	0	+	1:40	+	1:40							
10	-	0	-	0	-	0							
11	+++	1:128	+++	1:80	+++	1:80							
12	-	0	+	1:80	+	1:80							
Mean titres		1:24		1:48		1:50			1:394		1:29		1:141

is more sensitive (181). Formalinized, tanned sheep erythrocytes were coated with:

(i) serum albumin + ∝-fetoprotein, prepared as in Part 2.IV.4.(ii);

(ii) Serum albumin prepared from adult male serum by the same method (HSA);

(iii) Bovine serum albumin (BSA);

(iv) Goose serum albumin (GSA);

(v) Amandin.

These preparations were titrated, as described previously, against sera of 20 mothers of healthy infants, of 5 mothers of infants with IRDS, a monospecific antiserum to ∝-fetoprotein and a monospecific antiserum to human serum albumin.

Controls were set up as follows:

(i) Titrations duplicating the above, but with prior addition of 0.2mg ∝-fetoprotein + albumin to each well.

(ii) Similar titrations, with prior addition of 0.2mg HSA to each well.

(iii) Similar titrations, with prior addition of BSA, GSA or amandin to each well.

It was found that:

(a) All the mothers of IRDS infants had agglutinins

against formalinized sheep erythrocytes, coated with α-fetoprotein + albumin, titres ranging from 1:20 to 1:2560;

(b) Of the 20 mothers of healthy infants 14 had agglutinins against cells sensitized with α-fetoprotein + albumin, titres ranging from 1:20 to 1:1180. The other 6 mothers had no agglutinins.

(c) The mean agglutinin titre was higher in the IRDS group. However, the number of cases studied to date is not sufficient to draw statistically valid conclusions.

(d) All sera which agglutinated cells coated with α-fetoprotein also agglutinated cells coated with HSA, but to a lower titre.

(e) α-Fetoprotein + albumin and HSA were equally effective in inhibiting agglutination, if added to the sera prior to the addition of sensitized cells.

(f) Agglutinating sera reacted to a far lower titre with cells sensitized with BSA and GSA. These proteins were correspondingly less effective in inhibiting agglutination.

(g) BSA and GSA were shown to share certain antigenic determinants with HSA (Fig 3.18).

(h) None of the sera tested agglutinated cells coated with amandin and amandin was ineffective in inhibiting

agglutination.

(i) Monospecific antisera to ∝-fetoprotein (RAFT) and to HSA agglutinated cells coated with ∝-fetoprotein + albumin and with HSA to the same titre. The mean titre observed with these antisera was higher than that observed with maternal sera.

(j) The above antisera did not agglutinate cells coated with amandin and gave minimal agglutination with cells coated with BSA and GSA.

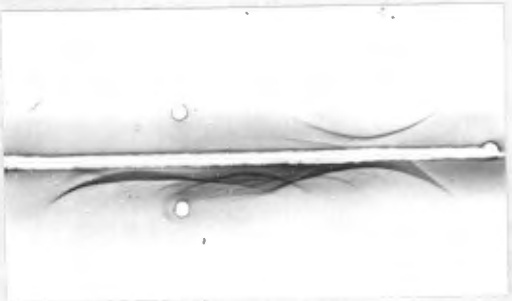
Further investigations of these findings are in progress. Preliminary observations are summarized in Table 3.15.

TABLE 3.15

Mean agglutinin titres in maternal sera

Group	No. in group	Mean gestational age of infants	Titres (mean)				
			vs ∝-feto protein + albumin	vs HSA	vs BSA	vs GSA	vs Amandin
Normal mothers	20	34.1	1:394	1:133	1:18	1:8	0
IRDS mothers	5	34.5	1:670	1:180	1:20	1:10	0
Control (RAFT)	5	--	1:1010	1:1010	1:40	1:20	0
Control (Anti-HSA)	5	--	1:1280	1:1280	1:40	1:20	0

FIGURE 3.18 : DEMONSTRATION, BY MEANS OF IMMUNOELECTROPHORESIS, THAT HUMAN SERUM ALBUMIN AND BOVINE SERUM ALBUMIN HAVE RELATED ANTIGENIC GROUPINGS.

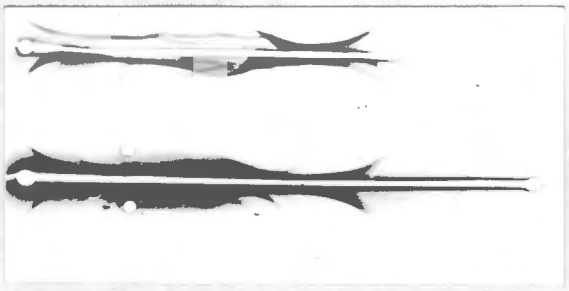


UPPER WELL : MIXTURE OF HUMAN SERUM ALBUMIN AND BOVINE SERUM ALBUMIN.

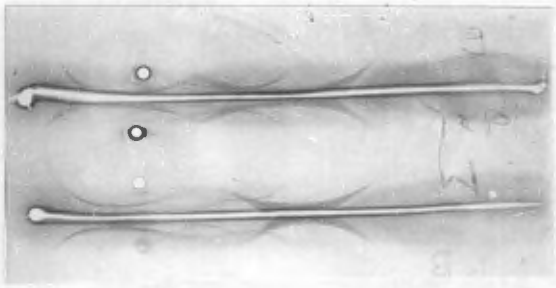
LOWER WELL : ADULT MALE SERUM.

POLYVALENT ANTISERUM TO ADULT HUMAN SERUM IN SLOT.

FIGURE 3.19 : IMMUNOELECTROPHORETIC PATTERN OF SERA OF IRDS INFANTS AND THEIR MOTHERS.

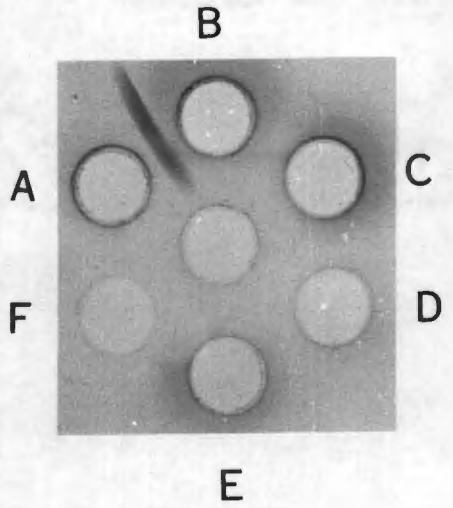


MOTHERS



INFANTS

FIGURE 3.20 : DEMONSTRATION BY MEANS OF AN OUCHTERLONY PLATE OF A PRECIPITIN REACTION BETWEEN HEPATOMA SERUM AND A PLACENTAL EXTRACT.



A. PLACENTAL EXTRACT , B. HEPATOMA SERUM ,
C. ADULT MALE SERUM , D, E. SERA OF A FOETUS AND A HEALTHY PREMATURE INFANT , F. HUMAN GROWTH HORMONE.
ANTISERUM TO HUMAN GROWTH HORMONE IN CENTRE WELL.

3.VI. Plasma cortisol estimations in maternal sera

Plasma cortisol was measured at intervals throughout pregnancy in 4 mothers who delivered infants with IRDS and in 5 mothers of healthy infants.

The results are in Table 3.16.

TABLE 3.16

Plasma cortisol concentrations during pregnancy and post-partum in mothers of IRDS and healthy infants

Duration of pregnancy (weeks)	0-20	21-28	29-31	32-34	35-37	38-40	Post-partum
Plasma cortisol in IRDS mothers (g/100ml)	43.2	45.9	51.6	44.9	59.5	68.5*	21.0
Plasma cortisol in normal mothers (g/100ml)	27.8	33.0	33.7	33.7	34.5	39.1	17.0

* Mothers of IRDS infants generally deliver before 37 weeks. This figure is based on 2 observations. The period of gestation may have been miscalculated.

As can be seen, mothers of IRDS infants had raised plasma cortisol concentrations, when compared with mothers of healthy infants. The concentration was raised from the earliest stage at which they came under observation and remained so throughout pregnancy. The differences between the groups were significant at the 5% level.

