"PLACENTAL TRANSFER OF IRON IN THE RABBIT"

by

JEAN P. RENTON, M.R.C.V.S.

Thesis submitted for the Degree of Doctor of Philosophy
in the Faculty of Medicine
The University of Glasgow.

1966.

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

"PLACENTAL TRANSFER OF IRON IN THE RABBIT"

CONTENTS

Page

SECTION TWO

SECTION THREE

THE ^{59}Fe CONTENT OF THE RABBIT PLACENTA

ACKNOWLEDGMENTS

I wish to express my gratitude to Professor Sir William Weipers for giving me the opportunity to carry out this work in his department, and for his constant help and encouragement. I also wish to express my thanks to Dr. T. A. Douglas, Dr. A. Goldberg and Mr. P. G. Mignett, for their interest and advice throughout the course of my studies. I would like to thank Dr. I. Dawson for the preparation and advice with regard to the electronmicroscopy and Mrs. E. Aughey for her co-operation during this part of the work. I would also like to thank Mr. R. Wright for his technical assistance during the entire course of the work, Miss M. Gracie for her part in the preparation of the electron micrographs, Mr. A. Finnie for the photography and Miss G. Keddie for the typing of this thesis. In conclusion I would like to express my thanks to Professor J.N. Davidson for facilities granted to me in the Biochemistry Department and the Agricultural Research Council who supported the work throughout.

IRON METABOLISM

Anaemia, as a result of deficiency of iron, is still one of the hazards of human pregnancy. A study of patients in a low income group, in New Orleans, indicated that half of the pregnant patients suffered from iron deficiency anaemia. (Curtis, Lund, Thomas, Sisson, 1958). In the veterinary field, iron deficiency anaemia is found frequently in the young suckling piglet and constitutes a major problem in the pig industry (Martin, 1959). The human foetus satisfies its need at the expense of the mother. In the pig, on the other hand, the iron transferred from the mother to the foetus, during gestation, is insufficient to meet the needs of the young piglets during the first few weeks of life. In both circumstances an understanding of the mechanism of placental transfer of iron is of importance. It is difficult, for obvious reasons, to collect data from the human species and, unfortunately, pregnant sows are expensive experimental material. The placentation in the rabbit, however, is similar to that of the human and the rabbit is a suitable experimental animal. Several workers (Bothwell, Pribilla, Mebust and Finch, 1958; Davies, Brown, Stewart, Terry and Sisson, 1959) have studied the absolute quantities of iron

transferred across the rabbit placenta and the rates at which this is accomplished. As yet the actual mechanism involved in the transfer of iron across the placenta of the rabbit is not understood and this aspect seemed worthy of study.

Distribution of iron in the body

Iron is present in varying quantities within every cell in the body. (McCallum, 1891). In the form of ferrous iron it is associated with porphyrin in the formation of haem, which in turn becomes attached to the protein globin, forming haemoglobin. Iron is also found in myoglobin, the oxygen storage compound found in muscle. In the ferric form iron combines with the protein apoferritin, in different quantities, to form either ferritin or haemosiderin, which are the storage forms of iron found in the liver, spleen and bone marrow. Small quantities are also found in haem enzymes.

The absorption of iron

The absorption of iron is not a continuous process and takes place from the food as it passes along the

intestinal tract. Not all the iron in the diet is absorbed, the greatest amounts being absorbed from the jejunum and duodenum. Green, Brook & Veldman,(1947); and Reichberg (1956) suggested that pH might play a part in determining the areas where absorption is greatest. Moore (1958) on the other hand, suggested that the reason for most absorption taking place in the upper part of the intestinal tract was that, by the time the dietary iron has reached the ileum, it was in the form of complex insoluble salts. In humans, iron in the ferrous state is more easily absorbed than in the ferric state (Green, et al. 1947) but this difference would appear not to apply in the case of certain animals, e.g., sheep and goat (Grollman, 1963).

Regulation of iron absorption

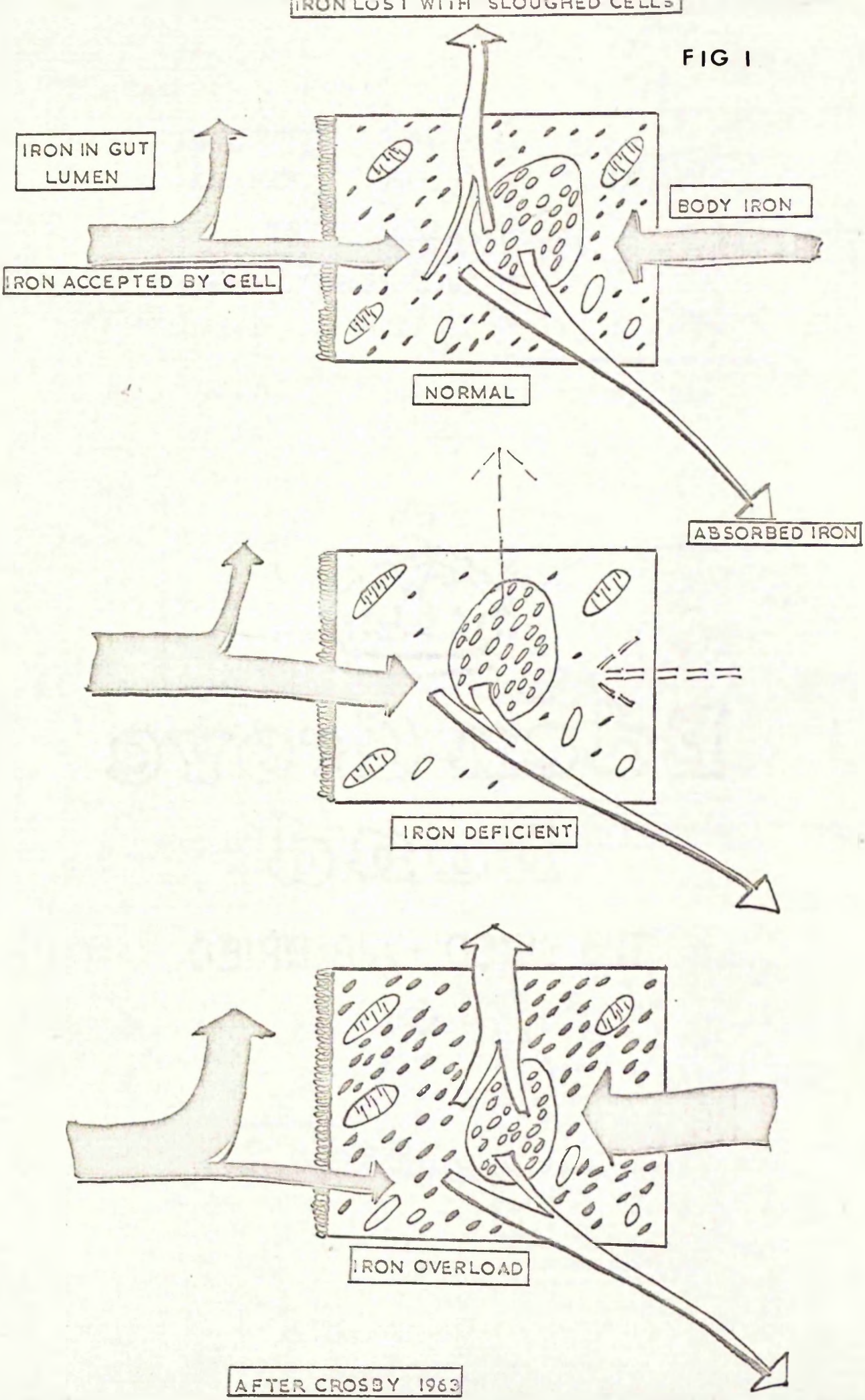
McCance and Widdowson wrote in 1937. "There are indeed indications that in man and certain animals that the bowel excretes practically no iron. If this is the case the amount of iron in the body must be regulated by controlled absorption". These same workers in 1938 published experimental evidence showing that, when humans were injected intravenously with large doses of iron, there was no significant increase in the total amount of iron excreted.

In 1943, Hahn, Bale, Ross, Balfour and Whipple, published evidence of increased iron absorption in humans suffering from iron deficiency anaemia and suggested "the mucosal block theory". This theory requires the presence of an acceptor for iron within the mucosal cells. They thought that this acceptor might be apoferritin and when saturated with iron would prevent the entry of more iron into the body. Granick (1946) published evidence in support of this theory in that, when he administered large doses of iron to guinea-pigs, ferritin could be crystallised from the mucosal cells of the intestine of these animals. On the other hand, Brown, Dubach and Moore (1958) using radioactive iron in humans concluded that the mucosal block theory was artificial, since the amount of iron required to stop absorption was far above that ever found in the diet of a normal person. They also claimed that the theory of a mucosal block did not account either for the higher rate of absorption or the greater amounts absorbed by people with iron deficiency anaemia. Heinemeyer (1956) reported that in long term experiments in which 15 mg. of ferrous iron were given daily to humans for twenty eight days, liver iron continued to increase up to the end of the experiment. This fact he felt proved conclusively

that there was no block preventing further absorption of iron. In 1961, Stewart and Gambino using radio-active iron found that the initial fast rate of absorption was not maintained throughout the period of absorption, but became markedly reduced by the end of the period. This reduction in the rate could not be completely explained by the passage of the diet along the intestinal tract or by the fast rate of removal of the iron from the cells by the circulating plasma, and they suggested that some regulating mechanism must be present within the mucosal cells themselves. Brown and Rother (1963) studied the mechanism of iron absorption in the rat. They measured the uptake of radio-iron by the upper small intestinal mucosa, its subcellular fractions and various other tissues. They further fractionated the iron particular fraction of the intestinal mucosa into protein and non-protein parts, and isolated the iron binding materials in each. They found two pathways of uptake. One an early rapid form of transfer which was associated with the amino acids glycine and serine. Secondly, a slow release mechanism with the iron bound to a protein which was not ferritin. Hartman, Conrad, Hartman, Joy and Crosby (1963) using the electron microscope identified ferritin in the absorbing cells of

the jejunum from iron replete humans. In a few sections, the iron was found scattered throughout the cytoplasm, but for the most part the ferritin molecules were contained within inclusion bodies in the cytoplasm. In 1965, Charlton, Jacobs, Torrance and Bothwell, agreed with the findings of Hartman, et al. (1963), in humans in that, when working with rats, they described a ferritin transport mechanism associated with the passage of iron through the intestinal cells. They did, however, also agree in part with the work of Brown et al. (1963) in that they identified an initial fast moving form of transport succeeded by a much slower form, but they could show no evidence that the first form was associated with either glycine or serine. In their work Charlton et al. (1964) identified the presence of ferritin by precipitation with a ferritin antiserum. On the other hand, Davies, et al (1959) using not so highly specific techniques of identification, failed to demonstrate the presence of ferritin. In a recent review, Crosby (1963) summarised the control of iron absorption by the intestinal mucosa as follows. (Figure 1). There are two routes taken by the iron entering the cells of the intestinal mucosa. First, the iron passes through the intestinal cells rapidly and enters the circulation and, secondly,

FIG 1


IRON LOST WITH SLOUGHED CELLS
IRON IN GUT LUMEN
BODY IRON
IRON ACCEPTED BY CELL
NORMAL
ABSORBED IRON
IRON DEFICIENT
IRON OVERLOAD
AFTER CROSBY 1963

some of the iron entering the cell combines with apoferritin within the intestinal cell to form ferritin. This author proposes that once the iron has become incorporated into ferritin it cannot be released but is permanently retained within the cell and later lost with the cell when it is exfoliated. In iron deficient patients, this second route is non-existent since intestinal cells maturing at this time do not synthesise ferritin. Any iron which is absorbed by these patients crosses the cell directly and enters the circulation. Crosby further postulates that in the case of the systemic-iron-overload subject the ferritin capacity of the absorptive cell has become saturated from the excess of iron already in the body. The shedding of these cells reprieves the overloaded system. In cases of haemochromatosis, he suggests a genetic inability to form ferritin and therefore, there is a direct route of entry of the absorbed iron into the circulation of these cases. The recent work of Smith, Drysdale, Goldberg and Munro (1966) takes this evidence a step further in that, in rats which have been given an injection of labelled leucine and then fed quantities of iron by mouth, an increased production of ferritin incorporating the labelled isotope can be demonstrated in the mucosal cells of the intestine of these animals.

Iron absorbed from the intestine and transferred to the circulation becomes attached almost immediately to the plasma protein transferrin (siderophilin). Transferrin is a B_1 globulin which carries the iron to and from its various sites within the body. Although it was recognised as early as the nineteen twenties that small amounts of iron were to be found circulating in the serum, it was not until 1949 that the iron carrying protein transferrin was recognised by Surgeron, Keechlin and Strong, working in America. Transferrin has a molecular weight of approximately 90,000 (Bothwell and Finch, 1962) and each molecule of transferrin can bind two atoms of iron in the ferric state (Laurell and Ingelman, 1947). The iron can be removed from transferrin by reducing it to the ferrous form (Davis, Salman and Benson, 1962). Shade, Reinhard & Levy (1949) demonstrated that the iron transferrin complex broke up below pH 6 and that there was an approximate 50% breakdown by pH 5, and by pH 4 complete breakdown of the complex had occurred. Studies carried out by Shiraswa (1964) using human serum demonstrated that, in vitro, the optimum conditions for the binding of iron to transferrin were using buffers of ionic strength less than 0.05 and a varying pH between 7.5 and 7.0. When dialysis was carried out against buffers of a relatively low ionic strength for long

periods, denaturing of the protein occurred. Shirasawa also demonstrated that binding was inhibited when buffers of a relatively high ionic strength were used. Binding was also inhibited to some extent in the presence of chelating agents such as diaminoethanetetra-acetic acid.(E.D.T.A.) Cleton, Turnbull and Finch (1963) showed that, in cases where the circulating plasma was completely saturated with iron, any chelating agents present such as E.D.T.A. would bind the available free iron. Azari and Feeney (1958) showed the similarity between transferrin in the plasma and the iron containing protein found in egg albumin called conalbumin. They also demonstrated that both these proteins were much more resistant to denaturing by heat and hydrolysis by chymotrypsin than the metal free proteins. Since iron binding would appear to be the property specific to transferrin in the circulating plasma, and since iron cannot exist unattached for any length of time in the circulating plasma, it follows that the amount of transferrin in the plasma represents the amount of iron that can be transported by the plasma. Transferrin in humans is normally one third saturated with iron (Laurell, 1947). In iron deficiency anaemia, the saturation value may drop as low as 15% and in severe cases even lower. In 1961, Morgan reported a decrease in serum iron levels in rats

and rabbits towards the end of pregnancy, and a marked increase immediately after parturition. This increase apparently does not occur in humans. Total Iron Binding Capacity levels (T.I.B.C.) (i.e. the total amount of iron that could be transported by the serum were all the available transferrin per 100 ml saturated) increase in the rabbit and human during pregnancy. This increase, however, does not occur in the rat, and Morgan (1964) suggests that this might be due to the fact that appreciable quantities of transferrin cross the placental barrier in the rat entering the foetal circulation. In the rabbit and human, on the other hand, only small amounts cross the placenta. In recent years many workers have been engaged in the study of the mechanism involved in the transfer of the iron from transferrin to the various types of cell. Jandl, Inman, Simmons and Allen (1959) reported that radio-active iron injected intravenously in humans was found in the circulating reticulocytes within a few minutes and this has been confirmed by the subsequent work of Jandl and Katz (1963). They reported that iron bound transferrin had much more attraction for circulating reticulocytes than did iron free transferrin, and suggested that the iron bound transferrin of the serum selectively attaches to the surface of the reticulocyte allowing the active removal of the iron which then enters

the cell. The iron free transferrin is then displaced from the cell membrane by iron bound transferrin. The dislodged transferrin acquires more iron from the body stores or from the mucosal cells of the intestine and the plasma-to-cell cycle is repeated. Jandl and Katz demonstrated by labelling the transferrin with iodine^{131} that it did not permeate the cell membrane but remained attached to the cell membrane throughout the transfer process. Morgan (1963) also studied the exchange of iron across endothelial membranes in the rat and rabbit. In the rat he showed that absorption of iron from the peritoneal cavity involved the participation of transferrin. It was also demonstrated that in the rabbit the iron remains bound to transferrin during its passage through the capilliary walls and that the transferrin bound iron leaving the plasma in the liver, spleen and bone marrow did so by ready transport through the fenestrated sinusiodal walls.

During the last five years, several workers have suggested that transferrin is not the only iron containing compound in the serum of rabbits and humans. (Faber and Jordal, 1961; Bern, Monti and Gunn, 1963). These contentions, however, have not been universally accepted.

Hossain and Finch (1964) concluded that the previous workers had been mislead by contamination of the serum with haemoglobin. In all species, most of the iron carried by the transferrin of the circulation is delivered to the bone marrow to be used in haemoglobin synthesis. In pregnancy, however, a large proportion of the iron is transferred to the foetus. In humans, 50% of the body iron is present as circulating haemoglobin (Hahn, 1937). However, in some animals, notably the dog and horse, the proportion of the total body iron present as haemoglobin is materially lower. This is probably due to the presence of high levels of myoglobin. Haemoglobin has a molecular weight of 68,000 and each molecule contains four atoms of iron (Drabkin, 1951). Its iron content is 0.34% of the total molecule. The iron released from the effete red blood corpuscles is for the most part stored in the liver and spleen (Hayes, Bothwell and Finch, 1960), the daily requirements of the bone marrow being met by the plasma iron turnover.

The next most important destination for the transferrin bound iron of the circulating plasma is the storage depots of iron found for the most part in the liver and spleen.

Storage occurs in two main forms, a) ferritin and b) haemosiderin.

Ferritin is a protein complex having a specific yellowish-brown colour. It was first isolated by Laughburger (1937), from the liver of a horse. As much as 23% of the ferritin complex is iron. Ferritin has a molecular weight of 400,000. Recent electron microscope studies (Hartman, et al. 1963) indicate that the iron within the protein molecule adopts a specific pattern. The protein portion of ferritin is apoferritin and this has the same iso-electric point as ferritin. Ferritin has been demonstrated in the cells of the placenta of both humans and rabbits during passage of iron from mother to foetus (Wohler, 1955), and in the erythroblast (Bessis and Breton-Gorius, 1959). While ferritin has been accepted as being present in the spleen and liver of most species, Kaldor (1953), working with sheep and cattle, failed to demonstrate its presence in the spleen and liver of either of these species. He suggested, therefore, that care should be taken in assuming that ferritin played the same role in all situations. Ferritin is soluble and in this respect differs from the other storage form of iron, haemosiderin. Haemosiderin is also composed of iron

attached to the protein apoferritin but in the case of haemosiderin there is much more iron present in the complex than in the case of ferritin. Haemosiderin appears as golden yellow granules in tissue sections and smears. Granick (1949) suggested that haemosiderin represented iron in excess but Sheden, Cabric and Finch (1953) felt that this was an over-simplification of the situation. Haemosiderin is still demonstrable in cases where humans have suffered a recent loss of blood through haemorrhage and are showing dangerously low serum iron levels. It is obvious, therefore, that the part played by haemosiderin in iron metabolism within the body is far from understood.

There are several other iron containing compounds found within the body but there is still confusion as to the function of the iron in these compounds and no great benefit would be gained from discussion of them at this stage.

Only very small quantities of iron are excreted, and mainly by way of the nails, sweat and urine. The largest quantities may be lost where there is extensive haemorrhage, e.g., in bowel ulceration or at parturition.

Most of the iron found in the excreta from the bowels has not been absorbed, or has only been absorbed into the mucosal cells of the intestine and shed into the intestinal lumen when these cells are exfoliated. A significant amount of iron is lost daily through the desquamated skin cells. Hawkins and Hahn (1944) showed that 0.1 to 0.2 mg. of iron are excreted in the bile. In women there is a regular loss of iron during the monthly menstrual period, but this does not occur in animals. The pro-oestral bleeding which occurs normally twice yearly in the bitch is not a cause of great blood loss.

Placental Transfer of Iron

For many years it was accepted that the red cells were the main source of foetal iron. These were supposedly broken down in the placental labyrinths, and the iron released. In 1942, Pommerenke, Hahn, Hale and Balfour, using radio-active iron in pregnant women near term, showed that the iron reached the foetus so quickly that the source of foetal iron must be other than the maternal cells. Similar results were obtained by Vosburgh and Flexner (1950) working with guinea pigs. These workers went even further

and reported that the iron was transported in the maternal serum by a plasma protein fraction and that the same fraction was responsible for transporting the iron in the foetal circulation. Hagberg (1953) identified the B_1 globulin transferrin as the specific carrier of iron in both maternal and foetal circulations. In 1958, Bothwell, Pribilla, Meburat and Finch, investigated the transfer of iron from mother to foetal rabbit and demonstrated that the amounts increased as gestation advanced. The age and weight of the foetus played a part in the amount of the iron transferred to each. By the end of pregnancy, 90% of the plasma iron turnover was transferred to the foetus. The transport of iron across the placenta in the rabbit takes place against a concentration gradient. They also showed that the uptake of iron by the placenta was an active process. They concluded that the amount of iron in the serum played a part in determining the amount of iron transferred. They also concluded that, since the plasma transferrin in the foetus is constantly saturated, this played no part in attracting the iron across the placenta. This work has been subsequently confirmed by Davies, Brown, Stewart, Terry, and Sisson (1959). In addition, these workers demonstrated a dramatic rise in

the amount of iron transferred at day 20 of gestation and showed that the amount transferred continued to increase to term. They found that the greatest proportion of the transferred radio-active iron was in the yolk sac placenta during the first third of gestation and that in the later stages of gestation the greatest amounts were to be found in the foetal liver. They also found that during the later stages of pregnancy the iron was transferred solely by the allantoic placenta. They concluded, therefore, that the part played by the yolk sac in the early stages of gestation was that of a storage organ, a function which was later taken over by the foetal liver as pregnancy advanced.

Nylander, in 1953, studied the placental transfer of iron in the rat and demonstrated the importance of the yolk sac placenta in this species. Laurell and Morgan (1964) carried out a series of in vitro experiments using slices of rat placenta. They investigated the mechanism which operates when the iron is being transferred from the serum to the placenta and found that the placenta took up transferrin irrespective of whether or not it contained iron. They also found that the uptake of iron by the placenta was not governed by the percentage saturation of the medium.

When they added enzyme inhibitors, e.g., sodium cyanide, sodium fluoride and sodium arsenite the amount of iron absorbed was decreased. Sodium cyanide, however, was the only inhibitor which had any effect on the ability of the yolk sac placenta to take up iron. Laurell and Morgan, also demonstrated the presence of ferritin in the placental cell of the rat but only in very small quantities. They felt that the amounts were too small to be of significance in the transfer mechanism. They concluded that the high concentration of transferrin in the serum of both humans and rabbits during the later stages of gestation aids iron transfer by inhibiting the laying down of storage iron, thereby allowing a greater amount of iron to be circulated and subsequently transferred across the placenta. Bothwell and Pribilla in their work of 1958 had reported a decrease in maternal erythropoiesis towards the end of pregnancy.

The work to be described in this thesis is a study of the mechanism involved in placental transfer of iron across the chorio-allantoic placenta in the rabbit using subcellular fractionation by centrifugation, gel filtration and electrophoretic techniques.

6 The term placenta used throughout the thesis refers to the chorio-allantoic placenta of the rabbit.

SECTION ONE

The study of

a) the haematology

b) the Serum Iron and Total Iron Binding Capacity levels

in a series of the rabbits used.

Introduction

Female rabbits of several breeds were used, all mating was observed and recorded. Pregnancy diagnosis by abdominal palpation was found to be reliable from the ninth day. To establish normality of the rabbits, blood samples were taken from a group of rabbits immediately before use. Routine haematology and serum iron estimations were carried out on these samples.

Six of the rabbits used were sampled at intervals from the fourteenth day of term to establish any change taking place in their haemoglobin, serum iron, and T.I.B.C. levels during this period.

Experimental

Blood samples were obtained from a small lengthwise incision in a marginal ear vein. When this method was adopted it was not necessary to dilate the vein by local application of xylol. The blood was collected in heparinised tubes. Haemoglobin concentration, packed cell volume, total white cell counts and differential white cell counts were carried out on each sample. The serum iron estimation and T.I.B.C. levels were carried out by the method of Ramsay (1955) (see appendix of methods and techniques).

Results and Discussion

All the haematological results from the twelve rabbits examined fell within the range of the results reported in the literature (Table 1). It would seem, therefore, that with regard to their haematological values, the rabbits examined were normal.

The results of the serum iron estimations are shown in Table 2. Since little indication as to the normal levels of these two estimations is found in the literature no comparisons could be drawn. However, the more important point in this work with regard to these results was to confirm that the amount of iron being injected during experimental procedures could be bound to the serum protein transferrin. The amount of iron injected either as ferric citrate or ^{59}Fe never exceeded 20 µg. The results shown indicate that in these instances this amount would certainly become bound by transferrin since the saturation level of the samples examined was never higher than 70%.

TABLE 1.

Comparison between haematological results of Archer (1965); Wintrobe (1936); Casey (1936); Scarborough (1931) and the results collected from twelve rabbits.

No. of rabbits	Hb.	P.C.V.	W.C.C.	M.C.H.C.	Differential				
					N.	L.	E.	B.	M.
12	13.3	38	7,000	37	42	54	0	1	3
	10.4-18	33-44	5,000-11,000	33 -44	23-79	18-71	-	0-4	0-4
Not stated (Casey, 1936)	11.9	-	7,700	-	49.4	32.9	1.5	6.7	9.5
Not stated Males only (Scarborough,1931)	-	-	7,900	-	43.4	41.8	2	4.3	9
Not stated (Archer, 1965)	12.0	40	9,000	36	-	-	-	-	-
Not stated (Wintrobe, 1936)	13.0 ± 4.3	39.8 ± 1.5	-	33 ± 1.7	-	-	-	-	-

TABLE 2

Mean Serum Iron and Total Iron Binding Capacity levels from Twelve Rabbits 11 - 26 days pregnant.

Serum Iron ug %	T.I.B.C. ug %	% Saturation
173[x]	361	51.6
132 - 246[xx]	204 - 438	32 - 75

x = Mean

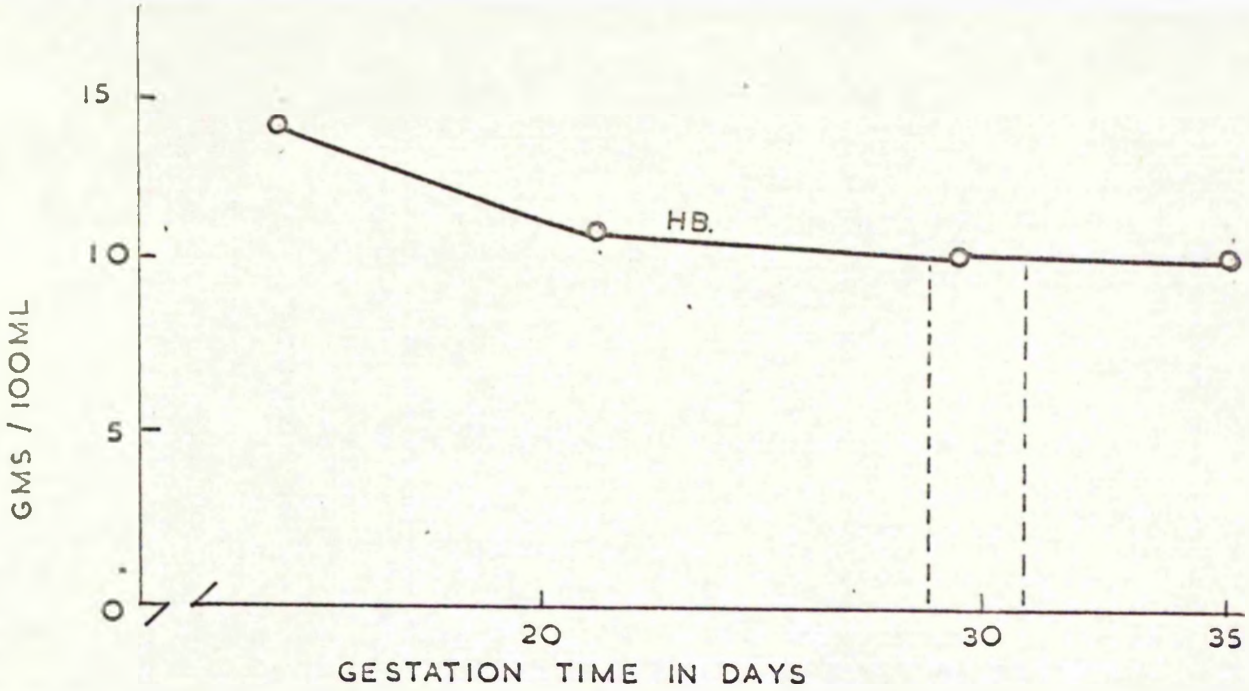
xx = Observed range

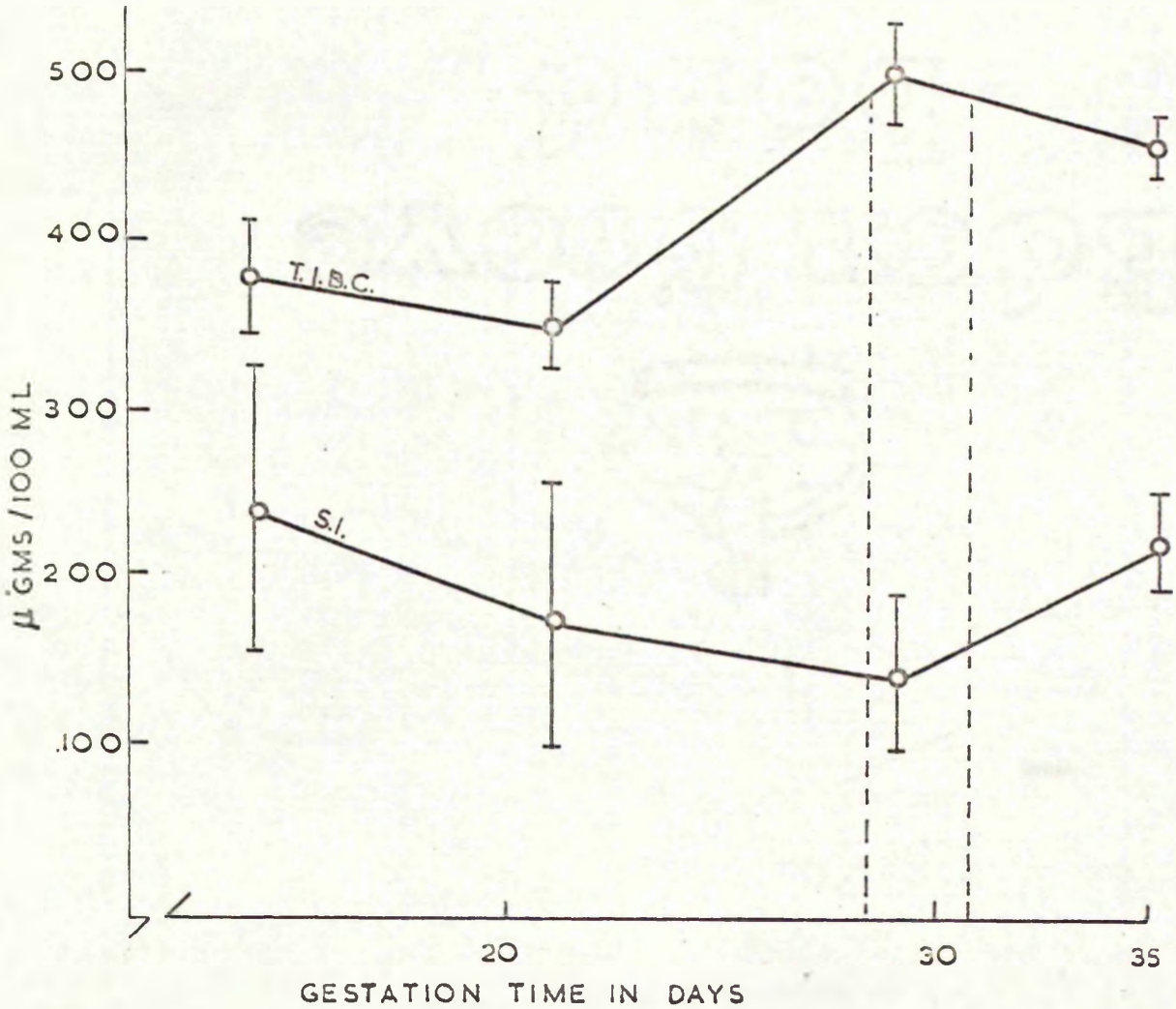
The results of the estimation of haemoglobin, serum iron and total iron binding capacity levels carried out on blood samples collected from six rabbits at periods throughout gestation are seen in Figures 2 & 3. The first graph shows the means of the six samples at each of the sampling dates and the vertical lines represent $\pm$ one standard deviation of the mean. The two other graphs in Figure 3 represent the T.I.B.C. and the serum iron levels shown in the same manner. These results as shown in the various graphs suggest a) there is a slight drop in the haemoglobin levels towards the end of gestation, b) a rise in the T.I.B.C. levels just before parturition, and c) slight drop in the serum iron levels towards the end of pregnancy. However, when the student "t test" was applied it was found that a) there was no significant difference between the haemoglobin levels just prior to parturition, b) nor was there any significant difference between the points 2 and 3 of the serum iron graph, but c) there was a highly significant difference in the Total Iron Binding Capacity levels between day 20 and 30 of the graph.

FIGURE 2 Mean levels of haemoglobin at different stages of gestation.

FIGURE 3 Mean levels of Serum Iron (S.I.) and Total Iron Binding Capacity (T.I.B.C.) at different stages of gestation

Dotted lines represent period of parturition.


15
10
5
0
GMS / 100ML
HB.
20
30
35
GESTATION TIME IN DAYS


500
400
300
200
100
μ GMS / 100 ML
T.I.B.C.
S.I.
20
30
35
GESTATION TIME IN DAYS

Morgan (1961) using seven rabbits and comparing those with eight non-pregnant rabbits reported a decrease in the blood haemoglobin and plasma iron levels towards the end of pregnancy and a considerable increase in the plasma iron immediately after parturition. He also reported a considerable increase in the T.I.B.C. levels in rabbits in the last few days of pregnancy.

SECTION TWO

A. Development of Extra-embryonic Membranes in the Rabbit.

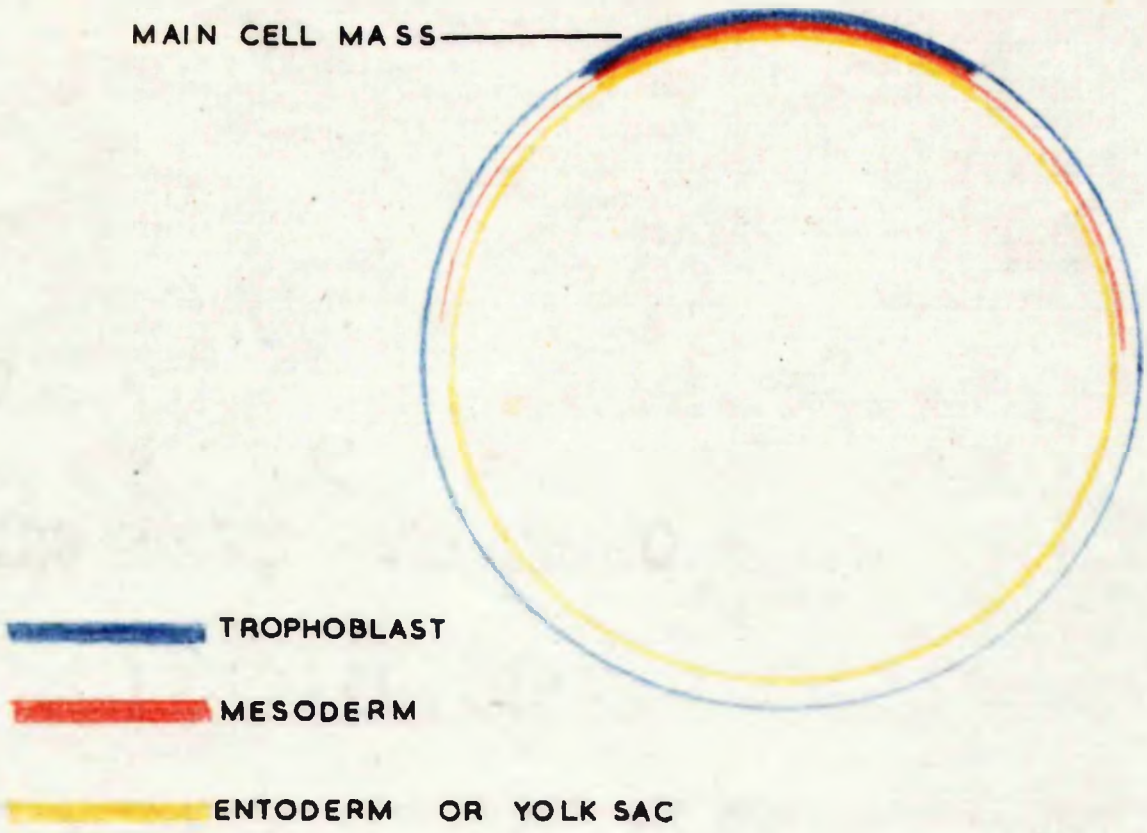
B. A Study of the Histology and Ultra-structure of the Rabbit Placenta.

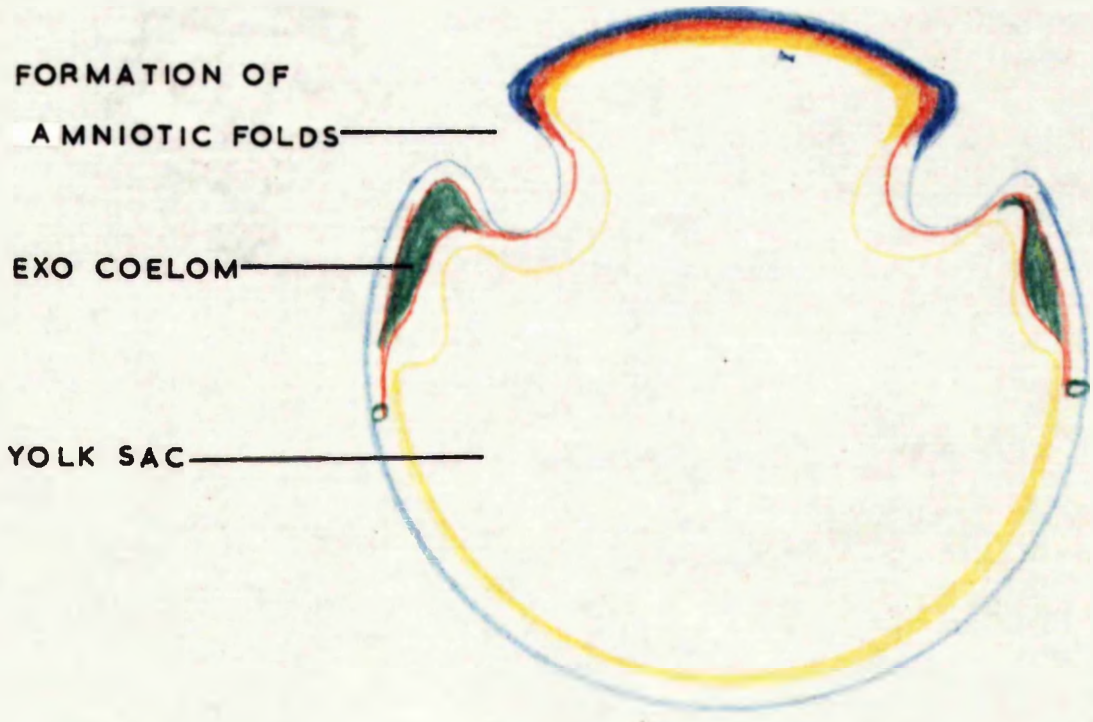
C. Autoradiography in the Rabbit Placenta.

