ERYTHROCYTE SURVIVAL STUDIES BY DIFFERENTIAL AGGLUTINATION IN NORMAL PREGNANCY AND IN PRE-ECLAMPTIC TOXAEMIA OF PREGNANCY.

T h e s i s

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by

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Contents.

PART I.

REVIEW AND CRITICISM OF THE METHODS OF DETERMINING ERYTHROCYTE SURVIVAL TIMES.

General Introduction

This thesis is a description of some original work performed to determine the span of life of human erythrocyte in women who develop pre-eclamptic toxaemia of pregnancy as compared to normal pregnant women.

This work was carried out during the tenure of a post-graduate exchange Fellowship in the Department of Obstetrics and Gynaecology, McGill University, Montreal, Canada, under the guidance of Dr. N.W. Philpott, Professor of Obstetrics and Gynaecology at McGill University and Dr. L. Lowenstein, Assistant Professor of Medicine at McGill University and Chief of Haematology at the Royal Victoria Hospital, Montreal.

In the course of studying anaemias of pregnancy, a slight but definite reticulocytosis was noticed in the peripheral blood and an increased phagocytosis of erythrocytes and erythrocyte pigment by reticulum cells in the bone marrow was observed in several patients who developed anaemia associated with pre-eclamptic toxaemia of pregnancy (Lowenstein, 1949, 1950). These observations suggested the possibility that an anaemia occurring in pre-eclamptic toxaemia of pregnancy had a haemolytic component. A series of experiments were carried out to test this assumption and the value of this present thesis lies in the fact that this assumption was disproved. It is original work. Nowhere

in the literature could there be found references to erythrocyte survival studies on normal pregnant women nor on women suffering from pre-eclamptic toxaemia of pregnancy. All the experimental work performed during the course of the investigation was done by the author himself, with the exception of the screening test for syphilis which was done by the technicians of the Blood Bank Laboratory at the Royal Victoria Hospital on each bottle of blood used for transfusion and the red and white blood cell counts which were done by the head technician in the Bessborough research laboratory in the Women's Pavilion, Royal Victoria Hospital.

The thesis is arranged in two main parts, the first is a review of the various methods used in determining erythrocyte survival and the second is a description of the experimental work performed.

REVIEW AND CRITICISM OF THE METHODS OF DETERMINING ERYTHROCYTE SURVIVAL TIMES.

Early Investigations.

In the early nineteenth century Scheel (1802) performed transfusion experiments on animals of the same species, by bleeding one animal until it became unconscious and then transfusing it with blood from the other. The first animal revived and continued to live normally with no apparent disruption of its usual routine and although no definite time is set for the erythrocyte survival, this particular investigator suggested that it was probably longer than had been previously assumed. Making use of blood cells of different morphology, which could be detected by a microscope, Marfels & Moleschott (1856) transfused sheep's red blood corpuscles into a frog and by studying the frog's blood microscopically were able to state that the sheep's red blood cells were still in the frog's circulation three and a half months later and concluded that this was the life span of the erythrocyte in this particular case. Brown Sequard (1857) used this technique, but in his experiments, different animals, transfusing blood from various mammals into birds. The mammal cells were visible in the birds' circulation at the end of forty five days. Twelve years later a new technique

was used to study erythrocyte survival times. Worm-Müller (1875) transfused normal animals with large quantities of blood of the same species, thereby inducing a plethora. Observations were made as to the time interval necessary for the blood values of such animals to return to normal. The average time for the restoration of these values to a mean was considered indicative of the red blood cell survival time and was found to be two weeks in this particular series. Quincke (1880) repeated these experiments on the same animals using the same method and his results were the same as Worm-Müller. Hunter (1885) reproduced both Quincke's and Worm-Müller's results by a slightly different technique, although after these experiments he concluded that the life span of the erythrocyte was approximately three weeks.

These earlier results show a diversity of opinion as to the length of time erythrocytes survive in various situations, but have not a practical application in human physiology. Scheel's early experiments in blood letting and transfusion did not take into account the stimulus of such a procedure to the bone marrow, which would increase its erythropoetic activity to combat the profund anaemia, nor the blood volume changes which would take place after such a severe, experimentally induced haemorrhage. Later workers, such as Marfels and Brown Sequard, when introducing

foreign erythrocytes into an animal's circulation have not taken into account the destruction of these transfused cells by the recipient's reticulo endothelial system, which would treat the cells as a foreign protein and presumably destroy them at a greater rate than compatible erythrocytes, thus the investigation did not follow the pattern of red blood cell survival but rather the elimination of a foreign protein reaction (Ashby, 1921). Worm-Müller, Quincke and Hunter, by inducing a plethora and noting the time for the blood values to return to normal, have been criticised by later authors on several grounds. A patient's or animal's organism, when subjected to a plethoric state, reacts by decreasing the number of circulating reticulocytes to a minimum (Robertson, 1917) and also by increasing blood destruction (Robertson & Rous, 1917). Later workers, using the differential agglutination technique, also found that the curve of elimination of transfused red blood cells in plethoric states was a steeper slope than in normal conditions and thus the transfused cells were being eliminated faster in patients with a plethora (Brown, Hayward et al., 1944). These considerations were not known during the earlier work of red blood cell survival and the results produced by Worm-Müller, Quincke and Hunter show a shorter life-span for the erythrocyte than is, in fact, true.

Reticulocyte Study as a Method of Calculating the Erythrocyte Survival Time.

It was felt that by studying the behaviour of reticulocytes, it would be possible to calculate the life span of the erythrocyte and this technique was utilised in 1930 by Eaton and Damren, who subjected rabbits to the withdrawal of large quantities of blood and then noted the daily response of the reticulocyte count to this. They found that there was an initial peak of the number of reticulocytes counted, followed by a definite cyclical increase of these cells thereafter. These cyclical peaks were set at eight and a half day intervals and on the basis of this observation, the authors assumed that the blood cells produced in response to the loss of blood had a life of eight and a half days and when these cells were all eliminated at this time, the bone marrow responded by another burst of activity and thus another number of reticulocytes, giving rise to a peak on their graphs. Graam (1942), repeated these experiments, again using rabbits as the experimental animal and by the same procedure found that the cycle of reticulocyte peaks in that particular series was nine days. Therefore, he concluded that the rabbit erythrocyte had a life span of nine days. Macacus Rhesus monkeys were used for experiments based on Graam's methods by Harne et al. in 1945 and in these experiments it was found that there was

an average of one hundred days from one peak of reticulocyte counts to another. They stated that this was the average life span of the erythrocyte in these monkeys, although in their series some results had been obtained showing a life span of ninety four days and some showing a life span of one hundred and seventeen days. This method of using the number of reticulocytes counted as an indication of the life span of the red blood cell was put into clinical practice by Berlin (1950), who selected patients with pernicious anaemia, treated these patients with intensive liver therapy until their blood contained a high percentage of reticulocytes and then withdrew some of this blood for transfusion experiments. Normal patients were selected and transfused with the blood containing the high percentage of reticulocytes and these cells were counted. By this method the author was able to calculate the red blood cell life span as one hundred and twenty five days.

It would seem that the peaks found in the reticulocyte curves in Eaton and Damren's work, Graam's work and also the work of Harne et al. is evidence of an inherent rhythmicity of blood metabolism and not necessarily an estimate of the red blood cell life span, Ashby (1948), Broun, McMaster, Rous (1923). The number of reticulocytes produced in response to blood letting is not an accurate

method of determining the erythrocyte life span as the reticulocyte count depends not only on the powers of production of the bone marrow but also on the quality of the stimuli that are acting on the bone marrow. Thus these experiments have taken no account of the ability or lack of ability of the bone marrow to produce reticulocytes and the various factors that affected this production, for example, the diet habits of the animals; nor were the varied changes in blood volume after blood letting taken into account in the expression of the results.

Studies of Erythrocytes Containing Material Foreign to a Normal Erythrocyte as a Method of Calculating the Red Blood Cell Life Span.

During the war, in the course of the manufacture of war materials, workers had to handle large quantities of trinitrotoluene over a prolonged period of time. In a number of cases this chemical led to the development of sulfhaemoglobinaemia and as it was possible to demonstrate this substance in the circulation by the use of spectrophotometry, Jope (1946) collected a series of cases suffering from this condition and by following the fate of the erythrocytes containing the sulfhaemoglobin, calculated the life span to be one hundred and sixteen days.

Recently it has been possible to "label" red blood cells by means of subjecting them to the action of radio-active isotopes and by transfusing these cells to a patient, follow the fate of the radio-active cells with a scintillometer, which counts the outflow of radioactivity from a blood specimen of the patient. In this manner the slow decrease in the radioactivity reading of each succeeding sample gives a slope of elimination for the "labelled" cells, from which can be calculated the life span of the erythrocyte. This method of "labelling" erythrocytes was used by Hevesy and Hahn (1940), who prepared cells containing radio-active phosphorous and also by Hahn et al. (1942), who used cells containing radio-active iron. Shemin and Rittenberg (1946) subjected glycine to the action of the heavy nitrogen isotope N^{15} and administered this "labelled" substance to volunteers. London et al. (1947, 1948) showed that this substance was then incorporated in the synthesis of the protoporphyrine molecule and used in the production of haemoglobin. With this substance taking part in the haemoglobin formation of the red blood cell, the elimination of these cells could be studied with the use of a scintillometer and the length of time of erythrocyte survival calculated. Using this method, these workers found that the erythrocyte lifespan was one hundred and twenty seven days.

Bale et al. (1949) made similar experiments with dogs, using a synthesized lysine as a method of studying the erythrocytes. The amino-acid lysine is one of the constituents of the globin molecule and is used in the synthesis of haemoglobin in the erythrocyte. In Bale's series, the lysine was subjected to the action of the carbon isotope C^{14} and the concentration of this substance in the dog's corpuscles calculated over an extended period. The slope of elimination of C^{14} from the dog's circulation and thus the elimination of erythrocytes was found to be one hundred and fifteen days.

These varied methods of following the survival of erythrocytes by using foreign material that is incorporated into the metabolism of the red blood cells, are open to criticism. In Jope's series of cases it is obvious that the production of a sulfhaemoglobinaemia is not an ideal method of studying the survival of erythrocytes, due to the risk of exposing volunteers to a potentially dangerous substance and also to the fact that sulfhaemoglobin is metabolised in the erythrocyte. There is no way of knowing the exact amount of sulfhaemoglobin present in the circulation, as different patients develop different degrees of sulfhaemoglobinaemia when exposed to the same quantity of trinitrotoluene. It is also possible that the presence of sulfhaemoglobin in the red blood cells shortens its

normal life span by interfering with the normal metabolic process. Radio-active phosphorous is not a suitable isotope for use in the study of erythrocytes labelled with it, as the phosphorous atoms are metabolised in the red blood cells and take part in the molecular exchanges that occur between these cells and the plasma, thus producing radio-active readings that are fallacious, in so far as the phosphorous atom may no longer be in the erythrocyte but are present in the plasma. This criticism can also be levelled at the use of radio-active iron, as this substance is liberated from the erythrocyte after the cell is sequestered from the circulation. The liberated radio-active iron atoms are then used again in the synthesis of haemoglobin and finds its way into new red corpuscles, which give a fallacious reading of the quantity of radioactivity initially introduced into the circulation.

Studies by Differential Agglutination.

Todd and White (1912) evolved a new method of following the survival time of transfused red blood cells. Polyvalent isohaemolytic serum was treated with red blood cells until the serum no longer agglutinated these cells, but would agglutinate the blood cells of another animal of the same species, in this particular case, Cyprian bulls. After transfusion, the previously treated serum was used to

differentiate the blood cells of the transfused blood, from the blood cells of the recipient, by agglutination of the recipient's erythrocytes, thus leaving the transfused cells free and available for study. In this particular experiment, Todd and White found that the transfused cells survived for four days in the circulation of the recipient. This principle of differential agglutination of red blood cells as a method of distinguishing between transfused cells and those of the recipient, was applied to human physiology by Ashby (1919), who made use of the ability of one human serum to agglutinate a specific human blood cell. Blood of one ABO group was transfused to a patient of a different ABO group and with a potent antiserum that corresponded to the patient's own blood type, the patient's red blood cells were selectively precipitated in the laboratory, leaving the transfused cells still in solution. For example, if blood of type O was transfused to a patient of type A, a specimen of blood from the patient, after transfusion, contained both O and A blood group cells and by using a potent anti-A serum, it was possible to agglutinate the type A cells from the blood specimen, leaving type O cells free to be counted. This procedure was then repeated at various time intervals after transfusion and by graphing the number of unagglutinated type O cells counted, against the time

interval after transfusion, a slope of elimination of transfused cells was produced, the end point of which denoted the maximum time that the transfused cells remained in the patient's circulation. The life span of these cells varied between thirty and a hundred and ten days in Ashby's cases, which consisted of patients suffering from anaemia after haemorrhage and anaemia coincidental with carcinoma.

Ashby (1921) investigated the problem of erythrocyte survival time in patients suffering from pernicious anaemia, using the method of differential agglutination as described in broad detail above, in an effort to determine the presence of a shorter life span for the red blood cells as part of the aetiology of this blood disease, but concluded from her experiments that erythrocyte destruction was not an aetiological factor in pernicious anaemia. The life span of the transfused erythrocytes in these patients was approximately a hundred days. Wearn, Warren and Ames (1922) repeated this work on patients with pernicious anaemia and reproduced Ashby's figures of the length of survival of cells from a normal patient, transfused into one suffering from pernicious anaemia. These workers concluded that the transfused cells survived for a normal period of time and thus abnormal destruction was not part of the aetiology of pernicious anaemia. Although Ashby and Wearn et al. had produced results independently that appeared to be the answer to the

problem of estimating the life span of human erythrocytes, the technique and methods were not used much between 1920 and 1940 due to divergent results produced by other authors, who estimated the erythrocyte life span as much less than Ashby. Jervell (1924), Clausens (1934). This was also due to the criticism of this particular method by Isaacs (1924), who stated that reticulocytes and nucleated red blood cells were not agglutinated by the antiserum used and that a transfusion to a person suffering from pernicious anaemia would cause a high percentage of reticulocytes to be released into the patient's circulation within a few days after the transfusion. As the nucleated cells were not agglutinated, the number of the transfused cells counted would contain a percentage of the patient's own reticulocytes. The number of unagglutinated cells present in the cell counts would then be fallacious and lead to a misrepresentation of the life span of the transfused cells. This criticism was unfounded as shown by Ashby (1924) and Maizels and Paterson (1940), who studied the occurrence or non-occurrence of reticulocytes among the unagglutinated cells and found no increase after transfusion. At a later date Callender, Powell and Witts (1945) and Young et al. (1947) demonstrated that reticulocytes and nucleated red blood cells agglutinated in the same manner as did the patient's erythrocytes.

In 1939, the problem of using blood stored in various temperatures and in various solutions was investigated, as a war-time necessity, and Wiener and Schaefer (1939, 1940) revived the Ashby technique of following the fate of transfused cells in an endeavour to find the length of time that such cells may be stored and in what type of preservative. These investigators made use of a modification of Ashby's original technique, previously described by Wiener (1934), in which he used the blood groups M and N. Thus, blood of group OM was injected into a recipient of group ON and the recipient's red blood cells agglutinated with anti-N serum, leaving the transfused OM cells unagglutinated and free to be counted. Using this method, the investigators determined the life span of erythrocytes preserved in sodium citrate for varying periods of time and found that blood preserved thus and stored for one week behaved in the recipient's circulation as though the blood was fresh. The blood existed in the patient's circulation for a hundred to a hundred and twenty days. Lengthier periods of storage, say for twenty one days, caused the transfused erythrocytes to be eliminated from the recipient's circulation within twenty six hours. These experiments awakened new interest in the Ashby method and numerous investigators performed transfusion experiments, using the differential agglutination technique to follow

the transfused cell's life span. It was found that a combination of sodium citrate and dextrose gave longer survival times for the transfused erythrocytes. Using this mixture of preservatives, Mollison and Young (1940), Maizels and Paterson (1940), Bushby et al. (1940), Belk and Barnes (1941), Belk and Rosenstein (1942), Denstedt et al. (1944) showed that it was possible to store blood for transfusion up to fourteen days, without the cells becoming damaged, as evidenced by the normal slope of elimination of these transfused cells obtained, using the differential agglutination method. Not only was the survival of transfused cells previously stored in various preservatives studied, but the Ashby technique was also used to study the erythrocyte survival times in various pathological states. As has been mentioned already, much of Ashby's original work was performed on hypochromic anaemias developing after haemorrhage, on patients suffering from anaemia due to carcinoma and on patients with pernicious anaemia. Brown, Hayward, Powell and Witts (1944) studied one patient with pernicious anaemia and found a normal life span of a hundred to a hundred and twenty days. Loutit (1945/46) investigated five cases with this blood condition and found the life span of the erythrocyte to be normal. Also, he performed the reverse experiment, withdrawing blood from two cases of untreated pernicious anaemia,

which he transfused to two patients with hypochromic anaemia. In these cases, the elimination of the transfused cells was rapid, fifty per cent of the transfused cells having been destroyed after ten and twelve days, that is, a life span of twenty and twenty four days. Because of these findings, Loutit postulated that the blood cells from an untreated pernicious anaemia case had a faulty protoplasm and were destroyed rapidly. The short survival times noted by Loutit when blood from pernicious anaemia patients was used for transfusion were reproduced by Singer, King and Robin (1948), who transfused erythrocytes from three cases of untreated pernicious anaemia into normal subjects and observed survival times for these erythrocytes of twenty seven, seventy five and seventy two days respectively. Using the cells for transfusion from a patient who had received adequate treatment, a normal survival time of a hundred and ten days was found.

Hypochromic anaemias, due to iron deficiency and as a result of haemorrhage have been studied, firstly by Ashby (1919) and then by Mollison and Young (1940). The latter authors studied the hypochromic anaemia that is found after haemorrhage and reported that the survival of transfused cells in these cases was within the normal range of a hundred to a hundred and twenty days. The hypochromic iron deficiency anaemias were studied by the Ashby technique

by Brown et al. (1944), who found that the graph of their results was linear and showed a constant rate of destruction of one per cent per day of the transfused cells and the average survival time of the cells was one hundred days.

Hypochromic anaemias associated with other pathological conditions have been investigated, anaemia in chronic sepsis, for example, chronic osteomyletis, pyelitis of pregnancy and empyema thoracis, Mollison (1947). In these diverse conditions the erythrocyte survival time was found to be within the normal limits of one hundred to one hundred and twenty days.

The haemolytic anaemias, both familial and acquired, have been studied with reference to the life span of erythrocytes in these conditions, by Dacie and Mollison (1943), who investigated the life span of transfused normal erythrocytes in six cases of familial haemolytic anaemia and in five cases found the life span to be within normal limits of a hundred to a hundred and thirty days. The remaining case showed a life span of only sixty days for the transfused erythrocytes and when investigated fully, this was found to be associated with an accidental Rh sensitization. The authors also reversed the procedure, using blood taken from one of their cases of familial haemolytic anaemia, for transfusion into a normal subject. The life span of the transfused erythrocytes in this case

was only fourteen days. Loutit (1945), Loutit and Mollison (1946), Mollison (1947) and Owren (1947) were able to confirm these results, both of the life span of the normal transfused erythrocytes and of the life span of the erythrocytes used for transfusion which were obtained from patients with familial haemolytic anaemia.

Chronic haemolytic anaemia was also investigated using differential agglutination methods after transfusion. Dacie and Mollison (1943), Mollison (1947), and Dacie (1948) calculated the survival of transfused normal erythrocytes into patients with nocturnal haemoglobinuria (Marchiafava-Micheli Disease) and in these cases found a normal life span of a hundred and ten days. Dacie and Mollison (1949) removed blood from a patient suffering from this type of chronic haemolytic anaemia and transfused these erythrocytes into a normal adult and a normal infant. It was found that the transfused blood cells were eliminated from the recipients' circulation in approximately twelve days. In view of these findings and the findings of the previous investigators, it was felt that there was no mechanism causing abnormal destruction of normal erythrocytes and that in cases of nocturnal haemoglobinuria, the fault lay in an abnormality of the patient's erythrocytes, whereby they were destroyed more rapidly than normal.

The problem of the aetiology of the anaemia in cases

of acquired haemolytic anaemia was extensively investigated by Loutit and Mollison (1946) by the Ashby technique and eight cases were subjected to transfusion experiments. In each of these cases a decreased survival time for the transfused erythrocytes was found, the average time for survival of fifty per cent of these cells was 6.3 days denoting a total average life span of 12.6 days. Mollison (1947) investigated a further eleven cases of acquired haemolytic anaemia and was able to reproduce the short life spans for transfused cells found by himself and Loutit. In several of the later cases, elimination of the erythrocytes was complete in five or six days and in all cases the elimination curves were not linear, but of exponential shape leading to the supposition that the transfused cells were being subjected to the action of some extracorpuscular factor that affected, not only the patient's own erythrocytes, but also the transfused cells. This conclusion has been reached by several workers, Damesnek and Estren (1947), Owren (1947), (1949), Evans and Duane (1949), and it has been suggested that erythrocyte survival studies using the differential agglutination method was a means of distinguishing cases of familial and acquired haemolytic anaemias, Mollison (1947), Berlin (1950).

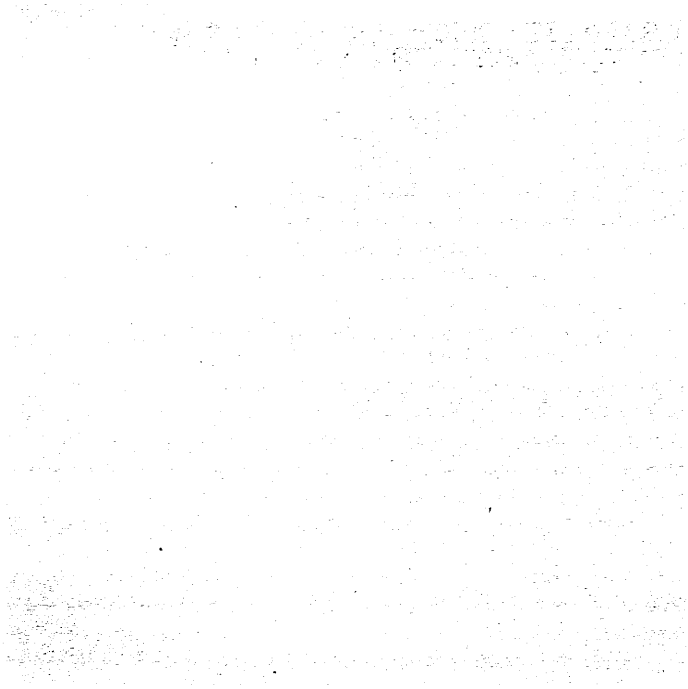
Other pathological conditions have been studied using the time of survival of transfused cells as a guide

to the aetiology of these conditions. Polycythaemia vera was studied by Merskey (1949), who found that the survival of normal cells transfused into a patient with this disease was normal. The transfusion of blood from this patient into a normal recipient also gave normal survival times. Singer et al. (1948) subjected patients suffering from sickle cell anaemia and sickle cell trait to transfusion experiments, as did Altmann (1947) and Callender and Nickel (1947), who found that there was a normal survival pattern for the normal transfused cells while in the diseased patient's circulation.