6 Weeks or more post-partum, there was no significant difference between the groups.

3.VII. Serum electrolyte and blood urea concentrations

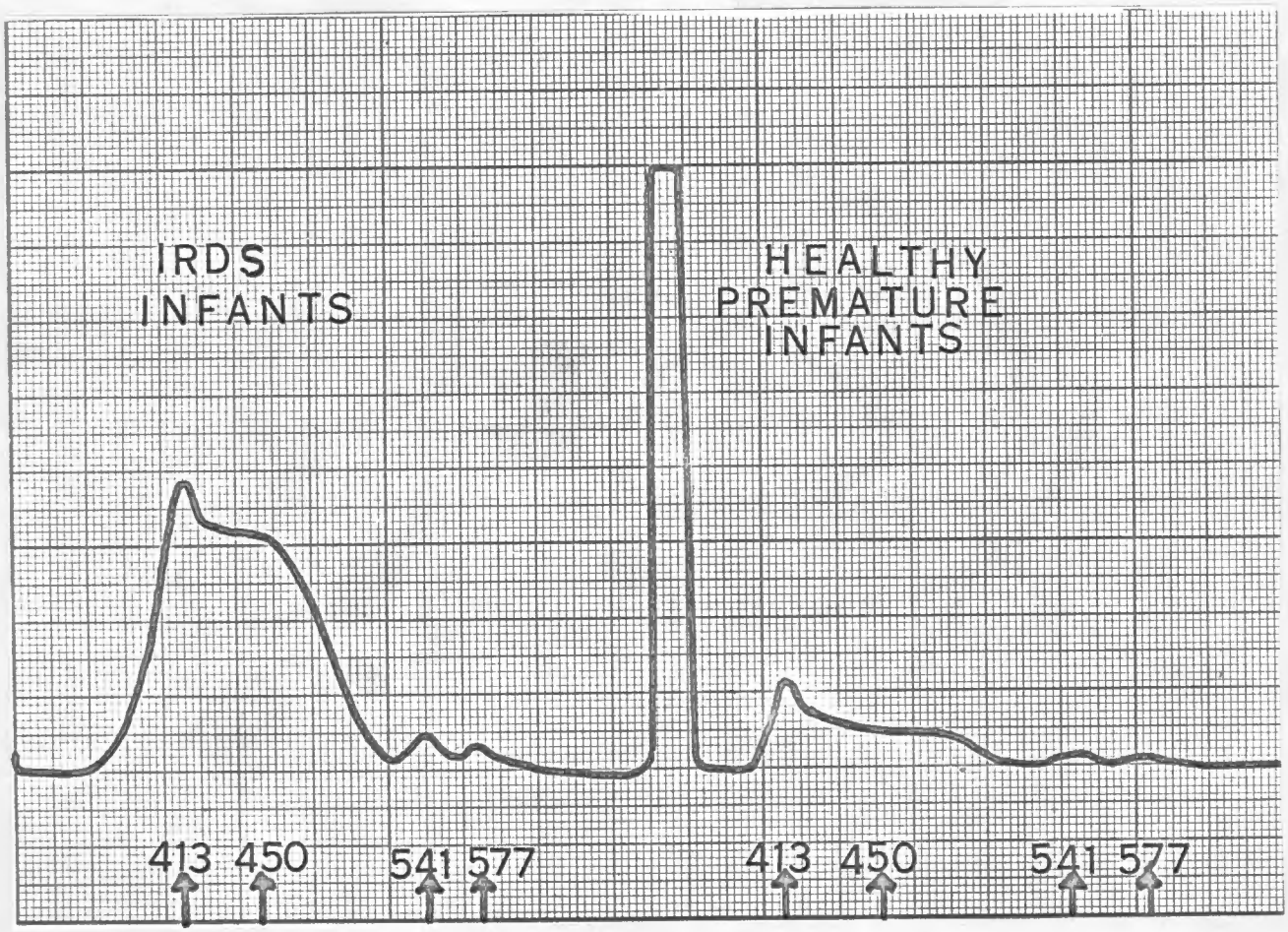
These were determined in the sera of a small number of affected infants and their mothers during pregnancy and at delivery. Blood urea concentrations were within normal limits in all instances.

The serum electrolyte pattern in the mothers was normal. In the infants, it varied, depending on what treatment the infant had received. Untreated cases had raised serum potassium and phosphate levels and a metabolic acidosis, as observed by other workers (2,3,4). The serum electrolyte pattern returned to normal on treatment with intravenous glucose and sodium bicarbonate.

3.VIII. Spectrophotometry of sera

Sera of 5 IRDS infants were scanned, using a Beckman DU recording spectrophotometer. The main absorption peaks corresponded to oxyhaemaglobin (i.e. λ max = 413,541, 577),(197) and bilirubin (i.e. λ max = 450), (198).

FIGURE 3.21: SPECTROPHOTOMETRY OF SERA OF AN IRDS INFANT AND A HEALTHY PREMATURE INFANT. ABSORPTION MAXIMA ARE MARKED BELOW.



3.IX. Examination of hyaline membranes

These were prepared for analysis as in Part 2.II.6. The lipid of the hyaline membranes was found to be mainly cholesterol. Amino-acid analysis of the centrifuged deposit did not reveal a striking preponderance of any one amino acid.

PART 4

DISCUSSION

Infants suffering from IRDS appear to be in a state of general prostration which is more severe than is warranted by their respiratory difficulty alone. (2,3). A generalized capillary lesion is present (22) and this cannot be attributed only to anoxia, as it is frequently found, at birth, in IRDS infants who have suffered no obvious anoxic episode in utero.

IRDS is probably a disease of multiple aetiology and it is well-documented that predisposing causes include perinatal asphyxia, antepartum haemorrhage, multiple pregnancy, maternal diabetes, Rh-isoimmunization and possibly other, as yet unidentified, factors.

The only known method of prevention is by preventing premature birth. It is also accepted by many authorities (182, 183) that the disease process has its origins in utero.

The experiments outlined in earlier sections were performed in an attempt to unearth biochemical and/or immunological abnormalities underlying this condition, both in affected infants and in their mothers.

An intravascular immune reaction in affected infants, accompanied by the release of substances such as histamine, heparin, and 5-hydroxy-tryptamine, would account, at least partially, for the pulmonary pathology and the capillary lesion.

Initially, a study of the serum-proteins was made, as it had been reported (32,33,34) that total serum-protein concentrations are abnormally low in IRDS. The first outcome was the finding of an extremely low total protein concentration, accompanied by a marked absolute and relative decrease in γ-globulins in IRDS infants, when compared with healthy infants of the same gestational age. (35,36). Although the deficiency of total protein had been observed previously by other workers (31,32,33,34), the relative decrease in γ-globulins had not been reported before. This finding was later corroborated by Greenberg and Smith. (183).

A less marked, but significant deficiency of serum γ-globulins with a normal total protein concentration, was later observed to be present in mothers of IRDS infants. (184). Experiments with immunoplates and gel filtration (Part 3.I.3), demonstrated that the deficiency was one of IgG only, and of IgG and IgM in their mothers. The IgG was of normal molecular weight. In the case of the mothers, the deficiency of IgG and IgM was present from the earliest stage at which they came under observation at the antenatal clinic (generally 16 - 20 weeks after the last menstrual period) and persisted until delivery. The other maternal serum-protein-fractions followed the normal pattern of change during pregnancy, i.e. a slight fall in total serum-protein concen-

tration, reaching lowest concentrations at about 34 weeks, and a relative increase in the concentrations of α- and β-globulins. (169,180,184,185). (See Tables 3.6, 3.7).

IRDS mothers were also found to have raised plasma cortisol levels throughout pregnancy (Table 3.16). By six weeks post-partum, the maternal IgG and plasma cortisol concentrations had returned to normal levels, but the IgM levels remained low. IgA levels were normal throughout pregnancy and post-partum. The explanation for the low levels of maternal IgM is, as yet, uncertain.

Oedema fluid, amniotic fluid, 24-hourly specimens of urine, meconium and faeces were examined for γ-globulin content, in affected mother-and-child pairs. The oedema fluid had a high protein content (1-2Gm/100ml), but more than 90% of this was serum albumin. Only the meconium and faeces of the infants investigated contained significant quantities of -globulins. However, the concentrations of γ-globulin in meconium from normal and affected infants were similar. This γ-globulin was not antigenically identical with serum IgG and was probably the "coproantibody" of Lipton and Steigman (63,64) and Roulet and von Muralt. (194).

The experimental findings supported the suggestion that leakage of serum albumin into the oedema fluid might account for the low concentration of total protein. It was not

possible to check this quantitatively, as turnover studies with radioactive tracers were not permissible in these infants. The disproportionately low IgG levels, however, could not be explained on the basis of leakage into the interstitial fluid, urine, amniotic fluid, or the gut. Further, the IgG concentration was observed to fall more rapidly than normal during the first 48 hours of postnatal life and then to return to levels similar to those found in cord blood by the 10th day. (Tables 3.2 and 3.3).

In view of these data and reports (65,66,67,68,69,71, 72,74,160) of sensitization of certain individuals against foreign IgG allotypes, with production of anti-IgG agglutinins, it was decided to investigate the possibility of the occurrence of an immune reaction in IRDS to account for the low IgG concentrations.

Instances of immunization of the mother by foetal antigens of paternal origin during pregnancy have been under investigation for many years (167,168,192), but only a few examples (e.g. Rh-isoimmunization and immunization with genetically foreign plasma proteins have been well-documented. (65,66,67,68,69,71,72,73,74,160,191)).

The observation was made earlier (Part 3.I(b)) that IRDS infants, unlike healthy premature infants, had, in serum specimens obtained between the ages of 3 weeks and

2 months, concentrations of IgG which were not significantly different from those observed in the cord blood. This suggested that these infants were either catabolizing IgG more slowly than normal, or else synthesizing larger amounts of IgG than normal infants of the same age. The question might be settled by determining the Gm phenotypes of affected infants and their mothers. This could not be done in the present study, as typing reagents were not available. Increased IgG synthesis by the infant could be a response to antigenic stimulation by maternal IgG of a foreign allotype.

Initial experiments were concerned with attempts at demonstrating a precipitin reaction, in solution or in agarose gel, between maternal IgG and the infant's serum. These trials were not consistently successful. Hence, experiments utilising passive haemagglutination were attempted. These demonstrated a striking difference between IRDS and healthy infants: all the IRDS infants had anti-IgG agglutinins directed against their maternal IgG, whereas these were present in only a minority of healthy premature infants. The mean anti-IgG titre observed in the IRDS group was twenty-seven times that of the mean of the normal group. Furthermore, the agglutinins reacted with only certain maternal IgG preparations. In a series of ten sera tested for cross-reactions with other maternal IgG preparations, there were 63% negative reactions and 37% positive. 3 Of

these sera agglutinated only cells coated with their own maternal IgG. (See Table 3.11).

It was also observed that, in follow-up specimens obtained at between 2 and 40 days after birth, the mean anti-IgG titre had increased considerably. (Table 3.12).

Similar agglutinins have been demonstrated in a minority of healthy, full-term infants and in children and adults, by other workers. (66,68,74). These cases had received no blood transfusions, or infusions of plasma. It has been shown that, in such persons, the agglutinins are directed at an antigenic determinant foreign to that individual and present on the IgG of his or her mother. It is hoped to include similar investigations in future studies.