D. Histochemistry of the Rabbit Placenta.

The fertilised ovum of the rabbit reaches the uterus four days after coitus. At this stage it is still surrounded by the zona pellucida and the albumin coat. Those two layers become increasingly thinner and two days later disappear so that the trophoblastic layer of cells which comes into contact with the endometrium of the uterus. The blastocyst consists of an outer layer of cells (trophoblast) which contains the inner cell mass, the latter being a flattened disc of cells in the meso-metrial region. From the inner cell mass arises the entoderm, giving origin to the yolk sac, and the mesoderm (Figure 4.). The mesoderm grows between the entoderm and trophoblast till it reaches half way round the circumference of the blastocyst. The mesoderm is split into two layers by the formation of the exocoelom and the area of the mesoderm nearest the embryo becomes the area vasculosa. With the separation of the mesoderm into two layers the inner vascular layer becomes closely applied to the upper portion of the yolk sac giving rise to the area vasculosa of the yolk sac. The lower area of the yolk sac is a-vascular. This is the stage reached seven days after coitus when implantation occurs. Implantation takes place in the anti-mesometrial region. The blastocyst

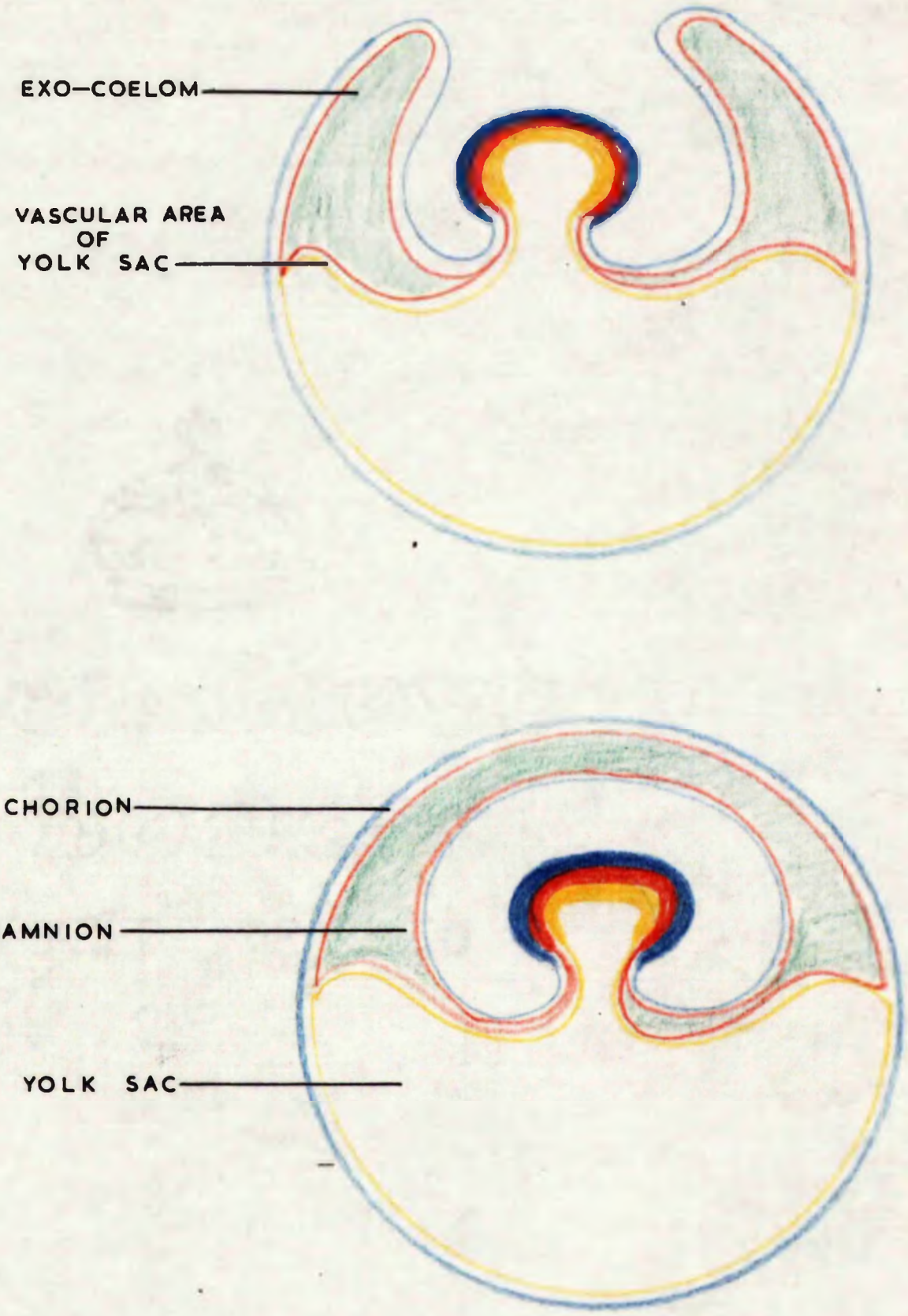
FIG 4


MAIN CELL MASS
TROPHOBLAST
MESODERM
ENTODERM OR YOLK SAC


FORMATION OF
AMNIOTIC FOLDS
EXO COELOM
YOLK SAC

comes into contact with the obplacental folds, which are thickenings projecting into the uterine lumen in this area. These folds become shortened forming a slight depression in which the blastocyst rests. The blastocyst then increases rapidly in size until it fills the entire diameter of the uterus and comes in contact, in the mesometrial region, with the placental folds, which are similar in form and placed opposite to the obplacental folds. The trophoblast with the closely applied non-vascular yolk sac makes contact with the mucosa of the endometrium, which becomes greatly swollen and vascular. By the ninth day the trophoblastic cells overlying the yolk sac in this region disappear, and the outer surface of the non-vascular yolk sac comes to lie against the very vascular region of the endometrium. Meanwhile, the exocoelom increases in size and the amniotic folds appear (Figure 5). These are foldings of the trophoblastic cells and the layer of mesoderm, around the embryo. The two folds meet and fuse to form the amnion. The outer trophoblastic cells in this region, lying in apposition to the placental folds of the uterus, are the beginnings of the chrio-allantoic placenta. The exocoelom increases in size as a result of the growth of the amnion and the embryo.

FIG 5


EXO—COELOM
VASCULAR AREA
OF
YOLK SAC
CHORION
AMNION
YOLK SAC

This causes the vascular area of the yolk sac to become narrowed and pushed downwards so that eventually its inner surface meets the inner surface of the non-vascular area of the yolk sac. At day fourteen the non-vascular area disintegrates so that the inverted area of the vascular yolk sac is open to the uterine lumen, thus forming the inverted yolk sac placenta (Figure 6).

Meanwhile, the allantois has grown out from the entodermic layer of the embryo into the exocoelom and along with a layer of mesoderm extends around the amnion. Its outer surface comes into contact with, and fuses with, the inner surface of the chorion, forming the allanto-chorionic placenta (Figure 7).

The allantoic placenta is functioning by the twelfth day of gestation, and grows rapidly. The invading trophoblast induces a decidual reaction and placental folds are greatly increased in size, being now referred to as cotyledons. The uterine epithelium disappears at the ninth day of gestation and the placentation is now syndesmo-chorial. With the disappearance of the connective tissue on the tenth day, the placentation becomes endothelio-chorial. By the fourteenth day of gestation the invasion zone of the

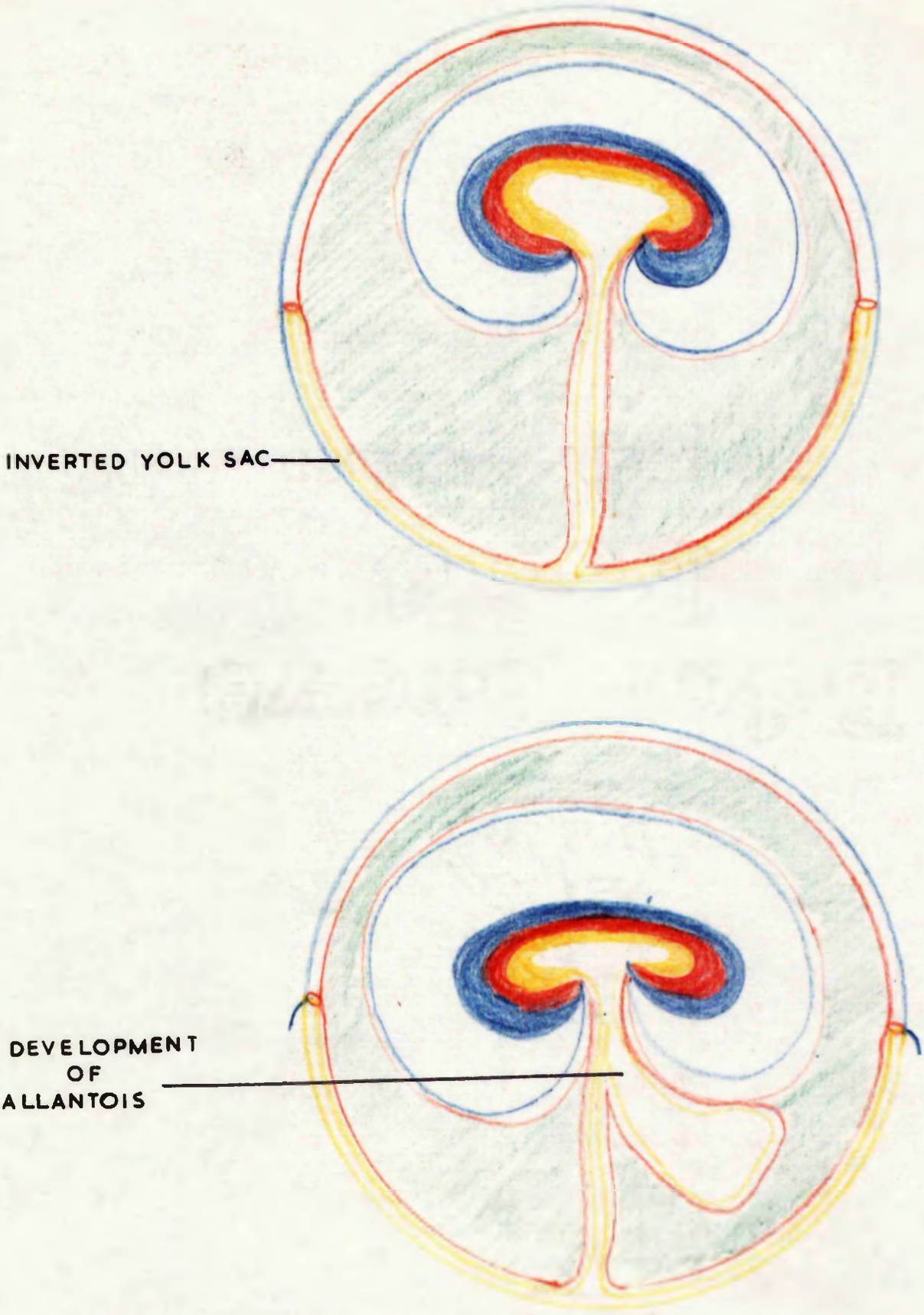
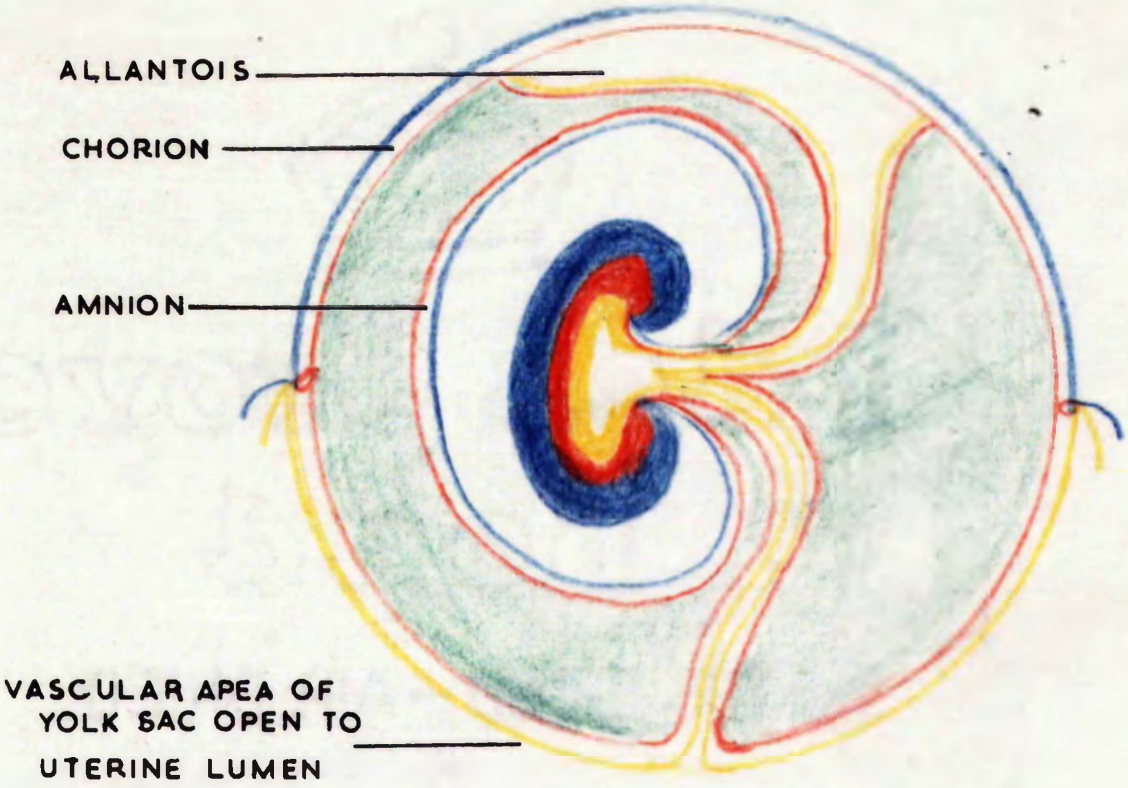

INVERTED YOLK SAC
DEVELOPMENT
OF
ALLANTOIS

FIG 7


ALLANTOIS
CHORION
AMNION
VASCULAR APEA OF
YOLK SAC OPEN TO
UTERINE LUMEN

placenta consists of foetal lamellae surrounding maternal blood, the maternal endothelium having disappeared. Placentation is now haemo-chorial. The trophoblast at this stage has two layers of cells, an outer syncytio-trophoblast and an inner cyto-trophoblast. The trophoblastic cells form column which are hollow tubes surrounding maternal blood, blind at the foetal end and open at the maternal end. In the second half of pregnancy the placenta is an elaboration of this with deeper penetration of the endometrium by these columns and the development of additional ones.

Amoroso (1952) using the light microscope described the disappearance of the cyto-trophoblast on the fourteenth day of gestation and of the syncytio-trophoblast at the twenty-second day of gestation. In his opinion, placentation becomes haemo-endothelial by the twenty-second day of gestation, i.e., the foetal endothelium being the single layer of cells between maternal and foetal blood. Wislocki & Dempsey (1955) Larsen (1962 & 1963) & Enders (1965) using the electron microscope, have recently reported that, although the trophoplastic cells undergo thinning from the fourteenth day to term, the two layers of cells persist so that the placentation remains haemo-chorial from the fourteenth day onwards.

Introduction

An adequate knowledge of structure is a necessary preliminary to any investigation of function. This is particularly so when dealing with the transfer of materials across the placenta.

The chorio-allantoic placenta of the rabbit has been classified as epithelio-chorial at the twelfth day of gestation and then undergoing progressive modifications until it finally becomes haemoendothelial by the twenty-second day of gestation, remaining so until term (Amoroso, 1952). Recently, two American workers using electron microscopy have reported that although the trophoblastic cells undergo thinning from the fourteenth day of gestation they persist to term so that placentation would seem to be haemo-chorial. (Larsen, 1962[a], 1963[b]; & Enders, 1965). In some cases the cells become so thin that they are only long tendrils of cytoplasm. Since there is still controversy as to the type of placentation present in the rabbit during the later stages of pregnancy, the opportunity was taken to carry out a complete histological examination of the rabbit placenta during the later stages of gestation using light microscopy and electron microscopy.

Experimental

A placenta was removed from the pregnant rabbits at either fifteen or twenty-five days of gestation. Each placenta was carefully dissected from its associated membranes and fixed in 10% formol, Kenkers Formol, or Bouin. The different fixatives were used in order to find the best fixative for placental tissue. While in fixative each placenta was quartered and each quarter dehydrated, cleared and embedded in paraffin wax. 5 μ sections were cut and stained in the normal way (see appendix of methods and techniques).

Electron microscopy

Originally 1 mm cubes of placental tissue (15 or 25 days) were removed from anaesthetised rabbits and fixed within two minutes in 1% buffered isotonic osmic acid (Zetterquist, 1956). Within two hours the tissue blocks were trimmed and placed again in 1% osmic acid for up to 30 minutes. The blocks were then washed in water, dehydrated through ascending grades of methanol, cleared in propylene oxide and embedded in aryldite (Luft, 1961).

On another occasion the uteri of two pregnant rabbits one fifteen and the other twenty-five days pregnant were perfused with 0.5% solution of gluteraldehyde (Muir, 1965). One mm cubes of placental tissue were removed and fixed in 1% buffered osmic acid and embedded in araldite as above.

Orientation was carried out by first cutting "thick" sections at 1 µ, staining with Toluidine blue and Pyronin, and viewing under the light microscope. Ultra thin sections were then cut on a microtome (L.K.B.) and stained with alcoholic lead acetate or uranul acetate.

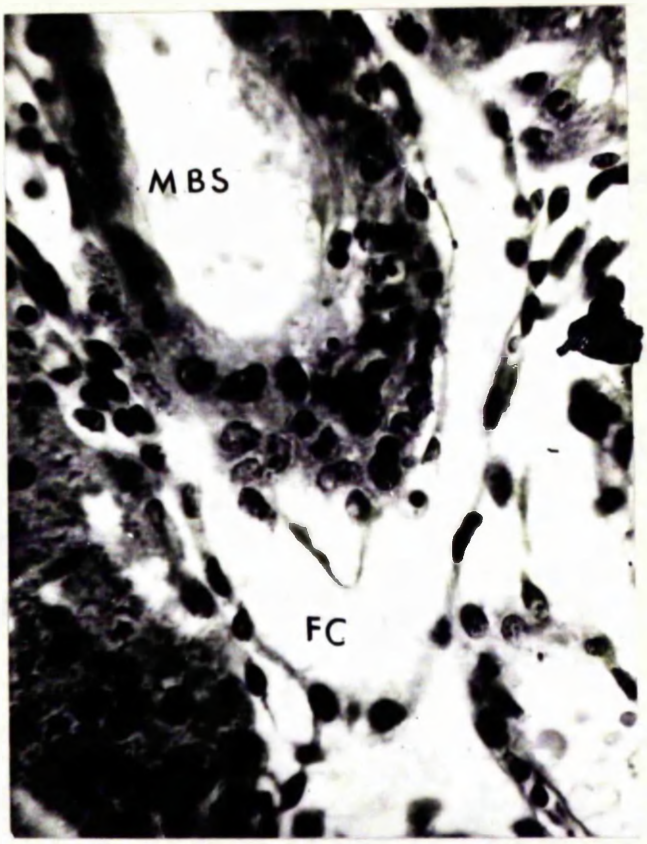
The histological sections shown in the photographs in Figures 8 - 12, illustrate thinning of the cellular barrier separating foetal and maternal blood between the fifteenth day and twenty-fifth day of gestation. It would appear from the histological sections, Figures 9 & 10, that only the endothelium of the foetal capillaries separates foetal and maternal blood. This is in agreement with the findings of Amoroso (1952). Incidentally, these sections demonstrate the considerable quantity of blood that is contained in the rabbit placenta at twenty-five days of gestation. This is of importance in the later investigation into the identification of the iron-containing compounds within the placenta.

The results of the examination of the ultra-structure of the rabbit placenta at day fifteen and day twenty-five of pregnancy are seen in Figures 13, 14, 15 and 16. In both instances three layers of cells are seen to separate maternal and foetal blood, i.e., two layers of trophoblastic cells and the endothelium lining the foetal capillaries. However, there appears to be a definite thinning of the trophoblastic cells by the twenty-fifth day of pregnancy and in some cases the cellular barrier consists of only

FIGURE 8

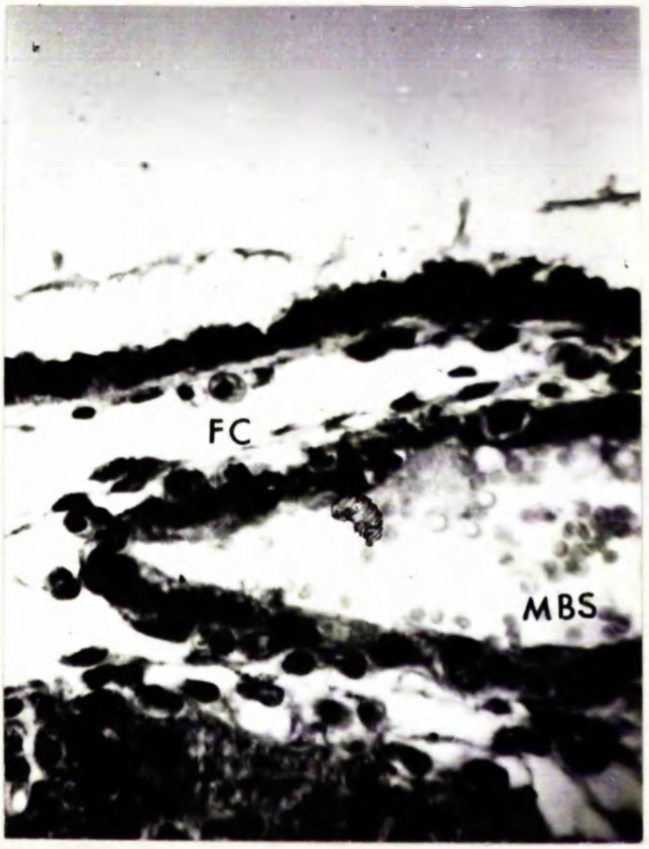
X450

Placental tissue from rabbit 15 days mated showing foetal capilliary lying against trophecto-derm which in turn surrounds a lake of maternal blood.



X 450

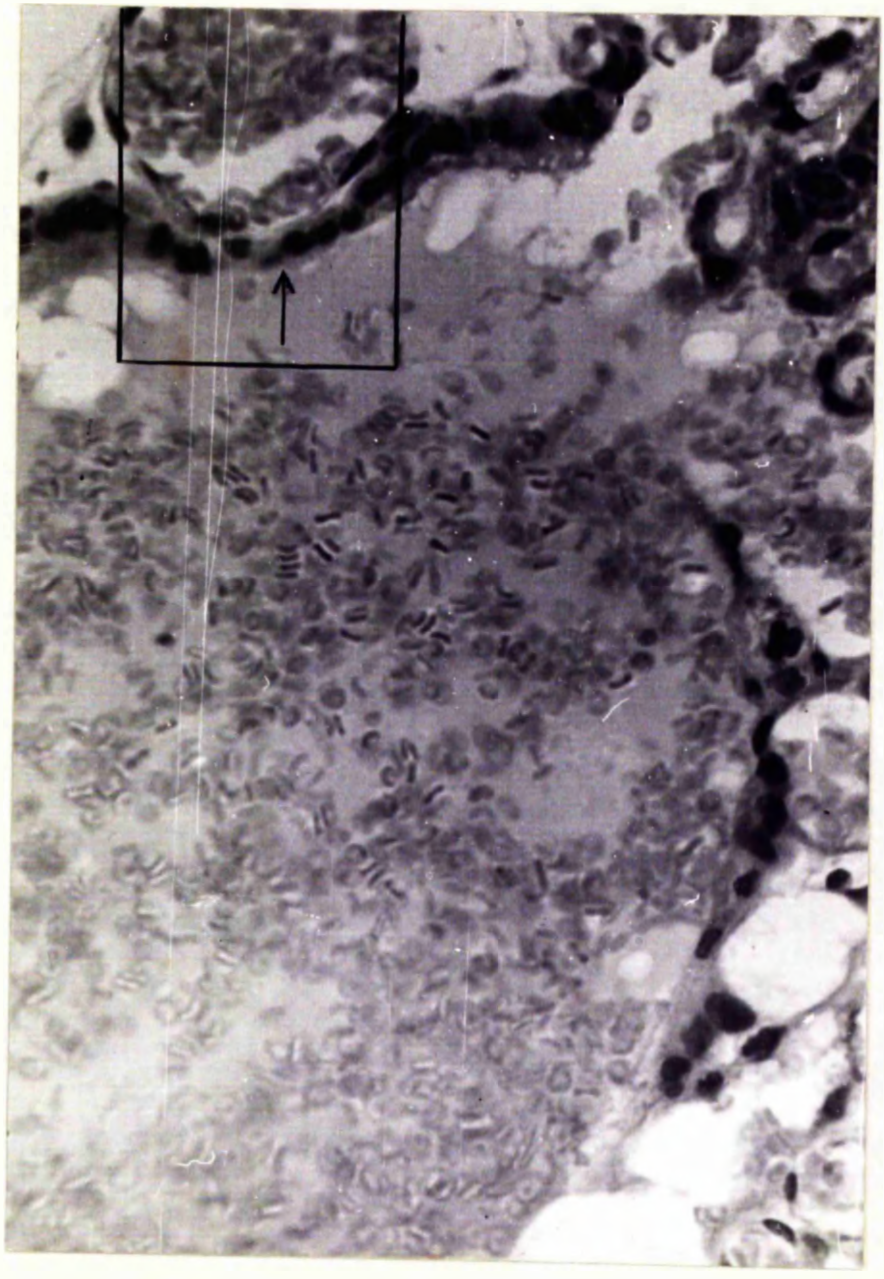
Also from a 15 day pregnant rabbit showing separation of foetal and maternal blood.



F.C. = foetal capilliary

M.B.S. = maternal blood space

FIGURE 9

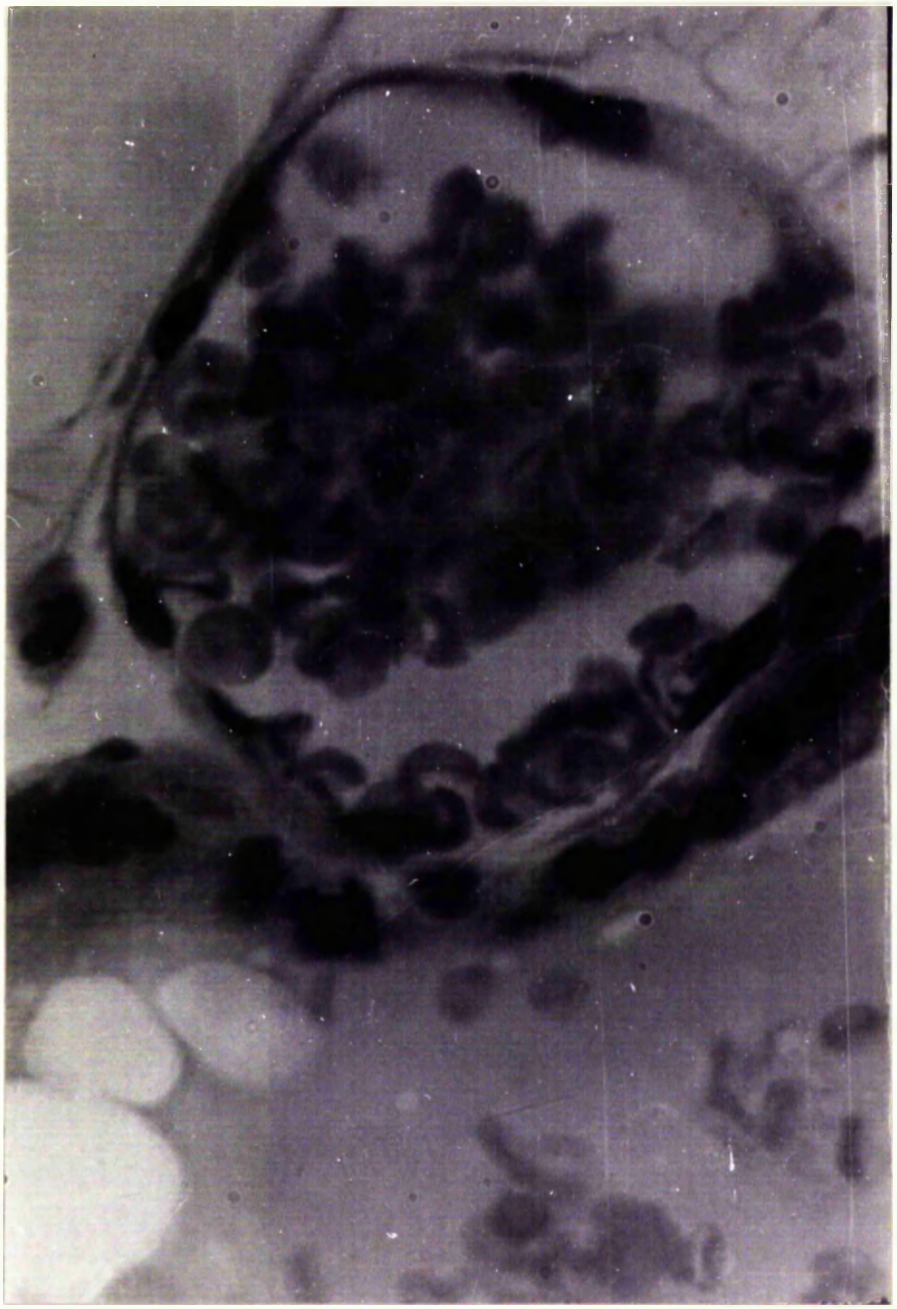


X 450

25 DAY RABBIT PLACENTAL SECTION.

TO ILLUSTRATE THE APPROXIMATION OF FOETAL AND MATERNAL BLOOD. ARROW INDICATES THINNEST AREAS OF TROPHOBLAST AND INTRA-EPITHELIAL FOETAL CAPILLIARY.

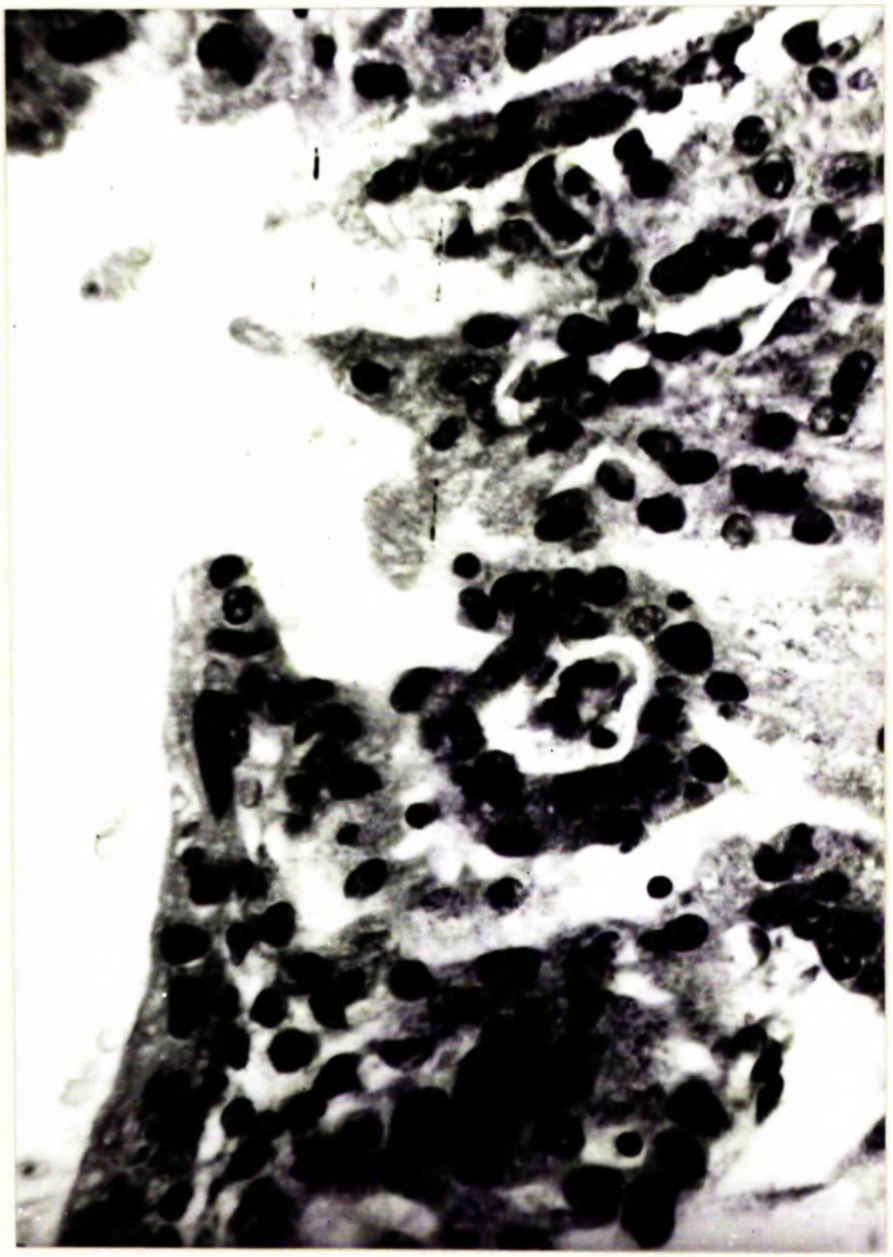
FIGURE 10



X 1500

HIGH POWERED VIEW OF FIGURE 9.

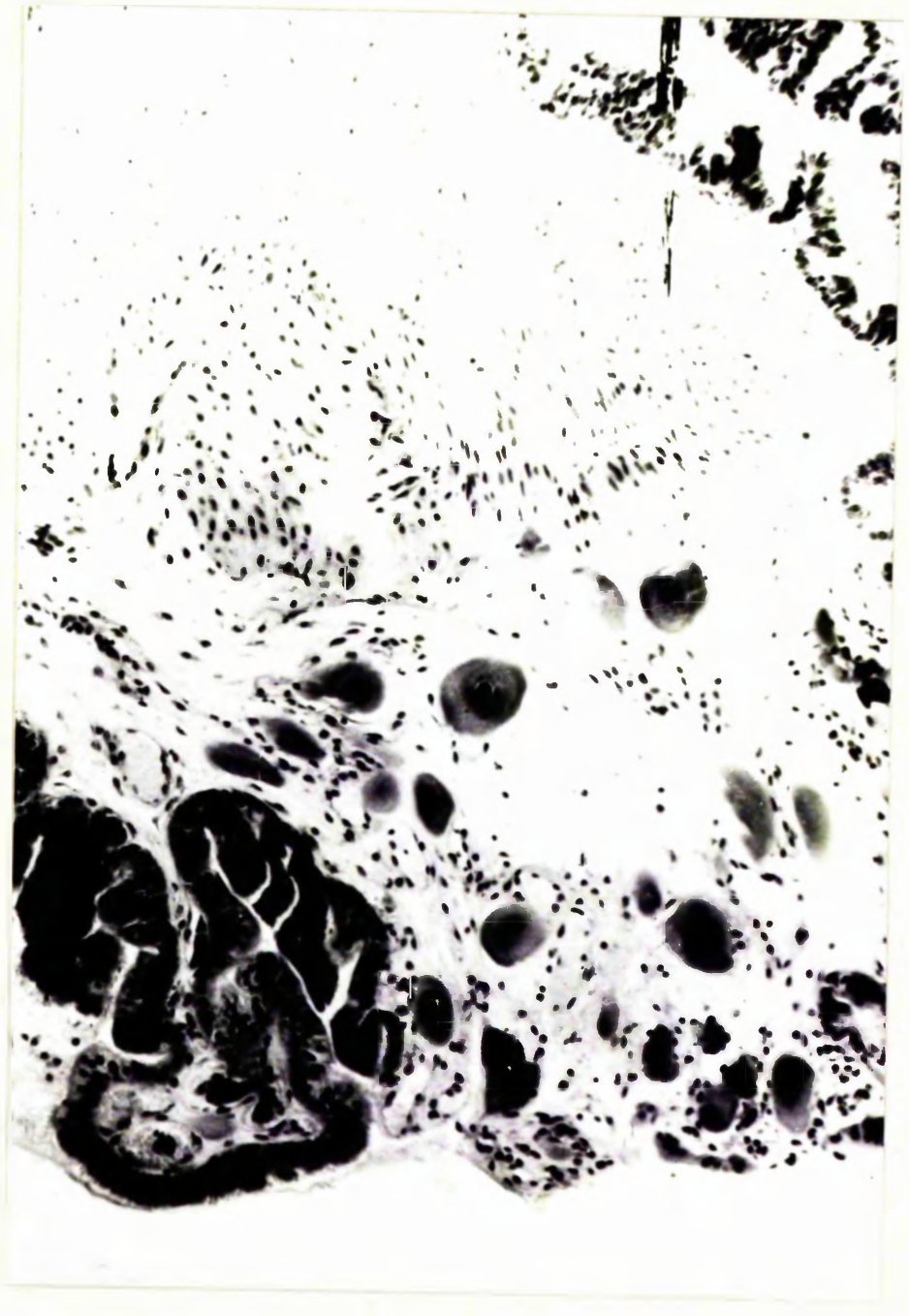
FIGURE 11



X 450

FIFTEEN DAY PLACENTAL SECTION SHOWING INVADING FOETAL CAPILLARY.

FIGURE 12



15 DAY PLACENTAL SECTION .

PERIPLACENTAL AREA SHOWING GIANT DECIDUAL CELLS PRESENT IN MATERNAL EPITHELIUM.

FIGURE 11

X 5000

ELECTRON MICROGRAPH OF PLACENTAL SECTION OF RABBIT

18 DAYS PREGNANT.

B = Maternal Blood Space

F = Invading Foetal Capillary.

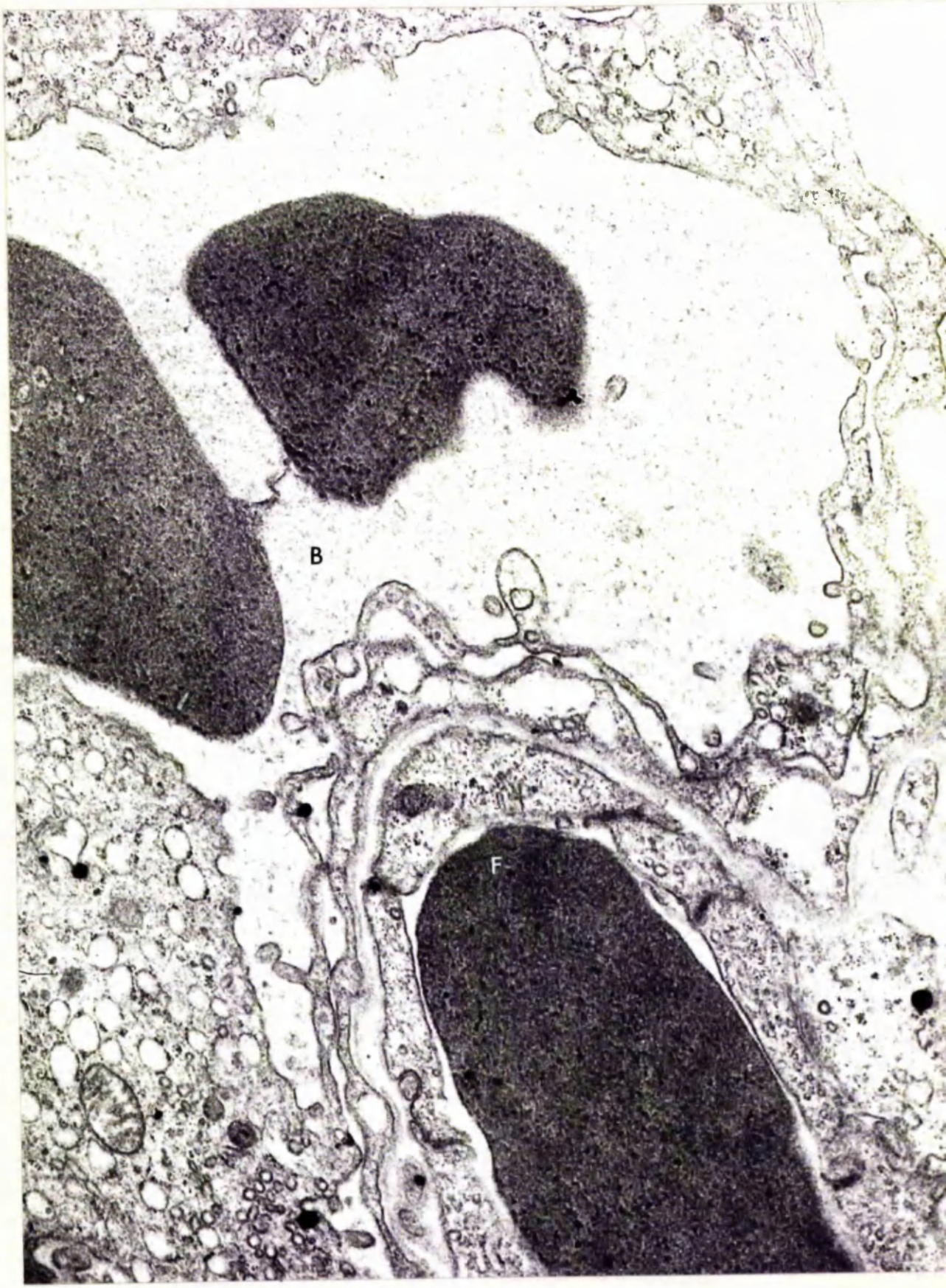

B
F

FIGURE 14

X 5000

ELECTRON MICROGRAPH OF 18 DAY RABBIT PLACENTAL SECTION.

B = Maternal Blood Space

F = Invading Foetal Capillary.

