UNIVERSITY OF EDINBURGH

BLOOD GROUPS OF PIGS

A DISSERTATION PRESENTED FOR

THE DEGREE OF

DOCTOR OF PHILOSOPHY

OF

UNIVERSITY OF EDINBURGH

BY

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OCTOBER, 1963

INDEX

INTRODUCTION:-

It is fascinating to observe that in the history of scientific achievement, the advancement of knowledge within one discipline has invariably been assisted by a comparable advancement in other disciplines. The identification of bacterial organisms in infectious diseases by Koch and his students, could not have advanced so dramatically without the aid of a simultaneous advance in the knowledge about aniline dyes and optical instruments.

In the demonstration of A and B isoagglutinins in the serum of man by Landsteiner (1900), his work must have benefited from the knowledge already gained in the study of immunity to bacterial organisms. This knowledge stemmed from the outstanding work by the bacteriologists of the last century, such as Pasteur, then Nuttall, von Behring and Kitasato and finally Bordet.

At the same time as Landsteiner's discovery of human blood groups, the principles of Mendelian genetics were rediscovered by three independent observers, namely de Vries, Correns and Tschermak. Their discoveries soon found application in the inheritance studies of human blood groups by Epstein and Ottenberg (1908), also von Dungern and Hirszfeld (1910). At that time biologists and students of Darwinism had generally accepted that variability existed within organisms, and were aware that even man showed certain uniqueness in colour, shape and aptitudes. The discovery of blood groups in man, however, was the first demonstration, that this uniqueness extended beyond the confines of physiognomy and mental attributes.

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The exposition of variability between the cells of different individuals arose from the knowledge of immunity and technical demonstration of the antibody response. The first demonstration of chemical individuality in man, however, was shown by Garrod (1902) in his study of "inborn errors of metabolism", such as the condition of alkaptonuria. This observation followed very closely on the heels of the blood group discovery, and was subsequently shown to be the first example of recessive inheritance to be recognised in man.

Since the beginning of the century, the knowledge of variability in many species has grown on several fronts. First, through the application of old and new immunological techniques; secondly, through the considerable advance in biochemical knowledge, and finally through the expert guidance of men like Fisher and Haldane, in the application of statistical methods to genetic analysis. Thus, the advances in the study of inherited differences gained from the knowledge of the immunologist and geneticist in the beginning, has within the last decade been given fresh impetus by the work of biochemists. It is interesting to record a passage from Putnam in the book on 'Plasma Proteins', in which he states, "Indeed, it has been said to be startling to immunologists, that the first clear-cut evidence for hereditary serum protein groups in normal humans came from electrophoretic rather than serological methods". This advance has once again amalgamated disciplines, and has brought knowledge to a new front with the study of immunoelectrophoresis. These discoveries have also coincided with the establishment of the basic chemical formula of deoxyribonucleic acid by Watson and Crick (1953), and that this compound and genes are synonymous. Perhaps within another decade or more, the present knowledge of variability will extend beyond test-tubes, electrophoretic techniques and arbitrary genes /

genes to the basic arrangement of phosphoric acid, pentoses and purines.

At this stage in our knowledge, it is generally agreed that the blood group characters on the erythrocytes of man and animals, and the different serum proteins and groups should all be classified as gene markers, or blood groups. This view is held by Race and Sanger (1959), who introduced a paper on the subject of 'the inheritance of blood groups' with this statement,""Blood groups", in a broader sense could nowadays include not only the differences due to the antigens of red cells, white cells and platelets, but also the distinctions made by the various haemoglobins, haptoglobins and Gm serum groups."

The knowledge and value of blood groups in man has been manifest in clinical transfusion, and in solutions to medicolegal problems of disputed parentage, and forensic medicine. They have also been applied in anthropological studies. In domestic animals, however, the knowledge of blood groups has not found comparable benefit in application to clinical transfusion, but has in solving problems of parentage. It has also found application in the detection of heterosis in highly inbred lines of chickens (Briles, 1953 and Gilmour, 1954), and in helping to distinguish two egg from one egg twins in cattle. With the advent of progeny testing of pigs on a national scale in this country, the necessity for a systematic study of heritable characters such as "blood groups" seemed essential in this species. In this respect it could be applied as an independent check on pedigree registrations, and to other fields of interest, such as heterozygous advantage and linkage with production characters. The dissertation presented for examination describes the application of immunological, serological and biophysical /

biophysical techniques to the discovery and characterisation of "blood groups" in the pig. The independence and interrelationship of these groups has also been investigated and is discussed.

PART I - IMMUNOLOGICAL STUDY

REVIEW OF LITERATURE

There appear to have been three main approaches to the subject of pig blood groups. The earlier workers seem to have been influenced by Landsteiner's work on the A and B isoagglutinins in human sera (1900). The first recorded investigation was fifty years ago by Fishbein (1913), who looked at the natural isoagglutinins in 60 pig sera. He showed that the red cells of some pigs were agglutinated by sera from other pigs. Although isoagglutination was extensive, no positive grouping could be made. His work was confirmed by Weszeczky (1920), who looked at the sera obtained from 11 pigs at the time of slaughter.