The results were consistent with the idea that an intravascular immune reaction, involving the maternal IgG as the antigen, plays a part in the aetiology of IRDS and may account for the observed deficiency of IgG and also for the generalised capillary lesion (22) and the appearance of general physical prostration exhibited by these infants.(27). Such a reaction would remove maternal IgG from the foetal circulation,as it crosses the placenta and also possibly accelerate the passage from mother to foetus, by increasing the concentration gradient across the placenta. Hence, IgG concentrations on both sides of the placenta might be

expected to fall.

Abolition of agglutinating activity by addition to the infant's serum of an antiserum to IgM, or by incubation of the serum with 2-mercapto-ethanol, revealed that the agglutinins were in the infant's serum and of the IgM type. Observations on similar agglutinins by other investigators (65,66,67,68,72) have confirmed that anti-IgG agglutinins are generally of the IgM type and persist as such for years, in the absence of any further antigenic stimulation.

It has also been reported that cord sera of a proportion of normal infants contain macroglobulin agglutinins against Bence Jones proteins (71) and that human sera contain agglutinins against papain- and pepsin-digested IgG, the incidence and titre of these rising with age. (69,70, 72).

Sera of normal and IRDS infants were titrated against formalinized sheep erythrocytes, coated with Bence Jones proteins of types K or L, or with papain-digested IgG. Approximately 50% of all sera tested contained agglutinins to these preparations. There was no obvious difference between the two groups of infants as regards the reaction against the papain digest and Bence Jones protein type K. IRDS infants had a significantly raised incidence of agglutinins against Bence Jones proteins type L, but the

mean titre, although higher in IRDS than in controls, was not significantly so, as there was a wide scatter in the observations. (See Tables 3.13 and 3.14).

The possible importance of this is discussed later.

The presumed function of agglutinins against IgG fragments is to remove from the circulation the end-products of IgG catabolism (69,70,72).

A further observation was that IRDS infants had a consistently raised concentration of α-fetoprotein in the cord serum (36). In cases of twins where only one had IRDS, it was usual to find a raised α-fetoprotein concentration only in the affected twin. The concentration of this protein was seen to fall during the first week of post-natal life and, in non-fatal cases, there was none detectable by 10 days of age. In the case of one 8-month-old infant who had suffered from severe IRDS, α-fetoprotein reappeared in the serum, following a slow intravenous infusion of pooled IgG. This was accompanied by a recurrence of his respiratory distress and oedema, presumably due to a recrudescence of the capillary lesion. In addition, his serum IgG concentration fell instead of rising. This child appeared to be suffering from an allergic reaction to the IgG.

The characteristic serum-protein pattern of IRDS is:

Total serum-protein 2.5 - 3.5 gm/100ml

IgG 0.1 - 0.5 gm/100ml

and a raised concentration of α-fetoprotein.

This pattern was also seen in infants suffering from Rh-isoimmunization. The striking similarity in the serum-proteins in these two conditions, lends further support to the evidence for the presence of an immune process in the aetiology of IRDS. In 90% of cases, it is possible to make the diagnosis of IRDS on inspection of a cellulose-acetate electrophoretogram of the serum. The changes cannot be attributed to prematurity alone, despite the fact that the serum-protein pattern resembles that of extremely immature infants. The control group of healthy premature infants was of the same mean gestational age as the IRDS group, but did not exhibit this abnormal serum-protein pattern. (35,36).

Spectrophotometry of the sera of affected infants revealed main absorption peaks corresponding to oxyhaemoglobin and bilirubin (201,202). This, together with the observation (196) that the levels of unconjugated bilirubin are raised in sera of IRDS infants, indicates that there is increased intravascular haemolysis, which would account for the low haematocrit. This phenomenon may be linked with an immune reaction, or it may be secondary to anoxia.

Sera from mothers of both normal and IRDS infants were often found to contain appreciable quantities of α-fetoprotein, especially during the second trimester of pregnancy. This observation is contrary to those of other investigators (51,96,98).

It was further decided to examine sera of mothers of IRDS infants and of healthy premature infants to discover whether or not they had agglutinins against α-fetoprotein. This study was undertaken for the following reasons:

(a) 11% Of pregnant women have been observed to develop skin reactions after subcutaneous injection of foetal serum and/or amniotic fluid extracts (193).

(b) α-fetoprotein was often present in maternal serum in this study.

(c) It has been reported (185) that sera of women in the latter half of pregnancy are extremely effective in inhibiting the growth of HeLa cells in vitro. The inhibitory activity is associated with the presence in the maternal serum of an abnormal α_1-globulin, which disappears shortly after delivery. These investigators suggested that the function of the protein was to protect the mother from growth-promoting factors produced by the foetus and also possibly to curtail the rate of foetal growth, as this protein appears at a time when the foetal growth-rate is

decreasing.

As it was not possible, for technical reasons, to separate α-fetoprotein from serum albumin, a preparation containing both proteins was coated onto tanned sheep erythrocytes and a preparation of sheep erythrocytes coated with pure human serum albumin (HSA) was utilized as a control. When tested in this way, sera of all mothers of IRDS infants in the present series were found to contain agglutinins against α-fetoprotein + albumin, and this was also true of sera of 70% of mothers of healthy infants. (See Table 3.15).

Sera which agglutinated sheep erythrocytes coated with α-fetoprotein + albumin also agglutinated similar cells coated with pure HSA, but to a lower titre. Prior addition to the serum of α-fetoprotein + albumin or of HSA, were equally effective in inhibiting agglutination.

In view of this puzzling observation, the agglutinating sera were then titrated against sheep erythrocytes, coated with bovine serum albumin (BSA) and with goose serum albumin (GSA). These agglutinated less markedly and to a low titre (1:20 with BSA and GSA, as compared with 1:670 with α-fetoprotein + albumin and 1:180 with HSA in the case of IRDS mothers). BSA and GSA were correspondingly less effective in inhibiting agglutination when added to the serum prior to

titration. BSA and GSA share certain antigenic determinants with HSA. (See Fig 3.18). Hence some degree of cross-reaction could be expected. Sheep erythrocytes coated with amandin (a protein extracted from almonds), provided a more satisfactory control: maternal sera had no agglutinating activity when titrated against these cells and amandin was ineffective in inhibiting agglutination of cells coated with the various proteins already examined.

It was also remarked that the sera of infants born to mothers with agglutinins against α-fetoprotein + albumin, had no agglutinating activity against α-fetoprotein + albumin or against HSA. Nonetheless, these infants' sera agglutinated sheep erythrocytes coated with maternal IgG. While the agglutinating activity of the infants' sera was abolished by incubation of the serum with 2-mercapto-ethanol, as described before, this procedure had no effect on the activity of maternal sera.

It seemed reasonable to conclude that maternal agglutinins were directed specifically against α-fetoprotein + albumin, rather than towards some other antigenic grouping on the sheep erythrocyte surface and that this specific agglutinating activity was confined to the maternal serum or extracellular fluid.

The agglutinins were likely to be of the IgG rather than

the IgM type, as they were not destroyed by incubation with 2-mercapto-ethanol. A majority of mothers of healthy infants, as well as all mothers of IRDS infants, possessed such agglutinins.

Their significance remains uncertain: they may provide a mechanism for removal of α-fetoprotein from the maternal circulation. The function of α-fetoprotein has not yet been defined, but it has been suggested (102) that mammalian foetal proteins may function as growth factors. Anti-α-fetoprotein agglutinins would protect the mother against such growth-promoting propensities. If the agglutinins were to cross the placenta in significant quantities, an immune reaction would ensue. Transplacental passage of these is feasible, as they are presumably not of the IgM type. As noted previously, sera of women in the second half of pregnancy have marked growth-inhibiting activity on cells in tissue culture and this is associated with the presence of an abnormal serum α_1-globulin (185). The agglutinins described may be responsible for the growth-inhibiting properties of these sera. The abnormal α_1-globulin was thought to be an α_1-lipoprotein, on the basis of the immunoelectrophoretic pattern.

Infants with IRDS have been noted to have a consistently raised serum concentration of α-fetoprotein.

There would thus be an increased tendency for α-fetoprotein to cross the placenta into the maternal circulation in these cases, with resultant immunization of the mother. The chain of events could be as follows:

(i) Increased rate of synthesis, or diminished rate of catabolism of α-fetoprotein by the foetus;

(ii) Increased passage of α-fetoprotein across the placenta to the mother;

(iii) Antibody production against α-fetoprotein by the mother;

(iv) Passage of maternal anti-α-fetoprotein (IgG) via the placenta to the foetus;

(v) Immune reaction between α-fetoprotein and maternal antibodies in the foetal circulation;

(vi) Removal of the antibody-antigen complex by foetal agglutinins against maternal IgG.

An immune reaction of the type outlined above, could account for the clinical picture presented by IRDS infants. The raised plasma cortisol concentration present in their mothers during pregnancy, may be a response to the reaction. The excess cortisol is unlikely to be protein-bound, as the plasma-protein concentration in these mothers is not elevated (184).

The other observation of interest in this connection was that placental extracts formed precipitin lines in agarose gel when placed in wells adjacent to preparations containing α-fetoprotein, although similar reactions could not be demonstrated with maternal sera. It seems likely that anti-α-fetoprotein activity is concentrated in the placenta and may serve as a barrier against entry of excessive amounts of the protein into the maternal circulation. (See Fig 3.20).

The reason for the raised concentration of α-fetoprotein in IRDS infants has not been established. If this protein functions as a growth factor and is being removed by maternal antibodies, increased synthesis may be a compensatory mechanism in the foetus. (It has been reported (28) that affected infants are, on average, small for their gestational age).

