Isoimmunization in the human subject to the blood group factors A, B and Rh

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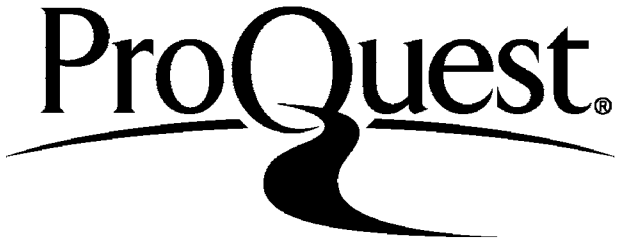

ISO-IMMUNIZATION IN THE HUMAN SUBJECT TO THE BLOOD GROUP FACTORS A, B & Rh.

The process by which specific antibodies are formed in an individual in response to stimulation by an antigen is called immunization. An antigen which is contained in some individuals of a species may, under suitable conditions, stimulate the production of specific antibodies when injected into other individuals of the same species who do not already possess the antigen. This process is called iso-immunization and the antibodies produced are iso-antibodies.

The first experiments on this subject were made on animals by Ehrlich and Morgenroth (6) who discovered that when one goat was injected with the blood of another goat, immune iso-haemolysins became apparent in its serum. In 1933 Irwin and Hill (8) made use of the phenomenon of iso-immunization for the purpose of studying the cellular individuality of erythrocytes in doves. Back cross-hybrids were joined by parabiosis and it was found that each member of a pair of parabiotic twins developed antibodies against the erythrocytes of the other, due to mutual iso-immunization. Dienst (3) first suggested that there might be iso-immunization within the human species. He showed that following pregnancy in some cases there was an increase in the Anti-A or anti-B iso-agglutinin titre of the mother's serum when the infant's erythrocytes contained a corresponding A or B agglutinogen.

Landsteiner, Levine and Janes [13] examined the sera of 7 individuals who had been repeatedly transfused with the blood of donors of the same group as their own. In two of these cases, after the transfusion, iso-agglutinins developed in the serum of the patient which reacted with the blood given.

A case was described by Neter [18] in which an immune iso-agglutinin and iso-haemolysin appeared in the recipient's serum after a blood transfusion. At 37°C the fresh serum of this patient haemolysed certain other bloods of the same group and when inactivated the serum gave agglutination reactions. At low temperatures only weak agglutination was obtained. In the same year, Zacho [33] investigated a transfusion reaction and demonstrated the presence of an immune atypical antibody in the serum of the recipient.

The human erythrocyte contains many antigenic substances or agglutinogens in its cell envelope. The well-known agglutinogens are A, B, O, M, N and Rh and their corresponding agglutinins are termed anti-A, anti-B, anti-O, anti-M, anti-N and anti-Rh respectively. The anti-A and anti-B iso-agglutinins are found in both natural and immune form. Individuals belonging to groups A and O, except in extremely rare cases, possess a naturally-occurring anti-B iso-agglutinin and similarly individuals belonging to groups B and O possess a naturally-occurring anti-A iso-agglutinin in their sera. Under certain conditions when stimulated by the homologous agglutinogen immune anti-A and anti-B iso-agglutinins are produced. The

anti-M and anti-N iso-agglutinins do not usually occur in a natural form and only rarely do individuals become immunised to these factors, (Paterson, Race and Taylor.)(7) The anti-Rh iso-agglutinins with one exception (Diamond)(2) are immune agglutinins which, in certain circumstances, are produced in the sera of Rh negative individuals, (i.e. indivuals who lack Rh agglutinogen in their erythrocytes).

The object of this present work is to study immune responses in the human subject. As far as possible the titres of the immune iso-agglutinins have been determined both before and after stimulation by the homologous agglutinogen and have been traced to the peak of the response and throughout a subsequent decrease to approximately the prestimulation level. The work is divided into sections according to the source of the agglutinogens providing the stimulus for the immune response.

Section (A) describes immune responses produced after transfusion of incompatible blood. In these cases the stimulating agglutinogen is contained in the transfused erythrocytes.

Section (B) describes immune responses after transfusion of sera which contain the agglutinogen in a soluble form.

In Section (C) the immune response is due to a foetus in utero and immune iso-agglutinins specific for the infant's erythrocytes are produced in the mother's serum.

Section (D) describes immune responses following a double stimulation which is provided by both a blood transfusion and a

foetus in utero.

Section (E) describes an entirely new phenomenon, the activation of immune iso-agglutinins by a factor contained in human serum.

EXPERIMENTAL METHODS.

Collection of Serum Samples. The serum to be titrated was obtained from a sample of blood withdrawn from the patient's arm by means of a dry sterile syringe. The blood was then allowed to clot and the serum separated from the red cells. If it was not possible to titrate the serum in a fresh state it was frozen solid at 5°F. until required.

Determination of Blood Group. The method adopted for determining the ABO group of erythrocytes was that of Taylor et al.(25) A suspension of the blood to be grouped (approximately 5% in terms of whole blood) was made in 3.8% sodium citrate solution. One volume of this, as measured with a pasteur pipette, was placed in each of two precipitin tubes. To one was added two volumes of anti-A serum and to the other two volumes of anti-B serum. (Sera diluted 1 in 2 with saline were used in order to reduce rouleaux formation). The tubes were shaken and allowed to stand for 2 hours at room temperature. The results were read by tapping the tubes sharply with the finger and recording the presence or absence of agglutination. Tubes in which no agglutination was apparent macroscopically were checked by making a smear of their contents on a slide and examining microscopically. To confirm the grouping of the erythrocytes a cross-grouping test was also carried out on

the blood serum. Equal volumes of the serum and standard red cells of groups A and B were mixed and allowed to stand for at least two hours. The readings of this test were made with the naked eye.

Determination of Rh type. The erythrocytes were always tested against 3 anti-Rh typing sera. A 2% suspension of erythrocytes was mixed with an equal quantity of each anti-serum in small precipitin tubes and incubated at 37°C., for 2 to 3 hours. When reading the tests the contents of each tube were treated with great care and gentleness owing to the ease with which Rh agglutination is broken down by rough handling. The tubes were never sharply tapped, but some of the sediment was gently removed by means of a pasteur pipette and placed on a microscope slide with only the minimum amount of spreading. The reading of the agglutination on slides prepared in this manner is sometimes a little difficult owing to the fact that careful manipulation of the cell sediment tends to cause the cells to cling together in "drifts" and "nests" which, when examined superficially, may be mistaken for genuine agglutination. However, when the worker becomes experienced, there is no difficulty in distinguishing between these phenomena and true agglutination.

Technique of Titration of Iso-agglutinins. The method of performing and reading the titrations was similar to that described by Taylor and Ikin.(23) Titrations were carried out in 2 x ¾" round-bottom precipitin tubes. A .85% solution of sodium chloride

in distilled water was used as diluent. The unit volume employed for the titration was measured by means of a Pasteur pipette and was equal to .04 c.c. approximately. The standard red cells necessary were obtained from the same donors throughout and were always less than 24 hours old when used. A 2% suspension of red cells in 3.8% sodium citrate was used for the titrations. The cell suspension was not made up accurately but judged by eye.

The precipitin tubes were placed in rows in small wooden blocks. The number of tubes in each row varied according to the expected length of the titration. Each titration was performed in duplicate. The serum dilutions were made by placing one volume of diluent in each tube of a row except tube 1, and one volume of the serum to be titrated in tubes 1 and 2. Tube 2, therefore, contained two volumes of fluid. The contents of this tube were thoroughly mixed by quickly drawing the fluid up and down within the stem of the Pasteur pipette. One volume of the contents was then withdrawn and placed in tube 3 and the process repeated along the entire row, one volume from the last tube being discarded. The result was a series of serum dilutions which were doubled, at each stage. An equal volume of the appropriate red cell suspension was added to each serum dilution and the tubes were then shaken to make the suspensions even. The titrations were read after standing for 2 hours at room temperature. Table I shows some typical titres of iso-agglutinins and the method of scoring the strength of a reaction is indicated in the footnote to this table. Readings were made after the cell deposit had been dispersed by tapping the tube sharply two or three times with the

finger. No reaction was scored as negative until it had been examined microscopically. For this purpose a drop of the contents of the tube was spread evenly over a microscope slide and examined.

The titre of a serum is denoted by the reciprocal of the greatest dilution at which agglutination occurs.

The experimental error of this technique in the hands of an inexperienced worked is usually not more than one dilution, in spite of the fact that the unit volume employed is very small. Table I shows fourfold titrations of the same Anti-A and Anti-B iso-agglutinins performed by two different workers. It can be seen that there is very good agreement both between the four titrations of each agglutinin carried out by the same worker and between the titrations of each worker.

The titration values are higher than those obtained when the serial dilutions are made with standard graduated pipettes using a separate pipette for each dilution. This is because using the Pasteur pipette method there is a slight carry-over from tube to tube, which is to be expected when one pipette is used for the whole titration. For this investigation, however, this was unimportant for when titrating iso-agglutinins to follow an immune response the titres are always comparative.

Modification of Technique for Titration of Anti-Rh Agglutinins.

A slightly different technique was employed for the titrations of anti-Rh iso-agglutinins. Each serum was titrated against standard Rh positive erythrocytes from two individuals, one of whom belonged to sub-group Rh_1Rh_1 (Race and Taylor)[19] and the other to sub-group Rh_2 (Race, Taylor, Boorman and Dodd).[20] Control tubes containing

the serum against Rh negative erythrocytes from 2 donors were included in each titration. The titrations were incubated at 37°C for 2 to 3 hours and then the reactions read, taking the same precautions as for Rh typing. Table Ia shows the titration of an anti-Rh agglutinin in triplicate carried out by the same two workers as in Table I. The footnote to Table I indicates the manner in which the strength of the agglutination reaction was recorded. Most of the sera investigated showed higher titration values with erythrocytes of sub-group Rh_2 than with those of sub-group Rh_1Rh_1, although an occasional serum was found in which the titre was higher with the latter. The majority of anti-Rh iso-agglutinin titres recorded in this work are those obtained with Rh_2 erythrocytes.

Comparison between Titrations of a Serum using Red Cells taken from the same donor on different days.

As mentioned above, the red cells necessary for the titrations were always obtained from the same donors throughout the experiments. This necessitated bleeding the donors on ~~several~~ many different occasions. Therefore in order to determine whether there was a serious variation in the agglutinability of red cells withdrawn on different days, a single Anti-A serum was titrated against red cells taken from the same group A donor on 4 successive occasions. The results of this experiment are shown in Table Ib. The end points of the titrations vary slightly but not enough to be significant of any change in the agglutinability of the red cells on the different occasions. It should be added that the experiment was carried out within a period of 8 days.

INHIBITION TECHNIQUE FOR ESTIMATION OF SERUM AGGLUTINOGEN.

To obtain some idea of the amounts of soluble A or B agglutinogen present in sera of persons belonging to groups A and B inhibition tests were performed. These tests were based on the ability of the A and B agglutinogens to inhibit specifically the corresponding anti-A and anti-B agglutinins.

Equal parts of the serum to be tested and the appropriate anti-serum were mixed. The mixture and the original anti-serum were then titrated and the titration values obtained were compared. The inhibition of the ~~titre~~ capacity to agglutinate of the anti-serum by the agglutinogen contained in the test serum was expressed as an inhibition index. The inhibition index is the number of times the original titre of the anti-serum is reduced, e.g. an inhibition index of 8 indicates that the titre has been reduced to one-eighth of its original value.

SECTION (A)

PRODUCTION OF IMMUNE ISO-AGGLUTININS AS A RESULT OF TRANSFUSION OF INCOMPATIBLE BLOOD

1. Production of Immune Anti-A and Anti-B Iso agglutinins after Transfusion of Blood containing Agglutinogens A and B.

Only a few accounts of incompatible blood transfusion in the literature mention the accompanying changes in iso-agglutinin titre. Rø[22] describes the case of patient of group O who received 500 c.c. of group A blood. The anti-A iso-agglutinin increased considerably in the period following the transfusion.

Wiener et al.,[31] have given details of two cases. In the first case the patient belonged to group O and was transfused with 500 c.c. of group B blood. The titre of the anti-A iso-agglutinin remained almost constant at ~~approximately~~ 32, while the titre of the anti-B iso-agglutinin, which was only 1 two hours after the tramsfusion, rose to 512 by the thirteenth day after the transfusion. The initial low titre of the anti-B iso-agglutinin was no doubt due to the absorption of this iso-agglutinin by the injection B blood and the subsequent rise was an immune response to stimulation by the B agglutinogen. On the twenty-fourth day after the transfusion the titre was still 512.

In the second case, where a group A patient was given 300 c.c. of group AB blood, there was a similar rise in the titre of the anti-B iso-agglutinin, but the changes were not so closely observed.

Mollison and Young[17] have recorded in detail the iso-agglutinin changes following the transfusion of 540 c.c. of blood

of group B to a recipient of group O. They showed an anti-B titre of 2 eight hours after the transfusion, rising to the extremely high level of 262,144 at the tenth day, gradually falling to 128 by the fifty-second day.

Recently, a similar case has been reported by Drummond (4) in which an immune anti-A iso-agglutinin, reaching a titre of 25,600 on the fifteenth day, was produced in a group B individual following an incompatible transfusion of blood of group A.

In the following cases varying amounts of blood of incompatible ABO group were inadvertently transfused, thus affording an opportunity for studying the effect of these transfusions on the iso-agglutinins in the sera of the patients concerned. In some of the cases the data are incomplete owing to the difficulty of obtaining samples of blood and often it was impossible to record pretransfusion titres as attention was not drawn to the patient until after the transfusion had been given.

A summary of the findings is shown in Table II. In all cases there was a specific immune response to the transfused blood denoted by an increase in titre either of the anti-A or anti-B iso-agglutinin according to whether the transfused erythrocytes contained the A or B agglutinogen.

In case 1, in which a small amount of group AB blood was given there was an increase in titre of both the anti-A and the anti-B iso-agglutinins. By the eighth day after transfusion, the anti-A iso-agglutinin titre had risen to 1,000 and the anti-B to 512. Owing to the death of the patient no further samples of blood could be obtained.

A graphical representation of case 2, which is ~~typical~~ striking, is shown in fig. 1. In this case there was an increase in titre of the anti-A iso-agglutinin which reached a maximum (32,000) on the eleventh day after the transfusion, then decreased to a steady level by the twentieth day after transfusion. During this period there were some variations also of the anti-B iso-agglutinin but compared with the response of the anti-A agglutinin, these were slight (see also fig. 1. which is on a true scale). Thus the increase in titre of the anti-A iso-agglutinin was a specific immune response to the A agglutinogen contained in the transfused erythrocytes. ~~The low anti-A titre which was found immediately after the transfusion was no doubt due, as in the case described by Wiener, to the absorption of the anti-A iso-agglutinins by the transfused erythrocytes. That low anti-A titres were not recorded immediately after transfusion in cases 2 and 5 was probably due to the fact that in these cases only 50 c.c. of group A blood were given.~~

In case 4, in which there was an increase in the titre of the anti-B iso-agglutinin, a smear made from the recipient's blood on the first day after transfusion revealed numerous small agglutinates. Although the recipient's serum already contained anti B iso-agglutinins (titre 8), these agglutinates increased in number and size when more anti-B serum was added to the smear. This is no doubt an example of "balanced reaction", which is a characteristic of the interaction between antigens and antibodies.

Fig. 2 is chosen from a number of controls and demonstrates that when compatible blood was given there was no appreciable change in titre of either the anti-A or anti-B iso-agglutinin. [see also Table II a]

2. Production of Immune Anti-A_1 Iso-agglutinins in Individuals of Sub-groups A_2 and A_2B after Transfusion with Blood of Sub-group A_1

In 1910 von Dungern and Hirszfeld[5] discovered that the A agglutinogen was not a single substance but was of at least two kinds which were later termed A_1 and A_2 by Landsteiner and Levine.[12] There are, therefore, two different sorts of group A blood and individuals of group A belong either to the sub-group A_1 or A_2. Similarly, there are two different AB groups, A_1B and A_2B. The anti-A iso-agglutinin contained in the sera of persons of group O and B reacts with both A_1 and A_2 ~~agglutinogens~~ erythrocytes, but if a group B serum is first absorbed with group A_2 erythrocytes until it will no longer agglutinate these, the resulting serum will agglutinate A_1 erythrocytes specifically. By the use of this absorbed serum it can be determined to which of the sub-groups A_1 and A_2 an individual belongs. Titration of an anti-A serum against A_1 and A_2 erythrocytes will also differentiate between the sub-groups, because the titration value is lower with A_2 than with A_1 erythrocytes.

α_1 iso-agglutinins occur naturally in some bloods of sub-groups A_2 and A_2B, but they are not active at 37°C and

are therefore called "cold agglutinins". It is also occasionally possible to demonstrate anti-O (α_2) cold agglutinins in bloods of sub-groups A_1, A_1B and B.

The fact that the A_2 agglutinogen reacts less strongly with an anti-A serum has led some authors (Lattes)[14] to the conclusion that the difference between sub-groups A_1 and A_2 is merely a quantitative one and is caused by different amounts of the same agglutinogen in the erythrocytes. Landsteiner and Levine,[10] on the other hand, consider that there are two qualitatively different agglutinogens A_1 and A_2. The following two cases present conclusive evidence in support of their theory, because they demonstrate the production of immune α_1 iso-agglutinins which become active at 37°C in two individuals of groups A_2 and A_2B respectively. If the difference between the A agglutinogens were quantitative only, no iso-agglutinin active at 37°C specific for either agglutinogen, could be produced.