Foy, Kondi et al. (1945) found that, in cases of blackwater fever, when the patients were transfused with normal cells, there was a marked difference in survival times immediately after an attack of fever compared to the period between attacks. The survival of cells in the patient's circulation immediately after the febrile episode was short, while the survival of cells in the periods between the febrile episodes was nearly normal. They concluded that there were extracorpuscular factors present in the patient's circulation immediately after the febrile period that acted on the erythrocytes, haemolysing them.

As can be seen, there appears to be a definite place for the use of the Ashby method of estimating the erythrocyte survival, in elucidating some clinical problems and as a

method of investigating the possibility of a haemolytic component being present in various disease processes. With a view to proving or disproving the presence of a haemolytic component in the anaemia that so often accompanies pre-eclamptic toxaemia of pregnancy, which had been postulated by a review of clinical and haematological findings Lowenstein (1950), this present series was undertaken.



PART II.

DESCRIPTION OF MATERIAL, METHODS AND EXPERIMENTAL WORK PERFORMED.

MATERIAL AND METHODS

I. LABORATORY EQUIPMENT.

Pipettes. A Thoma's white blood cell counting bulb type pipette, with markings of 0.5, 1 and 11 at various levels on the barrel, was used for mixing the necessary proportions of blood, saline and serum. Two pipettes of this type were certified by the Laboratory of Standards and used exclusively throughout the entire investigation.

A small bore Pasteur pipette was used for removing the suspension of unagglutinated cells lying above the agglutinates and for transferring the suspension to both sides of the haemocytometer. At the completion of each step in the technical procedure, these pipettes were washed out by suction through them of distilled water, followed by methyl alcohol and finally ether.

Haemocytometer. One haemocytometer and cover glass were used exclusively throughout the investigation. This was a Spencer Bright line haemocytometer, certified by the United States Bureau of Standards. The cleansing of the counting area and cover glass was done by distilled water and a soft cotton cloth used for drying purposes.

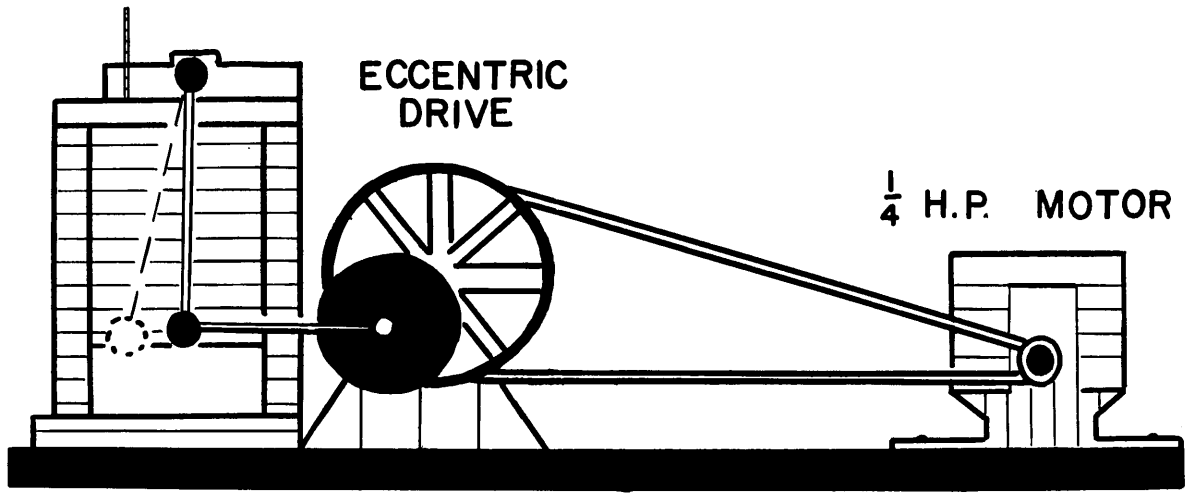
Tubes. Only one type of tube was used, this being a 7 mm. x 75 mm. serology tube. These tubes were cleaned by distilled water and dried by inverting them on a rack in a

bacteriological oven.

Waterbath. For regulation of the temperature at which the agglutination process took place, an electric International Clinical Waterbath with a variable rheostat was used. Its ability to keep a set temperature for prolonged periods was tested before the clinical aspect of the investigation was started and the rheostat was fixed so that an even temperature of 20° Centigrade was maintained. A clinical thermometer graduated in $^{1}/_{10}$ths from 0° Centigrade to 70° Centigrade was immersed in the water.

Agitator. A machine was designed and built to shake the serology tubes while they were in a constant temperature in the water bath. The necessity of this machine and for its number of vibrations per minute are explained on page 49, paragraph 2 . The apparatus consisted of a Delco ¼ horse power electric motor which, by means of a belt, turned a large wheel on which was set an eccentric drive. Two jointed arms set at a right angle, one end of the arm being attached to the eccentric and the other to a horizontal plastic bar immersed in the water, were moved through a 35° arc by means of the eccentric drive on the main wheel. The horizontal plastic bar in the water bath had 14 holes bored in it to accommodate the serology tubes. The electric motor ran at a constant speed and agitated the serology tubes

in the water bath, at a constant rate of 240 vibrations per minute (see drawing below).



Centrifuge. A variable speed International Clinical Centrifuge, with regular head capable of speeds ranging from 100 r.p.m. to 2000 r.p.m. was used. Prior to being used for this investigation, the speed of the head corresponding to the revolution indicator was tested. It was found that when the revolution indicator was set at 1500 revolutions per minute this was indeed the speed at which the head was revolving. Every three months after the start of the various experiments connected with this series, the centrifuge was tested and found to be correctly timed.

Microscope. The microscope used in this series was a Bausch and Lomb three objective model. The medium objective and 10x eye lenses were used for the counting of the cells in the haemocytometer. Light was furnished by a 60 watt bulb with a piece of blue matt glass in front of it.

II. BLOOD USED IN TRANSFUSION EXPERIMENTS.

The blood used for transfusion was obtained from the Blood Bank of the Royal Victoria Hospital. It was less than four days old, and was comparable to fresh blood with respect to red cell survival after transfusion, Mollison and Young (1942), Denstedt et al. (1944). The volunteers who gave the blood were young and healthy, had no previous history of attacks of jaundice and no family history of jaundice, had never been transfused themselves and had not given blood in the three month period prior to the present donation. Four hundred mls. of blood was taken from the antecubital vein into a commercial blood flask, the "Baxter vacuo-liter flask", which was produced with a vacuum inside the flask to facilitate the ease and speed of blood donation. The flask contained 150 mls. of diluent of dextrose U.S.P. 2.30% and sodium citrate U.S.P. 1.70%. During the period of blood donation, the flask was rotated gently to ensure adequate mixing of the blood and the diluent. The Blood Bank Laboratory carried out a screening

test for sypnilis and tested the ABO and Rh groups of the blood. Before transfusing this blood into a patient, the author repeated the tests for ABO and Rh grouping, tested the MN group of the blood and performed a red blood cell count. The average red blood cell count of the bloods used in transfusion was 3,800,000 per cu.mm. The stored blood cells were tested with the patient's serum, and the patient's blood cells with the serum of the preserved blood. These tests were carried out at 37° Centigrade using centrifugation to accelerate any reaction that might have been present. (See page 35 , paragraph g).

III. PATIENTS USED IN THIS SERIES.

A total of nine normal pregnant women were selected for study. Three of these were primigravidas and six were multigravidas. The patients' ages ranged from twenty to thirty two years and blood transfusion was performed near the end of pregnancy. Eight patients were transfused in the second trimester of pregnancy and one was transfused in the first trimester. Two of the patients developed pre-eclamptic toxaemia of pregnancy, fifty six and sixty nine days respectively after they had been transfused. Six of the remaining seven patients delivered spontaneously after normal labours, with a minimal blood loss and the seventh patient was delivered by Caesarean Section. General medical

histories and obstetrical histories of each patient were taken and each patient was subjected to a physical examination. None of the patients had received a previous transfusion. Haematological studies were performed as previously outlined by Tysoe and Lowenstein (1950), and in all cases were within the normal range.

The studies performed on each patient were as follows:-

a) Haemoglobin.

A haemoglobin estimation was performed on the patient before transfusion and regularly thereafter throughout the course of the investigation. It was done from a finger puncture, the blood being drawn up to the 20 cubic mm. mark in a suitably calibrated Bureau of Standard pipette. The 20 cubic mm. of blood was expelled into a tube containing exactly 10 mls. of ammonia water, the tube was shaken and inserted into an Evelyn galvano-photocolorimeter in which there was a 540 mu filter, Evelyn (1935). Before each reading was made for the haemoglobin on the galvanometer scale, a blank tube of distilled water was used as a control to set the scale at the reading 0. The ammonia water was made up with 0.48 mls. concentrated ammonium hydroxide in 1000 mls. of distilled water, Wintrobe (1946).

b) Haematocrit.

This investigation was also performed on each patient at regular intervals. Wintrobe's haematocrit and method were employed, Wintrobe (1946), whereby 5 mls. of venous blood were withdrawn with minimal stasis and delivered into a bottle containing dry anticoagulant. A Pasteur capillary pipette was filled with this blood and expelled slowly into the haematocrit tube up to the 0 graduation. The tube was centrifuged at 3000 r.p.m. for thirty minutes and the packed cell volume read off from the graduated scale marked on the tube.

The dry anticoagulant used in these estimations was made up of 6 mgs. ammonium oxalate and 4 mgs. potassium oxalate, prepared by mixing 1.2 g. ammonium oxalate and 0.8 g. potassium oxalate in 100 mls. of distilled water, pipetting 0.5 ml. into a bottle and allowing the solution to dry.

c) Red blood cell counts.

These counts were performed using Hayem's solution as a diluent. This solution consists of sodium sulphate 2.5 g.; sodium chloride 0.5 g.; mercuric chloride 0.25 g. in 1000 mls. of distilled water. The blood was obtained either from capillary blood or from oxalated venous blood if this was available, drawn up to the 20 cubic mm. mark in

a standardised pipette and expelled into 1.0 ml. of Hayem's solution in a serology tube. This was shaken and a suspension of the cells plated on to both sides of the counting chamber of the Spencer Bright Line haemocytometer. The cells on both sides were counted as in a normal red blood cell count.

d) The anti-globulin test first described by Coombs (1946) was performed on each patient prior to transfusion. The direct Coombs anti-globulin test was performed on the patient's blood cells, in an effort to demonstrate the presence of an immune globulin combined with its specific antigen on the erythrocytes, as for example, the non-specific autoantibody of certain acquired haemolytic anaemias.

A 2% suspension in normal saline of the erythrocytes to be tested was made in a serology tube and the cells washed three times with tubefuls of fresh saline. After the last washing the suspension was made up to 2% concentration again with saline and by means of a Pasteur pipette two to three drops of this were added to the anti-human serum prepared commercially by Ortho Laboratories. This was mixed in another serology tube. The tube was allowed to stand for 15 minutes at room temperature, centrifuged at 1000 r.p.m. for 2 minutes and examined by a hand lens for the presence of agglutination.

The indirect Coombs anti-globulin test was also performed on the patient's serum as an added protection against circulating antibodies, which had not combined with an antigen on the erythrocyte and thus would not agglutinate the saline suspensions of cells used in cross-matching the preserved blood against the patient's blood. A 2% suspension of the preserved blood for transfusion, in clean normal saline, was prepared and two drops of this were added to an equal amount of the patient's serum in a serology tube. This mixture was incubated in a water bath at 37° Centigrade for 60 minutes and examined for the presence of agglutination. If agglutination was absent then the suspension was washed three times with normal saline, centrifuging the tubes between each washing. After the last centrifugation all the saline was decanted, two drops of anti-human serum added to the sediment of red blood cells and this mixture shaken well. The tubes were allowed to stand at room temperature for 15 minutes, centrifuged at 1000 r.p.m. for 2 minutes and the cells examined by a hand lens for evidence of agglutination.

Each time the Coombs anti-globulin test was used, a negative and positive control test was performed as follows. A 2% suspension of known Rh positive and Rh negative bloods in saline was prepared in different serology tubes and one drop of anti-D typing serum was added to two drops of each

suspension. Each tube was incubated for sixty minutes in a water bath set at 37° Centigrade, during which time the Rh positive cells became coated with the antibody, while the Rh negative cells remained free of antibody. After incubation, the cell suspensions were diluted with fresh normal saline, centrifuged at 1500 r.p.m. for one minute and the saline decanted. This procedure was repeated three times and after the last of the saline had been removed, two drops of anti-human serum were added to the sediments, the resulting mixtures shaken well and allowed to stand at room temperature for fifteen minutes. The tubes were then centrifuged at 500 r.p.m. for one minute and shaken lightly. Provided that the sera used in the control tests were potent, then gross agglutination was noted in the tube containing the suspension of Rh positive cells and was absent from the tube containing the Rh negative cells.

e) ABO grouping, MN grouping and Rh grouping.

Each patient had these tests carried out before transfusion. The serum used for the ABO and Rh grouping was commercial liquid serum produced by Ortho Research Laboratories. The serum used for MN grouping was powdered blood typing serum prepared from rabbits by Lederle Laboratory Ltd.

A two per cent suspension of the patient's blood in

normal saline was prepared and two drops of this were put into four labelled serology tubes. One drop of anti-A serum was added to the first tube, one drop of anti-B serum to the second, 4 mgs. of anti-M to the third and 4 mgs. of anti-N serum to the fourth. The tubes were shaken thoroughly by hand and incubated for fifteen minutes at 37° Centigrade in a water bath. After incubation, the tubes were centrifuged at 500 r.p.m. for one minute and shaken gently again to dislodge the sediment. The presence or absence of agglutination was judged using a small hand lens.

To test the Rh properties of the blood, a 2% suspension of red blood cells was prepared using the patient's own serum as a diluent. Two drops of this suspension were added to one drop of anti-D typing serum in a serology tube and incubated for fifteen minutes at 37° Centigrade. The suspension was centrifuged for one minute at 500 r.p.m. and resuspended by shaking. Agglutination was noted using a small hand lens.

f) Testing of stored blood for transfusion with patient's blood. (Crossmatching procedure).

i) Saline testing.

Two suspensions in isotonic saline were prepared, one containing 5% of the stored blood cells and one containing

5% of the patient's blood cells. Two serology tubes were labelled A and B. Two drops of the stored blood cell suspension were added to tube A and also an equal amount of the patient's serum. Two drops of the patient's blood cell suspension were added to tube B and also an equal amount of plasma from the bottle of stored blood. Both tubes were shaken by hand, incubated in a water bath at 37° Centigrade for 60 minutes, centrifuged at 500 r.p.m. for 1 minute and shaken gently to suspend the agglutinates. Whether or not agglutination had taken place was determined by examining a small amount of the mixture under a microscope.

ii) Albumin testing

A similar set of tests, using 22% Bovine Albumin commercially prepared by Ortho Laboratories, as a dilution medium instead of saline were performed on the stored blood, the stored plasma, the patient's blood and the patient's serum. These tests were performed to obviate the presence of antibodies that would not agglutinate saline suspended cells. Two serology tubes were labelled A and B and two drops of a 5% suspension of stored blood cells in plasma were added to an equal amount of the patient's serum in tube A. Two drops of a 5% suspension of the patient's blood cells in their own serum and two drops of stored plasma were pipetted into tube B. To each of A and B were added two drops of 22% Bovine Albumin and the tubes were

centrifuged at 1000 r.p.m. for three minutes. After centrifugation, the tubes were incubated at 37° Centigrade in a water bath for sixty minutes, recentrifuged at the same speed and for the same period of time as before, the sediments resuspended by shaking gently and a small amount of the suspension examined on a slide under a microscope for agglutination.

g) Counting of the unagglutinated cells left after interaction between the patient's blood and a corresponding antiserum.

Before transfusion of a patient, a sample of the patient's blood was subjected to the agglutination procedure described further on (V), with a view to finding the number of cells left unagglutinated after the action of a potent antiserum. It has been stated, Osborne and Denstedt (1947), Mollison (1947), that it is impossible to agglutinate a hundred per cent of cells of a random sample of blood even using a potent, avid antiserum in proper dilution. This number of cells left unagglutinated varies from patient to patient and depends on the strength of the antiserum used. These authors suggested that this value of free cells should not exceed 10,000 per cu.mm. in the patient to be transfused. If this arbitrary number was exceeded then the antiserum was probably at fault and a more potent serum should be substituted. Values below

10,000 cells per cu.mm. were of negligible importance in calculating the erythrocyte survival after transfusion and could be ignored in the final expression of results. It was felt that each patient in this series should have this procedure performed on a random sample of blood as a method of checking the validity of the serum for that particular patient.

As has been stated in the first part of this thesis, it is possible to follow the fate of transfused cells by making use of the ability of a human antiserum to agglutinate a group specific erythrocyte, Ashby (1919). A modified version of the Ashby technique was used in this present work in an attempt to elucidate the problem of red blood cell survival in pre-eclamptic toxaemia as compared with normal pregnancy. Blood of one group was transfused into a patient of a different blood group and a potent antiserum used to agglutinate the patient's own erythrocytes. This left the transfused cells free and unagglutinated and these were counted at various time intervals after transfusion, giving a value for the life span of the erythrocytes transfused. This procedure is possible by using anti-A serum, anti-B serum, anti-Rh serum, Mollison and Paterson (1949), anti-M serum, or anti-N serum, Weiner (1934). In some cases in the present series, the survival of the transfused erythrocytes was followed by using two

different sera coincidentally. For example, blood of group OM was transfused to a patient of group AM and both anti-A and anti-M sera used to agglutinate the patient's own erythrocytes in separate serology tubes.

IV. TECHNIQUE OF TRANSFUSION AND COLLECTION OF SAMPLES.

The patient was requested to lie down for half an hour before the transfusion was started. Exactly 5.0 ml. of blood was withdrawn with minimal stasis from the antecubital vein into a sterile all glass syringe through a No.18 needle. This preliminary sample was delivered slowly into a clean, dry bottle containing dried potassium oxalate and ammonium oxalate (Wintrobe) and the bottle gently rotated to ensure adequate mixing of the blood and anticoagulant. The blood transfusion was started through the same No.18 needle, the blood having previously been allowed to reach room temperature. 400 mls. of the appropriate blood was given to the patient during the course of 1 hour. After the transfusion had finished, the patient had to remain supine for a further hour so that further blood specimens could be collected. By previous investigations it had been proved by the author and by Berlin (1951) that samples could be collected either from venous blood or capillary blood from a finger prick and to save the patient the distress of numerous venepunctures, the samples, with

the exception of the pre-transfusion one, were all taken from the finger. The finger was first cleansed with alcohol which was dried off completely and the pulp of the finger then stabbed with a Hagedorn needle. The first blood show was wiped off with a dry swab and once a free flow of blood was established, a Thoma's pipette was filled to the mark 0.5 with this. Any excess blood at the tip of the pipette was wiped off and the pipette was filled to the mark 11 with clean isotonic sodium chloride solution. The blood and saline mixture was then expelled from the pipette into a chemically clean serology tube, labelled with the patient's name, date and time of collection. Specimens, as outlined above, were collected at ½ hour intervals after the start of the transfusion and again 1 hour after the transfusion had finished. Further samples were taken 24 hours, 48 hours, 96 hours after the end of the transfusion and thereafter once a week. The specimens collected during the time of the actual transfusion were placed in the refrigerator till the transfusion was finished and then investigated.

V. AGGLUTINATION PROCEDURE.

The procedure used throughout this investigation is a slight modification of that described by McKerns and Denstedt (1950).

As described above, a Thoma's white blood cell counting pipette was filled to the 0.5 mark, either from venous blood suitably oxalated, or directly from a free-flowing puncture wound in the finger and isotonic saline solution added to the mark 11. This suspension was expelled into a serology tube. The same pipette was cleaned and dried and the original red blood cell and saline suspension drawn up to the 1.0 mark. Temperature equilibrated antiserum of the correct type was then drawn up to the 11 mark and the pipette rotated through all planes, to ensure adequate mixing. The contents of this pipette were then expelled into another serology tube of the same dimensions as the first one, which was transferred to the rack in the water bath and shaken through a 35° arc at 240 vibrations per minute for 20 minutes in a constant temperature of 20° Centigrade. After shaking, the tube was centrifuged at 1500 r.p.m. for two minutes, the cell sediment broken up by flicking the tube with the finger and the suspension again centrifuged at 1500 r.p.m. for 2 minutes. The tube was then transferred to the agitator and shaken at the 240 vibrations per minute for a further twenty minutes and after removal from the agitator, the agglutinate was allowed to settle. A small amount of fluid above the agglutinate was removed with a Pasteur pipette, placed on either side of a haemocytometer and a

cover glass put on top of the counting area. The fluid in the haemocytometer was allowed to settle and the total number of cells in the whole field (9.0 sq.mm.) of one side of the haemocytometer was counted. This was repeated for the other side and the average of the two counts calculated. The haemocytometer and cover glass were cleaned and dried and another amount of fluid from above the agglutinates in the serology tube was spread on the counting chamber and both sides counted. Clumps of cells, or two cells touching each other, were ignored and if the cell counts on each side of the chamber varied within 10%, the counts were repeated.

In those cases where dried, powdered anti-M and anti-N sera were used, the procedure was modified to suit these. The original dilution of blood and saline was made as before and expelled into a serology tube; the second dilution of this suspension with liquid serum was omitted and saline substituted for the liquid serum. The resultant suspension was expelled into another serology tube containing 4 mgs. of the dried anti-serum and then shaken and centrifuged as described above.