The first definite classification along these lines was made by Szymanowski and co-workers (1926). On examining blood samples from the slaughterhouse, they came to the conclusion that there were three blood groups distinguishable in pigs on the basis of combinations of a blood cell antigen, and the antibody acting against it. The erythrocytes of pigs belonging to the first group had an isoantigen they called A, but there were no isoantibodies in their sera (A_a). On the red cells of the second group (0_a), there were no isoantigens, but their sera contained isoantibody against antigen A occurring in the first group. The third group of animals had neither isoantigen or isoantibody, and were called group 0_o. Similar type groupings were confirmed by Kayser (1929). At the same time Szymanowski et al. (1926) and Kaempffer (1932a) compared the A antigen on the erythrocytes of pigs with the isoantigens of humans. They found it to be very similar to the A isoantigen in man, for with human A red cells it was possible to absorb a large portion of the /

the antibodies from pig sera containing anti-A. The other human erythrocytes of group B and O did not give similar results. The last mentioned worker (Kaempffer, 1932a), also established that the A factor was inherited as a dominant character. Later in 1927, Szymanowski and Wachler showed that the A factor had serological similarities to the A factor in sheep. The latter is now known as the R substance.

In the first paper, Szymanowski et al. (1926) also indicated the occurrence of normal antibodies with reactions other than anti-A. This observation was confirmed by Kaempffer (1932b), who by means of absorption was able to isolate a natural antibody different from anti-A.

The investigation for normal isoagglutinins was continued by Hardt (1937), who found that the classification was not as simple as the three groups given by Szymanowski, but he failed to establish a system. It was not until 1950, that Kuhns described four distinct normal agglutinins in pig sera. He called them P_1, P_2, P_3 and P_4 and assumed the existence of 14 blood group combinations. The occurrence of at least four different normal antibodies was confirmed by Eyquem (1953). In the next year, Szent-Ivanyi and Szabo (1954) described in greater detail by absorption techniques, the existence of four normally occurring agglutinins. They established the frequency of four antigens and antibodies as follows:-

Antigens			Antibody	
A	47.8%		anti a	9.6%
B	22.7%		anti b	10.4%
C	8.2%		anti c	0.4%
D	26.5%		anti d	8.0%
O	15.1%	Type O (no antibody or antigen)		67.6%

In /

In their paper, they state that immunised pigs were not used for this investigation. They also established a serological relationship between their anti A and human anti-A. It is believed that the other three antibodies they isolated are no longer in existence, and little or no work has been done since then to confirm or reject the existence of normal antibodies other than anti-A.

The first indication of a new approach to the subject was given by Szymanowski and Wachler (1926), who observed that serum from pigs vaccinated against swine fever occasionally contained antibodies which agglutinated pig red cells other than type A. They concluded that as the vaccine was prepared from pooled pigs' blood, that type O blood was antigenic and capable of producing antibody if injected into a suitable recipient of the same species.

It was not until 1949, that Bruner et al. demonstrated that experimental isoimmunisation of pregnant sows with blood from the mated boar could produce haemolytic disease of their newborn piglets. This was followed quickly by reports of clinical cases of haemolytic disease in piglets by Kershaw (1950), then by Szent-Ivanyi and Szabo (1953); Buxton and Brooksbank (1953); Doll and Brown (1954) and Newberne (1957). The report by Buxton and Brooksbank stimulated the interest of Coombs, who helped to diagnose the condition by demonstrating antibody coating on the red cells of haemolytic piglets by using the direct antiglobulin sensitisation test. The findings were summarised in a paper by Buxton, Brooksbank and Coombs (1955), in which it was mentioned that the one important factor involved was the injection of animals with crystal violet vaccine.

The occurrence of haemolytic disease initiated a wide scale investigation of pig blood groups by the Cambridge workers. In the first paper by Saison, Goodwin and Coombs (1955) on the detection of the natural occurring A isoantibody by the antiglobulin test, it was suggested that the O_c group of Szymanowski probably all belonged to group O_a. In the same year Goodwin, Saison and Coombs (1955) demonstrated that a large number of pigs which had received more than one injection of swine fever vaccine had a high titre of red cell iso-antibodies. Later Goodwin and Saison (1956) reported a breed difference in the immune isoantibody response to this vaccine. Goodwin and Coombs (1956) also failed to establish that the A antigen-antibody system of pigs was involved in haemolytic disease of the newborn piglet. They did establish, however, that the A antigen is absent from the red cells of the newborn, but appeared in increasing strength on the cells at varying times during the first month of life.

Saison (1958) demonstrated that pure testing reagents could be isolated from the immune antisera of vaccinated sows. With one other antibody donated by Gershowitz, she described a blood group system of pigs involving four factors called E_a, E_b, E_c and E_d. These factors were shown to be heritable, and Saison believed that the system formed was consistent with the existence of four alleles. The four alleles were made-up of the following groups of factors; (E_a), (E_b E_c E_d), (E_b E_d) and (E_c E_d). The factors E_a and E_b were considered to be equivalent to Andresen's factors E_a and E_b. In a later communication, Saison (1962) amended the previous report and removed E_c from the new system, because it was believed to be a

weak /

weak E_d. She also isolated a further seven antibodies from the sera of seven vaccinated sows. The additional factors were called K_a, K_{a1}, DK, B111, P, Q and R. Factor K_a was believed to be similar to the K_a described by Andresen (1959c), but Saison produced evidence for the existence of two antibody fractions in the original K_a antiserum. Two sub-groups K_{a1} and K_{a2} were postulated. Four of the other factors listed above were detected by Löw's papain method (1955).

Joysey, Goodwin and Coombs (1959) also defined 11 antigens by absorption of antisera from six vaccinated sows producing haemolytic litters. These factors were numbered 1 to 11. The distribution of these antigens and the A factor were calculated in seven different breeds by the same authors (1959). Inheritance studies on these factors were not carried out, and comparison with reagents identified by other workers was not made.