The first of these cases was a patient suffering from haemolytic anaemia who was given a transfusion of 1,000 c.c. of concentrated red cells of group O followed four days later by 1,000 c.c. of concentrated red cells of group A. A further transfusion of 1,000 c.c. concentrated group A red cells was given after another three days had elapsed. On each occasion cross-matching tests at 37°C had shown the donor red cells to be compatible with the patient's serum. However, six days after the first transfusion of group A blood, an atypical iso-agglutinin was found in the patient's serum. This reacted with a number of

group A bloods at 37°C. The recipient was then tested and found to belong to sub-group A_2. Seven of the eight donors who had contributed to the transfusions of group A blood were traced and all of them were found to belong to sub-group A_1. This suggested that the atypical iso-agglutinin was α_1 and this was confirmed when the patient's serum was tested with a larger series of group A bloods wherein only those of sub-group A_1 were agglutinated. Fig. 3 represents the changes in titre of the α_1 iso-agglutinin for a long period after the transfusion. The peak of the response occurred on the fifteenth day after the first group A transfusion and the peak titre attained at 37°C was 32. Forty-nine days after the transfusion the α_1 iso-agglutinin was still active in the patient's serum (titre 2). No satisfactory titration values could be obtained at room temperature because the patient's serum also contained a very strong auto-agglutinin which masked the reaction of the α_1 iso-agglutinin at this temperature. The auto-agglutinin, however, was not active at 37°C.

The second case was also one in which the patient was suffering from haemolytic anaemia. The patient belonged to group A_2B and she received several transfusions of blood of groups O and A at close intervals. Examination of her serum on the fifteenth day after the last of these transfusions revealed the presence of an α_1 iso-agglutinin, the titre of which was 32 at room temperature and 8 at 37°C. Fig. 4 is a graphical representation of the subsequent changes in titre of this iso-agglutinin. Between the fifteenth and seventieth days after transfusion there was a gradual decrease in titre which resulted in the complete disappearance of the activity

of the antibody at 37°C, although a titre of 4 was maintained at room temperature. Although it was not known to which sub-group of A the transfused blood belonged, it seems likely, since only one in five group A bloods are of sub-group A_2, that some of it at least must have been of sub-group A_1. It was most probably the latter, therefore, that caused an immune response of the α_1 iso-agglutinin. This received confirmation from the fact that 140 days later, after a further transfusion of two bottles of group A_1 blood, the α_1 iso-agglutinin by the twentieth day after the transfusion, was again slightly active at 37°C (see fig. 4.).

In these two cases there was no record of whether the patients' sera contained natural α_1 iso-agglutinins before the first transfusion of A_1 blood was given. In the first case the rapidity of the response to the transfused blood is very suggestive that the α_1 iso-agglutinin was present prior to the blood transfusion, although it was not then active at 37°C, because the cross-matching test at this temperature did not reveal any incompatibility.

Two other cases have been described in the literature in which immune α_1 iso-agglutinins active at 37°C have been reported, (Wiener,[27] Race et al[21]). In neither of these, however, is there such a complete record of the immune response at 37°C as in the cases described above.

3. Production of Immune Anti-Rh Iso-agglutinins in Rh negative Individuals following Transfusion of Rh positive Blood.

Landsteiner and Wiener[11] discovered the Rh factor and published their first paper on this subject in 1940. They found that by injecting the red cells of the rhesus monkey into rabbits, an anti-

serum was produced which would agglutinate the red cells of 85% of the human population, while the remaining 15% were not agglutinated. Those persons whose erythrocytes were agglutinated were, therefore, termed Rh positive (85%) and the remaining 15%, Rh negative. Shortly after this discovery it was found that Rh negative individuals could be immunised against the Rh factor or antigen contained in the blood of Rh positive individuals and an agglutinin serologically identical with the anti-rhesus agglutinin was produced. Wiener and Peters (30) described cases in which Rh negative individuals developed anti-Rh iso-agglutinins following repeated transfusion of Rh positive blood. They stated that these iso-agglutinins which they found were not active at 37°C.

Five cases have been studied here in which Rh negative individuals became immunised to the Rh agglutinogen contained in the Rh positive blood with which they were transfused. A summary of these is given in Table III. Case 159 in which titrations were made at close intervals is depicted graphically in fig. 5. In this instance, an anti-Rh iso-agglutinin was shown to be present in the serum by the fifth day after transfusion. By the seventh day a titre of 32 was recorded, and after the ninth day the titre gradually decreased until on the twentieth day after transfusion it was reduced to 2. In the remaining 4 cases the data are not so complete, but there is definite evidence for the development of an anti-Rh iso-agglutinin in the recipient's serum. It is interesting to note that in all cases, except No. 161, the patients had received several previous transfusions. In No. 161, an immune anti-Rh iso-agglutinin was produced following a first

transfusion. In contrast to Wiener and Peters' (30) first cases all these anti-Rh agglutinins were active and indeed stronger at 37°C than at room temperature.

SECTION B.

PRODUCTION OF IMMUNE ISO-AGGLUTININS FOLLOWING TRANSFUSION OF SERUM.

In this series, nine individuals of group O received injections of either group A or group B serum which contained the A or B agglutinogen. The amount of agglutinogen contained in the sera was measured by the inhibition test. The results are recorded in Table IV and figs. 6 and 7.

In the five cases in which group A serum was injected, there was a specific increase in the titre of the anti-A iso-agglutinin and in four out of the five cases in which group B serum was in jected there was a specific increase in the titre of the anti-B iso-agglutinin. One individual of group O (case 1) received two injections of serum at an interval of four weeks, the first of group A and the second of group B. Each injection produced a definite response of the corresponding antibody. Figs. 6 and 7 are graphical representations of cases 2 and 3 which have been chosen as typical examples of the whole series. In both of these there was a steep rise in titre until the peak of the response was reached at 6 and 13 days respectively and then a subsequent diminution. In fig 6 the responding iso-agglutinin is shown well above its prestimulation level as long as thirty-four days after the injection. In fig. 7 the responding iso-agglutinin is a little above normal forty-four days after the injection.

Fig. 8 is the control case and shows the effect on the anti-A and anti-B iso-agglutinin of the injection of group O serum which contains no A or B agglutinogen. It can be seen that there was a slight variation in the titres of both iso-agglutinins, but no appreciable increase in the titre of either. Accompanying the specific response in five cases there was a small rise in the titre of the "other" agglutinin, e.g. in case 3, while the anti-A titre rose from 256 to 16,000, the anti-B titre also increased from 64 to 256. However, in comparison with the specific response, the response of the anti-B iso-agglutinin was small. The only exception to this is case 8 in which the individual received 150 c.c. of group B serum and yet the anti-A titre increased to a greater extent than the anti-B. The reason for this is not understood.

It is interesting to note (see Table IV) that although the injected sera contained varying amounts of agglutinogen as measured by the inhibition technique, there is no apparent correlation between the amount of serum agglutinogen injected and the degree of immune response. In fact an amount of agglutinogen, too small to be detected <u>in vitro</u> by the inhibition technique, produced quite a good response <u>in vivo</u>, (case 9).

This method of injection of serum containing the A and B agglutinogens is an excellent one for studying the antigenic action of the group specific substances A and B in human beings, without harm to the individuals who receive the injections. It is also of value as a means of preparing high titre sera for group mg purposes and in this capacity, after the publication of the above experiments, has recently been adopted by some workers in America.

SECTION C.

PRODUCTION OF IMMUNE ISO-AGGLUTININS AS A RESULT OF PREGNANCY

As mentioned previously, Dienst (3) first demonstrated an increase in the anti-A or anti-B iso-agglutinin in the sera of some women who had been delivered of infants of groups A or B.

In 1936 Jonsson (9) observed that in the sera of group O mothers who had been recently delivered of group A infants there was usually an anti-A haemolysin. Similarly, in the sera of group O mothers, with group B infants, an anti-B haemolysin was present.

Levine, Katzin and Burnham (15) suggested that immunisation of a pregnant woman to an antigen contained in her foetus might explain the presence of atypical iso-agglutinins found in her serum. They also discovered that the majority of atypical iso-agglutinins occurred in Rh negative women with Rh positive infants and gave reactions identical with the anti-Rh sera of Landsteiner and Wiener. (11) Similar observations have been made since by Wiener, (27) Boorman et al. (1) and Race et al. (21)

In the following cases the immune response to the agglutinogens A, B and Rh as a result of pregnancy have been investigated.

1. Immune Anti-A Iso-agglutinins in Cases in which the Mother Was Group O or B and the Foetus group A or AB.

Twenty-nine cases were examined and of these 25 showed a specific increase in the titre of the anti-A iso-agglutinin shortly after the birth of an infant of group A or group AB. A typical example is shown in fig. 9. There was a rapid rise in the anti-A iso-agglutinin titre from 128 on the second day after delivery to the high level of 8,000 on the fifteenth day after delivery. On the

thirtieth day the anti-A iso-agglutinin titre was still far above its initial level. During the whole of this period the fluctuations of the anti-B titre were comparatively small.

Fig. 10 is a summary of the findings in all twenty-five cases in which there was an anti-A response. The degree of immune response in each instance is expressed by the number of times the peak titre is greater than the original titre. This ranges from 4 to greated than 64. It should be pointed out that the original titre may be higher than normal, as there is evidence that the responding iso-agglutinin sometimes increases during pregnancy as well as after delivery. The time of attainment of the peak titre varied between less than five days to more than twenty days after delivery, but the majority occurred between the eleventh and twentieth days.

2. Immune Anti-B Iso-agglutinins in Cases in which the Mother was Group O or A and the Foetus Group B or AB.

In this section 15 cases were examined and of these 11 showed an increase in the titre of the anti-B iso agglutinin following delivery of an infant possessing the B agglutinogen. Fig. 11 is a graphical representation of one of these. The first sample of maternal serum was obtained 7 days before delivery when an anti-B titre of 256 was recorded. This increased rapidly after the birth of the infant and a peak titre of 8,000 was attained on the fourteenth day after delivery. Titrations were made at intervals until 3 months after delivery by which time the anti-B titre had diminished considerably, but was still 1,000. The anti-A titre during this period varied between 256 and 1,000. The results in all 11 cases are summarised in fig. 12. As in the anti-A responses the degree of immune response

ranged from 4 to greater than 64. Some of the peak titres attained were extremely high and they ranged from 512 to over 256,000.

3. Control Cases.

13 cases were studied in which there was no incompatibility between the mother's iso-agglutinin and the infant's erythrocytes. Detailed protocols of these are shown in Table XVI and one of them is depicted graphically in fig. 13. It can be seen that the titres remained extremely steady. In fact there was no variation greater than would be expected with the particular titration technique employed (see Tables 1, 1a, and 1b).

4. Immune Anti-Rh Iso-agglutinins in Cases in which the Mother was Rh Negative and the Foetus Rh Positive.

Immune anti-Rh iso-agglutinins were found in the sera of a large seried of Rh negative women who had recently given birth to an Rh positive infant affected with haemolytic disease of the foetus. A typical example of the response in one of these cases is depicted in fig. 14. On the sixth day after delivery of the infant, when the maternal serum was first tested, the titre of the anti-Rh iso-agglutinin was 64 and by the fourteenth day had risen to 256, which was the highest titre recorded. At the thirtieth day the titre had fallen to 32 and after 3 months an anti-Rh iso-agglutinin was still active at a titre of 8.

79 cases are summarised in fig. 15. The lowest titre recorded was 1 and the highest greater than 4, 000 and the majority of these occurred between the sixth and twentieth days after delivery. As the anti-Rh iso-agglutinin is not present in the serum in the form of a natural antibody, fig. 15a can be regarded as also representing

the degree of immune response. However, this may not be true for all cases, because in some there may have been an Rh antibody present in the serum which had been produced as a result of a previous pregnancy. Usually the anti-Rh iso-agglutinin remained in the maternal serum for some months after the delivery of the infant. In one instance, an antibody was detected as long as 5 years after the birth of the last child.

All the titration values recorded in fig. 15 are those which were obtained at 37°C, because these were usually higher than the values obtained at room temperature. This is well shown in Table V, which summarises the findings in 61 cases in which the anti-Rh iso-agglutinins were titrated both at room temperature and at 37°C. Among these 61 cases, 3 are included in which the anti-Rh iso-agglutinin was stimulated by transfusion of Rh positive blood. In all the cases the titre was found to be greater or at least as great at 37°C than at room temperature. In none of these was the anti-Rh iso-agglutinin inactive at 37°C as in the first cases described by Wiener.

In 9 cases there was an opportunity of studying the changes in titre of the anti-Rh iso-agglutinin during pregnancy as well as after delivery (Table VI and fig. 16). In 3 of these, (nos. 71, 109 and 157) there was no increase in titre during pregnancy or after delivery, although the infant, when born, was found to be Rh positive . The reason for this is not clear. It is possible that in case 71 the peak of the response was missed as no sample of serum was obtained between the second and twelfth days after delivery. Similarly, in case 113, in which the serum was not titrated later than 7 days after delivery, the peak of the response might not yet have been attained.

The two cases (82 and 142), which exhibited an increase in titre of the anti-Rh iso-agglutinin after pregnancy, are the only two in which there was also an increase in titre during pregnancy. The significance of this, however, cannot be determined from such a small series.

5. Immune Anti-Rh_2 Iso-agglutinins in Cases in which the Mother is Rh positive.

Not long after the discovery of the Rh factor, it was realised that this factor was not as simple as at first supposed. Wiener in 1941 (27) found a human serum which agglutinated only 70% of bloods instead of the usual 85%. Rh negative bloods did not react with the new serum and the 70% which did so were said to belong to sub-type Rh_1

In 1943 Race et al. (20) found another type of anti-Rh agglutinin which occurred in the serum of the mother of an infant suffering from haemolytic disease of the foetus. This agglutinin, which reacted with 30% of bloods was called anti-Rh_2. This serum, the first of its type to be discovered and 5 other similar sera found later are described briefly below.

In all 6 cases both mother and infant were Rh positive, but each belonged to a different Rh sub-group. Without exception, the blood of the infant contained the Rh_2 antigen (inherited from the father), and the mother, lacking this antigen, became immunized to it and an anti-Rh_2 agglutinin was formed in her serum. The highest titration values of the anti-Rh_2 agglutinins found in the 6 cases were 256, 128, 16, 16, 8, and 1,000 respectively. The chief interest of the finding of these anti-Rh_2 antibodies lies in the fact that they show that it is possible to obtain immunization within the sub-groups of Rh owing to the fact that there is more than one Rh antigen and, therefore, examination of the sera of Rh positive as well as Rh negative individuals will sometimes

reveal the presence of Rh antibodies.

SECTION D.

IMMUNE ANTI-Rh ISO-AGGLUTININS IN Rh NEGATIVE WOMEN PRODUCED AS A RESULT OF AN Rh POSITIVE BLOOD TRANSFUSION AND AN Rh POSITIVE FOETUS.

The immune anti-Rh iso-agglutinin in these cases was produced as a result of a double stimulation which was caused by an Rh positive foetus and an Rh positive blood transfusion. The titres of the anti-Rh iso-agglutinins found are shown in Table VII. In spite of the double stimulation, the degree of immune response is not remarkable. The highest titre recorded is 128.

In cases 134, 153, and 154, the titres found were not greater than 2. In order to demonstrate the significance of titres of 2, the details of the titres found in case 153 are shwon in Table VIIa. It is apparent from these that although the titration value is small there was very definite agglutination of the Rh positive red cells. Therefore it is legitimate to conclude that an Rh antibody was present in the maternal serum. Moreover in all the cases shown in Table VII the infant suffered from haemolytic disease of the newborn - a disease associated with the presence of Rh agglutinins in the maternal serum.

Case 138 is of particular interest becayse an anti-Rh iso-agglutinin did not appear in the serum until 42 days after the delivery of the infant. The reason for this is to be discovered in the fact that the transfused Rh positive erythrocytes were surviving in the circulation for a long time after the transfusion and did not entirely disappear until just after the anti-Rh iso-agglutinin was detected.

SECTION E.

THE ACTIVATION OF IMMUNE ISO-AGGLUTININS BY A FACTOR CONTAINED IN HUMAN SERUM

While making preliminary experiments to determine whether human complement plays any part in the specific agglutination of immune sera, an interesting phenomenon was observed. The titration value of the immune iso-agglutinin chosen for the experiment was noticed to be considerably increased when human serum instead of saline was used as a diluent for the titrations. The protocol of this finding is shown in Table VIII. Example (1) of this table demonstrates that the titration value of the immune iso-agglutinin when serum is the diluent is 1,000 against a value of 64 when the same iso-agglutinin is titrated in saline. Example (2) shows comparative titres of another immune iso-agglutinin in saline and serum diluents. In this instance, titrating in serum has not increased the length of the titration,)the titration value being 4,000 in both saline and serum), but there is, however, a tightening of the agglutination in some of the dilutions of the serum titration, e.g. at a dilution of 1,000 the agglutination in saline is only visible microscopically and is fairly weak [(+)], whereas at the corresponding dilution made in serum the agglutination is clearly visible macroscopically (V). When the same experiment was carried out, using a naturally-occurring or non-immune antibody (Table VIII, example 3), there was no appreciable difference between the titrations in saline and in serum. When the serum to be used as a diluent was heated at 56°C for half-an-hour, its amplifying property was still retained.

From these first experiments, therefore, it seemed probable that a

method for distinguishing between immune and natural antibodies had been discovered. Further experiments were carried out with this end in view.