The cross-reaction between anti-α-fetoprotein and HSA could be demonstrated only by means of a passive haemagglutination technique. No precipitin line formed in agarose gel, when the protein and antiserum were allowed to diffuse toward one another. Laron and Assa (116) made similar observations with regard to HSA and an antiserum to component 3 (C3) of Human Growth Hormone (HGH). HGH, prepared by the method of Raben (194), was separated into 3

components (C1,C2,C3) by preparative electrophoresis on starch gel and antisera were prepared against each of these components. All three antisera gave rise to precipitin lines, when tested in agar gel against a standard HGH preparation. None of the antisera elicited a precipitin line with HSA, in this medium. However, the antiserum to C3 cross-reacted with HSA 1:1000 (w/v), when coated onto tanned sheep erythrocytes, while the other antisera did not. C3 Migrates electrophoretically as an α_1-globulin; C1 and C2 are electrophoretically slower.

The immunochemical interrelationships between α-fetoprotein, HSA, IgG and HGH were investigated for the following reasons:

(a) Foetal serum had been reported to be superior to adult serum in promoting spreading of cells in tissue culture (102).

(b) The similarity observed in the cross-reactions of anti-α-fetoprotein and anti-HGH,C3, with HSA by the Passive Haemagglutination procedure.

(c) The similar electrophoretic mobilities of HGH,C3 and of α-fetoprotein.

(d) The fact that, in the second half of pregnancy, growth-inhibiting properties of the serum are coupled with

the presence of antibodies against α-fetoprotein.

(e) The observation, in IRDS infants, that a low serum IgG concentration was present together with a raised α-fetoprotein concentration.

A placental extract containing Human Placental Lactogen (HPL) was included in the study, as HPL is related antigenically to HGH, although it possesses little growth-promoting activity (117,118,119).

Also included were: a preparation of Human Chorionic Gonadotropin (HCG), as this is another hormone synthesized by the placenta, sera from pregnant and lactating women (as sources of HCG and Lactogenic Hormone (LH)) and sera of foetuses, premature infants (healthy and with IRDS) and cases of primary Carcinoma of the Liver (Hepatoma), as containing α-fetoprotein.

As is evident from Table 3.4, there were definite cross-reactions between preparations of HGH and antisera to α-fetoprotein, IgG and Bence Jones protein type L, but not between HGH and anti-Bence Jones protein type K, or antisera to IgA and to IgM. From this it may be concluded that HGH shares antigenic determinants with α-fetoprotein, with the IgG (γ) heavy chain and with type L light chains, but not with type K light chains, or with α or μ heavy chains.

The antiserum to HGH was too dilute to produce a visible precipitin arc in agar gel, when tested against HGH. (The antiserum supplied by the N.I.H. was diluted 1:2,000 v/v with 0.15M saline, as it was intended for use in a radio-immunoassay). However, it generated faint precipitin lines with IgG, α-fetoprotein + albumin and foetal serum, when examined by means of Ouchterlony plates. All of these preparations had previously been found to react with anti-α-fetoproteim. The anti-HGH gave no visible reaction with HSA.

It was also observed that, on immunoelectrophoresis the albumin arc in sera containing α-fetoprotein, although distinct from the α-fetoprotein arc, had an abnormally blurred appearance, similar to that seen in certain cases of bisalbuminaemia (201). This was suggestive, either of increased microheterogeneity (187,188,189,190,191,192) of the albumin molecules, or of complex-formation between serum albumin and other substances. (See Fig 4.1).

The results obtained in this study, viz. that α-fetoprotein, serum albumin, HGH, the γ-heavy chain and type L light chains are immunologically related, may be interpreted in various ways. The cross-reactions observed may be due to formation of complexes between serum albumin and smaller molecules: complex-formation between serum albumin and

FIGURE 4.1 : IMMUNOELECTROPHORESIS OF 16-WEEK FOETAL SERUM AND HUMAN GROWTH HORMONE AGAINST A POLYVALENT ANTISERUM TO ADULT HUMAN SERUM.

NOTE THE BLURRED APPEARANCE OF THE FOETAL SERUM ALBUMIN ARC, SUGGESTING MICROHETEROGENEITY OF THE PROTEIN MOLECULES.

immune globulins, immune globulin fragments, and between immune globulins and other proteins has been reported frequently (54,56,57,195). Such complexes generally have an electrophoretic mobility which is intermediate between that of the two (or more) substances involved and where immune globulins are concerned, complex-formation of this type has been put forward as a suggested explanation for the wide range of electrophoretic mobilities encountered in this group of proteins. It is conceivable that α-fetoprotein may be formed as a result of complexing of serum albumin with HGH or certain fragments of the IgG molecule.

However, when utilizing immunochemical systems such as those employed in the present study, traces of other protein contaminants, not readily detectable by the methods described before, may be present. It follows that preparations of protein fractions migrating electrophoretically in any particular region (e.g. α-fetoprotein, HGH, serum albumin complexes, etc, all of which migrate as α_1-globulins) may contain small amounts of other proteins with similar physicochemical properties. This may give rise to incorrect interpretation of the cross-reactions observed between the protein preparations and antisera studied. For this reason, any discussion concerning cross-reactions between these

proteins and antisera, is subject to this reservation. However, it seems unlikely that a preparation of HGH made from pooled adult human pituitaries would contain appreciable amounts of α-fetoprotein.

An observation of interest was that infants with IRDS had a raised incidence of agglutinins against Bence Jones protein type L, but not against type K. (See Table 3.14). Further, the antiserum to Bence Jones protein type L, but not the antiserum to Bence Jones protein type K, gave cross-reactions with HGH and with α-fetoprotein + albumin, but not with HSA.

This could be interpreted as meaning that in IRDS there is selective catabolism of a protein, or proteins, containing type L light chains, possibly preceded by interaction of these light chains with agglutinins. Catabolism of light chain fragments, in normal individuals, has been reported to take place mainly in the kidney (173). The relationship between this finding and the increased protein content in the urine of IRDS infants during the first three days of extra-uterine life, has not been investigated in the present study.

The preparation of α-fetoprotein + albumin was observed to cross-react with antisera to type L light chains, whereas the HSA preparation did not react in this way.

As all reasonable precautions had been taken to exclude contaminants from the preparations under investigation, a possible conclusion is that α-fetoprotein was undergoing abnormally rapid catabolism in the IRDS infants studied in this series.

The presumed mechanism for this, i.e. maternal antibodies against α-fetoprotein crossing the placenta and causing an immune reaction, with generalised systemic effects, including impaired growth in the affected infant, has already been discussed. The rapid disappearance of α-fetoprotein from the serum during the first week of postnatal life is consistent with a raised catabolic rate for the protein in these infants.

A raised serum concentration of α-fetoprotein, despite increased catabolism, would indicate that the rate of synthesis in utero is increased to a greater extent than the catabolic rate.

The cause of such increased synthesis, if present, remains to be elucidated.

Further investigations are in progress to determine the following:

(i) The incidence and mean titre of agglutinins against α-fetoprotein, in sera of a larger series of mothers of

premature and full-term infants.

(ii) The significance of such agglutinins, as regards foetal metabolism and development.

(iii) The biological function(s) of α-fetoprotein.

(iv) Further details concerning the structure of α-fetoprotein and its antigenic similarities to, and differences from, the other proteins listed in Table 3.4.

(v) The importance of the anti-α-fetoprotein activity observed in placental extracts.

(vi) Reasons for the presence of α-fetoprotein in sera only of foetuses, neonates and cases of primary carcinoma of the liver.

(vii) The incidence and mean titre of agglutinins against maternal IgG in full-term infants. (Although this has been studied by other investigators, it is of interest to compare findings on premature and full-term infants in the system employed here).

(viii) Similarities to and differences between the IgG genotypes and phenotypes of IRDS infants and of their mothers.

(ix) Physicochemical properties of the agglutinins in sera of infants and of their mothers.

PART 5

SUMMARY AND CONCLUSIONS

(1) A characteristic serum-protein pattern, from which the diagnosis can be made in 90% of cases, has been established for IRDS infants.

The main features are:

(a) A low concentration of total serum-protein (2.5 - 4.5 gm/100ml; normal 5.5 gm/100ml).

(b) A concentration of IgG which is low out of proportion to the low total serum-protein concentration (0.1 - 0.5 gm/100ml), i.e. about 10% of the total; normal 1.0 gm/100ml, i.e. about 15% of the total).

(c) A raised concentration of α-fetoprotein as compared with healthy premature infants matched for gestational age.

Infants suffering from Rh-isoimmunization exhibited a similar serum-protein pattern.

(2) Mothers of IRDS infants had significantly decreased concentrations of serum IgG and IgM throughout pregnancy, as compared with mothers of healthy premature infants. IgG levels, in mothers of affected infants, returned to normal within six weeks after delivery, but the IgM concentration remained low. The IgA levels remained normal during pregnancy and

post-partum in mothers of IRDS infants and controls.

(3) Infants with IRDS did not lose appreciable amounts of IgG from the circulation in secretions into the lung or gut, or in urine or oedema fluid.

(4) Affected infants had agglutinating antibodies, probably of the IgM type, directed against the intact maternal IgG molecule. Similar agglutinins were present in a minority of healthy premature infants. At present, similar data are not available for full-term infants.

(5) The titre of anti-maternal-IgG agglutinins showed a significant increase during the first three months of post-natal life in all the infants studied.

(6) The titre of agglutinins against fragments of the IgG molecule was found to be similar in IRDS infants and in healthy controls, but the incidence of antibodies against Bence Jones protein type L was raised in IRDS.

(7) Pregnant women were frequently observed to have appreciable quantities of α-fetoprotein in their serum, especially during the second trimester.