The third and probably most definite approach to this subject was made about the same time as the investigation by the Cambridge workers. Andresen working in Copenhagen adopted the positive technique of producing isoimmune and heteroimmune antisera by repeated injections of non pooled donor blood. In 1957, he described seven different reagents against factors called E, F, G, H, I, J and K. All were obtained from antisera produced by isoimmunisation. Family data was presented to show that these factors were inherited as dominant characters. In 1959 a whole series of papers by Andresen were published, establishing systems and genetic control of several antigenic factors. In the first paper /

paper from Wisconsin, Andresen and Irwin (1959a), found two factors E_a and E_b, and proposed that they were controlled by allelic genes in a multiple allelic system. The E_a factor was the same as the previous E Andresen had defined. Then Andresen and Wroblewski (1959b) with a third factor E_e, produced at the Pasteur Institute, established that all E_a animals had the E_e factor as well, also that E_e was a contrasting factor to E_b. The theory was advanced that the E system constituted a closed genetic system formed by three alleles E^{ae}, E^e and E^b.

A further paper by Andresen and Irwin (1959c) demonstrated that by heteroimmunisation of rabbits with pig red cells and absorption experiments, two haemolysins against pig blood group factors K_a and K_b were specified. The K_a factor was similar to the K factor previously obtained by isoimmunisation. These two factors were concluded to be controlled by two allelic genes in a multiple allelic system.

In a paper on studies of pig blood groups in connection with progeny testing given at the Blood Group Congress in Munich, Andresen et al. (1959d) indicated that preliminary studies on 16 blood group factors had been concluded. On the basis of his investigations he stated that, "the 16 blood groups under study belong to nine different blood group systems". The systems were called A, E, F, G, H, I, J, K and L. Two additional factors E_f and E_d were added to the existing E system. The G system was also found to be a closed system, in that all pigs had either factor G_a or G_b or both. Another system called H had two factors H_a and H_b, but /

but was not a closed system. It was found that the H^a gene showed a dosage effect in that cells from homozygous individuals were more reactive than heterozygotes. The existence of the G and H systems was later confirmed in a paper by Andresen and Wroblewski (1961). The remaining factors mentioned at Munich were F_a, I_a, J_a and L_a (= DK_5), all single factors representing different systems. Two additional factors called K_c and K_d were also added to the K system.

Bräuner Nielsen (1961) another Danish worker, added another system called M to the above classification. This new system arose from isoimmunisations using pigs of the Pietrain breed as donors against Landrace as recipients. It was represented by a single factor called M_a, and no association with any other factor could be found. Test crossings involving all other systems except the L and J systems confirmed its independence.

In the most recent communication, again by Andresen (1962), he has established the existence of ten blood group systems on the basis of segregation studies within relevant families. An additional system called B containing factor B_a has been found. This factor appeared on investigation of an antiserum from a sow which had given birth to pigs affected with haemolytic disease. It is the first time that a specific antigen has been implicated in this disease. Anti A and E_a were also present in the antiserum. The use of the letters B, C and D had previously been avoided by Andresen in his notation, in view of the work by Szent-Ivanyi and Szabo (1954). As the stock of their reagents had disappeared, the necessity for reserve was overlooked. In addition, within the E system /

system another factor called E_g was proposed, and evidence was presented for the existence of three pairs of contrasting characters within a multiple allelic system. The contrasting factors proposed are E_b and E_e; E_a and E_d also E_f and E_g. Inheritance studies demonstrated the existence of five E alleles controlling the following groups of these factors: E_{bdg}; E_{edg}; E_{aeg}; E_{efd} and E_{bfd}. A shorthand notation has been adopted by Andresen to identify these groups. The other new additional factors given in the above paper were L_b and L_c. It is suggested by Andresen, that these factors along with L_a form a closed system comprising three alleles L^a, L^b and L^{bc}. A comparison of the frequencies of the various blood group alleles was also presented for three different breeds. Several alleles showed significant differences in their frequency between the different breeds.

2. METHODS AND MATERIAL:-

1. Terminology: (a) Antigen An antigen is defined as a substance capable of stimulating antibody formation, when introduced parenterally into an animal. The antibody produced is capable of combining with the antigen in some detectable way. In blood group studies antigens have been found to occur on the surface of the erythrocyte. They are often referred to as blood group substances and chemically are conjugated proteins of a mucopolysaccharide type.

(b) Antiserum This refers to the serum of an animal in which antibodies may be found. They are modified serum globulins usually belonging to the gamma range. Each antibody has usually a unique specificity for the antigen or group of antigens it was stimulated by, although cross specificities do occur. This specificity by in vitro tests manifests itself in the form of a particular reaction, whereby the antibody combines with the antigen and causes the vehicle (i.e. red cell) to agglutinate or lyse.

(c) Specific antibody For blood grouping the isolation of specific antibodies from heterogeneous antiserum is the primary aim, so that each antibody will detect its reciprocal antigen. Thus animals can be grouped according to the type of antigens they have on their red cells. The level at which any worker can assess the unit specificity of any antibody isolated is to study the ACTIONS OF THE genes which controls the antigens or group of antigens detected. If the inheritance studies comply with the simple rules of Mendelian segregation, then the worker may justify the existence of /

of such an antibody by calling it a reagent. The antigen a reagent detects is often called an antigenic factor, or blood group factor.

(d) Blood group system This may be defined as a region of a chromosome within which one or several genes may control several antigenic factors. By the study of inheritance within reliable pedigreed animals, association between blood group factors may be found, so that they may be assigned to the same region or system. Those which segregate independently cannot belong to the same system. If the region of the chromosome is confined to a short distance, then it has less chance of becoming divided up due to the phenomenon of crossing over at meiosis. By measuring the recombinant values for two linked factors, then the percentage crossing over within a defined system may be assessed.