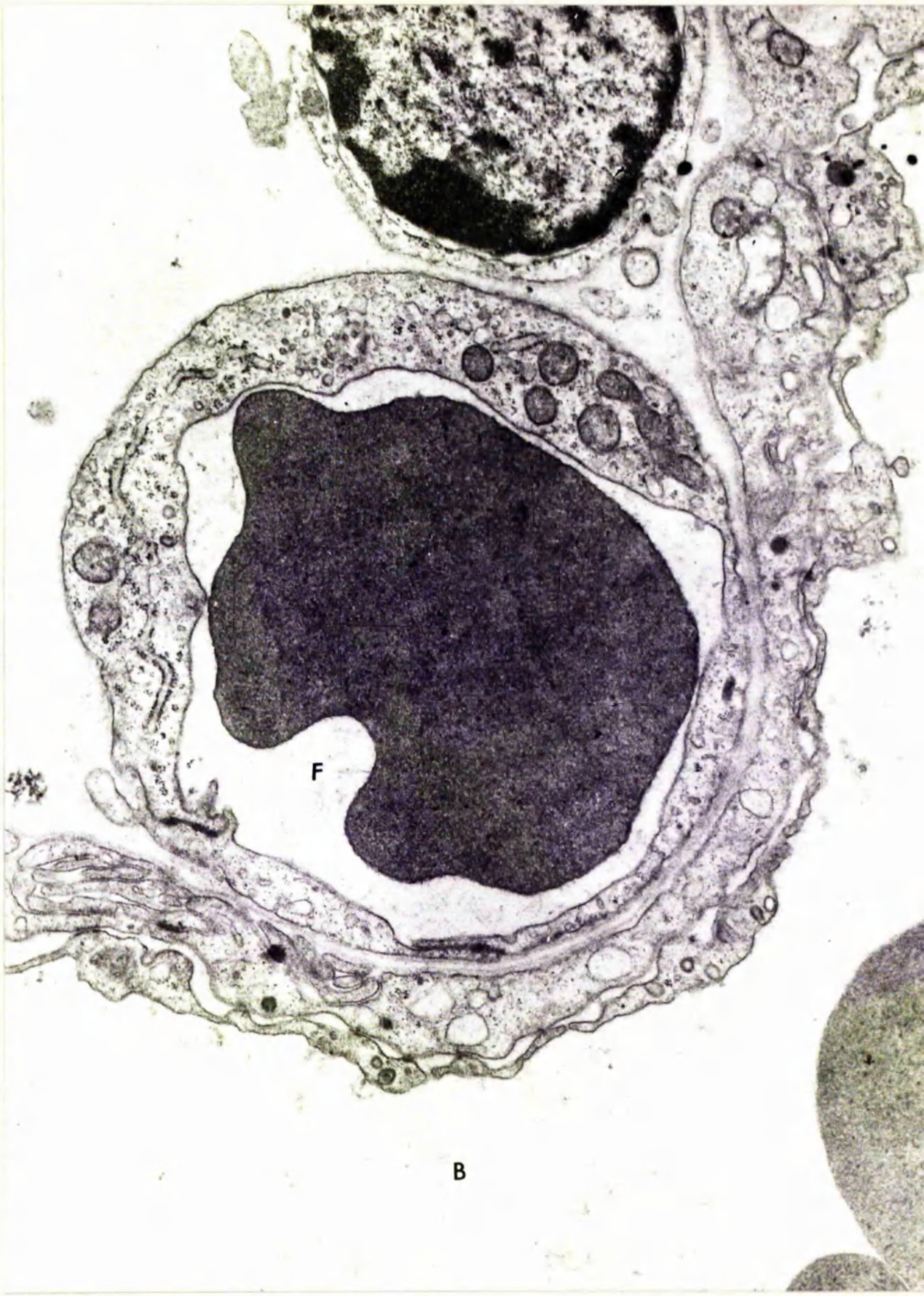

F
B

FIGURE 15

X 13,750

ELECTRON MICROGRAPH OF 28 DAY RABBIT PLACENTAL SECTION

B - Maternal Blood Space

F - Invading Foetal Capillary.

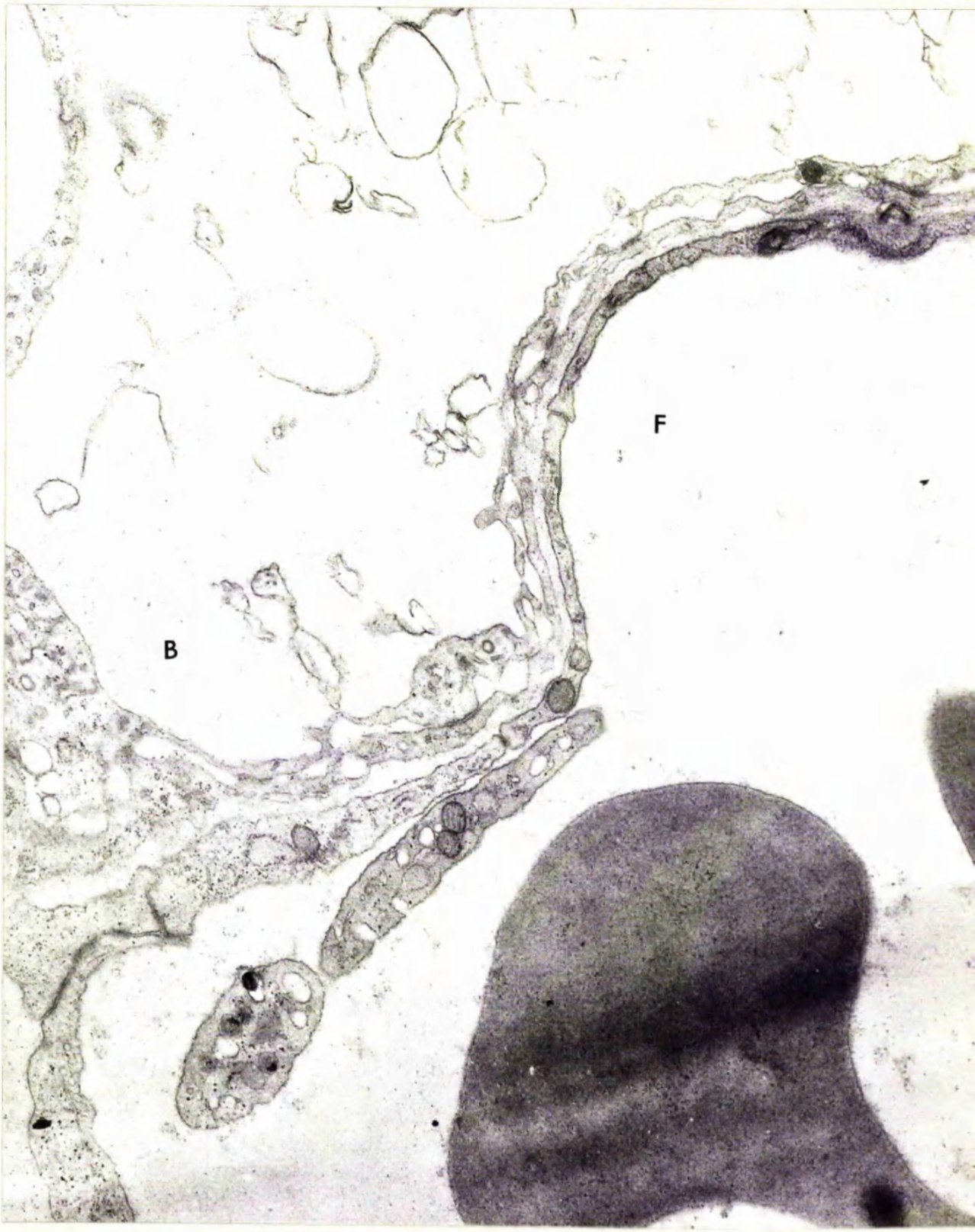
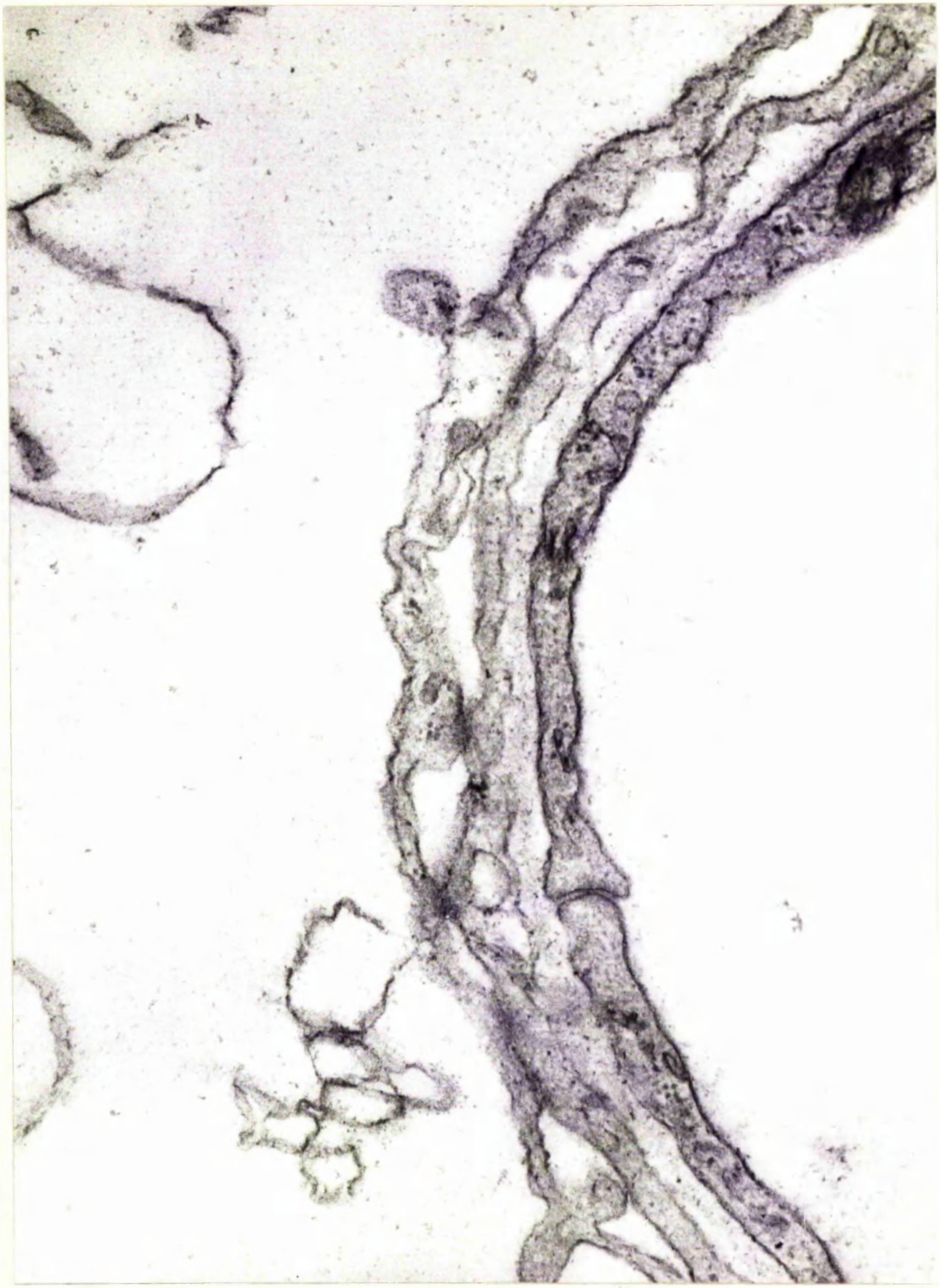

F
B

FIGURE 16

X 55,000

ELECTRON MICROGRAPH OF 25 DAY RABBIT PLACENTAL SECTION.

ENLARGEMENT OF THREE CELLULAR LAYERS SEPARATING MATERNAL AND FOETAL BLOOD.



tongues of cytoplasm (Figure 16). In interpreting electron photomicrographs one must bear in mind the very limited areas that are being scanned in each field since these sections are 800 Å thick. It follows that these findings together with the recent reports by Larsen (1962 & 1963) and Enders (1965) must be viewed with some reservation. On the other hand, the areas shown in Figures 15 and 16 are those in which obvious thinning was seen to have taken place when viewed at the lowest power of the electron microscope. The delicate nature of the cellular strands separating the maternal and foetal blood indicate that only the electron microscope would be capable of demonstrating their existence. Bothwell, et al.(1958) reported that the dramatic increase in the rate of placental transfer of iron in the rabbit takes place from the twentieth day of gestation. It would seem, therefore, that this cannot be explained by the disappearance of cells separating the maternal and foetal blood although the obvious thinning of these cells might well play a part.

When preparing and obtaining the placental tissue from rabbits at different stages of gestation these sections

prepared from fifteen day pregnant rabbits required more careful handling since the cells at this stage are more friable and readily distorted.

Introduction

Since it had been decided that the investigation into the mechanism involved in the transfer of iron from mother to foetal rabbit would incorporate the use of radio-active iron as ^{59}Fe, it was obvious that the histological investigation could profitably be taken a stage further by the use of autoradiographic techniques. Autoradiography is the demonstration of radio-active isotopes in tissue sections by means of their ability to reduce silver salts in a photographic plate or emulsion. By this method it was hoped to detect the position of the radio-active iron within the histological sections of the rabbit placenta. Histochemistry, whereby the iron in the tissue is identified by various staining techniques, was also undertaken.

Autoradiography

Experimental

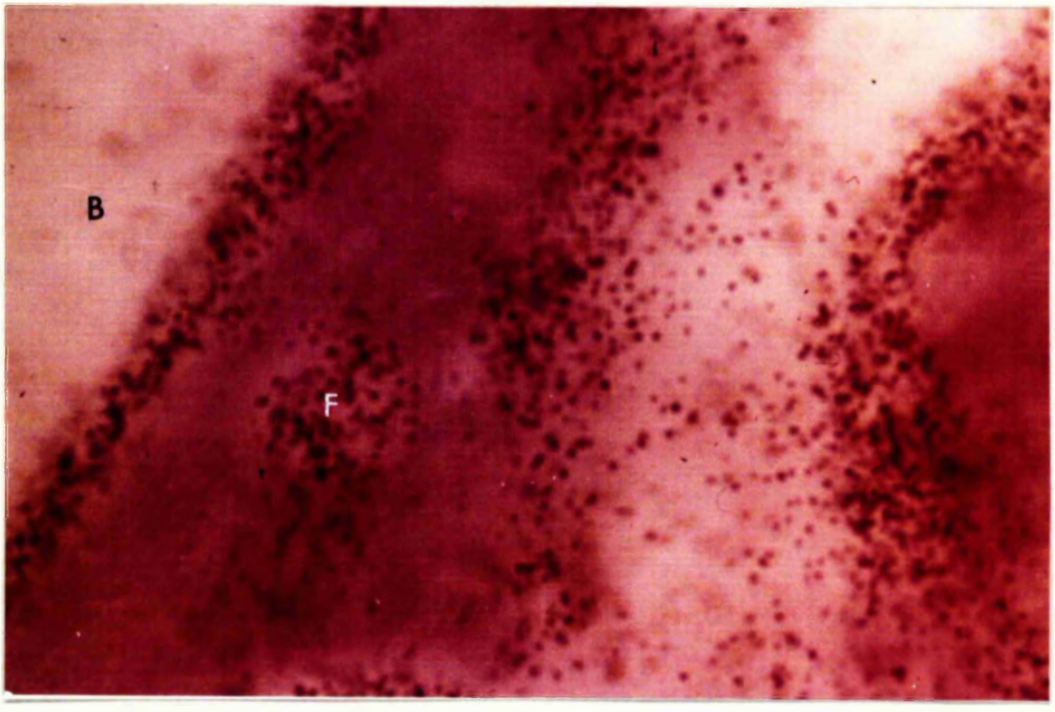
Two hours after injecting rabbits at either 15 or 25 days of gestation with known quantities of ^{59}Fe placentae were removed from each and fixed in 10% formalin.

Sections were removed and prepared as for histology. Pieces of Kodak A.H. 10 stripping film were left in apposition to these sections for at least three months. The technique is described in detail in the appendix of methods and techniques. On developing the overlying stripping film and staining the underlying sections it was possible to relate the exposed areas of the stripping film to regions in the placental tissue.

Results and Discussion

The results of the autoradiography of placental sections from rabbits 25 days pregnant are shown in Figures 17, 18 & 19. Sections prepared from 15 day pregnant rabbits gave negative results with the autoradiographic technique. In Figure 17 the positive particles are seen in quantity in both foetal and maternal spaces and the linear arrangement adopted by the radio-active iron in the maternal spaces is of particular importance as it may well be a necessary part of the transport mechanism. In the case of the autoradiographs of the two trophoblastic cells shown in Figures 18 and 19 the associated positive radio-active particles are again easily seen. However, since the histological sections are 5 μ thick and since a normal cell is approximately 10 μ thick a portion of the cell membrane is almost certainly present in each section. The conclusions that can be drawn from these sections, therefore, are that the radio-iron has been shown to be associated with the trophoblastic cells separating the maternal and foetal blood, and that the allantoic placenta in the rabbit is actively associated with the placental transfer of iron. This agrees with the findings of Davis, et al. (1959).

FIGURE 17



X 10000

Autoradiograph of placental section showing active particles in maternal and foetal spaces.

B = Maternal Space

F = Foetal Space.

FIG. 18. Autoradiograph of placental trophoblastic cells (stripping film in focus).

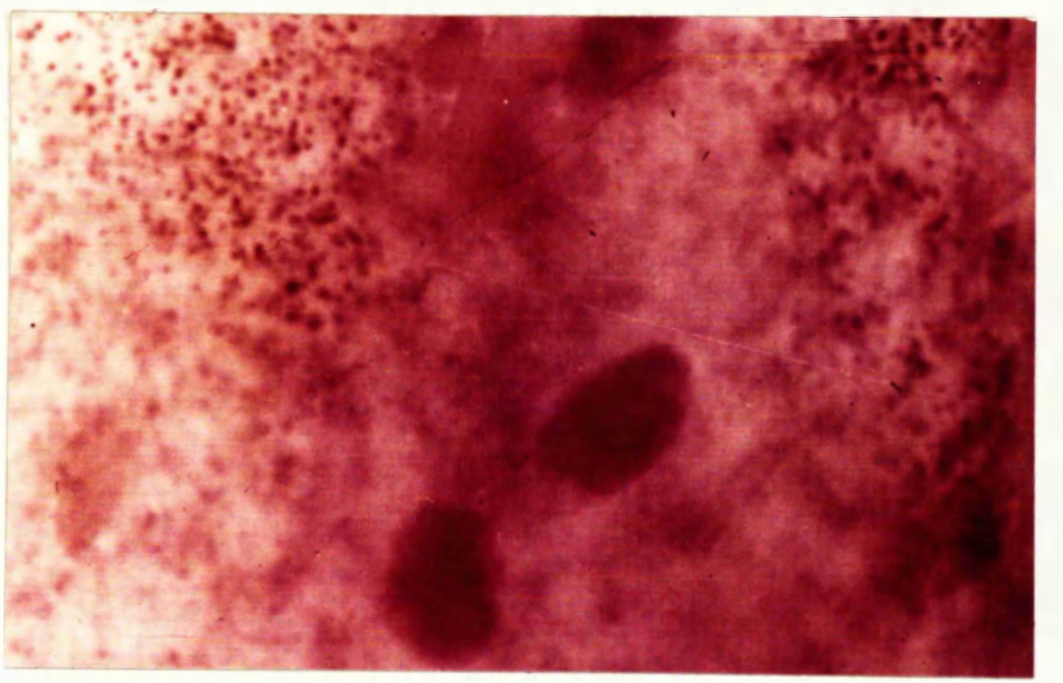


FIG. 19. Autoradiograph of placental trophoblastic cells (section in focus).

Histochemistry

The placental sections used for histochemistry were prepared in the same way as those for histology. The iron in the sections was stained blue by the method of Perl (1867). Other sections were stained with amido black for the demonstration of the tissue proteins. Both staining techniques are described in detail in the appendix of methods and techniques.

Results and Discussion

The results of the histochemical technique for the detection of iron within the placental sections from rabbits 25 days pregnant are shown in Figures 20, 21, 22 & 23. As with the autoradiographs (Figures 17,18 & 19) positive results were found in those sections from these rabbits that were 25 days pregnant. No iron was detected in those sections from 15 day placentae. This agrees with Davies, et al. (1958) who reported that approximately 2.2% of the initial amount of ^{59}Fe injected was transferred from mother to foetal rabbit at day 15 of gestation, compared with 48.1% at day 25 of gestation.

The actual location of the iron within the placenta is of interest. From the autoradiographs (Figures 17, 18 & 19) it will be seen that the iron in the maternal spaces has a close association with the luminal surface of the trophoblastic cells. This agrees with the location suggested by the histochemical technique.

FIGURE 20

X 1200

25 DAY PLACENTAL SECTION SHOWING PRUSSIAN BLUE REACTION

THIS SHOWS THE POSITIVE REACTION IN TROPHOBLASTIC CELLS.

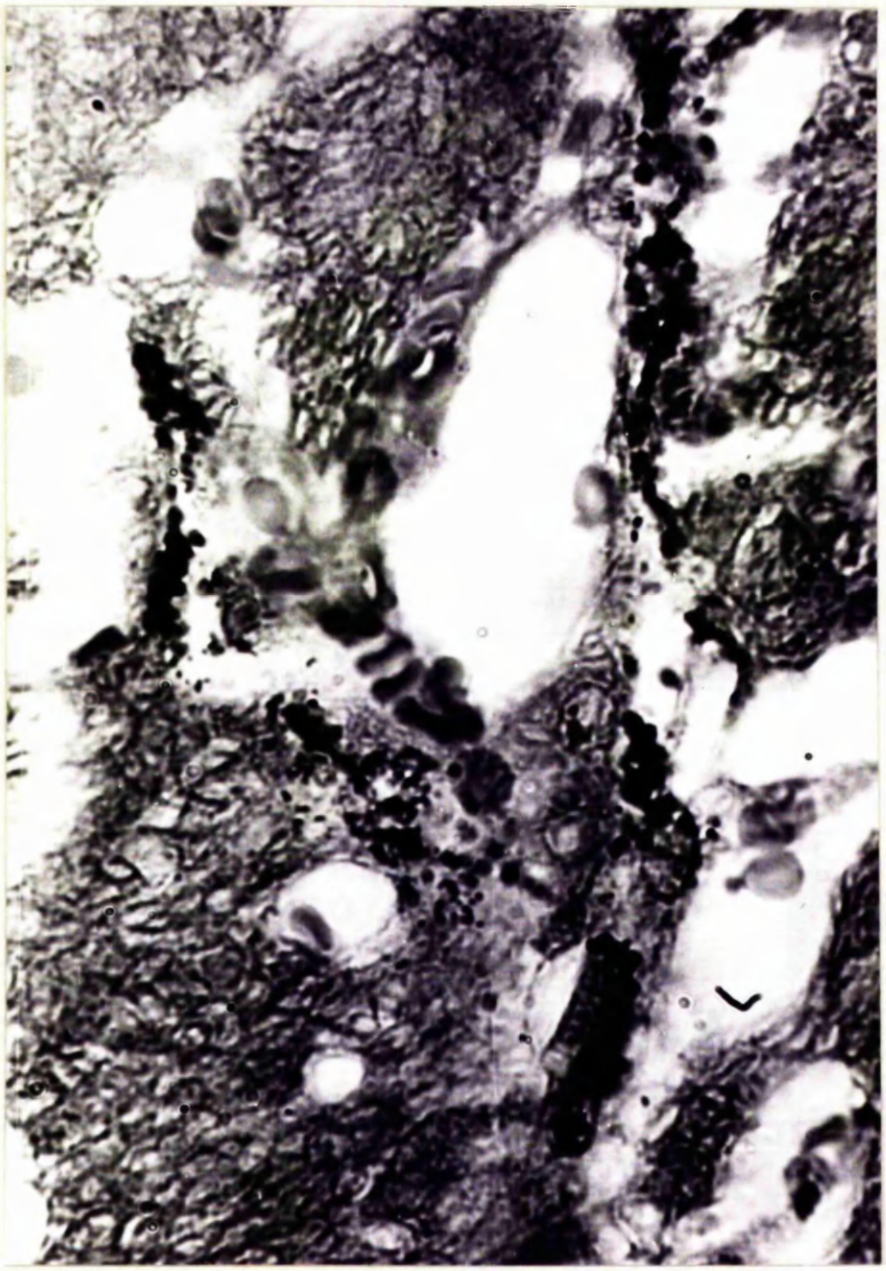


FIGURE 21

X 1200

25 DAY PLACENTAL SECTION

ILLUSTRATES THE ALMOST LINEAR ARRANGEMENT OF THE PRUSSIAN POSITIVE GRANULES.

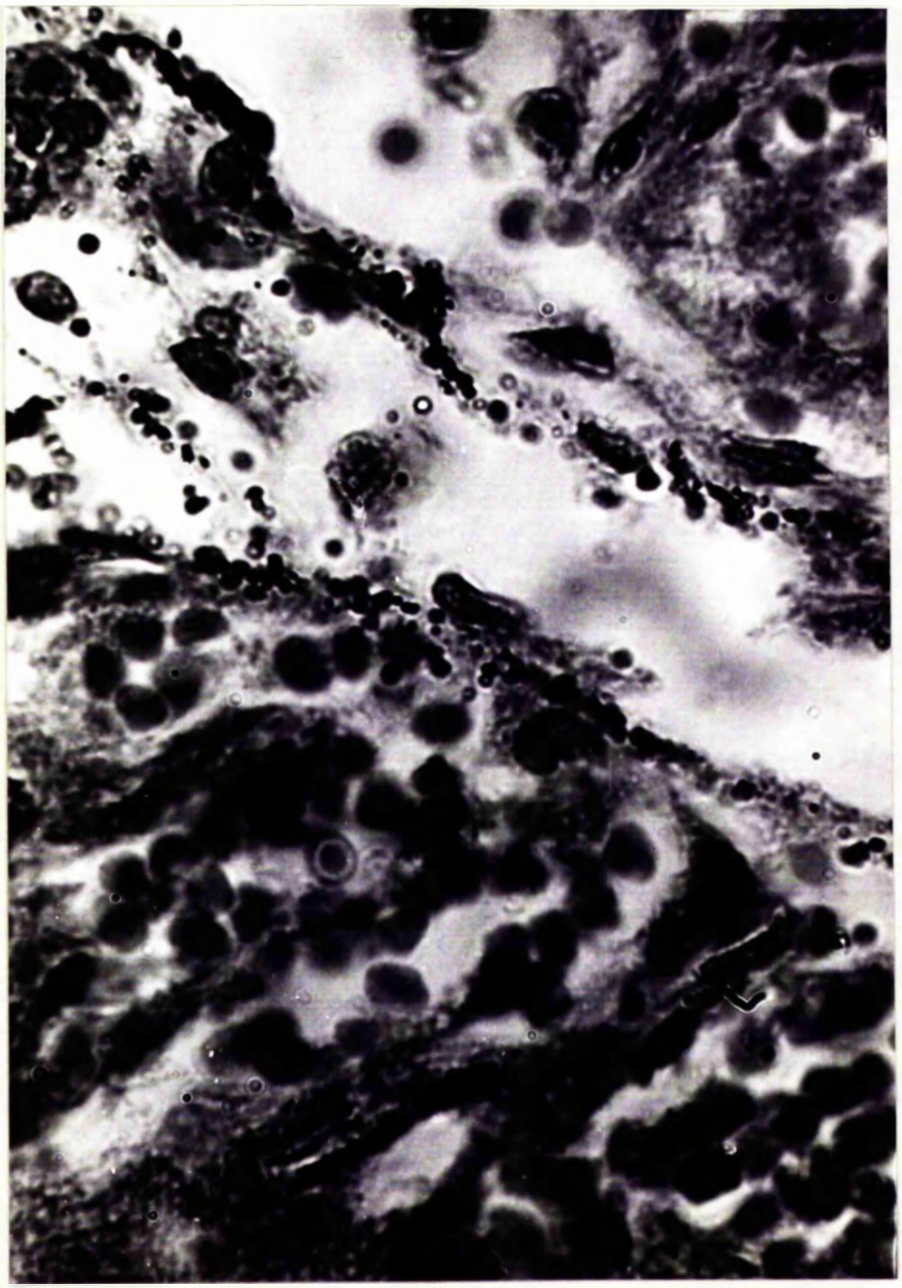


FIGURE 22

25 DAY PLACENTAL SECTION

HERE THE POSITIVELY STAINED GRANULES CAN BE SEEN LINED ALONG THE TROPHOBLASTIC CELLS IN THE MATERNAL BLOOD SPACES.

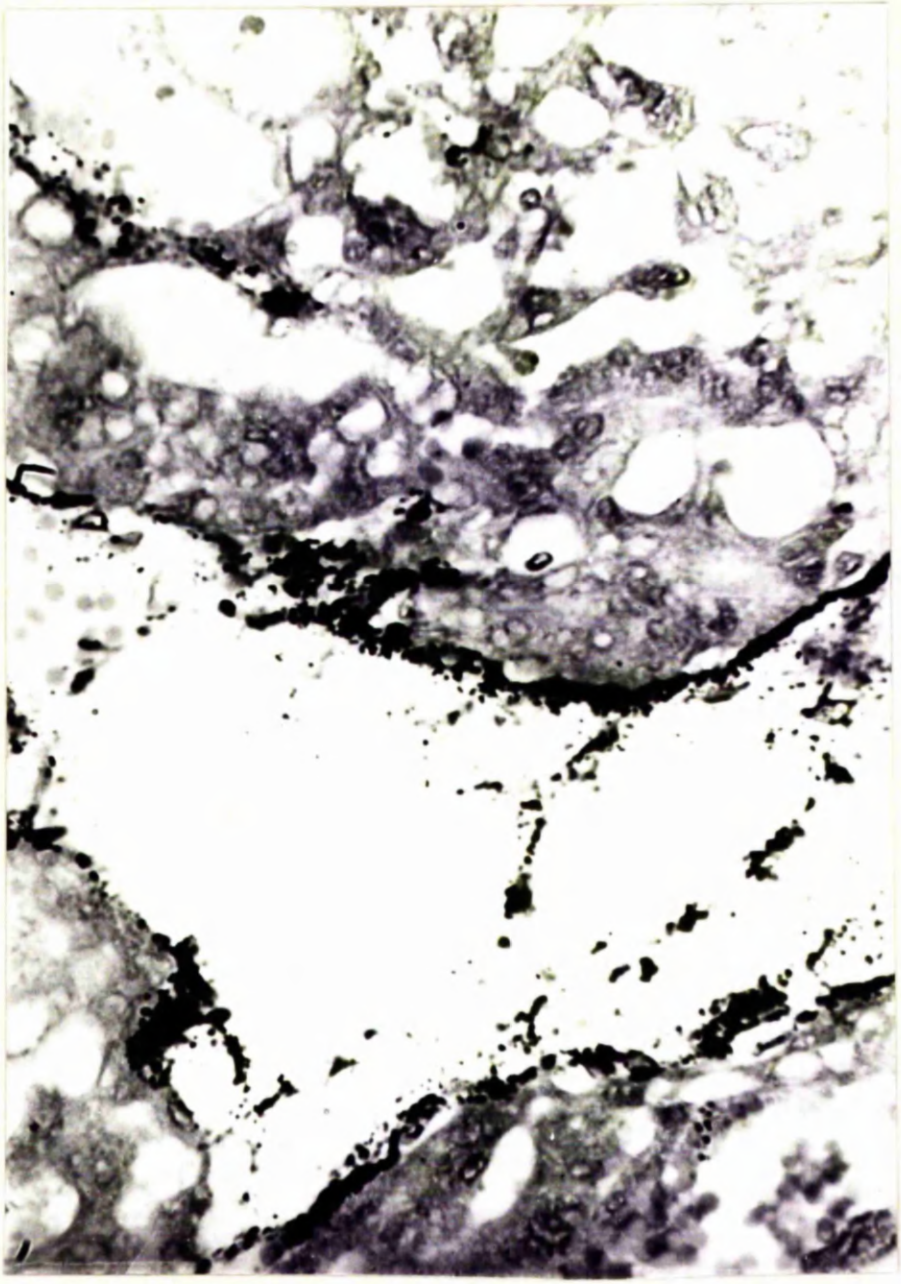


FIGURE 25

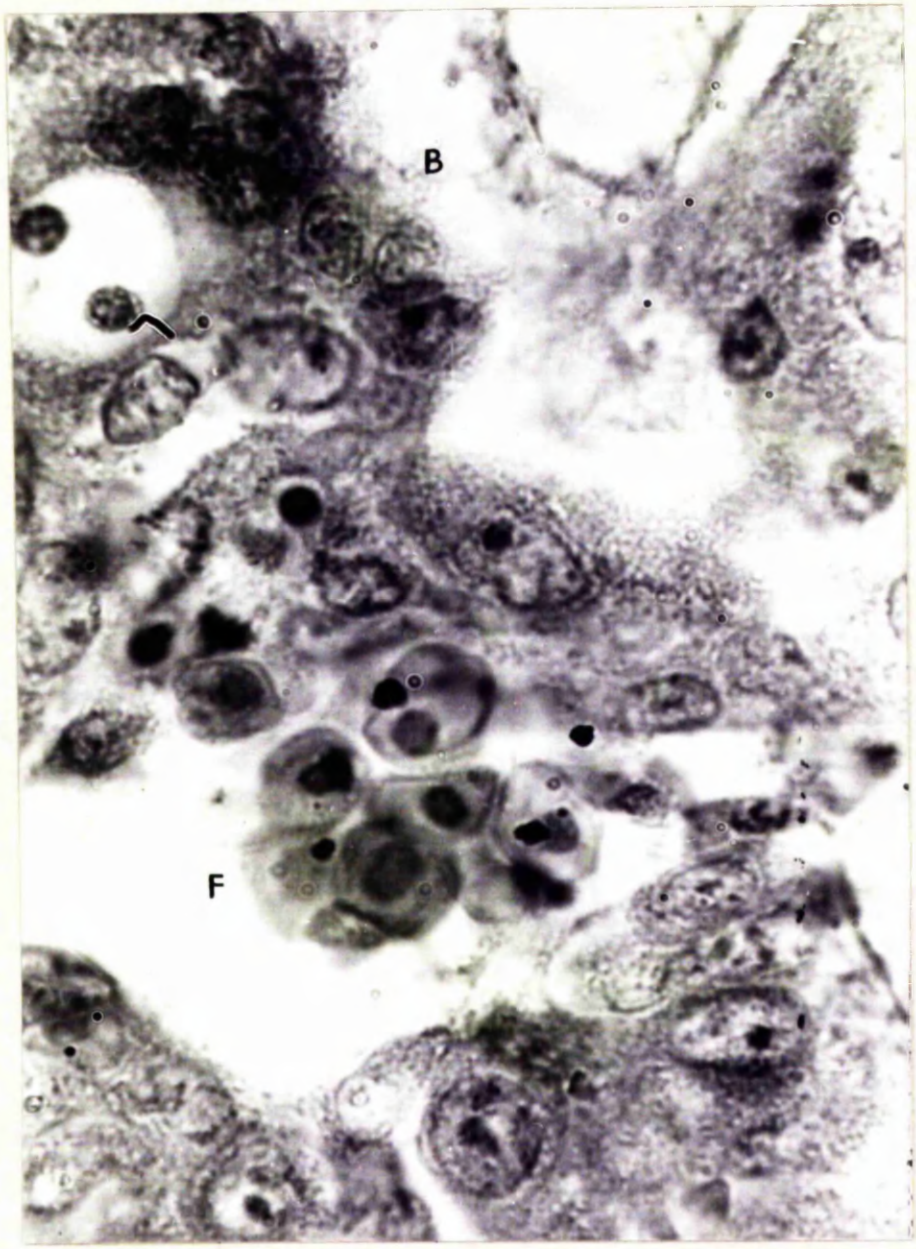
X 1200

15 DAY RABBIT PLACENTAL SECTION

HERE IT IS OF INTEREST TO NOTE THE POSITIVE STAINING WITHIN THE FOETAL ERYTHROCYTES CAUSED BY THE BOUIN FIXATIVE. MATERNAL BLOOD SPACES ARE NEGATIVE.

B = Maternal Space

F = Foetal Space.


B
F

SECTION THREE

The ^{59}Fe content of the rabbit placenta.

Gestation Time

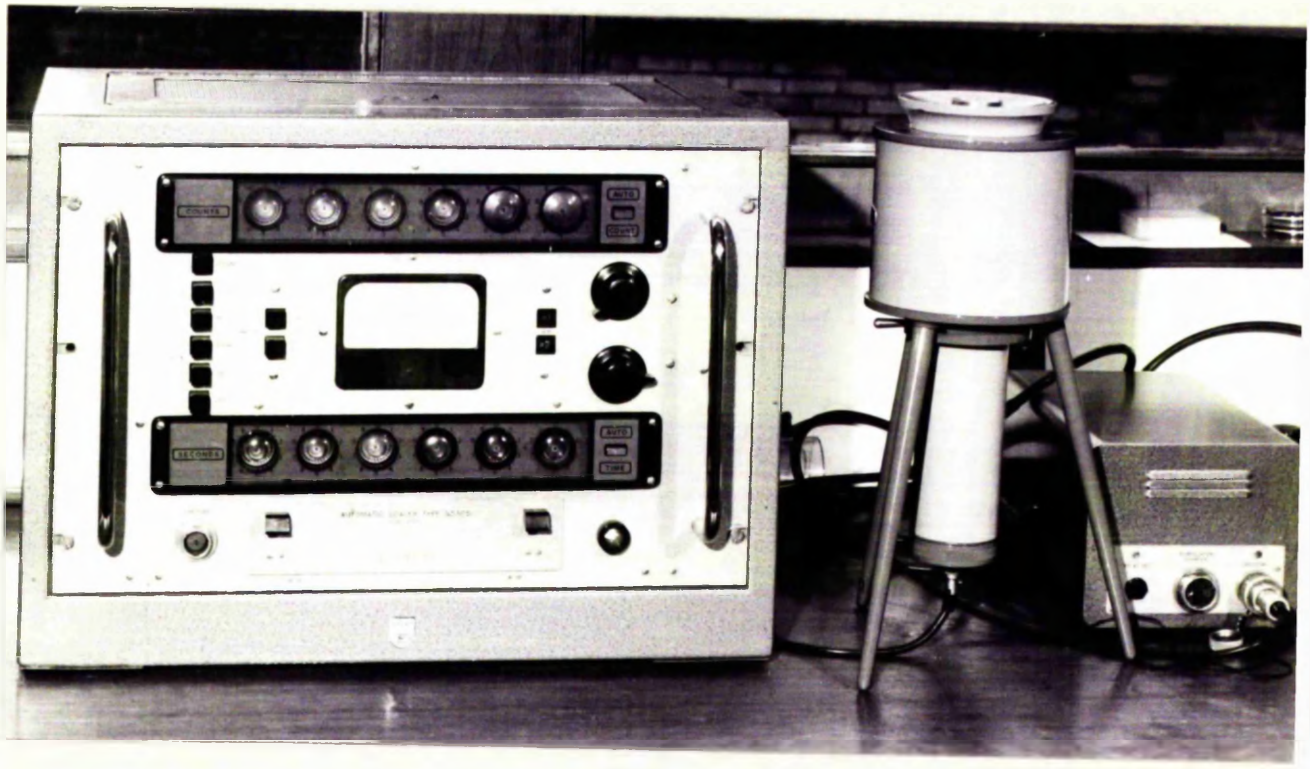
The amount of iron transferred from mother to foetal rabbit increases as gestation advances (Davies, et al. 1959). These workers described a rapid increase in the amount transferred from the 20th day to term. There are several possible explanations for this sudden increase in the rate of transfer. The histological changes taking place at this time might well be significant. On the other hand, the increase might be due to the involvement of a different form of transfer. No detailed information with regard to the placental uptake of ^{59}Fe at different stages of gestation is available. In a study of the mechanism involved in the transfer of iron by the rabbit placenta such information is necessary.

Experimental

Pregnant rabbits of known breeding history were injected intravenously with 5 µc of ^{59}Fe as ferric citrate. Two hours later the rabbits were sacrificed by an intracardial injection of Euthatal sodium pentobarbitone (May & Baker). All the foetuses and attached placentae were removed without delay. Each placenta was carefully

separated from its associated foetus and surrounding membranes, washed in ice cold saline, blotted dry with filter paper and weighed. The placenta was then finely chopped with scissors, added to 10 ml. of 0.25M-sucrose and the mixture homogenised in an ice-cold Potter-type perspex-glass homogeniser (Potter and Elvjhem, 1936). The total ^{59}Fe activity of each homogenate was measured in a scintillation counter with a well-type crystal (Figure 24), and the results expressed as a percentage of the initial activity injected into the mother rabbit.

FIGURE 24



PANAX SCINTILLATION COUNTER

Results and Discussion

The results obtained from the rabbits sacrificed during this part of the work are shown in Table 3. It would appear that there is no correlation between the stage of gestation and ^{59}Fe uptake by the placental tissue. However, when comparisons are made between rabbits carrying the same number of foetuses at different stages of gestation (Table 4), it is obvious that there is an increase in the ^{59}Fe uptake by the rabbit placenta as gestation advances. In a study of the mechanism of ^{59}Fe transfer by the placenta it would be advantageous to use rabbits in the last third of gestation. However, even at this stage of gestation the ^{59}Fe activity of the placental homogenate is very low compared with the initial amount injected (Table 4). The previous study of the histology of the rabbit placenta (Section 2) indicated that the placenta contained a significant amount of blood during the last third of gestation. This blood would contain ^{59}Fe bound to transferrin. Under these circumstances it would be difficult to detect the presence of any other iron-containing compound which may be important in the iron transporting process.

TABLE 3

Days of gestation	Number of foetuses	Total placental ^{59}Fe uptake
11	8	0.15
14	2	0.69
15	5	0.47
16	5	0.75
21	8	3.60
23	5	4.00
23	7	3.30
25	10	6.60
	6	2.70
27	8	8.10

The above table shows results from rabbits at different stages of gestation. The placental activity is expressed as a percentage of the initial activity injected.