(8) The majority of pregnant women studied were found to have agglutinating antibodies against α-feto-

protein. The titre of these was raised in IRDS mothers. Using the passive haemagglutination technique, it was found that the agglutinins in maternal serum cross-reacted with serum albumin, prepared from adult male serum and to a lesser extent with bovine serum albumin and goose serum albumin.

(9) Placental extracts were found to form strong precipitin arcs against preparations containing α-fetoprotein in agarose gel. The placenta may contain a concentration of anti-α-fetoprotein activity as a barrier to entry of α-fetoproteins into the maternal circulation.

(10) α-fetoprotein was found to share antigenic determinants with human serum albumin, human γ-heavy chains, Bence Jones protein type L and Human Growth Hormone.

(11) The possible importance of these observations, concerning the aetiology of IRDS and the structure and functions of α-fetoprotein have been discussed.

PART 6

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PART 7

ADDENDA

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SERUM-PROTEINS IN THE IDIOPATHIC RESPIRATORY-DISTRESS SYNDROME OF THE NEWBORN

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FROM time to time it has been reported that newborn babies with the idiopathic respiratory-distress syndrome (I.R.D.S.) have abnormally low total serum-protein concentrations (Cooke 1960, Usher 1961b, Fraillon and Kitchen 1962, Felbo et al. 1963). Normal premature infants have lower serum-protein levels than full-term infants, but in distressed infants the values are even less than those in prematures. Infants with I.R.D.S. are generally œdematous and the œdema fluid has been found to have a high protein content (Usher 1961b).

There is also some evidence that such infants are in a hypercatabolic state as a result of the severe stress of laboured respiration and that the plasma-proteins are being catabolised together with other body constituents (Usher 1961a).

We have investigated the serum-proteins in infants with I.R.D.S. to determine whether any specific abnormalities are present.

Some infants with hæmolytic disease due to rhesus incompatibility were included in the study. With this condition the serum-antibody titre parallels the serum-γ-globulin as measured chemically and rhesus-incompatible infants show a fairly rapid fall in antibody titre

after birth (Wiener 1961). The pattern of the serum-proteins in these infants has been compared with that of infants with I.R.D.S.

Clinical Materials and Methods

Specimens of blood were obtained from the umbilical cord at birth and from the external jugular vein or a catheterised umbilical vein on the three days after birth from a series of normal full-term and normal premature infants born in the teaching hospitals of the University of Cape Town. Similar samples were obtained from a series of infants suffering from I.R.D.S. Where possible, these infants were followed up for ten days or longer. Infants included in this category showed at least two of the following features for which no obvious cause could be found: (1) cyanosis, (2) rib recession, (3) expiratory grunting, (4) œdema, (5) " ground-glass " opacity of the lung fields with an air bronchogram on X-ray.

Total serum-proteins were determined by the biuret method (Gornall et al. 1949).

Paper electrophoresis was done on 0·01 ml. of serum in a Durrum-type tank using barbitone buffer of pH 8·6, ionic strength 0·075. A potential difference of 150 V was applied for sixteen to eighteen hours. The strips were then heated and stained with lissamine-green according to the method of Gorringe (1957). Bands corresponding to the protein-fractions were eluted, the eluates were filtered, and the optical density was read in a Klett-Summerson photometer using a red filter ($\lambda=660$). Optical densities were taken to be proportional to the concentration of the protein fraction.

Experimental error was determined by serial measurements on the same serum. The 95% confidence range was approximately 5% of the mean value for each fraction.

The results by the above method were also checked against values obtained by precipitating the protein fractions with a 23% w/v solution of sodium sulphate and found not to differ from them by more than 6% over a wide range of albumin and globulin concentrations. Serial determinations were performed on the same serum on twelve successive days to ascertain whether the relative proportions of the various protein fractions changed with storage. The proportions of the various fractions exhibited little change for about ten days and after this the relative proportions of albumin and β-globulins tended to fall. None of the samples used in this study was more than five days old.

Results

Normal Infants

Concentrations of total protein, albumin, and γ-globulins have been calculated for different weight-groups of normal infants (full-term and premature) and expressed in table I

TABLE I—SERUM-PROTEINS IN NORMAL INFANTS AND IN INFANTS WITH I.R.D.S.: MEANS (±STANDARD ERROR) OVER FIRST SEVENTY-TWO HOURS IN EACH WEIGHT GROUP

Group	Weight (kg.)	No.	Total protein (g. per 100 ml.)	Albumin		γ-globulin	
				(g. per 100 ml.)	% of total	(g. per 100 ml.)	% of total
Normals	<2·0	30	4·9±0·9	3·19±0·30	64·5±6·1	0·71±0·25	14·2±2·7
	2·0–2·5	16	5·5±0·8	3·74±0·25	67·9±4·5	0·83±0·14	15·0±2·5
	2·5–3·5	38	5·4±0·7	3·62±0·22	67·6±4·2	0·77±0·15	14·4±2·6
	3·5–4·5	30	5·5±0·7	3·72±0·15	68·2±3·5	0·77±0·14	14·0±2·7
	4·5	16	5·7±0·7	3·75±0·43	65·9±7·7	0·88±0·14	15·5±2·4
	—	All (mean)	5·3±0·8	3·54±0·24	66·8±4·6	0·77±0·14	14·5±2·7
I.R.D.S.	<2·0	9	3·8±0·6	2·75±0·18	72·3±4·9	0·33±0·07	8·4±1·9
	2·0–2·5	5	4·6±0·8	3·32±0·29	72·3±6·4	0·38±0·15	8·3±3·3
	2·5–3·5	5	5·4±0·7	3·97±0·20	76·2±3·8	0·45±0·20	8·6±3·9
	—	All (mean)	4·4±0·7	3·20±0·23	73·4±4·2	0·37±0·14	8·4±3·3

as means of all observations made during the first seventy-two hours of life.

The full-term infants (over 2·5 kg.) and larger premature infants (over 2·0 kg.) had approximately the same concentrations of total protein, albumin, and γ-globulins.

Smaller prematures (under 2·0 kg.) had a lower total-protein than larger infants but the difference was not statistically significant. The proportions of albumin and γ-globulins in the sera of these prematures did not differ significantly from those in larger babies.

The sera were also divided into groups according to the age of the infants when the blood-specimens were collected (see table II). There was a slight drop in the concentrations of total protein, albumin, and γ-globulins over the first seventy-two hours of life. These are of doubtful significance but are similar to results obtained by other workers (Saito et al. 1956, Tudvad et al. 1957, Felbo et al. 1963).

Infants with I.R.D.S.

19 cases were included in the study. Cord-blood samples were obtained at birth in most cases and, thereafter, specimens were taken at intervals of one day or more until the child recovered or died. As for the normal infants, mean serum-protein values were calculated for the first seventy-two hours in the various weight-groups and also for all weights in a particular age-group (tables I and II). The concentrations of total protein and albumin were significantly lower than normal in the smaller distressed infants ($P < 0{\cdot}01$), but not in those over 2·5 kg. There were only 5 infants over 2·5 kg. in this series. None of these were infants of diabetic mothers.

The mean concentrations of γ-globulins were considerably lower in distressed infants of all weight-groups than in the comparable group of normal infants; this difference was highly significant ($P < 0{\cdot}001$). The cord blood contained an abnormally low concentration of γ-globulin and the fall in γ-globulin concentration during the first twenty-four hours of life was much more notable than that in normal infants, despite the fact that the majority of the more severely affected infants had received 1 g. of a γ-globulin preparation by intramuscular injection during the first twelve hours of life as part of their treatment. One severe case did not receive γ-globulin and here the concentration fell from 0·43 g. per 100 ml. in the cord blood to 0·07 g. per 100 ml. at thirty-six hours.

TABLE II—SERUM-PROTEINS IN NORMAL INFANTS AND IN INFANTS WITH I.R.D.S.: MEANS (±STANDARD ERROR) OVER ALL WEIGHTS IN EACH AGE-GROUP

Group	Age (hr.)	No.	Total protein (g. per 100 ml.)	Albumin		γ-globulin	
				(g. per 100 ml.)	% of total	(g. per 100 ml.)	% of total
Normals	Cord blood	88	5·4±0·8	3·61±0·25	66·8±4·6	0·82±0·15	15·1±2·7
	0–24	19	5·4±0·8	3·56±0·27	66·0±5·0	0·78±0·16	14·4±3·0
	24–48	16	5·3±0·7	3·55±0·23	66·9±4·3	0·76±0·14	14·4±2·6
	48–72	17	5·1±0·8	3·49±0·23	68·4±4·5	0·73±0·13	14·4±2·5
I.R.D.S.	Cord blood	12	4·4±1·0	3·20±0·20	72·8±4·5	0·43±0·13	9·8±2·9
	0–12	6	4·2±0·9	3·11±0·21	74·0±6·1	0·38±0·15	9·0±2·9
	12–24	8	4·0±0·7	3·02±0·15	75·5±3·5	0·26±0·12	6·6±2·5
	24–48	6	4·1±1·1	2·94±0·22	71·9±4·5	0·29±0·15	7·1±2·8
	48–72	4	4·7±1·9	3·36±0·25	71·4±6·5	0·39±0·15	8·3±3·0

Subsequent changes have been followed in a small series of patients. 4 infants between forty-eight and seventy-two hours old exhibited a rise in the concentrations of total protein, albumin, and γ-globulins, the proportion of the latter remaining depressed.

Follow-up specimens were obtained at about one month from 5 babies who had recovered from I.R.D.S. In these, total proteins were within normal limits, but γ-globulin still comprised only about 3% of the total—i.e., about 0·16 g. per 100 ml.