Methods.

Tests performed with several different human sera showed that they each possessed the power to increase the titration value of an immune anti-serum, although to a varying degree. It was, therefore, decided that before extending the investigation further, standard sera must be chosen for use as diluting fluids. In order to select satisfactory sera it was necessary to consider three points:-

(a) The amplifying power of the serum. Different sera were found to vary markedly in their power to increase or amplify the strength of an iso-agglutinin titre. Therefore, for the purpose of selecting standard sera with good amplification properties, an anti-Rh agglutinin was titrated in many sera of groups A, B and AB. The amplification found in each case was expressed as an index similar to the inhibition index. Thus, if a given serum increased the strength of the immune serum eight times, the amplification was said to be 8. As shown in Tables IX and X, there was a very wide variation of amplification, ranging from 0 to 64.

(b) The ABO group of the serum. When erythrocytes

of group O were used for titration purposes, (e.g. for titration of anti-Rh sera), the diluting serum could be chosen irrespective of its ABO group, but for titration of anti-A and anti-B sera obviously it was essential to take the ABO group of the diluting serum into account. Anti-A agglutinins were titrated in a standard serum of group A and anti-B agglutinins in a standard serum of group B. In many cases both anti-A and anti-B agglutinins were titrated in a serum of group AB.

(c) The inhibition index of the serum.

The fact that all or almost all sera of groups A, B and AB contain varying quantities of soluble A or B agglutinogen has been discussed above. (Section B). Since this soluble serum agglutinogen would in part neutralise the amplification effect when immune anti-A and anti-B sera were titrated in sera of groups A and B respectively, standard sera with no inhibition index were selected for these experiments. This precaution minimised but did not entirely elimate the neutralising action of the serum agglutinogen, because even sera showing no inhibition index contain small quantities of agglutinogen. It must, therefore,

be borne in mind, when assessing the increase in titre of anti-A and anti-B agglutinins in serum, that the amplification would be even greater were it possible to elimate entirely the neutralising effect of the serum agglutinogen. The amplification phenomenon takes place in spite of the presence of serum agglutinogen in the serum diluent.

Titration of Agglutinins in Saline and in Serum.

1. Anti-Rh.

49 anti-Rh sera were titrated in saline and in serum (No.11 table 10) using the technique already described and the titration values compared. (Table XI). In all but three cases these were higher in serum than in saline, the amplifications varying from 2 to 32. A comparison of the size of the agglutinates at the same dilution in the two diluents almost invariably showed them to be larger and firmer in serum. This seemed to be a characteristic and fairly constant phenomenon and was displayed even in the cases where no actual increase in the titre could be demonstrated, (see Table VIII, example 2).

Several anti-Rh sub-group sera were tested in saline and in serum in the same manner. All of these (Table XII) showed a definite increase in titre in the serum diluent.

2. Immune and Natural Anti-A and Anti-B.

9 anti-A and anti-B agglutinins which were natural antibodies were tested. None of these showed any increase in titre in serum,

nor was there any difference in the degree of agglutination at the same dilution in serum and saline.

The same test was next carried out using immune anti-A and anti-B agglutinins. 13 of these were produced by stimulation due to a foetus in utero and 2 by injections of A group specific substance (Loutit and Morgan, 1945, unpublished experiments). In most cases the individuals belonged to group O and their sera contained in addition to an immune agglutinin, a non-immune agglutinin which formed an excellent control. Table XIII records the values obtained. In all but case 3 the immune agglutinin was anti-A and it can be seen that the titration value of this agglutinin was nearly always markedly increased in the serum diluent. In case 7, although the amplification was 0, the characteristic tightening of the agglutination in serum was observed. Although in all these cases the titre of the anti-B, which was the non-immune agglutinin, showed no increase in serum. In three cases (5,12, and 15) it was inhibited. The reason for this is not known. It might be accounted for by the presence of B group specific substance in the diluent serum but this is not a likely explanation since other anti-B agglutinins were not affected in the same way, and also the diluent serum was chosen because it had no inhibiting effect on an anti-B titre. The anti-B titration in case 15 was repeated on a subsequent occasion and the result was found to be unaltered.

In case 3, in which the anti-B was the immune agglutinin, there was an amplification of its titre in the serum diluent.

A detailed protocol of one of the individuals of group O who received injections of A group specific substance is shown in Table XIV. An examination of the results given for the anti-A agglutinin

reveals no appreciable difference between the titres in the two diluents up to the third day. By the eighth day after the injection of the A substance there was a very definite tightening of the agglutination in the serum diluent and the amplification was 2. No such tightening of agglutination was present in the corresponding anti-B titration. By the eleventh day after the injection a very striking difference between the saline and serum titrations of the anti-A agglutinin was apparent and an amplification of 8 was recorded. This is to be expected if the agglutinin activating substance in serum increases the activity of immune agglutinins only and does not affect those which are non-immune. By the eleventh day after the injection the production of immune anti-A agglutinins is usually near its peak, therefore the maximum difference between the saline and serum titrations will be at this time.

3. Immune and Natural α Agglutinins.

3 natural and 2 immune α_1 agglutinins were titrated in saline and serum. 2 of the 3 natural agglutinins were found in the sera of persons belonging to group A_2B and the third was prepared from the serum of a group B individual by absorption with red cells of sub-group A_2. None of these α_1 agglutinins showed any increase in titre in the serum diluent, (see Table XV).

Of the 2 immune α_1 agglutinins, one was produced by stimulation caused by foetus in utero and the other by transfusion of A_1 blood to an individual of sub-group A_2. Both these immune α_1 agglutinins, in contrast to the natural α_1 agglutinins, showed a definite increase in titration value in the serum diluent.

DISCUSSION.

From the above results it is apparent that the agglutinating power of some agglutinins is enhanced if they are titrated in a serum instead of a saline diluent. In some cases very great amplification of titre was found (e.g. Table XI nos. 3, 16-19) although in many others the amplification was small. In table XI 15 out of the 49 examples shown have amplifications of 2 only.) Nevertheless these slight amplifications are considered to be significant because in practically all cases they were accompanied by a tightening and increase in size of the clumps of cells which, as has been mentioned, above, was an almost constant phenomenon when these agglutinins were titrated in a serum diluent.

The only reference to an agglutinin activating substance in human serum to be found in the literature is that of Waaler.(26) He reports the occurrence of a factor in human serum capable of activating the specific agglutination of sheep blood corpuscles. A small quantity of human serum was added to a series of dilutions of anti-sheep corpuscle serum with the result that the agglutination titre increased many times, the actual figures given being 2,000 without human serum and 64,000 with .01 c.c. of human serum added to each tube. Waaler did not correlate the phenomenon with immune antibodies and apparently confined his attention to animal sera. It seems likely, however, that the factor which causes the increase in agglutinating power of human immune sera is identical with that described by him.

Most human sera seem to contain this factor, although in amounts

which vary considerably from individual to individual. Very little is known about the nature of the factor. That the amplifying power of serum is not destroyed by heating at 56°C. for half an hour proves that it cannot be complement as a whole, nor one or more of its heat labile fractions. This is confirmed by the fact that the property is retained even after the serum is stored frozen solid for many months.

The increase in titre in the serum diluent was not caused by the extra viscosity of the serum, otherwise the titre of naturally occurring antibodies would also be greater in the serum diluent than in saline. The differentiation between immune and naturally occurring agglutinins is probably the most important point which emerges from the work in this section. It would seem to indicate a definite qualitative difference between immune and non-immune agglutinins.

COMPARISON BETWEEN IMMUNE RESPONSES TO THE AGGLUTINOGENS A, B AND Rh.

Although there are points of dissimilarity between the immune responses produced by the blood group factors A, B and Rh, the response to each antigen conforms with the same general pattern. In every instance immune iso-agglutinins are produced in the serum which rapidly increase in titre until the peak of the response is attained between 5 and 20 days after blood transfusion or delivery. The disappearance of these immune iso-agglutinins from the serum is usually gradual and they may be found months or even years after the immune response has been started. All three antigens stimulate the production of antibodies which are active at 37°C, but whereas this temperature is the optimum for the activity of the anti-Rh iso-agglutinin, the anti-A and anti-B iso-agglutinins are more active at lower temperatures.

The A and B agglutinogens almost always seem to cause an immune response when injected into the appropriate individuals. In all cases in which there was a blood transfusion of incompatible ABO group there was an increase in titre of either the anti-A or anti-B iso-agglutinin or both. Only one case in the seried of serum injections failed to respond and 36 out of 44 cases, in which the stimulation was due to an A or B foetus in utero, showed an increase in titre of the corresponding anti-A or anti-B iso-agglutinin. On the other hand, a first stimulation by the Rh agglutinogen rarely evoked an immune response. In 4 of the 5 cases examined in which Rh antibodies were produced following transfusion of Rh positive blood in Rh negative individuals, there had been several previous transfusions. Similarly,

out of more than 100 pregnancy cases investigated, only 4 were found in which anti-Rh iso-agglutinins were produced as a result of a first pregnancy. The above facts lead one to the conclusion that the A and B agglutinogens are more strongly antigenic in man than the Rh. The production of immune anti-A and anti-B iso-agglutinins, however, may be facilitated because, in contrast to the anti-Rh iso-agglutinin, these iso-agglutinins are already present in the serum before the immune response takes place. A more complete knowledge of the antigenicity of the blood group factors in man could be obtained if it were possible to immunise deliberately a large number of individuals to these factors. Up to the present time the optimum doses of an agglutinogen necessary to produce the optimum response and the time interval at which these doses should be given have not been investigated.

THE CLINICAL SIGNIFICANCE OF ISO-IMMUNISATION IN THE HUMAN SUBJECT.

A detailed account of the clinical significance of iso-immunisation is beyond the scope of this work, but owing to its great interest and practical importance a brief summary is included here.

The formation of immune iso-agglutinins following blood transfusion may lead to serious or fatal reactions. If an immune iso-agglutinin has been produced as a result of one transfusion, and a further transfusion of the same kind of blood is given, an incompatible transfusion reaction is likely to occur. Moreover, the reaction will be of greater severity if the time of the blood transfusion coincides with the time of the peak of the immune response. In order to minimise this danger the donor's erythrocytes should be cross-matched with the recipient's serum at 37°C, before each transfusion. This is particularly important if several transfusions are given at intervals, for by this method immune antibodies which may appear after any one of the transfusions are likely to be detected.

The most important aspect of immunisation as a result of pregnancy is the effect of the immune iso-agglutinins on the clinical condition of the infant. It is now established (Levine et al.)(15) that haemolytic disease of the foetus or newborn is caused by immune antibodies produced in the maternal serum which are incompatible with the infant's erythrocytes. In the majority of cases it has been found that the antibody responsible is the anti-Rh and that the typical serological picture associated with the disease is:-

Mother Rh negative (with anti-Rh iso-agglutinins in her serum)

Father Rh positive

Infant Rh positive

The way in which the Rh agglutinogen of the foetus enters the maternal circulation is not clear. It has been considered, (Wiener, Blood groups and Transfusion), (29) that the infant's erythrocytes are able to cross the placenta, thus entering the maternal circulation and thereby stimulating the production of anti-Rh antibodies. In a recent publication Wiener[32] contends that Rh substance is present in a soluble form in amniotic fluid. If this is so, it is possible that this soluble substance might cause the sensitization in the mother.

77 of the 79 families studied in section C (3) [fig.17] are arranged diagrammatically in groups according to the peak titre of the anti-Rh iso-agglutinin found in the maternal serum after the birth of the last infant. Each narrow strip represents one family and is sub-divided according to the number of children in the family. The children are arranged in chronological order and their clinical condition at birth is indicated as shown in the key. The arrangement of families in this manner reveals the following points of interest:-

(a) The majority of first infants are unaffected with haemolytic disease. In fact in the entire series only 4 families were found in which the first infant was definitely affected. This indicates that it is usually necessary for the mother to receive more than one Rh positive stimulus in order to become immunised to the

Rh agglutinogen. In this series of families just over half of the siblings were Rh typed. Of these only 2 were found to be Rh negative, and as indicated in fig. 17 both were of the same family. This is no doubt correlated with the fact now established by Taylor and Race (24) that in families in which haemolytic disease of the foetus occurs, most of the infants are Rh positive.

(b) Once an affected infant has been born, most of the subsequent children in the family will also be affected with haemolytic disease.

(c) There is no direct correlation between the titre of the anti-Rh iso-agglutinins in the maternal serum and the number of stimuli the mother receives. This is true whether all Rh positive infants are regarded as stimuli or only those affected with haemolytic disease. This point is illustrated by the similarity between the group of families in which the titre of the anti-Rh iso-agglutinin was 2 and the group in which the titre was 256; also by the fact that the highest titre of all was found in a family in which there were only 2 children, the first of which was normal and the second affected with haemolytic disease.

It is extremely important when it becomes necessary to transfuse Rh negative mothers whose sera contain Rh iso-agglutinins, that they should receive Rh negative blood. If Rh positive blood is given, not only is there a risk of causing a serious transfusion reaction, but there is also a great possibility of the Rh positive blood acting as a further stimulus for the production of immune anti-Rh iso-agglutinins. It is obvious that this would be undesirable. Ideally, every Rh negative woman of child-bearing age when in need of a transfusion, should receive Rh negative blood in order to avoid sensitisation to the Rh agglutinogen.

SUMMARY.

Iso-immunisation to the blood group factors, A, B and Rh have been studied in the human subject.

The immune anti-A, anti-B and anti-Rh iso-agglutinins which were produced as a result of the immunisation were titrated at intervals during the immune response.

The agglutinogens which were responsible for the production of these immune iso-agglutinins were contained in (a) transfused erythrocytes, (b) transfused serum, (c) a foetus in utero.

(a) Transfused erythrocytes.

Ten cases have been studied in which immune iso-agglutinins were produced as a result of an incompatible blood transfusion. In five of these cases immune anti-A or anti-B iso-agglutinins were demonstrated in the serum of the recipient. In the remaining five cases the recipients were Rh negative and immune anti-Rh iso-agglutinins were found in their sera. Two cases are described in which anti-A_1 iso-agglutinins, active at 37°C, were formed in individuals of groups A_2 and A_2B in response to transfusion of A_1 blood.

(b) Transfused serum

In this series immune anti-A and anti-B iso-agglutinins were produced as the result of the transfusion of serum containing varying amounts of soluble A or B agglutinogens.

(c) A Foetus in utero

Evidence of iso-immunization, due to pregnancy, was obtained in 118, cases. Of these, 25 immune iso-agglutinins

were anti-A, 11 anti-B, 79 anti-Rh and 3 anti-Rh_2. Control cases were included in each group

A section describing a factor contained in human serum which causes increase in titre of immune agglutinins is included.

The outstanding features of iso-immunization to the blood group factors A, B, and Rh have been discussed and a summary of the clinical significance of iso-immunization is given with particular reference to the important role of the anti-Rh iso-agglutinin in haemolytic disease of the foetus.

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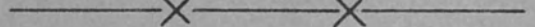
KEY TO FIGURES

1, 2, 5, 6-7-8-9, 11-13-14, & 16.

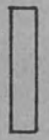
ANTI-A



ANTI-B

ANTI-Rh

KEY TO FIGURE 17



= INFANT- NORMAL.

= INFANT- WITH PHYSIOLOGICAL JAUNDICE

= INFANT- STILL-BORN.

= INFANT- AFFECTED WITH HERMOLYTIC DISEASE OF THE FOETUS.

Fig. 1.

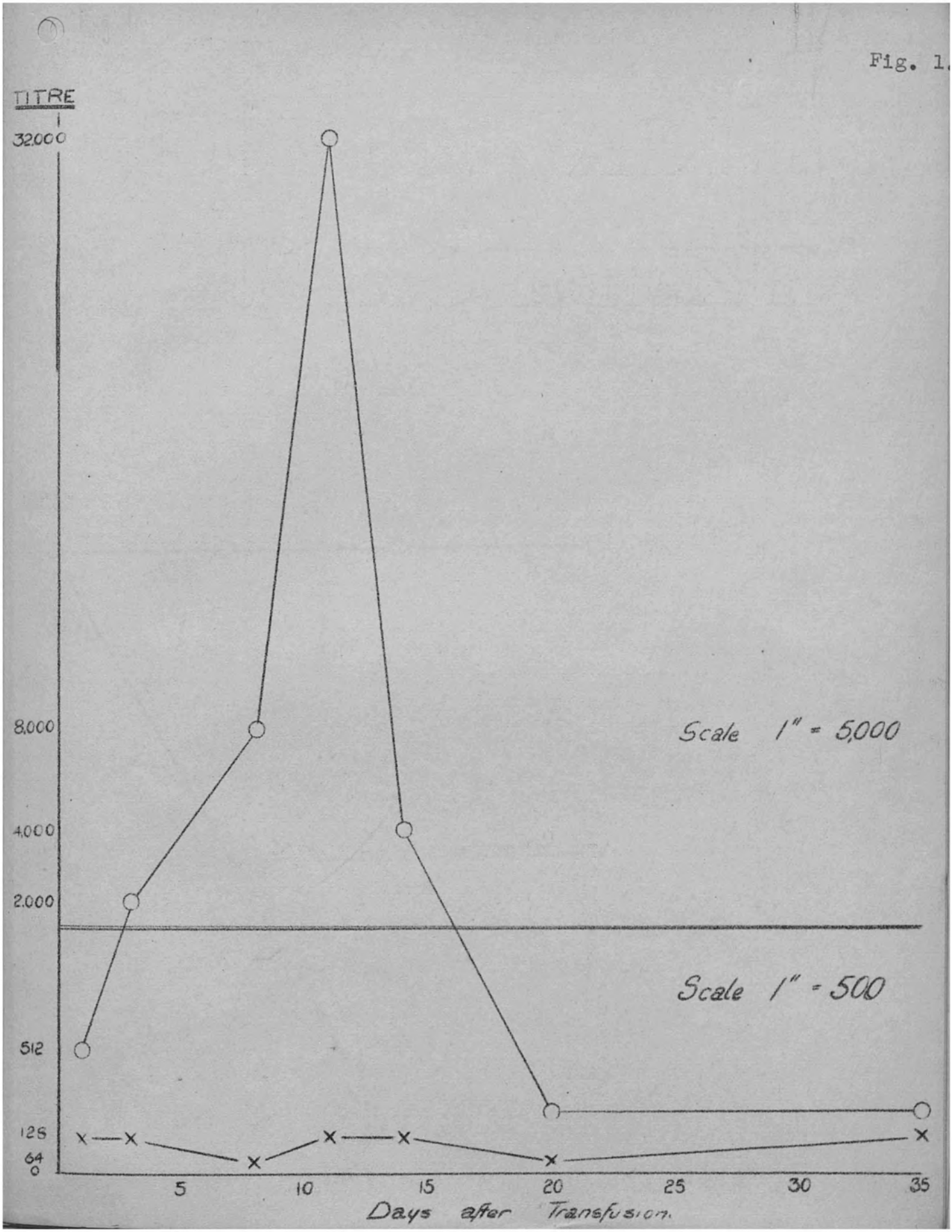

TITRE
32,000
8,000
4,000
2,000
512
128
64
0
Scale 1" = 5,000
Scale 1" = 500
5
10
15
20
25
30
35
Days after Transfusion.