To calculate the number of unagglutinated cells per cubic millimetre in each specimen, the average of the four counts of the cells in each side of the haemocytometer was multiplied by a factor of 269, which was derived from the

following computation:-

Original dilution of capillary blood with normal saline

= 22 times.

Second dilution of blood and saline with antiserum

= 11 times.

Depth of counting chamber = 0.1 mm.

Total area in which the unagglutinated cells were counted

= 9.0 sq.mm.

Thus the number of cells per cubic millimetre = $\frac{11 \times 22}{9.0 \times 0.1}$

= 269.

VI. DESCRIPTIONS OF VARIOUS TECHNICAL ASPECTS OF THE AGGLUTINATION PROCEDURE.

i) Investigation of the method of collecting samples.

Most investigators, Osborne and Denstedt (1947), Dacie and Mollison (1943), have used venous blood in a suitable anticoagulant mixture for making the original blood cell and saline mixture. Berlin (1951) proved that capillary blood was acceptable for this phase of the procedure. In this investigation it was felt that the number of venepunctures performed on the patients should be minimal in view of the fact that the patients were disinclined to travel into the laboratory for what they considered to be a frightening aspect of the investigation. Consequently, a series of tests were performed to test the feasibility

of using capillary blood from a finger prick instead of venous blood.

5.0 mls. of venous blood were removed from a volunteer and transferred to a bottle containing dried oxalate (Wintrobe). At the same time, blood from a stab wound in the pulp of a finger was taken up to the 0.5 mark in a Thoma's pipette and diluted with saline as previously described. This was expelled into a labelled serology tube. Using the same cleaned and dried pipette, the oxalated venous blood was diluted with saline to the same proportions and placed in another serology tube. The agglutination procedure as described on page 38 was then completed on both samples and the number of unagglutinated cells counted. This investigation was repeated using three volunteers of group A and three volunteers of group M. Results are shown in Chart (i).

ii) Serum.

Previous workers, Mollison, Dacie, Berlin, have stressed the fundamental need for a serum that is powerful enough to agglutinate nearly all of the patient's own blood cells. If the serum used was not potent, then erroneous results were obtained as the number of transfused red cells at any one time during the course of the investigation was the basis for this method, and if the serum did not con-

stantly agglutinate the majority of the patient's red cells, then the cells left in solution were a variable mixture of transfused red cells and patient's red cells. As has been pointed out by Osborne and Denstedt, it is impossible to agglutinate all of the patient's blood cells even using high potency serum, although the number of unagglutinated cells can be reduced to a statistically unimportant level by the careful selection of antiserum. Not only was the potency or strength of the serum of importance, but also the avidity or speed at which reaction between the serum and red cells occurred. Commercial serum was not potent nor avid enough to give satisfactory agglutination of the corresponding blood group and thus it was decided to stimulate the production of highly potent, avid serum in a group of volunteers by means of intravenous injections of 1.0 ml. of A and B group specific substance (Witebsky) at intervals of three weeks. Stimulation of antibody formation by this method varies in individual cases and numerous volunteers from McGill University Medical School were tested before selecting three whose serum was of the correct potency and avidity. The criteria for a satisfactory serum were based on three experiments; the avidity, which must be less than four seconds, the dilution of serum with a saline suspension at which agglutination occurs and could be observed microscopically must be more than 1:5000, and the number of cells

not agglutinated, after interaction between the serum and the blood of a control patient, must not be more than 10,000 cells per cu.mm., Mollison (1947), Osborne and Denstedt (1947). If a volunteer's serum fulfilled these conditions then five hundred mls. of blood were removed by way of an antecubital vein into a sterilised flask containing no anticoagulant. The blood was allowed to coagulate and after twelve hours' refrigeration at 4° Centigrade, the serum was removed and stored in ampoules under sterile conditions. Serum stored at ordinary refrigeration temperatures of 4° Centigrade loses its avidity and potency over a period of weeks, but if kept in a frozen state at a temperature of -20° Centigrade will maintain its important properties for the time necessary to perform investigations of a prolonged nature (Berlin). The serum used in this investigation was kept in a constant frozen state at -20° Centigrade except when in use and control tests throughout the period of the experiments showed no loss of avidity nor of potency. Chart (ii). Commercially prepared dried serum of groups anti-M and anti-N were made available to the author by Lederle Laboratory Ltd. These sera were subjected to the same tests as the liquid sera and were found to be just as potent and avid and gave an adequate level of unagglutinated cell counts. These sera were stored in small bottles with a rubber stopper in an atmosphere free

of moisture in a desiccator vacuum chamber with a layer of calcium chloride. The quantity of sera used was approximately 4 mgs., which was measured on a small wooden toothpick.

Dilution of serum.

Two main stages in the process of agglutination of red blood cells by a specific antiserum have been postulated, Weiner (1943), Landsteiner (1945), Boyd (1943), Eagle (1930, 1932), Duncan (1938) and Kendall (1942). Firstly, a rapid process of combination of antigen (agglutinogen) and antibody (agglutinin) to form sensitized cells and secondly, a slower process of aggregation or clumping of these cells. There is a direct relationship between the potency of the antibody and the number of cells that are agglutinated, but this relationship is maintained only until the potency of the serum or antibody reaches a certain level. Very potent serum may not produce a maximum agglutination and this zone of inhibition is called the prozone. If a potent serum is diluted, then maximum agglutination will take place, Jones and Orcutt (1934). As agglutination is an antigen-antibody reaction and as this takes place extremely rapidly with potent avid serum, it is a possibility that numerous cells become imprisoned in the rapidly formed agglutinate, without coming in contact with the antibody and thus, without becoming sensitized. If

the agglutinate of cells is disturbed the non-sensitized cells are liberated and remain so because of the absence of the antibody, which has been used to cause the initial agglutination, Berlin (1950), Osborne and Denstedt (1946). A potent agglutinating serum contains a moderate concentration of isohaemolysins, which possess the power of reducing the ability of the blood cells to agglutinate. If the serum is diluted this action of the isohaemolysins no longer occurs and complete agglutination may therefore take place, Berlin (1950).

In this present investigation it was found that constant maximum agglutination, as shown by the unagglutinated cell counts on unselected bloods, was not occurring and in view of the opinions expressed above and of the prozone phenomenon, a series of experiments was carried out to determine the optimal dilution for maximum agglutination. Two batches of frozen serum, one of type anti-A and one of type anti-B, were allowed to liquefy and come to room temperature. Dilution of these sera was then made using a constant amount of serum (0.1 ml.) and a varying amount of isotonic saline (0.1 ml., 0.2 ml., 0.3 ml., 0.4 ml., 0.5 ml., etc.). A blood and saline mixture was made as before in a Thoma's pipette and various dilutions of serum with saline used for agglutination. The mixture was subjected to agitation and centrifugation and the number of unagglutin-

ated cells counted in the haemocytometer. The maximum agglutination, as shown by the lowest unagglutinated cell counts, was when the serum was diluted by saline to a proportion of one of serum to eight of saline. Chart (iii). Thus prior to the start of each investigation the appropriate serum was diluted in saline in the aforementioned proportions.

Centrifugation.

In an endeavour to produce maximum agglutination by repeated contact between antigen and antibody and therefore maximum sensitisation of the red blood cells, various methods have been used - shaking by hand, Ashby (1919), the use of small serological tubes, Wiener and Schaefer (1940), Bushby, Kekwick et al. (1940), Belk and Barnes (1941), the use of small beakers, Maizels and Paterson (1940), the use of small bottles, Loutit (1945), by light centrifugation, Dacie and Mollison (1943), Osborne and Denstedt (1947), in a red blood cell pipette using shaking, Thalnimer and Taylor (1945).

Eagle (1932) has stated that mechanical agitation accelerates the rate of agglutination, by increasing the incidence of collision between antibody and antigen and Osborne and Denstedt state that centrifugation will increase the potency of a serum up to ten times that of the same serum used without centrifugation. Before the start of the

present investigation, a series of tests were run to determine the effect of repeated centrifugations on the agglutinating power of the serum, as evidenced by the number of unagglutinated red blood cells after various periods of centrifugation. As most of the foregoing authorities had insisted on light centrifugation, the revolutions per minute were kept constant at 1500 and the period of time that the red blood cell and serum mixture was subjected to centrifuging was varied between one minute and eight minutes.

Capillary blood from a finger puncture was drawn up to the 0.5 mark in the Thoma's white blood cell pipette and saline added to the 11 mark. This mixture was expelled into a serology tube and using the same, cleaned and dried pipette, a quantity of the blood and saline suspension drawn up to the 1.0 mark and the pipette filled to the 11 mark with anti-A serum of optimal dilution. This suspension was then expelled into another serology tube and centrifuged at 1500 r.p.m. for one minute. The centrifuge was turned off and allowed to coast to a standstill. The tube was flicked once with the finger to resuspend the sediment and after allowing the agglutinates to settle, the fluid above these clumps was drawn off with a Pasteur pipette and transferred to both sides of the counting chamber. Counts were made in duplicate of the

unagglutinated cells and the result was plotted against the time of centrifugation. This procedure was repeated for various periods of centrifugation.

The investigation was repeated using dried, powdered anti-M serum. In this case the technique used for liquid serum was modified. The original blood and saline mixture in the pipette was made as before and expelled into a serology tube. The second dilution of the suspension with liquid anti-serum was omitted and saline drawn up to the 11 mark. This suspension was then expelled into another serology tube containing 4 mgs. of the dried serum, shaken once by hand to mix the serum and blood/saline suspension and then centrifuged as before. The results of both liquid anti-A serum and dried anti-M serum are shown graphically in Chart iv.

Shaking.

It was thought that not only centrifugation, but also shaking of the blood and serum mixture would influence the percentage of agglutination and thus the number of unagglutinated cells present in the supernatant fluid. Ashby, Dacie and Mollison, Berlin had made use of either tapping the mixture or shaking by hand. Osborne and Denstedt, McKerns and Denstedt (1950) had used this principle and devised a mechanical shaker with a Graham variable speed motor for agitating the mixture at varying speeds.

A similar machine was designed and built for this investigation. It has been described in detail already under "Laboratory Equipment". The electric motor of this machine ran at a constant speed and agitated the serology tubes in the water bath at a constant rate of 240 vibrations per minute.

The rate at which vibrations occur influenced the completeness of the agglutination process. Thus, in Osborne and Denstedt's work, the rate of vibrations per minute was altered from 140 to 190 in an attempt to insure maximum agglutionation. It was found that the higher rate of agitation produced more complete agglutination. In McKerns and Denstedt's experiments, a higher rate of agitation, namely 230 vibrations per minute, was used with good results. In a personal interview with Dr. Denstedt, it was suggested that a mechanical shaker vibrating at 240 vibrations per minute would increase the percentage of agglutination and the present machine was built to this specification.

A series of investigations was carried out to find the effect of time of agitation of the blood and serum mixture on the completeness of agglutination. Two series of tests were performed on blood of a group AN volunteer, using both anti-A and anti-N serum. The tests ran concurrently and not only yielded information about the effect

of agitation on agglutination, but afforded a comparison between the behaviour of dried antiserum and liquid antiserum. The Thoma's white blood cell pipette was filled to the 0.5 mark with capillary blood and normal saline added to the 11 mark and mixed. This was expelled into a serology tube and after cleaning and drying the pipette, the suspension was drawn up to the 1.0 mark and liquid anti-A serum added to the mark 11. After mixing, this second mixture of blood, saline and serum was expelled into another serology tube and the tube placed in the lucite bar of the agitator. The agitator was switched on for two minutes and after this length of time, the tube was removed and the agglutinates allowed to settle. Some of the fluid, lying above the clumps of cells, was removed with a Pasteur pipette and transferred to the haemocytometer, where the unagglutinated cells were counted. As before, the counts were performed in duplicate and the average of four counts taken as a value for the unagglutinated cells. When using dried anti-N serum, saline was substituted for the liquid serum and this mixture expelled into a serology tube containing 4 mgs. of powdered serum. The above scheme of investigation was repeated, keeping all steps in the procedure as constant as possible, but varying the time that the blood and serum mixture was subjected to agitation. The values for the unagglutinated cells were plotted against

the time of agitation and were recorded graphically in Chart v.

Temperature.

There seemed to be a lack of agreement between various workers on the problem of red blood cell survival, as to the temperature at which the agglutination process should be carried out. In the original method described by Ashby, the tubes containing the antigen-antibody mixture were incubated at 37° Centigrade for forty minutes. Mollison and Young, in 1940, disagreed with this temperature and felt that room temperature was the correct environment for accelerating the agglutination process and that one hour was the correct length of time for the process to be completed. Taylor (1938) suggested that the temperature described by Ashby was adequate, but that the time of reaction at this temperature was not and suggested two hours. Berlin (1950) did not agree with either of these suggestions and during the course of preliminary investigations, found that a temperature of 4° Centigrade increased the agglutination of the red blood cells by the serum. The length of time that was necessary for the reaction to be completed was also investigated in Berlin's series and by varying the length of time necessary for the interaction between the red blood cells and the serum, all other factors being kept

constant, it was found that the optimal reaction time was 1½ - 2 hours. From these observations, Berlin suggested that the blood cell and serum mixture should be allowed to stand in a refrigerator at 4°C for 2 hours. The risk of cold agglutinins taking part in this process was obviated by determining the erythrocyte sedimentation rate on each sample. If the erythrocyte sedimentation rate was increased, a titration for cold agglutinins was carried out. No evidence of interference with the agglutination reaction by cold agglutinins was found during the course of Berlin's investigation. Other investigators, McKerns and Denstedt, have produced satisfactory results using a constant temperature of 20°C during the course of the agglutination reaction and in view of the fact that it was deemed desirable to have both centrifugation and agitation incorporated in the present investigation, it was difficult to see how this could be accomplished and yet maintain a temperature of 4°C.

To test the feasibility of using a constant temperature of 20°C, a number of investigations were carried out. The length of time of agitation and the number of vibrations per minute were kept constant, the only variable factor being the temperature of the water bath in which the serology tubes containing the blood cell and serum mixture were being agitated. The temperature range in which these investigations were carried out was from 4°C to 45°C. As previously

described, a Thoma white blood cell pipette was used to mix the original blood and saline mixture which was then expelled into a serology tube. The suspension was then mixed with the appropriate antiserum, the pipette was rotated in planes and the contents of the Thoma's pipette expelled into another serology tube. This was centrifuged at 1500 r.p.m. for 4 minutes, agitated in a water bath at 4°C. for 40 minutes and after allowing the large agglutinates to settle, the fluid above these agglutinates was removed with a Pasteur pipette. The fluid in this pipette was used to fill both sides of the counting changer and the un-agglutinated cells were counted. The counting was then repeated with another aliquot of fluid and the average of the four counts taken as the unagglutinated cell count. In succeeding experiments, the temperature of the water bath was raised 5°C, and the agitation of the specimen repeated at that temperature. The results were graphed and were shown in Chart vi.

R E S U L T S.

(1) Mode of expression of the results.

The results of studies of the survival of transfused red blood cells by the Ashby differential agglutination technique have been expressed by several methods. As an absolute number of unagglutinated erythrocytes surviving in the recipient's circulation at different intervals after

transfusion, Ashby (1919), Wearns et al. (1922), as the total survival time, that is, as the maximum time that transfused erythrocytes could be detected in the patient's circulation, Martinet (1938), Dekkers (1939).

However, the number of surviving cells at any one time is not always inversely proportional to the time interval since transfusion, that is to say that, when the results are represented graphically, the slope of elimination is not always linear. In haemolytic anaemias, the slope of elimination is curved, Brown, Hayward, Powell and Witts (1944), and even in normal patients not suffering from this condition, but rendered plethoric by transfusion, there is an initial curve followed by a linear slope of elimination, Brown, Hayward et al. (1944), and there is an increased blood destruction in plethoric states, Robertson and Rous (1917). Also, if old, stored blood is used for the transfusion, there is an initial phase of rapid destruction followed by a phase of slower destruction, Mollison and Young (1940, 1942).

According to Mollison (1947), direct determination of the point at which transfused cells finally disappear from the patient's circulation was technically difficult, as the Ashby method was least sensitive when there were fewest surviving cells. Also, he suggested that the total survival time was not an accurate method of expressing

results, due to the blood volume changes that occurred after transfusion, the blood volume being only slowly stabilised. In a series of experiments, he presented evidence that these blood volume changes were complete in 48 hours in normal subjects and consequently based his results on the counts obtained at this particular time. The results were then plotted graphically, using a percentage survival time, instead of a total survival time. The count of surviving cells at 48 hours was taken as 100%.

In an effort to overcome any fallacies in the unagglutinated cell counts due to blood volume changes that can occur in the immediate post transfusion period, the subject to be transfused has been subjected to venesection before transfusion and the value of a sample count taken immediately after transfusion, used to represent 100% survival of the transfused cells, Callender, Powell and Witts (1945). Other workers, Young, Platzer and Rafferty (1947), and Dornhost (1951), have treated the matter of elimination of the transfused blood as a highly mathematical and statistical problem, using equations to find the probable life span of the erythrocytes at any particular time interval after transfusion, but without any inference to the kinetics of erythrocyte destruction.

Sheets, Janney, Hamilton and De Gowin (1951) dealt extensively with the subject of kinetics of red blood cell

destruction and suggested that the values for the unagglutinated cell counts should be plotted on Cartesian co-ordinates, on a chart one meter square, with the cell counts on the ordinates and the time in days after transfusion on the abscissae. These workers assumed that there were several possibilities that could be postulated in erythrocyte survival studies. Firstly, a normal ageing process of a normal erythrocyte which gave a linear slope of elimination when represented graphically. If the disappearance of normal cells from the circulation was complete in 120 days, then each day, $\frac{1}{120}$ths of the original number of transfused cells disappeared and the plotted curve was rectilinear. Secondly, there was a condition of accelerated senescence, such as occurs when blood from a person with severe Cooley's trait was transfused into a normal patient. In this case, when the unagglutinated cells were plotted as suggested, the slope of elimination was again a rectilinear curve which attained zero concentration in sixty days. Thirdly, there was a condition of slow random destruction by extrinsic or intrinsic factors, affecting the transfused cells at a constant rate, plus the added factor of normal loss from ageing, for which these workers produced an equation in the notation of differential calculus. This was expressed as

$$N = N_o\left(1 - \frac{t}{T}\right)e \quad - \quad \frac{\log_e 2}{r}\, t$$

on the abscissae, would be suitable. However, at the time of charting these results, it was found that a marked drop in the unagglutinated cell counts occurred during the first ninety hours after transfusion and thus Mollison's method of expression of results would lead to fallacious interpretation. The reason for this drop in the number of unagglutinated cells was thought to be one or a combination of several factors. The blood and plasma volumes during pregnancy and the puerperium show wide fluctuations, Tysoe and Lowenstein (1950), and as has been discussed before, subjects rendered plethoric by transfusion have an initial curved slope followed by a linear slope of elimination of transfused erythrocytes, Brown et al. (1944). Also a variable time is required for stabilization and readjustment of blood volume changes, Mollison (1947), and it was thought possible that this time required for equalisation might differ in the pregnant and non pregnant state. Lastly, the blood for these red cell survival studies was drawn into Baxter vacuum flasks and it was felt that, during the course of venesection, it was possible that a percentage of blood cells might have been damaged by subjecting them to a decrease in pressure from that of their normal environment, with the consequence that they were sequestered from the patient's circulation immediately post transfusion. This last hypothesis was tested using vacuum drawn blood for

transfusion and comparing it to blood drawn by gravity. The results of this experiment are shown after the case studies.

In view of these possible factors and in view of the fact that blood volume studies had not been done on these pregnant women, it was decided to plot the results of the present studies in two ways; the first, using the absolute number of unagglutinated or free cells as the ordinate of a graph and the second, using the ratio of unagglutinated cell counts to the total red cell count as the ordinate of a graph. The abscissa of the graph was the length of time, in hours and days, for which these patients were followed after transfusion. It was thought that this last named method of using a ratio would minimise the error introduced by blood volume changes due to the pregnant state and to the transfusion. Included in the representation of the results are the haematocrit, the haemoglobin and the red blood cell count values.

(2). RESULTS OF PRELIMINARY EXPERIMENTAL WORK.

a) Comparison of the unagglutinated cell counts, using two types of serum and two different methods for the collection of blood samples.

The results of an experiment to compare the unagglutinated cell counts obtained, using oxalated venous blood and capillary blood from a finger prick, are shown below. This experiment was performed using three volunteers of both group A and M, the antiserum in the first test being liquid and in the second test, dried antiserum.

Antiserum used	Liquid Anti-A		Dried anti-M	
Blood used	Capillary	Venous	Capillary	Venous
Volunteer H.S.	8265	8333	7963	7820
	8671	8261	7562	7461
Volunteer B.R.	7216	7358	7023	7866
	6997	6892	7658	7621
Volunteer B.S.	7821	8000	7986	7066
	8026	7991	7021	7523

Chart (i). Comparison of oxalated venous and capillary bloods, using anti-A and anti-M sera.

These results agree with Berlin's work (1951) on the same problem and in view of the fact that there was close agreement in the numbers of the unagglutinated cell counts, it was decided to use the blood collected by finger puncture for determinations of the unagglutinated cells when the transfusion experiments started.

b) Experimental evidence that liquid antiserum, stored in sterile ampoules at -20° Centigrade, will keep its potency and avidity for a prolonged period of time.

Two ampoules, one containing liquid anti-A serum and one containing anti-B serum, were stored in the deep freezing unit in the Women's Pavilion, Royal Victoria Hospital, at a constant temperature of -20°C. These ampoules of sera were random samples of the batches of sera produced in volunteers by using group specific substance (Witebsky) intravenously. Throughout the course of the experiments designed to follow the fate of transfused erythrocytes, these sera were tested to find out if they maintained their properties of avidity, potency and low, unagglutinated cell counts.

At the same time as these tests were performed on the liquid sera, unagglutinated cell counts were performed on the dried anti-M and anti-N sera. These sera were kept in small rubber capped bottles in a vacuum desiccator con-

taining calcium chloride. The results for both types of sera are shown below

Serum	Liquid anti-A			Liquid anti-B		
Tests	Avid. (secs)	Potency	Unaggl. cells	Avid.	Potency	Unaggl. cells
June 1952	3.5	1:8192	7654	2.75	1:10,230	8163
July 1952	3.25	1:8192	7863	2.75	1:10,230	8311
Aug. 1952	3.5	1:8192	7721	3.0	1:10,230	8378
Sept.1952	3.5	1:8192	7016	3.0	1:10,230	8260
Oct. 1952	3.5	1:8192	7866	3.0	1:10,230	8516
Nov. 1952	3.5	1:8192	7341	3.0	1:10,230	8181
Dec. 1952	3.5	1:8192	7912	3.0	1:10,230	8678

Chart (iia). Potency, avidity (in seconds), and unagglutinated cell counts of anti-A and anti-B sera after storage at -20°C. for prolonged periods.