The cord-blood sera of 3 newborn infants with severe hæmolytic disease due to rhesus incompatibility showed similar changes, with an even lower γ-globulin concentration than that of infants with I.R.D.S. (mean total-protein 4·5 g. per 100 ml., mean γ-globulin 0·24 g. per 100 ml.). Since these infants were treated by exchange transfusion, serial specimens could not be obtained to discover a further fall in γ-globulin.

Discussion

I.R.D.S. patients have a relative and absolute deficiency of γ-globulins as well as abnormally low concentrations of total protein and albumin in the cord blood at birth. The abnormal pattern tends to become more notable over the first twenty-four hours after which the concentrations of all fractions start to rise. At one month of age the γ-globulin concentration has again fallen to very low levels as compared with normal infants of the same age.

We suggest two possible explanations for these findings. Firstly the low γ-globulin concentration in the cord blood may be due to defective transfer across the placenta. All the $7S\gamma_2$-globulins present in the infants' circulation at birth are obtained by selective transplacental transfer from the maternal circulation (Gitlin 1957, 1965, Janeway and Gitlin 1957), and synthesis in the infant does not commence until two to three months of age, at which time a concentration of 300–600 mg. per 100 ml. is reached in normal infants (Gitlin 1957, 1965). Little $7S\gamma_2$-globulin, if any, reaches the circulation from colostrum or breast milk (Hassan and Gunther 1958, Vahlquist 1958). Impaired transfer from the mother may be due to, or associated with, a low concentration of γ-globulins in the maternal circulation. Secondly, the further fall in protein concentrations in the first twenty-four hours of life might arise from:

(1) Leakage of protein through capillary walls damaged by anoxia. This is unlikely to account for the selective disappearance of γ-globulins since the œdema fluid contains a higher proportion of albumin than does serum (Usher 1961b).

(2) Excessive catabolism of γ-globulins.

(3) An immune reaction involving maternal antibodies to a fœtal antigen (as is the case in infants suffering from hæmolytic disease of the newborn). The similarity between the serum-protein patterns in I.R.D.S. and rhesus hæmolytic disease is consistent with this suggestion.

Summary

The serum-proteins of a series of normal full-term and premature infants were compared with those of infants with the idiopathic respiratory-distress syndrome, using paper electrophoresis. Infants with respiratory distress have abnormally low total-protein concentrations in the serum. The proportion of γ-globulins is abnormally low. Both these abnormalities are present in the cord blood at birth and tend to become more pronounced as the disease progresses.

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MATERNAL SERUM-PROTEINS IN IDIOPATHIC RESPIRATORY DISTRESS SYNDROME OF THE NEWBORN

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Summary Serum-proteins were studied in healthy women and in women who delivered infants with idiopathic respiratory-distress syndrome (I.R.D.S.) at intervals of 2–3 weeks during pregnancy; at delivery in a series of mothers of healthy premature infants and in a series of mothers whose infants had I.R.D.S.; and in some of the same women 6 weeks or more postpartum. I.R.D.S. mothers had significantly lower levels of γ-globulins than the control group. This deficiency was present from about 16–20 weeks and throughout pregnancy, and was rectified within about 6 weeks after delivery. I.R.D.S. mothers and controls did not differ significantly with respect to the concentrations of total serum-protein and serum-albumin.

Introduction

OUR finding of diminished γ-globulin concentrations in infants with the idiopathic respiratory-distress syndrome of the newborn (I.R.D.S.) (Hardie, Heese, and Kench 1965) prompted us to investigate the serum-proteins of women giving birth to affected infants.

Patients and Methods

Specimens of blood were obtained at delivery from a series of mothers of infants with I.R.D.S. and also from a series of mothers of healthy premature infants born at the teaching hospitals associated with the University of Cape Town.

TABLE I—MEAN VALUES IN CASES FOLLOWED THROUGH PREGNANCY

Group	—	Duration of pregnancy (weeks)						Delivery	
		0–20	21–28	29–31	32–34	35–37	38–40		
Healthy mothers (40)	Total serum-proteins (g. per 100 ml.)	5·4 ± 0·7	5·4 ± 0·6	5·5 ± 0·6	5·4 ± 0·7	5·3 ± 0·7	5·5 ± 0·8	5·7 ± 0·9	
	Serum-albumin (g. per 100 ml.)	3·24 ± 0·30	3·19 ± 0·29	3·14 ± 0·22	3·08 ± 0·25	2·96 ± 0·19	3·08 ± 0·29	3·20 ± 0·33	
	γ-globulin (E) (electrophoretic) (g. per 100 ml.)	0·77 ± 0·19	0·72 ± 0·15	0·70 ± 0·20	0·68 ± 0·14	0·71 ± 0·15	0·73 ± 0·15	0·83 ± 0·10	
	γ-globulin (I.P.) (immuno-plate) (g. per 100 ml.)	..	1·81 ± 0·60	1·67 ± 0·56	1·62 ± 0·62	1·73 ± 0·53	1·99 ± 0·78	1·92 ± 0·65	6 cases
	Ratio I.P./E	..	2·5	2·4	2·4	2·4	2·7	2·3	6 cases
I.R.D.S. mothers (10)	Total serum-proteins (g. per 100 ml.)	5·8 ± 1·0	5·3 ± 0·7	5·2 ± 0·5	5·2 ± 0·6	5·2 ± 0·6	..	5·2 ± 0·8	
	Serum-albumin (g. per 100 ml.)	3·36 ± 0·43	3·08 ± 0·18	3·12 ± 0·20	2·92 ± 0·31	2·76 ± 0·38	..	3·17 ± 0·21	
	γ-globulin (E) (electrophoretic) (g. per 100 ml.)	0·68 ± 0·11	0·65 ± 0·14	0·59 ± 0·09	0·60 ± 0·12	0·61 ± 0·15	..	0·60 ± 0·10	
	γ-globulin (I.P.) (immuno-plate) (g. per 100 ml.)	1·71	2·10	1·85	2·01	..	..	..	2 cases
	Ratio I.P./E	2·5	3·2	3·1	3·4	..	..	..	2 cases

In addition, blood-specimens were taken at intervals of 2–3 weeks from women attending the antenatal clinics. The investigation was begun at the earliest time that they came under observation (generally 16–20 weeks) and continued until delivery. In this way 10 women who gave birth to premature infants with I.R.D.S. as well as a large number of controls could be followed through pregnancy. Where possible, follow-up blood-specimens were obtained 6 weeks or more postpartum from the same women for comparison with the findings during pregnancy.

24-hour collections of urine and fæces were obtained from two mothers of infants with I.R.D.S. during the last 10 days before delivery. The urines were dialysed against distilled water, freeze-dried, and redissolved in a small volume of water. Fæces were homogenised in water, centrifuged, and the deposit discarded. Both were examined for γ-globulin content by electrophoresis.

Large-scale cellulose-acetate electrophoresis was carried out according to the method of Kohn, using barbitone buffer, pH 8·6 (Kohn 1960). Strips were stained with lissamine-green and washed and dried in air at room temperature. Bands corresponding to the protein fractions were dissolved in chloroform/ethanol 9:1 v/v and the optical density was read in a Klett-Summerson photometer, using a red filter ($\lambda = 660$). Protein fractions and total serum-protein were determined as before (Hardie, Heese, and Kench 1965). The experimental error was calculated to be about 5%.

Immunoelectrophoresis was carried out on the extracts of stools and urine and some of the sera according to the method of Grabar and Williams (1955).

In some women the concentrations of the three main immune globulins (IgG, IgM, and IgA) were determined through pregnancy and at delivery using ' Immuno-plates ' (Hyland Laboratories). The stated error for this method is 10%.

Results

Patients Followed from Early Pregnancy to Delivery

Mothers giving birth to healthy infants.—There were 40 women in this series who subsequently delivered healthy premature or full-term infants. The total serum-protein concentration, as well as the concentrations of albumin and γ-globulins, show minor fluctuations throughout pregnancy, with lowest levels within the period 32–37 weeks, but the variations are not statistically significant (table I). The concentrations of γ-globulin (IgG+IgM+IgA) as measured by immuno-plates showed the same general pattern, but the values obtained were considerably higher than the electrophoretic readings. We have been unable to account for the discrepancy.

Mothers giving birth to infants with I.R.D.S.—Similar serial studies were made on ten women, including three

diabetic patients, who delivered premature infants with I.R.D.S. In these, the total serum-protein and serum-albumin concentrations did not differ significantly from those found in healthy mothers at the same stage of pregnancy. The lower values at delivery are due to the fact that these women were all delivered before 37 weeks—a stage at which healthy women also have lower values. The γ-globulin level, however, was significantly lower than in healthy women and remained consistently so throughout pregnancy. Lowest levels were reached at 29–31 weeks and remained low thereafter (table I). Two cases were followed by means of immuno-plates. Here, the ratio of immuno-plate reading to electrophoretic reading was even higher than in healthy women.

Blood-specimens Taken at Delivery

These constitute a larger series than the one already described. Here, thirty mothers of infants with I.R.D.S. are compared with twenty-five mothers of healthy premature infants, the blood-specimens being taken at or just after delivery. Again the two groups did not differ significantly with respect to the concentrations of total serum-protein

TABLE II—MEAN VALUES IN BLOOD-SPECIMENS AT DELIVERY FROM I.R.D.S. MOTHERS AND MOTHERS OF HEALTHY PREMATURE INFANTS

Group	No.	Total protein (g./100 ml.)	Serum-albumin (g./100 ml.)	γ-globulin (electro-phoretic) (g./100 ml.)	γ-globulin (immuno-plate) (g./100 ml.)
Mothers of healthy premature infants	25	5·3 ±0·7	3·12 ±0·25	0·90 ±0·20	1·78 ±0·61
Mothers of I.R.D.S. infants	30	5·2 ±0·8	2·90 ±0·20	0·70 ±0·13	1·69 ±0·80

and serum-albumin (table II), but the γ-globulin concentration was lower in the I.R.D.S. group, the difference being very highly significant ($P < 0{\cdot}001$).