Fig 2.

Fig. 2

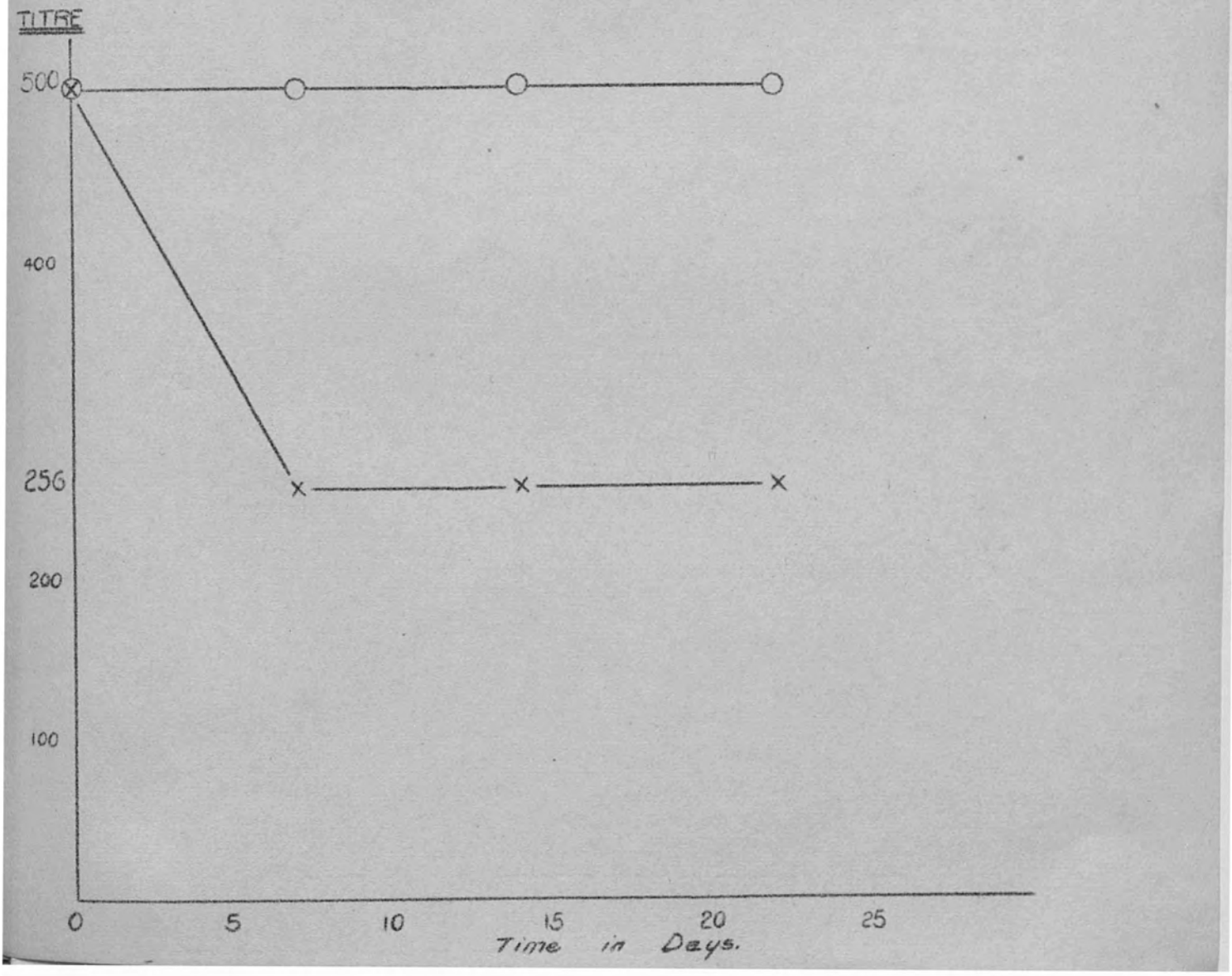


Fig. 3.

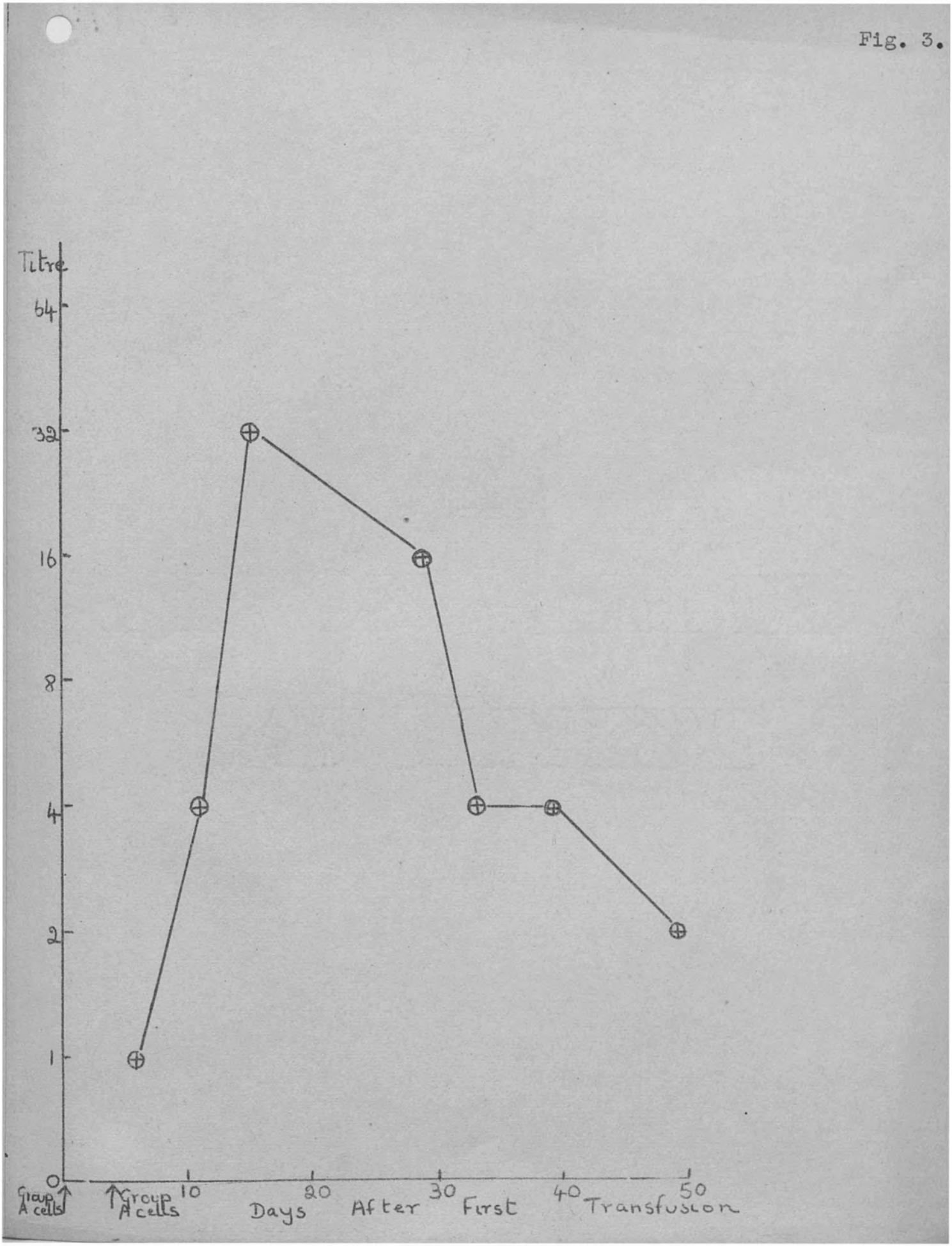

Titre
64
32
16
8
4
2
1
0
Group A cells
Group A cells
10
20
30
40
50
Days After First Transfusion

Fig. 4.

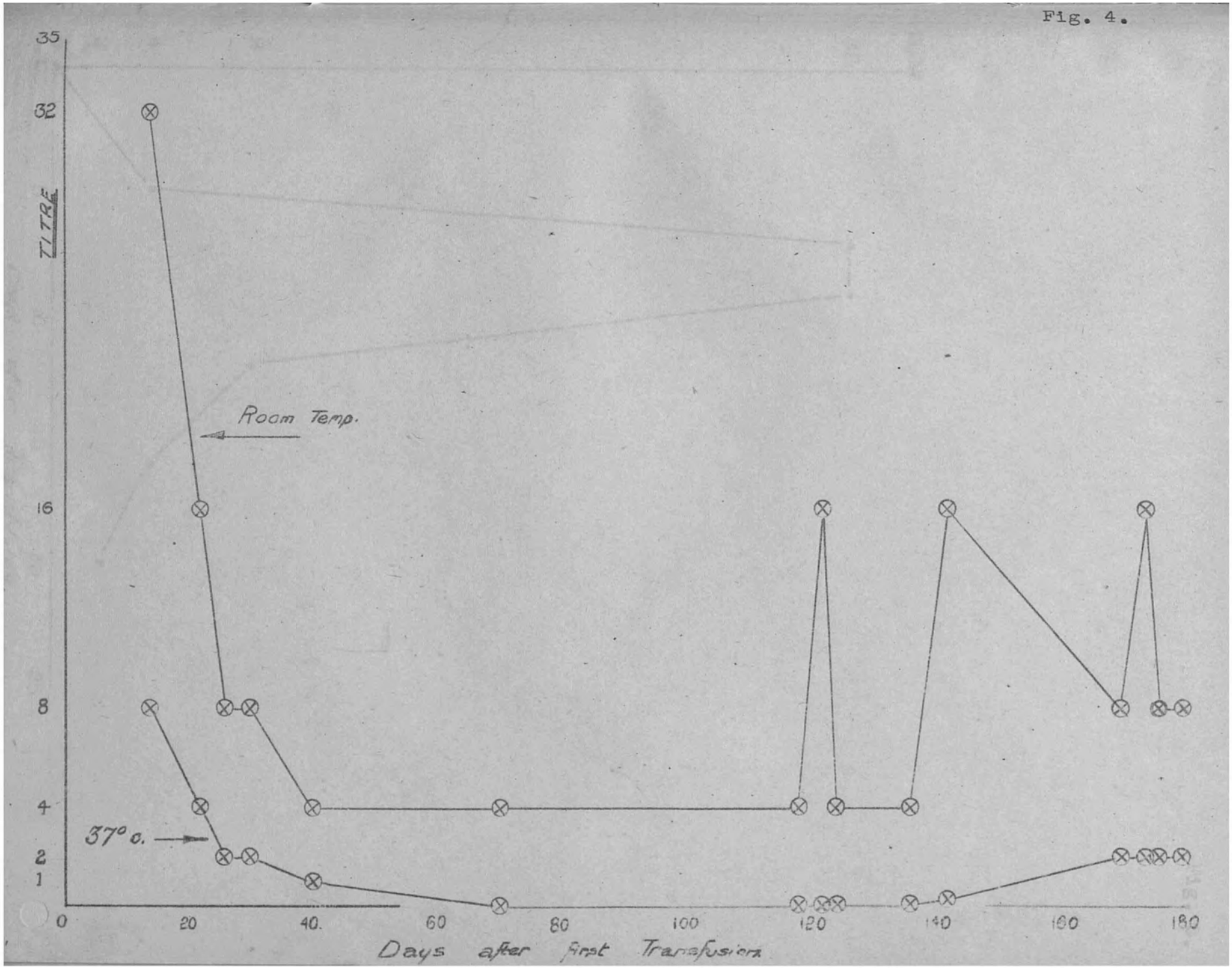

35
32
TITRE
16
8
4
2
1
Room Temp.
37° c.
0
20
40.
60
80
100
120
140
160
180
Days after first Transfusion

Fig. 5.

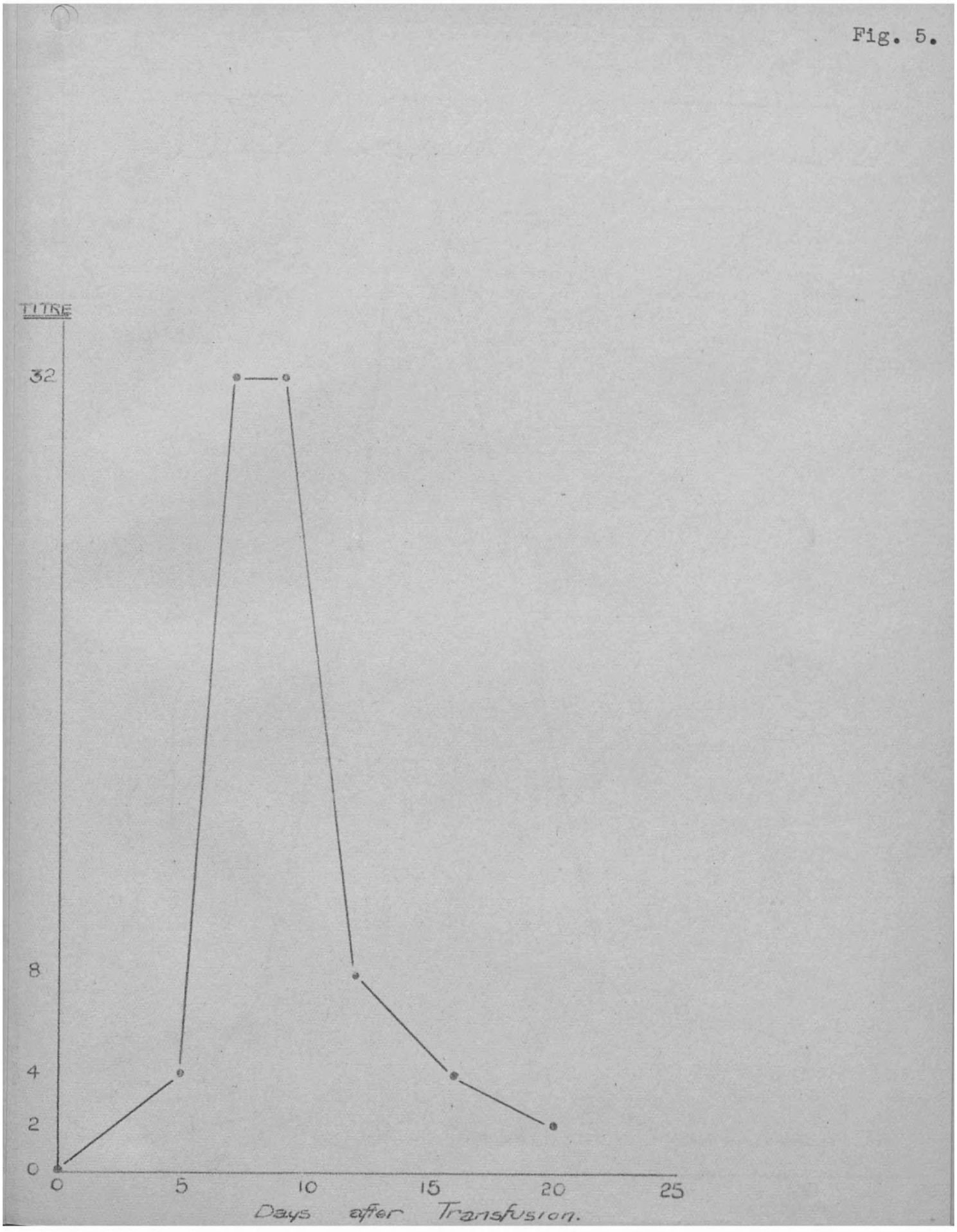


Fig. 6.