In the notation used so far to define pig blood groups, each system is characterised by a different letter of the alphabet. The antigenic factors within a system are written as subscripts to this letter and are used to indicate the phenotype, whereas the genotype is indicated by using superscripts. For example, a blood system called A may have antigenic factor A_a in the phenotype, and the genotype may be expressed as A^a/A^a in the homozygote and A^a/A^- in the heterozygote. If an animal is negative for the antigenic factor A_a, then the phenotype may be expressed simply as A-ve, and the genotype as A^-/A^-, that is the double recessive. Most antigenic factors are inherited as dominant Mendelian characters revealing themselves when in the homozygote and heterozygote form.

2. /

probability of obtaining a deviation from expected greater than the observed when taken in the direction of the observed deviation. The two tail significance tests correspond to the single tail tests at the conventional probability levels 0.05 and 0.01 respectively. This means that in assessing the probability value obtained by the Exact treatment the significance placed can only be applied at half the probability obtained by the conventional two tail test.

'Yate's correction of continuity' can be applied. By this method χ^2 is calculated by making the observed frequencies less extreme, by adjusting them by half a unit. The probability significance is applied at the same level as for the two tail test.

In this study for frequencies less than five the method used is the Exact Treatment by Fisher (1950). By this method the probability values are calculated exactly, and this is illustrated in the results involving the Reagents Nos. 5 and 11. This example shows that the two figures to be reduced stepwise by one until 0 is reached are those which are smaller than the expected values, or are those forming the smallest cross-product. The expected values are shown in brackets:-

		Factor 11 or E_f		
		+ μ	- μ	Total
Factor 5 or E_a	+ μ	2 (7.3)	34 (28.7)	36
	- μ	19 (13.7)	49 (54.3)	68
		21	83	104

$$p = \frac{36!68!21!83!}{104!} \cdot \left[\frac{1}{2!34!19!49!} + \frac{1}{1!35!20!48!} + \frac{1}{0!36!21!47!} \right] \simeq 0.0057$$

The above method may be more easily calculated by using Fisher and Yates (1948) Tables for the logarithm of the factorial numbers.

When the standard χ^2 test is applied to a 2 x 2 contingency table the distribution is called two tail, because the test is based on the probability of obtaining a difference in either direction, which can be greater than the actual difference between observed and expected frequencies. The Exact Method given above however, is called a single tail test, and indicates the

In the four cells of the table, a, b, c and d represent the actual number of animals observed within the population to react with Reagents X and Y. The values expected by chance alone for any cell can be calculated by taking the product of the corresponding marginal totals and dividing by N.

eg. $\frac{(a + c).(a + b)}{N.}$

However, without calculating the expected values for each cell Fisher (1950) showed that χ^2 may be calculated directly by the following formula:-

$$\frac{(ad - bc)^2 . N.}{(a + b).(c + d).(a + c).(b + d)}$$

If the cross product of (a.d) is greater than (b.c), it is called a positive association, and may suggest a mixture of antibodies, or possibly a genetic relationship, such as a linkage in coupling. Alternatively, if (b.c) is greater than (a.d), it indicates a negative association and could suggest linkage in repulsion.

It is pointed out by Fisher (1950), that "The distribution of χ^2 is a continuous distribution. The distribution of frequencies must, however, always be discontinuous. Consequently, the use of χ^2 in the comparison of observed with expected frequencies can only be of approximate accuracy, the continuous distribution being in fact the limit towards which the true discontinuous distribution tends as the sample is made ever larger". In order to avoid the irregularities produced by small numbers, Fisher recommended that in no group should the expected number be less than five.

In cases of frequences less than five the method of

Statistical Analyses:-

1. Chi-squared test:- This statistical test has been frequently applied to the data given in this thesis. The basic chi-squared formula is taken from Fisher (1950), and is as follows:-

$$\chi^2 = S\left(\frac{x^2}{m}\right)$$

where 'm' is equal to the hypothetical expected values, and 'x' is the deviation between the observed values and the expected.

The chi-squared value has also been calculated in 2 x 2 contingency tables, which have also been extensively applied to the data. The 2 x 2 table has been used to investigate the possibility of serological and genetical association between various blood group factors by calculating the χ^2 value, or the corresponding probability that the association observed could reasonably occur by chance alone. The assumption that chance alone is responsible for any association observed, may be found to be very unlikely (ie. the Null Hypothesis), and the alternative view, that some other factor is involved is rendered more acceptable. In general, where the probability is greater than 0.05 the association has been ignored, but where the probability value is less than this limit further serological or genetical investigations have been pursued in search of a more likely cause than chance. The 2 x 2 table is applied in the following manner, when the distribution of reactions for two reagents called X and Y within the same random population are compared in a contingency table:-

		Y Reagent reaction		
		+ve	−ve	Total
X Reagent reaction	+ve	a	b	a + b
	−ve	c	d	c + d
		a + c	b + d	N = (a+b+c+d)

2. Experimental animals: (a) Pigs The majority of the pigs described in this study were provided by the Agricultural Research Council and located at two of their experimental animal breeding research farms. These farms were (1) The Pig Breeding Research Station, Mountmarle, Roslin, Midlothian and (2) Skedsbush Farm, Gifford, East Lothian.

For the section on Sodium and Potassium blood values the material was obtained from pigs at the National Pig Progeny Testing Station, Bridge of Allan, Stirling. The remainder of the material was obtained from pigs of unknown origin being slaughtered at the Edinburgh City Corporation Abattoir, Gorgie, Edinburgh.

(b) Rabbits These were supplied by the Agricultural Research Council and were located at their Dryden Field Laboratory, Roslin, Midlothian.