The mothers of healthy premature infants had at delivery (i.e., at 30–37 weeks) a higher γ-globulin concentration than mothers of healthy full-term infants at this stage of pregnancy. This difference is highly significant and suggests that mothers of healthy premature infants constitute a different population.

Follow-up Specimens 6 weeks or more Postpartum

Blood-specimens were obtained from twenty of the mothers of healthy infants and from fifteen who had delivered infants with I.R.D.S. In both groups, the

rise in the concentrations of total serum-protein and serum-albumin were of the same order. The control mothers showed a slight but non-significant rise in γ-globulins, whereas in mothers of infants with I.R.D.S. the rise between the time of delivery and the time when the follow-up specimen was taken was pronounced and significant.

Postpartum γ-globulin levels were actually slightly higher in the I.R.D.S. mothers than in the control group, but the difference was not statistically significant (tables II and III).

Discussion

Mothers of infants with I.R.D.S. clearly showed a specific deficiency of γ-globulins during pregnancy and at delivery as compared with mothers of healthy premature and full-term infants. This cannot be explained on the basis of hæmodilution, since the concentrations of the other serum-protein fractions did not differ significantly from normal. Moreover, the deficiency in γ-globulins was present from the earliest time that these patients come under observation and disappeared within 6 weeks after the termination of pregnancy.

Although the variations, throughout pregnancy, in the concentrations of all the serum proteins of both normal and I.R.D.S. mothers are not statistically significant, lowest levels are generally observed within the period 32–37 weeks. This may be partly due to hæmodilution since the plasma-volume reaches its peak at about this time. Correcting for the increase in plasma-volume (Hytten and Paintin 1963) the total amount of circulating γ-globulin can be shown to remain fairly constant in both groups but

TABLE III—MEAN VALUES 6 WEEKS OR MORE POSTPARTUM

Groups	No.	Total protein (g./100 ml.)	Serum-albumin (g./100 ml.)	γ-globulin (electrophoretic) (g./100 ml.)
Mothers of healthy infants	20	5·7 ± 0·6	3·82 ± 0·31	0·93 ± 0·14
Mothers of I.R.D.S. infants	15	5·8 ± 0·5	3·83 ± 0·22	1·01 ± 0·15

is maintained at a lower level in I.R.D.S. mothers. This finding is of particular interest, because the infants with I.R.D.S. had a similar deficiency of γ-globulins at birth. The deficiency is likely to be mainly one of IgG, since this makes up most of the γ-globulin in healthy adults.

Preliminary immunoelectrophoresis and column chromatography with ' Sephadex G200 ' (Flodin and Killander 1962) support the idea that the sera are deficient in 7S globulins and in IgG rather than IgM or IgA. The immuno-plate results were difficult to interpret; they gave concentrations of proteins much greater than techniques dependent on measurements of dye-binding. This may account for the higher values recorded by Gitlin and Boesman (1966).

Examination of urine and fæces of these women and their infants disclosed little or no γ-globulin. This makes it unlikely that the γ-globulins are being lost through the kidneys or gut. There seem to be a number of possible explanations for our findings:

(1) There is present in these women a pregnancy-induced defect in the synthesis of γ-globulins resulting in lowered maternal serum concentrations, and consequently diminished placental transfer to the infant. Against this idea is our observation that in several sets of twins of whom one had I.R.D.S. and the other not, the healthy twin had normal γ-globulin levels.

(2) The fœtus is catabolising the maternal γ-globulin at a rate faster than normal as it comes across the placenta.

(3) An immune reaction is in progress, with the maternal γ-globulin as either an antibody or an antigen, and a large proportion of it is removed from the fœtal circulation soon after it has crossed the placenta.

We are indebted to Prof. D. A. Davey, head of the department of obstetrics and gynæcology, for permission to take blood-specimens from his patients; to Dr. H. de V. Heese and his unit for identifying infants with I.R.D.S.; to Dr. M. C. Botha of the blood-transfusion service for allowing us access to his samples; to Mrs. M. Torrington, of the department of medicine, for invaluable help with the follow-up studies; and to Dr. J. G. Burger, superintendent of Groote Schuur Hospital, for permission to publish.

Requests for reprints should be addressed to G. H., C.S.I.R. Protein Research Unit, Department of Chemical Pathology, Medical School, University of Cape Town.

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FURTHER OBSERVATIONS ON SERUM PROTEINS IN RESPIRATORY DISTRESS SYNDROME OF THE NEWBORN

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Further Observations on Serum Proteins in Respiratory Distress Syndrome of the Newborn

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In our initial study of newborn infants suffering from the respiratory distress syndrome (RDS) (Hardie, Heese, and Kench, 1965), it was found that affected infants had significantly lower serum concentrations of total proteins than normal premature infants, and that the relative and absolute concentrations of γ-globulins, as determined by paper electrophoresis, were much diminished. These abnormalities were present in the cord blood and became more marked in the first 2 to 3 days.

In that paper we suggested that the γ-globulin deficiency could be due to either (1) defective placental transfer of γ-globulins, or (2) excessive γ-globulin katabolism by the affected fetus and newborn, or (3) an immune reaction taking place in the fetus and newborn involving the maternal γ-globulins as either antibodies or antigens.

With these possibilities in mind a more detailed and extensive investigation of the serum proteins in this condition is described.

Clinical Material and Methods

Subjects of the study were either normal premature infants or premature babies suffering from RDS. The mean gestational age of the normal premature infants for whom data were available (72 of the 88 in the series) was 33·9 weeks. All the RDS infants (33) were traced, and these had a mean gestational age of 35·2 weeks. The clinical criteria for diagnosis of RDS were as before (Hardie *et al.*, 1965). They were the presence of at least two of the following features for which no other obvious cause could be found: (1) cyanosis; (2) rib recession; (3) expiratory grunting; (4) generalized oedema; and (5) a 'ground glass' opacity on *x*-ray of the lung fields with an air bronchogram.

Where the patient presented with cyanosis and oedema, it was established that cyanosis was not due to any cause other than respiratory.

Specimens of blood were collected, as previously reported, from the umbilical cord at birth, and from a peripheral vein or a catheter in the umbilical vein on each of the first 3 days after birth, and again between the 4th–10th days. Where possible, follow-up specimens from infants were examined between the ages of 3 weeks and 2 months. Sera were also obtained by cardiac puncture from a small number of fetuses at hysterotomy.

24-hourly specimens of urine were obtained from normal and affected infants. Urine was dialysed, lyophilized, and the residue redissolved in a small volume of 0·15 M NaCl. Meconium and faeces were homogenized in water, centrifuged, and the deposit discarded. The extracts were examined for γ-globulins by electrophoresis on cellulose acetate, and quantitation of the protein fractions was performed as previously described (Hardie and Kench, 1967). The standard curve for recovery of known quantities of γ-globulins added to the cellulose acetate strip is shown in Fig. 1.

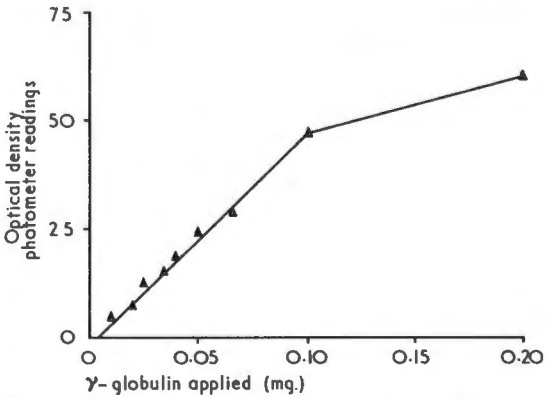

FIG. 1.—*Standard curve for recovery of known quantities of γ-globulins applied to the cellulose acetate strip, and subjected to electrophoresis, staining, and extraction in the usual way.*

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TABLE I

Concentrations of Serum Proteins in Normal Premature Infants and in Infants with RDS at Birth and in First 2 months of Life (electrophoretic readings ± standard deviation)

Group	Age	No.	Total Protein (g./100 ml.)	Albumin (g./100 ml.)	γ-globulin	
					(g./100 ml.)	(% of total protein)
RDS	Cord	33	3·6±0·5	2·76±0·19	0·37±0·10	10·3±2·0
	1st day	33	3·7±0·6	2·73±0·27	0·38±0·11	10·3±2·5
	2nd day	17	3·6±0·6	2·60±0·23	0·32±0·14	9·1±2·1
	3rd day	14	3·7±0·8	2·68±0·23	0·35±0·12	9·5±2·2
	4th–10th day	14	4·0±1·0	2·95±0·25	0·40±0·13	10·0±2·2
	Later (3 wk.–2 mth.)	8	4·4±0·9	3·13±0·29	0·45±0·10	10·2±2·6
Normal premature infants	Cord	88	5·4±0·8	3·61±0·25	0·82±0·15	15·1±2·7
	1st day	19	5·4±0·8	3·56±0·27	0·78±0·16	14·4±3·0
	2nd day	16	5·3±0·7	3·55±0·23	0·76±0·14	14·4±2·5
	3rd day	17	5·1±0·8	3·49±0·23	0·73±0·13	14·4±2·5
	4th–10th day	15	5·1±0·7	3·49±0·21	0·72±0·15	14·3±3·1

All observations of unknown concentrations of γ-globulins lie on the linear part of the standard curve. The intercept on the abscissa is due to a small quantity of dyed protein firmly bound to the strip and not extractable by the solvent (9 : 1 v/v chloroform/ethanol).

In a number of cases, the concentrations of the three main types of immunoglobulin (IgG, IgM, and IgA) were individually determined in the sera and also in the extracts of urine, meconium, and faeces, using immunoplates (Hyland Laboratories).