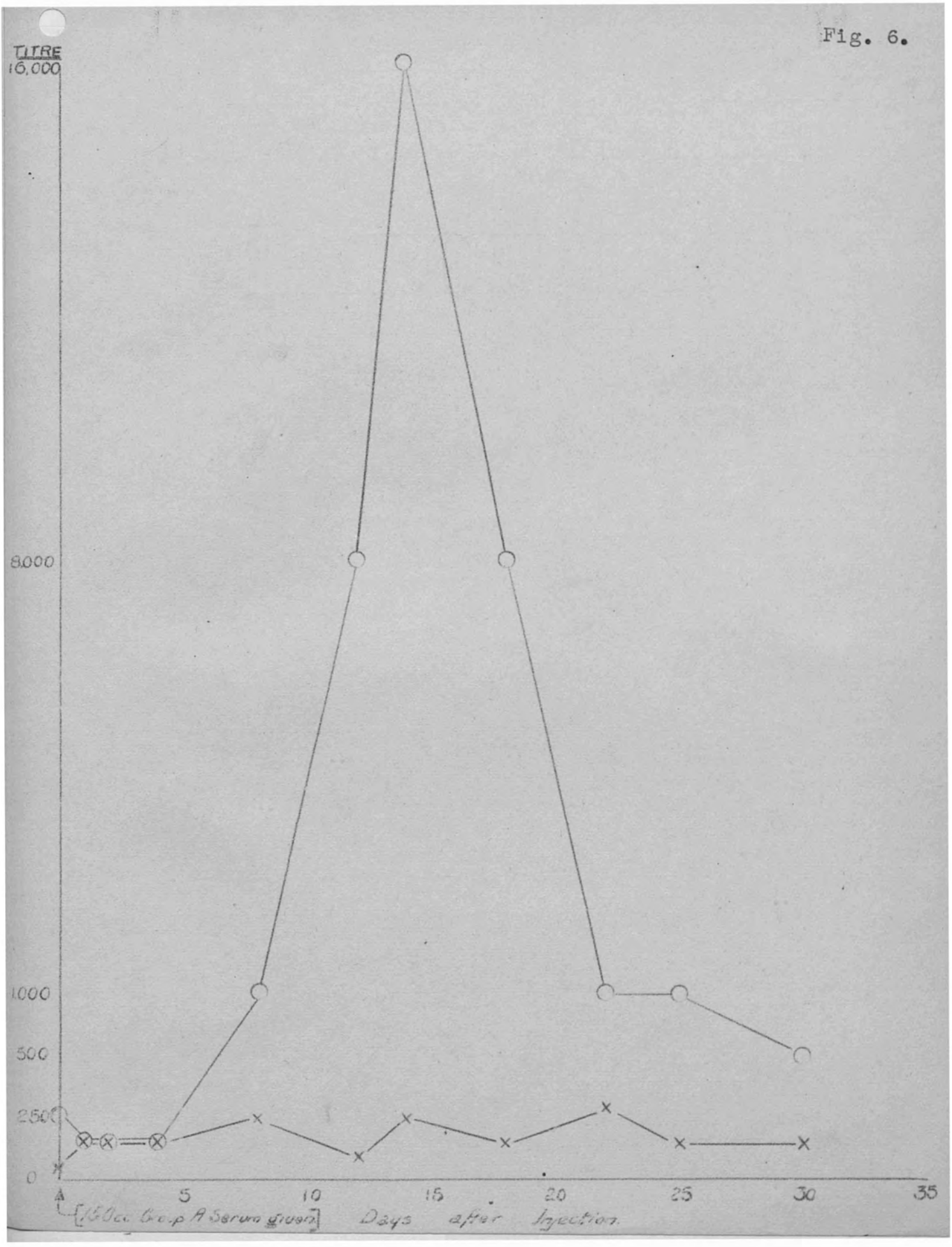

TITRE
16,000
8,000
1,000
500
250
0
A
5
10
15
20
25
30
35
[150 cc. Grp A Serum given]
Days after Injection.

Fig. 7.

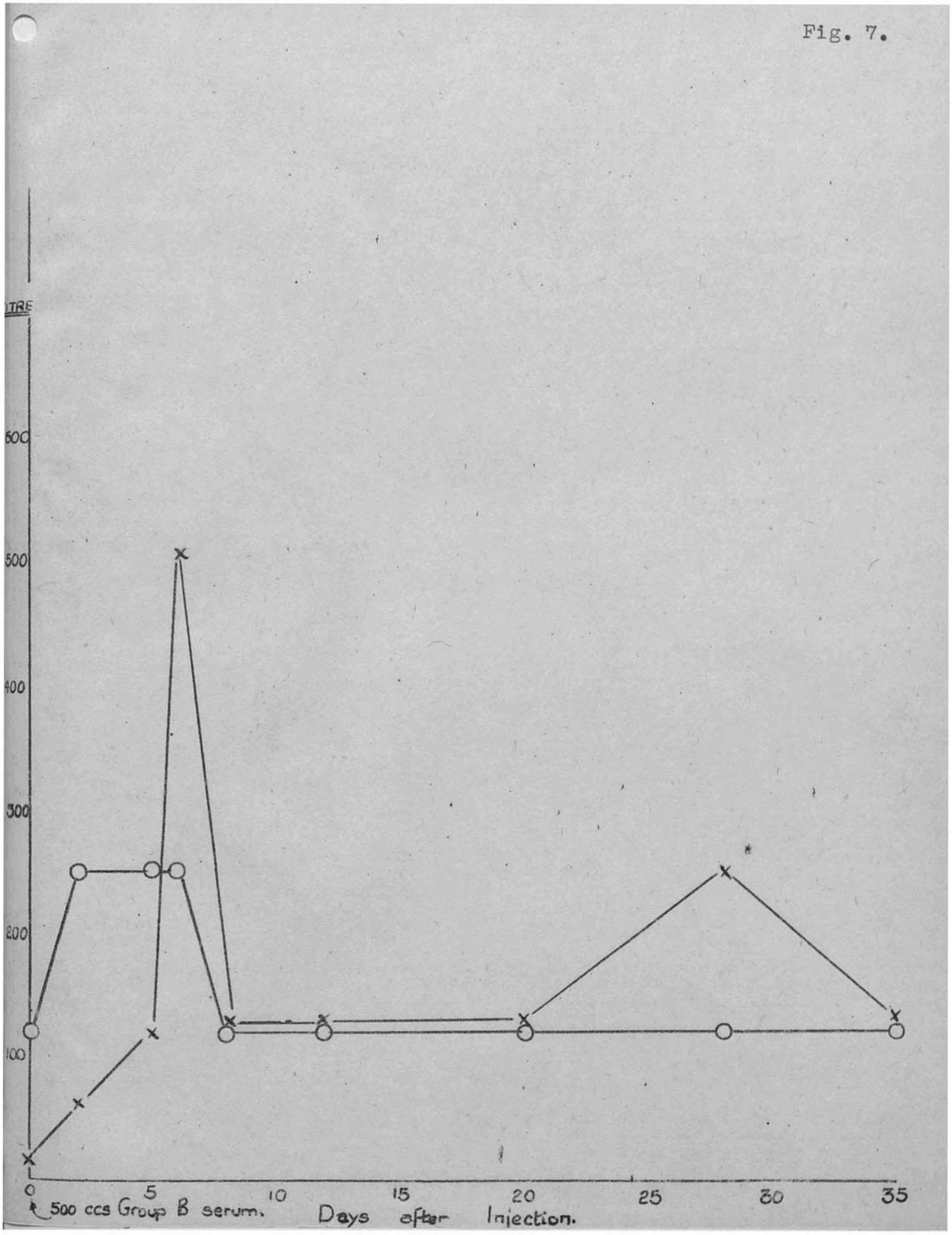

ITRE
600
500
400
300
200
100
0
5
10
15
20
25
30
35
500 ccs Group B serum.
Days after Injection.

Fig. 8.

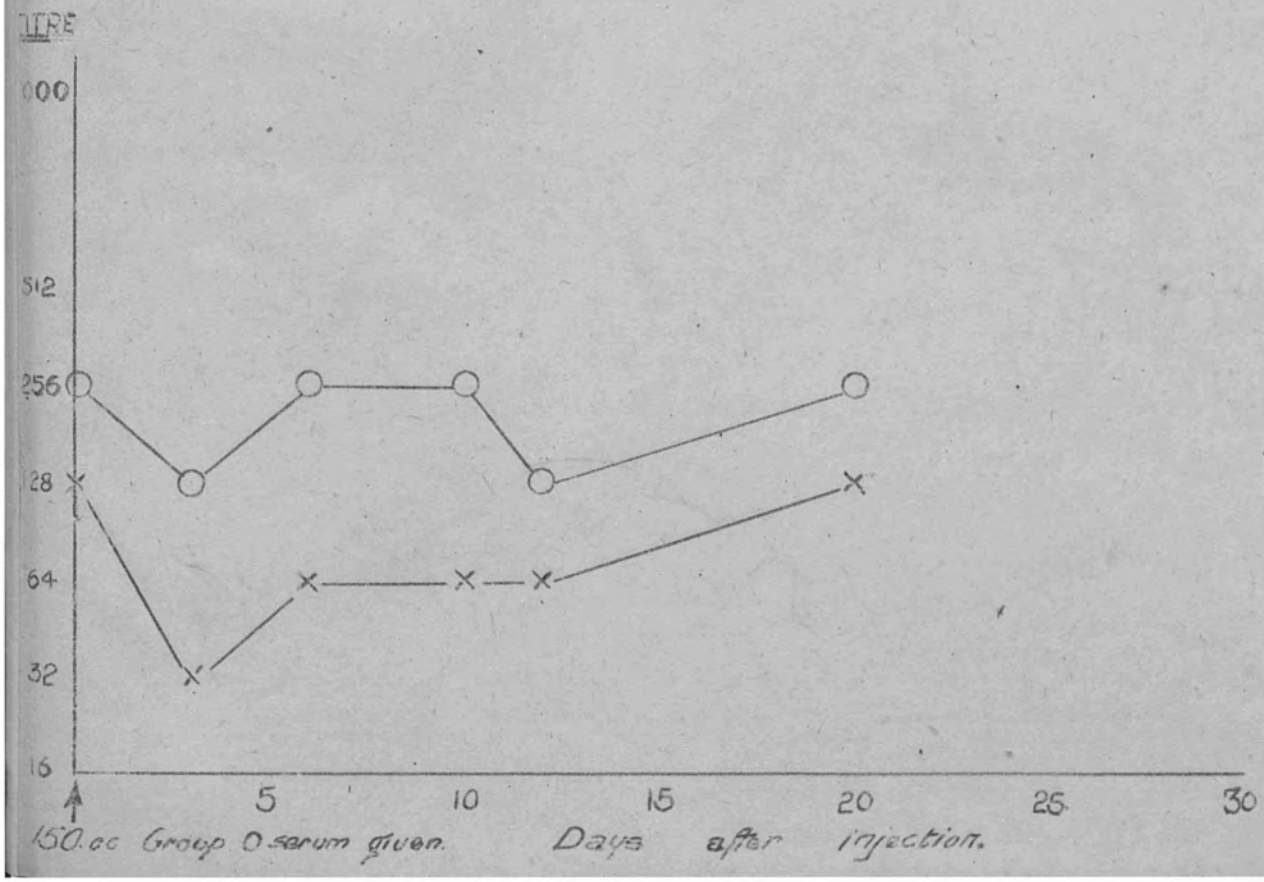

TITRE
000
512
256
128
64
32
16
5
10
15
20
25
30
150 cc Group O serum given.
Days after injection.

Fig. 9.

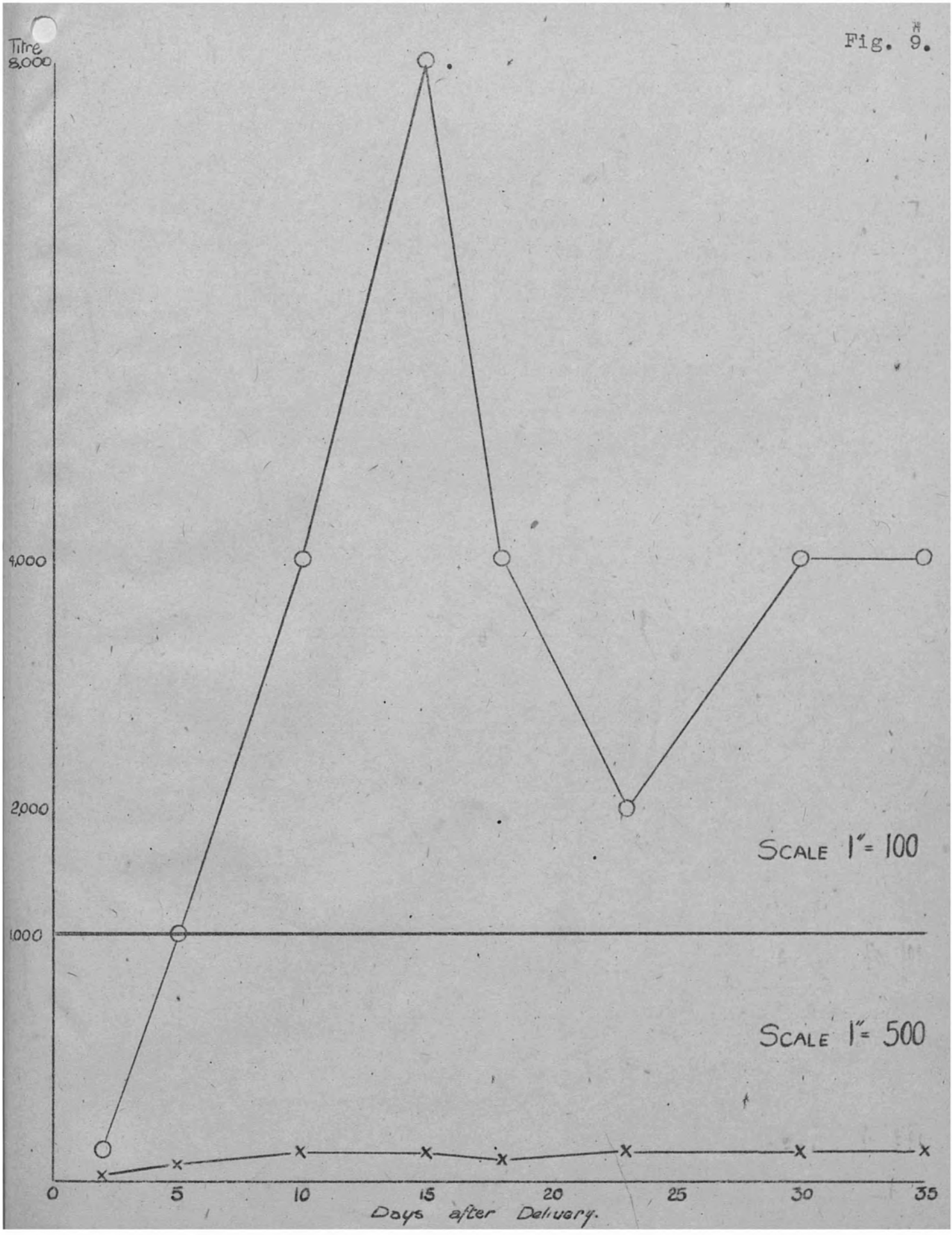

Titre
8,000
4,000
2,000
1000
SCALE 1" = 100
SCALE 1" = 500
0
5
10
15
20
25
30
35
Days after Delivery.

Fig. 10

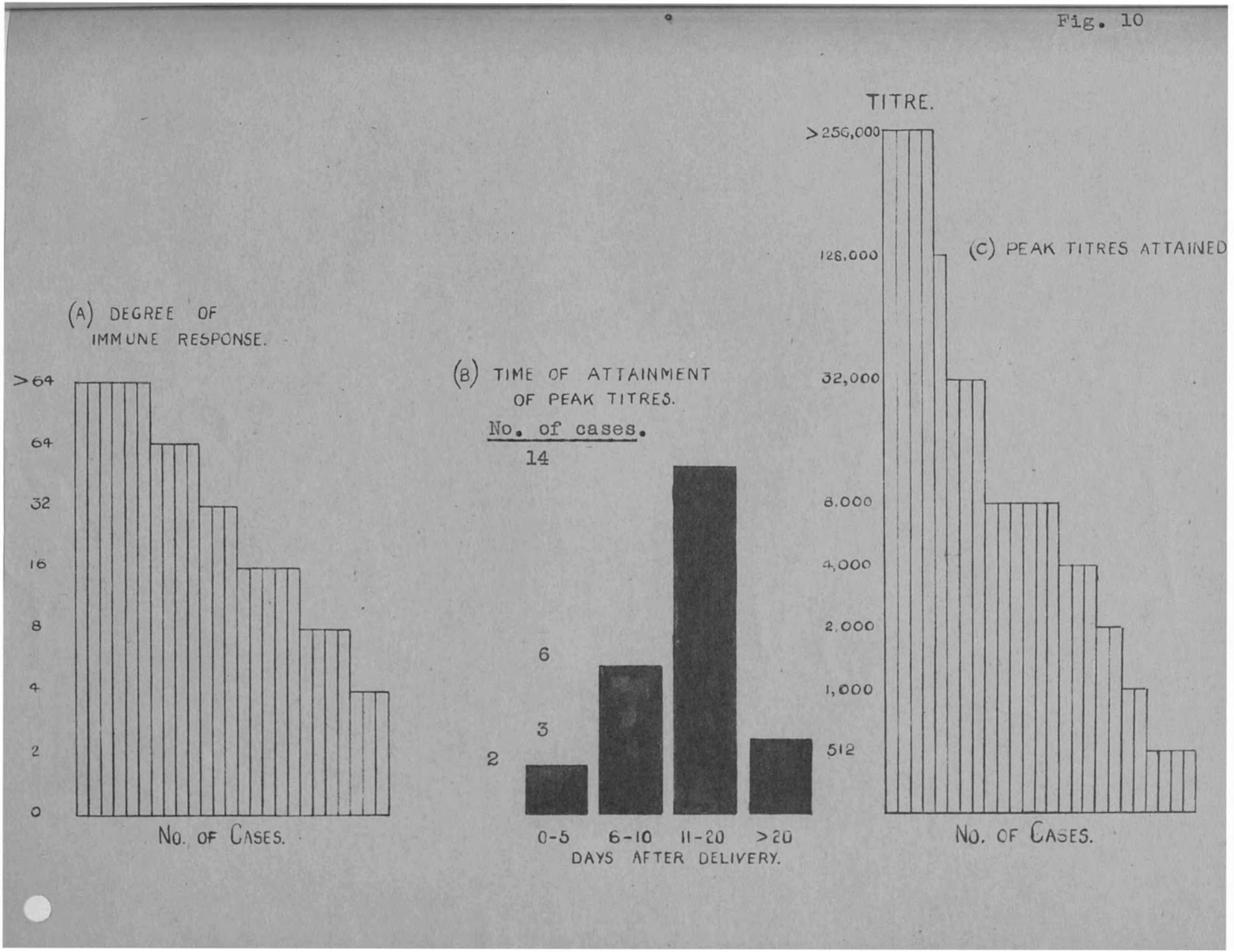

(A) DEGREE OF IMMUNE RESPONSE.
>64
64
32
16
8
4
2
0
No. of Cases.
(B) TIME OF ATTAINMENT OF PEAK TITRES.
No. of cases.
14
6
3
2
0-5
6-10
11-20
>20
DAYS AFTER DELIVERY.
TITRE.
>256,000
128,000
32,000
8,000
4,000
2,000
1,000
512
(C) PEAK TITRES ATTAINED
No. of Cases.