TABLE 4

Days of gestation	Number of foetuses	Total placental activity
11	8	0.13
21	8	3.69
27	8	8.10
15	5	0.47
16	5	0.73
23	5	4.00

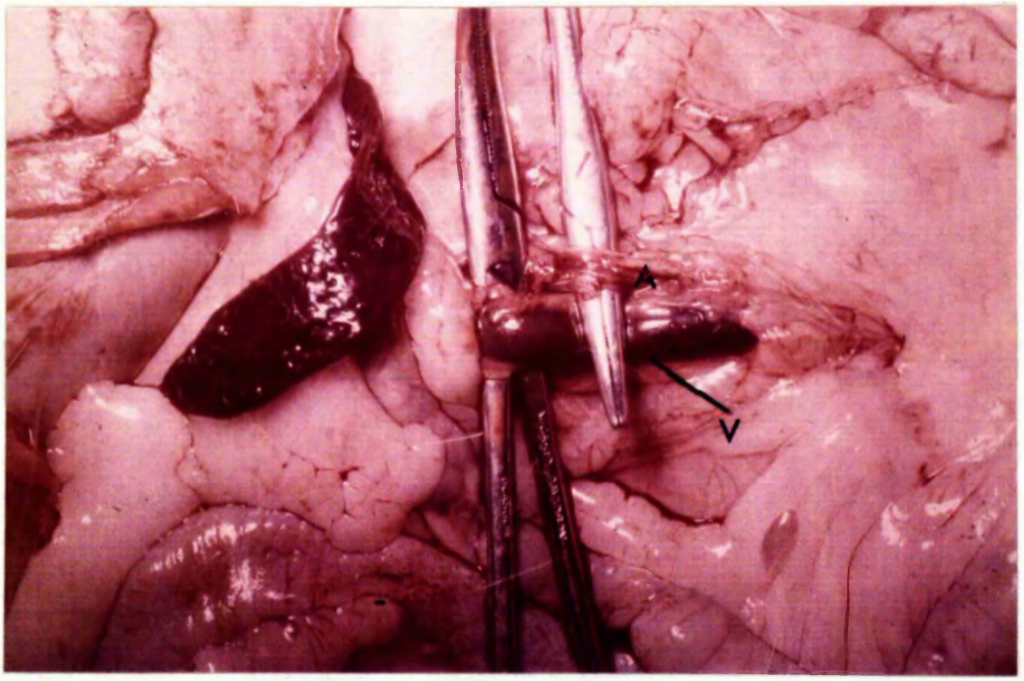
The results above are from rabbits containing either eight or five foetuses at different stages of gestation. The placental uptake of ^{59}Fe is expressed as a percentage of the initial amount of ^{59}Fe injected.

As much blood as possible was removed from the placenta by perfusing the uterus and its contents with ice cold physiological saline.

Technique of perfusion of the placenta

The pregnant rabbits were anaesthetised with intravenous nembutal and then intubated and anaesthesia continued with Fluothane (Halothane, I.C.I.). Thirty thousand international units of heparin were injected intravenously and the abdomen opened. The pregnant uterus was carefully exteriorised and placed on warm moist sterile swabs. The intestines were drawn to one side. Several methods of placental perfusion were attempted including the canulation of two ventricles of the heart (Amoroso, 1965), but it was found that the pressure necessary to pump the perfusing fluid as far as the uterus caused shredding of the placental tissue. The most satisfactory results were obtained by canulating the abdominal vena cava and aorta (Holmes, 1965). The aorta and vena cava were exposed on the floor of the abdominal cavity (Figure 25). These vessels were very closely allied to one another, the aorta lying underneath and to the left of the vena. It was found that it was

FIGURE 25

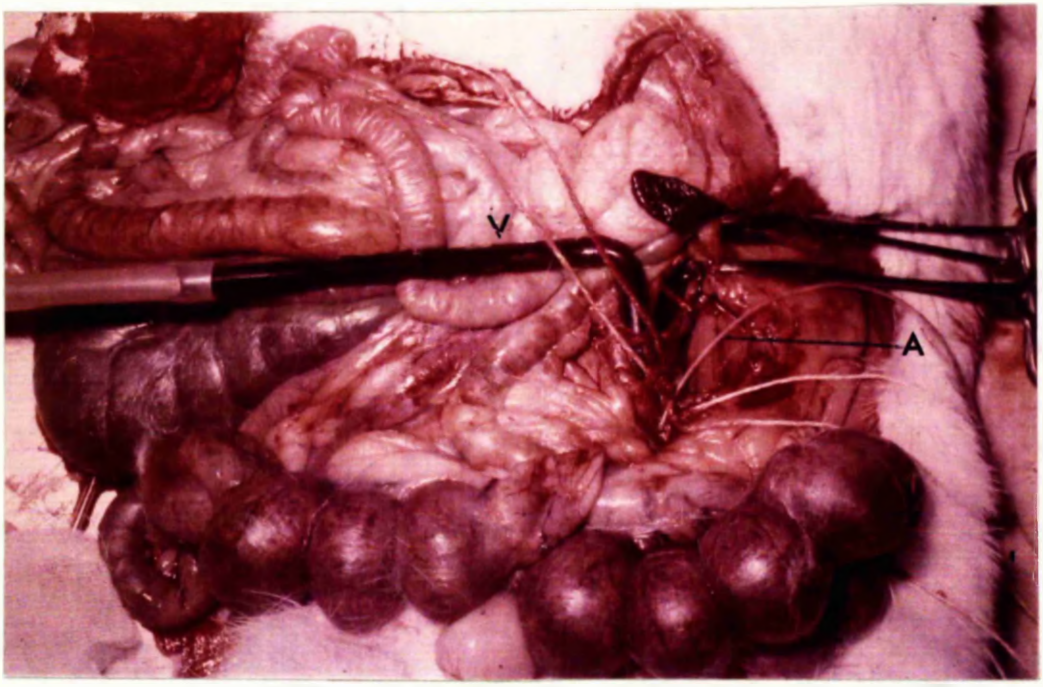


Exposure of aorta (A) and

vena cava (V).

better to canulate the aorta first since the inflowing saline prevented the thin walled vena cava from collapsing on sectioning. Pieces of soft twine were passed under each vessel, one above and one below the site of incision. Twine was used because it was not likely to damage the vessels especialy the thin walled vena cava. An assistant gently raised the aorta from the abdominal floor by means of the lower piece of twine, and the pressure applied in doing this was sufficient to stop an outflow of blood from the vessel when it was incised. The aorta was cut with a sharp pointed pair of scissors and a nylon catheter (Portex pattern, size 3) was inserted into the lumen and passed along its length for approximately 2 cm. The canula was secured in position by means of the lower piece of twine and, by tying off the upper piece of twine, the upper circulation was stopped. (Figure 26). The canula was now connected to a bottle of ice cold saline suspended above the operating table and the saline allowed to flow freely under gravity. The vena cava was now canulated with a sharp pointed glass canula. When in position this was secured by the lower piece of twine and the upper circulation again tied off. The outflowing blood from the vena cava was collected. As soon as the saline was flowing freely into the aorta

FIGURE 26



Canulae in Position

in

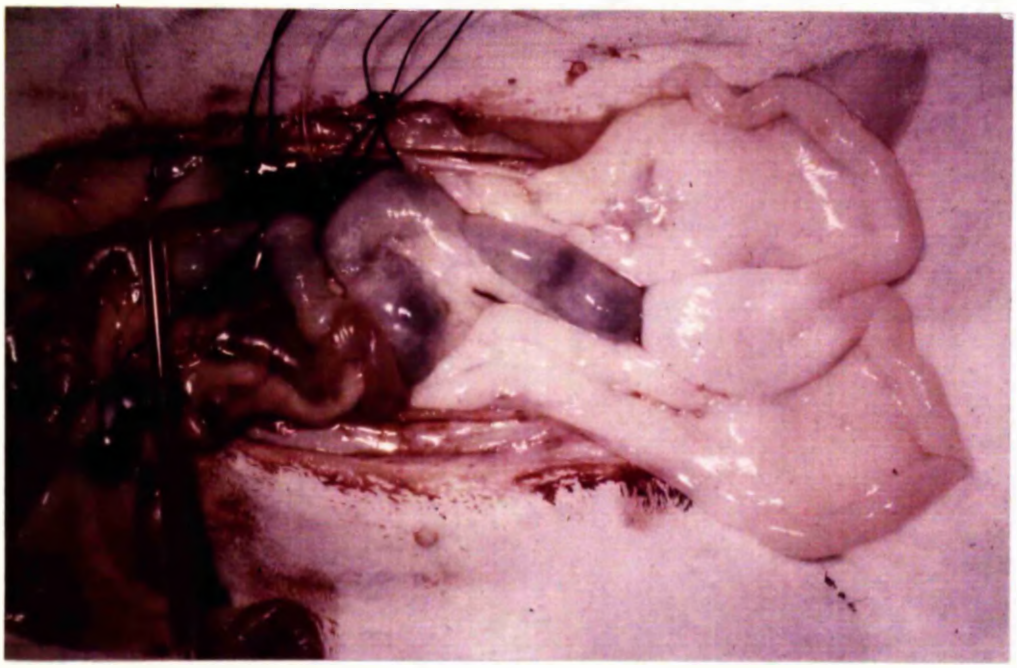
(A) AORTA

(V) VENA CAVA

the rabbit was sacrificed by intracardial Euthatal.

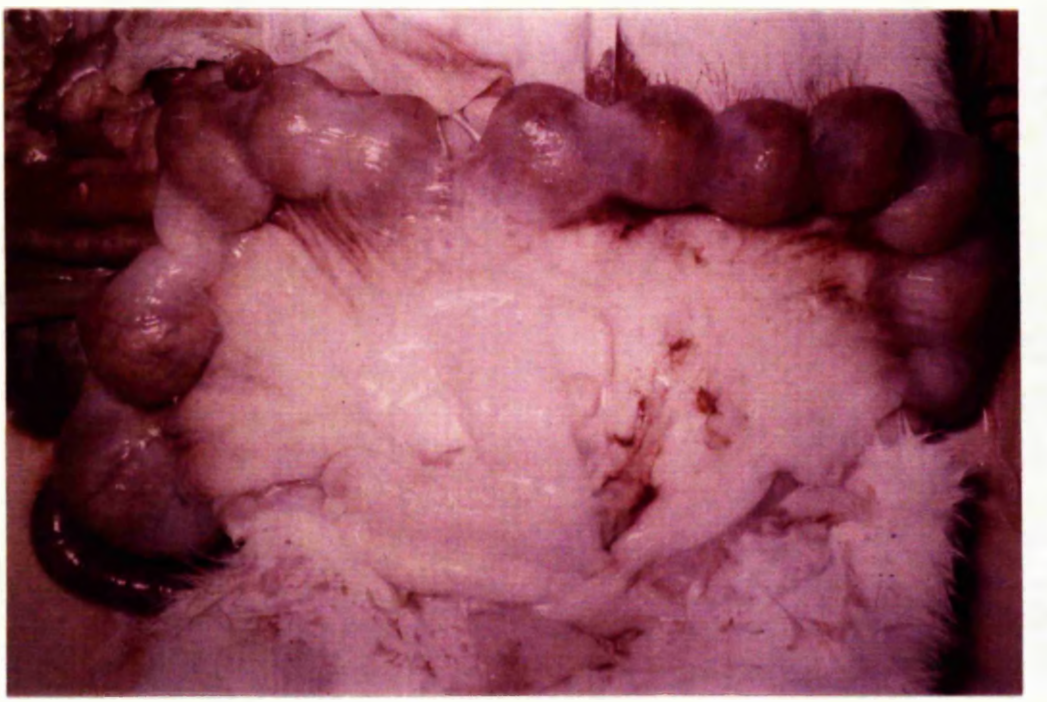
It is known that in human surgery if a patient suffers from cardiac arrest but is resuscitated within five minutes no apparent cellular damage takes place (Holmes, 1965). On this evidence it was decided to perfuse the uterus and its contents for no longer than five minutes. The results obtained using this perfusion technique are shown in Figure 27, and illustrate a definite blanching of the non-pregnant uterus. However, since the foetal blood is not removed in the perfusion technique a pinkish hue of the uterine contents is still apparent (Figure 28) and is due in part to the presence of foetal blood. Comparison, however, of the ^{59}Fe activity of placental homogenates with and without perfusion is shown in Table 5. The results obtained indicate a marked reduction in the ^{59}Fe content of the perfused placenta. Histological sections prepared from perfused placentae indicated that while much of the blood had been removed from the maternal spaces a marked amount of blood remained in the sinusoids (Figure 29). Moreover, this finding was further substantiated by demonstrating that there was still radio-activity in the fluid flowing from the canulated vena cava five minutes after the

FIGURE 27



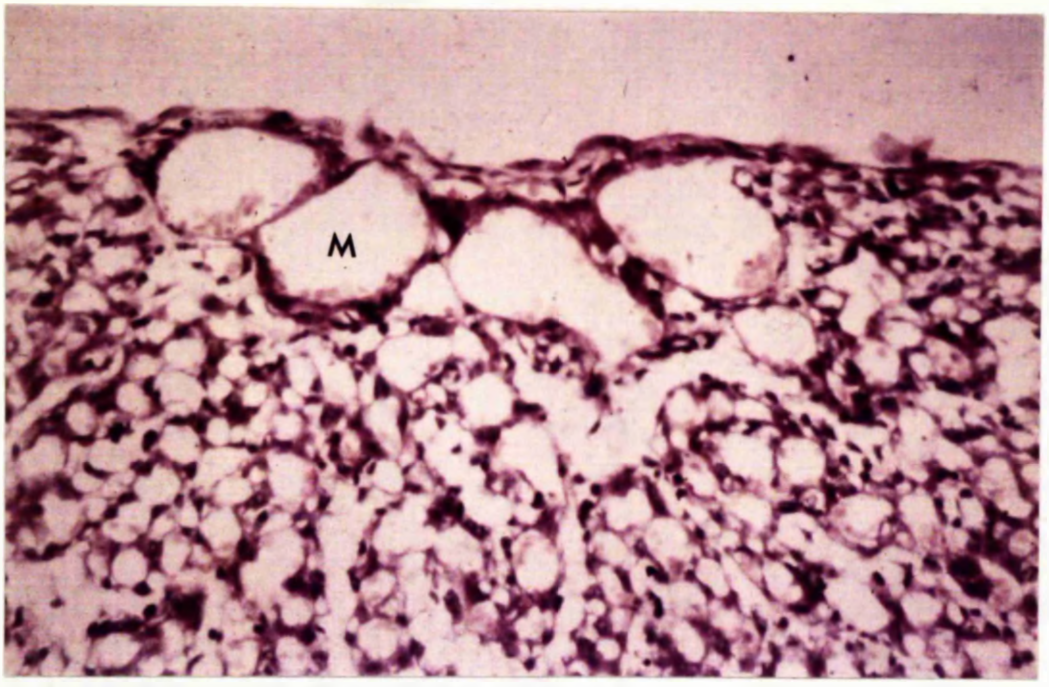
Non-pregnant uterus after perfusion

FIGURE 28



Pregnant uterus after perfusion.

FIGURE 29



Histological section of rabbit placenta after perfusion

M = maternal space.

TABLE 5

Placental activity expressed as % of initial amount of ^{59}Fe injected.

Placenta No.	Perfused	Non-perfused
1	0.55	1.1
2	0.4	1.1
3	0.35	0.8
4	Taken for histology	1.0
5	-	1.0
Total	1.2	5.0
Average	0.4	1.0

Comparison between the ^{59}Fe activity of placental homogenates from two rabbits one of which had been perfused. Each of the rabbits was twenty-two days pregnant and all the placentae were removed two hours after administering the radio-active iron.

beginning of perfusion. To further minimise the contamination of the placenta with maternal blood containing radio-active iron it was decided to investigate the plasma clearance rate of ^{59}Fe in rabbits in late pregnancy. This would afford information as to the period of time which should be allowed to elapse between the injecting of the ^{59}Fe and the removal of the placenta.

Introduction

A study of the plasma clearance rate was carried out in several pregnant rabbits during the last third of gestation. The plasma clearance rate was also studied in two non-pregnant rabbits. After injecting each of the rabbits with a known amount of ^{59}Fe ranging from 5 to 30 μc., blood samples were withdrawn from the marginal ear vein at approximately 15 minute intervals. The first sample was withdrawn between 10 and 15 minutes after administration of the isotope. As the investigation into placental activity continued, it became obvious that much greater amounts of radio-active iron would have to be injected into the mother rabbit in order to study the radio-active compounds within the placenta. Hence the reason for the variation in the amounts of isotope injected into the different rabbits studied. At the end of the experiments of plasma clearance rate in the pregnant animals the placentae were removed for investigation. The radio-activity of each of the blood samples was measured in the scintillation counter. The P.C.V. of each of the samples was also measured by the method described previously. From the two results the ^{59}Fe activity of equal amounts of plasma at the different sampling times were calculated. Since

different amounts of radio-active iron had been injected into the different rabbits studied, the first sample was arbitrarily taken to represent 100% activity and the subsequent samples expressed as a percentage of the first amount.

Results

The results shown in Figure 30 are typical of those obtained from the rabbits examined. They indicate the plasma clearance rate in two pregnant rabbits one 27 days pregnant and the other at term. The graph also includes results from one non-pregnant rabbit. It will be seen the the plasma clearance rate of ^{59}Fe is greater in the pregnant rabbit compared with the non-pregnant. Moreover, the plasma clearance rate increased with gestation. It would seem, therefore, that if half an hour were allowed to elapse after administering the radio-iron and before removing the placentae, then the circulating plasma ^{59}Fe of the dam would be markedly reduced. Little information was available about the uptake of ^{59}Fe by the rabbit placenta as a function of time. Therefore, the ^{59}Fe uptake by the rabbit placenta at intervals ranging from six minutes to two hours after injecting the radio-iron was investigated in rabbits in the later stages of gestation.

Experimental

Pregnant rabbits in the last third of gestation were anaesthetised with intravenous nembutal (sodium pentobarbitone containing 1 gr. per ml.). They were

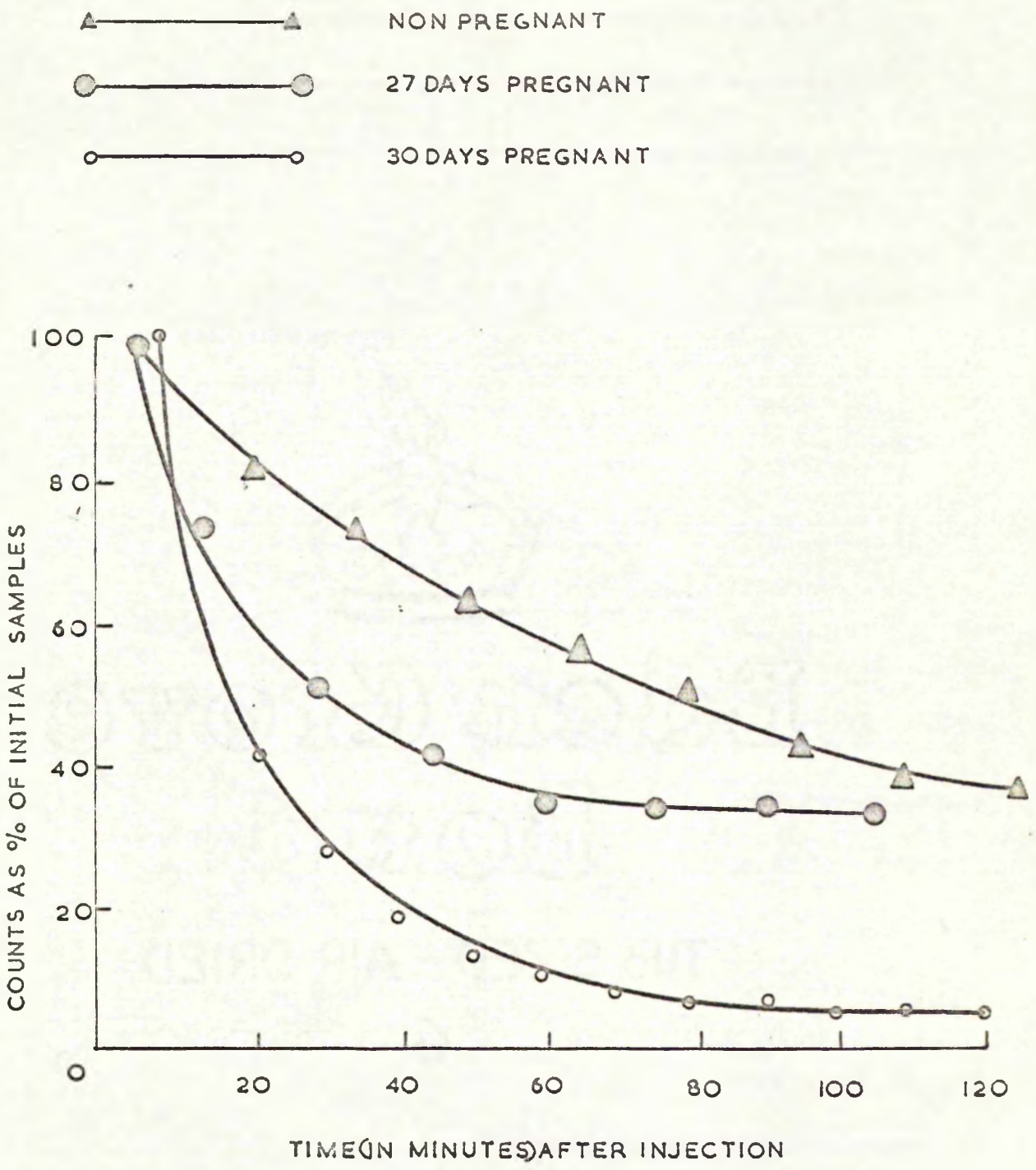
FIGURE 30

PLASMA CLEARANCE RATE IN

A. ONE NON-PREGNANT RABBIT

B. ONE 27 DAY PREGNANT RABBIT

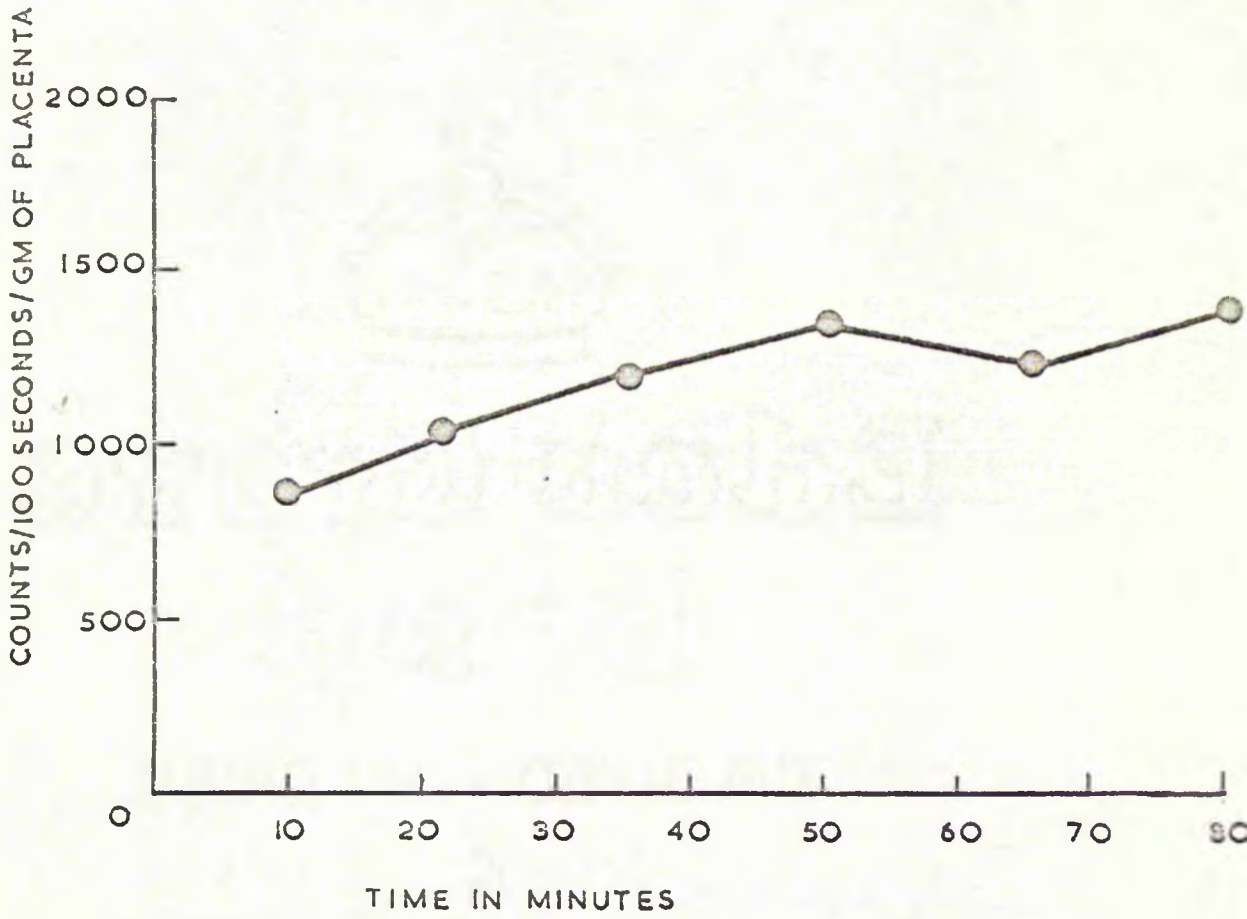
C. ONE 30 DAY PREGNANT RABBIT


NON PREGNANT
27 DAYS PREGNANT
30 DAYS PREGNANT
COUNTS AS % OF INITIAL SAMPLES
100
80
60
40
20
0
20
40
60
80
100
120
TIME (IN MINUTES) AFTER INJECTION

then intubated and and anaesthesia continued with Halothane (Fluothane) in a to and fro apparatus. The abdomen was opened and the uterus exposed. A known amount of ^{59}Fe as ferric citrate was injected intravenously. Six minutes later one foetus with its attached placenta was removed from the uterus. The incision made in the uterine wall was closed with artery forceps and the uterus containing the remaining foetuses covered with warm moist swabs. This procedure was repeated at 15 minute intervals until all the foetuses and their placentae had been removed. Each placenta on removal was washed in ice cold saline and blotted dry with filter paper. It was then weighed and homogenised in 0.25M-sucrose as previously described. The total ^{59}Fe activity of each placental homogenate was determined in the well-type scintillation counter, and the results expressed as counts per 100 seconds per g. of placental tissue. Over the range of time studied there was no notable difference in the ^{59}Fe uptake by the placenta. A typical example of the results obtained is shown in Figure 31. From this it would appear that the best opportunity for investigating the mechanism involved in the transport of iron from mother to foetal rabbit would be by using animals in the later stages of pregnancy.

FIGURE 31

UPTAKE OF ^{59}Fe by RABBIT PLACENTA AS A FUNCTION OF TIME AFTER ADMINISTRATION OF THE ISOTOPE.


COUNTS/100 SECONDS/GM OF PLACENTA
2000
1500
1000
500
0
10
20
30
40
50
60
70
TIME IN MINUTES

Furthermore, by waiting for at least 30 minutes after intravenously injecting the ^{59}Fe followed by perfusion before removal of the placentae, the amount of contamination from the mother's blood would be reduced.

SECTION FOUR

Subcellular fractionation of the rabbit placenta

Introduction

When cells are disrupted in a suitable medium and subjected to a process of differential centrifugation, the various subcellular fractions are separated in accordance with their size and density (Bell, Davidson and Scarborough, 1956). Hogeboon, Schneider and Palade (1948) and Hogeboon and Schneider (1950) demonstrated that the morphological and cytological properties of the particulate components of the cell were preserved when the homogenates were prepared in a medium containing sucrose and the homogenates could be separated by means of differential centrifugation into a nuclear fraction, a mitochondrial fraction, a microsomal fraction, and a non-particulate fraction or cell sap. The cell fractionation technique has been applied to various tissues and the resulting cellular components studies using the electron microscope. No evidence could be found in the literature of this technique having been applied to the placenta.

Rabbit placentae were fractionated by the method of Hogeboon, Schneider and Palade (1948), with modifications by Hogeboon and Schneider (1950) and electron micrographs were prepared of the different cellular components.

The distribution of the ^{59}Fe within the placental fractions was also measured.

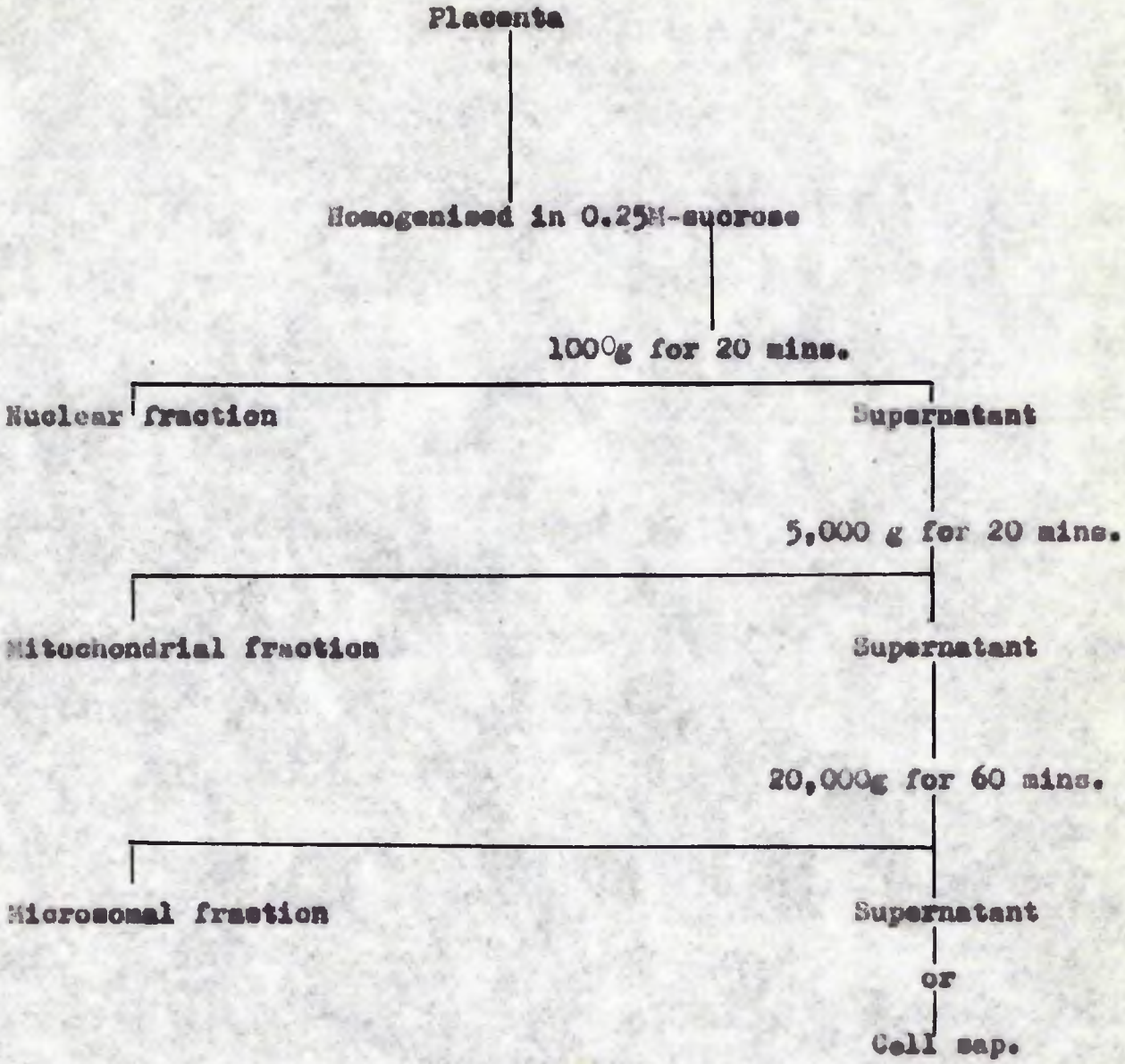
Experimental

Pregnant rabbits during the last third of gestation were injected intravenously with 5 µc of ^{59}Fe as ferric citrate in a solution of 1% sodium citrate. Two hours later the rabbits were sacrificed and all the placentae removed. Each placenta on removal was homogenised in 10 ml 0.25 M-sucrose in the usual way (see appendix of methods and techniques). The homogenates were then separated into nuclear, mitochondrial, microsomal, and non-particulate fractions by centrifuging in the M.S.E. refrigerated centrifuge at the different speeds indicated on the flow sheet in Figure 32. Prior to centrifugation a sample was taken from each of the homogenates. The radio-activity of each of the fractions and of the homogenate was measured in the scintillation counter and the ^{59}Fe activity of each fraction expressed as a percentage of the initial homogenate (see appendix of methods and techniques). On one occasion, the mother rabbit was anaesthetised with intravenous nembutal, intubated and anaesthesia continued with Fluothane in a to and fro

FIGURE 32

FLOW SHEET OF SUBCELLULAR FRACTIONATION OF

RABBIT PLACENTA


Placenta
Homogenised in 0.25M-sucrose
1000g for 20 mins.
Nuclear fraction
Supernatant
5,000 g for 20 mins.
Mitochondrial fraction
Supernatant
20,000g for 60 mins.
Microsomal fraction
Supernatant
or
Cell sap.

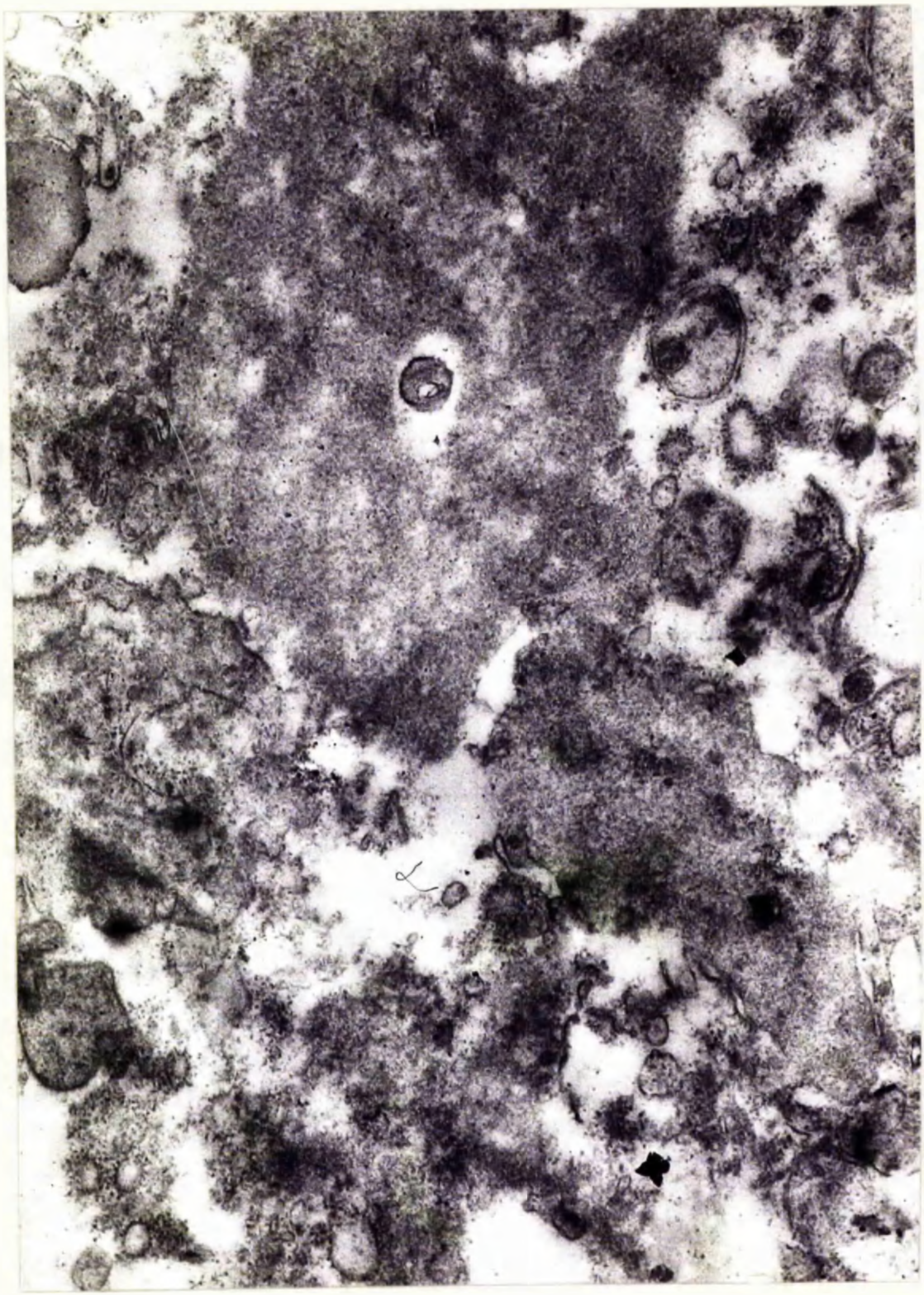
apparatus. The abdomen was opened and the uterus exposed. A known amount of ^{59}Fe as ferric citrate was injected intravenously. Fifteen minutes later the first foetus and placenta was removed. This procedure was repeated at 15 minute intervals until all the foetuses and placentae were removed. On removal the placentae were treated as described in the first part of this procedure.

Results and Discussion

The electron micrographs, Figures 33, 34 and 35 demonstrate that the placental tissue was successfully separated into a deposit containing the nuclei of the cell and cell debris (Figure 33), a mitochondrial fraction (Figure 34) and a fraction containing the endoplasmic reticulum of the cell broken down into microsomes (Figure 35), leaving a final supernatant or cell sap.

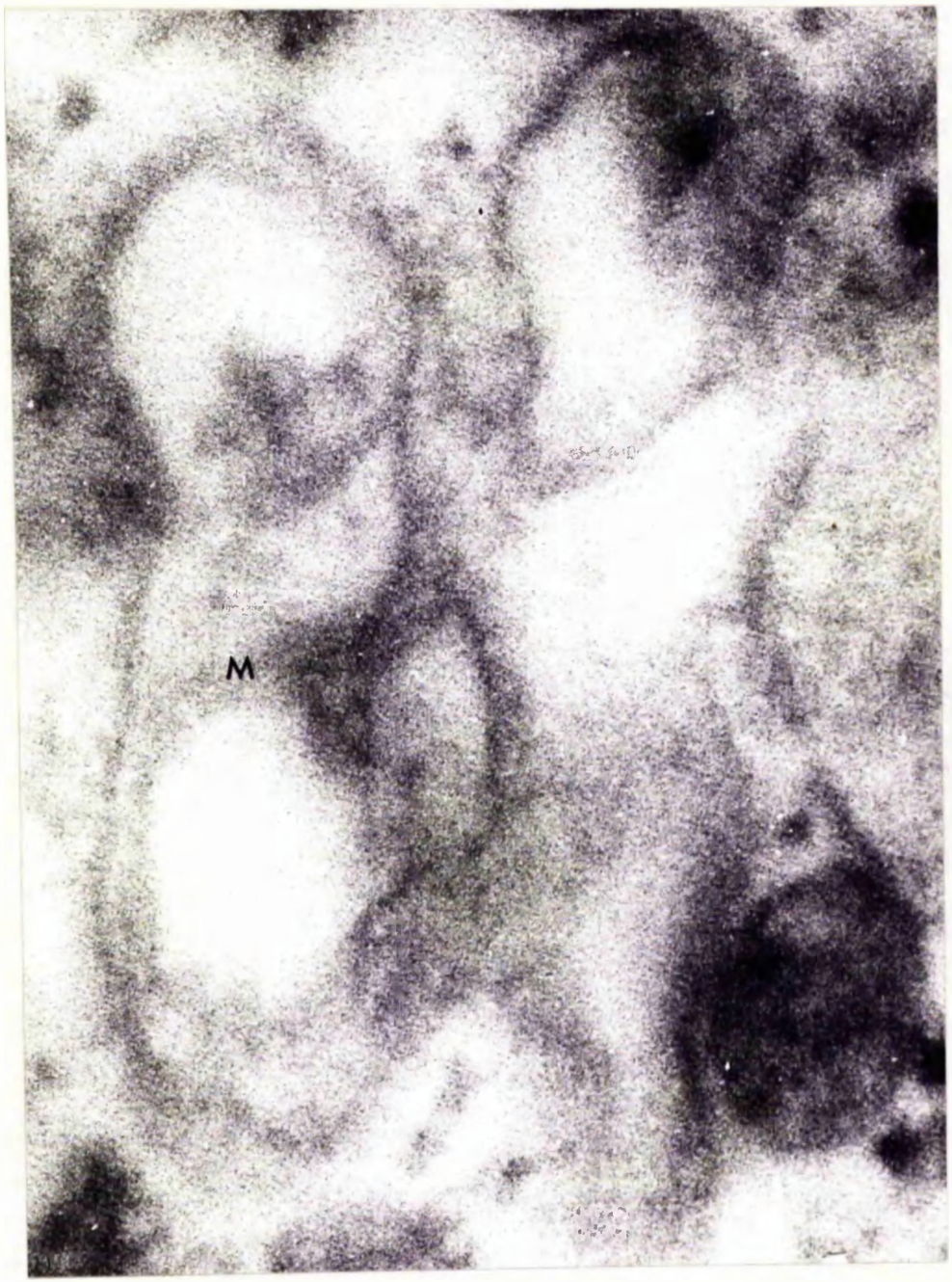
The distribution of the ^{59}Fe in the different fractions of the cell are shown in Tables 6, 7 & 8. By examining Table 6 with Table 7 and 8, the ^{59}Fe uptake by the placental fractions as a function of time can be studied. There is no obvious difference between the three sets of results. In most instances the highest concentration of ^{59}Fe is found in the non-particulate part of the cell. On the two occasions when this is not so, the nuclear layer contains the greatest proportion. The nuclear layer contains the nuclei of the cell and some unbroken cells. The number of unbroken cells depends on the efficiency of the homogenising technique. These results, therefore, might have resulted from inefficient homogenising.