Serum	Dried anti-M	Dried anti-N
Tests	Unaggl. cell counts	Unaggl. cell counts
July 1952	6218	7180
Aug. 1952	6066	7021
Sept.1952	6601	7632
Oct. 1952	6281	7810
Nov. 1952	6366	7218
Dec. 1952	6412	7068

Chart (iib). Unagglutinated cell counts of dried anti-M and anti-N after storage in a desiccator at room temperature for prolonged periods.

From these results, it would seem that liquid or dried antiserum will maintain its important properties for the length of time necessary to study erythrocyte survival by the Ashby technique, provided it is stored in a suitable environment.

c) Experimental evidence of the optimal dilution of serum withsaline at which maximal agglutination takes place.

As described previously, potent sera may exhibit a "zoning" phenomenon, and the experimental results, shown below, determined the dilution of the serum withsaline at which maximal agglutination, as shown by the lowest unagglutinated cell counts, took place. Liquid anti-A and liquid anti-B sera were used and the results illustrated graphically, the unagglutinated cell counts on the ordinate and the dilution of serum on the abscissa.

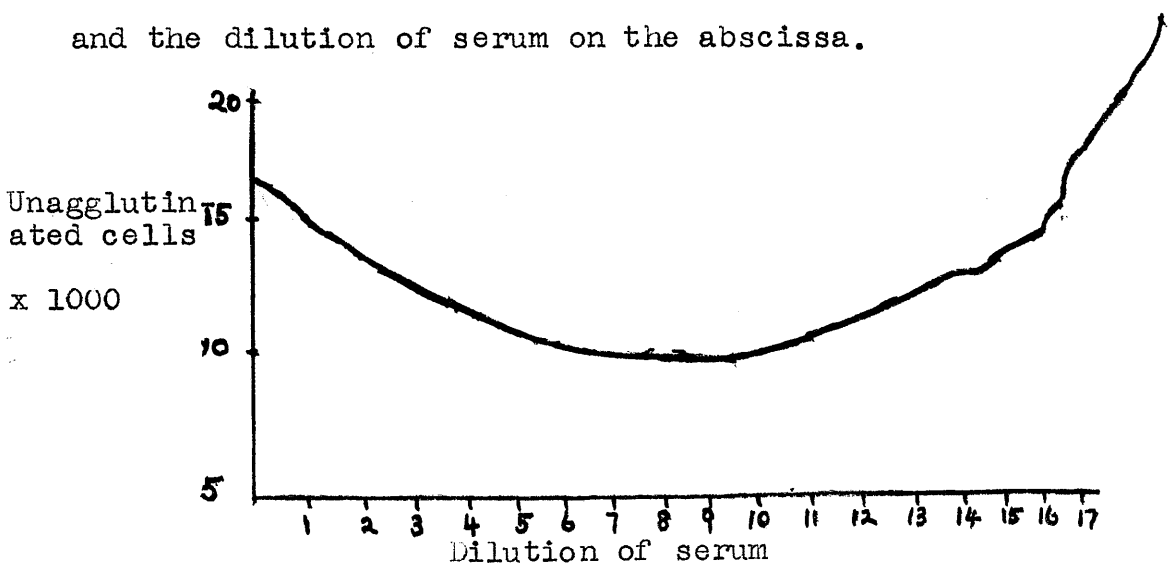


Chart (iii). Dilution of serum at which maximal agglutination was produced.

As can be seen from these results, the lowest free cell counts were obtained when the sera were diluted in the proportions of one of serum to eight of saline. These results agree reasonably well with Berlin (1951) and Mollison (1947), who found that maximal agglutination occurred in dilutions ranging from one in eight to one in sixteen.

d) Experimental work on the effect of time of centrifuging at 1500 r.p.m., on the unagglutinated cell counts of a mixture of blood, saline and specific antiserum.

A series of tests were conducted to find the period of time of centrifugation that would give maximal agglutination of a blood, saline and group specific antiserum mixture. The blood selected was from a group AM volunteer and concurrent tests were made with liquid anti-A and dried anti-M sera. The results are shown graphically on the next page, maximum agglutination being the lowest number of unagglutinated cells counted.

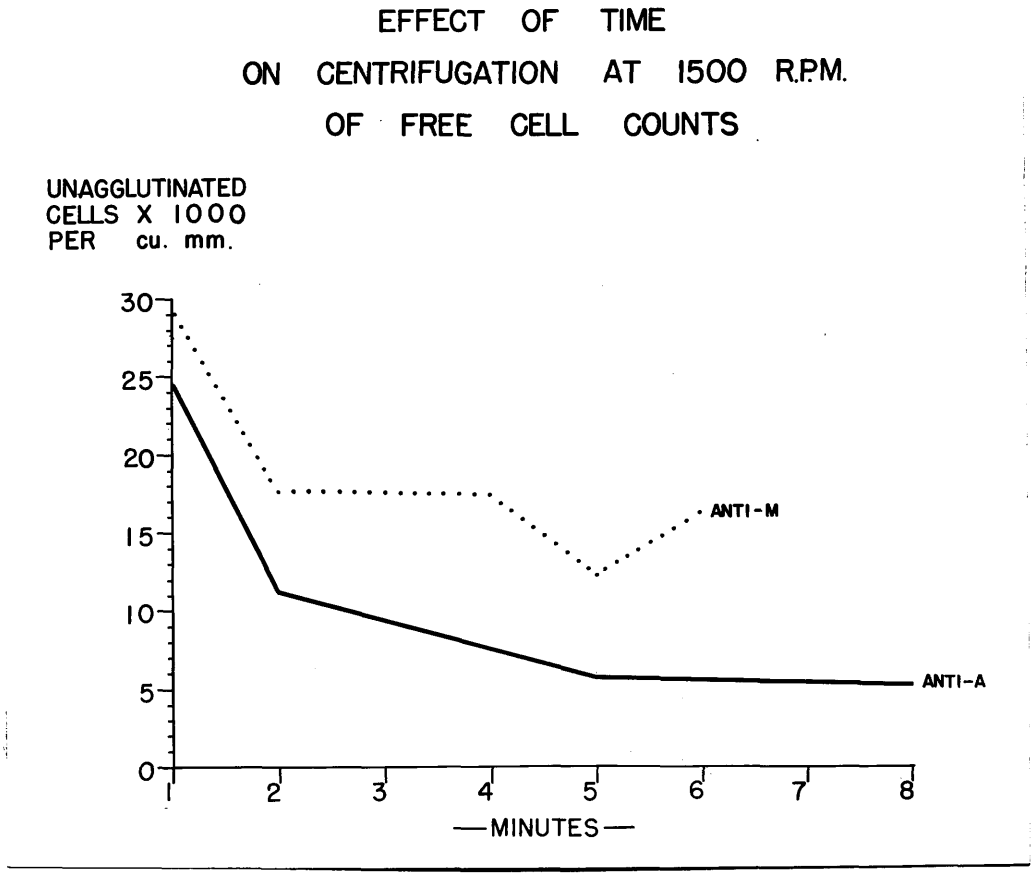


Chart (iv).

The period of centrifugation that gave the lowest unagglutinated cell counts with both anti-M and anti-A sera was four minutes at 1500 r.p.m. This time for centrifugation was incorporated in the agglutination procedure. Other authors, McKerns and Denstedt (1950), Dacie and Mollison (1943), and Berlin (1951), have found that periods

of centrifugation of three to four minutes gave optimum agglutination, although Dacie and Mollison felt that centrifuging anti-M and anti-N sera might lead to non-specific agglutination. This was not found in this series, perhaps due to the use of powdered serum.

e) Experimental work on the effect of time of agitation on the unagglutinated cell counts, the speed of agitation being kept constant at 240 vibrations per minute.

A series of experiments using blood of group AN and liquid anti-A and dried anti-N sera was performed to find the length of time that a blood and serum mixture should be shaken, in order to produce the optimal agglutination of the red blood cells by the specific serum. The specially built agitator, previously described, was used and the results of the unagglutinated cell counts plotted against the period of time that the erythrocyte/serum mixture was subjected to agitation, Chart (v).

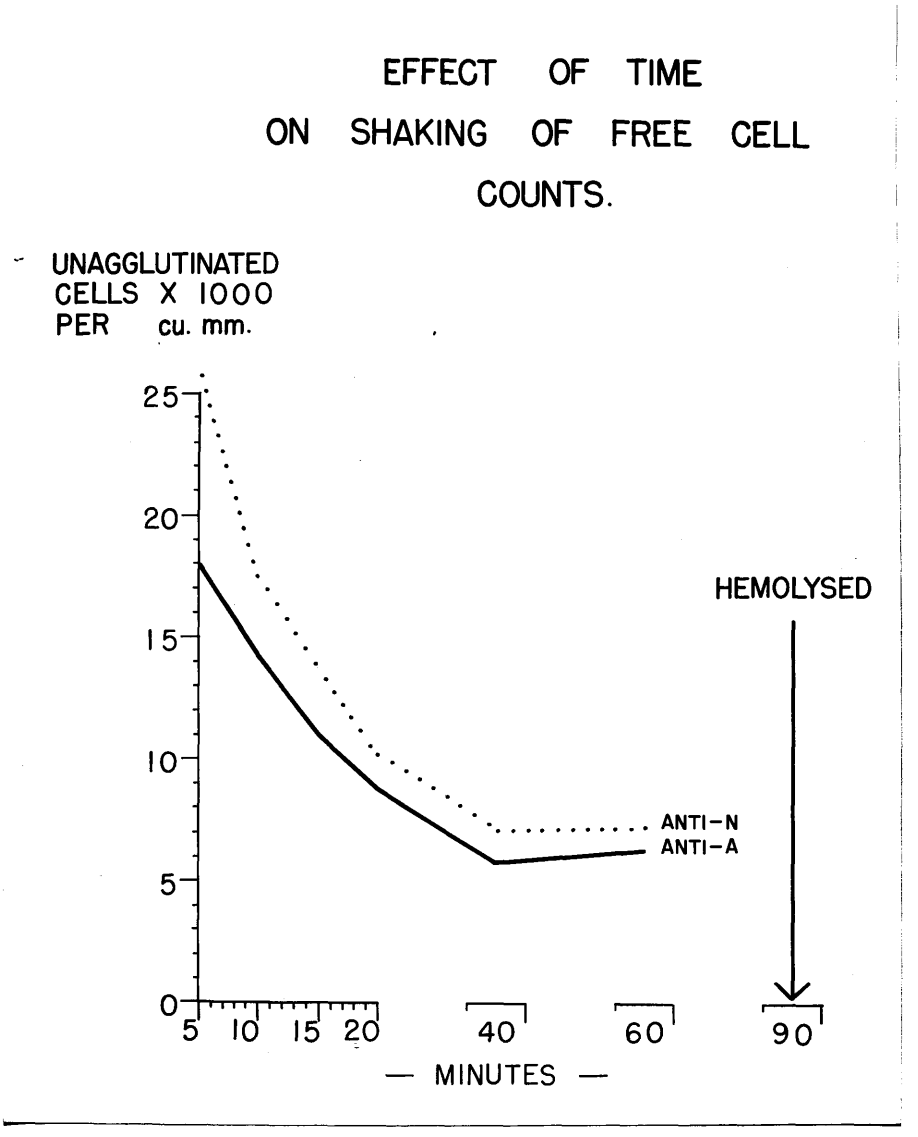


Chart (v).

The results of this series of experiments showed close agreement between the graph of the unagglutinated cell counts after the action of anti-A serum and the graph

of the cells counted after the action of anti-N serum. No results of this type of experiment could be found in other authors' works, although Osborne and Denstedt (1947) have subjected erythrocyte and serum mixtures to varying rates of agitation in an attempt to produce optimal agglutination.

From the results obtained in these experiments it was decided to agitate the blood cell and serum mixture for a total period of forty minutes at 240 vibrations per minute.

f) Experimental work on the effect of temperature changes on the completeness of agglutination.

Blood of group A was selected for study and using liquid serum anti-A, the agglutination procedure, described previously, was carried out at temperatures varying from 4°C. to 45°C. The length of time of centrifugation and the period of agitation of the blood and serum mixture were kept constant. The number of unagglutinated cells per cu.mm. was counted and plotted graphically against the temperature of the water bath at which the reaction occurred. The results are shown in Chart (vi).

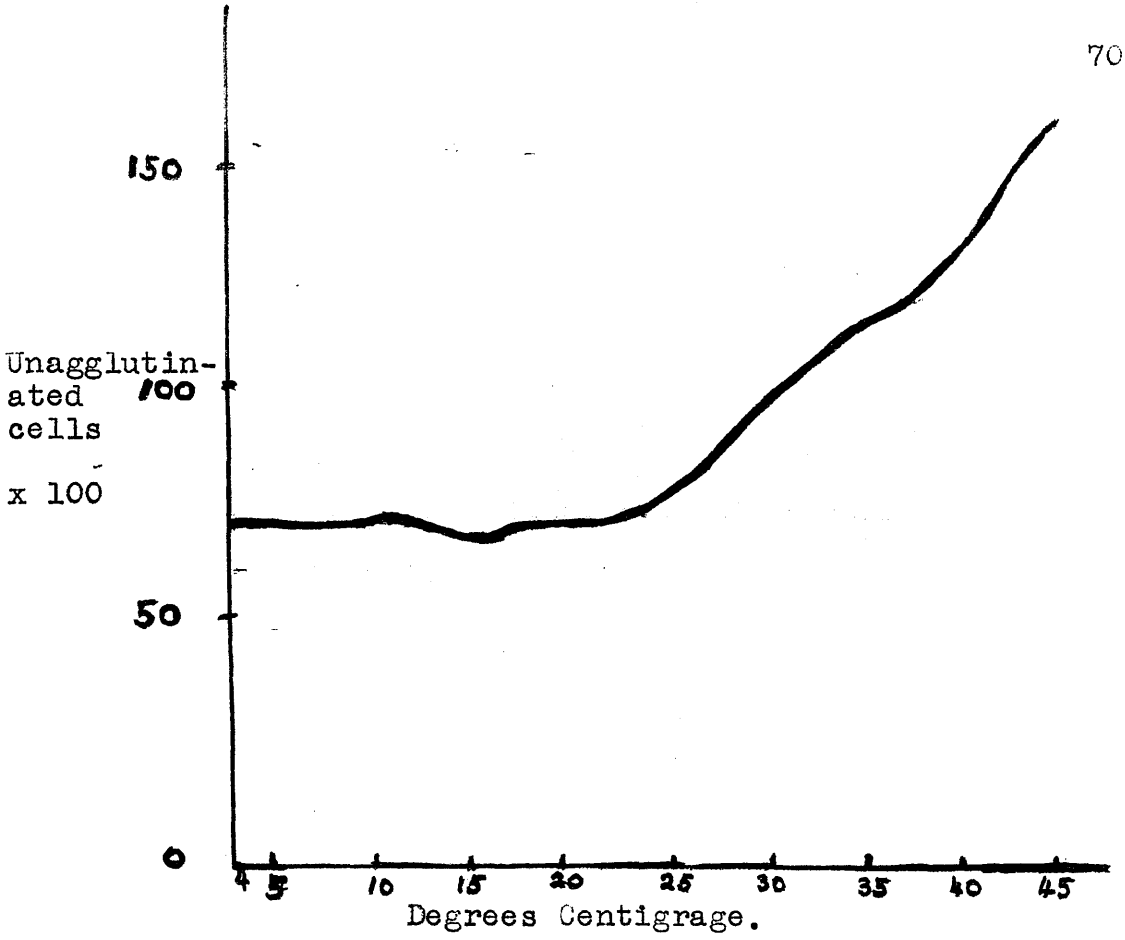


Chart (vi). Effect of temperature on agglutination.

In contradistinction to Berlin's work (1951) in which he noted a marked rise in unagglutinated cell counts between 16° and 24°C., no such rise was evident in this series. In fact, it was not until the temperature reached 25°C that any appreciable increase in the unagglutinated cell counts was seen. This seems to be borne out by Osborne and Denstedt (1947) and Mollison (1947), who allow the agglutination reaction to take place at room temperature, and by McKerns and Denstedt (1950), who used a water bath at a constant temperature of 20°C for the reaction.

Due to the difficulty of maintaining the low temperature (4°C) suggested by Berlin and due to the results of the experiments described above, the agglutination reaction in this series was carried out at 20°C.

(3). PATIENTS' CASE. STUDIES AND GRAPHICAL REPRESENTATIONS OF THE RESULTS IN THIS SERIES.

Case I. M. Anderson.

This 26 year old primigravida was first interviewed at the clinic in her seventh month of pregnancy. The patient's previous medical history was normal, except for the removal of her appendix in 1947. She had no previous history of anaemia, nor jaundice, nor blood transfusion. Physical examination of the various systems was completely normal. Her obstetrical history was normal and examination showed a foetus of twenty eight gestation in a vertex position in an apparently normal pelvis. Examination of her urine was negative for albumin and sugar, she had no oedema and her blood pressure was 130/80 mms. of mercury. Haematological findings were as follows:-

Haemoglobin: - 12.5 grams.

Red blood cell count:- 3.8 million per cu.mm.

Packed cell volume (haematocrit):- 39 volumes per cent

Blood group:- A Rh positive.

She was given 400 mls. of whole blood of group ORh positive on 9th June, 1952 and the transfused cells followed by the Ashby differential agglutination technique. Partial haemograms and Coombs tests were carried out at intervals.

On the 28th July, 1952, forty nine days after transfusion, she was found to be suffering from mild pre-eclamptic toxaemia of pregnancy with oedema of her hands and legs, albumin in her urine and a hypertension of $^{150}/_{100}$ mms. of mercury. She was treated, on an outpatient basis, with sedation by the barbiturates, ammonium chloride by mouth and a low salt, low fluid diet.

One week after this the patient's general condition had improved and her blood pressure was now down to within normal limits, being $^{120}/_{70}$ mms. of mercury, but she still had oedema of her extremities and albumin in a catheter specimen of urine. She continued to attend the clinic as an outpatient on the same treatment as before and two weeks after this examination she was found to be much worse. Her blood pressure had risen to $^{170}/_{90}$ mms. of mercury, she had clinical oedema of her legs as far as the knees and a routine specimen of urine showed marked albuminuria. She was confined to hospital on complete bed rest, sedation and a strict low salt, low fluid regime. Physical examination, apart from the hypertension and oedema, was normal. The

optic fundi were examined and appeared to be normal.

With hospital treatment, the patient's condition remained unchanged, but she went into labour on 23rd August, 1952: four days after admission to hospital, seventy four days after transfusion and delivered a baby assisted by a low forceps extraction and episiotomy. The estimated blood loss at delivery was 400 mls. In the first few days of the puerperium, the patient's condition improved, the oedema of her legs disappeared, the albumin in the urine was estimated at 1.0 gram by Esbach's test and although her blood pressure remained above normal, it fell from the previous reading of $^{170}/_{100}$ to $^{150}/_{100}$ mms. of mercury. During the next ten days, the patient apparently recovered, and the blood pressure fell to $^{120}/_{80}$ mms. of mercury, albumin disappeared from the urine and the oedema was no longer clinically demonstrable. Four and a half days after delivery, on 28th August, 1952, the haematologic findings in this case were:-

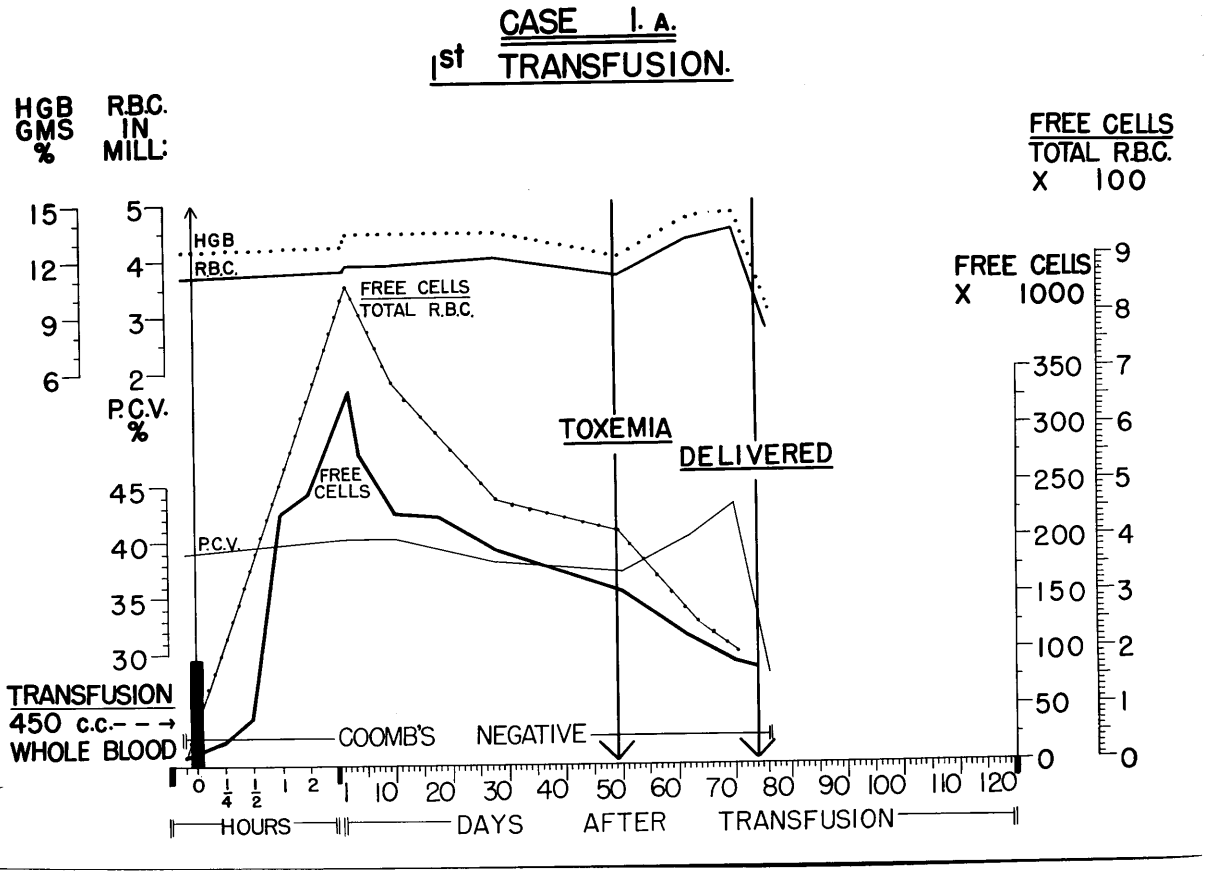
Haemoglobin:- 8.9 grams.