Fig. 11.

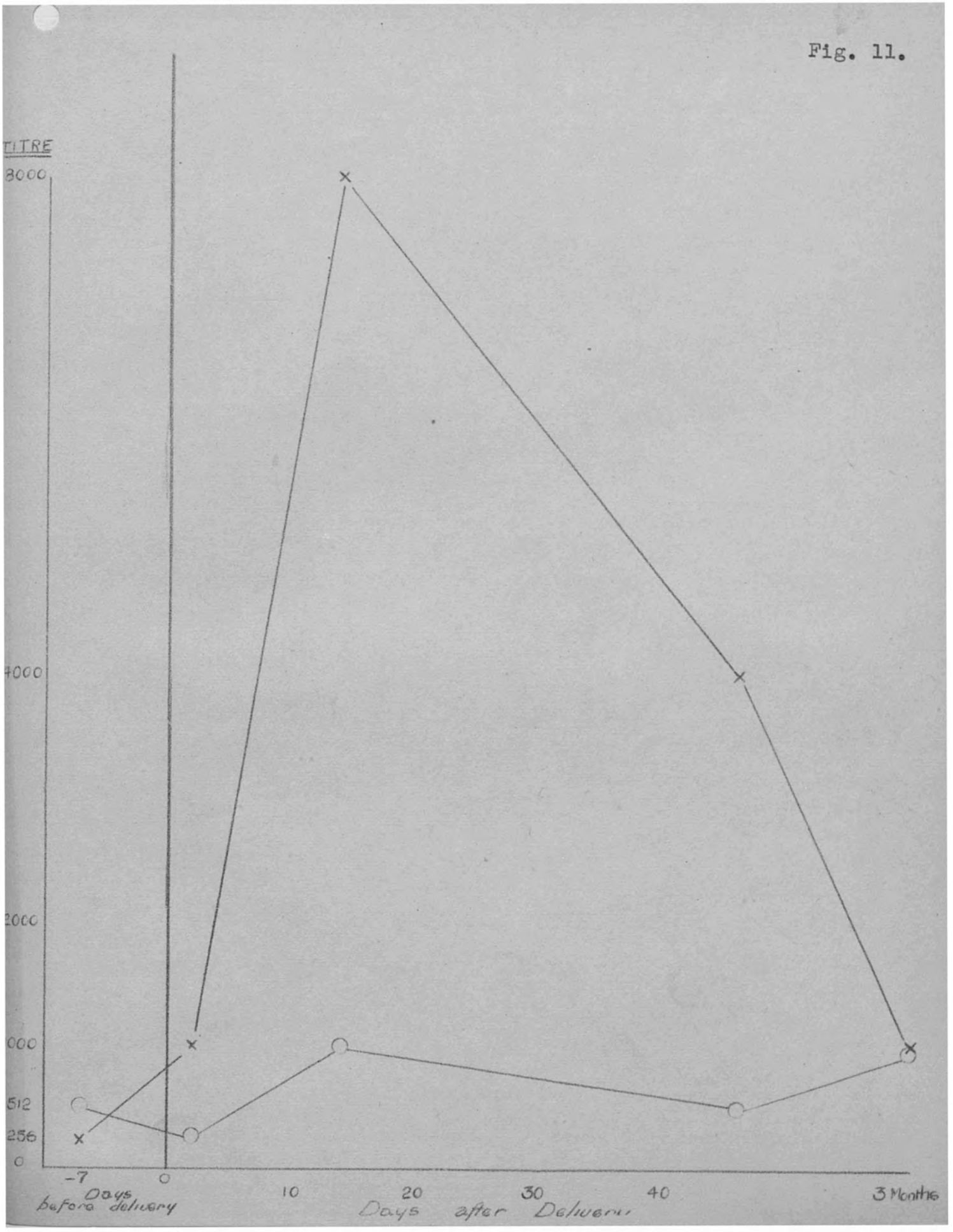

TITRE
8000
4000
2000
000
512
256
0
-7
0
10
20
30
40
3 Months
Days before delivery
Days after Delivery

Fig. 12

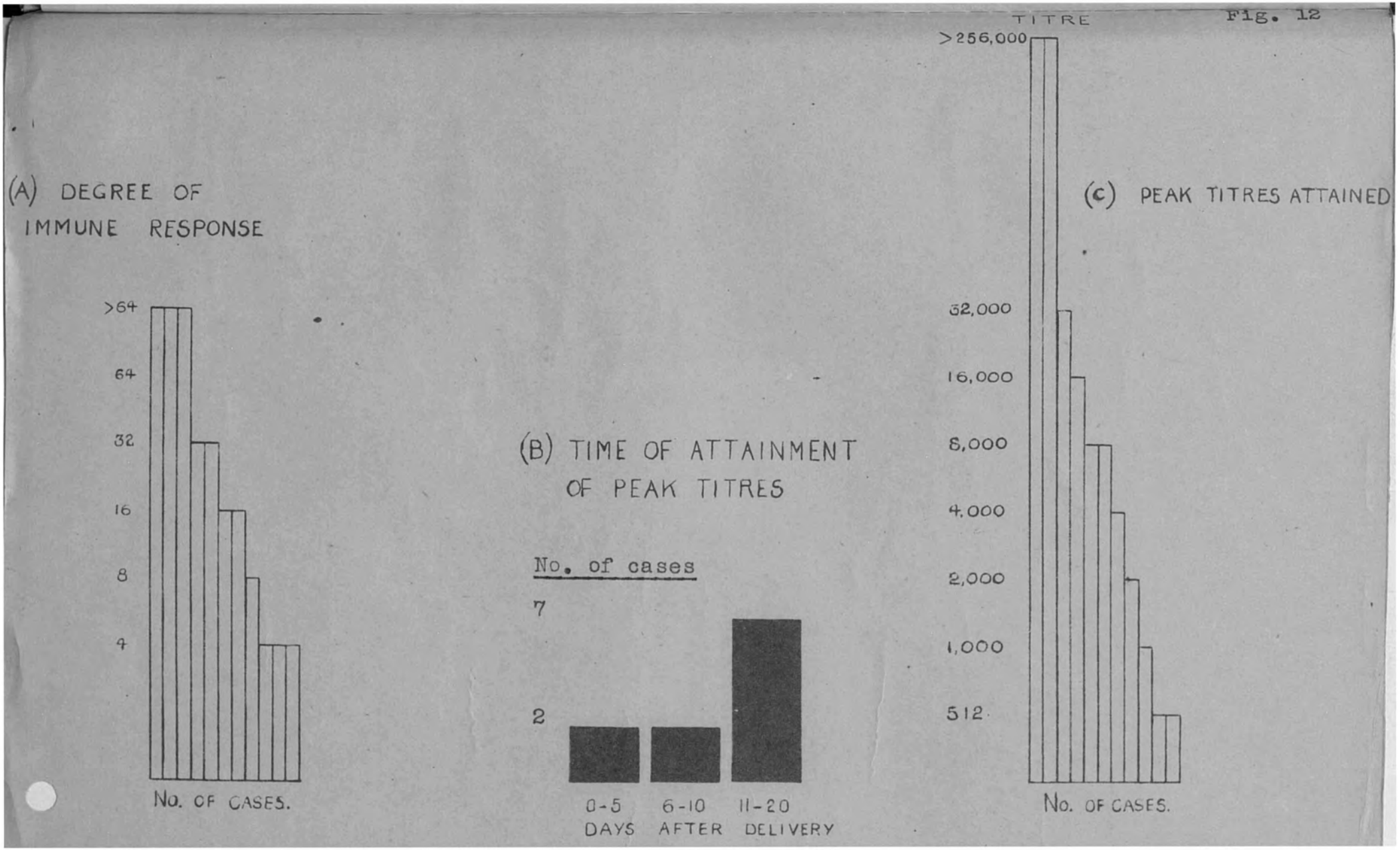

(A) DEGREE OF IMMUNE RESPONSE
>64
64
32
16
8
4
No. OF CASES.
(B) TIME OF ATTAINMENT OF PEAK TITRES
No. of cases
7
2
0-5 DAYS
6-10 AFTER
11-20 DELIVERY
TITRE
>256,000
32,000
16,000
8,000
4,000
2,000
1,000
512
(C) PEAK TITRES ATTAINED
No. OF CASES.

Fig. 13.

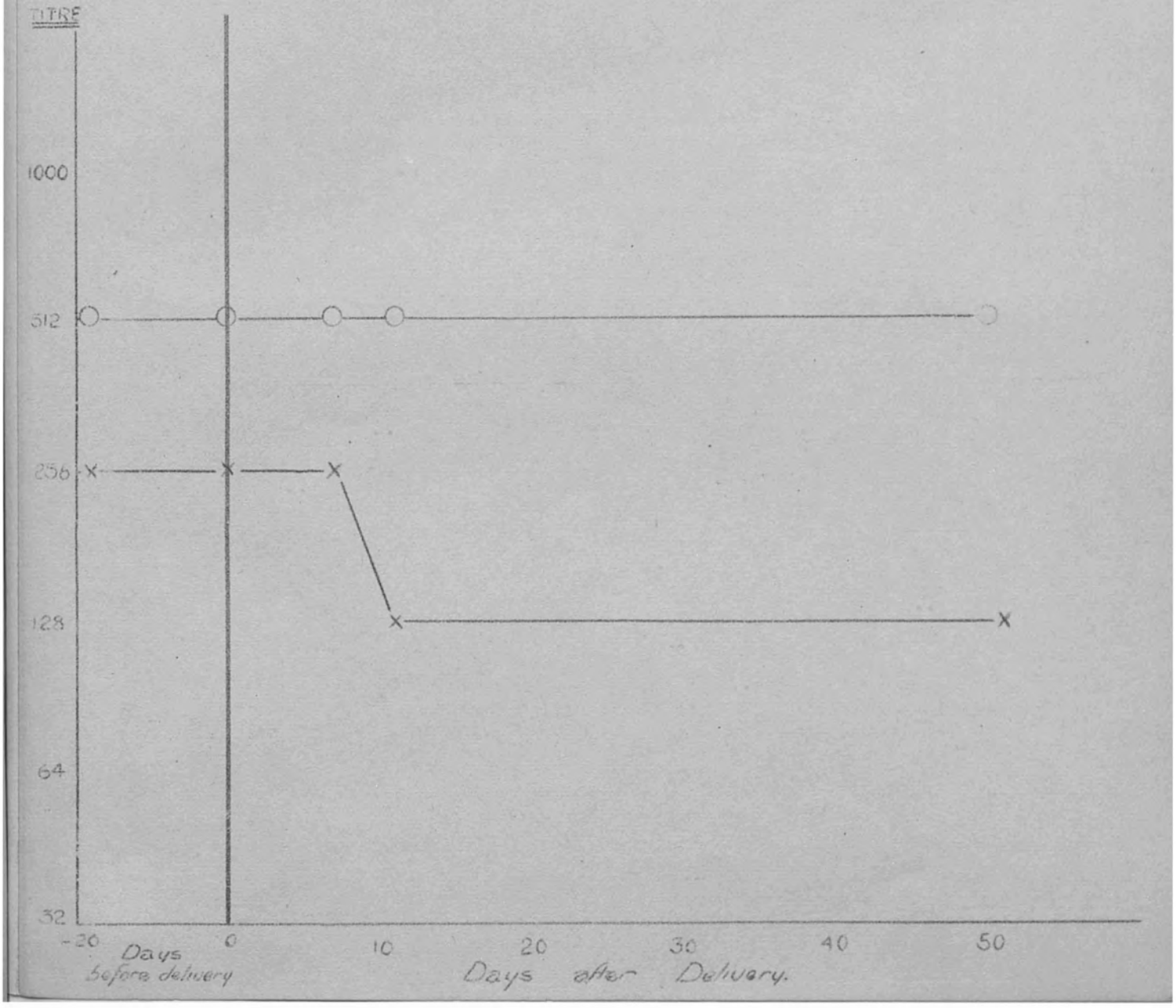
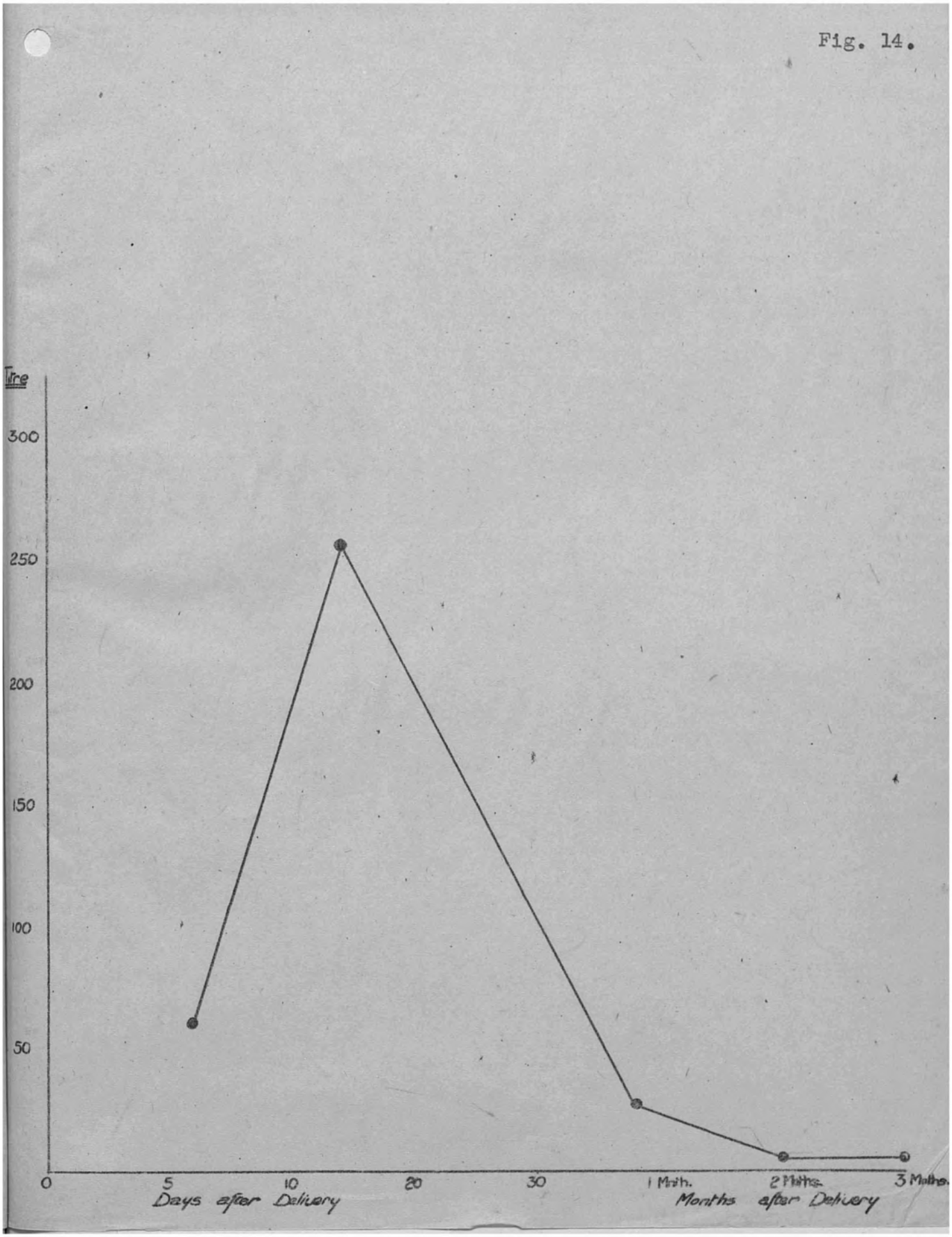

TITRE
1000
512
256
128
64
32
-20
Days
before delivery
0
10
20
30
40
50
Days after Delivery.