FIGURE 33



ELECTRON MICROGRAPH OF NUCLEAR LAYER

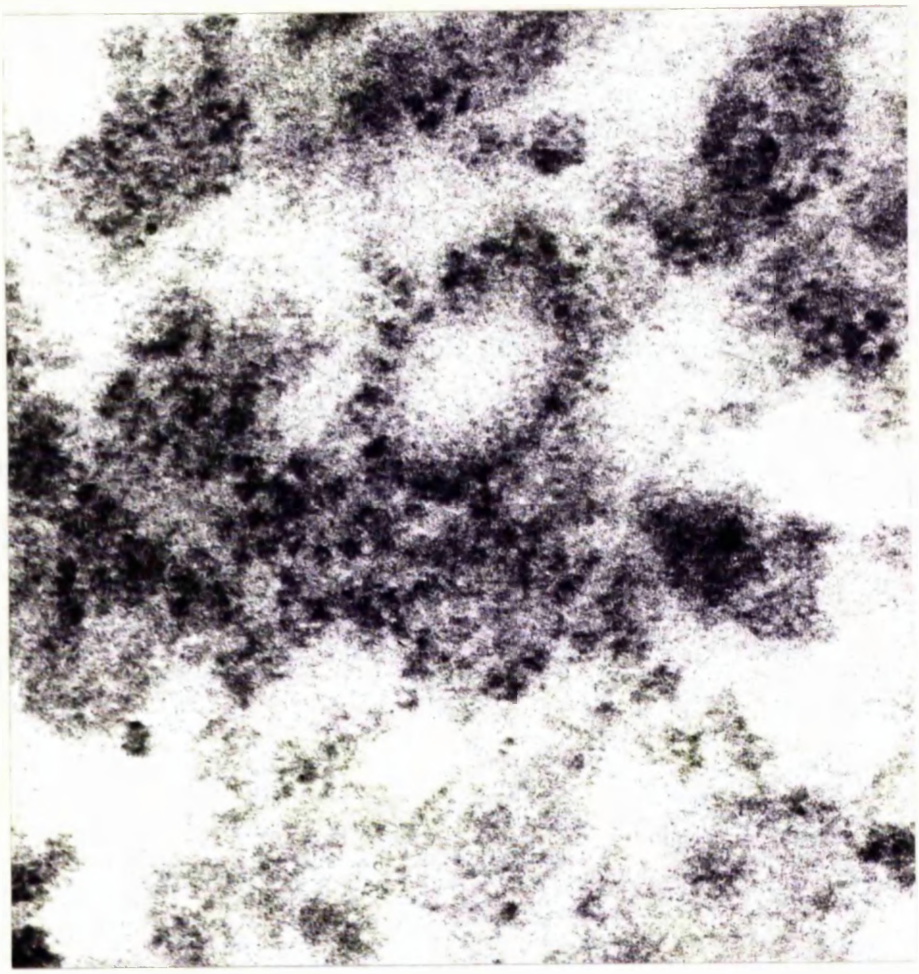
FIGURE 34



ELECTRON MICROGRAPH OF MITOCHONDRIAL LAYER

M = mitochondria

FIGURE 35



ELECTRON MICROGRAPH OF CHROMOSOMAL LAYER

TABLE 6

Distribution of ^{59}Fe in the subcellular fractions of the rabbit placenta.

Time of removal of placenta after injecting the ^{59}Fe (in minutes)	The ^{59}Fe activity of the subcellular fractions (cts/100 sec) as % of total activity.				
	Nuclear	Mitochondrial	Microsomal	Cell Sap	% Recovered
15	26.7	0.9	8.8	57	93
30	30.6	2.0	8.8	52	93
45	31.4	0.7	7.5	55	95
60	27.0	2.9	9.2	46	85
75	34.0	3.9	10.1	49	97
90	30.8	4.2	10.9	54	100

The above results are from a rabbit 25 days pregnant.

TABLE 7

Distribution of ^{59}Fe in the subcellular fractions of the rabbit placenta.

Time of removal of placenta after injecting the ^{59}Fe (in minutes)	The ^{59}Fe activity of the subcellular fractions (cts/100 sec.) as % of total activity.				
	Nuclear	Mitochondrial	Microsomal	Cell Sap	% Recovered
120	29	3.8	3.6	51	87
120	26	5.3	7.4	55	94
120	29	4.3	1.5	57	92
120	33	6.6	4.7	49	93
120	26	4.6	4.4	60	95

The above results are from a rabbit 22 days pregnant.

TABLE 8

Distribution of ^{59}Fe in the subcellular fractions of the rabbit placenta

Time of removal of placenta after injecting the ^{59}Fe (in minutes)	The ^{59}Fe activity of the subcellular fractions (cts/100 sec.) as % of total activity.				
	Nuclear	Mitochondrial	Microsomal	Cell Sap	% Recovered
120	48	4.0	6.0	33	91
120	28	5.9	7.0	50	91
120	34	5.5	6.7	51	97
120	32	2.9	5.4	48	88
120	35	3.0	7.3	54	99
120	40	4.7	5.0	33	83

The above results are from a rabbit 25 days pregnant

These results indicate that the cell sap is the subcellular fraction of choice for further investigation into the transfer of iron across the placenta.

Introduction

Further investigation of the chemical composition of the radio-active iron compounds of the placental cell sap was carried out using the chromatographic technique of Porath and Flodin (1959) and Porath (1959), who used cross-linked dextrans which acted as a sieve for molecules of different size. The cross-linked dextrans are available commercially as Sephadex (Pharmacia Ltd., Sweden). It was decided to use the G25, G75 and G200 types of Sephadex. In order to minimise possible interference from enzymatic activity all the chromatographic work was carried out in the cold room at 4°C.

Experimental

The G25 Sephadex slurry was prepared and the glass columns packed as described in the appendix of methods and techniques. Cell sap was prepared after placentae were removed from rabbits during the last third of gestation and homogenised followed by centrifugation for 40 minutes at 40,000g in the Model L Spinco. A sample of this cell sap amounting to approximately 3% of the total bed volume was applied to the column. The samples were eluted from

the column with 0.01M phosphate buffer pH 7.3. 3 ml samples were collected in a fraction collector and were examined for the presence of radio-active compounds.

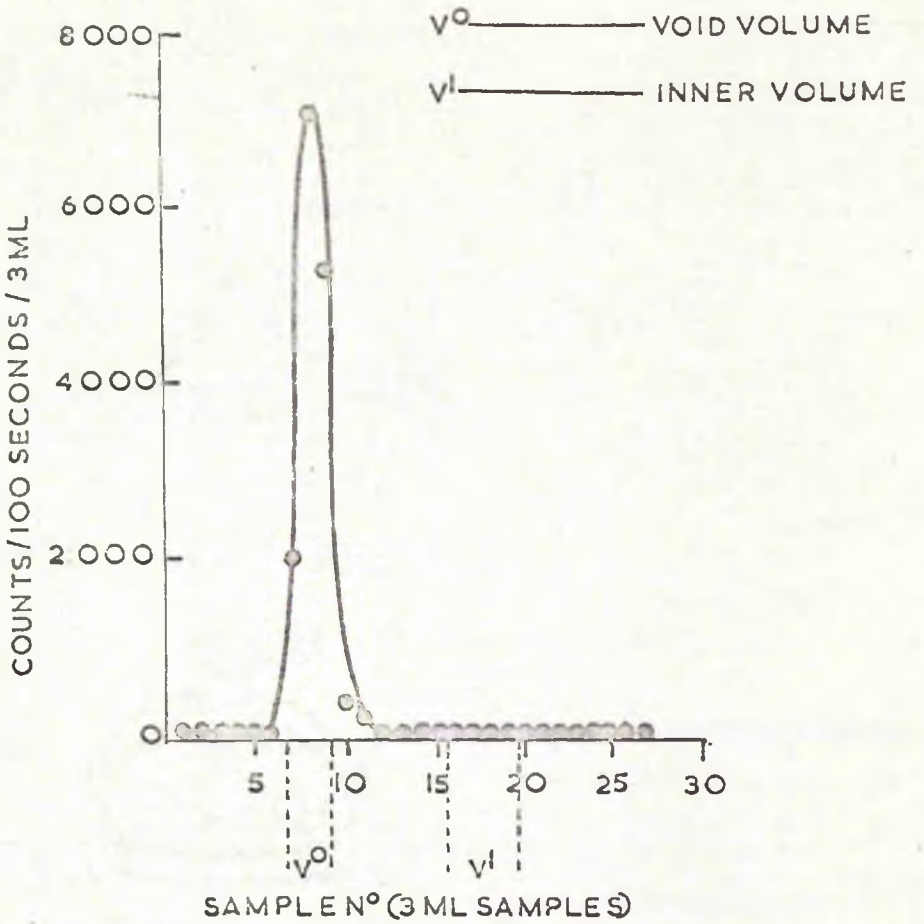
Results and Discussion

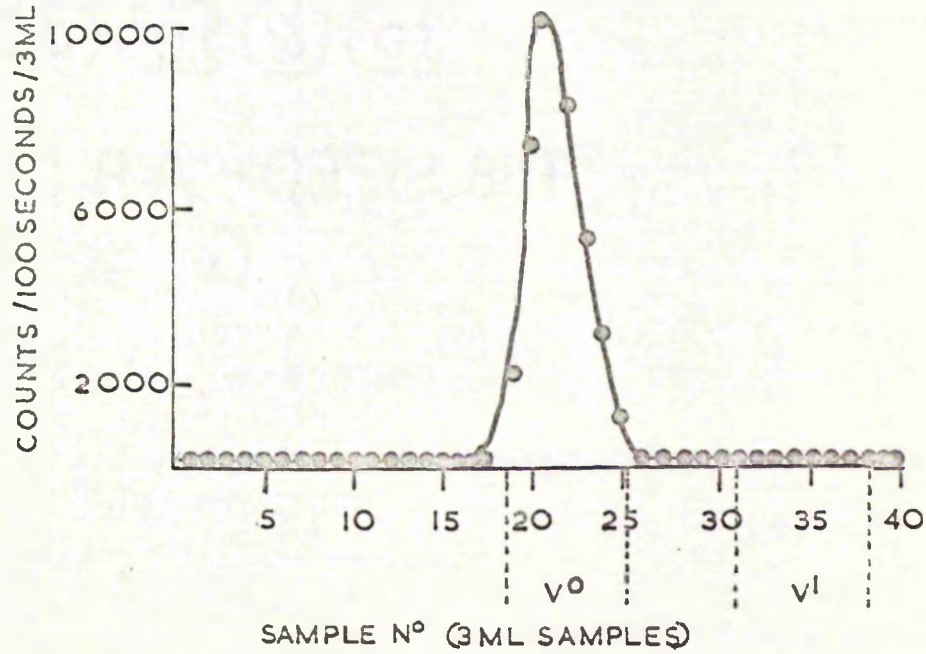
A typical example of the results obtained from the G25 Sephadex column work is shown in Figures 36, 37, 38 & 39. All the radio-active iron was found in the void volume from the columns. (The void volume is the volume of liquid required to elute a substance through a Sephadex column if the molecules are completely excluded from the gel particles). Similar results were obtained irrespective of the time interval between injecting the radio-iron into the mother rabbit and removal of the placentae. The radio-active iron in the placental cell sap would seem to be associated, therefore, with compounds whose molecular weight is greater than 5,000 since the exclusion volume of the G25 type of Sephadex is approximately 5,000. This would rule out the possibility that radioactive iron is associated with free amino acids in the placental cell sap from rabbits in the last third of gestation.

In order to obtain further information as to the molecular range of the iron containing compounds of the placental cell sap columns of G75 Sephadex were used.

FIGURE 36. RABBIT PLACENTAL CELL SAP (Placentae removed 30 minutes after injecting ^{59}Fe)

FIGURE 37. RABBIT PLACENTA CELL SAP (Placentae removed 40 minutes after injecting ^{59}Fe)

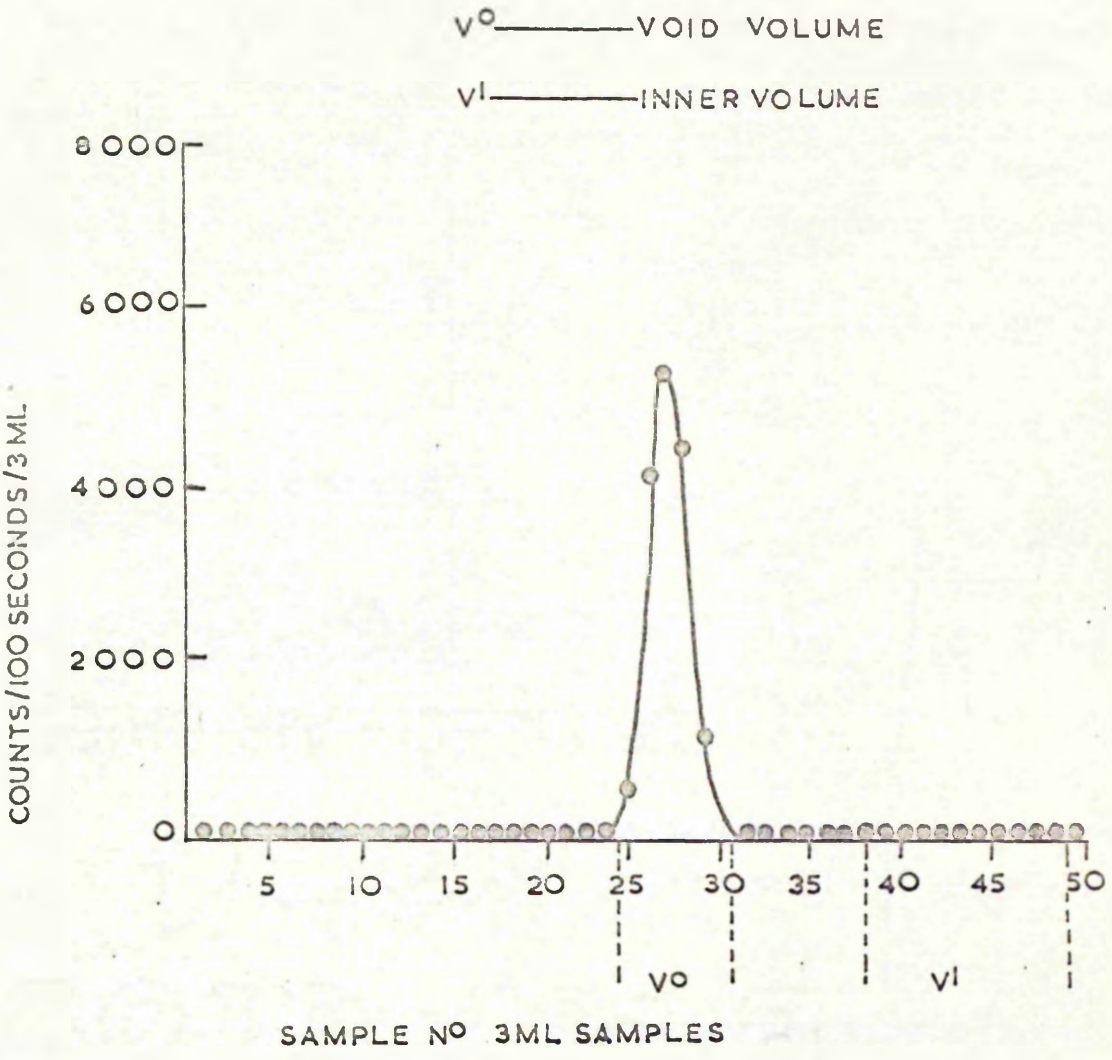

V^o VOID VOLUME
V^i INNER VOLUME
8000
6000
4000
2000
0
COUNTS/100 SECONDS / 3 ML
5
10
15
20
25
30
V^o
V^i
SAMPLE N° (3 ML SAMPLES)

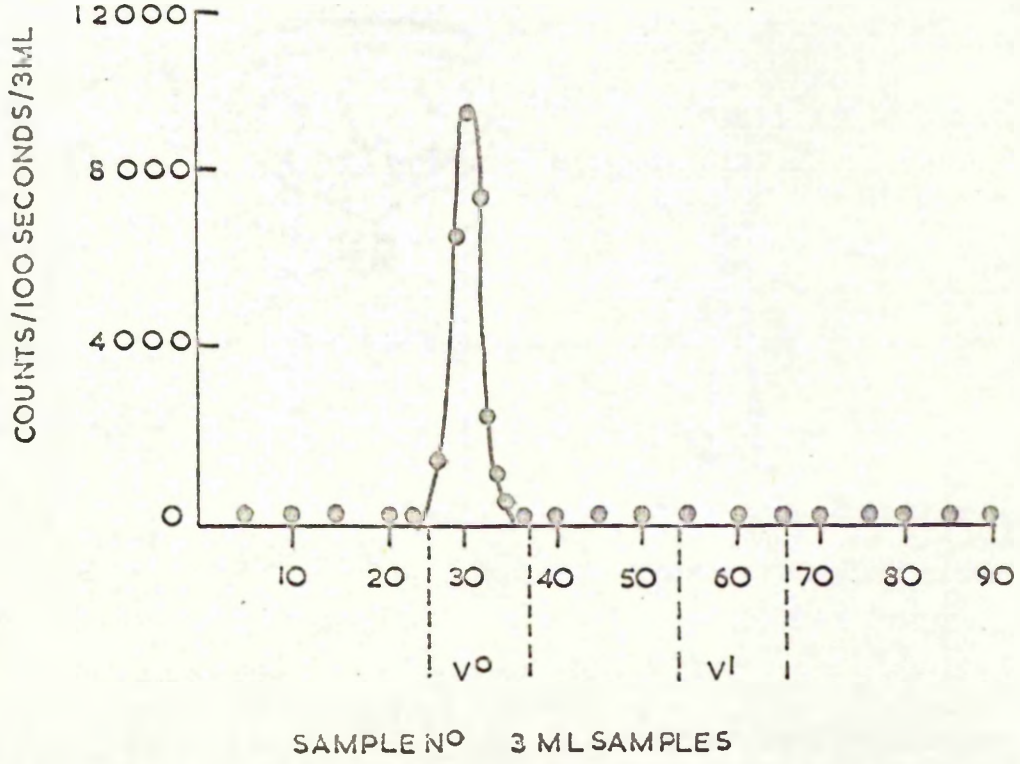

10000
6000
2000
COUNTS /100 SECONDS/3ML
5
10
15
20
25
30
35
40
V^o
V^i
SAMPLE N° (3 ML SAMPLES)

RESULTS OF CHROMATOGRAPHY USING SEPHADEX G 25

FIGURE 38. RABBIT PLACENTAL CELL SAP (Placentae removed 120 minutes after injecting ^{59}Fe)

FIGURE 39. RABBIT PLACENTAL CELL SAP (Placentae removed 180 minutes after injecting ^{59}Fe)


V° ——— VOID VOLUME
Vᴵ ——— INNER VOLUME
8000
6000
4000
2000
0
COUNTS/100 SECONDS/3 ML
5
10
15
20
25
30
35
40
45
50
V°
Vᴵ
SAMPLE N° 3ML SAMPLES


12000
8000
4000
0
COUNTS/100 SECONDS/3ML
10
20
30
40
50
60
70
80
90
V°
Vᴵ
SAMPLE N° 3 ML SAMPLES

With Sephadex G75, compounds of molecular weight greater than 50,000 would appear in the void volume from the columns.

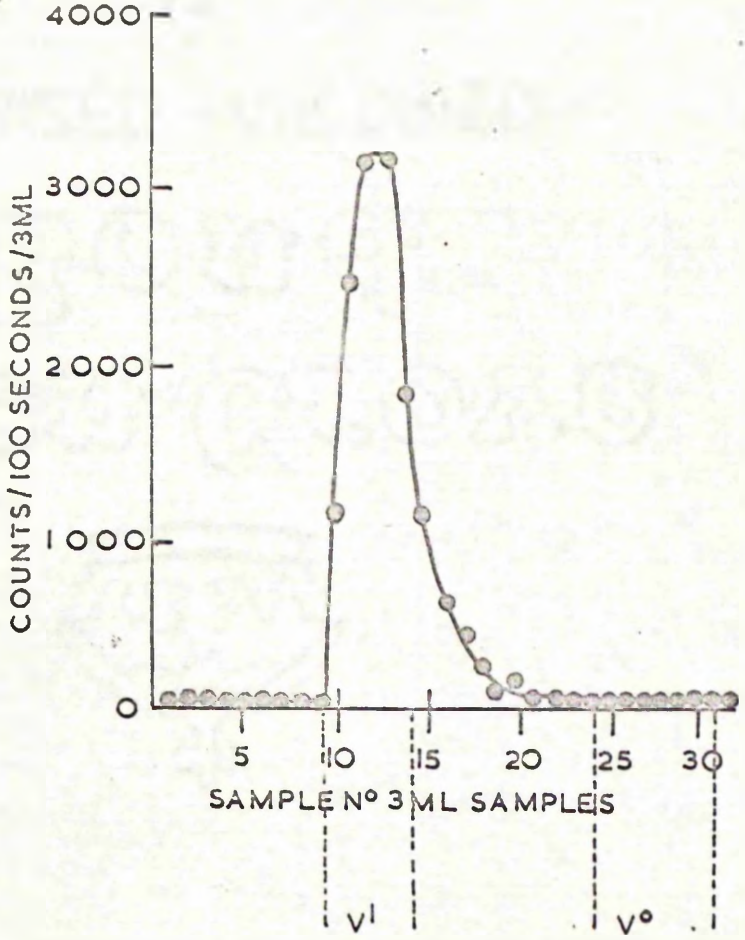
The results from the chromatography using the G75 type of Sephadex are shown in Figures 40 and 41., and the ^{59}Fe activity was found to be contained within the outer volume of the column in each case. This would indicate that the radio-active iron compounds of the placental cell sap had a molecular weight greater than 50,000.

A typical example of the results obtained from the work using G200 is shown in Figure 42. This indicates that, in this case, the activity was found in both the inner and void volume from the column suggesting that the molecular weight of some of the radio-active iron containing compounds falls between 50,000 and 200,000. (200,000 is the exclusion volume of the G200 type of Sephadex). On the other hand, since radio-activity was also found in the outer volume from the column some of the radio-active compounds of the placental cell sap must have molecular weights greater than 200,000. The same results were obtained irrespective of the time interval between

RESULTS OF CHROMATOGRAPHY USING SEPHADEX G 75

FIGURE 40

RABBIT PLACENTAL CELL SAP (Placentae removed 120 minutes after administration of ^{59}Fe)


4000
3000
2000
1000
0
COUNTS/100 SECONDS/3ML
5
10
15
20
25
30
SAMPLE Nº 3 ML SAMPLES
V¹
V°

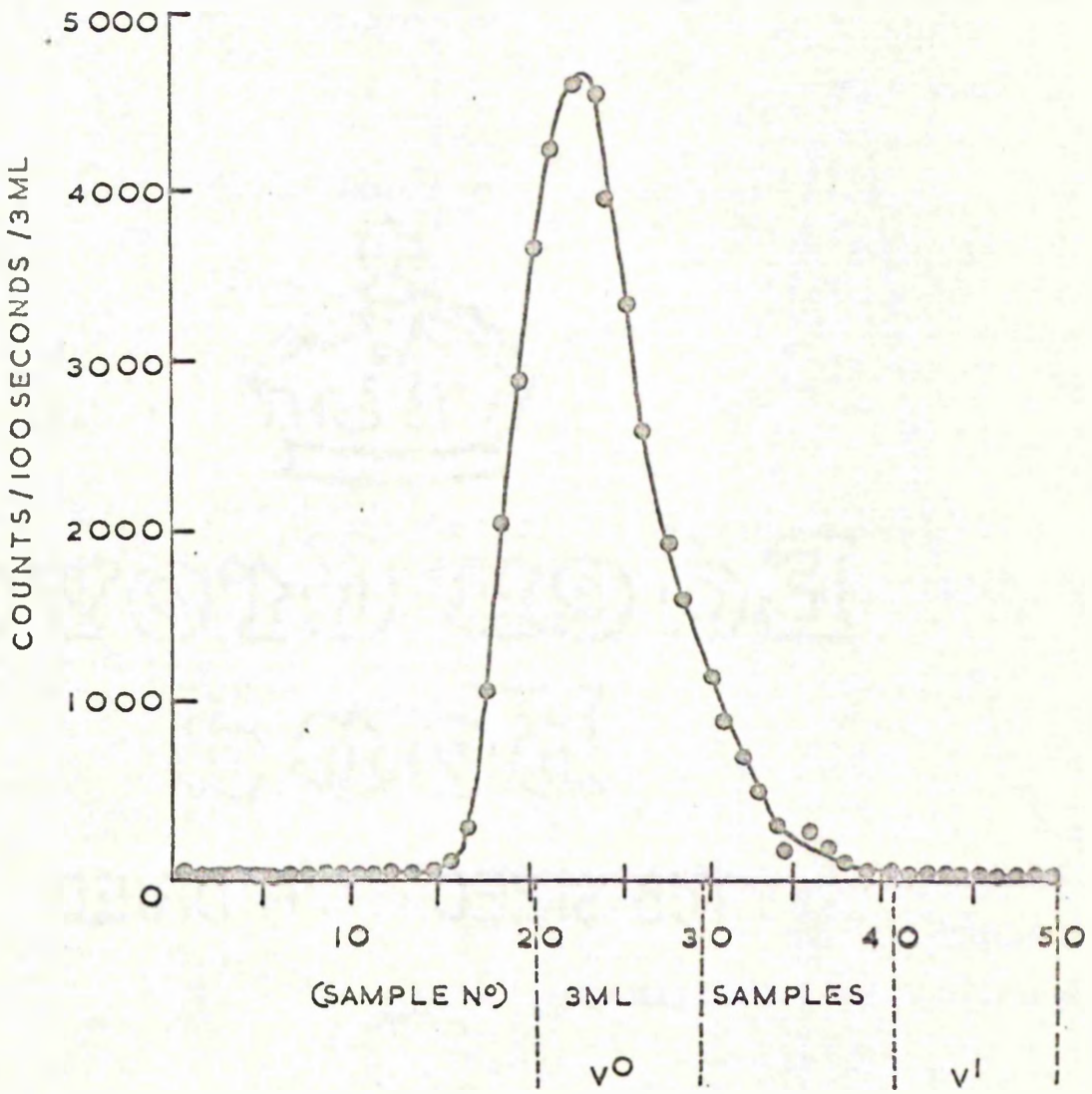
RESULTS OF CHROMATOGRAPHY USING SEPHADEX G 75

FIGURE 41

POSITION OF RADIOACTIVITY IN ELUTE FROM COLUMN WHEN RABBIT PLACENTAL CELL SAP, PREPARED FROM PLACENTAE REMOVED 40 MINUTES AFTER INJECTING ^{59}Fe, WAS APPLIED.

V^o —— VOID VOLUME

V^I —— INNER VOLUME



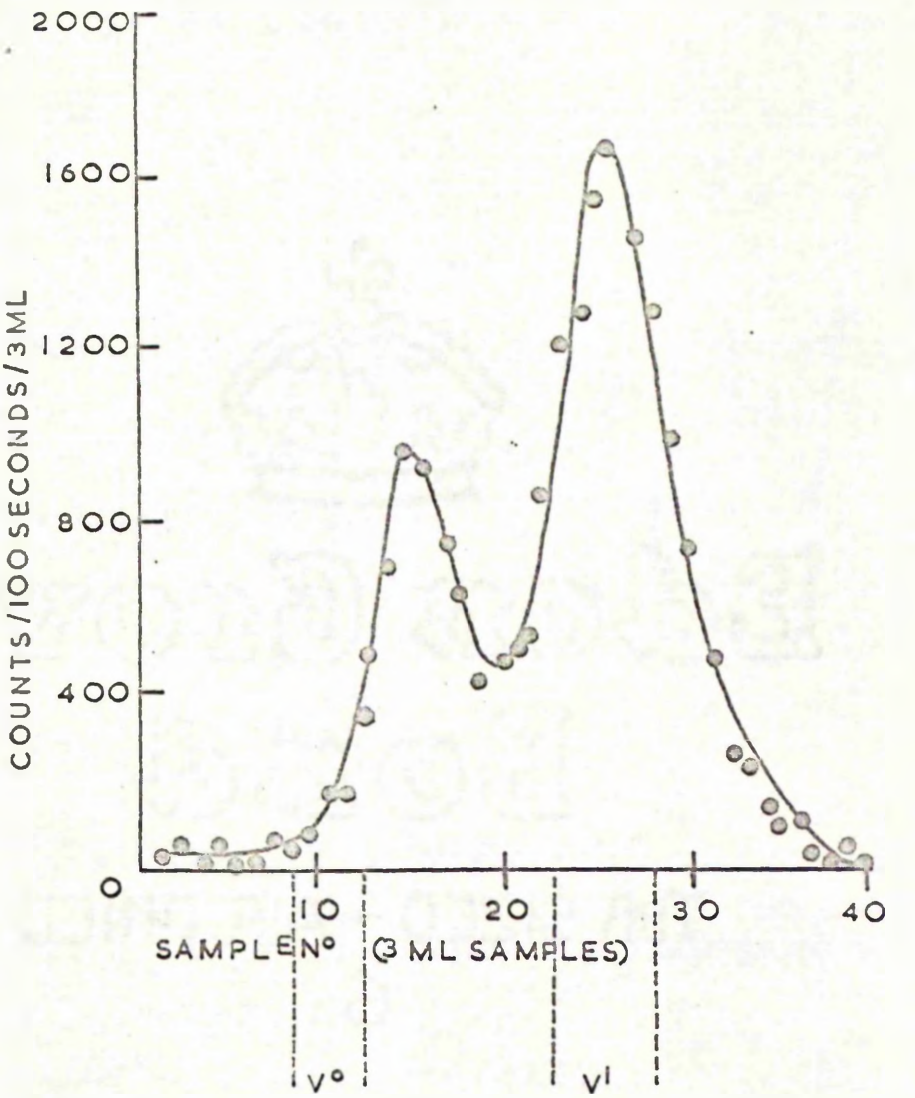
RESULTS OF CHROMATOGRAPHY USING SEPHADEX G200

FIGURE 42

POSITIONS OF RADIO-ACTIVITY IN EFFLUENT FROM COLUMNS WHEN PLACENTAL CELL SAP WAS APPLIED.

V^o ——— VOID VOLUME

V^i ——— INNER VOLUME



injecting the radio-active iron into the mother and removal of the placenta.

Work already described in this thesis indicates that the placental cell sap contains both foetal and maternal blood (Section 3). It is known that this blood contains radio-active iron attached to transferrin. This protein has a molecular weight of approximately 90,000 and would therefore be found in the inner volume from a column of Sephadex G200. This would explain at least some of the radio-activity found in this position in samples of placental cell sap applied to columns of Sephadex G200. The compound of molecular size greater than 200,000 which might be associated with radio-active iron could be ferritin (molecular weight 400,000). Granick, 1946).

It was decided to further investigate whether or not ferritin was one of the radio-active components of the placental cell sap.

Rabbit ferritin was prepared from livers and spleens of mature rabbits using, with minor modifications, the method of Granick (1943) (see appendix of methods and techniques).

A sample of rabbit ferritin (Figure 43), was run in conjunction with a sample of horse ferritin on starch gel electrophoresis and the results are seen in Figure 44. It is obvious that the rabbit ferritin adopts a similar pattern within the starch gel to that of the horse ferritin. It is also clear that the rabbit ferritin contains very little, if any, impurities.

A sample of the rabbit ferritin was now passed through a G200 Sephadex column, and its position in the samples collected from the column was identified by its obvious reddish brown colour. A sample of radio-active placental cell sap was then passed through the same column and the position of the radio-active iron in the samples noted.

Results and Discussion

The results of the work using the pure rabbit ferritin and the placental cell sap on the Sephadex G200 columns are shown in Figures 45, 46 and 47. The radio-activity found in the void volume from the columns agrees with the position of the ferritin which previously has been passed through the same column. This would suggest that at least some of the radio-active iron found in the

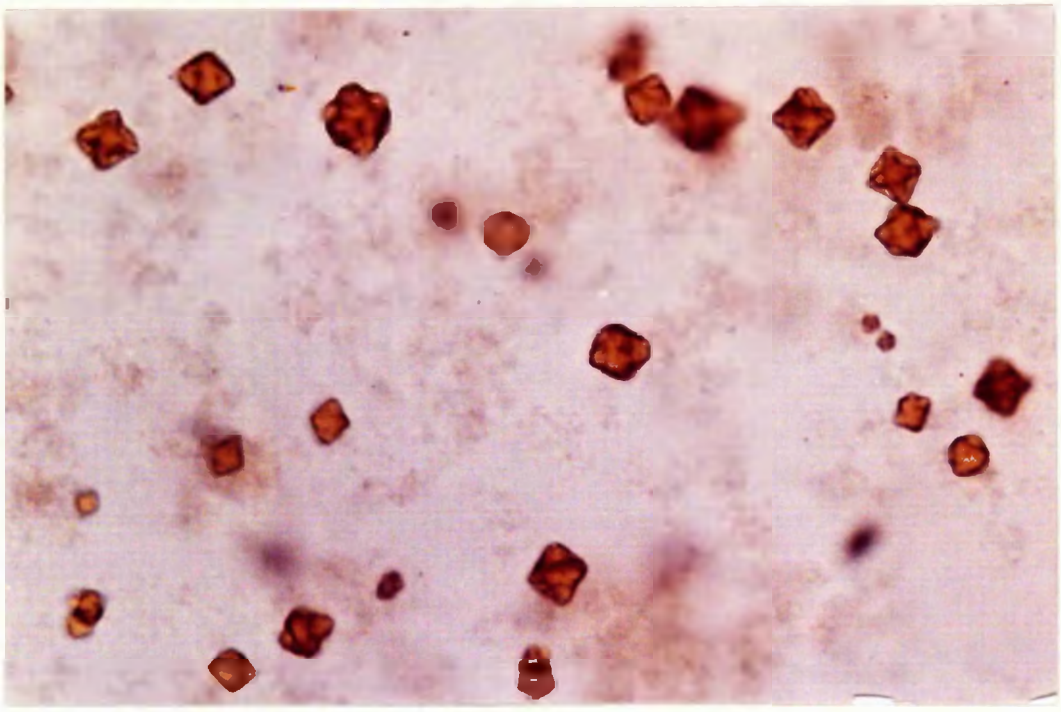
FIGURE 43

CRYSTALS OF PREPARED RABBIT FERRITIN.

FIGURE 44

ELECTROPHORESIS OF PREPARED RABBIT FERRITIN (R)

AND HORSE FERRITIN (H)



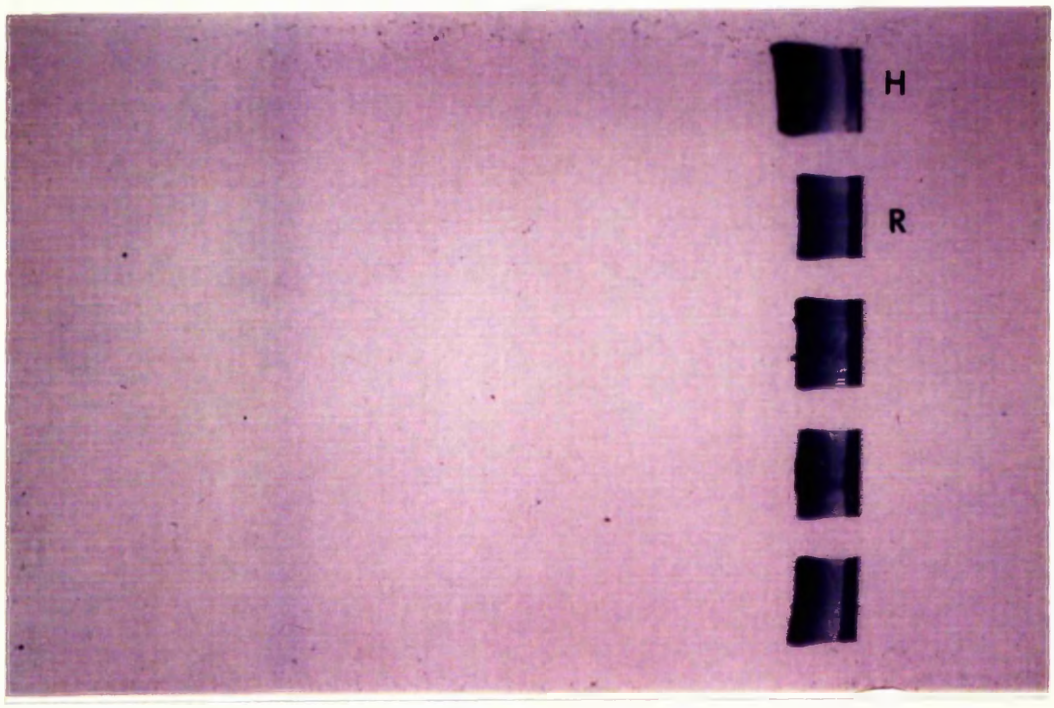
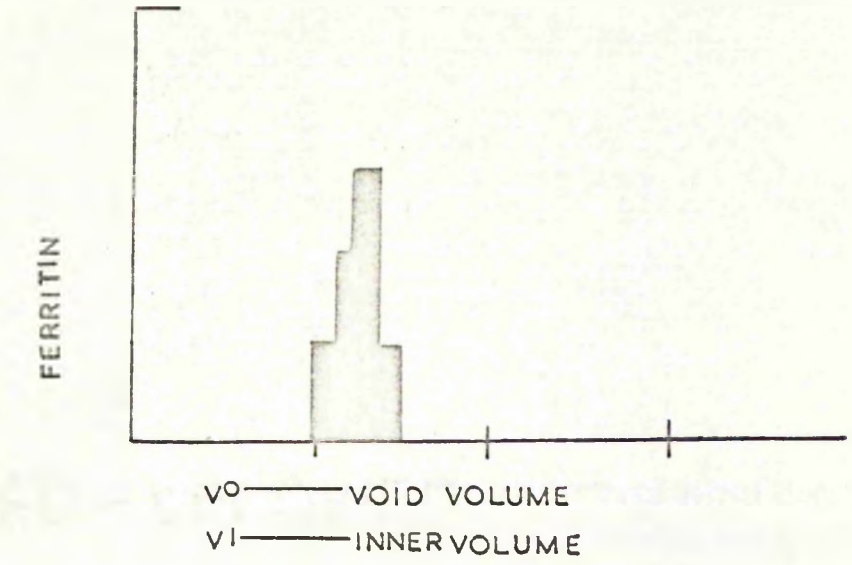
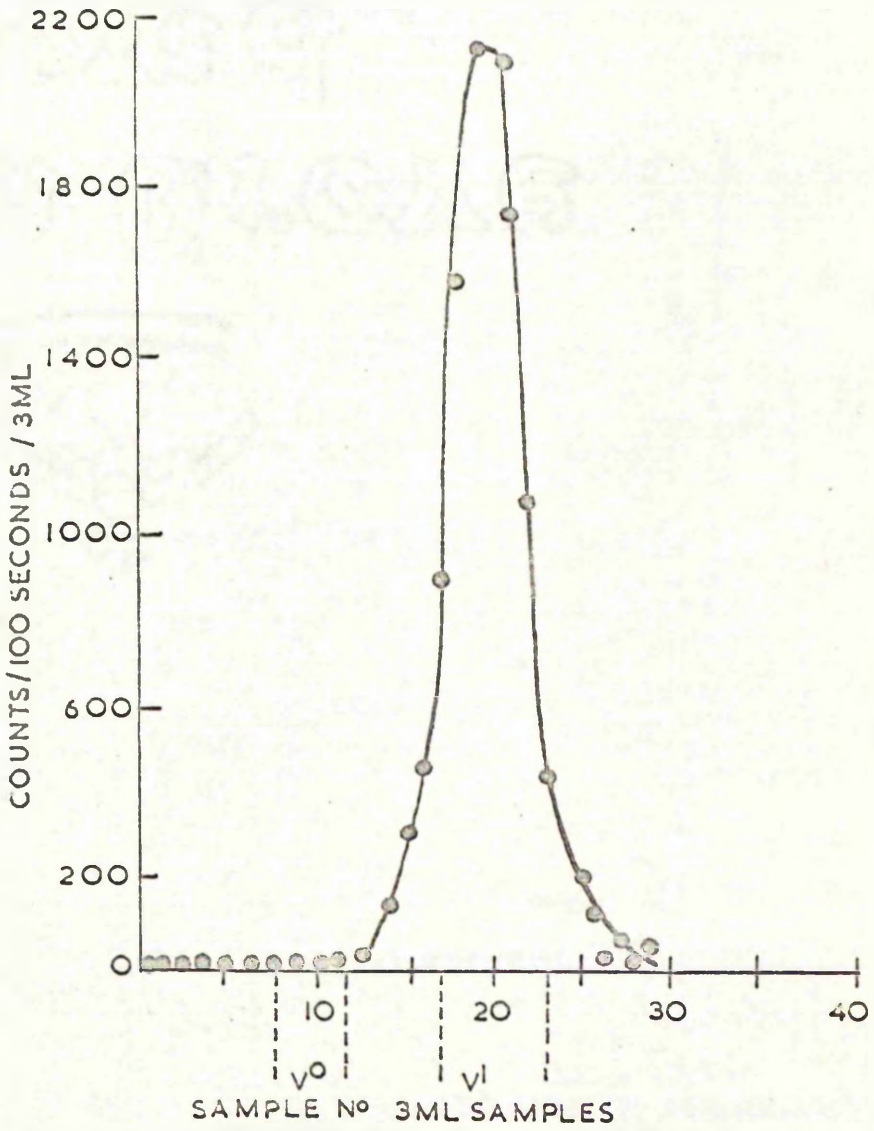

H
R

FIGURE 45. POSITION OF RABBIT FERRITIN (ESTIMATED VISUALLY) IN ELUTE FROM COLUMN.

FIGURE 46. POSITION OF RADIOACTIVITY IN ELUTE FROM COLUMN WHEN MATERNAL SERUM CONTAINING ^{59}Fe WAS APPLIED.