(c) Reference panel In selecting animals for blood grouping by serological techniques, a considerable amount of assistance was provided as the result of a previous experiment. This occurred indirectly through Dr. Andresen in Copenhagen, who in 1960 wished to collect data on red cell antigen frequencies within the Large White breed. The blood from several animals of this breed were collected for him at the farms mentioned above, and sent to Denmark. At that time Andresen had by isoimmunisation and fractionation of antisera obtained sixteen antibody reagents each capable of detecting a different red cell antigen. On the basis of a survey of the Landrace breed and segregation study within families, he had grouped these antigens into nine blood group systems, as shown in the table below:-

Blood group system	Blood group antigen or factors
A	A
E	E_a, E_b, E_e, E_f
F	F_a
G	G_a, G_b
H	H_a, H_b
I	I_a
J	J_a
K	K_a, K_b, K_d
L	L_a

Of the Large White pigs which were blood grouped on the basis of sixteen antibodies, only a small proportion remained when this work first began. Over a period of several months, nineteen of these pigs became available and were selected for use as a reference panel. Between them they represented a total of fifteen antigenic factors, the only factor absent being K_d. Only three systems were not represented by double recessive animals, that is the -/- genotype. They were the E, G and I systems. Of these systems E and G are believed by Andresen to be closed systems, and double recessive animals have not as yet been found (Andresen, 1962).

The purpose of the reference panel was twofold:-

(1) They were used as donors and recipients in immunisation courses in an effort to duplicate some of the antibodies isolated by Andresen. Also, they were a source of known donor cells.

(2) /

(2) Their main function was as a source of known reference cells to be used in the determination of some of the antibodies present in unknown antisera.

(d) Animals for inheritance study

The definition of a particular antibody unit capable of detecting one antigenic factor is not complete without a study at the level of the gene determining this factor. Bernstein (1924) first conclusively showed that the simple Mendelian segregation ratios could be applied to the human AB antigens. To test the segregation ratios of the genes determining the factors detectable by:

(1) Specific antibody units as isolated by serological techniques.

(2) Biophysical methods.

A large number of pig families were bled and grouped. These families were selected to give a mixture of breeds, so that a maximum distribution of the genes involved could be obtained. The number of animals involved are grouped in breeds as shown in the table below:-

Breed	Boars	Sows	No. of offspring
Large White	6	21	88
Landrace	3	6	22
Gene pool*	6(+3)	21	133
Total	15	48	243

*This was a pool of animals from Large White, Landrace, Wessex and Tamworth breeds. Nine boars were included in the gene pool mating, of which three were the Landrace boars.

3. Blood sampling and storage: (a) Bleeding For general purposes when small volumes of blood were required (i.e. 30 to 100 ml.) the external auricular vein was used. The ear was scrubbed and shaved and swabbed with spirit, then a fine film of petroleum jelly was rubbed over the ear from the proposed site of bleeding to the edge of the ear. A small slit was made along the longitudinal axis of the vein, and blood was collected by free flow from the edge of the ear. Sometimes the tip of the ear was rubbed with a swab dipped in xylol to help vasodilate the vein.

Larger volumes of blood could sometimes be obtained from the ear vein, but generally it has been more successful to collect by puncture of the anterior vena cava. Sows were restrained in the standing position by a loop of rope around the upper snout. The rope was stretched upwards to a ring in a wall and tied. All bleeding was performed on the right side of the animal from a site just in front of the manubrium of the sternum and to the right of the trachea. The site for puncture was scrubbed in the usual manner and the actual point of entry was on a line taken from the manubrium to the base of the ear approximately 1 to 1½" from the manubrium entering at the supraclavicular fossa. A 4 to 5" needle of 14 S.W.G. was inserted upwards, slightly inwards and slightly backwards. On entering the skin negative pressure was applied to a syringe attached to the needle. Entry of the anterior vena cava is usually at a point approximately 3" from the surface of the skin.

(b) /

(b) Collection and storage of whole blood The acid-citrate-dextrose solution recommended by Loutit and Mollison (1943) was used for this purpose. Four volumes of blood were mixed with one volume of this solution. The blood was then stored at +4°C. The erythrocytes in blood collected from the living animal generally kept for a period of three weeks in this solution at this temperature, and if they were not disturbed could keep for one month. The cells in blood collected at the abattoir, however, only kept for one week to ten days and were generally unsatisfactory.

(c) Clotted blood and storage of serum Serum from immunised animals which contained antibodies and serum for biophysical investigation was obtained by first collecting blood in a clean, dry, sterile bottle. This blood was allowed to clot, then the clot was freed from the surface of the container and given time to contract at room temperature. Contraction could be anhanced by incubation for one hour at 37°C. After this the blood was allowed to stand in the refrigerator at 4°C. for three to four hours. The serum was then decanted into centrifuge bottles or test-tubes and spun at 1,500 G for 30 minutes. The supernatant serum was then sucked off with a clean pipette and placed in bottles or test-tubes, and stored at -15 to -20°C. It was found that pig cells within clotted blood tend to haemolyse quickly if left too long at room temperature. For the collection of relatively clear unhaemolysed pig serum it was found necessary to separate the serum within four to five hours of collection.

4. /

4. Transfusion for antibody response

(i) Isoimmunisation: (a) Method Blood from the selected donor pig was collected into acid-citrate-dextrose solution and 10 ml. of this mixture was injected intramusculary as a sensitising dose in the neck of the recipient pig. Prior to the commencement of immunisation the cells of the donor animal were tested with the serum of the recipient for the presence of pre-injection antibody. After the sensitising dose subsequent injections of the same volume and mixture of fresh whole blood were given at weekly intervals for four to six weeks. Test samples of serum from the recipient were taken prior to each injection, and were tested against the donor cells for the presence of antibody. This enabled one to follow the antibody response and decide at what stage immunisation should stop. If there was a suitable response a large volume of blood was taken from the recipient animal ten days after the last injection.