Fig. 14.


Titre
300
250
200
150
100
50
0
5
10
20
30
1 Mnth.
2 Mths.
3 Mths.
Days after Delivery
Months after Delivery

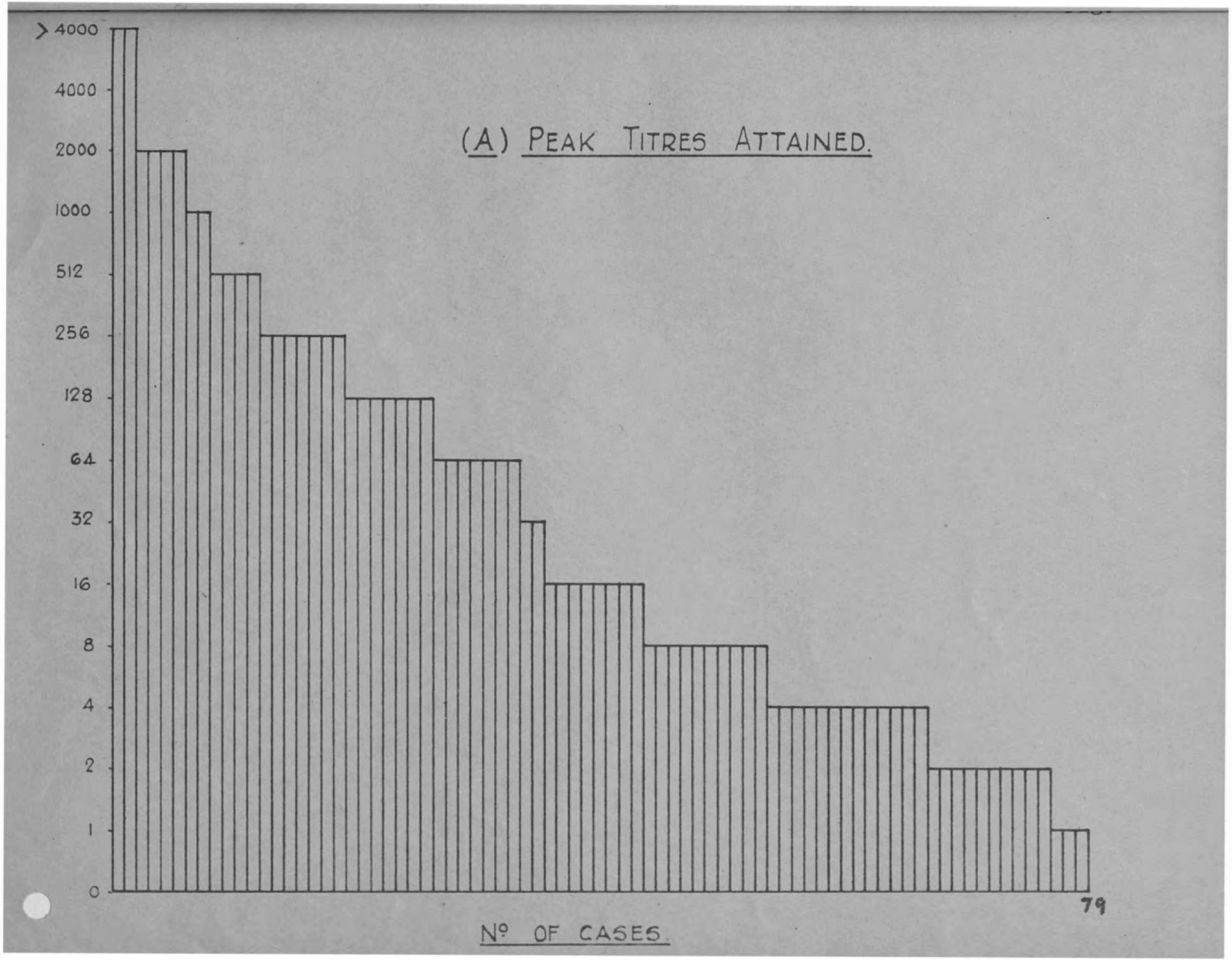

(A) PEAK TITRES ATTAINED.
> 4000
4000
2000
1000
512
256
128
64
32
16
8
4
2
1
0
79
Nº OF CASES.

Fig. 15b

(B) TIME OF ATTAINMENT OF PEAK TITRES.

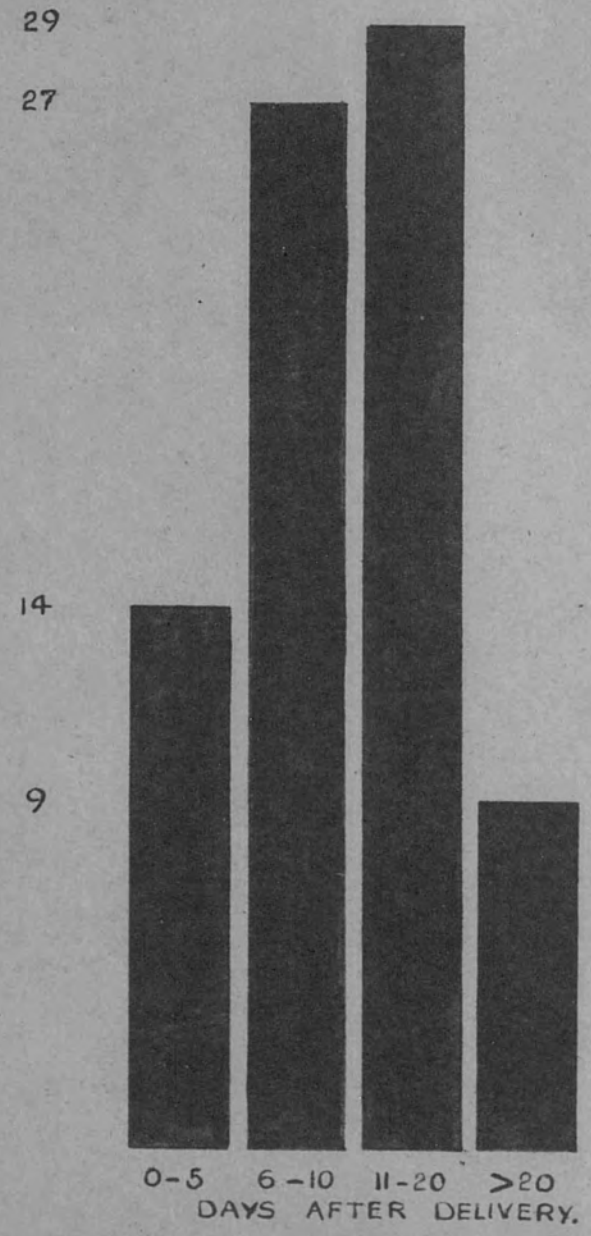
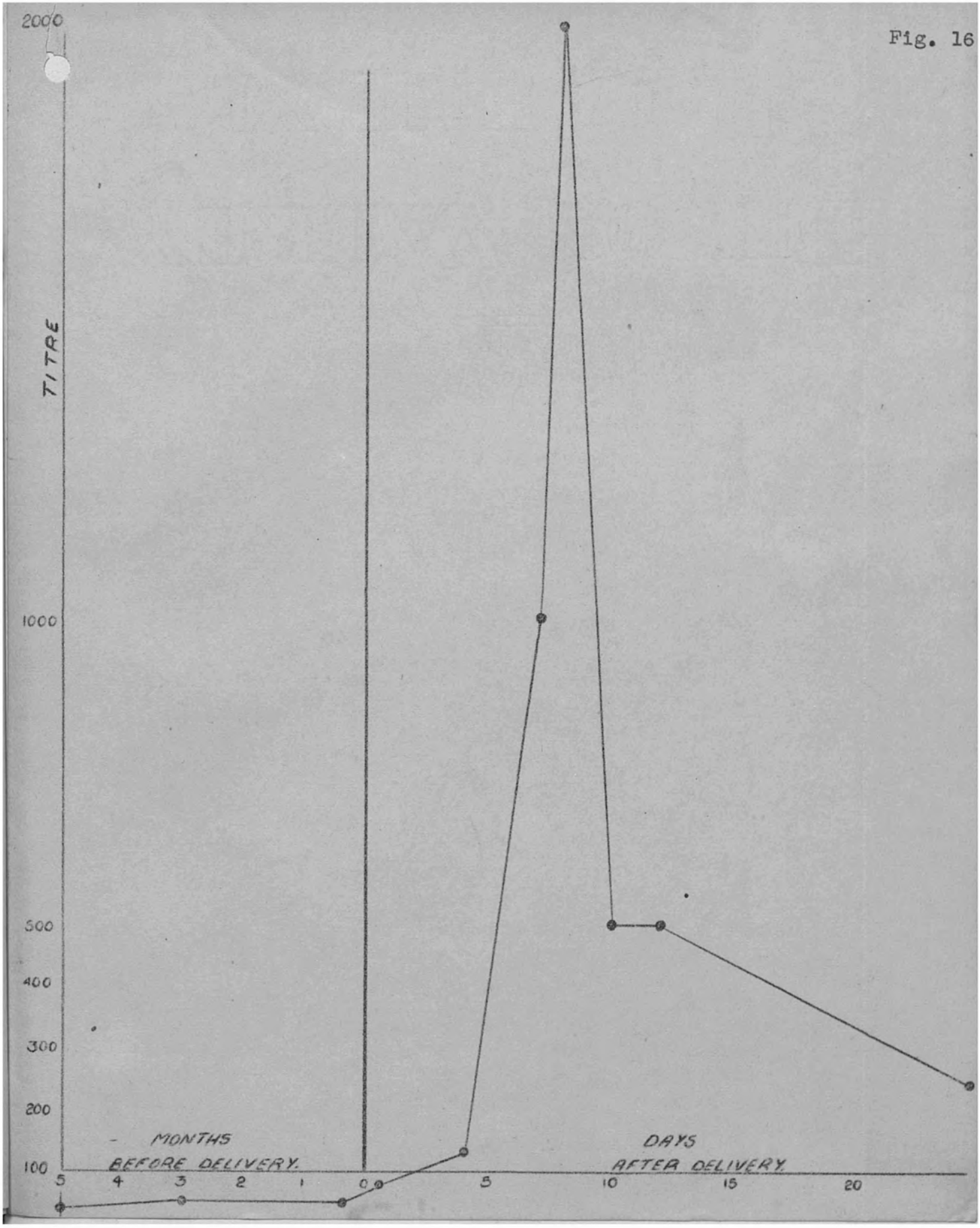


Fig. 16


2000
TITRE
1000
500
400
300
200
100
MONTHS
BEFORE DELIVERY.
5
4
3
2
1
0
DAYS
AFTER DELIVERY.
5
10
15
20

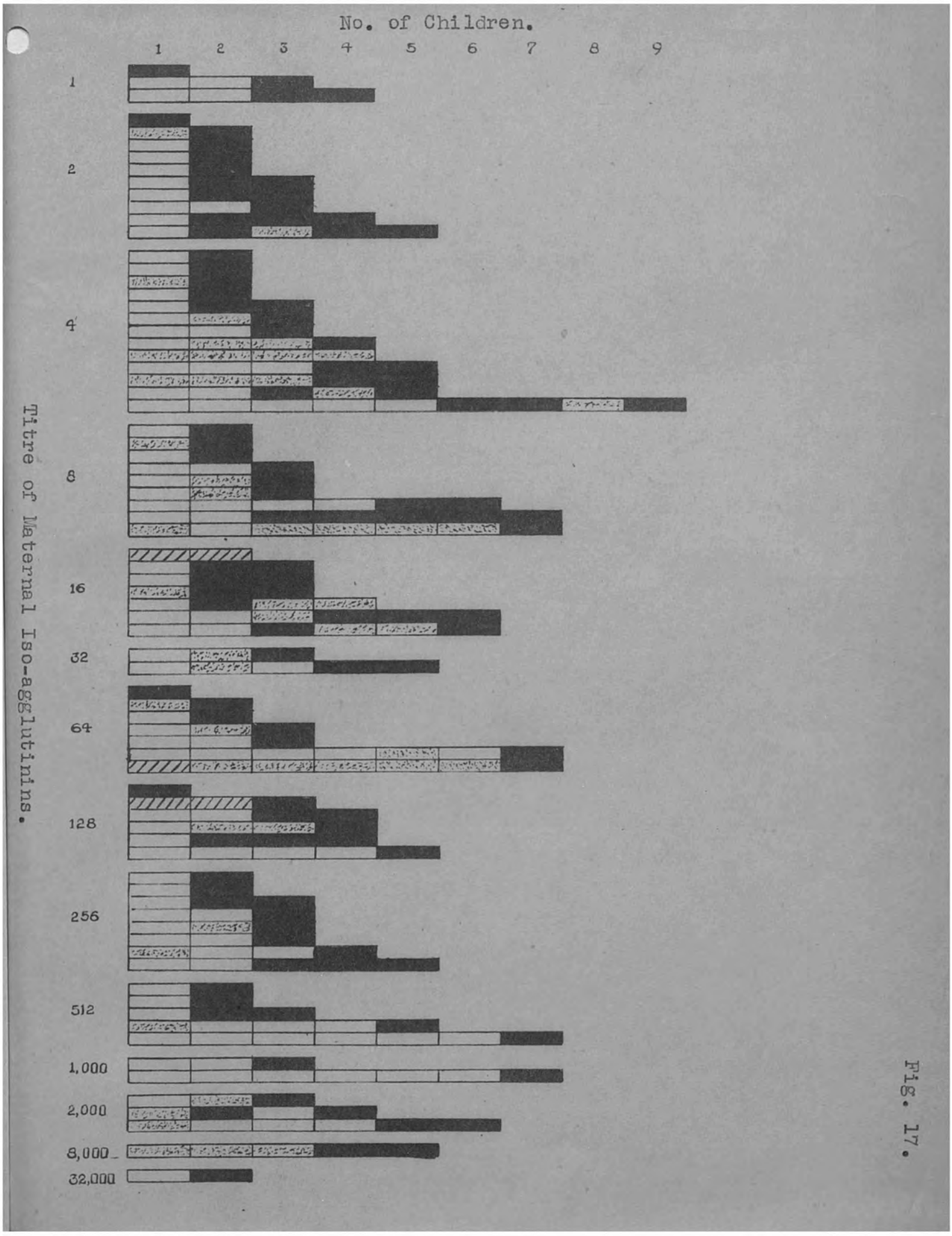

No. of Children.
1
2
3
4
5
6
7
8
9
Titre of Maternal Iso-agglutinins.
1
2
4
8
16
32
64
128
256
512
1,000
2,000
8,000
32,000

Fig. 17.

Table I

TITRES OF ANTI-A AND ANTI-B ISO-AGGLUTININS IN A SERUM OF GROUP O.PERFORMED BY TWO INDIVIDUALS - K.B. AND B.D.

	K.B.								B.D.							
Serum Dilutions	Anti-A				Anti-B				Anti-A				Anti-B			
1: 1	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c
1: 2	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c
1: 4	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c
1: 8	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c	c
1: 16	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v
1: 32	v	v	v	v	++	+	++	++	++	++	+	++	++	++	++	++
1: 64	++	+	+	+	(+)	(+)	(+)	(+)	+	+	(+)	+	(+)	(+)	(+)	(+)
1:128	(+)	(+)	(+)	(+)	w	w	w	w	w	w	w	(+)	w	gw	gw	w
1:256	w	w	w	w	-	-	-	-	-	-	-	-	-	-	-	-
1:512	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Read macroscopically.

c= complete agglutination; surrounding fluid clear.
v= visual clumps; surrounding fluid pink.

Read microscopically.

++= very large clumps.
+= large clumps.
(+)= smaller clumps with many free cells.
gw= good weak agglutination; clumps of 6-8 cells
w = weak agglutination; clumps of 4 or 5 cells
vw= very weak agglutination; clumps of 2 or 3 cells, only accepted as evidence of agglutination if the smear from the succeeding tube is clear.

Table Ia

TITRES OF AN ANTI-Rh ISO-AGGLUTININ PERFORMED IN TRIPLICATE BY WORKERS K.B. AND B.D.

	K.B.			B.D.		
Serum Dilutions	Anti-Rh			Anti-Rh		
1: 1	c	c	c	c	c	c
1: 2	c	c	c	c	c	c
1: 4	c	c	c	c	c	c
1: 8	c	c	c	v	v	v
1: 16	v	v	v	v	v	v
1: 32	+	+	++	++	v	++
1: 64	(+)	(+)	+	+	+	+
1:128	w	vw	gw	(+)	(+)	+
1:256	-	-	vw	w	w	w
1:512			-	-	-	-

Table Ib

TITRATION OF ANTI-A SERUM AGAINST RED CELLS TAKEN FROM THE SAME DONOR ON DIFFERENT OCCASIONS.

Serum Dilutions	1st Occasion		2nd Occasion		3rd Occasion		4th Occasion.	
1: 1	c	c	c	c	c	c	c	c
1: 2	c	c	c	c	c	c	c	c
1: 4	c	c	c	c	c	c	c	c
1: 8	c	c	c	c	c	c	c	c
1: 16	c	c	c	c	c	c	c	c
1: 32	v	v	v	v	c	c	c	c
1: 64	v	v	v	v	c	c	c	c
1: 128	v	v	v	v	c	c	c	c
1: 256	v	v	v	v	v	v	v	v
1: 512	++	++	+	++	++	++	++	++
1:1,024	+	+	(+)	(+)	+	+	+	+
1:2,048	(+)	w	w	w	(+)	+	+	+
1:4,096	w	w	-	-	w	gw	(+)	(+)
1:8.192	-	-			-	-	w	gw
							-	-

TABLE II.