FERRITIN
V^o —— VOID VOLUME
V_I —— INNER VOLUME


2200
1800
1400
1000
600
200
0
COUNTS/100 SECONDS / 3ML
10
20
30
40
V^o
V_I
SAMPLE Nº 3ML SAMPLES

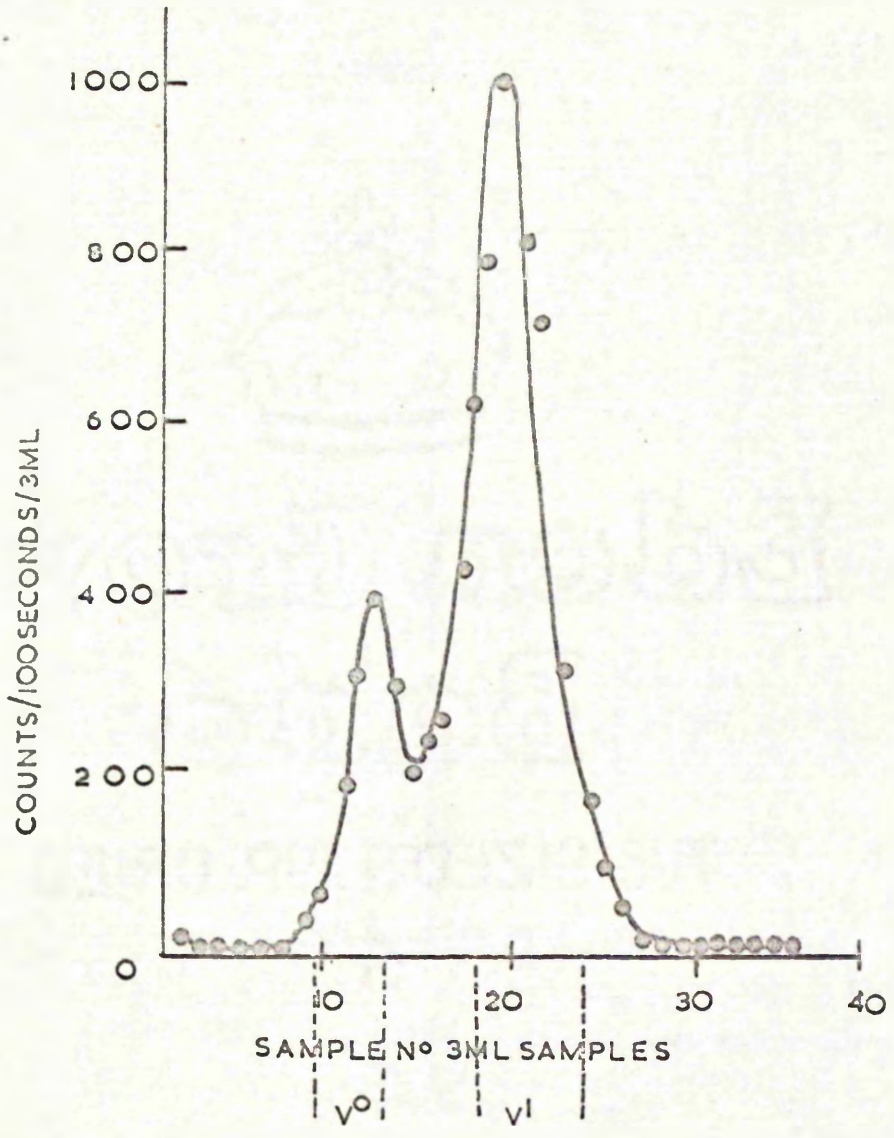
RESULTS OF CHROMATOGRAPHY USING SEPHADEX G200

FIGURE 47

POSITION OF RADIO-ACTIVITY IN ELUTE FROM COLUMN WHEN PLACENTAL CELL SAP WAS APPLIED.

V^o ——————— VOID VOLUME

V^i ——————— INNER VOLUME



void volume from the G200 Sephadex columns when a sample of placental cell sap is applied to such a column is associated with ferritin.

SECTION SIX

Starch Gel Electrophoresis

Introduction

The gel filtration studies discussed in section 5 indicated that all the radio-active iron of the placental cell sap of rabbits in the last third of pregnancy was in compounds of molecular weight greater than 50,000. This suggested that the iron was associated with protein, and in fact the work using the G200 Sephadex had suggested that ferritin was one of the iron-containing compounds in the placental cell sap.

It was, therefore, decided to investigate further the radio-active components of the placental cell sap by electrophoresis. The starch gel zonal electrophoresis method of Smithies (1955) using the discontinuous system of Poulik (1957) with minor modifications by Ashton (1957) was used. This resulted in good separation and high resolution of the serum proteins.

Experimental

The starch gels were prepared and moulded as described in the appendix of methods and techniques. The first series of gels contained samples of placental

cell sap and maternal serum. The latter was collected two hours after injecting the mother rabbit with ^{59}Fe intravenously. The placenta and serum were from the same rabbit. The gels were run for 16 hours at a constant voltage. Each gel was sliced horizontally with the gel slicer (Shandon Instrument Makers) and each half stained with amido black. A pattern of the stained bands in each of the gels was drawn on graph paper. The area of the gels containing the placental cell sap was divided into half centimetre sections and each section digested in nitric acid. The radio-activity of each sample was measured in the scintillation counter and the results drawn out on graph paper. A similar graph was constructed for the maternal serum run in each gel.

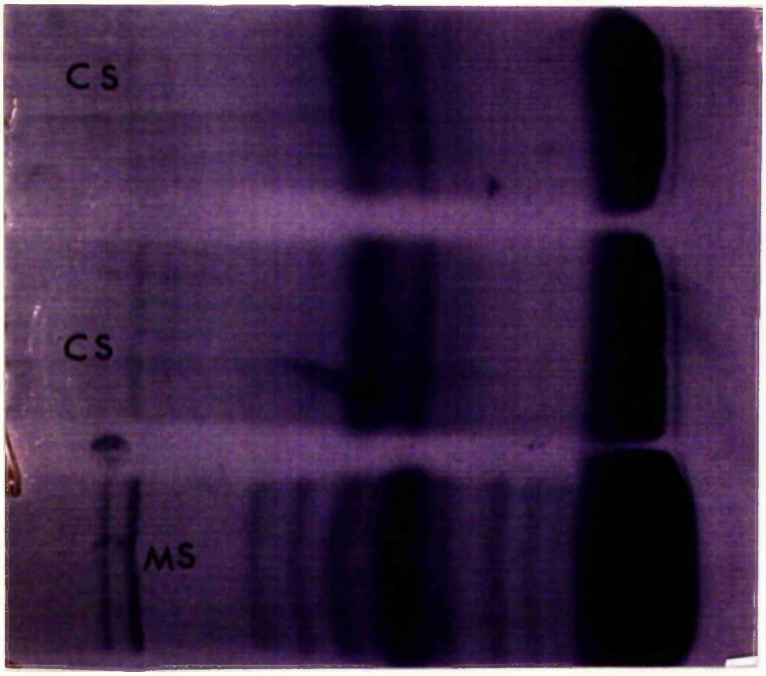
In the next series of gels, two of the slots in the gel contained samples of placental cell sap. The third slot contained a sample of rabbit ferritin. The gels were again run for 16 hours and stained. Some of the gels containing the rabbit ferritin and placental cell sap were stained with amido black and the activity in the different sections of placental cell sap measured as before. Under these conditions, the rabbit ferritin which contained

no radio-active iron was identified in the gel by the position of the single stained band which was seen easily in the area of the gel in advance of the slot which originally contained the rabbit ferritin. The rest of the gels containing the placental cell sap and the rabbit ferritin were stained by covering the gel with 2N-HCl and then sprinkling it with crystals of potassium ferrocyanide (Drysdale, 1965). By this method the iron of the ferritin was stained blue.

Results and discussion

A typical example of the results of the electrophoresis carried out using placental cell sap and maternal serum is seen in Figure 48. The results of the activity in the maternal serum and placental cell sap section are shown in Figure 49. Above the graph is shown a diagram of the pattern of stained bands seen in the gel before sectioning. It is seen that the serum contains only one peak of activity and there was activity in the corresponding region of the separated proteins of the cell sap. It was known that the peak of activity in the maternal serum represented radio-active

FIGURE 48

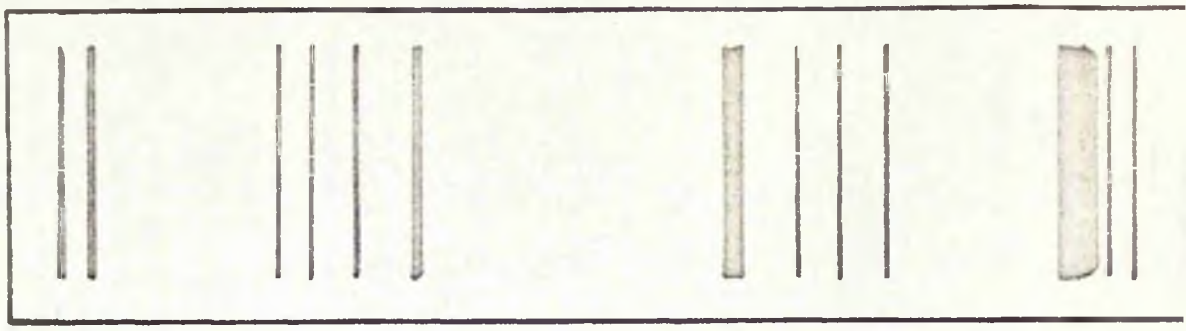


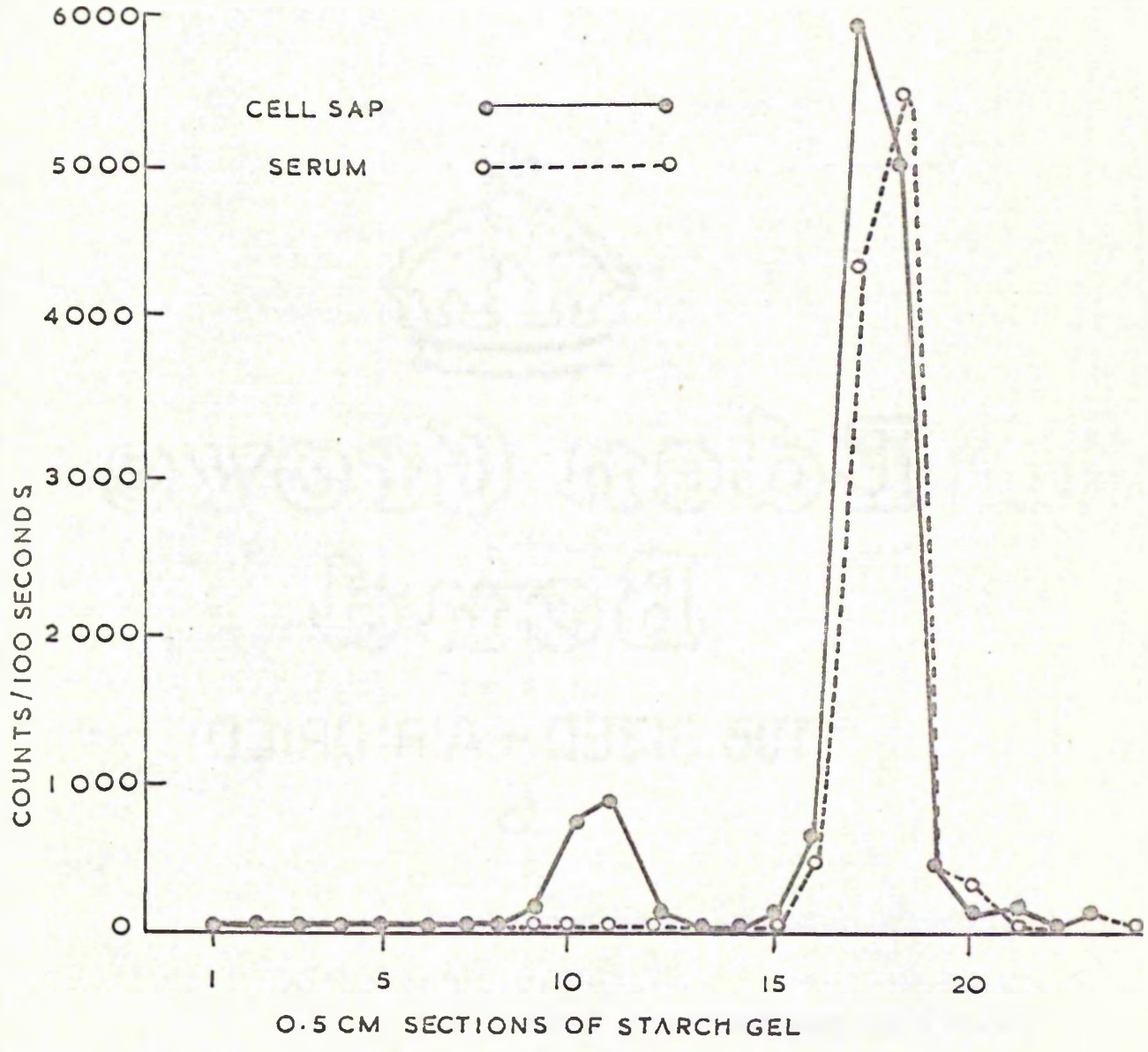
STARCH GEL OF PLACENTAL CELL SAP AND MATERNAL SERUM

MS = Maternal serum - CS=Placental cell sap.

FIGURE 49

POSITION OF RADIO-ACTIVITY IN STARCH GEL CONTAINING PORTIONS OF MATERNAL RABBIT SERUM AND RABBIT PLACENTAL CELL SAP.

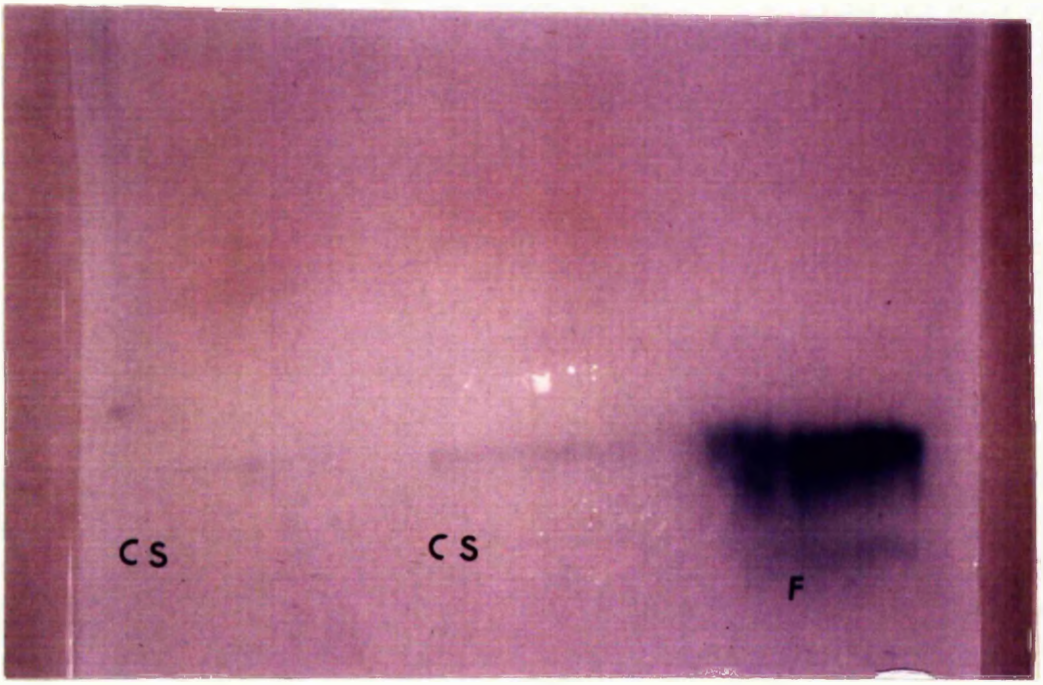



6000
5000
4000
3000
2000
1000
0
CELL SAP
SERUM
COUNTS/100 SECONDS
1
5
10
15
20
0·5 CM SECTIONS OF STARCH GEL

iron associated with the serum protein transferrin which is a B_1 globulin. It was also known that there was some maternal and foetal blood in the placental cell sap. The electrophoretic mobility of the radio-active compound in the cell sap is the same as the transferrin of the serum and suggests that this peak of activity in the cell sap represents radio-active transferrin. There is, however, an additional peak of activity within the cell sap graph. This peak is situated nearer the origin anddoes not amount to as much radio-activity as was found in the B_1 globulin region. The gel filtration work using G200 had indicated the presence of ferritin. It was decided to investigate whether or not this peak of activity represented ferritin and therefore the next series of starch gels contained rabbit ferritin and samples of placental cell sap.

An example of the results of the electrophoresis of the starch gels containing pure rabbit ferritin and placental cell sap is seen in Figure 50. These gels were stained with a mixture of NH_4Cl and potassium ferrocyanide crystals. The protein zone of the pure rabbit ferritin has stained darkly and there appeared to be two separate bands in the gel in this region. Only one palely staining

FIGURE 50



STARCH GEL ELECTROPHORESIS OF PLACENTAL CELL SAP AND PURE RABBIT FERRITIN.

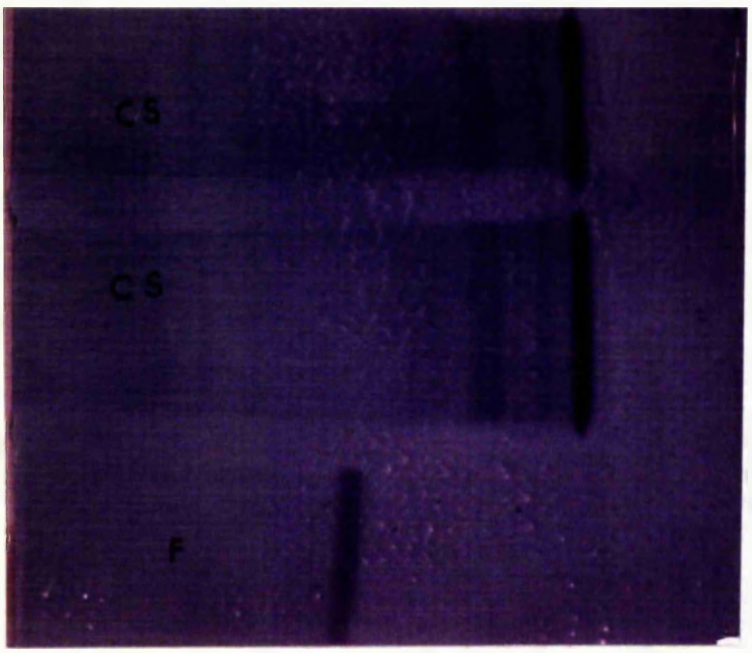
F = Ferritin - C.S. = Cell Sap

band was seen in the placental cell sap run but it coincides with the position of the darkly stained band of the pure rabbit ferritin.

Since a considerable amount of ferritin is required to be present before positive iron-staining reaction is obtained these results suggest the presence of ferritin in the placental cell sap. In order to determine whether or not the ferritin demonstrated in the placental cell sap is involved in the mechanism of placental transfer of iron it was necessary to demonstrate that it contained radio-active iron. Gels were therefore run containing rabbit ferritin and placental cell sap and the activity in the radio-active protein zone of the cell sap, other than that representing the transferrin, compared with the position of the rabbit ferritin in the same gel.

An example of the amido black stained gel containing rabbit ferritin and cell sap is shown in Figure 51. The position of the ferritin is seen easily as the only stained band in the area of the gel continuing from the slot which contained the sample of rabbit ferritin. The graph shown in Figure 52, indicates the activity found in the half centimetre sections of the gel containing the separated

FIGURE 51



Starch gel electrophoresis of rabbit ferritin and placental cell sap stained with Amido Black

F = Pure ferritin

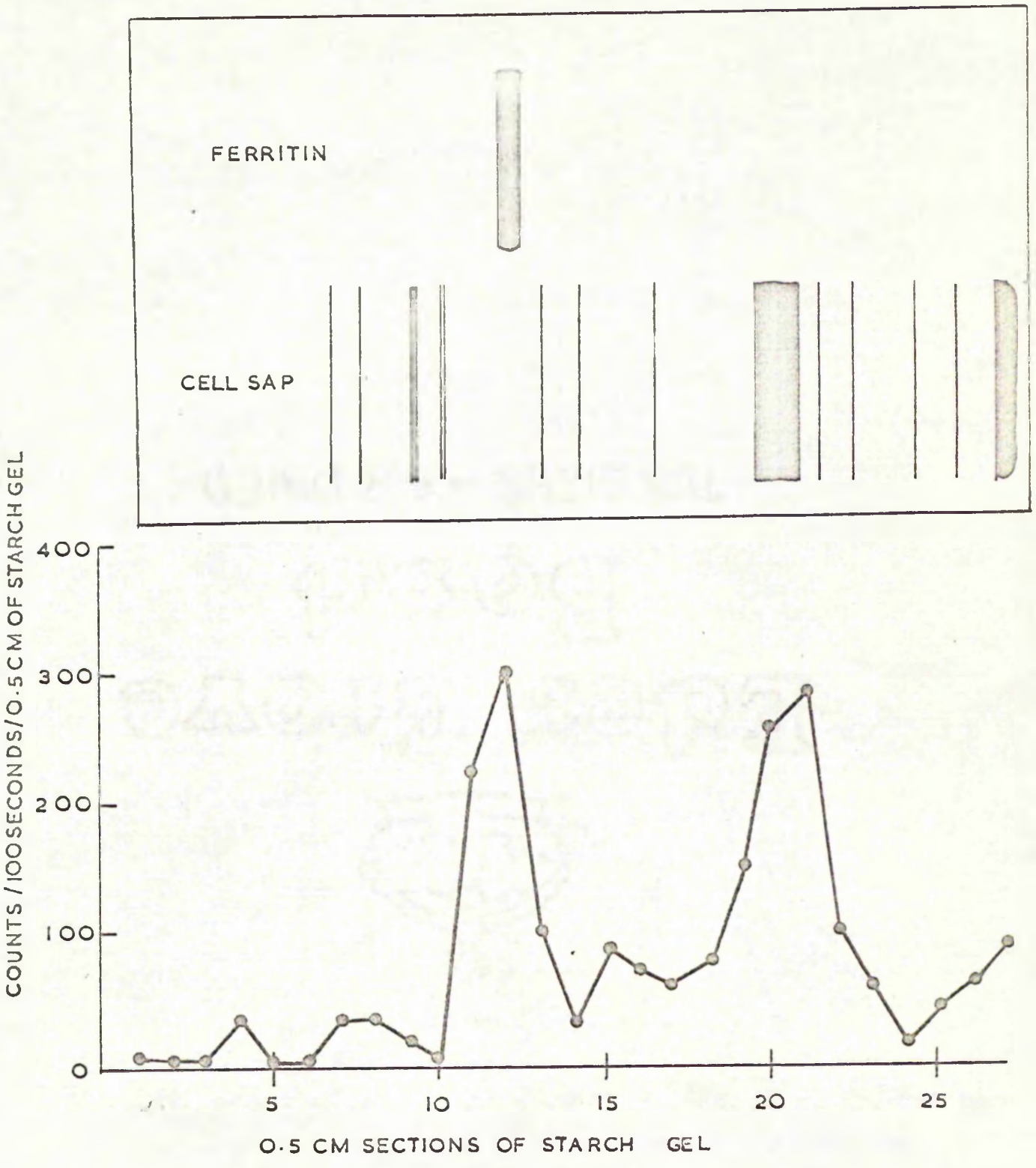
C.S. = Placental cell sap.

FIGURE 52

POSITION OF RADIO-ACTIVITY IN STARCH GEL ELECTRO-PHORESIS OF PLACENTAL CELL SAP COMPARED WITH POSITION OF PURE RABBIT FERRITIN.

A = Albumin

B = B_1 Globulin


FERRITIN
CELL SAP
COUNTS / 100 SECONDS / 0·5 CM OF STARCH GEL
400
300
200
100
0
5
10
15
20
25
0·5 CM SECTIONS OF STARCH GEL

proteins of the placental cell sap. This time the peak of activity nearer the origin of the gel corresponds with the position of the stained band representing the protein zone of the rabbit ferritin within the same gel. This suggests that the ferritin in the cell sap contains radio-active iron.

Conclusions

The electrophoresis technique used in the investigation of the iron containing compounds of the placental cell sap indicated the presence of radio-active transferrin and ferritin.

SECTION SEVEN

Further investigation of the role played by transferrin in placental transfer of iron.

Introduction

Studies using the gel filtration and electrophoresis had indicated that the injected ^{59}Fe was associated with ferritin and transferrin within the rabbit placental cell sap. Several reasons migh explain the presence of the radio-active-iron-bound-transferrin in the cell sap.

a). That the ^{59}Fe was bound to the transferrin of the maternal blood trapped in the placenta.

b). That the ^{59}Fe was attached to transferrin in the foetal circulation. This ^{59}Fe might have (1) become detached from the maternal transferrin, crossed the placenta and become attached to foetal transferrin, or, (2) crossed the placenta still attached to maternal transferrin and as such entered the foetal circulation.

c). The ^{59}Fe bound transferrin might represent a constituent of the placental cells.

If the transfe rin is capable of crossing the placenta of the rabbit it might do so by one of two routes:

one by way of the yolk sac placenta or, two, by way of the allantoic placenta. Brambell, Hemmings and Henderson (1951) demonstrated that all antibodies, which are relatively large molecules, cross from mother to foetal rabbit by way of the yolk sac placenta during the later stages of gestation. On the other hand, several workers (Davies, et al. 1959 ; and Rothwell, et al. 1958) have demonstrated that all the iron crossing from mother to foetus in the rabbit did so via the allantoic placenta.

In order to elucidate the role played by the transferrin molecule in the mechanism of placental transfer of iron in the rabbit it was decided to trace-label pure rabbit transferrin with ^{131}I, and to measure the amount of the transferrin which crosses the placenta and enters the foetus.

Methods

Rabbit transferrin greater than 97% pure (Manns Chemicals, America) was labelled with ^{131}I by the method of McFarlane (1958), as described in the appendix of methods and techniques. Enough ferric chloride was added to the labelled protein to saturate completely the transferrin with iron. The mixture was left on the bench for at least 30 minutes to ensure complete binding of the iron. The solution was dialysed against normal saline to remove any unbound iron.

Experimental

Pregnant rabbits during the last third of gestation were injected intravenously with ^{131}I -transferrin saturated with iron. Two hours later the rabbits were sacrificed with intravenous Euthatal, and all the foetuses removed quickly. Each foetus was carefully and thoroughly washed in warm saline to ensure that no contaminating maternal blood remained. A blood sample was collected from the jugular vein of each foetus. 0.1 ml. of blood from each foetus was mixed with 2 ml of distilled water in a polythene tube and the ^{131}I activity of each sample measured in a

scintillation counter. Each foetus was then digested in a mixture of concentrated nitric acid and Hydrochloric acid 2:1, and the ^{131}I activity of each measured as before.

Results and Discussion

The results of the experiments carried out using ^{131}I-labelled transferrin saturated with iron are shown in Tables 9 and 10. These results indicate that at day 22 and day 25 of gestation 5% and 2.5% respectively of the iron bound labelled protein injected into the mother rabbit crossed the placenta and entered the foetal rabbits. This transferrin might have reached the foetuses by either of the following routes:

a) the yolk sac placenta, which in the rabbit persists to term and has been shown to play a part in the transfer of certain serum proteins and the antibodies from mother to foetal rabbit during the later stages of pregnancy (Hemmings, 1958). However, Hemmings (1965) suggested that the transfer of antibodies via the yolk sac placenta in the rabbit was a process that took at least 24 hours before evidence of their presence in the foetuses was available. This would seem to suggest that in the present experiments which lasted two hours at the most, that the transferrin found in the foetuses had crossed from mother to foetus by

b) the chorio allantoic placenta of the rabbit which

TABLE 9

Foetus No.	^{131}I activity in total foetal digest as counts per 100 secs.
1	19,296
2	12,852
3	9,756
4	5,957
Total foetal activity	47,861
Total amount injected into mother rabbit	9,572,000
∴ Percentage of initial amount in foetuses	0.5

The above results are from a rabbit 22 days pregnant containing 4 foetuses.

TABLE 10

Foetus No.	^{131}I in total foetal digest - counts per 100 seconds
1	63,500
2	101,246
3	77,880
4	110,000
5	15,158
6	88,060
7	94,780
8	86,220
9	24,811
Total foetal ^{131}I activity	661,765
Total injected into mother rabbit	25,254,000
∴ Percentage of the initial injection found in foetuses	2.5

The above results are from a rabbit 25 days pregnant and containing 9 foetuses.

has been shown by several workers (Bothwell et al. 1958 and Davies et al. 1959) to be the main route taken by the iron crossing from mother to foetal rabbit, during the last third of gestation. If the transferrin is crossing by this route it might well be involved in the mechanism of placental transfer of iron.

However, one other explanation for the presence of ^{131}I in the foetal digest must be considered. It was assumed that this ^{131}I was attached to transferrin but this could in fact be free ^{131}I which had not become bound to the protein during the labelling process and had not been subsequently removed by dialysis. Electrophoresis of the foetal serum would show clearly whether or not the ^{131}I was associated with the B_1 globulin fraction of the plasma proteins.

Experimental

Starch gel electrophoresis was carried out on serum samples collected from foetuses at 25 days gestation which were removed two hours after their mother had been injected intravenously with ^{131}I iodinated transferrin saturated with iron. For comparison, samples of normal

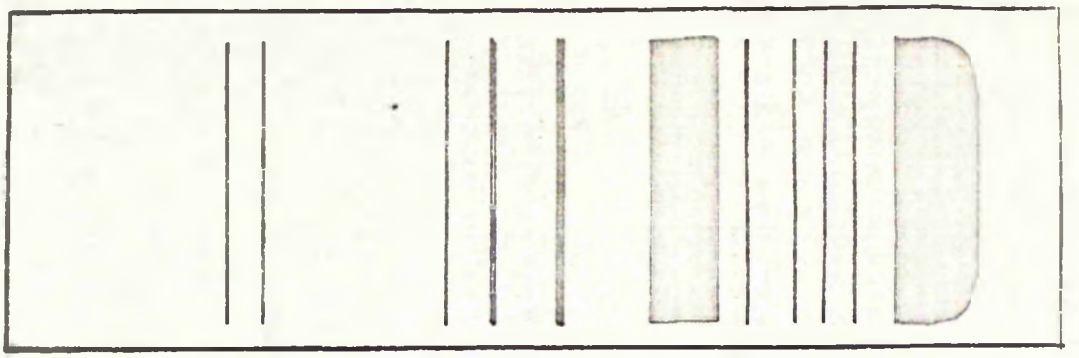
rabbit serum were run at the same time. Staining and measurement of the activity in the gels was carried out as described previously (see appendix of methods and techniques).

Results and Discussion

The results of the ^{131}I activity found in the electrophoretogram of the foetal and maternal serum are shown in Figures 53 and 54. Above the graph, a diagram of the stained protein bands of the normal rabbit serum is shown. Activity was present in the area of the foetal serum adjacent to the B_1 globulin region of the normal rabbit serum. However, activity was also present in the region of the foetal serum corresponding to the albumin region of the normal rabbit serum. This suggested that whilst there was definitely some ^{131}I bound to transferrin there was also ^{131}I associated with albumin in the foetal serum. This indicated that the transferrin used in this group of experiments was not pure. It was necessary, therefore, before proceeding with this part of the work to purify the transferrin.

FIGURE 53

RESULTS OF ^{131}I ACTIVITY IN ELECTROPHORETOGRAPH OF FOETAL SERUM



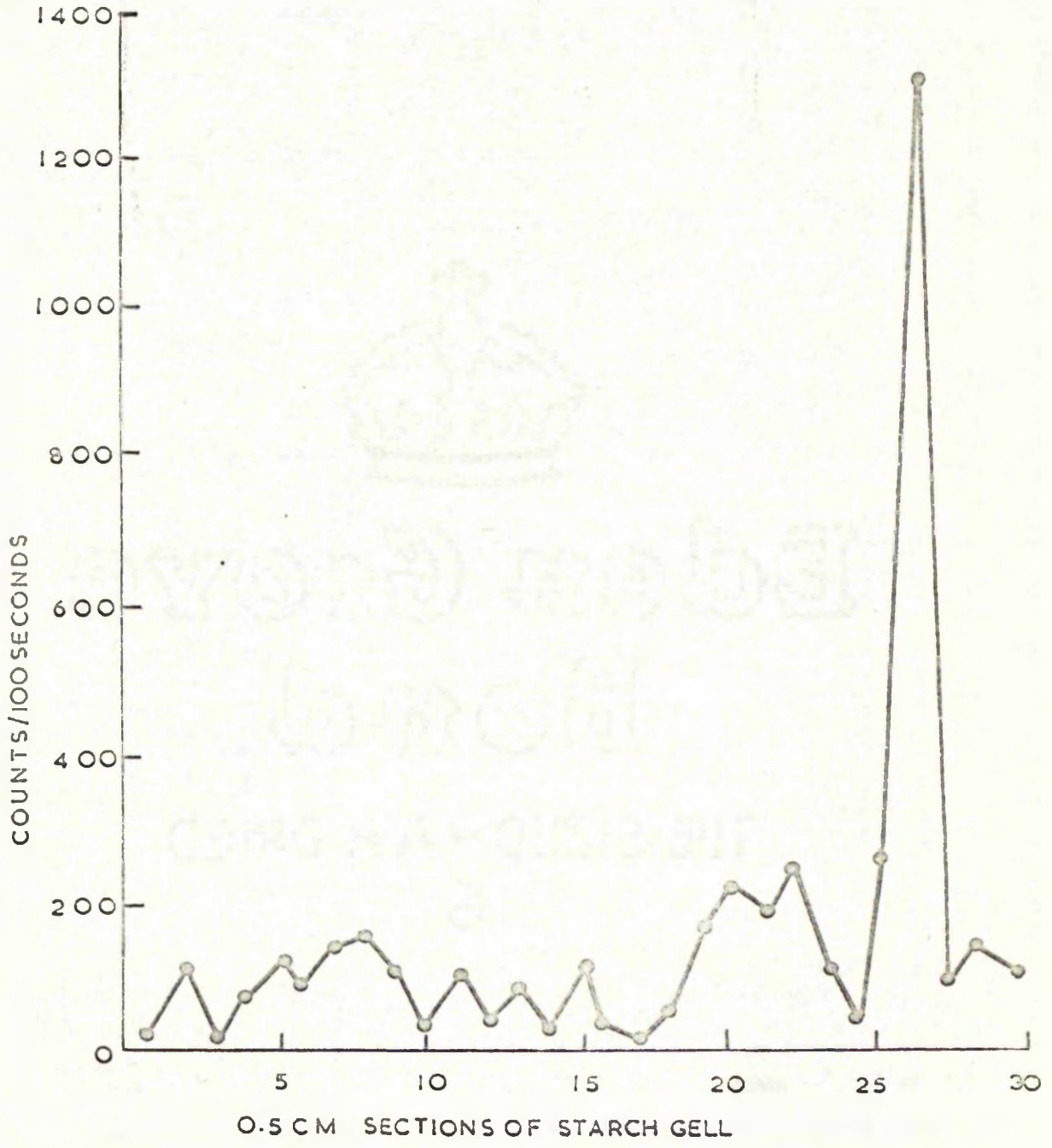
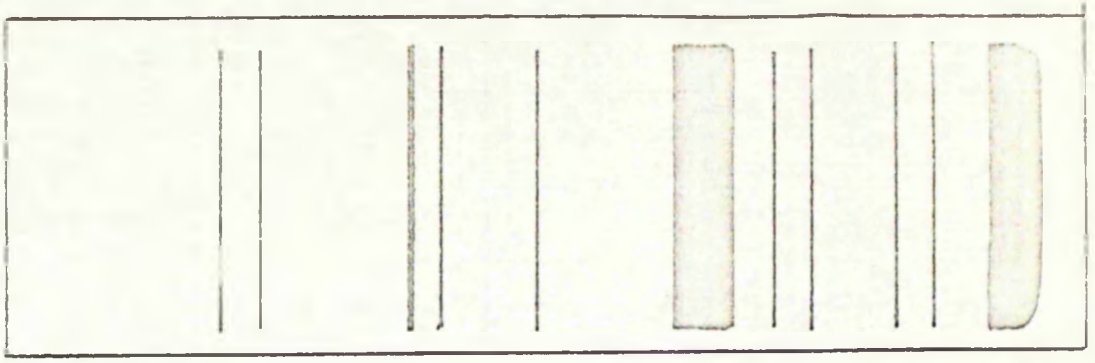
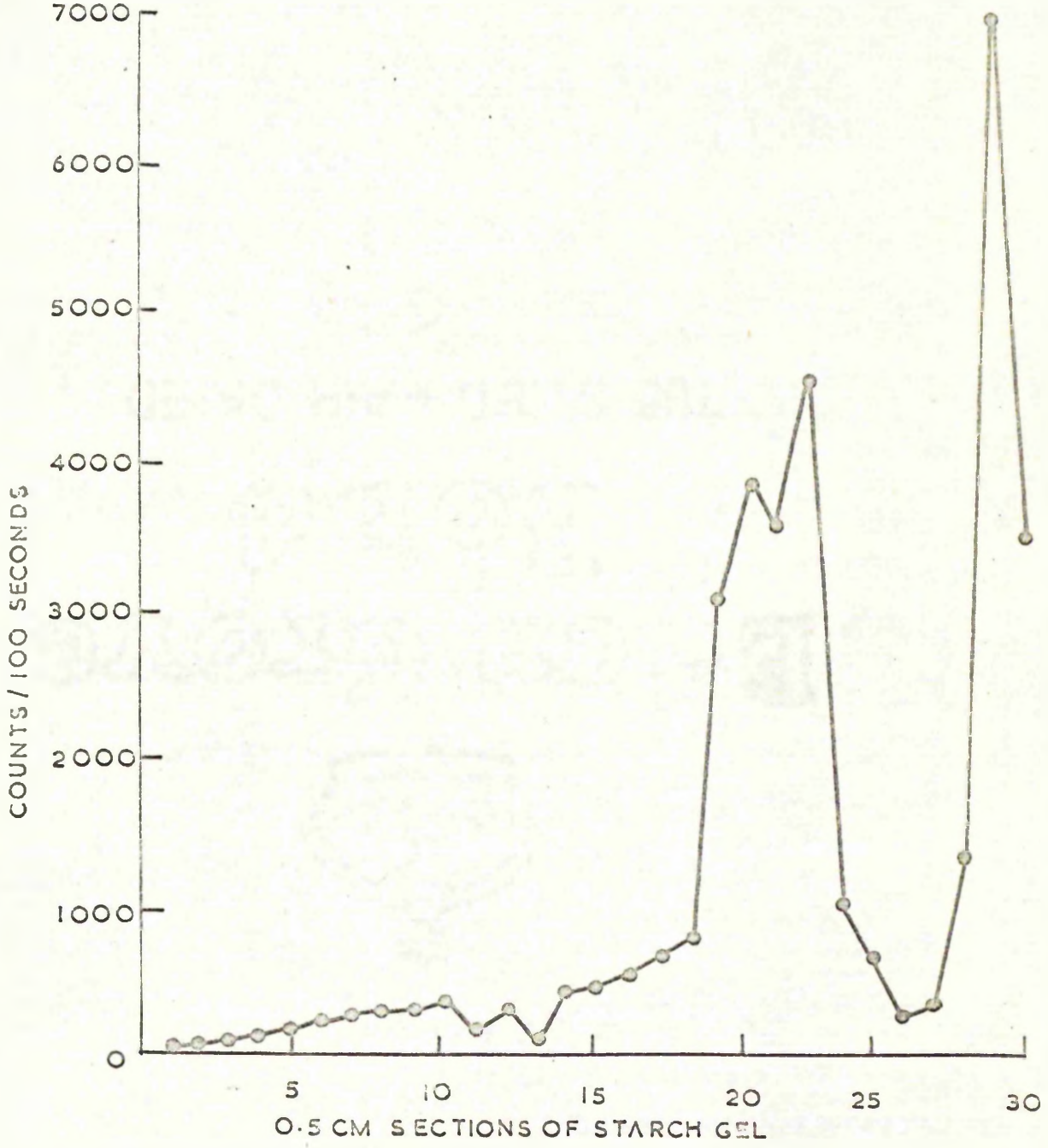

1400
1200
1000
800
600
400
200
0
COUNTS/100 SECONDS
5
10
15
20
25
30
0.5 CM SECTIONS OF STARCH GELL

FIGURE 54

RESULTS OF ^{131}I ACTIVITY IN ELECTROPHORETOGRAM OF MATERNAL SERUM.




7000
6000
5000
4000
3000
2000
1000
0
COUNTS / 100 SECONDS
5
10
15
20
25
30
0·5 CM SECTIONS OF STARCH GEL

Purification of Transferrin

The rabbit transferrin was dissolved in 0.02M-phosphate buffer, pH 6.6, and passed through the D.E.A.E. Sephadex A50 anion exchange column. The method used was that of Patterson & Sober (1959), Porter (1961) and Fleck (1963) (see appendix of methods and techniques). The different proteins were separated and eluted from the column by an increased gradient of NaCl solution set up as shown in Figure 55. The outflow from the column was collected in 3 ml amounts in a fraction collector. The position of the proteins in the samples collected was found by measuring the optical density of each of the samples in the spectrophotometer at 280 mμ . The optical density results are shown in Figure 56. It is seen that there are two peaks of which the first is the greater. This peak is transferrin and the other peak is albumin. The samples comprising the first peak shown on the graph were pooled, dialysed against ammonium bicarbonate and freeze-dried overnight. A sample of the freeze-dried powder was electrophoresed on cellulose acetate strips along with a sample of the original mixture and a sample of normal rabbit serum. When the strips were stained it was seen that the purified transferrin now contained only one

FIGURE 55

APPARATUS USED FOR PURIFICATION OF TRANSFERRIN.