In seven different transfusions adjuvant in the form of sorbital monoleate ('Crill 16'), as recommended by Dresser (1961) was given with the sensitising dose. The site of injection of the adjuvant was different from the site for the injection of blood. It was given subcutaneously behind the elbow of the animal. This was to avoid the possibility of sterile abscess formation in the neck region.

All the transfusions were performed in mature sows of at least two years of age and over.

The choice of intramuscular injection as the route of administration was decided for the following reasons:-

1. It avoided damage to ear veins, which were being used for bleeding purposes.
2. It allowed slower absorption of the transfused blood and this helped to avoid possible transfusion reaction. In fact, none of the sows which were injected showed a transfusion reaction.
3. Previous reports have shown that antibody stimulation by the injection of crystal violet vaccine, which contains pooled whole blood of pigs is extremely good (Goodwin, 1955). This vaccine is administered subcutaneously or intramusculary.

A comparison of the results of the transfusions within and between different breeds are given in Table I. A total of 49 sows were immunised and studied, of which 47 were selected animals and the remaining two were obtained at slaughter 14 days after their second injection of crystal violet vaccine.

Table I

Breed of donor	Breed of recipient	Total no. of sows immunised	No. giving antibody response of titre 1/2 and over	% response	No. giving antibody response of titre 1/64 and over	% response
LW	LW	22	12	54.5	6	27.3
LW	Wessex	15	10	66.7	4	24.5
Wessex	LW	3	3	100	0	0
Wessex	Wessex	7	7	100	0	0
Crystal Violet Vaccine	Wessex	2	2	100	1	50
Total		49	34	69	11	22.4

Of /

Of the 22 Large White sows immunised, seven were given sorbital monoleate with the sensitising dose and a summary of the results obtained are shown in Table II.

Table II

No. of sows immunised	No. giving antibody response of titre 1/2 and over	% response	No. giving antibody response of titre 1/64 and over	% response
7	6	85.7	4	57

The approach adopted in the first instance for the isolation of specific antibodies was based on the work by Rendel (1958). He used Wisconsin reagents to type Swedish cattle, and then selected animals which were similar in blood type apart from one or two factors. These animals were then used as donors and recipients in immunisation courses. In this way he was sometimes able to produce uncomplicated antisera containing only one or two antibodies. The reference panel of 19 Large White sows which were available were selected in a similar manner for immunisation. Only a few combinations showing a limited antigen difference were found. Even in those instances where the only apparent difference was one factor, it did not necessarily follow that this was the only factor; but within the limits of Andresen's grouping, it was the only factor. The results in Table III show the different types of antibody and titre of antisera produced within the different breeds selected for immunisation. Where the number of antigen differences are known it is shown, also the specific antibodies isolated from the antisera produced are shown. The first part of this thesis is concerned with a study of the specific antibodies which were isolated.

Details /

Table III

Breed of donor	Breed of recipient	No. of antigen differences if known	No. of antisera obtained	Distribution of antiserum showing type of antibody and titre response																		Specific antibody isolated
				Saline Agglutinin						Haemolysin						Incomplete Agglutinin						
				1/4	1/16	1/32	1/64	1/128	1/256	1/4	1/16	1/32	1/64	1/128	1/256	1/4	1/16	1/32	1/64	1/128	1/256	
LW	LW	At least 1	5	1	1	1[c]	1	1	-	All negative						Not typed						No. 2
LW	LW	At least 2	2	-	-	-	-	-	1	-	<1[c]	-	-	-	-	-	-	1[c]	-	-	-	No. 5 & 14
LW	LW	At least 3	4	-	-	<2[c]	1>[c]	-	-	<1[c]	-	-	1>[c]	-	1>[c]	-	-	-	1>[c]	-	-	No. 11, 12, 13 & 15
LW	LW	At least 4	1	-	-	<1[c]	-	-	-	Not typed						Not typed						No. 10
LW	W	Not known	10	4	2	-	2	2	-	1	-	1	-	1	-	1	2	-	2	1	1>	No. 8 & 9
W	LW	Not known	3	<1	-	2	-	-	-	All negative						Not typed						None
W	W	Not known	7	2	5	-	-	-	-	-	2	-	-	-	-	Not typed						None
CVV	W	Not known	2	<1	<1	-	-	-	-	-	-	-	-	-	1>	-	1>	-	<1	-	-	No. 6

Note:- 1. Antibodies marked with a 'c' were obtained from antisera of animals immunised with whole blood plus 'Crill 16' adjuvant.

2. A proportion of the antisera were not investigated for incomplete agglutinins. because at the time of immunisation a suitable antiglobulin serum was not available.

Details of all the immunisations carried out are shown in the Appendix 1A and 2A.

(b) Heteroimmunisation The laboratory animal used for the production of hetero-antibody was the rabbit. This animal was immunised for two purposes:-

1. Stimulation of antibody to particular pig red cell antigens.
2. Obtaining an anti-pig globulin serum for Coomb's test.

1. The method adopted for immunisation was intravenous injection of 1 ml. of a 20% suspension of thrice washed pig cells at two day intervals for six injections. Pre-injection samples of blood were taken from the rabbits before and after sensitisation. Each animal was bled from the marginal ear vein at ten days after the sixth injection. Two specific antibodies Nos. 3 and 4 were obtained as a result of these immunisations, and they will be described under the section on results.