Case no.	Blood group of recipient.	Group of blood given.	Approx. amount of blood given (c.c.s)	Recipient's iso-agglutinins.	TITRE OF ISOAGGLUTININS IN RECIPIENT'S SERUM — Days after Transfusion.												
					1 - 5				6 - 10			11 - 20				20	
					1	2	3	5	7	8	10	11	14	18	20	30	35
1	0	AB	10	anti-A anti-B				128 32		1000 512							
2	0	A	45	anti-A anti-B	512 128		2000 128			8000 32		32000 128	4000 128		256 64		256 128
3	0	A	1000	anti-A anti-B							256000 512		32000 2000	8000 512			
4	0	B	600	anti-A	256 8	256 8	256 64	256 4000									
5	0	A	50	anti-A anti-B	128 128		1000 256		1000 256				8000 256			2000 128	

Case No.	Blood Group of Recipient.	Group of Blood Given.	Recipient's Iso-agglutinins.	Titre of Iso-agglutinins in Recipient's Serum — Days after Transfusion.							
				0	3	6	7	8	14	21	31
1	O	O	Anti - A	512		512			512	512	
			Anti - B	512		256			256	256	
2	O	O	Anti - A	512			512		512	512	1024
			Anti - B	256			256		256	256	512
3	A	A	Anti - B	64	64		64				
4	O	O	Anti - A	512	256			512			
			Anti - B	256	128			256			

TABLE III.

Case no.	Type of recipient.	Type of blood given.	Approx. amount of blood given.	TITRE OF ANTI-Rh IN RECIPIENT'S SERUM. Days after Transfusion.								
				0	1 - 5	6 - 10	11 - 15	16 - 20	> 20			
				0	4 5	7 9	12	16 20	3 mths	6 mths	8 mths	9 mths
159	Rh-	Rh+	1000	0	4	32 32	8	4 2				
160	Rh-	Rh+	500	2	8	16						4
161	Rh-	Rh+	500							2	1	
162	Rh-	Rh+	1000		16	8						
163	Rh-	Rh+	500						2			

TABLE IV.

IMMUNE RESPONSES FOLLOWING INJECTION OF SERUM OF GROUPS A & B INTO INDIVIDUALS OF GROUP O.

Case No.	Blood Group of Recipient	Group of serum given	Approx. amount of Serum given in c.c.s.	Inhibition index of serum given	Recipient's iso-agglutinins	Titre of iso-agglut-inins before injection of serum	Titre of iso-agglut-inins at peak of response.	No. of times titre is increased beyond original titre.	Time of attainment of peak titre in days after injection.
1 (a)	O	A	150	8	Anti - A	256	2,048	8	12
					Anti - B	128	256	2	
(b)	O	B	150	not known	Anti - A	256	256	0	16
					Anti - B	256	2,048	8	
2	O	B	150	16	Anti - A	128	256	2	6
					Anti- B	16	512	32	
3	O	A	150	8	Anti - A	256	16,284	32	13
					Anti - B	64	256	4	
4	O	A	125	not known	Anti - A	64	1,024	16	12
					Anti - B	32	64	2	
5	O	A	150	2	Anti - A	64	512	8	6
					Anti - B	32	32	0	
6	O	A	300	8	Anti - A	64	1,024	16	10
					Anti - B	16	64	4	
7	O	B	150	8	Anti - A	256	1,024	4	10
					Anti - B	128	2,048	16	
8	O	B	150	2	Anti - A	32	256	8	11
					Anti - B	32	128	4	1
9	O	B	400	0	Anti - A	256	256	0	8
					Anti - B	16	512	32	

Table V

COMPARISON OF TITRATION VALUES AT ROOM TEMPERATURE AND AT 37°C of 61 ANTI-Rh SERA

A.

No. of times titre at 37°C is stronger than at room temperature.	No. of Cases.
0	13
2	26
4	15
8	4
16	2
32	1
Weaker at 37°C than at room temperature	0

B.

Typical Example of Comparative Titres at Room Temperature and 37°C.		
Serial Dilution	R.T.	37°C.
1: 1	v	c
1: 2	++	v
1: 4	++	v
1: 8	(+)	++
1: 16	w	++
1: 32	-	+
1: 64		w
1:128		-

Table VI.

Case No.	Rh type. Mother	Rh type. Infant	TITRE OF MATERNAL ANTI-Rh ISO-AGGLUTININS																														
			Time before delivery														Days after delivery																	
			6 - 1 month						30-20 days		19-10 days			9 - 1 days			1 - 5				6 - 10			11 - 20				20					
			6	5	4	3	2	1	30	27	19	18	17	5	3		1	2	4	5	6	7	10	11	12	13	16	21	23	28	39	58
71	Rh-	Rh	2	2	2	2	2							2				2							2							
81	Rh-	Not tested			4	2	2	4			4								2			2		4			2	2	4			4
82	Rh-	Rh		4		16							16				64		128		1000	2000	512		512					256		
109	Rh-	Rh	2		2	2						2						2			2					2					1	1
112	Rh-	not tested			1	1	2	1									2					1				1						
113	Rh-	Rh					4		4	4						8						8										
142	Rh-	not tested											8		16		1000		8000													
156	Rh-	Rh-		2			2							2		2				2												
157	Rh-	Rh-							4		4			2			4					2										

TABLE VII.

Case no.	Rh typing: Mother	Rh typing: Infant	Blood given to mother	Time of transfusion: Days after delivery	Time of complete elimination of donor blood: Days after delivery	Titre of maternal anti-Rh: Days after: - 20 to - 1			0	1 - 5				6 - 10						iso - agglutinin. delivery: 11 - 20				21 - 30				31 - 60				Mths after delivery. > 2 mths			
						-20	-17	-13		1	2	3	4	6	7	8	9	10	12	13	14	16	17	21	24	25	29	31	42	47	52	3	4	5	
59	Rh-	* not tested	Rh+	0	> 3								8																					
131	Rh-	Rh+	Rh+	6	> 3 hrs					8		8		8	32		32			64		128			16									
134	Rh-	Rh+	Rh+	0	> 7										1			2	2					1				1				1		
135	Rh-	Rh+	Rh+	0	8-12		1		1	0	0				8	8		8	4				4											
138	Rh-	Rh+	Rh+	-20	29-42	0		0	0	0		0				0											0		8		64	3			
153	Rh-	Rh+	Rh+	+ 3 4	> 5				1												2		2			2			1			1	1	1
154	Rh-	Rh+	Rh+	0	> 2					2													2										=		

* affected with Haemolytic Disease, therefore most probably Rh+

Table VIIa.

TITRATIONS AGAINST STANDARD RH POSITIVE RED CELLS										
Serial Dilutions	DAYS AFTER DELIVERY									
	0		14		17		25		60	
1:1	+	+	v	v	++	++	+	+	++	++
1:2	-	-	w	w	w	w	w	-	-	-
1:4			-	-	-	-	-			

TABLE VIII

Dilutions	Example 1.		Example 2.		Example 3.	
	Saline	Serum	Saline	Serum	Saline	Serum.
1:1	c	c	c	c	c	c
1:2	c	c	c	c	c	c
1:4	c	c	c	c	c	c
1:8	v	c	c	c	c	c
1:16	+	v	c	c	c	c
1:32	(+)	v	c	c	c	v
1:64	w	v	v	c	v	v
1:128	-	+	++	c	++	++
1:256		(+)	+	c	(+)	(+)
1:512		gw	+	c	w	w
1:1024		w	(+)	v	-	-
1:2048		-	(+)	++		
1:4096			w	w		
1:8182			-	-		

TABLE IX.

	Anti-Rh titre in saline	TITRE OF SAME ANTI-RH ISO-AGGLUTININ IN VARIOUS SERA.																																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37
1:1	V	v	v	v	v	c	c	v	c	c	v	v	v	v	v	v	c	c	c	c	c	c	v	c	c	c	c	c	c	c	c	v	c	v	v	c	v	v
1:2	+	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	c	v	v	c	v	v	v	c	v	c	c	v	v	c	c	v	v	v	v	v	v	v
1:4	w	v	+	v	v	v	v	++	v	v	v	v	v	v	v	v	v	v	+	v	v	+	v	v	v	v	v	v	v	v	+	v	v	v	+	++	(+)
1:8	-	+	(+)	+	+	v	++	+	(+)	+	v	+	(+)	(+)	+	++	v	v	w	v	v	gw	+	v	v	v	v	v	v	v	v	(+)	++	+	v	+	++	w
1:16	-	(+)	w	+	(+)	+	(+)	(+)	(+)	(+)	+	w	(+)	w	(+)	+	(+)	(+)	w	+	+	w	w	v	(+)	v	++	+	v	+	v	(+)	gw	+	++	+	+	w
1:32	-	(+)	-	gw	w	(+)	w	w	w	gw	(+)	-	vw	w	w	w	w	w	-	(+)	w	-	w	(+)	-	+	(+)	(+)	++	(+)	++	w	-	gw	+	gw	w	w
1:64	-	w		vw	-	w	vw	w	w	w	w		-	-	-	-	w	vw		w	-		-	w		w	w	(+)	+	w	gw	-		-	-	w	w	-
1:128	-	-		-		-	-	-	-	-	-						-	-		-				-		-	-	w	(+)	-	-					-	-	-
1:256																												vw	w									
1:512																												-	-									
Amplification		16	4	16	8	16	16	16	16	16	16	4	8	8	8	8	16	16	4	16	8	4	8	16	4	16	32	64	64	16	16	8	4	8	8	16	16	8

TABLE X.

	Anti-Rh titre in saline.	TITRE OF SAME ANTI-RH ISOAGGLUTININ IN VARIOUS SERA.																										
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1:1	o	o	o	v	v	o	v	v	v	v	v	v	v	v	o	v	v	v	o	o	v	o	o	o	v	v	v	v	v	v	o
1:2	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v	v
1:4	+	v	v	++	v	++	+	++	++	+	++	v	++	v	v	v	++	v	v	v	v	v	v	v	v	v	v	v	v	++	v
1:8	gw	++	v	+	++	+	(+)	+	+	+	+	++	+	++	+	++	++	++	v	++	+	++	++	v	++	+	++	++	v	+	+
1:16	w	+	++	(+)	+	+	(+)	(+)	gw	(+)	+	+	+	+	+	+	+	+	v	++	+	+	+	+	++	(+)	+	+	+	+	+
1:32	-	gw	+	-	+	gw	w	w	w	w	gw	(+)	(+)	(+)	gw	w	(+)	(+)	+	+	(+)	(+)	+	(+)	++	(+)	+	+	+	gw	(+)
1:64		vw	gw	-	+	w	-	-	-	-	w	++	w	gw	-	w	w	gw	+	(+)	w	w	w	w	+	gw	(+)	(+)	gw	-	gw
1:128		-	-	-	w	-	-	-	-	-	-	gw	-	-	-	-	-	vw	(+)	vw	-	w	-	-	+	w	gw	w	w	-	vw
1:256		-	-	-	-	-	-	-	-	-	-	w	-	-	-	-	-	-	w	-	-	-	-	-	(+)	-	w	-	-	-	-
1:512		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	w	-	vw	-	-	-	-

TABLE XI.

Anti-Rh sera	Titre in saline	Titre in serum	Amplification	Anti-Rh sera	Titre in saline	Titre in serum	Amplification.
1	256	2,000	8	24	1	2	2
2	1,000	4,000	4	25	2	4	2
3	256	8,000	32	26	1	2	2
4	4	8	2	27	2	4	2
5	16	64	4	28	8	8	0
6	128	256	2	29	1	1	0
7	16	256	16	30	2	4	2
8	32	64	2	31	8	32	4
9	256	512	2	32	32	256	8
10	4	16	4	33	64	256	4
11	64	512	8	34	256	2,000	8
12	4	16	4	35	4	8	2
13	2	4	2	36	128	512	4
14	128	1,000	8	37	256	1,000	4
15	4	8	2	38	64	256	4
16	32	512	16	39	128	1,000	8
17	64	1,000	16	40	64	512	8
18	8	128	16	41	64	256	4
19	1,000	16,000	16	42	256	1,000	4
20	16	64	4	43	64	128	2
21	64	1,000	16	44	2	8	4
				45	2	16	8
22	1	2	2	46	64	1,000	16
				47	1	2	2
23	2	2	0	48	1	8	8
				49	2	8	4

TABLE XLL.

Anti-Rh sub-group sera.	Titre in saline	Titre in serum	Amplification
Anti-Rh_1(H)	8	64	8
Anti-Rh_2(K)	64	2048	32
Anti-Rh_2(K_2)	16	64	4
Anti-Rh_2(K_3)	16	64	4
Anti-rh 1)	8	32	4
2)	16	64	4
3)	16	32	2

TABLE XIV.

Anti-body	Serum dilution	Before injection: Saline	Before injection: Serum	Days after Injection: Immed:after Sal.	Immed:after Ser.	1 day Sal.	1 day Ser.	3 days Sal.	3 days Ser.	8 days Sal.	8 days Ser.	11 days Sal.	11 days Ser.
ANTI-A	1:1	v	v	c	c	v	v	H	H	H	H	H	Hv
	1:2	v	v	c	c	v	c	v	v	H	Hv	Hv	v
	1:4	c	c	c	c	c	c	c	c	Hv	v	v	v
	1:8	c	c	c	c	c	c	c	c	cv	v	v	c
	1:16	c	c	v	v	c	v	c	c	c	c	c	c
	1:32	c	c	v	++	c	v	c	c	c	c	c	c
	1:64	v	v	++	+	v	v	c	c	c	c	c	c
	1:128	++	++	(+)	w	+	+	v	v	c	c	v	c
	1:256	+	(+)	gw	vw	gw	(+)	v	v	c	c	v	c
	1:512	(+)	gw	vw	-	vw	gw	+	+	c	c	v	c
	1:1024	w	w	-	-	-	vw	(+)	(+)	v	c	v	c
	1:2048	-	-				-	w	w	v	c	++	c
	1:4096							-	-	++	c	++	c
	1:8192									++	v	+	c
	1:16284									(+)	v	(+)	c
	1:32568									w	(+)	(+)	v
	1:65136									-	(+)	gw	v
	1:130272										-	w	++
	1:260544											-	gw
	1:521088												w
	1:1042176												w
													-
ANTI-B	1:1	c	c	c	c	c	c	c	v	v	v	v	v
	1:2	c	c	c	c	c	c	c	v	v	v	v	v
	1:4	c	c	c	c	c	c	c	c	v	c	c	v
	1:8	v	c	c	v	c	c	c	c	c	c	c	c
	1:16	v	v	v	v	v	v	c	c	c	c	c	v
	1:32	++	++	++	++	++	++	v	++	++	++	v	v
	1:64	(+)	gw	+	(+)	+	(+)	++	(+)	+	+	++	+
	1:128	w	-	gw	w	(+)	w	(+)	w	(+)	(+)	+	(+)
	1:256	-		w	-	w	vw	w	vw	gw	w	gw	w
	1:512			-		-	-	-	-	vw	vw	vw	vw
	1:1024									-	-	-	-
Amplification: Anti-A		0		0		2		0		2		8	
Anti-B		0		0		0		0		0		0	

TABLE XV.

ANTIBODY.	Titre in saline	Titre in serum	Amplification.
1. Natural anti-A_1 found in group A_2B.	16	16	0
2. Natural anti-A_1 found in group A_2B	32	32	0
3. Anti-A_1 prepared from group B	8	8	0
4. Anti-A_1 prepared from group B	16	16	0
5. Immune anti-A_1 stimulated by blood transfusion	8	16	2
6. Immune anti-A_1 stimulated by foetus in utero			
(a) Room temperature	64	1000	16
(b) 37° C	16	64	4

Case No.	Group of Mother	Group of Infant	Maternal Agglutinins	TITRE OF AGGLUTININS IN MATERNAL SERUM.																				
				Months before delivery								Days after delivery										Weeks after delivery			
				8	6	5	4	2	1½	1	Less than 1 Month	0	1	2	4	5	6	7	8	9	10	1 - 2	2 - 4	4 - 6	6 - 8
1.	A	O	Anti - B									256					256				128			128	
2.	A	A	Anti - B								256									256			256		256
3.	A	A	Anti - B							512					512						1024			256	
4.	O	O	Anti - A Anti - B								512 256	512 256						512 256				512 128			512 128
5.	B	B	Anti - A					256			256								256			256			256
6.	A	A	Anti - B						128				128							64					
7.	A	A	Anti - B								128					128								128	
8.	O	O	Anti - A Anti - B								64 32	64 32						32 64							
9.	O	O	Anti - A Anti - B								1024 256		2048 512							1024 256					
10.	A	O	Anti - B							256				256				128				256			256
11.	O	O	Anti - A Anti - B					1024 512						256 256								256 256			
12.	O	O	Anti - A Anti - B				128 32					256 64									128 64				
3.	O	O	Anti - A Anti - B											1024 128								512 512		512 256	
14.	O	O	Anti - A Anti - B		16 8			16 8	32 8			32 8									32 8				
15.	O	O	Anti - A Anti - B			128 64		128 32				128 32										64 32			
16.	O	O	Anti - A Anti - B	256 256						512 512		256 256									128 128				

Table XVI.