A. 680 ml. 0.02M phosphate buffer

B. 680 ml. 0.02M phosphate buffer

C. 566 ml. 0.02M 3.9%M NaCl

D. Column containing DEAE A50 Sephadex

E. Fraction collector

F. Magnetic stripper

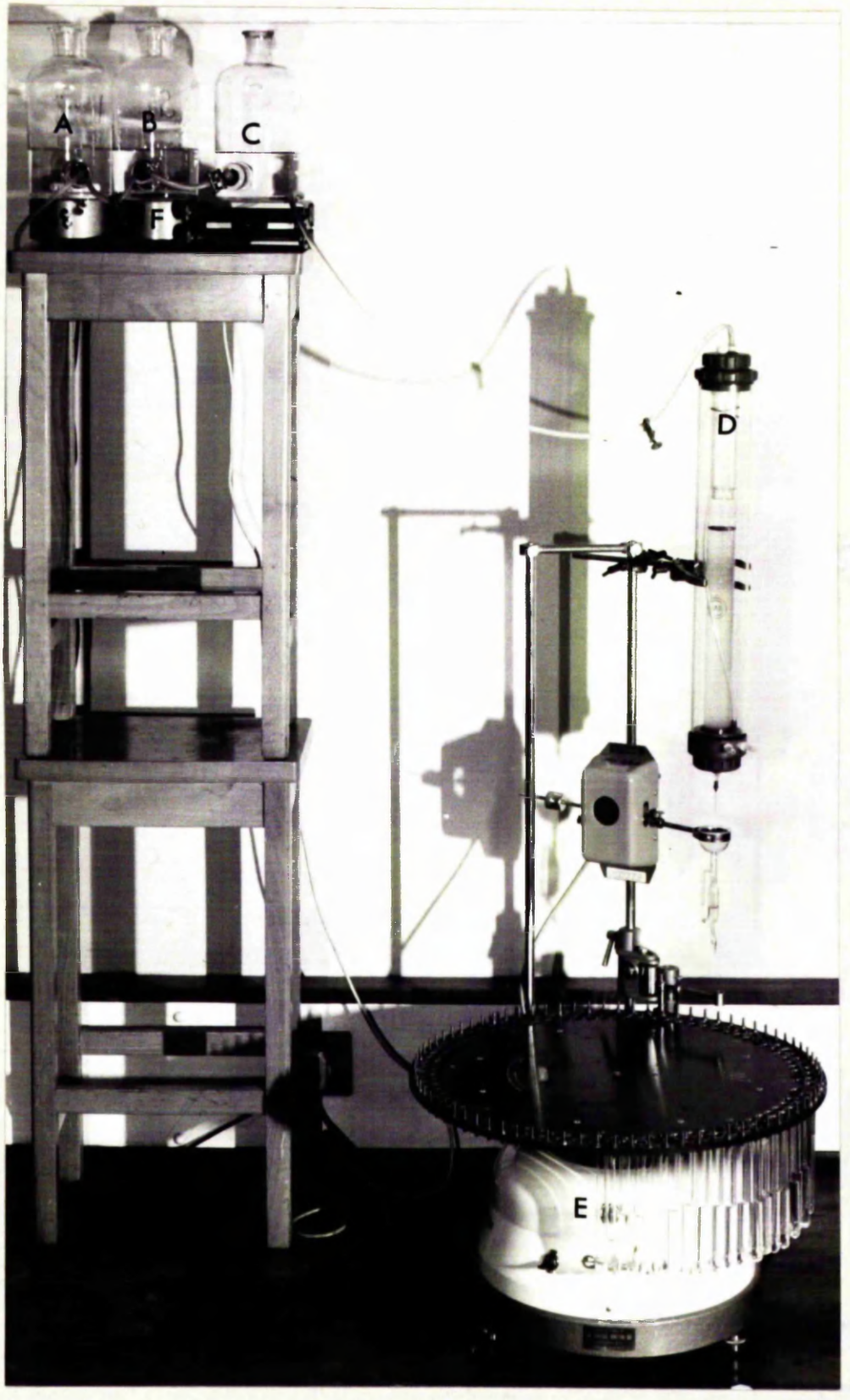
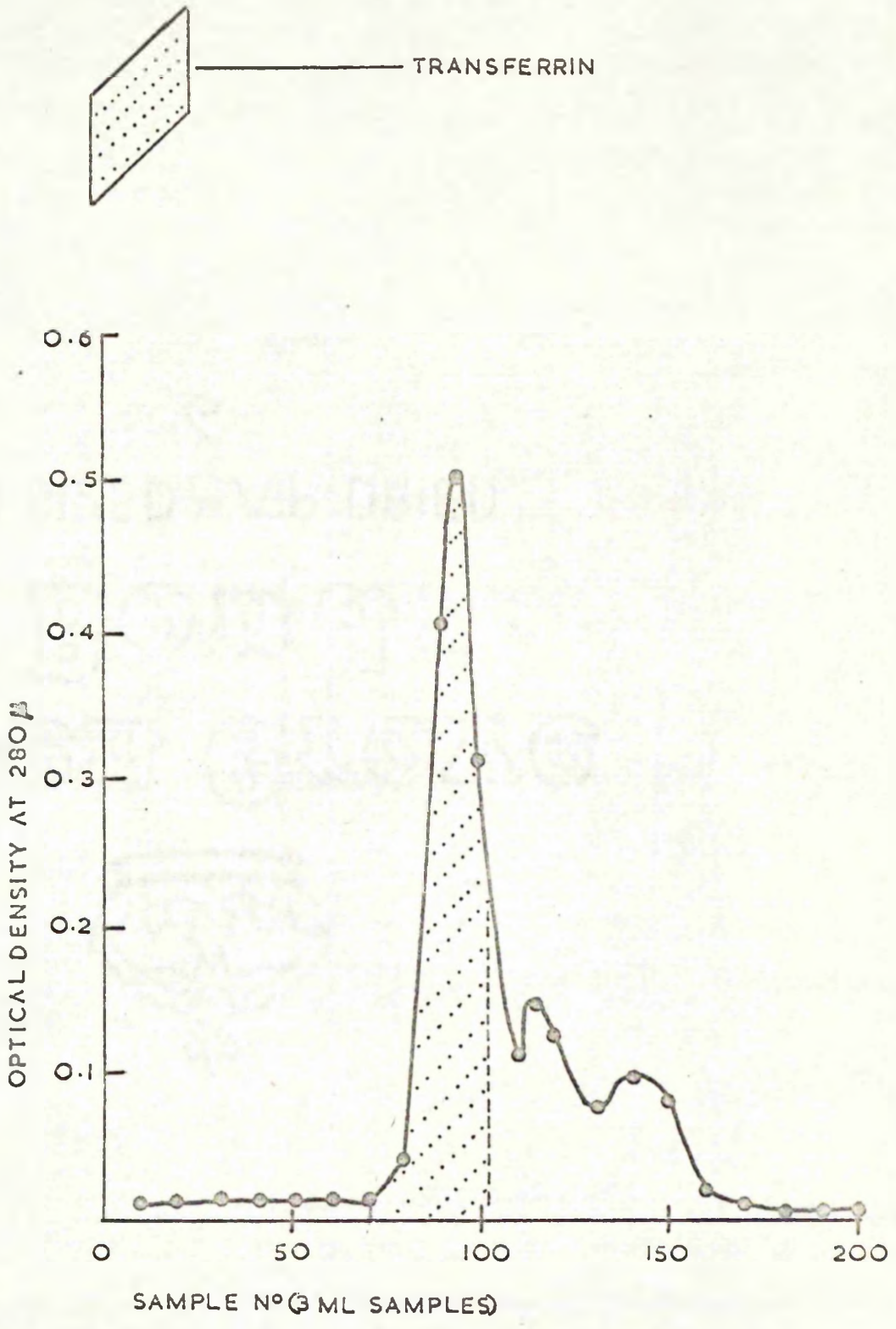

A
B
C
F
D
E

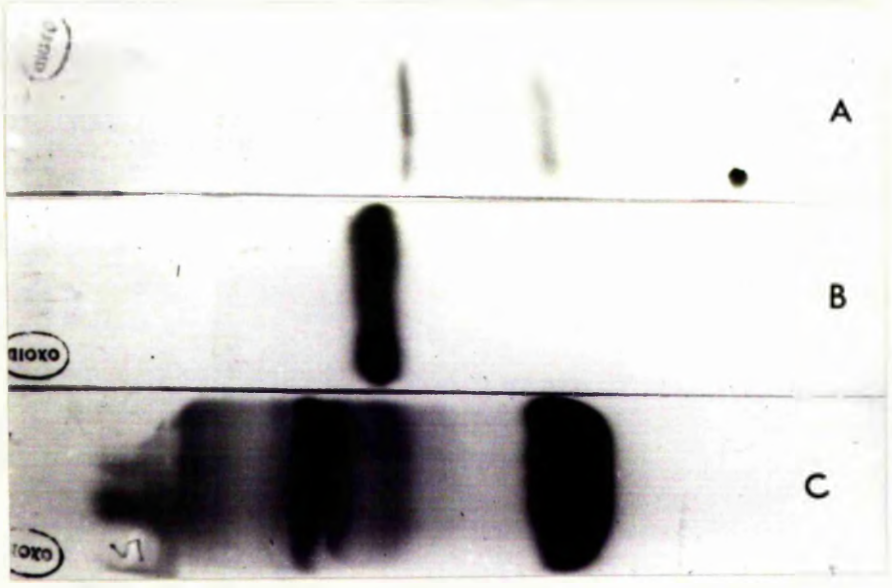
FIGURE 56

POSITION OF PROTEINS IN SAMPLES COLLECTED FROM DEAE A50 SEPHADEX COLUMN.


TRANSFERRIN
0·6
0·5
0·4
0·3
0·2
0·1
OPTICAL DENSITY AT 280μ
0
50
100
150
200
SAMPLE Nº (3 ML SAMPLES)

stained band which corresponded to the position of the B_1 globulin fraction in the normal rabbit serum (Figure 57). Iodination of the purified transferrin was carried out as previously described. Enough radioactive iron and cold iron, as ferric chloride, was added to completely saturate the transferrin and the unbound iron was removed by dialysis against normal saline. Finally, a sample of the ^{131}iodinated transferrin, saturated with the radio-active and cold iron, was passed through a Sephadex G25 column. The samples collected from this column indicated that all the radio-activity was confined to the outer volume of the column indicating that all the radio-active iron and iodine was protein bound.

FIGURE 57



Starch Gel Electrophoresis of Rabbit Transferrin before and after Purification.

A = Contaminated rabbit transferrin

B = Purified rabbit transferrin

C = Normal rabbit serum

Electrophoresis of the foetal serum had indicated that it contained some ^{131}I attached to transferrin at day 25 of gestation. This demonstrated that the transferrin molecule was capable of crossing the rabbit placenta at this stage of pregnancy. However, since it was of interest to know whether or not the transferrin molecule was involved in the transfer if iron from mother to foetal rabbit and, since the work already done did not indicate whether or not the transferrin was associated with iron whilst crossing the placenta, it was decided to adopt another method of approach to the problem.

Experimental

Rabbits in the last third of gestation were injected with a known amount of ^{131}iodinated transferrin saturated with a mixture of ^{59}Fe and ferric chloride. 0.005 ml of the mixture injected was diluted with 2 ml of distilled water in a polystyrene tube and a drop of 4N-NaOH added to stop precipitation of the protein. Two hours later the mother rabbit was sacrificed by intravenous Euthatal. A blood sample was taken from the mother rabbit just prior to death. 0.2 ml of this blood was diluted in 2 ml of

distilled water plus a drop of 4N-NaOH in a polystyrene tube. The foetuses were removed rapidly, washed thoroughly in normal saline and from a blood sample collected from their jugular veins 0.5 ml was diluted in 0.2 ml of distilled water in a polystyrene tube. The foetuses were then digested in a mixture of concentrated nitric acid and sulphuric acid and a sample of the digest transferred to a polystyrene tube in order to measure the radioactivity in the foetuses. The total radioactivity of each of the samples collected, i.e. the mixture injected, the maternal and foetal blood and the foetal digest, and of each of the isotopes, i.e. ^{59}Fe and ^{131}I, was measured in the scintillation counter daily for several days. The decay factors for the ^{59}Fe and the ^{131}I were calculated from the daily readings of the standards and those readings agreed with the work of Francis, Mulligan and Wormall (1959) Table 11. The ratio of the ^{59}Fe to ^{131}I in each of the samples collected was calculated. A typical example of the method of calculation used is shown on the following page. Finally an Echo-counter was set to measure ^{59}Fe activity only in each of the samples on the last day of the readings.

Example of Calculation

Maternal blood from rabbit one.

Let x = the ^{59}Fe activity of the mother's blood on day one.

Let y = the ^{131}I activity of the maternal blood on day one.

25427 = the total activity of the maternal blood on day one.

$\therefore$ $x + y = 25427$

97.7% of the ^{59}Fe activity remains after 24 hours

90.5% of the ^{131}I activity remains after 24 hours (Table 11)

20994 = the radio-activity expressed as counts per 100 seconds in the maternal blood on day two.

$\therefore$ $0.977x + 0.905y = 20994$

By multiplying equation one by 0.977

$0.977x \times 0.977y = 22838$

and by subtracting equation two from equation three

$0.72y = 1894$

$\therefore$ $y = 26305$

and $x = 0$

This indicates that there is no radio-active iron in the maternal blood sample.

PERCENTAGE DECAY OF ^{59}Fe and ^{131}I OVER 72 HOURS

Radioactivity Expressed as Counts/100 Sec.

DECAY TIME (Hrs.)	^{59}Fe	% REMAINING	^{131}I	% REMAINING
0	602,502		155,794	
24	588,797	97.7	141,107	90.5
48	141,107	95.9	128,953	82.4
72	202,340	90.5	119,238	76.2

TABLE II

Results and Discussion

The total radio-activity expressed as counts per 100 seconds for each of the samples recorded on the 5 days following their collection are shown in Table 12. The calculations made from these results of the ratio of the $^{59}Fe/^{131}I$ in each of the samples on each of the 5 occasions that they were counted are shown in Tables 13 & 14. These indicate that:

a) the amount of ^{59}Fe bound to transferrin left in the mother's blood, two hours after injecting it intravenously, is very small. In fact, in two cases out of four, the results indicate that no iron was retained in the maternal blood, and when the scintillation counter was set to read only the counts from ^{59}Fe, the counts recorded from the maternal blood were only a background reading. This confirmed that there was no iron remaining in the mother's blood.

b) There was both ^{59}Fe and ^{131}I in the foetal blood. Since the ^{131}I was known to be bound to transferrin this indicated that transferrin had crossed the rabbit placenta at this stage of gestation.

TABLE 12

COMPARISON OF TOTAL RADIOACTIVITY (^{59}Fe + ^{131}I) IN MATERNAL AND FOETAL BLOOD AND ORIGINAL ISOTOPE MIXTURE INJECTED

Total Radioactivity Expressed as Counts/100 Sec.

DECAY TIME (Hrs)	MATERNAL BLOOD (1)	FOETAL BLOOD (1)	(^{59}Fe + ^{131}I) MIXTURE	MATERNAL BLOOD (2)	FOETAL BLOOD (2)
0	23,427	31,574	216,403	14,787	8,595
24	20,994	30,760	201,294	13,567	8,200
48	19,624	30,281	189,903	12,602	7,966
72	17,789	29,577	181,265	11,565	7,647
	(3)	(3)		(4)	(4)
0	19,000	16,478	216,403	30,067	21,180
24	17,480	16,059	201,294	27,422	20,826
48	16,305	15,715	189,903	25,188	20,298
72	15,309	15,309	181,265	23,336	19,657

Rabbits 1 & 4 - 26 days mated - maternal and foetal blood removed ONE HOUR after administering isotope

Rabbit 2 - 26 days mated - " " " " " TWO HOURS " " "

Rabbit 3 - 20 days mated - " " " " " TWO HOURS " " "

Discriminator settings such that both ^{59}Fe and ^{131}I being counted.

COMPARISON OF RATIO OF $^{59}Fe/^{131}I$ IN MATERNAL & FOETAL BLOOD AND IN ORIGINAL ISOTOPE MIXTURE INJECTED.

Radioactivity Expressed as Counts/100 Sec.

DECAY TIME (Hrs)	$^{59}Fe/^{131}I$ in MATERNAL BLOOD (1)	$^{59}Fe/^{131}I$ in FOETAL BLOOD (1)	$^{59}Fe + ^{131}I$ MIXTURE INJECTED	$^{59}Fe/^{131}I$ in MATERNAL BLOOD (2)	$^{59}Fe/^{131}I$ in FOETAL BLOOD (2)
24	0/26,305	29,005/2,569	75,695/140,708	0/14,787	5,859/2,736
48	2,370/21,057	31,574/0	85,833/130,570	3,007/11,780	6,551/2,044
72	0/23,781	29,577/0	94,959/122,344	1,724/13,063	6,314/2,281
	(3)	(3)		(4)	(4)
24	3,959/15,041	15,973/555	76,595/140,708	2,924/27,125	20,692/0
48	4,808/14,192	15,834/644	85,833/130,570	3,060/27,502	20,207/96
72	4,776/14,274	15,851/627	94,959/122,344	2,447/27,620	18,698/959

Rabbits 1 & 4 - 26 days mated - maternal and foetal blood removed ONE HOUR after administering isotope

Rabbit 2 - 26 days mated - maternal " " " " TWO HOURS " " "

Rabbit 3 - 20 days mated - " " " " " TWO HOURS " " "

TABLE 13

TABLE 14

RATIO OF $^{59}Fe/^{131}I$ PRESENT IN MATERNAL BLOOD, FOETAL BLOOD AND ORIGINAL MIXTURE

EXPRESSED AS A FACTOR OF 1

DECAY TIME (Hrs)	MATERNAL BLOOD (1)	FOETAL BLOOD (1)	^{59}Fe + ^{131}I MIXTURE INJECTED	MATERNAL BLOOD (2)	FOETAL BLOOD (2)
24	0/1	0.92/0.08	0.34/0.66	0/1	0.69/0.31
48	0.1/0.9	1/0	0.39/0.61	0.2/0.8	0.76/0.24
72	0/1	1/0	0.4/0.6	0.12/0.88	0.73/0.27
	(3)	(3)		(4)	(4)
24	0.2/0.79	0.97/0.03	0.34/0.66	0.1/0.9	1/0
48	0.26/0.74	0.96/0.04	0.39/0.61	0.11/0.89	0.99/0.01
72	0.26/0.74	0.96/0.04	0.4/0.06	0.09/0.91	0.92/0.08

Rabbits 1 & 4 - 26 days mated - maternal and foetal blood removed ONE HOUR after administering isotope

Rabbit 2 - 26 days mated - " " " " " TWO HOURS " " "

Rabbit 3 - 20 days mated - " " " " " TWO HOURS " " "

Discriminator settings such that both ^{59}Fe & ^{131}I being counted.

c) Comparison between the ratio of $^{59}Fe/^{131}I$ in the original mixture injected and the foetal blood indicated that, in each instance, the ratio was greater in the foetal blood than in the original mixture (Tables 13 and 14). This indicated that some of the radio-active originally bound to transferrin when injected into the mother rabbit had become detached from this transferrin during its passage across the placenta.

The results obtained using the "low background" Echo-Counter are shown in Table 15. These agree with the previous findings.

TABLE 15

COMPARISON OF RATIO OF $^{59}Fe/^{131}I$ in MATERNAL BLOOD, FOETAL BLOOD AND MIXTURE INJECTED DAY 7 USING ECHO-COUNTER.

Radioactivity Expressed as Pulses/Second

^{59}Fe and ^{131}I	^{59}Fe ACTIVITY	^{131}I ACTIVITY	RATIO to 1
MIXTURE	218.5	389.1	0.35/0.64
MATERNAL BLOOD 1	3.8	68.7	0.06/0.94
MATERNAL BLOOD 2	2.3	45.2	0.05/0.95
MATERNAL BLOOD 3	8.4	42.4	0.17/0.83
MATERNAL BLOOD 4	5.6	87.2	0.07/0.93
FOETAL BLOOD 1	62.7	9.0	0.87/0.13
FOETAL BLOOD 2	14.6	5.75	0.71/0.29
FOETAL BLOOD 3	32.5	3.7	0.89/0.11
FOETAL BLOOD 4	40.4	6.8	0.85/0.15

SAMPLE READ AT SEPARATE OPTIMAL THRESHOLDS FOR IODINE AND IRON.

SECTION EIGHT

A study of iron-containing compounds of placental cell sap when 'Desferal' was incorporated in homogenising medium.

Introduction

Transferrin is capable of binding iron when the two are mixed *in vitro* at room temperature. The results of Sections 1 - 6 indicated the presence of radio-iron associated with only transferrin and ferritin in the placental cell sap. However, the experiments carried out in Section 7, indicated that some of the radio-iron crossing from mother to foetal rabbit did so unaccompanied by the transferrin of the maternal serum. This would suggest that this iron either combined with ferritin within the placental cells and, by some unknown mechanism, was transferred from the placental cells to the foetal circulation, or that some other form of radio-active iron was present in the placental cell sap which had not been identified. There was never any evidence that, in the various techniques used, the radio-active iron was present in any form smaller than molecular weight 5,000. However, there was always the possibility that there might have been unattached iron within the placenta on removal, which during the homogenising technique and the centrifugation carried out, became attached to unsaturated transferrin.

It is generally accepted (Rothwell and Finch, 1962), that iron within the body rapidly becomes attached to some complex and is seldom, if ever, found in the free state.

Most of the work of homogenising and centrifugation was carried out at 4°C., which in itself was inhibiting to the binding process. Nevertheless, if some chelating agent could be used that would not remove the iron already bound to other compounds within the placental homogenate but would compete with the transferrin for any unbound iron, results might prove of interest. E.D.T.A. has been reported as being an iron chelating agent which does not remove iron from either ferritin or transferrin and this was the first agent used. Even at concentrations of only 0.05% it was found that the E.D.T.A. removed the iron from the transferrin of the normal rabbit serum. The next attempt was with Desferal (desferrioxamine B mesylate, CIBA), which is a chelating agent widely used in the human field in iron storage disease. It is superior to the other agents known in that it is highly specific for iron and is rapidly excreted by the kidneys. At 2% concentration it is capable of removing 10-15% of the iron both from transferrin and ferritin (Wohler, 1962 and Neilson, 1962 & 1963). No report could be found of its ability to remove iron from the transferrin of rabbit serum.

Experimental

In the first series of experiments normal rabbit serum was incubated *in vitro* with ^{59}Fe for at least 30 minutes to allow total binding of the iron to the transferrin. A solution of desferal was added so that the final concentration was 0.05%. This mixture was allowed to stand on the bench for a further hour. A sample was then passed through a Sephadex G25 column and the column eluted with 0.01M-phosphate buffer pH 7.3. The radio-activity of the samples collected was measured in a scintillation counter. The inner and outer volumes of each of the columns used had been determined as described in appendix of methods and techniques. In this way, it was possible to demonstrate whether or not radio-active iron had been stripped from the transferrin of normal rabbit serum.

In the second series of experiments the rabbits were injected intravenously with the radio-active iron. Two hours later, all their placentae were removed and divided into two groups differing in their subsequent treatment as follows:

Group A - homogenised in 0.025M-sucrose in the normal way.

Group B - homogenised in a solution of 0.05% desferal in 0.25M-sucrose.

A sample of the resulting cell sap from each group was passed through a G25 Sephadex column. The position of the ^{59}Fe in the samples from each of the columns was noted.

In the third series of experiments placental cell sap from rabbits which had been injected previously with ^{59}Fe was either:

a) mixed immediately with desferal at 0.05% concentration or

b) mixed with desferal 0.25% 24 hours later.

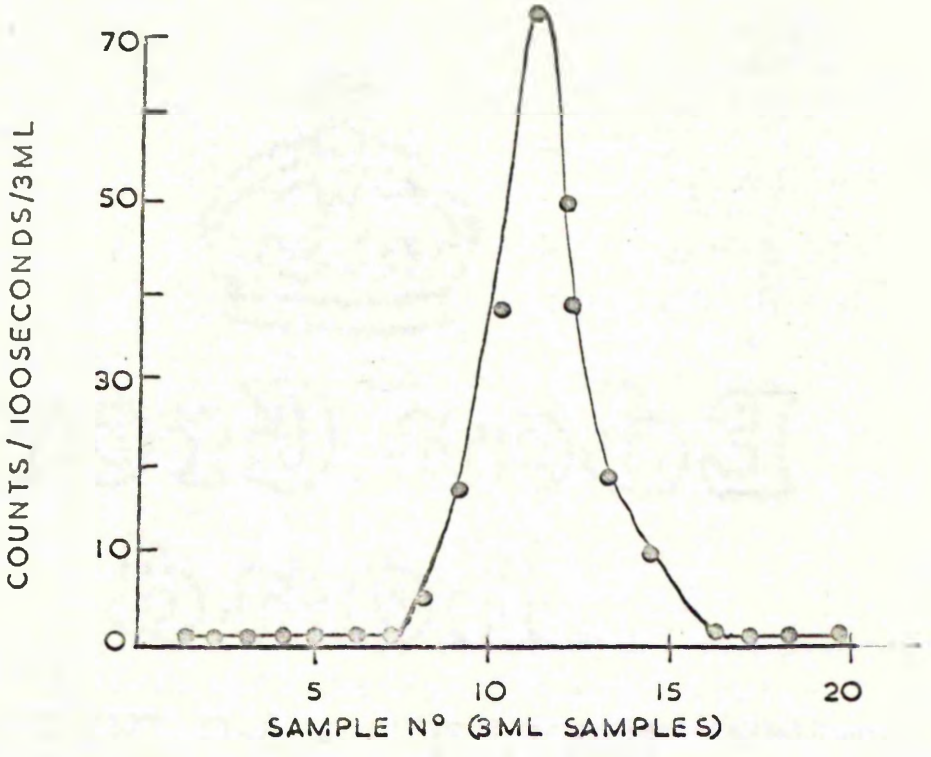
Results and Discussion

A typical example of the results of the first series of experiments where the desferal was added to a sample of normal rabbit serum and left for a period of one hour are shown in Figure 58. It will be seen that there was a single peak of activity in the outer

CHROMATOGRAPHY USING G25

FIGURE 58

POSITION OF RADIO-ACTIVITY IN EFFLUENT AND FROM COLUMN WHEN NORMAL RABBIT SERUM CONTAINING DESFERAL (0.05%) WAS APPLIED.


70
50
30
10
0
COUNTS / 100SECONDS/3ML
5
10
15
20
SAMPLE N° (3ML SAMPLES)

volume from each of the columns. The radio-active iron had remained attached to the transferrin of the serum thus showing that desferal at this concentration was incapable of removing the radio-active iron from the transferrin of normal rabbit serum. These results suggested that here is a means of showing whether or not unbound iron is present in placental tissue on removal.

The results from the second series of experiments show that:-

a) there was one peak of activity in the normally produced cell sap indicating that all the radio-activity was protein bound. Figure 59.

b) There were two peaks of activity in the cell sap produced when the homogenate contained desferal. This indicated that some of the radio-active iron was attached to a compound or compounds of molecular weight less than 50,000. Figure 60.

Since desferal has a molecular weight of 656.8 it would seem that some of the radio-active iron of the placental cell sap had become attached to desferal. Two explanations might be offered:-

a) that some ^{59}Fe had been stripped from the compounds of the placental cell sap.

CHROMATOGRAPHY USING SEPHADEX G 25

FIGURE 59

POSITION OF RADIO-ACTIVITY IN EFFLUENT FROM COLUMN WHEN NORMAL PLACENTAL CELL SAP WAS APPLIED.

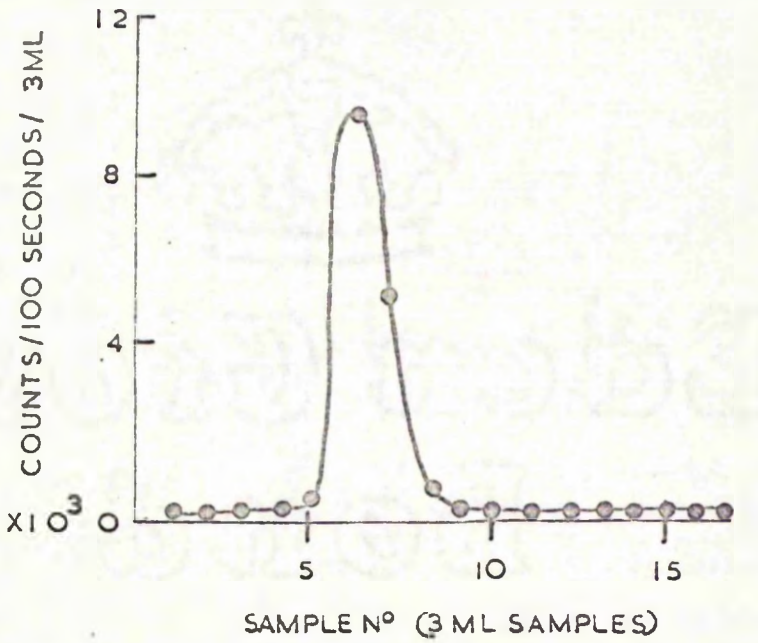
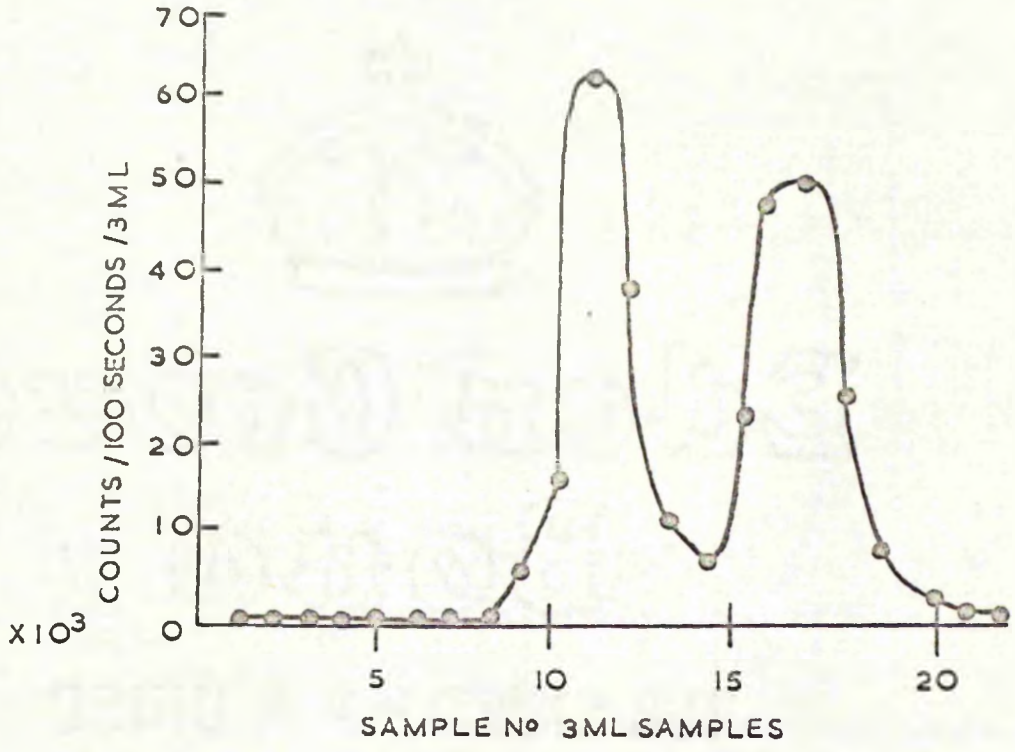

12
8
4
0
X10³
COUNTS/100 SECONDS/ 3ML
5
10
15
SAMPLE N° (3 ML SAMPLES)

FIGURE 60

POSITION OF RADIOACTIVITY IN EFFLUENT FROM COLUMN WHEN PLACENTA WAS HOMOGENISED IN MEDIUM CONTAINING DESFERAL (0.05%)


70
60
50
40
30
20
10
0
X 10³
COUNTS / 100 SECONDS / 3 ML
5
10
15
20
SAMPLE Nº 3ML SAMPLES

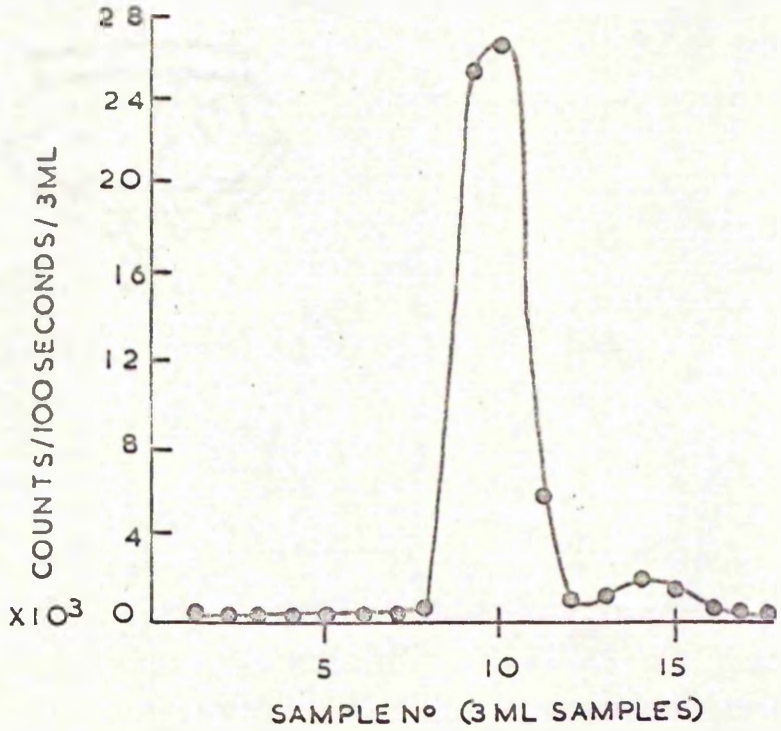
b) That there was some unbound iron in the placenta which was chelated by desferal. When desferal was not incorporated in the medium this iron became attached to transferrin.

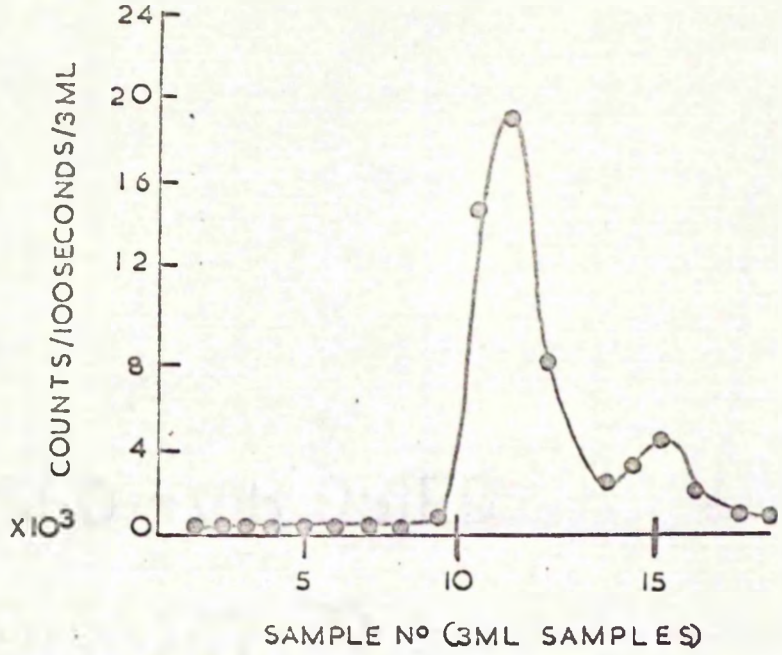
From the first series of experiments it would appear that the only compound contained in the cell sap from which the desferal might strip the radio-active iron was ferritin. Wonler (1965) claimed that 2% desferal could affect this separation when working with humans.

The results of the third group of experiments are shown in Figures 61 & 62. There are two peaks of activity in the cell sap of Group A (Figure 61). There are also two peaks in the cell sap of Group B (Figure 62). This indicated that in both groups there was radio-active iron in a molecule of size less than 50,000. This also would seem to suggest that desferal was capable of stripping iron from the compounds of the placental cell sap. A sample of normal placental cell sap when passed through the Sephadex G25 column had produced the usual one peak of activity in the outer volume from the column. One feature worth noting in the results from the third series of experiments is that the peak of activity in the inner

FIGURE 61 POSITION OF RADIOACTIVITY IN EFFLUENT FROM COLUMN WHEN DESFERAL (0.05%) WAS ADDED IMMEDIATELY.

FIGURE 62 POSITIONS OF RADIOACTIVITY IN EFFLUENT FROM COLUMN WHEN DESFERAL (0.05%) WAS ADDED 24 HOURS AFTER PREPARATION OF PLACENTAL CELL SAP.


28
24
20
16
12
8
4
0
X10³
COUNTS/100 SECONDS/3ML
5
10
15
SAMPLE Nº (3 ML SAMPLES)


24
20
16
12
8
4
0
X10³
COUNTS/100SECONDS/3ML
5
10
15
SAMPLE Nº (3ML SAMPLES)

volume from the columns through which cell sap, to which desferal had been added immediately, was passed, is noticeably smaller than the corresponding peak found when desferal had been added after an interval of 24 hours. This indicates that the iron was much more easily stripped from the compound or compounds to which it was normally attached in the cell sap when the desferal was added immediately, than when the cell sap had been allowed to stand for 24 hours.

GENERAL DISCUSSION

The iron which is required by the developing foetus must come from the mother by way of the placenta. Foetal iron was thought to come from the maternal red cells which were broken down in the maternal blood spaces of the placenta and the iron released and transferred to the foetuses. (Davies, 1961). Experiments using radioactive iron cast doubts on the validity of this theory, for it appeared that the iron bound to transferrin in the maternal serum was the source of foetal iron (Pommerenke, Hahn, Bale and Balfour, 1942).

As yet there is little information about the mechanism involved in the transfer of iron across the placenta. The absorption and transfer of iron by the cells of the intestinal mucosa has, however, been extensively studied by many workers and several theories have been advanced to explain the transfer of this iron. It is generally agreed that iron is transferred across the mucosal cells of the intestine in two ways: A. Rapidly, some workers have suggested an association with an amino acid (Brown and Rother, 1964). B. Slowly, the iron being incorporated into ferritin within the intestinal cells (Charlton et al. 1964). Recently, Crosby (1963) in a

review of the absorption of iron by the cells of the intestinal mucosa, suggested that all the iron which is incorporated into ferritin within these cells is returned to the lumen of the bowel when the cells are exfoliated and is eventually excreted into the faeces. He further suggested that in cases of iron deficiency anaemia the iron is freely absorbed into the circulation because little or no ferritin is present and that in haemotochomatosis this mechanism is upset. An alternative explanation of the regulating role of ferritin during iron absorption has recently been suggested by Smith, Brysdale, Goldberg and Munro (1966), who carried out experiments using labelled leucine as a means of indicating protein synthesis in normal rats and found that the administration of iron by mouth stimulated the synthesis of ferritin. They concluded from those results that the body iron controlled the amount of ferritin synthesised by the mucosal cells of the bowel.

It is possible that a similar mode of transfer exists in the placenta to that occurring in the mucosal cells of the bowel.

It is now accepted that the transferrin bound iron

of the maternal plasma is the source of foetal iron. Under normal conditions all the iron of the plasma is tightly bound to transferrin (Jandle & Katz, 1964). In experiments involving the use of ^{59}Fe, to simulate physiological conditions, it is necessary to be sure that all the isotope is attached to transferrin. If all the circulating transferrin is fully saturated then more iron injected will not become protein bound and will leave the circulation rapidly. This condition does not occur under physiological conditions. In experiments using ^{59}Fe it is customary to allow the ^{59}Fe to become transferrin bound by mixing it with serum or plasma approximately 30 minutes prior to injection. However, Loeffler, Rapport & Collins (1958) showed that if all the ^{59}Fe were in a solution of 1% sodium citrate then the plasma clearance rate of the radio-iron was similar to that of the disappearance of globulin bound radio-iron. They concluded that the iron injected in 1% sodium citrate becomes rapidly incorporated into the iron binding protein of the plasma and, therefore, no prior binding is necessary. Furthermore, Katz (1964) recently reported that the binding of iron and transferrin in vivo is a process that takes much longer than is generally assumed and that at least 12 hours should be allowed to elapse before assuming that complete binding has taken place.

In the studies described in this thesis, all the iron was administered as ferric citrate in a solution of 1% sodium citrate. The serum iron and T.I.B.C. levels were measured in several of the rabbits used and these results indicated that the transferrin was not completely saturated and was capable of binding the small quantities of radio-active iron used. (Table 2). Confirmation that all the ^{59}Fe was protein bound was established by electrophoresis and gel filtration (Sections 5 & 6).

The iron bound to the transferrin of the circulating plasma is carried to several destinations within the body, e.g., to the liver and spleen, where it is stored as ferritin, to the bone marrow where it is used in the production of haemoglobin, and during pregnancy, to the placenta where it is transferred to the foetuses. In the rabbit during the last third of pregnancy 70-90% of the plasma iron turnover is accountable by placental transfer of iron (Davies, et al. 1959).

One of the complications in a study of placental transfer of iron is the complex and ever changing cellular structure of the placenta. In the rabbit both a yolk sac and a chorio-allantoic placenta are functional to term.

However, all the observations on record suggest that during the last third of gestation all the iron transferred to the foetuses does so by way of the corio-allantoic placenta (Davies, 1961). It is at this stage that approximately 70% of the total iron of the foetus is accumulated (Bothwell, et al. 1958).

The chorio-allantoic placenta of the rabbit 20 - 30 days pregnant consists mainly of large blood filled maternal sinusoids into which the invading foetal capillaries dip. Thus the cellular layers surrounding these capillaries form the barrier across which the iron must pass. The structure of the cellular barrier at this stage is, therefore, of importance. Amoroso (1952) using the light microscope described placentation in the rabbit as undergoing a change from haemo-chorial to haemo-endothelial between the fourteenth and twenty-second day of gestation. These structural changes would be in accord with the sudden increase in the amount of iron subsequently transferred. However, recently electron microscopists have reported that although the trophoplastic cells undergo marked thinning during this stage of gestation both cellular layers are still present at term (Larsen, 1963; 1964 and Enders, 1966). The electron microscopy described in this thesis confirms

these recent findings and it appears, therefore, that iron which leaves the maternal blood in the placental sinusoids must cross three layers of cells in order to reach the foetal circulation.