2. The method first adopted was injection of 1 ml. of pooled pig serum intraperitoneally as a sensitising dose. A similar injection was given one month later and the rabbits were bled ten days after this injection. The first six rabbits injected did not yield any precipitating antibody and repeat injections were given at three monthly intervals. After the fourth injection, an antiserum to pig globulin was obtained.

The second method adopted was injection of 1 ml. of pooled serum plus 0.2 ml. of sorbitol monoleate subcutaneously as a sensitising dose. Three months later the rabbits were injected intraperitoneally with varying doses of pooled pig serum. The quantity injected varied from 0.2 ml. to 1.0 ml. with different rabbits. This was followed one week later by a similar injection of the same amount as given previously to each rabbit. The rabbits were then bled 14 days later. The antiserum obtained from these rabbits was not satisfactory. Some difficulty was experienced in trying to obtain a suitable rabbit antiserum against pig globulin, and this will be discussed further in the next section.

5. Blood grouping techniques: All whole blood obtained from isoimmunised sows was left in the refrigerator at 4°C. for three to four hours before spinning down cells and decanting the antiserum. This was to enable any naturally occurring cold auto-antibodies present to bind on to the red cells and therefore be removed from the serum on spinning down the cells. An aliquot of each antiserum to be investigated was then serially diluted in 0.9% saline, either in doubling dilutions from 1 in 2, or trebling dilutions from 1 in 3. The latter was eventually preferred and adopted routinely, because it gave sharper end-points, and was slightly more economical on antiserum. Cells from the donor pig and from the animal yielding the antiserum were then tested against the serially diluted serum. The inclusion of the recipient's own cells acted as a negative control.

Three standard techniques for the detection of antibody were investigated and used in this study. The techniques used were:-

1. /

1. Saline agglutination
2. Anti-globulin (Coombs) test
3. Haemolytic test

1. Saline agglutination This was the first test to be applied to any antiserum and it was carried out at two different temperatures, that is at room temperature (18 - 20°C.) and at 37°C. The use of unheated antiserum helped to preserve whatever complement factors there were present, and this allowed one to observe whether any of the antibody present besides being agglutinating was of a haemolytic nature as well. If haemolysis did occur it only appeared in the first two tubes. After this screening test, the antiserum was then heated to 56°C. and left for 30 minutes at this temperature. It was found that this was sufficient to remove the complementary effect of the pig serum. The saline agglutination tests were then repeated on the heated serum, and the other blood grouping techniques were applied when possible. In the isoantibodies to be described, there was a slight difference in the effect of temperature on the reaction of saline agglutinins, also a slight drop in the titre of the antiserum after heating to 56°C. The following example illustrates this effect:-

Effect of temperature on the reaction of saline agglutinins

Conditions of antiserum	Cells		Titre of antiserum AP 8914							
			1 in 2	1 in 4	1 in 8	1 in 16	1 in 32	1 in 64	1 in 128	SC
1. UNHEATED										
(a) Incubated at 37°C.	Donor	9091	a	a	a	a	a	sa	sa	-
for two hours	Recipient	8914	-	-	-	-	-	-	-	-
(b) Incubated at RT	Donor	9091	a	a	a	a	a	a	-	-
for two hours	Recipient	8914	-	-	-	-	-	-	-	-
2. HEATED										
(a) Incubated at 37°C.	Donor	9091	a	a	a	a	a	sa	sa	-
for two hours	Recipient	8914	-	-	-	-	-	-	-	-
(b) Incubated at RT	Donor	9091	a	a	a	a	sa	-	-	-
for two hours	Recipient	8914	-	-	-	-	-	-	-	-

Legend:- a - agglutination
sa - slight agglutination
sc - saline control

Two methods were investigated for detecting saline agglutinins. The first method used was the slide test. Pieces of white vitrolite glass measuring 13¼" x 6½" were cut, and each piece was then divided into 72 x 1" squares by means of a carborundum wheel which cut grooves of 1/10" wide by 1/32" deep. Thus each tile had 6 rows of 12 x 1" squares, which therefore enabled one to use one row of 12 squares for one antiserum in ten serial dilutions with a saline control. On any one tile, cells from six individuals could be tested against one antiserum in serial dilution. Conversely, six individual antisera in serial dilution could be tested against cells from one individual or each tested separately against other cells.

For each test, aliquots of red cells were washed in fresh saline and then spun in the centrifuge. Between each spinning the saline was removed by suction and the cells resuspended in fresh saline. At the end of the third wash the cells were spun for 30 minutes to pack the cells. A measured amount of these packed cells were sucked up into a pipette, and resuspended in a known volume of saline to make a 2% suspension. This 2% suspension was used in the direct test against the serially diluted antiserum.

A pipette calibrated to give a volume of approximately .03 ml. with each drop was used to put out the antiserum and the suspension of red cells. One drop of each dilution of antiserum was mixed with one drop of the red cell suspension on each 1" square on the tile. The mixing was completed with the corner of a microscope slide.

At /

At room temperature the plate was rocked gently for 15 minutes, and the reaction read by means of a hand lens. After this time at room temperature, the mixture began to dry out and the cells stuck to the bottom of the plate.

For tests at 37°C., the whole slide was placed in an incubator on top of a blotting pad soaked with water as in the technique described by Simmons et al. (1943). It was left at this temperature for 30 minutes, then taken out and read as above. There was no apparent evaporation during this period.