Red blood cell count:- 2.7 million per cu.mm.

Packed cell volume:- 28 volumes per cent.

The patient was given another whole blood transfusion of 400 mls. of group ORh positive blood on 28th August, 1952 and the cells of this transfusion followed for 85 days, thus giving an opportunity to study the fate of transfused cells, before delivery in a toxaemic patient and after delivery

in the same patient who had recovered from the toxaemic process. The findings are illustrated in Case IA and IB, IA being the graphical representation of the results before delivery and IB being the findings after delivery.



Case IA. M.A. Survival of transfused erythrocytes in a patient with pre-eclamptic toxaemia of pregnancy, studies performed before delivery.

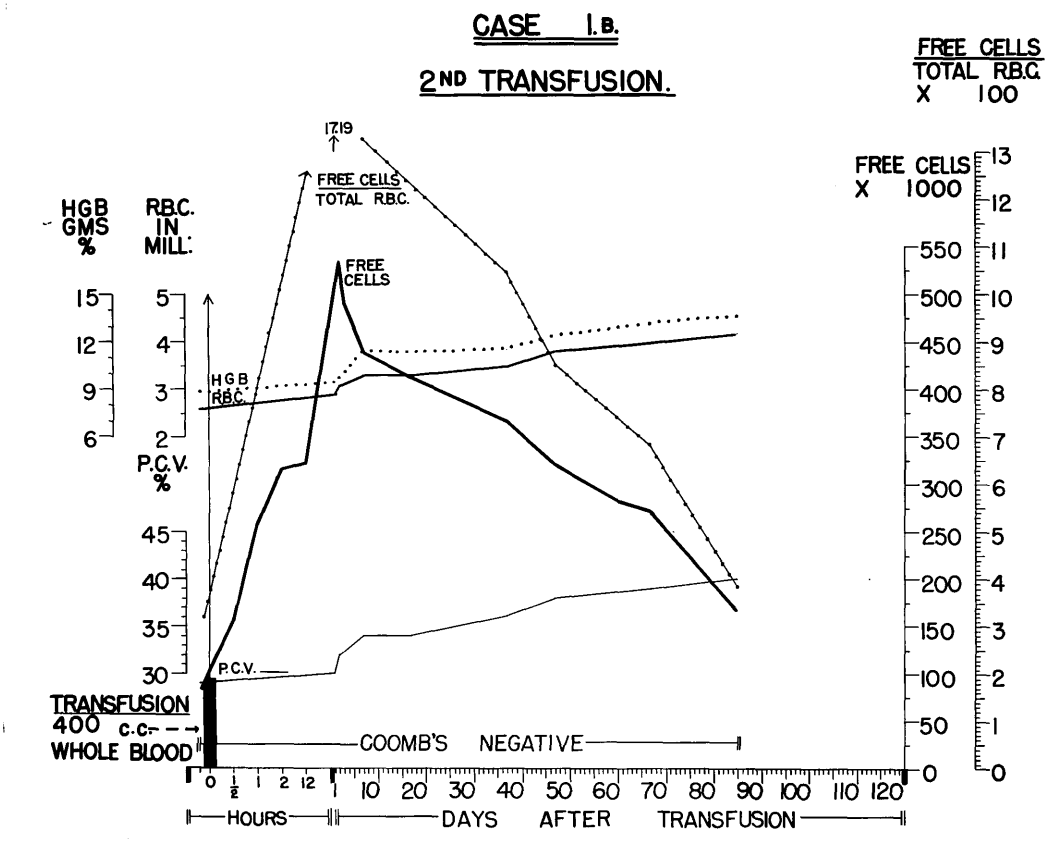
During the first four hours immediately after transfusion there was an understandable rise in the unagglutinated cell counts as the transfused cells were circulating in ever increasing numbers in the patient's circulation. This rise continued to be apparent for twenty four hours after transfusion and then, after reaching a peak at this time, the unagglutinated cell counts started to fall rapidly over the next five to six days. This fall was not only in the absolute number of surviving cells counted, but was reflected in the values obtained when plotting the ratio of free cells to total red blood cells. As this latter method had been used in an attempt to regulate fallacies in the cell counts due to blood volume changes, it was difficult to see how this sudden unexpected fall in the values for the ratio could be explained. If blood volume changes due to transfusion were to lead to misrepresentation of the results, then the curve of the values of free cells/total red blood cells would have been in an upward direction and not a fall as experienced in this case. This aspect of the case and the possible reasons for the phenomenon are discussed elsewhere ("Discussion").

After the immediate fall in both the absolute number of surviving erythrocytes and the values for the ratio free cells/total red cells, the results of this case conform to results found in normal patients. The graphs describe

a linear slope of elimination of erythrocytes and if these slopes were extrapolated to meet the base line, the length of time that the red cells survived would be approximately one hundred and twelve days.

From the chart it would seem that the haemoglobin, red blood cell count and haematocrit values had fallen rapidly from the seventieth to the seventy sixth day. This was not, in fact, true. This patient delivered a baby on the seventy fourth day after transfusion and the last recorded antepartum haematologic studies, other than unagglutinated cell counts, were performed on the seventieth day after transfusion and were quite normal (see Chart IA). Haematologic studies were done on the patient twelve hours after delivery and were found to have fallen to a marked degree. On the chart these values for the haemoglobin, red blood cell count and haematocrit, one antepartum and one postpartum, were joined up and thus gave a false curve.

The Coomb's test remained negative during the course of this survival study.



Case IB. M.A. Survival of transfused erythrocytes in a patient who apparently recovered from pre-eclamptic toxaemia. The transfusion was given four and a half days post-partum.

The pattern of the graphed results for both the absolute number of unagglutinated cells and for the ratio of unagglutinated cells/total red blood cells was the same in this case as in case IA. There was a considerable rise in the unagglutinated cell counts, coincidental with a rise in the value of the ratio for the first twelve hours,

the graph reaching a peak. After this time, the fall of both these values, noted in the previous chart, was again evident and occurred for the first six days after transfusion. After this initial fall, the slope of elimination levelled off and by extrapolating the curves to zero values the survival of the transfused erythrocytes would have been approximately one hundred and twenty two days. This was within the normal range for erythrocyte survival.

The haemoglobin, red blood cell count and haematocrit values were low in this case in the immediate few days post-partum, but rose steeply after transfusion. After this peak, all three haematologic studies showed a gradual upward rise towards normal thereafter. The Coomb's test remained negative throughout this second investigation, evidence that there was no production of an antibody in response to the stimulus of the first blood transfusion.

Day	Free Cell Count	F.C.C./R.B.C. x100	P.C.V. %	Hgb. in grams	R.B.C. in millions
Pre-transfusion	9,684	.0261	39		3.7
¼ hour	21,520	No R.B.C.			
½ "	43,038	counts			
1 "	225,950	available			
2 hours	241,830				
2 days	333,820	8.564	40	13.3	3.9
4 "	276,260				
10 "	224,350	5.743	40	13.3	3.9
18 "	221,120				
28 "	191,250	4.775	38	13.3	4.0
50 "	153,060	4.135	37	12.0	3.7
62 "	114,330	2.651	40	14.0	4.3
70 "	90,921	2.000	43	14.3	4.5
74 "	85,816				
76 (2 days post-partum)			28	9.2	2.7

Chart IA. Haematologic studies performed in case IA including one result performed two days after delivery. The unagglutinated (free) cell counts were produced after the action of the anti-A serum.

Day	Free Cell Count	F.C.C. x100 / R.B.C.	P.C.V. %	Hgb. in grams.	R.B.C. in millions
Pre-transfusion	84,197	3.237	29	8.9	2.6
¼ hour	108,611	No R.B.C. counts available			
½ "	159,248				
1 "	258,509				
2 hours	316,344				
1 day	322,262	11.600	30	9.5	2.9
2 days	533,427	17.900	32	9.8	3.1
3 "	492,539				
7 "	439,008	13.300	34	11.4	3.3
17 "	411,839	10.450	34	11.4	3.3
37 "	365,571	8.500	36	11.7	3.5
47 "	321,993	8.04	38	12.5	3.8
60 "	282,181				
67 "	272,228	6.800	39	13.3	4.0
85 "	163,228	3.880	40	13.6	4.2

Chart IB. Haematologic studies performed on Case IB (post-partum).

Case II. - L. Proszanski.

This 25 year old patient was first seen at the clinic in the seventh month of her first pregnancy. No abnormality was detected on general physical, obstetrical, or haematologic history and examination. The blood pressure was $^{120}/_{80}$ mms. of mercury and the uterus was enlarged to the size of a twenty eight week pregnancy in a vertex position in a normal pelvis.

The blood findings at this time were as follows:-

Haemoglobin:-	13.6 grams.
Red blood cell count:-	4.3 million per cu.mm.
Haematocrit:-	40 volumes per cent.
Blood group:-	A Rh positive.

The patient was given a transfusion of 450 mls. of whole blood of group ORh positive on 9th July, 1952 and followed by the Ashby technique, using liquid anti-A serum for a period of eighty four days. Partial haemograms and Coombs tests were carried out at intervals. On the 9th September, 1952 she complained of slight swelling of her ankles, but general physical examination demonstrated no apparent abnormality, her blood pressure being $^{130}/_{80}$ mms. of mercury and the urine was clear of albumin and sugar. On the 16th September she was seen again and at this time she had pitting oedema of her feet and ankles, the blood pressure was elevated to $^{145}/_{95}$ mms. of mercury and she was

excreting albumin in her urine. This was sixty nine days after transfusion of whole blood. The patient was admitted to hospital and was treated by bed rest, the barbiturates and a low fluid, low salt diet.

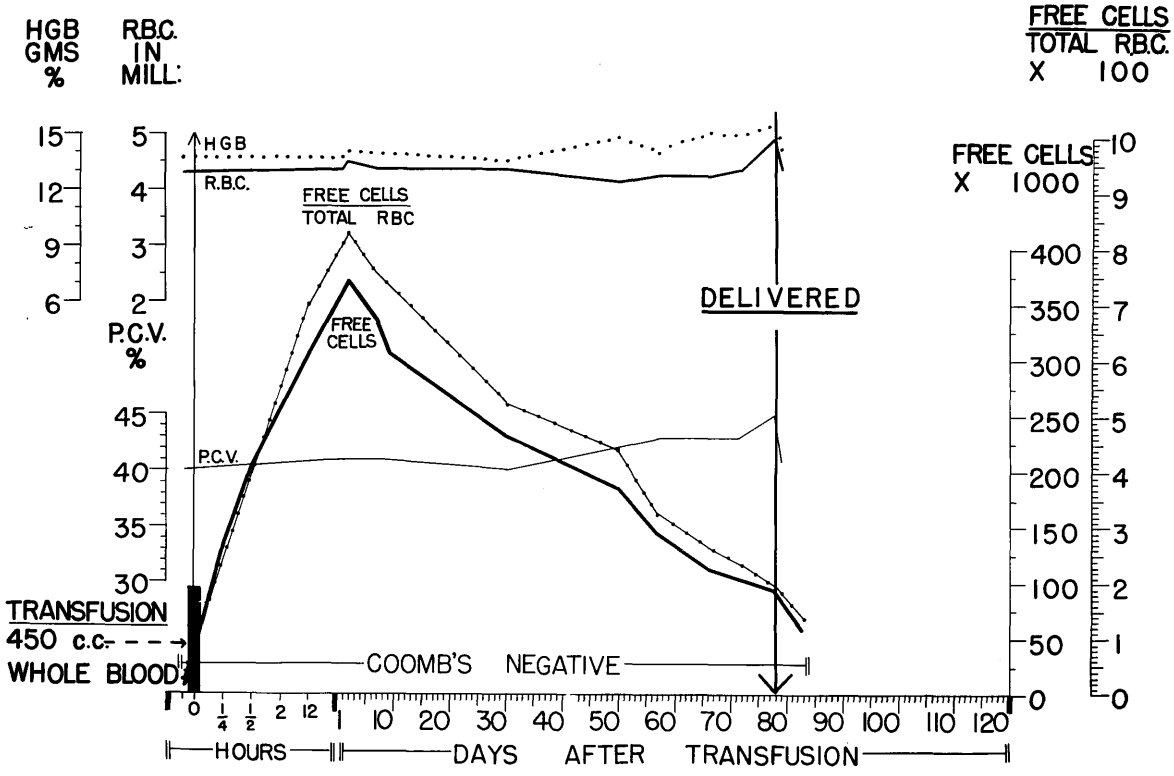
The day of admission to hospital her blood pressure rose to $^{150}/_{100}$ mms. of mercury and there were 6 grams of albumin per twenty four hours in her urine, as demonstrated by Esbach's test. Physical examination of the patient was essentially negative except for the presence of hypertension and oedema. The optic fundi were within normal limits. Liver flocculation tests were negative, and investigation of her blood chemistry showed a decreased total blood protein and albumin with a raised globulin fraction. Routine haematologic findings were within normal limits. The results of the blood chemistries and haematology studies were as follows:-

Haemoglobin:-	15.1 grams.
Red blood cell count:-	4.4 million per cu.mm.
Packed cell volume:-	43 volumes per cent.
Non-protein nitrogen:-	19 mgs. per cent.
Total protein:-	6.46 mgs. per cent.
Albumin:-	3.52 mgs. per cent.
Globulin:-	2.94 mgs. per cent.

With treatment by bed rest, sedation and diet, the patient improved, the oedema apparently disappeared and

her blood pressure fell to 120/80 mms. of mercury, but there were still traces of albumin in her urine. Nine days after admission to hospital and seventy eight days after transfusion, the patient started labour and was delivered of a baby spontaneously, without surgical interference and an estimated blood loss of 300 mls. After delivery, the patient's condition improved rapidly and she was discharged fit and well on the sixth post-partum day. She was seen at the follow-up clinic six weeks later and was normal in every respect.

An attempt was made to transfuse this patient again, after delivery, but the offer was declined.



Case II. L.P. Survival of transfused erythrocytes in a patient with pre-eclamptic toxaemia of pregnancy.

The curve of the graphed results for the survival of transfused cells in this patient was essentially normal in so far as the slope of elimination, if carried down to the base line, would intersect the line at approximately one hundred and five days, which was within the normal range for the absolute survival of erythrocytes. However, the five day period immediately after transfusion showed

the same type of initial curve as did cases IA and IB, with both the absolute count of erythrocytes and the ratio free cells/red blood cells reaching a peak within the twenty four hours after transfusion and then falling rapidly. This rapid fall was complete within six days after transfusion and then the graph of the results described a rectilinear curve (see "Discussion"). The toxaemic process did not seem to have any effect on the survival of the transfused cells as shown by the graph of the results for the unagglutinated cell counts during this time.

The haemoglobin, red blood cell counts and haematocrit values remained within normal limits during the study of this case and the Coomb's test did not demonstrate any evidence of immunisation.

Day	Free Cell Count	$\frac{F.C.C.}{R.B.C.} \times 100$	P.C.V. %	Hgb. in grams.	R.B.C. in millions
Pre-transfusion	9,515	0.221	40	13.6	4.3
¼ hour	136,383	No R.B.C.			
½ "	207,121	counts			
1 "	257,970	available			
12 hours	305,860	6.954	41	13.6	4.4
2 days	370,680	8.244	42	14.0	4.5
7 "	335,740	7.567	40	13.6	4.4
9 "	307,670				
30 "	232,410	5.272	40	13.6	4.4
50 "	185,340	4.404	42	14.8	4.2
57 "	145,250	3.295	43	14.8	4.3
66 "	114,330	2.651	43	15.1	4.3
72 "	104,100	2.363	43	15.1	4.4
78 "	94,686	1.915	45	15.6	4.96
79 " post-partum			41	14.3	4.4
83 "	59,987	1.370	43	14.3	4.38

Chart II. Haematologic studies performed on Case II. The unagglutinated cell counts were produced after the action of anti-A serum.

Case III. A. Menastryski.

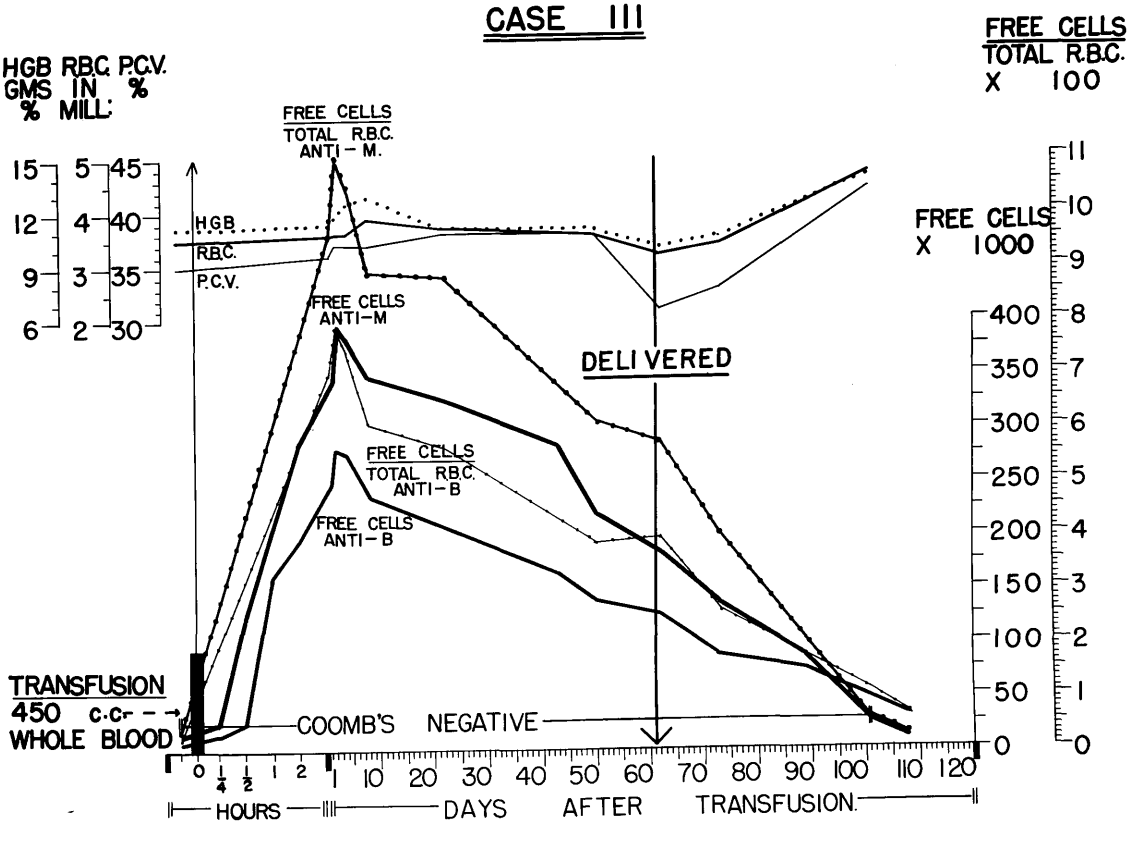
This 21 year old patient was pregnant for the second time and when first examined at the Outdoor Clinic, she was at the beginning of her seventh month of pregnancy. General physical and haematologic history and examination of this patient elicited no abnormality. Her obstetrical history was normal for the first pregnancy, but she had been hospitalised in England at the beginning of the present pregnancy for hyperemesis gravidarum. After six days' hospitalisation, she had been discharged fit and well and had remained so since then. Obstetrical examination revealed a uterus enlarged to twenty eight week period of gestation. The pelvis appeared adequate and of normal shape. Haematologic examination showed:-

Haemoglobin:-	11.2 grams.
Red blood cell count:-	3.5 million per cu.mm.
Packed cell volume:-	35 volumes per cent.
Blood group:-	BM Rh positive.

The patient was given a transfusion of 450 mls. of whole blood of group ON Rh positive on June 10th, 1952 and followed for erythrocyte survival for fifty eight days ante-partum and for forty two days post-partum. The survival of the transfused erythrocytes was followed concurrently, using both liquid anti-B serum and dried anti-M serum. During the course of the pregnancy the patient remained

perfectly well and on August 7th, 1952 she delivered a normal full term infant with a second degree perineal tear. The blood loss was estimated at 400 mls. The puerperium was normal and when seen at post-natal clinic six weeks after delivery, she was fit and well.

Haemoglobin, red blood cell counts and haematocrit studies done at intervals thoughout the pregnancy and the puerperium were within the normal range (Chart III). The Coomb's test remained negative during the course of the erythrocyte survival studies.



Case III. Survival of transfused erythrocytes before and after delivery, in an apparently normal pregnant woman.

The values for the absolute count of surviving erythrocytes and the ratio, unagglutinated cells to total red blood cells, have been plotted simultaneously for both the liquid anti-B serum and the dried anti-M serum. With both these values, there was the same type of graph produced in the immediate six day period after transfusion, as in the previous cases already described, namely, a peak of the curve and then a sudden, quite profound fall of the graph. After this fall, the values obtained for the unagglutinated cell counts using liquid anti-B produced a relatively straight line, with its terminal point, projected to the abscissa, at approximately one hundred and twenty five days. This was considered normal for the erythrocyte survival.

There were two factors noted about the curves obtained when using dried anti-M serum. Firstly, the discrepancy between the values obtained with this serum and those obtained when liquid anti-B serum was used. Using the absolute counts of unagglutinated cells, this discrepancy was found to be as high as 110,000 cells per cu.mm. in some counts (see Chart III), and in general the difference between the counts using these different sera remained fairly constant throughout the course of the investigation. If this were due to technical error in the procedure, one would have expected occasional discrepancies between the

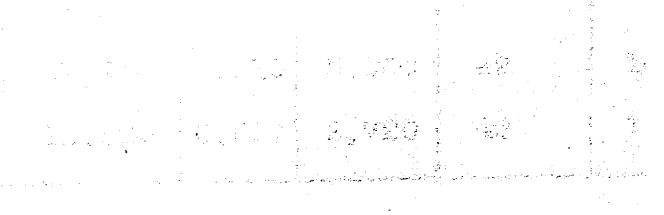
counts obtained with two sera to have been of this magnitude, but some other factor must have caused the difference in counts to have been so constant. A similar pattern was noted in another case to be described (Case IV). A feasible explanation of the difference in counts was that the liquid anti-B serum was of greater potency in respect to the low number of cells left unagglutinated after the agglutination procedure, than the dried anti-M serum.