The location of the iron within the chorio-allantoic placenta of the rabbit was investigated by histochemical and autoradio-graphic techniques (Section 2). It was found by examining sections of placenta from 15 and 25 day pregnant rabbits that only by 25 days of gestation could iron be demonstrated. In 25 day placental sections the iron was seen in the maternal and foetal blood spaces associated with the placental cells (Figure 17). It could not be demonstrated in any of the other vascular areas of the placenta. Within the maternal blood spaces it was seen lying in apposition to the luminal surface of the trophoblastic cells separating the maternal and foetal blood, suggesting that, in these areas, it leaves the circulating blood and becomes available for transfer across the placental cells. The function of the maternal trans-ferrin in this situation is reminiscent of that in the transfer of iron from the circulating plasma to the reticulocyte. In this case the transferrin becomes attached to receptor sites on the membrane of the reticulocyte

and the iron is released and enters the cells to be used in the formation of haemoglobin.

Much of the injected radio-active iron in the placental cell sap from rabbits during the later stages of gestation was found to be associated with transferrin (Section 5 and 6). Although placental perfusion of the rabbit placenta had been carried out prior to removal it was known that some of the maternal blood remained (Table 5) and, since the foetal placental circulation had not been included in the perfusion technique, there was also foetal blood present. It seems likely, therefore, that at least some of the transferrin associated with the radio-active iron in the placental cell sap is due to the presence of foetal and maternal blood. It is possible, however, that iron might cross the placenta attached to maternal transferrin. This would imply that transferrin molecules must be capable of crossing the placental barrier. Studies using iron saturated ^{131}I labelled transferrin in pregnant rabbits during the later stages of gestation indicated that some of the labelled protein reached the foetal blood two hours after administering the mixture intravenously into the mother rabbit (Figure 53). Since it has been shown

(Bothwell, et al. 1959) that iron crosses from mother to foetal rabbit only via the chorio-allantoic placenta during the last third of gestation then if this labelled transferrin carries iron it must pass via the chorio-allantoic placenta. Should it be unassociated with iron then the possibility of passing via the yolk sac placenta cannot be excluded. Hemmings and Oakley (1957) demonstrated that serum globulins which include transferrin cross from mother to foetal rabbit by way of the yolk sac placenta. Furthermore, subsequent studies using ^{131}I labelled transferrin saturated with radio-active iron indicated that at least some of the iron crossing the rabbit placenta does so unattached to maternal transferrin (Section 7). These results would seem to suggest, that although the transferrin molecule is capable of crossing the rabbit placenta during the later stages of gestation, its major role in the placental transfer of iron in this species is as a carrier of iron in both the maternal and foetal blood and the delivering of such iron to the trophoblastic cells which separate the foetal and maternal circulations.

When the iron has reached the trophoblastic cells some becomes detached from the transferrin and enters the cells and forms other associations. The other radio-active

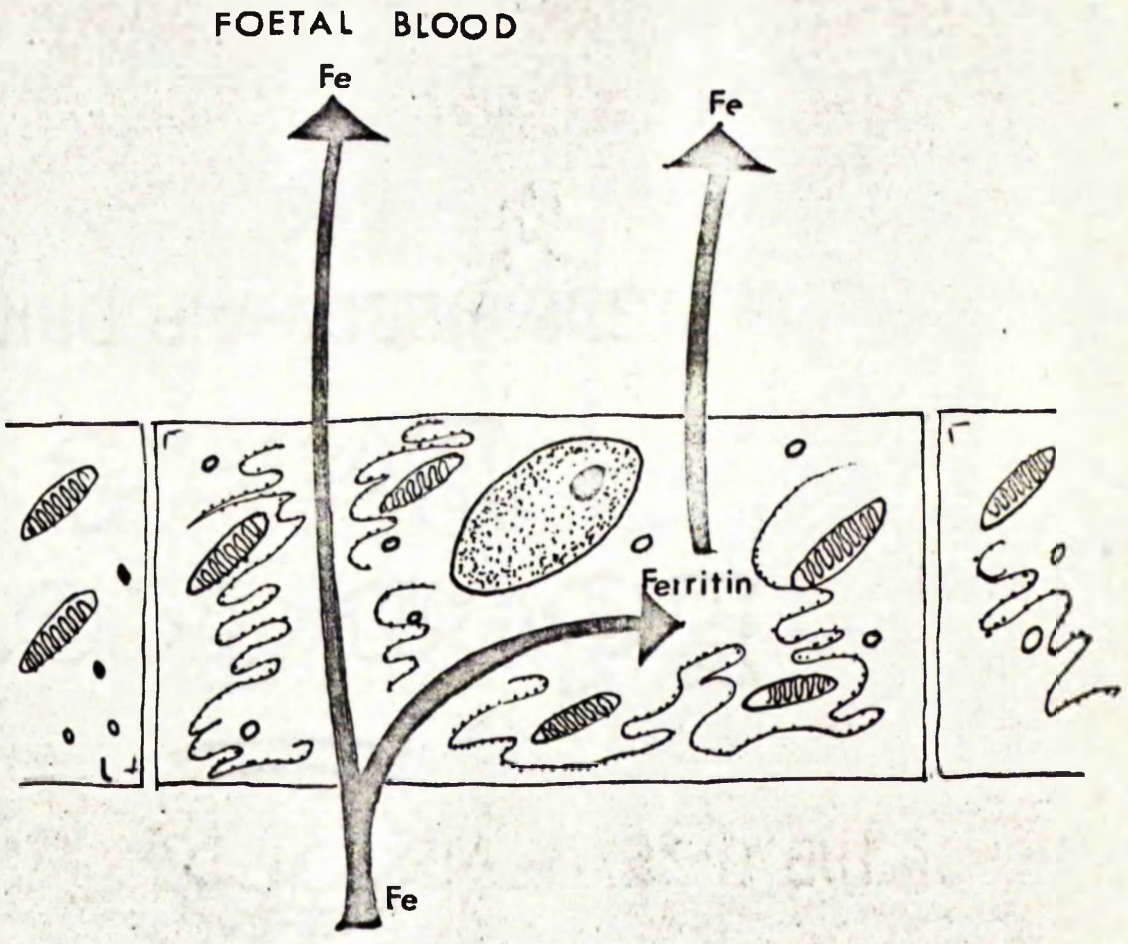
compound found in the placental cell sap, was ferritin (Sections 5 & 6). Since ferritin occurs in the circulating blood only under abnormal conditions it would appear that this ferritin is a component of the placental cells. Bothwell, et al. (1959) reported that iron is always transferred from mother to foetus and never in the reverse direction. Furthermore, the same authors reported that if the umbilical vessels of several of the foetuses were ligated and those foetuses removed prior to administration of the radio-active iron the amount of iron taken up by the tied off placentae almost equals that which would normally be transferred to the foetuses. The implication of this work is that once iron is taken up by the placenta of the rabbit it never returns to the maternal circulation, and must eventually go to the foetuses. Ferritin found within the placental cells thus contains iron which will be transferred to the foetuses and the placental ferritin therefore plays a part in the mechanism of iron transport across the placenta of the rabbit as distinct from the regulating role proposed for it in the mucosal cells of the intestine. Nevertheless, the possibility exists that as in the mucosal transfer of iron there are two forms of placental transfer. Ferritin under these circumstances might

play the role of the slow form of transfer giving up its iron as the foetus requires it. Ferritin is present in the erythroblast of the bone marrow and it is thought that some of its iron is eventually used in the synthesis of haemoglobin after the cell has used all the alternative sources of iron (Zail, Charlton, Torrance & Bothwell, 1964). This situation might be repeated within the placental cells.

With regard to the initial rapid form of transport this is most likely to be iron which is not protein bound. Attempts to isolate iron in such a form have not been entirely successful. However, results have been obtained which seem to suggest that in the placental cell sap from rabbits during the later stages of gestation, iron exists in a form which is initially less firmly associated with protein than after the passage of time. This would not rule out the possibility of iron being present very loosely associated with protein and this association being easily reversible and the iron becoming readily available for transfer to the foetuses. This loose association of the iron to protein might well take place during experimental procedure, e.g., the iron might become attached to transferrin which binds iron when the two are mixed together on the laboratory bench.

The state of knowledge of the mechanism involved in the placental transfer of iron in the rabbit is that the iron of the maternal transferrin reaches the trophoblastic cells associated with the foetal capillaries which invade the maternal blood sinusoids. Here most of the iron becomes detached and enters the placental cells. Within these cells some of the iron becomes part of the ferritin molecules but this iron is eventually transferred to the foetuses. There is, however, some indication that a non-protein form of iron is also present within these placental cells and such iron would play an important role in the transport mechanism of iron across the placenta.

POSSIBLE ROUTES TAKEN BY IRON CROSSING PLACENTAL CELLS



SUMMARY

The mechanism of placental transfer of iron was studied in rabbits. ^{59}Fe in 1% sodium citrate solution was injected intravenously into pregnant rabbits in amounts ranging from 5 - 50 uCs.

A histological investigation of the rabbit placenta at different stages of gestation was carried out in an endeavour to explain the increase in the amount of iron transferred from mother to foetal rabbit during the last third of gestation. Placental sections from rabbits either 15 or 25 days mated were examined using both the electron and light microscopes. No decrease in the number of cellular layers separating maternal and foetal blood at the later stage of gestation could be demonstrated, although there was an obvious thinning of the trophoblastic cells as gestation advanced.

Autoradiographic and histochemical techniques illustrated the presence of iron in certain areas of the 25 day placental sections.

The uptake of radio-active iron by the rabbit placenta at different stages of gestation was studied. The greatest amounts of radio-active iron were found to be present during the last third of gestation. The histological sections showed that a good deal of blood was present within the placental spaces at this stage of gestation. The uterus was perfused for 5 minutes by canulating the abdominal aorta and vena cava.

A study of the plasma clearance rates for ^{59}Fe in rabbits during the last third of gestation indicated that the contamination by the maternal blood could be further reduced by allowing at least 30 minutes to elapse after administering the radio-active iron before removing the placentae for study. A subsequent study of the placental uptake of ^{59}Fe as a function of time indicated that the amount of ^{59}Fe found within the placenta at intervals from 30 minutes to 2 hours after administration showed no significant difference.

The distribution of the ^{59}Fe in the placental subcellular fractions of placentae from rabbits during the last third of gestation removed between 30 minutes

and 2 hours after administering the isotope was measured. The non-particulate fraction or cell sap contained the greatest percentage of radio-active iron.

A study of the radio-active iron compounds of the placental cell sap was carried out using gel filtration and electrophoresis techniques. Transferrin and ferritin were found to be associated with the radio-active iron within the placental cell sap.

The role of transferrin in the mechanism of placental transfer of iron was further investigated by labelling pure rabbit transferrin with ^{131}Iodine. The labelled protein was injected into pregnant rabbits and its presence subsequently identified within the foetal serum. However, by double labelling the transferrin with both radio-active iron and iodine it was found that iron was capable of crossing the rabbit placenta unaccompanied by transferrin.

Finally an investigation using chelating agents was carried out, to find whether iron might normally be present in the placenta of the rabbit in some form than those already isolated. It was thought that this iron might be in an other form capable of being loosely bound

to protein (as transferrin) present in the placental cell sap, the binding taking place during experimental procedures. These studies suggested the presence of iron loosely bound to protein within the placental cell sap.

APPENDIX OF EXPERIMENTAL METHODS & TECHNIQUES

Preparation of histological sections of rabbit placenta

The placentae on removal were fixed in either 10% formalin, Bouin or Zenkers fluid. While in the fixative, each placenta was quartered, dehydrated, cleared and embedded in wax in the normal way. 5 μ sections were cut and stained with haemotoxylin and eosin in the standard way.

Prussian blue reaction for iron (Perl, 1867)

This method detects both free and protein bound iron in the tissues, the latter being made stainable by HCl. The characteristic blue colour of ferric ferrocyanide (Prussian Blue) indicates the presence of iron.

Method

The placental tissue was fixed and sectioned as before. The sections were then rinsed in distilled water and immersed in the following freshly prepared solution:

2% aqueous solution of potassium ferrocyanide - 2 parts

2% hydrochloric acid (w/v) - 2 parts

The sections were left immersed for 20 to 30 minutes. Once stained the sections were washed with water and counterstained with eosin and finally mounted.

Autoradiographic technique

Sections were prepared as described for histology and mounted on glass slides. The remainder of this technique was carried out in the photographic dark room. Pieces of Kodar AR.10 stripping film were cut into sections large enough to surround the area of slide occupied by the tissue section. These pieces of film were then dropped onto the surface of clean dust free water in a glass bowl, emulsion side downwards, and allowed to float until the initial wrinkling had disappeared. The slide with the section surface uppermost was then brought into contact with the undersurface of the floating film. The slide with the section now completely surrounded with stripping film was removed from the water placed in an upright position and allowed to dry in a drying cupboard. Once thoroughly dry the slide was placed in a thick black envelope and kept in a sealed metal box at $4^{\circ}C$ for three to four months. The film was then developed in Kodak 19B developer and fixed in acid fixative. The underlying section was now stained

with haemotoxylin and eosin in the normal way.

Determination of Serum Iron and Total Iron Binding Capacity (T.I.B.C.)

Serum Iron and Total Iron Binding Capacity were estimated by the method of Ramsay (1955), with a few minor modifications. In this method the ferric iron of the serum is reduced to the ferrous form by sodium sulphite. The ferrous iron then reacts with 2,2 - dipyridyl to give a pink colour the optical density of which is estimated in a spectrophotometer at 520 mμ.

Reagents

0.2M-Sodium sulphite (A.R.). Freshly prepared each day. 2,2 - dipyridil (A.R.) in 3% acetic acid (v/v). This was kept in a dark coloured bottle.

Chloroform (A.R.).

Stock Iron Solution (containing 100 μg per ml of iron conc.) was made by weighing 0.498 gm ferrous sulphite. 1 ml of concentrated sulphuric acid was added and made up to one litre with distilled water. The stock solution was diluted with distilled water to give a working standard.

Method

1 ml of serum or plasma, 1 ml of sodium sulphite and 1 ml of 2,2-dipyridyl were added to 2 ml of distilled water in a centrifuge tube. The mixture was heated in a boiling water bath for exactly five minutes, cooled immediately, and 1 ml of chloroform added. The mixture was thoroughly shaken for thirty seconds, and centrifuged at 2000 xg for five minutes. The optical density of the clear supernatant was measured in a spectrophotometer at 520 mμ. A standard graph was prepared in the same way using suitable dilutions of the stock solution.

Total Iron Binding Capacity

Iron was added to the serum in excess of that required to completely saturate the available transferrin and the unbound iron removed by adding magnesium carbonate. An iron estimation was carried out as previously described and the Total Iron Binding Capacity calculated.

Method

Four ml of stock iron solution containing 200 μg per ml were added to 2 ml of serum in a test tube and

allowed to stand for 5 minutes, 100 mg of magnesium carbonate for every ml of iron solution used were added and the mixture shaken thoroughly for the next 30 - 60 minutes, and centrifuged at 2000 xg for 5 minutes. Four ml of the clear supernatant were transferred to a clean test tube and an iron estimation carried out as previously described.

Routine Haematology

The haematological estimations carried out consisted of haemoglobin concentrations, Packed Cell Volume (P.C.V.), and white cell counts.

Haemoglobin Estimations were carried out by the alkaline haematin method using Gibson Harrison standard.

Packed Cell Volume (P.C.V.) estimations were carried out by using the Hawksley microhaematocrit centrifuge. The microhaematocrit tubes were filled directly from a punctured ear vein and were centrifuged for 6 minutes.

Total White Cell Counts were carried out using the haemocytometer. A differential white cell count was carried out on a blood smear with Leishmans and a total of 200 cells were counted.

Method of estimating the ^{59}Fe activity of the various placental subcellular fractions

Each placenta was homogenised in 0.25 M-sucrose in a Potter type homogeniser. The total volume of each homogenate was measured. One ml of the homogenate was pipetted into 2 ml of distilled water in a polystyrene tube. The ^{59}Fe activity of the homogenate was determined in a scintillation counter. The activity of the total placental homogenate was then calculated. The total activity of each placental homogenate was expressed as a percentage of the initial ^{59}Fe activity administered to the rabbit.

Activity of the nuclear fraction

The nuclear fraction was obtained by centrifugation at 1000 g for 20 minutes. The nuclear pellet was dissolved in two drops of 4M-NaOH and made up to 3 ml. with distilled water, transferred to a polystyrene tube and ^{59}Fe activity measured as before. The results were expressed as a percentage of the total activity of the relevant homogenate.

Activity of the mitochondrial fraction

The mitochondrial fraction appeared as a small granular button in the bottom of the tube after the second centrifugation at 5,000 g for 20 minutes. Three ml of distilled water were added, the whole transferred to a polystyrene tube and the ^{59}Fe activity measured as before. Once again, the ^{59}Fe activity was expressed as a percentage of the total placental homogenate.

Microsomal Fraction

This was obtained after centrifugation at 20,000 g for one hour at 0°C. It was suspended in 3 ml of distilled water and the ^{59}Fe activity measured as before. The results were expressed as a percentage of the total homogenate activity.

^{59}Fe activity of the maternal serum

0.1 ml of blood was withdrawn from the marginal ear vein of the rabbit and transferred to a polystyrene tube containing 2 ml of distilled water. The mixture was thoroughly shaken and the ^{59}Fe activity measured in the scintillation counter. At the same time as each

sample was withdrawn a microhaematocrit tube was filled and the P.C.V. estimated. From the two results the activity of 0.1 ml of plasma from each sample was calculated.

Gel Filtration Technique

The dry sephadex powder in bead form, either as type G25, G75 or G200 was stirred into a beaker containing 0.6% NaCl solution, and allowed to swell. In the case of the G25 this took 2 - 3 hours, whereas, in the case of G75 and G200 it took 2 and 3 days respectively. Glass columns 3 cm by 55 cm., with a sointered glass base were used. The flow from the columns was regulated by a glass stop-cock. The columns were mounted with a circle of filter paper which stopped the sephadex grains from clogging the pores of the sointered glass. Initially, 0.01M phosphate buffer, pH 7.2, was poured into the column to a height of approximately 10 cms. Then the sephadex slurry was added and the first few centimetres of the column allowed to settle. More slurry was added and the stop-cock opened and the eluant allowed to drain away. Slurry was added until the column had reached the desired height. Once the sephadex had finally settled the normal saline on top of the column was siphoned

off and phosphate buffer allowed to run through overnight. A circle of filter paper was dropped onto the uppermost surface of the sephadex.

Application of the sample

In the case of the G25 and G75 the eluant was drained away just to the level of the uppermost surface of the column. The sample, amounting to approximately 2 - 3% of the total bed volume of the column, was then slowly pipetted onto the filter paper on top of the column. This was then allowed to enter the column completely after which two or three ml. of the phosphate buffer was then pipetted onto the top of the column. Phosphate buffer was then added in small quantities until it was seen that the sample had travelled some distance down the column. At this stage the column was filled to the top with buffer. The samples of effluent from the column were collected by means of a 3 ml. siphon in a fraction collector (Figure 33). The ^{59}Fe activity of each of the fractions was measured in the scintillation counter.

Application of the sample in the case of sephadex G200

When using G200 a special column 24" high (Pharmacia Sweden) was used. This column consisted of a chromato-

graphic tube, sample applicator, and top and bottom caps with two flanges. The top and bottom caps and the sample applicator fitted the columns exactly. This column was packed and prepared exactly as described for the column used with the G25 and G75 types. The sample in this case, however, was applied to the top of the column by means of the special applicator which was a perspex tube of such a diameter that it fitted exactly into the column. The base was made of nylon net through which the sample drained evenly. The sample in this case amounted to approximately 1% of the total bed volume, of the column. The applicator was removed immediately after the sample was seen to have entered the column.

Checking the column prior to use

Before the columns were used for experiments, the outer and inner volumes of each were determined. This was done by passing a mixture of two substances, one of high molecular weight, and the other of low molecular weight, through the column and determining the respective volumes containing the substances. The mixture used was blue dextran and glucose. This preliminary run through the column also gave an indication of the packing. In a well

packed column the coloured high molecular weight substance should be seen to be descending the column as an even narrow band, whereas, in a badly packed column it is seen as a broad band. The position of the dextran in the collected fractions was easily recognised by the blue colour. In the case of the glucose this was determined by Benedict's test.

Checking the G200 column

This was done in the way described above for the G75 and G25 columns. It was also necessary to know the definite position of rabbit ferritin in the fractions collected. Therefore, samples of rabbit ferritin were also passed through the G200 column and the position of the brownish pigment noted in the fractions collected.

Preparation of rabbit ferritin

Since rabbit ferritin is not available commercially it was found necessary to prepare this in the laboratory. This was done by the method of Granick (1943) who prepared horse ferritin. Livers and spleens from rabbits whose

ferritin production had been stimulated by injecting inferon (Fison Iron Dextran), were homogenised in distilled water 1:4 w/v. The homogenate was then heat coagulated at 80°C. The supernatant was removed after centrifugation at 0°C in the M.S.E. refrigerated centrifuge. This was then precipitated with 50% ammonium sulphate and kept at 4°C for at least four hours. The precipitate was dissolved in distilled water and re-precipitated with 50% ammonium sulphate. The precipitate was then dissolved in distilled water and passed through a G200 sephadex column using 0.01 M sodium acetate buffer pH 6.5, as the eluting fluid. The ferritin in the elute was crystallised by adding sufficient 20% cadmium sulphate to make a 5% solution. The ferritin crystals were separated by centrifugation and recrystalised with cadmium sulphate. This process was repeated three or four times. Finally the redissolved ferritin was passed through a sephadex G200 column to remove the cadmium sulphate.

Starch Gel Electrophoresis

Starch gel electrophoresis was carried out by the method of Smithies (1955) using the discontinuous system

of Poulik (1957) with minor modifications by Ashton (1957).

Method

37.5 gm of hydrolysed starch (B.D.H.) were mixed with 500 ml. of 0.076 M-Tris Buffer pH 8.65. This was then heated over a bunsen flame until it became viscid. The mixture was then degassed, poured into a plastic mould 15 cm.by 20 cm. and covered with a piece of weighted glass. This was left in position for at least 4 hours. Slots were then made in the gel at a distance of 3 cm. from one end. Pieces of Whatman #17 filter paper were saturated with the sample to be run and inserted in the gel. The gel was then placed in a Kohn tank (Shandon Scientific Company). Wicks of three pieces of 3 cm. filter paper, saturated with tris buffer, were applied to each end of the gel. The buffer in the tank was 0.3M-borate buffer, pH 8.65. Finally the gel was covered with a strip of Melanex. The current was then switched on and the gel run at a constant voltage of 180 volts for 2 hours. After this period it was usually found that all the sample had left the filter paper inserts and these were now removed and the slots filled with a mixture of starch and tris buffer. The current passing through the gel was reduced to 120 volts and

the run continued for a further 12 hours. The gel was removed from the tank and stained with either amido black or a mixture of ferrocyanide and hydrochloric acid.

Staining the gel

Reagents

Amido Black

Amido Schwarts 10B 1 g.

Methanol /acetic / acid 50/20/50.

Decolourising fluid

Methanol / acetic acid / water 50/20/50

Method

The gel was removed from the plastic mould and halved horizontally using the Sandon gel slicer (Figure The two halves were transferred to an enamel tray and the amido black solution poured gently over them until each half was completely covered. The stain was poured off after one minute and the gel cleared with decolourising fluid.

Ponceau S.

Reagents

Ponceau S. 0.2% in 3% aqueous trichloracetic acid

Method

The ponceau solution was poured over the gel and left in contact for at least ten minutes. The stain was then poured off and the gel decolourised with 5% acetic acid.

Staining with ferrocyanide and hydrochloric acid

Normal hydrochloric acid was poured over the sliced gel and then ferrocyanide crystals were sprinked over the immersed gel. Staining took between half and one hour.

Estimation of the radio-activity in the different regions of the gel

Once the gel had been stained and decolourised, the two halves were placed together in their original position on a glass plate. The position of the stained bands in the gel were recorded on graph paper. The gel was then cut longitudinally to separate the different samples and each section was cut into 0.5 cm. portions. Each portion was transferred to a numbered polystyrene tube and the gel digested with concentrated nitric acid (A.R.). The activity of each sample was determined in the scintillation counter. The various active areas were then compared with the stained bands recorded on the graph paper.

Electrophoresis using cellulose acetate strips

This was carried out by the method of Kohn (1957) with a few minor modifications (Shandon Instrument Application, 1964).

Reagents

Ponceau S

Acetic acid 5%

Method

Oxoid cellulose acetate strips were saturated in veronal buffer and the excess buffer removed by blotting with filter paper. They were then placed in a tank and the sample applied about 2 cm. from one end, and the current switched on. A constant current of 0.4 milliamps per cm. length was passed for 2 hours. The strips were then removed from the tank, dried thoroughly and stained with Ponceau S. and decolourised with 5% acetic acid.

Method of detection of ^{59}Fe activity in paper strips

Once stained the cellulose acetate strips were put in contact with Industrial Kodak X-ray film in cassettes and left in apposition for at least 9 weeks. The X-ray film was then developed in the normal way and the exposed areas marked on the paper strips.

Trace labelling of transferrin with ^{131}Iodine

This was done by the method of McFarlane (1955) in which iodine monochloride is converted to hypoiodite which is readily incorporated into protein.

Iodine monochloride was prepared by the method described in Vogel's "Quantitative Inorganic Analysis" (1941). 5.0 gm. of potassium iodide and 3.22 gm. of potassium iodate were dissolved in 37.5 ml. of distilled water. 37.5 ml. of concentrated H Cl (A.R.) were added along with 5 ml. carbontetrachloride (C Cl_4). If, on vigorous shaking, the C Cl_4 layer did not become faintly pink some potassium iodide solution was added until the presence of iodine was seen in the C Cl_4 layer. If, on the other hand the C Cl_4 layer was more than faintly pink a little potassium iodate solution was added to convert some of the iodine to iodine monochloride. The solution then contained approximately 147 mg. I/ml. as iodine monochloride. When this is diluted 1/350 with M-NaCl the resulting solution contained approximately 0.4 mg. iodine/ml. and was approximately 0.01N with respect to Hcl. These aqueous solutions remained stable for months in the presence of a high concentration of chloride ions and a slightly acid reaction.

The radio-active iodine was free of reducing agents and was added before the conversion of the iodine monochloride to hypoiodite. This step which is indicated by the loss of colour of the iodine monochloride appears to be a necessary pre-requisite for substitution of iodine in the benzene ring of tyrosine. This was carried out by injecting a glycine buffer A* pH 8.5, into the ^{131}I monochloride solution just prior to mixing with the protein. The transferrin was then dissolved in buffer B** pH 9.0. The pH was not allowed to exceed pH 9.5 since hypoiodite is unstable above this. The calculated volume of iodine monochloride containing ^{131}I was buffered to pH 8.6 by the addition of buffer A, and rapidly mixed with the buffered protein. Provided that the molar ratio of iodine monochloride to protein is greater than 2, efficiencies of 60-80% labelling was obtained. The labelled protein was then dialysed against normal saline for 48 hours to remove any unbound ^{131}I and to obtain an isotonic solution for injection.

* Buffer A. 9 ml. M -glycine in N/4 NaCl + 1 ml. N NaOH.

** Buffer B. 8 ml. M -glycine in N/4 NaCl + 2 ml. N NaOH.

Purification of rabbit transferrin

Rabbit transferrin was obtained from Mann Chemicals Ltd., and was found to contain a percentage of impurities most of which appeared to be albumin. It was decided to remove the impurities by column chromatography using DEAE-Sephadex A-50.

Preparation of DEAE-Sephadex A-50 (Pharmacia Ltd., Sweden)

The method used was that of Peterson & Sober (1959) with modifications by Porter (1961 and Fleck (1963). 6 gm. of DEAE-Sephadex was allowed to swell in water overnight and fines were removed six times with re-suspension in water each time. The swollen grains were then placed in a large buchner funnel, through which were gently sucked equal volumes (500 ml.) of 0.5M-HCl., 0.5M-NaOH and 0.5M-H_3PO_4 (Analar). 0.2M-phosphate buffer, pH 6.6 (ca. 1 litre) was finally sucked through the grains until the pH of the elute was 6.6. The prepared DEAE-Sephadex was now poured into a glass column 100 x 2 cm. diameter; the column used was similar to that used for the G200 Sephadex work.

Chromatography using DEAE-Sephadex A-50

The transferrin to be purified was dissolved in 0.2M-phosphate buffer, pH 6.6, and applied to the top of the prepared column. The column was eluted with the phosphate buffer in a continuous concave gradient of NaCl. These conditions were achieved by using three identical one litre gradient bottles (See Figure 5's numbered 1, 2 and 3 and graduated 650, 650 and 566 ml. respectively. The bottles were linked by glass and plastic tubing with screw clips to allow the filling of the aspirator bottles without the mixing of their contents. The outlet from the aspirator No.1 was connected to the top of the column. The bottles were placed 1 metre above the top of the column, and the contents of the aspirators 1 and 2 were continuously stirred. 566 ml. of 0.3M-phosphate buffer pH 6.6, containing 3.93M-NaCl was added to aspirator No.3 and to each of the aspirators 1 and 2, were added 650 ml. of 0.02M-phosphate buffer pH 6.6. When the sample had been applied the screw clips were opened and the samples eluted under the conditions described above. 5 ml. fractions were collected using the fraction collector (Figure 5's) The used column was regenerated by elution with 0.02M - phosphate buffer pH 6.6, until the elute was pH 6.6, and contained no NaCl.

Detection of Protein in the Fractions

The amount of protein in each fraction was measured by transferring part of the fraction to a quartz cell and measuring the optical density at 280 mu (Bonvan and Holiday, 1952) using a Beckham DB Spectrophotometer (Beckham Instrument Ltd).

Freeze Drying of Samples

Those samples from the DEAE-Sephadex A-50 column which were known to contain transferrin were pooled and dialysed against ammonium bi-carbonate (Porath, 1955). The dialysed pool was freeze dried and pure transferrin remained. The ammonium bi-carbonate was removed during the freeze drying process.

BIBLIOGRAPHY

AMOROSO, E.C. (1952). Marshall's Physiology of Reproduction. Ed. A.S. Parkes. Pub. Longman, Green & Co., London. Page 127.

AMOROSO, E.C. (1965). Personal Communication.

ARCHER, R.K. (1965). Haematological Techniques for use on Animals. Pub. Blackwell Scientific Publications, Oxford. Page 120.

ASHTON, G.C. (1957). Nature, 180. No. 4592. p. 919

AZARI, P.R., and FERNEY, R.E. (1958). J. Biol. Chem. 232. No.1. p. 293.

BEHVAR, C.H., and HOLIDAY, E.R. (1952). Advances in Protein Chemistry. ed. Anson, M.L., Bayley, K., Edsall, J.T. N.Y. Academic Press. VII. Page 320.

BELL, G.H., DAVIDSON, J.N., & SCARBOROUGH, H.H. (1956). Textbook of Physiology and Biochemistry. Pub. E & S. Livingstone Ltd.

BESSIS, M., BRETON-GORIUS, Janine (1959). Blood, 14. No.4. 423

BOTHWELL, T.H., and FINCH, C.A. (1962). Iron Metabolism. J. & A. Churchill Ltd., London. p. 141.

BOTHWELL, T.H., PRIBILLA, W.F., MEBUST, W., & FINCH, C.A. (1958). Am. J. Physiol. 193. 3. 615-622.

BRAMELL, F.W., HEMMINGS, W.A., HENDERSON, N.T. (1951). Antibodies and Embryos. University of London Press.

BROWN, E.B., DURACH, E., & MOORE, C.V. (1958). J. Lab. & Clin. Med. 52. 335-355.

BROWN, E.B., & ROTHER, Mary L. (1963). J. Lab. Clin. Med. 62. No.3. 357.

CASEY, A.R., ROSAHN, P.D., & PEARCE, L. (1936). J. Exp. Med. 64. 453

CHARLTON, R.W., JACOBS, P., TORRANCE, J.D., & BOTHWELL, T.H. (1965). J. Clin. Invest. 44. No.4. 543

CLETON, F., TURNBULL, A., & FINCH, C.A. (1963). J. Clin. Invest. 42. No.3. 327

CROSBY, W.J. (1963). Blood (J. Haematol). 22. No.4. 415

CURTIS, J., LUND, M.D., THOMAS, R.C., & SIBSON, M.D. (1958). Iron in Clinical Medicine. Univ. Cal. Press.

DAVIES, J. (1961). Survey of Research in Gestation and the Developmental Sciences. Williams, Wilkins & Co., Baltimore, Maryland, U.S.A.

DAVIES, J., BROWN, E.B. Jr., STEWART, D., TERRY, C.W., & SISSON, J. (1959). Am. J. Physiol. 197. (1). 87-92.

DAVIES, B., SALTMAN, P., & BENSON, S. (1962). Biochem. & Biophy. Res. Comm. 9. No.1. 56

DERN, J., MONTI, A & GLYNN, F. (1963). J. Lab. Clin. Med. 61. 280

DRABKIN, D.L. (1951). Physiol. Rev. 31. No.4. 345

DRYSDALE, J. (1965) Personal communication.

ENDERS, J.(1965). Amer. J. Anat. 116. 29

FABER, M., & JORDAL, R. (1961). Nature. 192. 181

FLECK, A. (1965). Studies in Plasma Protein Metabolism. PhD. Thesis. Faculty of Medicine. University of Glasgow.

FRANCIS, C.E., MULLIGAN, W., & WORMALL, A. (1959). 'Isotpic Tracers'. 2nd Ed. Univ. of Lond. The Athlone Press.

GRANICK, S. (1943). J. Biol. Chem. 149. 157

GRANICK, S. (1946). J. Biol Chem. 164. 737

GRANICK, S. (1949). Bull. N.Y. Acad. Med. 25. 403

GROEN, J., VANDEN BROEK, W.A., & VELDMAN, H. (1947).
Biochemica et Biophysica acta. 1. 315

GROLLMAN (1963). The Functional Pathology of Disease. p 550.
2nd Ed. McGraw-Hill. The Maple Press
Co. York, PA.

HAGBERG, B. (1953). Acta Paed. 42. supp.93. 13.

HAHN, P.F. (1937). Medicine. 16. 3. 249.

HAHN, P.F., BALE, W.F., ROSS, J.F., BALFOUR, W.M., &
WHIPPLE, G.H. (1943).
J. Exp. Med. 78. No.3. 169

HARTMAN, Roberta S., CONRAD, M.E., HARTMAN, R.E., JOY, R.J.T.,
& CROSBY, W.H. (1963).
Blood. 22. No.4. 397

HAWKINS, W.B; & HAHN, P.F. (1944). J. Exp. Med. 80. No.1 31

HEMMINGS, W.A. (1958). in Transactions, Conference of
Placenta Assoc. for the Aid of Crippled
Children. N.Y.

HEMMINGS, W.A. (1965). Personal Communication.

HEMMINGS, W.A., & OAKLEY, C.L. (1957). Proc. Roy.Soc. Ser.B. Biol. Sci. 146 (925) 573-579.

HEIMEYER, L. (1956). Iron in Clin. Med. p.24. Univ. Cal. Press.(Ed. H.C. Walestein & S.R. Mettier)

HOCHBOOM, G.H., SCHNEIDER, W.C., & PALADE, G.E. (1948). J. Biol Chem. 172. 169

HOCHBOOM, G.H. & SCHNEIDER, W.C. (1950). J. Biol. Chem. 186. 417

HOLMES, M. (1966). Personal Communication

HOSAIN, F. & FINCH, C.A. (1964). J. Lab. Clin. Med. 64. 905

JANDL, J.H., INMAN, J.K., SIMMONS, R.L. & ALLEN, D.W. (1959). J. Clin. Invest. 38. No.1. 161

JANDL, J.H., & KATZ, J.H. (1963). J. Clin. Invest. 42. No.3. 314

JANDL, J.H., & KATZ, J.H. (1964). 'Iron Metabolism'. International Symposium. p.103. Ed. F. Gross. Assis. by E.R. Naegeli, & H.A. Philps. Pub. Springer-Verlag. Berlin.

KATZ, J.H. (1964). 'Iron Metabolism'. International Symposium. p.72.
Ed. F. Gross, Assis. by H.R. Knegeli, & H.D. Philps.
Pub. Springer-Verlag, Berlin.

KALDOR, J. (1953). Aus. J. Sci. 16. 111

LARSEN, J.F. (1962). J. Ultrastructure Research. 7. 535-549.

LARSEN, J.F. (1963). Amer. J. Anat. 112. 269-284.

LAUFBERGER, M. (1937). Bull. of Soc. Chem. Biol. 19. 1575

LAURELL, C.B., & INGELMAN, B. (1947). Acta. Chem. Scand. 1. 771

LAURELL, C.B. (1947). Acta. Physiol. Scand. 14. Suppl. 46.

LAURELL, C.B., & MORGAN, E. (1964). Acta. Physiol. Scand. 62. 271-279.

LOEFFLER, R.K., RAPPOPORT, D.A., and COLLINS, V.P. (1955). Proc. Soc. Exper. Biol. & Med. 88. 441-444.

LUFT, J.H. (1961). J. Biophys. & Biochem. & Cytol. 9. 409-414.

MACALLUM, A.B. (1891). Proc. Roy. Soc. 50. 277

MARTIN, W.B. (1959). Nutritional Iron Deficiency Anaemia of Piglets.
PhD. Thesis. Faculty of Medicine.
University of Glasgow.

McCANCE, R.A., WIDDOWSON, E.M. (1937). The Lancet. 2. 680

McCANCE, R.A., & WIDDOWSON, E.M. (1938). J. of Physiol. 94. 148

McFARLANE, A.S. (1958). Nature. 182. No.4627. 53

MOORE, C. (1958). Iron in Clinical Medicine. Univ. Cal. Press.

MORGAN, E.H. (1961). Nature. 192. No. 4801. 461

MORGAN, E.H. (1963). J. Physiol. 169. 2. 339

MORGAN, E.H. (1964). J. Physiol. 171. p55

MUIR. A.R. (1965). Personal Communication.

NEILSON, J.B. (1962). Schweiz. Med. Wschr. 92. 1295

NEILSON, J.B. (1963). Acta. Med. Scand. 173. 499

NOYES, W.D., ROTHWELL, T.H., & FINCH, C.A. (1960). Brit. Jour. of Haematology. 6. 43-55.

NYLANDER, G. (1953). Acta. Physiol Scand. 29. sup. 107

PERL, M. (1867). Arch. Path. Anat. 39. 42

PETERSON, E.A., & SOBER, H.A. (1959). Anal. Chem. 31. 857

POSCHENRIEDER, W.T., HAHN, P.F., BALE, W.F., & BALFOUR, W.M. (1942). Amer. J. Physiol. 137. No.1. 164.

PORATH, J., & FLODIN, P. (1959). Nature. 183. 1657

PORATH, J. (1959). J. Clin. Chim. Acta. 4. 776

PORATH, J. (1960). Biochem. & Biophys. Acta. 31. 195-207

PORTER, R.R. (1961). in "Biochemists Handbook". Ed. C. Long. Pub. E. & F.N. Spon.Ltd.Lond.

POTTER, V.R., & ELVEHJEM, C.A. (1936). J. Biol. Chem. 114. 495

POULIK, M.D. (1957). Nature. 180. No.4600. 1477

REICHBERG, J. (1956). Deutche Stsch Vendaugsche. 16. 223-229.

REYNOLDS, E.S. (1963). J. Cell. Biol. 17. 208

RAMSAY, W.N.M. (1957). Clin Chim. Acta. 2. 214-220.

SCARBOROUGH. R.A. (1931). J. Biol. & Med. 3. 63. 168-202

SCHADE, A.L., REINHART, E.W., & LEVY, H. (1949).
Arch. of Bioch. 20. No.1. 170

SHIRAWAWA, K. (1964). Proc. J. Acad. 40. 351

SHODEN, A. CARRIO. E.W., FINCH, C.A. (1955). J. Biol. Chem.
204. No.2. 823

SMITH, J., DRYSDALE, J., GOLDBERG, A. & MUNROE, H. (1966).
In Press.

SMITHIES, O. (1955). Biochem. J. 61. No.4. 629

STEWART, W.B., & GAMBINO, S.R. (1961). Amer. J. Physiol. 201. No.1. p.67.

BURGENOR, O.M., KOECHLIN, B.A., & STRONG, L.E. (1949). J. Clin. Invest. 28. No.1. 73

VOGEL, A.I. Quantitative Inorganic Analysis. (1941) 2nd Edit. p. 366. Longman, Green & Co.

WOSBURGH, G.J., & PLEYNER, L.B. (1950). Amer. J. Physiol. 161. No.2. 202

WINTROBE, M.M. (1946). Clinical Haematology. 2nd Edit. p.48 London Hampton.

WISLOCKI, G.B., DEMPSEY, E.W. (1955). Anat. Rec. 123. 42

WOHLER, F. (1955). Deutche Med. Vooh. 80. 30

WOHLER, F. (1962). Schweiz. med. Wschr. 92. 1295

WETTERQUIST, H. (1956). The Ultrastructure Organisation of Collumnar Absorbing Cells of the Mouse Jejunum. Dept. Anat. Karolinska. Inst. Stockholm.

ZAIL, S., CHARLTON, R.W., TORRANCE, J.D., & BOTHWELL, T.H. (1964) J. Clin. Invest. 43. No.4. 670