Column chromatography on dextran gel (Sephadex G200) with 0·1 M tris buffer *p*H 8·0 and a flow rate of 20 ml./hr. was performed on sera of cord blood of 6 affected infants and 6 normal premature infants.

Results

Findings on the serum are summarized in Tables I and II. Normal infants had a slight steady fall in the concentrations of all protein fractions during the first 3 days of life. The γ-globulins formed a relatively constant proportion (14–15%) of the total.

In infants with RDS the concentrations of all protein fractions were lower than normal, all differences being highly significant ($p<0·01$). The γ-globulins accounted for a significantly lower proportion (9–10%) of the total. On the average the γ-globulin concentrations reached their lowest concentrations during the 2nd to 3rd day of life, and rose slightly thereafter. This was not so with normal infants.

Immunoplate readings were obtained on a smaller number of patients suffering from RDS. As a rule IgG concentrations measured by this method were higher than electrophoretic readings but followed the same general pattern. IgM was always present in measurable quantities in the cord blood, though then, as later, there was a wide scatter in the observed concentrations. Levels had roughly doubled 2 months after birth.

In normal infants only the cord blood was studied by the immunoplate method. The IgG levels were significantly higher than in affected infants ($p<0·01$) but there was no significant difference in IgM concentration. IgM was not invariably present in measurable quantities in the cord blood of normal infants. None of the cord sera contained IgA in measurable amounts. Gel filtration of the sera on Sephadex G200 indicated a smaller area of the 7S peak in infants with RDS as compared with normals.

On inspection of the electrophoretic strips it was observed that as a rule sera of infants with RDS contained a protein, generally of low concentration, and migrating between the albumin and α_1-globulins (Fig. 2). This was not usually present in sera of

TABLE II

Concentrations of IgG and IgM in Normal Premature Infants at Birth and in Infants with RDS at Birth and During First 2 months of Life (Immunoplate readings ± standard deviation)

Group	Age	No.	IgG (g./100 ml.)	IgM (mg./100 ml.)
RDS	Cord	15	0·51±0·15	11±10
	1st day	11	0·54±0·15	16±5
	2nd day	5	0·54±0·06	16±4
	3rd day	5	0·44±0·15	10±6
	4th–10th day	10	0·57±0·12	22±10
	Later	5	0·49±0·11	34±7
Normal premature infants	Cord	17	0·87±0·21	9±7

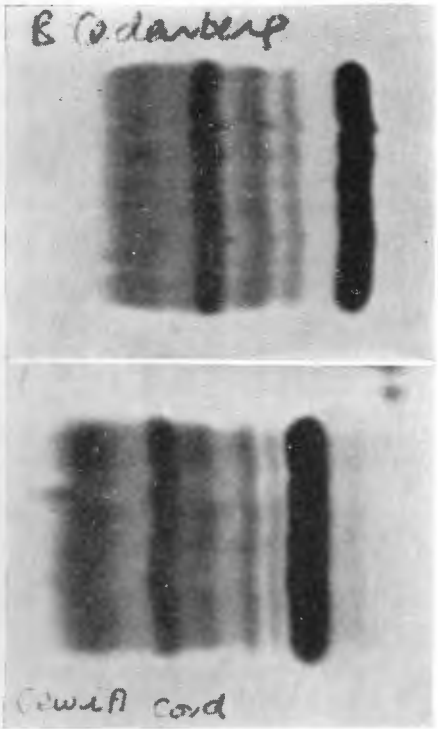

FIG. 2.—*Electrophoretic strip of serum of an infant with RDS (below) compared with the serum of a normal premature infant. The serum of the affected infant shows the presence of an α-fetoprotein band, immediately adjacent to the prominent albumin band.*

normal premature infants. Thus, of 49 cord sera of infants with RDS, it was observed in 43, while of 48 cord sera of normal premature infants it was seen in only 13. Further, in 4 sets of twins, in each of which only one twin had RDS the other being normal, it was detectable in the serum of the affected twin alone in 3 cases (Fig. 3). In the 4th set this post-albumin fraction was seen in both twins.

The band tended to grow fainter during the first days of life and was usually no longer apparent by 7–8 days of age.

The fetal sera were from fetuses ranging in maturity from 14–22 weeks. A protein migrating in a similar position was observed in all these sera, the intensity of its band being greater in the less mature fetuses.

No difference could be detected between the urinary proteins of normal and affected infants. Meconium and stools were likewise found to contain similar quantities of γ-globulins.

Discussion

In our previous study (Hardie *et al.*, 1965), we compared the concentrations of serum proteins of infants suffering from RDS with those of normal premature babies, matched according to birthweight and to age (hours) after birth. The mean concentrations of γ-globulins were considerably lower in distressed infants of all weight groups than in the comparable group of normal infants; this difference was highly significant ($p < 0{\cdot}001$). H. de V. Heese (personal communication, 1967) found that the birthweight/gestational age ratio of RDS babies was less than that of normal prematures, so probably our RDS patients were relatively older than normal premature infants of the same size at birth. In the larger series now reported, the mean gestational age of the infants with RDS (35·2 weeks) was slightly greater than that of the normal premature

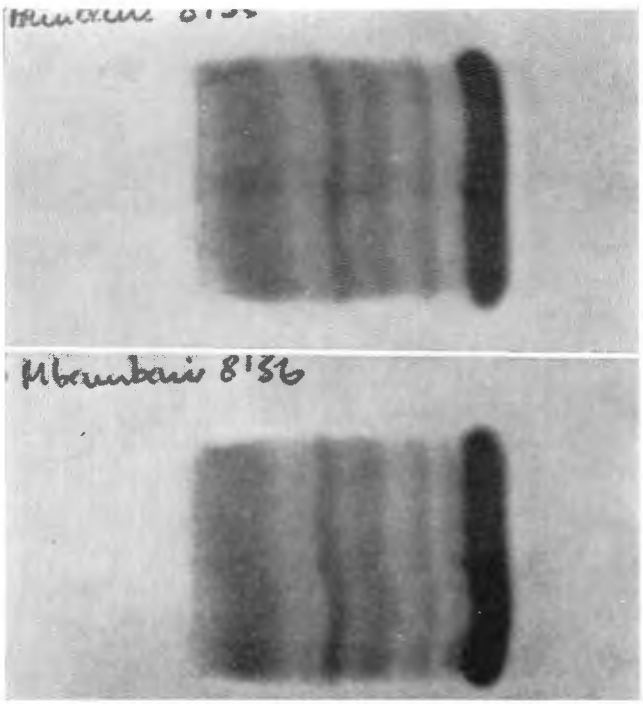

FIG. 3.—*Electrophoretic strips of sera of a pair of twins of which one had RDS and the other was clinically normal. The serum of the affected twin (below) shows the presence of an α-fetoprotein band, adjacent to the albumin band.*

infants (33·9 weeks). The electrophoretic and immunochemical determinations of the serum proteins have confirmed our earlier observations, and have shown that the deficiency was one of IgG. The finding of a lowered 7S peak on gel filtration of the sera supported this. We have attempted to exclude the possibility that abnormally large quantities of protein and particularly γ-globulins were being lost through the kidneys or alimentary canal. The γ-globulin present in the meconium and stools of both normal and affected infants was not identical antigenically with IgG and was probably the copro-antibody described by Roulet and von Muralt (1961) and Lipton and Steigman (1957).

The observed low concentrations of serum albumin in RDS subjects may have arisen, partly at least, by leakage through the capillary walls into the oedema fluid which had a high protein content (1·0–2·5 g./100 ml.), most of which was albumin. However, this mechanism cannot be held responsible for the deficiency of IgG, which seems likely to be due to defective placental transfer or to some reaction occurring in the fetal and neonatal circulation or tissues. This may be an immune reaction involving the maternal IgG either as an antibody or as an antigen. Such a reaction could cause the generalized capillary lesion which is a feature of the condition. The capillary lesion is unlikely to be anoxic in origin, as, in the lung, the capillary endothelial cells are damaged whereas the alveolar endothelial cells are frequently normal (Strang, 1966).

In contrast to the findings of Gitlin and Boesman (1966) and Bergstrand and Czar (1957) we have detected the presence of the α-fetoprotein not only in the sera of fetuses but also in the sera of most infants with RDS, and in an appreciable proportion of normal premature infants, some of whom were as mature as 36 weeks. It was present in the cord blood and even for a few days after birth. We are as yet unable to offer any explanation for the strong association of RDS with the presence of α-fetoprotein in the serum of affected infants.

Summary

Proteins in the serum, urine, and faeces of normal premature infants were compared with those of infants with respiratory distress syndrome (RDS) using electrophoresis on cellulose acetate, immunoplates, and column chromatography on dextran gel (Sephadex G200).

Infants with RDS had abnormally low concentrations of all serum proteins. The proportion of IgG relative to the other serum proteins was significantly less than normal. The presence of an α-fetoprotein in the serum was strongly associated with RDS. The protein content of urine and faeces was similar in infants with RDS and normal premature infants.

We are indebted to Professor F. J. Ford and Dr. H. de V. Heese of the Department of Child Health for their interest in this work, to Dr. M. C. Botha of the Blood Transfusion Service for permitting us to make use of his samples, to Miss S. van Olm of the Department of Bacteriology for providing fetal sera, and to Dr. J. G. Burger, Superintendent of Groote Schuur Hospital, for permission to publish.

Addendum

In a recent study, McKay, Thom, and Gray (1968) have been unable to detect significant differences between the concentrations of IgG, IgA, IgM, and albumin in the umbilical cord plasma of healthy infants and of neonates who developed respiratory distress syndrome. The reasons for the disagreement between McKay *et al.* and ourselves on this issue are not yet apparent.

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