The second factor noted about the curves obtained with dried anti-M, is that the general slope of elimination of the transfused cells was not a strictly linear curve as found in the counts produced by anti-B serum. This was not due to blood cell volume or plasma volume changes, as the shape of the curve was identical to that found when the ratio of unagglutinated cells counted/total red blood cells was plotted. If blood volume changes were to cause these fluctuations, they would have been reflected in a different type of curve for the ratio values. Osborne and Denstedt (1947) found similar fluctuations in unagglutinated cell counts and were not able to explain the phenomenon. These deviations do not necessarily vitiate the results in the present case, as they occurred only with the use of anti-M serum and not with anti-B serum.

The values obtained for the haemoglobin, red blood cell counts and packed cell volume show some deviations

immediately after transfusion and this was not considered abnormal in view of the fact that whole blood was transfused and the patients were not subjected to venesection before transfusion. Also Tysoe and Lowenstein (1950) have shown that there is a wide fluctuation in blood and plasma volumes during pregnancy. It would appear from the graph that the haematocrit value had fallen abruptly in the period immediately before delivery, but, as in Case IA, this was erroneous. The last ante-partum haematocrit reading was performed seven days before delivery and the next value obtained was that taken on the first day post-partum, which was much lower. Consequently, on joining up these results on the graph, it would appear that there had been a fall in the haematocrit reading.

The Coomb's test remained negative throughout the course of the investigation.



Days	Free cell count		F.C.C./R.B.C. x100		P.C.V. %	Hgb. in grams.	R.B.C. in millions
	B	M	B	M			
Pre-transfusion	9,415	14,795	0.2690	0.423	35	11.2	3.5
¼ hour	14,510	25,623	No R.B.C.				
½ "	26,001	125,231	counts				
1 hour	165,722	210,100	available				
2 hours	198,263	286,356					
24 "	251,616	346,110	7.000	9.600	36	11.4	3.6
2 days	282,240	396,250	7.840	11.000	37	11.7	3.6
4 "	276,530	384,610	7.450	10.400	37	12.5	3.7
8 "	238,050	348,289	6.100	8.930	37	12.9	3.9
22 "	210,310	326,297	5.670	8.800	38	11.1	3.7
43 "	165,435	285,162					
50 "	140,149	220,570	3.890	6.140	38	11.1	3.6
62 "	126,430	185,070	3.940	5.770	31	10.1	3.2
73 "	89,038	136,650	2.620	4.030	33	10.0	3.4
89 "	75,051	87,151					
101 "	47,343	27,936	1.010	5.930	42	14.0	4.7
108 "	30,935	12,820	0.657	2.720	42	14.0	4.7

Chart III. Haematologic studies in a normal pregnant (Case III) woman. Both ante-partum and post-partum values are included.

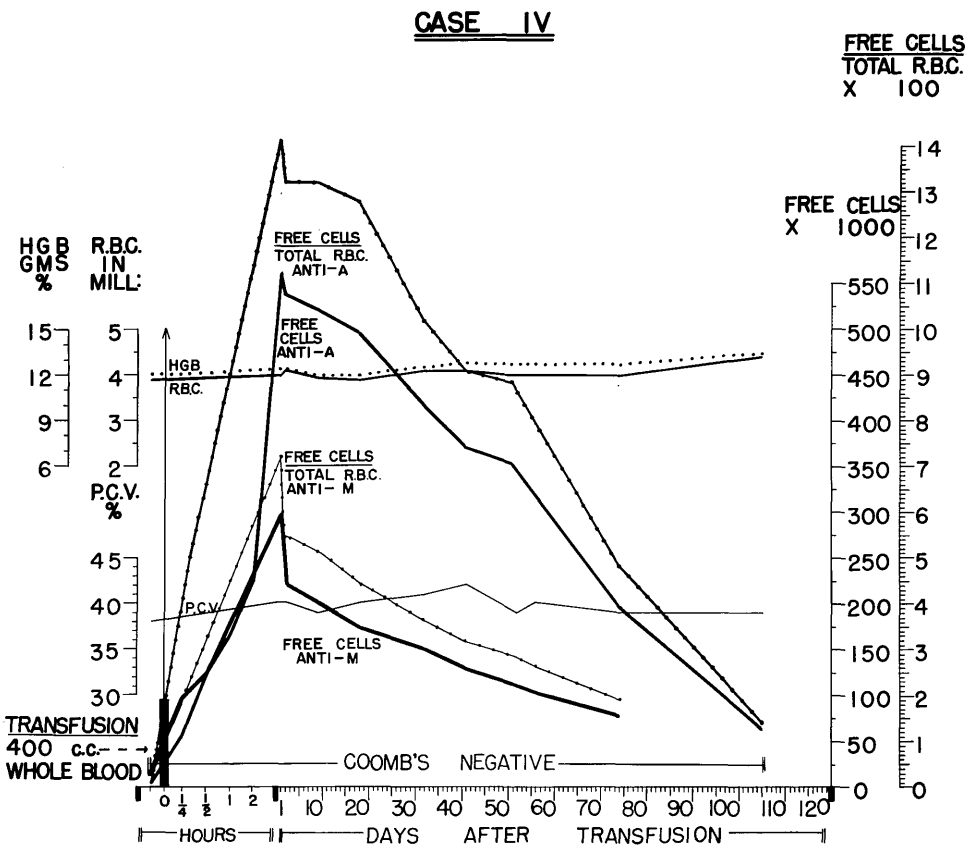
Case IV. M. Wrobel.

This 26 year old primigravida was examined on her first visit to the Antenatal Clinic. She was then thirteen weeks pregnant. The general medical history elicited a history of gastric upset for four years, comprising of an aching type of pain in the epigastrium, bearing no relation to meals, and a slight waterbrash prior to eating. There was no history of vomiting or malaena. Physical examination and obstetrical history and examination revealed no abnormality. The uterus was enlarged to approximately ten to twelve weeks' gestation and a routine specimen of urine was reported clear of albumin and sugar. Haematologic history was negative and the findings at the time of examination were as follows:-

Haemoglobin:-	12.0 grams.
Red blood cell count:-	3.9 million per cu.mm.
Packed cell volume:-	38 volumes per cent.
Blood group:-	AM Rh positive.

She was given a transfusion of 400 mls. of whole blood of group ON Rh positive on August 14th, 1952 and the survival of the transfused cells followed by the Ashby technique using liquid anti-A serum and dried anti-M serum, for one hundred and five days. By the time the survival experiments had finished, the patient had not delivered, but she remained perfectly well throughout the period of pregnancy

that the transfused cells were studied. Partial haemograms and Coomb's tests were performed at intervals.



Case IV. Survival of transfused cells in a normal pregnant woman who did not deliver before the end of the investigation. Survival followed using liquid anti-A and dried anti-M sera.

Unlike the graph of the results in the previous cases which showed a marked fall in the curve of the values of the unagglutinated cells counted over the six day period

immediately after transfusion, the results in this case showed a marked fall of these values for two days only. During the transfusion there was a rapid increase of unagglutinated cells as these entered the patient's circulation and a peak was reached on the graph at approximately twelve hours: thereafter the values decreased rapidly till the first day and then levelled off, to a more normal type of curve of elimination. This fall in the values of the surviving cells was accentuated when the ratio unagglutinated cells to total red cells was plotted and on this basis it was thought that in this particular case, the deviation in the curve might be due partially to a plethora. As has been said previously, it was unlikely to be due to a haemodilution, in which case an upward curve would have been experienced. The Ashby technique is not sufficiently accurate to justify deductions based on such small variations and it is possible that the fall in the curve noted in this case was due to technical error. The falls in the curves of previous cases were larger and lasted for a longer period of time and were more liable to have been due to causes other than technical error (see "Discussion").

In this graph, as with that of Case III, there was a marked difference noted between the counts obtained when using liquid antiserum and dried antiserum. In Case III, the counts obtained with anti-B were lower than those ob-

tained with anti-M sera. In this investigation, the counts made using anti-M serum were consistently lower than those made using liquid anti-A serum. As in Case III, this anomaly was noticed during the complete investigation and was not just one or two isolated instances. It would therefore seem to be due to a difference in potency between these sera and to technical error. The unagglutinated cell count values during the transfusion were sometimes 200,000 cells per cu.mm. lower with anti-M serum than with anti-A serum. It would seem then that anti-M serum is more potent, in terms of unagglutinated cell counts, than anti-A serum, but less potent than anti-B serum. This discrepancy was unexplained.

The general shape of the curves was rectilinear and when extrapolated to meet the base line, did so at approximately one hundred and twenty-five days, which was considered normal for the length of survival of the transfused cells.

Haemoglobin, red blood cell count and haematocrit values did not differ from the mean average for these values in pregnangy, Tysoe and Lowenstein (1950) (Chart IV).

Days	Free cell counts		F.C.C./R.B.C. x100		P.C.V. %	Hgb. in grams.	R.B.C. in millions.
	A	M	A	M			
Pre-transfusion	4,766	12,376	0.123	0.318	38	12.0	3.9
¼ hour	55,145	96,839					
½ "	126,210	126,900	No R.B.C.				
1 "	165,350	156,863	counts				
2 hours	224,070	216,836	available				
1 day	565,120	298,270	14.100	7.200	40	12.5	4.0
2 days	540,670	222,990	13.200	5.430	40	12.5	4.1
9 "	522,670	201,750	13.200	5.130	39	12.0	3.95
18 "	497,650	174,630	12.800	4.460	40	12.0	3.9
32 "	416,681	150,630	10.200	3.680	41	12.5	4.1
41 "	370,682	129,390	9.100	3.140	42	12.8	4.1
51 "	354,080	113,510	8.850	2.850	39	12.5	4.0
56 "	318,302	104,100	3.950	2.600	40	12.8	4.0
74 "	196,640	76,033	4.900	1.900	39	12.0	4.0
105 "	62,946	30,068	1.400	0.680	39	13.3	4.4

Chart IV. Haematologic studies performed on Case IV. Ante-partum only.

Case V. A. Rastynini.

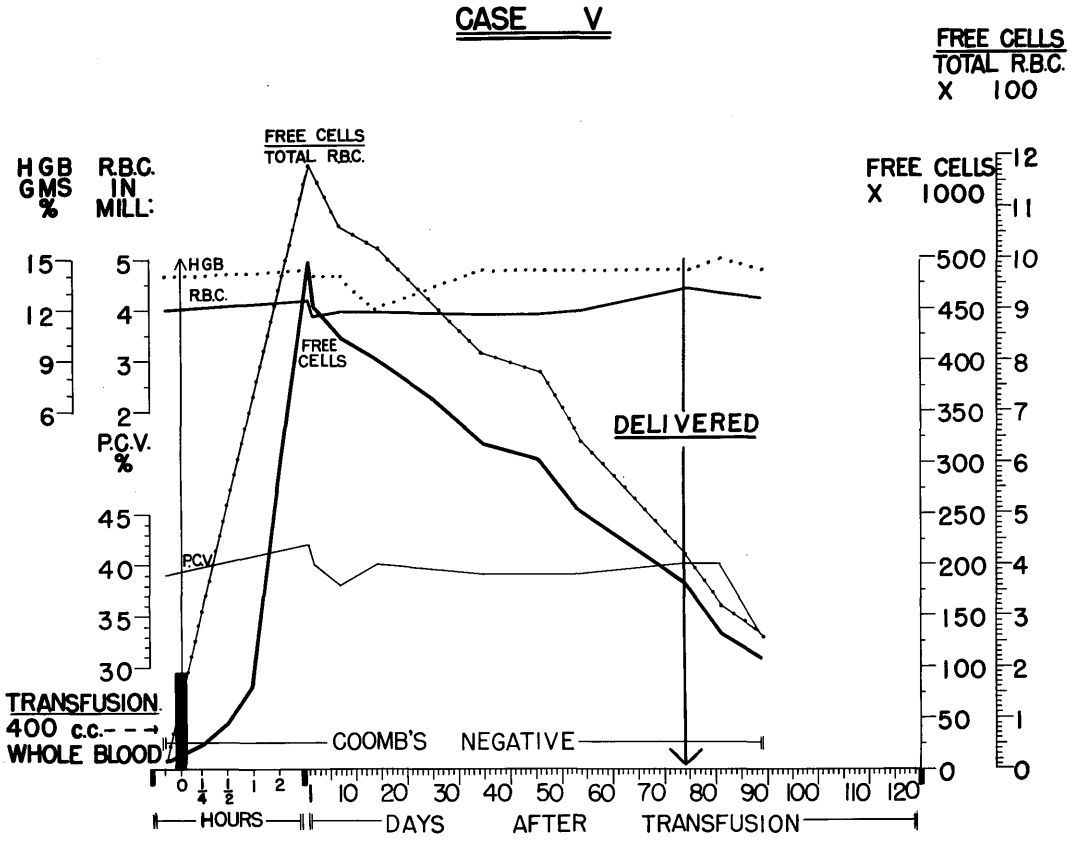
This thirty two year old multigravida was in the sixth month of her pregnancy when first examined. General physical, obstetrical and haematologic history and examination were considered to be normal. Examination of the abdomen revealed a twenty four week old foetus in a vertex presentation in a normal pelvis. Haematologic findings were:-

Haemoglobin:-	14.0 grams.
Red blood cell count:-	4.0 million per cu.mm.
Haematocrit:-	39 volumes per cent
Blood group:-	A Rh positive.

The patient was given a transfusion of 400 mls. of whole blood of group O Rh positive on September 20th, 1952 and the blood cells were followed by the differential agglutination technique for eighty nine days. She remained well throughout the pregnancy and on December 8th, 1952 the patient began labour, seventy nine days after transfusion. The labour was normal and she delivered a baby spontaneously without surgical interference and with an estimated blood loss of 300 mls. She remained fit and well in the puerperium and was physically normal at the post-natal clinic six weeks after delivery. The survival of the transfused cells was followed for ten days post-partum.

Partial haemograms and the Coomb's test were performed

throughout the period of investigation.



Case V. A.R. Survival of transfused cells in a normal pregnant woman, performed ante-partum and post-partum.

The curve of the elimination of the transfused cells, although showing some deviations at the beginning and the middle of the curve, was within normal limits for the absolute value of the end point of disappearance of the cells. In this case, this was at approximately 125 days. The

sudden disappearance of the transfused cells in the six day period immediately after transfusion was again noted and was again reflected in the ratio values for unagglutinated cells/total red blood cells. This was a constant feature in the cases reviewed previously.

The values for the haemoglobin, red blood cell count and haematocrit showed a fall in the immediate post-transfusion period and this was felt to be due to the haemodilution and blood volume changes that occur after transfusion of whole blood. Apart from these deviations, the values remained within the range of normal for pregnancy. The Coomb's test remained negative during the complete investigation.

Chart V. Haematologic studies in a normal pregnant woman. The la

Day	Free Cell Count	F.C.C. x100 / R.B.C.	P.C.V. %	Hgb. in grams.	R.B.C. in millions
Pre-transfusion	7,801	0.195	39	14.0	4.0
¼ hour	18,068				
½ "	120,867				
1 "	246,152				
2 hours	386,811				
24 "	498,457	11.850	42	14.3	4.2
2 days	454,341	11.620	40	14.0	3.9
7 "	424,131	10.650	38	14.0	3.98
14 "	402,108	10.230	40	12.0	3.92
25 "	364,226				
35 "	318,227	8.15	39	14.3	3.9
46 "	304,198	7.79	39	14.3	3.9
53 "	255,725	6.43	39	14.3	3.98
74 "	184,678	4.450	40	14.3	4.4
81 "	138,266	3.150	40	14.8	4.3
89 "	109,828	2.56	33	14.3	4.2

Chart V. Haematologic studies in Case V, a normal pregnant woman. The last two studies were post-partum.

Case VI. O. Daigle.

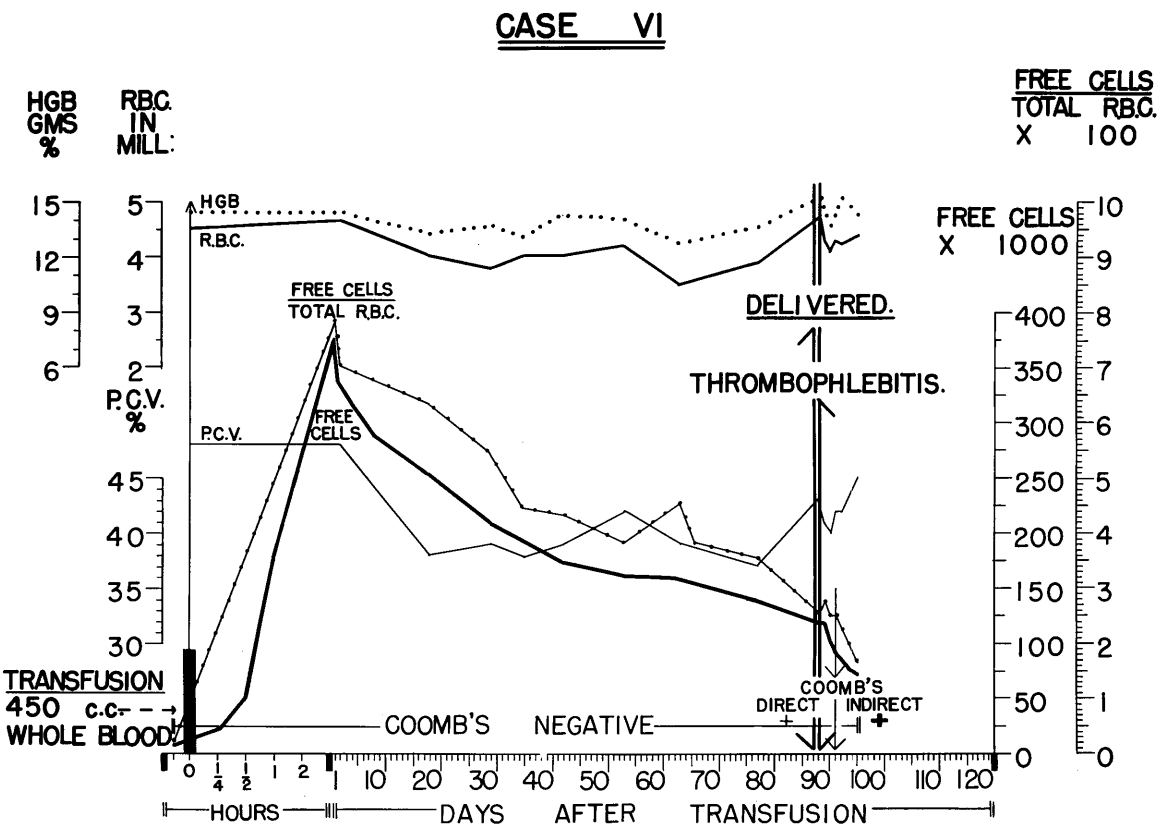
This patient was twenty six years old, a multigravida and six and a half months pregnant when she was first examined. General physical, obstetrical and haematologic history and examination was negative for any abnormality. The uterus was enlarged to approximately twenty four week period of gestation, the foetus was presenting by the vertex and the pelvis was normal. Haematologic examination showed:-

Haemoglobin:-	13.6 grams.
Red blood cell count:-	4.8 million per cent.
Packed cell volume:-	48 volumes per cent.
Blood group:-	A Rh positive.

The patient was given a transfusion of 450 mls. of whole blood of group O Rh positive on August 28th, 1952 and followed for eighty seven days ante-partum and seven days post-partum, by the differential agglutination technique.

The course of pregnancy progressed normally and the patient delivered a baby spontaneously without interference on November 24th, 1952, with an estimated blood loss of 200 mls. The day after delivery, she complained of pain and swelling of her left leg below the knee and on examination she was found to have a thrombophlebitis. This condition was treated by bed rest, elevation of the affected leg, moist heat and 600 mgs. of dicoumarol over a seven day period. During the next seven days the patient's condition

improved rapidly under this regime and she was allowed home on the eighth post-partum day. She refused to attend the laboratory as an out-patient for further studies and did not return to the post-natal clinic.



Case VI. O.D. Survival studies of transfused cell in a pregnant woman who developed a thrombophlebitis after delivery and a positive Coomb's test.

The type of initial curve seen in previous cases was repeated in this graph, with a sudden, relatively profound drop in the counts of the unagglutinated cells and in the values for the unagglutinated cell counts/total red cell

ratio. As before, this drop was in the six day period after transfusion and was probably due to the same causes as affected the counts in the other cases (see "Discussion").

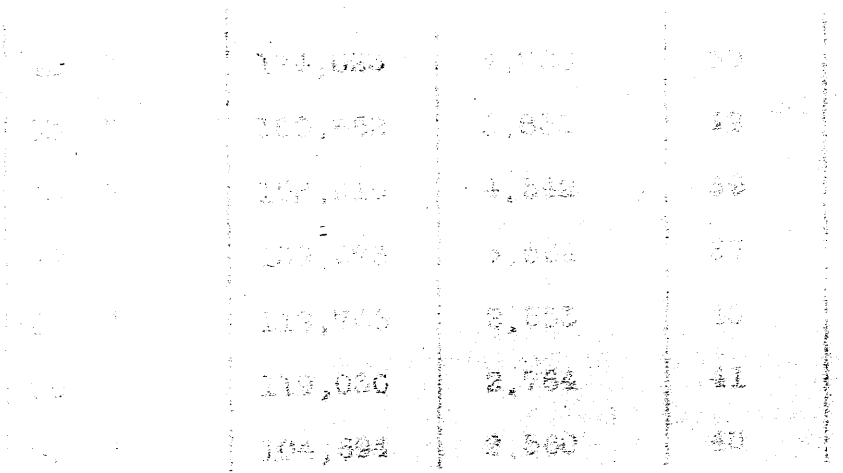
The rest of the curve of the absolute counts of the unagglutinated cells did not describe a rectilinear slope of elimination as did the previous graphs. It appeared to be roughly logarithmic or exponential in shape and corresponded to the shape of the curves described by Mollison (1947), of the survival of transfused cells in acquired haemolytic anaemia, although in these cases the total survival time was in the order of eleven to thirteen days. It also corresponded to the theoretical curves plotted by Sheets, Janney et al. (1951) for the disappearance of transfused cells lost by random destruction plus ageing, the random destruction occurring at a constant rate. It was possible that random destruction of the transfused erythrocytes was occurring in this case due to the action of an antibody produced in response to the transfusion. This supposition was borne out by the fact that a positive Coomb's test, both direct and indirect, was found after delivery. However, the occurrence of a positive Coomb's test was not necessarily indicative of this type of immunisation and such a positive test could be obtained from perfectly normal patients who were not suffering from either haemolytic anaemia or from an incompatible transfusion, but had a cir-

culating, normal, incomplete antibody. This usually gave a positive Coomb's test at temperatures below 10°C., but on occasions gave a false positive result at 37°C., Dacie (1950). The relationship between the exponential curve, the thrombophlebitis, the treatment with dicoumarol and the occurrence of a positive direct and indirect Coomb's test was not clear and could not be definitely discovered. The possible explanation that was suggested, of a transfusion immunisation, was considered the most feasible.

The extrapolated curve reached the baseline at one hundred and twenty days and was considered within the normal range for the total survival time for transfused cells.

The curve of the ratio free cells/total red cells, after an initial sharp drop, showed a well marked upward trend for the first thirty two days after transfusion, after which time it levelled off for a further twenty days and then described another peak. As these deviations were coincidental with fluctuations in both the haematocrit and red blood cell count values, it was postulated that these alterations in the ratio curve were due to blood volume changes during pregnancy. Fluctuations of this magnitude have been noted in the blood volume, the red blood cell counts and the haematocrit during pregnancy, Lowenstein, Pick and Philpott (1950), and were accentuated by graphing the free cell count/total red blood count values.

The values for the haemoglobin, red blood cell counts and haematocrit were within the normal range for pregnancy throughout the investigation. The Coomb's test was negative during the ante-partum period of the investigation, but became positive, both direct and indirect tests, on the first day post-partum and remained so until the patient was discharged from hospital. As she refused to have further investigations performed once she had been released from hospital, the persistence or otherwise of this positive test was not known.



Day	Free Cell Count	$\frac{F.C.C.}{R.B.C.} \times 100$	P.C.V. %	Hgb. in grams.	R.B.C. in millions.
Pre-transfusion	9,953	0.207	48	13.6	4.8
¼ hour	22,865	No R.B.C.			
½ "	49,765	counts			
1 "	178,885	available			
2 hours	196,201				
24 "	337,255				
2 days	336,519	7.020	48	14.0	4.8
4 "	317,151				
8 "	287,299				
18 "	253,398	6.325	38	13.3	4.0
29 "	208,475	5.473	39	13.6	3.8
35 "	179,154	4.475	38	13.3	4.0
42 "	174,023	4.328	39	14.3	4.02
53 "	160,862	3.833	42	14.0	4.2
63 "	158,910	4.542	39	12.8	3.5
77 "	139,073	3.564	37	13.6	3.9
88 "	119,705	2.553	43	15.1	4.7
89 "	119,030	2.764	41	14.3	4.3
90 "	104,694	2.560	40	13.6	4.1
91 "	92,100	2.560	42	14.3	4.3
94 "	79,810	1.90	42	15.1	4.27
95 "	72,010	1.632	45	14.3	4.4

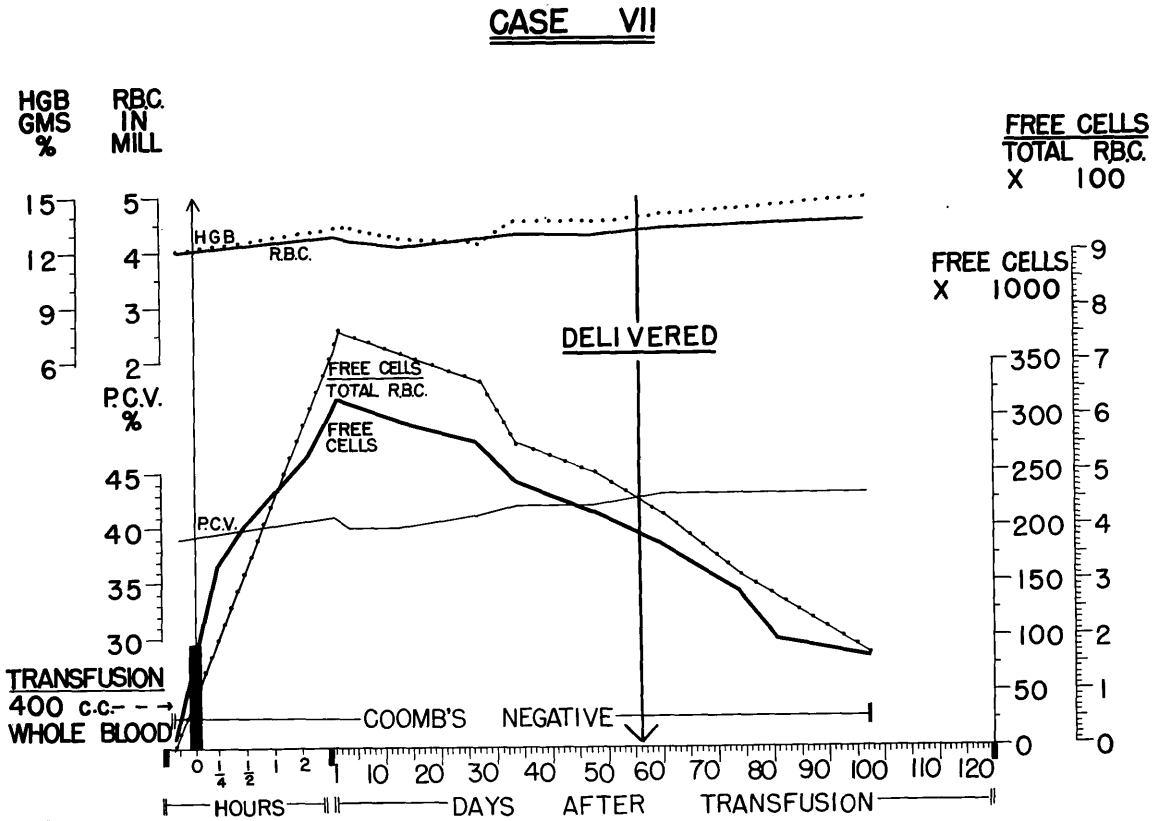
Chart VI. Haematologic studies in Case VI, a pregnant woman who developed thrombophlebitis and a positive Coomb's test post-partum.

Case VII. M. Carriere.

This case was a thirty year old multigravida, pregnant for the second time. She was examined when six and a half months pregnant. She was considered normal after investigation of her general medical, obstetrical and haematologic history. Obstetrical examination revealed a twenty eight week old foetus in a normal pelvis, presenting by the vertex. Haematologic examination was as follows:-

Haemoglobin:-	12.0 grams.
Red blood cell count:-	4.0 million per cu.mm.
Haematocrit:-	39 volumes per cent.
Blood group:-	A Rh positive.

The patient was given a transfusion of 400 mls. of whole blood of group O Rh positive on July 31st, 1952 and the survival of the transfused erythrocytes followed using anti-A serum for fifty six days before delivery and forty one days after delivery. On September 25th, 1952, the patient began labour and delivered a baby after a normal labour. There was no surgical interference and the blood loss was estimated at 100 mls. The puerperium was quite normal and she returned to the post-natal clinic six weeks after delivery, when she was considered fit and well. Partial haemograms and Coomb's tests were performed during the course of the investigation.



Case VII. Survival of transfused erythrocytes in a normal pregnant woman, before and after delivery of a baby.

The curve of the values for the absolute count of surviving erythrocytes and for the ratio of unagglutinated cells to total red cells in this case did not show the rapid fall in the six day period immediately after transfusion that was noted in the previous cases. The curve was of rectilinear shape, more in keeping with that of the

investigators who had worked on the problem of erythrocyte survival in normal adult males, Osborne and Denstedt (1947), Berlin (1951), and conformed to the theoretical curve postulated by Sheets, Janney et al. (1951), for the disappearance of normal transfused erythrocytes solely by ageing. Propagation of the general line of the curve gave a value of one hundred and twenty five days for the total survival time of the transfused erythrocytes and this was considered to be within the normal range.

The values for the haemoglobin, red blood cell counts and haematocrit estimations were all within normal limits for pregnancy and the Coomb's test remained negative during the course of the investigation.

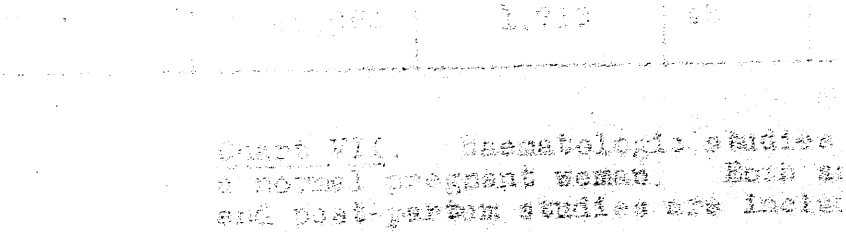


Chart VII. Haematological studies
a normal pregnant woman. Both a
and post-partum studies are inclu

Days	Free Cell Count	F.C.C. x100 / R.B.C.	P.C.V. %	Hgb. in grams.	R.B.C. in millions.
Pre-transfusion	8,339	0.002	39	12.0	4.0
¼ hour	166,780	No R.B.C.			
½ "	202,666	counts			
1 "	236,819	available			
2 hours	264,132				
24 hours	308,543	7.200	41	13.3	4.3
4 days	317,689	7.590	40	13.3	4.2
13 "	298,321	7.020	40	12.8	4.1
27 "	278,146	6.610	41	12.5	4.2
34 "	243,445	5.580	42	13.6	4.35
48 "	215,469	5.000	42	13.6	4.3
60 "	186,417	4.280	43	14.0	4.42
74 "	141,225	3.160	43	14.3	4.45
81 "	98,454				
97 "	83,263	1.710	43	14.8	4.55

Chart VII. Haematologic studies in Case VII, a normal pregnant woman. Both ante-partum and post-partum studies are included.

Case VIII. T. Cantafio.

This patient was transfused on December 6th, 1952 and six days later was subjected to Caesarian Section, during which she had several blood transfusions. In view of this, the results in this case were excluded from the results on normal pregnant women. However, the results obtained in this case were used to compare blood drawn for transfusion by two different methods (see "Discussion").

Case IX. M. Palardy.

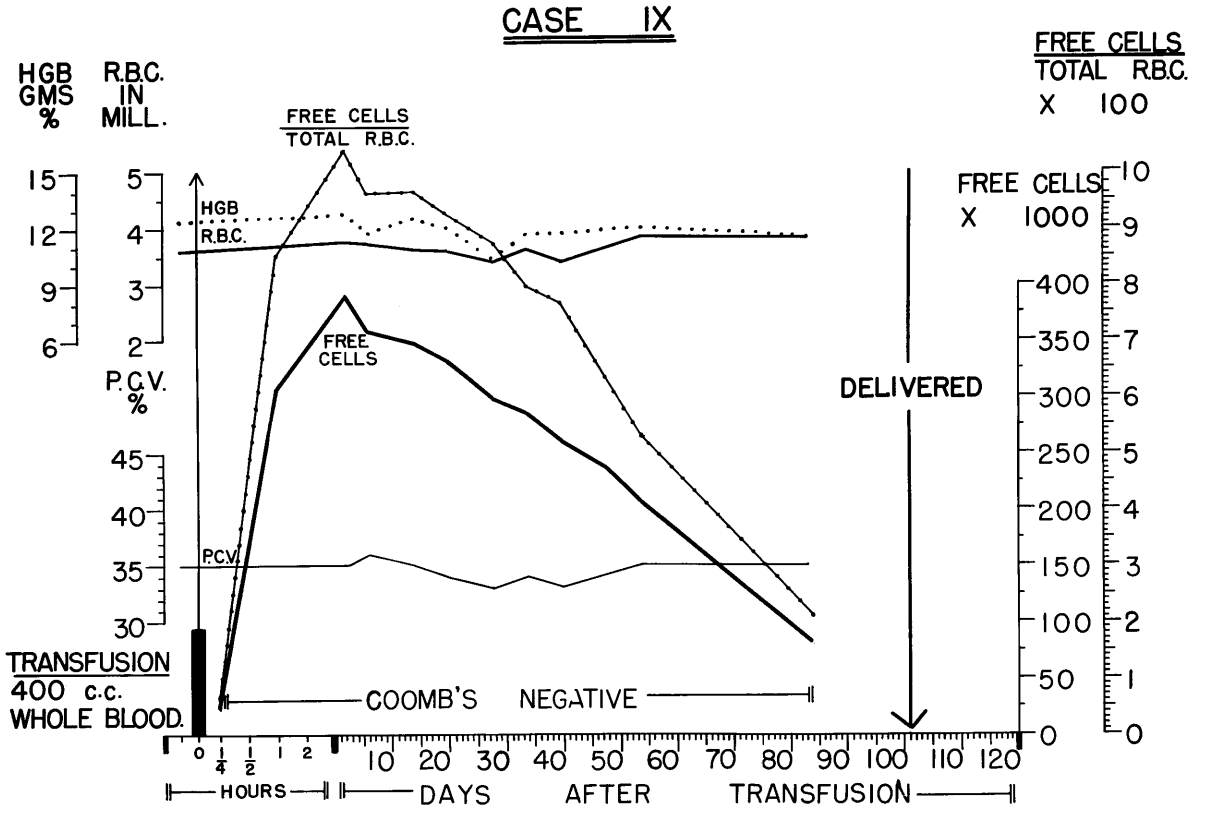
This twenty seven year old multigravida was transfused with 450 mls. of whole blood of group O Rh positive on September 18th, 1952. There was no abnormality detected in the medical, obstetrical and haematologic history and examination. The uterus was enlarged to twenty four week period of gestation and the foetus presented in the vertex in a normal pelvis.

Studies before transfusion gave the following haematologic values:-

Haemoglobin:-	12.6 grams.
Red blood cell count:-	3.7 million per cu.mm.
Haematocrit:-	35 volumes per cent.
Blood group:-	A Rh positive.

The survival of the transfused cells was followed using anti-A serum for eighty three days, during which time the

-patient remained normal. She went into labour spontaneously and delivered a baby without surgical interference on December 27th, 1952. This was eighteen days after the erythrocyte survival studies were finished.



Case IX. Survival of transfused erythrocytes in a normal pregnant woman. All the studies were performed before delivery.

The curve of survival of the transfused cells in this case was generally rectilinear with the exception of the period of six days immediately after transfusion. As

before, the curve reached a peak as the transfused cells increased to a maximum in the patient's circulation and then fell sharply for six days. This fall was reflected in a drop for the ratio of free cells to total red blood cells. In general, the curve of the absolute number of surviving erythrocytes was smoother and straighter than the curve obtained with the ratio values and this was due to the variation in the red blood cell count and haematocrit values which paralleled the deviations of the ratio curve. This was considered to be due to the blood volume changes occurring in the pregnancy state, Lowenstein, Pick and Philpott (1950). The projections of both the absolute number of erythrocytes counted and the ratio of free cells/total red blood cells met the base at approximately one hundred and fifteen days, which was considered normal for the total erythrocyte survival in this series.

Although the haemoglobin, red blood cell count and haematocrit values showed deviations and irregularities throughout the investigation, they were not considered abnormal. The Coomb's test remained negative during the experiment.

Day	Free Cell Count	F.C.C./R.B.C. x100	P.C.V. %	Hgb. in grams.	R.B.C. in millions
Pre-transfusion	8,138	0.226	35	12.6	3.7
¼ hour	22,120				
½ "	44,510				
1 "	236,000				
2 hours	307,000				
24 hours	401,632	10.50	35	13.0	3.8
2 days	390,050	10.370	35	12.8	3.71
6 "	357,232	9.622	36	11.7	3.71
14 "	348,355	9.666	35	12.5	3.6
20 "	332,484	9.222	34	12.0	3.6
28 "	298,321	8.764	33	10.4	3.4
34 "	285,140	7.916	34	11.7	3.6
40 "	260,123	7.647	33	11.7	3.4
48 "	236,451				
54 "	205,000	5.297	35	12.0	3.87
83 "	79,928	2.105	35	11.7	3.86

Chart IX. Haematologic studies on Case IX, a normal pregnant woman. All results obtained before delivery.

Case X. R. Howden.

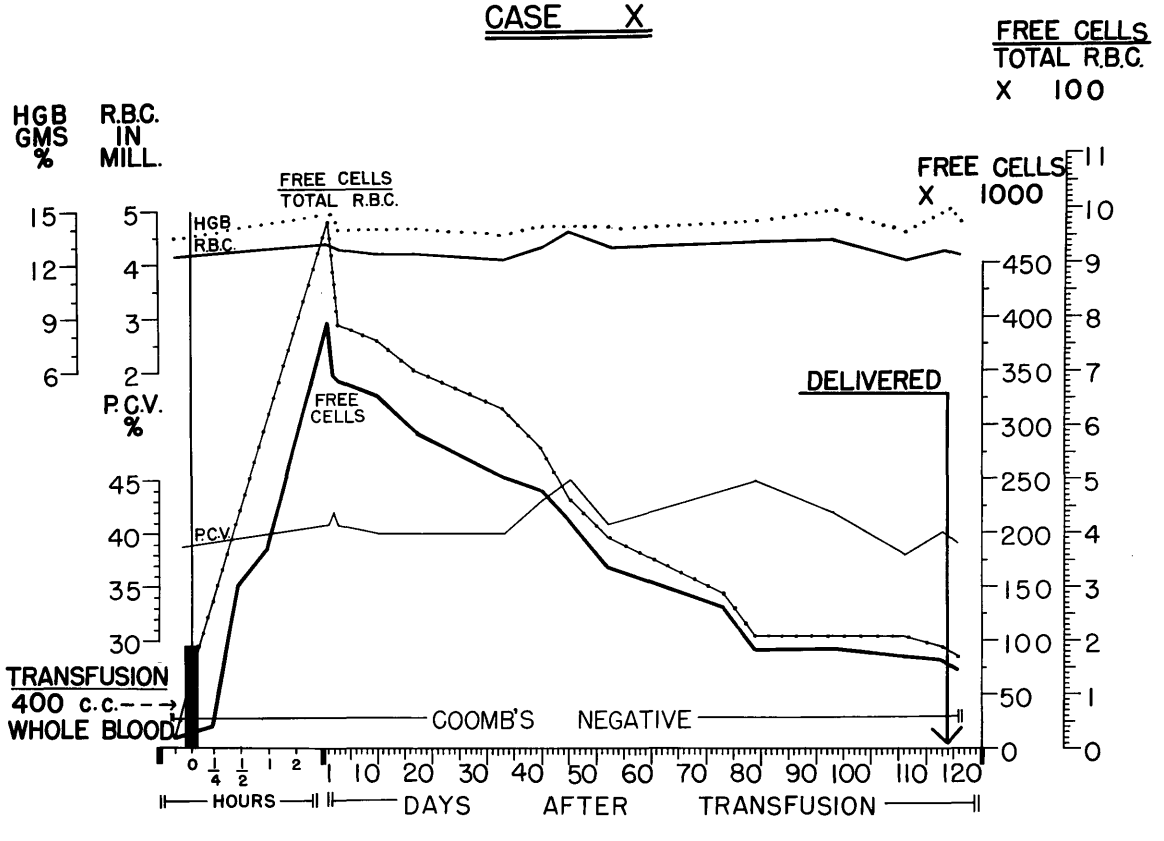
This twenty nine year old multigravida was seen in the sixth month of her second pregnancy. The medical history elicited the fact that the patient had had repeated attacks of what she called pneumonia. These attacks had comprised of a febrile illness, coughing and general malaise. She had never had a haemoptyses. Physical examination showed that there were suppressed breath sounds and a few coarse rates over the right lower lobe. X-Ray examination was negative: obstetrical and haematologic history and examination were negative. The uterus was enlarged to a twenty four week period of gestation and the foetus was in a vertex position in a normal pelvis. Haematologic studies were as follows:-

Haemoglobin:-	13.6 grams.
Red blood cell count:-	4.2 million per cu.mm.
Packed cell volume:-	39 volumes per cent.
Blood group:-	A Rh positive.

The patient was considered suitable for the erythrocyte survival studies and she was given 400 mls. of whole blood of group O Rh positive on August 9th, 1952 and the fate of transfused cells followed with anti-A serum. The study lasted for one hundred and sixteen days.

The patient remained well during the pregnancy and began labour on December 1st and delivered normally on

December 2nd, 1952, one hundred and fourteen days after transfusion. The labour and delivery were without incident and the estimated blood loss was 200 mls. The puerperium was normal and when seen at post-natal clinic the patient was fit and well. Partial haemograms and Coomb's tests were performed during the investigation



Case X. Survival of transfused cells in a normal pregnant woman.

The curve of the counts of the surviving erythrocytes in this case showed more irregularities than did the previous case results, but the deviations were not of such magnitudes that they could not be explained by either technical error or blood volume changes or a combination of both. Two of the major alterations in the slope of elimination were coincidental with major alterations in the blood cell counts and the haematocrit readings and it was felt that these irregularities did not vitiate the results of the total red blood cell survival time, which in this case was approximately one hundred and thirty days. However, the end of the curve showed a distinct flat appearance between the eightieth and the hundred and fifteenth day and this could not be explained satisfactorily by blood volume changes. As has been said previously, Mollison (1947) discussed the problem of the counts of surviving erythrocytes when these were fewest in the patient's circulation and stated that the Ashby technique of differential agglutination was least accurate when there were low numbers of transfused erythrocytes present in the blood specimens. It was thought that the shape of graph in this present case at the end part of the investigation, was due to the inherent difficulties in the Ashby technique of making exact counts of the absolute number of the surviving transfused cells when the number of these cells fell below 70,000 per

cu.mm. When the number of surviving cells was above 100,000 per cu.mm., it was possible to produce accurate counts. The curve of the ratio of unagglutinated cells/ total red cells showed the same type of irregularities as the curve for the absolute number of unagglutinated cells, although in a different direction. In the first portion of the curve this was almost certainly due to the fluctuations of the red blood cell counts, which reflected the blood volume changes and as can be seen from the graph, the haematocrit varied widely during the period of study.

The change in the curve in the last twenty days of the investigation was a reflection of the abnormality discussed in the preceding paragraph in relation to the total unagglutinated cell slope of elimination.

Although there were marked deviations in the red blood cell and haematocrit values in this case, it was felt that the readings were within normal limits. The Coomb's test remained negative during the course of study of the transfused cells.

Day	Free Cell Count	$\frac{F.C.C.}{R.B.C.} \times 100$	P.C.V. %	Hgb. in grams.	R.B.C. in millions.
Pre-transfusion	8,600	0.21	39	13.6	4.2
¼ hour	20,230				
½ "	151,638				
1 "	186,632				
2 hours	261,514				
24 "	393,009	9.80	41	14.3	4.4
2 days	349,700	8.70	42	14.8	4.35
3 "	342,168	7.95	41	14.0	4.30
10 "	330,601	7.80	40	14.0	4.2
17 "	295,093	7.02	40	14.0	4.2
33 "	253,398	6.17	40	13.6	4.1
40 "	240,217	5.58	43	14.0	4.3
45 "	214,931	4.67	45	14.0	4.6
52 "	170,546	3.94	41	14.0	4.33
73 "	131,003				
79 "	93,074	2.10	45	14.3	4.4
93 "	93,022	2.11	42	14.8	4.4
106 "	85,111	2.11	38	13.6	4.02
113 "	81,012	1.91	40	14.8	4.2
116 "	74,101	1.85	39	14.3	4.13

Chart X. Haematologic studies in Case X, a normal pregnant woman.

DISCUSSION.

During the years since Ashby first proposed a method of estimating the survival of the human erythrocyte by agglutination of the patient's own blood cells and studying the disappearance of transfused cells injected into the patient, every worker who has used this technique has modified some aspect of it. Some of these modifications have been of use, such as the introduction of centrifugation and agitation in an endeavour to produce maximum agglutination, and other modifications have been of dubious value, for example, performing the agglutination procedure at low temperatures and adding extra investigations to guard against the occurrence of non specific cold agglutinins. A great many of the more useful modifications have been incorporated in the technique used in this present series, but these are of no avail in securing maximum agglutination of the patient's erythrocytes, if a potent antiserum is not used. This antiserum, be it liquid or powdered, has to be investigated before the start of any investigation in order to determine not only the potency but the number of cells left unagglutinated after interaction with blood of the appropriate group. If this number of unagglutinated cells is above 10,000 per cu.mm., then the serum should be discarded because irregularities in the cell counts of up to 200,000

per cu.mm. may occur when using it. It has been shown by many workers in the fields of immunology that the most potent serum is not necessarily that which is used undiluted, but, due to a prozone or zone of inhibition, optimum action of the serum may occur at various dilutions. Thus, this aspect of the action of the serum used must be investigated.

The incidence of collision between the antibody and antigen has a definite influence on the ability of the serum to cause maximum agglutination and as complete agglutination of the patient's erythrocytes is the ideal situation in an investigation of this type, the influence of centrifugation and agitation on the process of agglutination must be examined. It is not possible by present techniques to produce complete agglutination of the patient's erythrocytes and therefore an unagglutinated cell count of zero, but it is possible to reduce the number of cells unagglutinated to a minimum and statistically unimportant in the expression or graphing of results.

The first part of the experimental work of this thesis has been a description of the various technical aspects of serum dilution, agitation, centrifugation and temperature control that were considered to have a practical application for the investigations conducted on patients subjected to erythrocyte survival studies.

The results of erythrocyte survival investigations in

the cases reviewed in the preceding paragraphs, in patients suffering from pre-eclamptic toxaemia of pregnancy and in apparently normal pregnant women, have been of a negative value only in so far as they demonstrated that there was no decrease in the erythrocyte life span in cases I and II, although the probability of a haemolytic component being present in the disease process of pre-eclampsia had been observed clinically. The patients who did develop pre-eclamptic toxaemia were not what could be classified as severe cases in that the degree of hypertension was not severe and the patients did not progress to active eclampsia. It was thought possible that extended erythrocyte survival of studies of patients suffering from severe pre-eclampsia would yield interesting information about the cause of the anaemia that occurs in this condition and that the haemolytic component, if present, could be more readily detected in these more severe cases. The positive value of this work lies in the fact that it showed that the survival of transfused erythrocytes was within the normal limits for patients suffering from a mild degree of pre-eclamptic toxaemia and for normal pregnant women.

The technique of differential agglutination for following the fate of transfused erythrocytes is not a mathematically exact method. It is open to technical errors, variations in blood volume of the patient transfused, and dependent

on various factors, such as the strength of the serum and the period of centrifugation used. Thus, it is inadvisable to lay too much stress on slight variations or deviations of the curves of results obtained by this method, but analysis of the graphs drawn in the present work gave a constant recurring fact, namely, there was a marked fall of the unagglutinated cell count in the first five to six days after transfusion. A method was used to test the assumption that this was due to blood volume changes and the ratio of unagglutinated cells/total red blood cells was charted. Contrary to expectation, this curve followed the lines of the original graph of free cells and thus the fall could not be due to blood volume changes. If it had been due to blood volume changes, even the wide fluctuations that have been recorded in pregnancy, the curve of the ratio would have described an upward arc due to the haemodilution consequent to the transfusion of whole blood.

Another possible explanation was that this was due to some aspect of the technical procedure, but as the whole method of investigation was kept constant and the cell counts done by the author himself, it did not seem feasible that such an error should occur in every case at the same time interval after transfusion.

As both the ratio values and the absolute values dropped at the beginning of each investigation, it seemed

likely that the cause lay with the blood transfused to the patient and that in some manner a percentage of stored erythrocytes were damaged before transfusion and sequestered from the recipient's circulation. On this assumption, the possibility that some of the red blood cells were damaged during removal from the volunteer donors was investigated. As has been said previously, the blood for transfusion was taken into a commercial Baxter "vacuo-liter" flask, which was manufactured with a vacuum inside the bottle to facilitate removal of blood from the donor's vein and on the basis that a vacuum such as this might have caused a change in either the permeability of the corpuscle or broken up an elderly erythrocyte, an experiment was carried out.

400 mls. of blood was drawn by gravity only into a Baxter flask, the vacuum having been released previously and this was stored for exactly twelve hours in a refrigerator at 4°C. Another 400 mls. of blood was drawn into a Baxter flask, the vacuum being kept intact, and this blood was stored under similar conditions for twelve hours.

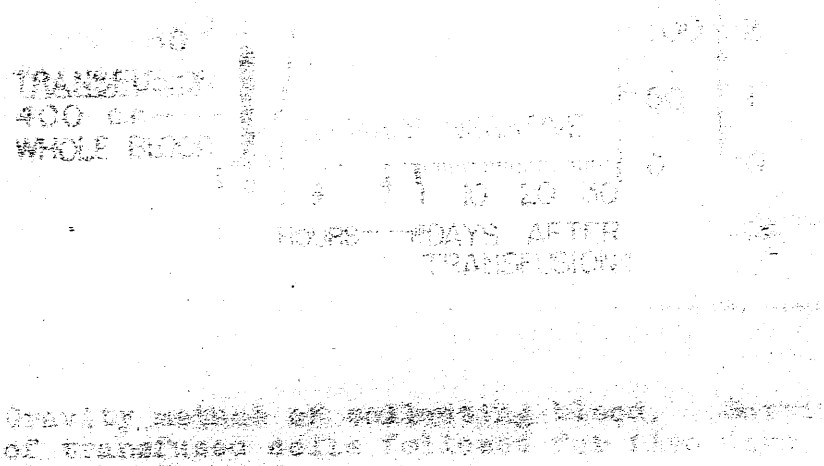
The blood drawn by gravity was given to Case VIII and the survival of the transfused cells followed for five days. Each time that a sample was collected for the agglutination procedure, venous blood was tested for the haemoglobin, red blood cell count and haematocrit values. This patient was eventually subjected to Caesarean Section

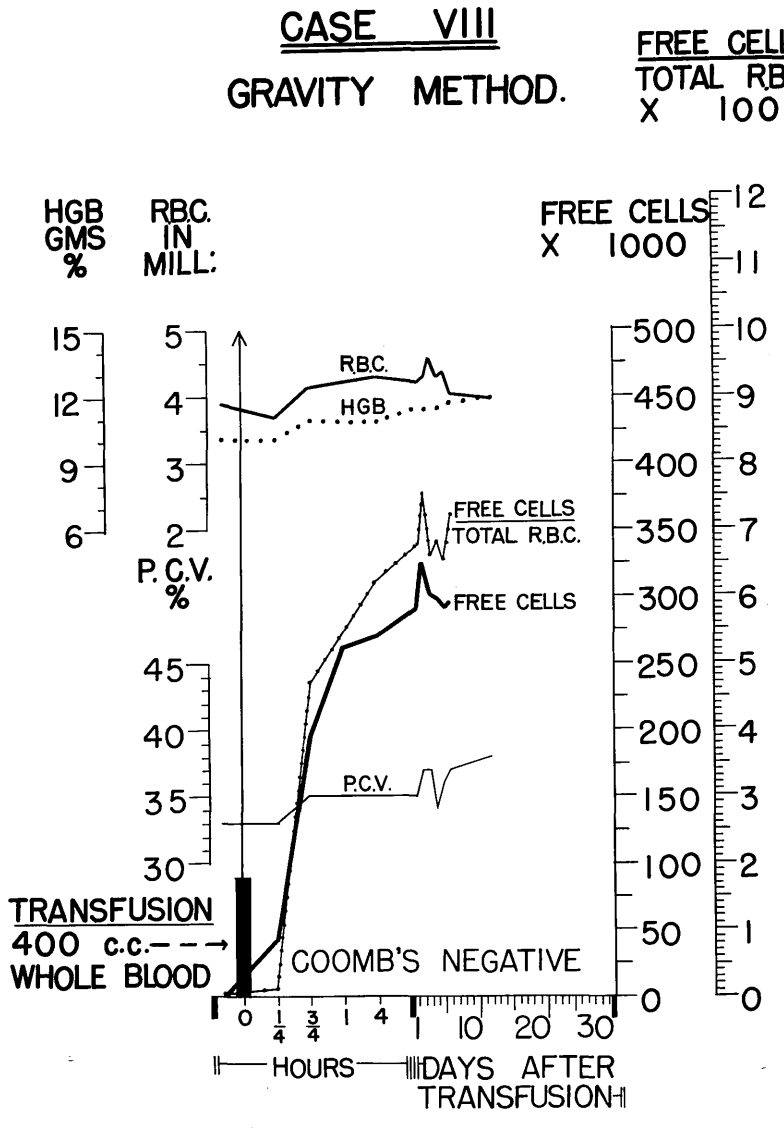
on the sixth day after transfusion.

Before transfusion, haematologic studies were performed on the patient and were as follows:-

Haemoglobin:-	10.1 grams.
Red blood cell count:-	3.9 million per cu.mm.
Haematocrit:-	33 volumes per cent.
Blood group:-	ABMN Rh positive.

She was given a transfusion of 400 mls. of whole blood of group AN Rh positive and the transfused cells were followed using anti-M serum for five days.





Gravity method of collecting blood. Survival of transfused cells followed for five days.

The disappearance of the transfused cells from the recipient's circulation did not appear to be so rapid in this case as in Cases I - X, nor as rapid as the cells transfused in Case XI. The curve of elimination of the

cells as shown by the absolute count of unagglutinated cells reached a peak in forty eight hours and then fall gradually over the next five day period. The total number of transfused cells that disappeared from the patient's circulation was approximately 30,000 per cu.mm. in the five days. This was a much lower number than occurred in the other cases where vacuum drawn blood was used. In some of these cases, for example Case I, the number of transfused cells that disappeared from the recipient's circulation over a comparable period of time was approximately 100,000 per cu.mm. Some of the irregularities that occurred in the graphs in previous cases were undoubtedly due to blood volume changes, as can be seen from the irregular curve described by the ratio of free cells to total red cells in this case. In this experiment, the red blood cell count, haemoglobin and haematocrit had been estimated at quarter hour intervals during transfusion and thereafter on each specimen that was removed for the agglutination procedure and definite irregularities occurred in the graph of these values, presumably due to the transfusion of the whole blood.

However, blood volume changes could not be wholly responsible for the magnitude of the number of cells that disappeared rapidly after transfusion and it was not expected that the blood volume changes would last for a period of five to six days. The results in this case gave a partial

explanation for the phenomenon, namely, the rate of disappearance of the transfused cells was connected with the method of drawing blood for the transfusion.

Day	Free Cell Count	$\frac{F.C.C.}{R.B.C.} \times 100$	P.C.V. %	Hgb. in grams.	R.B.C. in millions.
Pre-transfusion	3,000	0.769	33	10.1	3.9
¼ hour	43,478	1.156	33	10.1	3.76
½ hour	196,032	4.722	35	10.9	4.150
4 hours	270,140	6.250	35	10.9	4.32
12 "	290,295	6.807	35	11.4	4.26
2 days	325,121	7.558	37	11.4	4.3
3 "	301,010	6.600	37	11.4	4.56
4 "	296,163	6.883	34	11.4	4.3
5 "	293,610	6.569	36	11.4	4.4
6 "	295,100	7.230	37	11.7	4.08

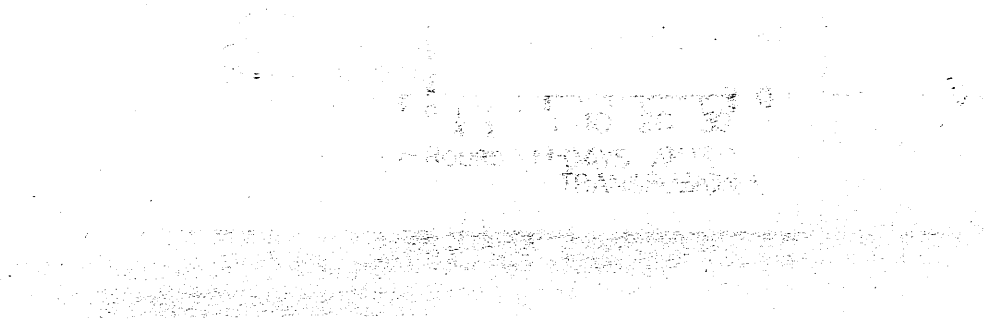
Gravity method of drawing blood.
Haematologic studies on a pregnant woman.

On the assumption that the method of drawing blood for transfusion was partially responsible for the fall in numbers of unagglutinated cells immediately after transfusion and on the assumption that blood volume changes might differ in the pregnant from the non-pregnant state, a further transfusion experiment was carried out. A non-pregnant,

gynaecological patient was selected and transfused with 400 mls. of whole blood that had been collected in a vacuum flask, the vacuum having been left intact and the survival of the transfused cells followed by using anti-M serum. Haematologic studies were as follows:-

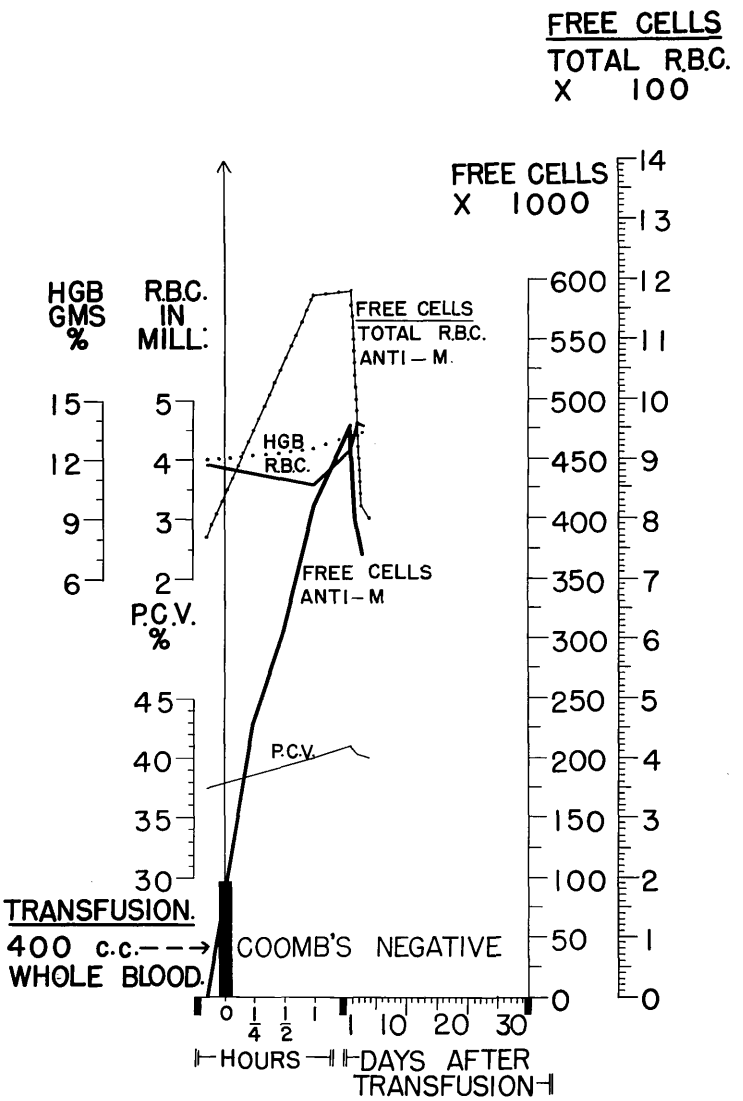
Haemoglobin:-	12.0 grams.
Red blood cell count:-	3.92 million per cu.mm.
Haematocrit:-	37 volumes per cent.
Blood group:-	ABMN Rh positive.

The transfusion of ON Rh positive blood was given on December 6th, 1952 and during the course of the investigation the patient remained well. After the studies had finished, she had a dilatation and curettage performed for investigation of sterility.



CASE IX. b.

VACUUM METHOD.



Vacuum drawn blood. Survival of transfused cells in a non-pregnant gynaecological patient.

There was a marked difference in the appearance of

the curve of results in this patient compared to the curve obtained with blood drawn by gravity. The behaviour of the slope of elimination of the transfused cells was the same as in the previous pregnant normals investigated. The number of unagglutinated cells increased rapidly to a peak on the graph and then started to fall during the immediate period after transfusion. The fall of the numbers of unagglutinated cells was much greater than the fall experienced using gravity drawn blood, being in the order of 100,000 cells per cu.mm. This was considered a statistically valid difference even using the Ashby technique, and it was thought that conclusions could be drawn from the comparison of the two cases investigated as to the cause of the constant, marked fall in the numbers of transfused erythrocytes immediately after transfusion. The explanation was put forward that a certain percentage of cells were damaged by being subjected to a sudden vacuum in the course of removing blood from a donor for transfusion. How many cells were damaged and by what exact mechanism this damage occurred was not known and it was felt that further investigation into the problem of drawing blood by using a decreased pressure was necessary before these questions could be answered.

It was also of interest to note that the ratio of the value for free cells/total red blood cells in the case of a

non-pregnant woman did not show such wide deviations and fluctuations as the ratios obtained in a pregnant patient, so it was possible that the blood and plasma volume changes differed in the pregnant state as compared to the non-pregnant state, although it was considered impossible to justify this on the basis of one experiment.

Day	Free Cell Count	$\frac{F.C.C.}{R.B.C.} \times 100$	P.C.V. %	Hgb. in grams.	R.B.C. in millions.
Pre-transfusion	3,000	0.763	37	12.0	3.92
$\frac{1}{4}$ hour	234,299	6.000	37	12.1	3.90
$\frac{1}{2}$ hour	313,771	8.250	37	12.3	3.8
1 hour	416,102	11.270	40	12.5	3.69
12 hours	482,584	11.690	41	13.3	4.13
2 days	392,691	8.524	40	13.3	4.61
3 days	363,442	7.925	40	13.3	4.58
4 days	354,101	7.86	40	13.1	4.5

Vacuum drawn blood. Haematologic studies using vacuum drawn blood for the transfusion. A gynaecological, non-pregnant, patient.

In two cases (III and IV) marked differences were noted between the cell counts obtained using dried anti-M serum and liquid serum. The liquid serum used in case III was anti-B type and in this case the unagglutinated cell

counts produced with this serum were lower during the course of investigation than the counts produced with anti-M serum. The pre-transfusion values for the unagglutinated cells differed with each serum, being 16,000 with anti-B and 25,000 with anti-M. A corresponding difference in free cell counts was noted throughout the course of study (Chart III). In this case it would have seemed that the anti-B serum was more potent than the anti-M serum.

A similar difference in the pre-transfusion values for the unagglutinated cell counts was present in Case IV, when both anti-A and anti-M sera were used. The values were 4,000 and 12,000 cells respectively and this difference was maintained during the course of the experiment.

In contradistinction to the high values obtained for the free cell counts in Cases III and IV, were the values obtained in the experiment designed to compare the difference in blood drawn by a vacuum and by gravity, where anti-M serum was used. The pre-transfusion counts of free cells were very low, being 3000 cells per cu.mm. in these cases.

The reason for this variation of counts in different patients was unknown, although at first it was thought to be due to the different antigenic properties of dried serum compared to liquid serum, and this explanation could have

been accepted if Cases III and IV only were compared. However, the occurrence of suitably low free cell counts in subsequent cases with the anti-M serum made this assumption invalid. Apart from an upset in the technical procedure, the only remaining explanation that was possible was that in some manner the dried anti-M serum varied in antigenic properties from patient to patient, or that the blood cells of different patients responded to the anti-serum with a different degree of sensitisation. This was unproven and purely speculative.

S U M M A R Y.

An attempt was made to elucidate the problem of the aetiology of the anaemia that often accompanies pre-eclamptic toxaemia of pregnancy and with a view to providing or disproving the occurrence of a haemolytic component in this anaemia, the length of survival of the human erythrocyte by a modification of the Ashby technique.

Nine pregnant women were transfused with blood of a different group from their own and the patients' erythrocytes agglutinated by a suitable antiserum. The non-agglutinated cells were counted and charted at various time intervals after transfusion and a slope of elimination of cells produced. Two of the patients developed mild pre-eclamptic toxaemia of pregnancy and of the remaining patients, one was subjected to Caesarean Section and rejected from the study and six patients remained well. The life span of the erythrocyte in the two patients who developed toxaemia was normal compared to the life span of the erythrocytes in the six normal patients and the conclusion from these studies was that in mild pre-eclampsia there was no decrease in the survival of the red blood cell and thus no haemolytic component present.

Suggestions were made for further research in more severe cases of pre-eclamptic toxaemia and for further investigations into the possible harm that might occur to

erythrocytes withdrawn for transfusion when subjected to a decreased pressure.

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