The other method investigated and finally adopted routinely was the test-tube technique. A finer bore Pasteur pipette capable of delivering approximately .015 ml. with each drop was used for this test. One drop of the cell suspension was mixed with one drop of the test serum in the bottom of a small tube measuring 5 mm. internal diameter and 50 mms. high. The two were mixed by shaking the tube. These tubes could be left for two hours at room temperature in a large metal rack capable of holding 600 similar size tubes. The whole rack could also be placed for a similar period in a large water bath at 37°C., so that the tubes were one third immersed in the water. There was no evaporation during this time. The reaction in each tube was estimated by very gently sucking up the contents into a long wide bore Pasteur pipette, then spreading them out carefully on a microscope slide. Each reaction was then interpreted under the low power of the microscope. This technique proved more valuable than the slide test, because it allowed a simple score system to be adopted for reading the degree of agglutination present. The /

The score system adopted is shown in the table below:-

Score system for interpreting degree of agglutination

Score	Interpretation	Approx. % of cell agglutinated
-	= no visible reaction	0
0	= very occasional clump of 3 or 4 cells	1 - 5%
1	= small clumps of 5 or 6 cells - 80% of cells free	20%
2	= small clumps of cells more numerous than 1	40%
3	= large clumps of cells with moderate number free	60%
4	= very large clumps with the occasional free cell	80%
5	= large unbroken clumps with no free cells	100%

2. Anti-globulin (Coombs) test This test was devised by Coombs, Mourant and Race (1945) for the detection of the incomplete agglutinating, Rhesus antibody. Unlike the complete agglutinin, which operates in two stages, by first combining with the reciprocal antigen on the red cells, and then bringing the cells together in a clump in the second stage, the incomplete agglutinin only accomplishes the first stage. So that the cells coated with antibody (which is usually a γ-globulin) can complete the second stage, it is necessary to introduce a further antibody, that is an anti-globulin serum. The type of anti-globulin serum used depends on the species from which the antiserum coating the cells was obtained. For the detection of incomplete agglutinins in pig antisera, an anti-pig globulin serum was necessary. The methods used to obtain such an anti-globulin serum in rabbits are described under the section on hetero-immunisation. As mentioned in this section some difficulty was experienced in obtaining such a serum, and consequently, the anti-globulin /

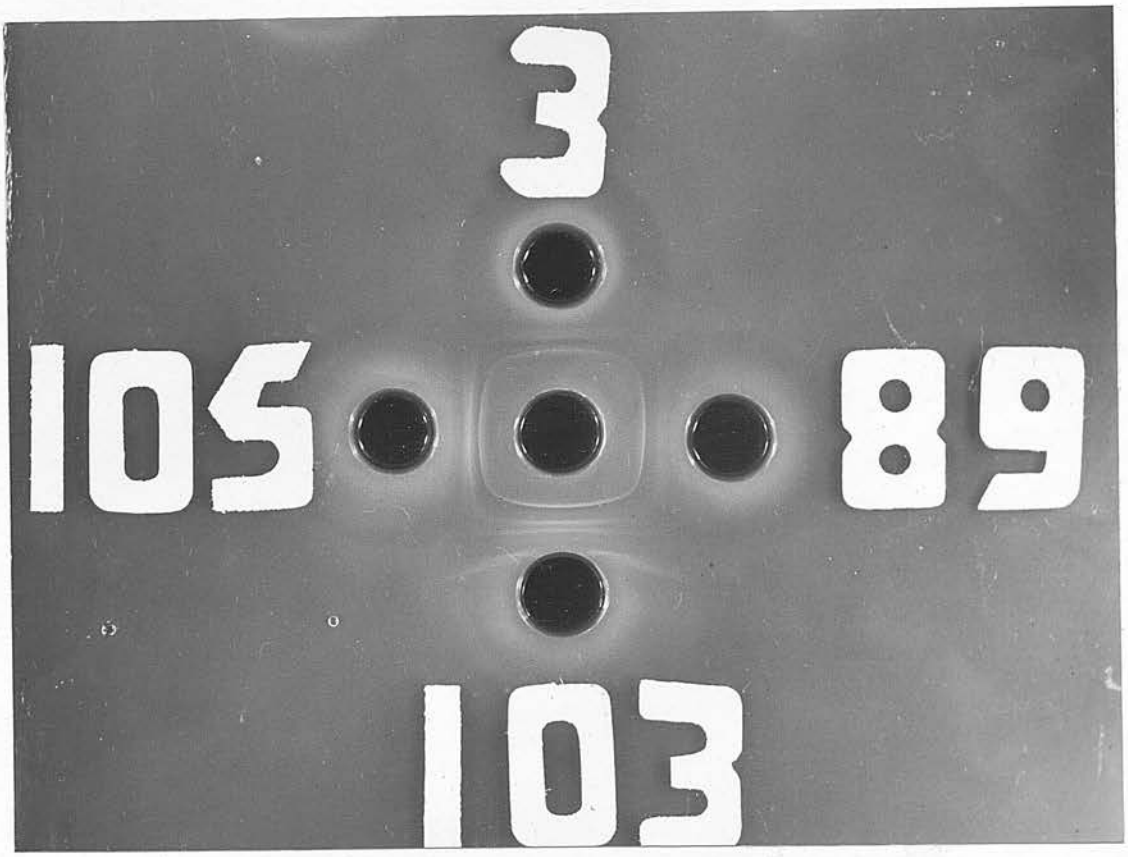
globulin test could not be applied to several of the isoantisera first investigated.

At this stage, it was not known whether the fault lay with the immunised rabbits, who had not produced anti-pig globulin, or with the isoimmunised pigs, who had not produced incomplete agglutinins. To establish that antibody to pig serum had been produced by the rabbits injected, agar gel diffusion on Ouchterlony plates was set up. Serum from four rabbits injected with pooled pig serum was placed in the four outer wells of an Ouchterlony plate, and allowed to diffuse at 37°C. against pooled pig serum in the centre well. The results of such a test are shown in Photograph No. 1.

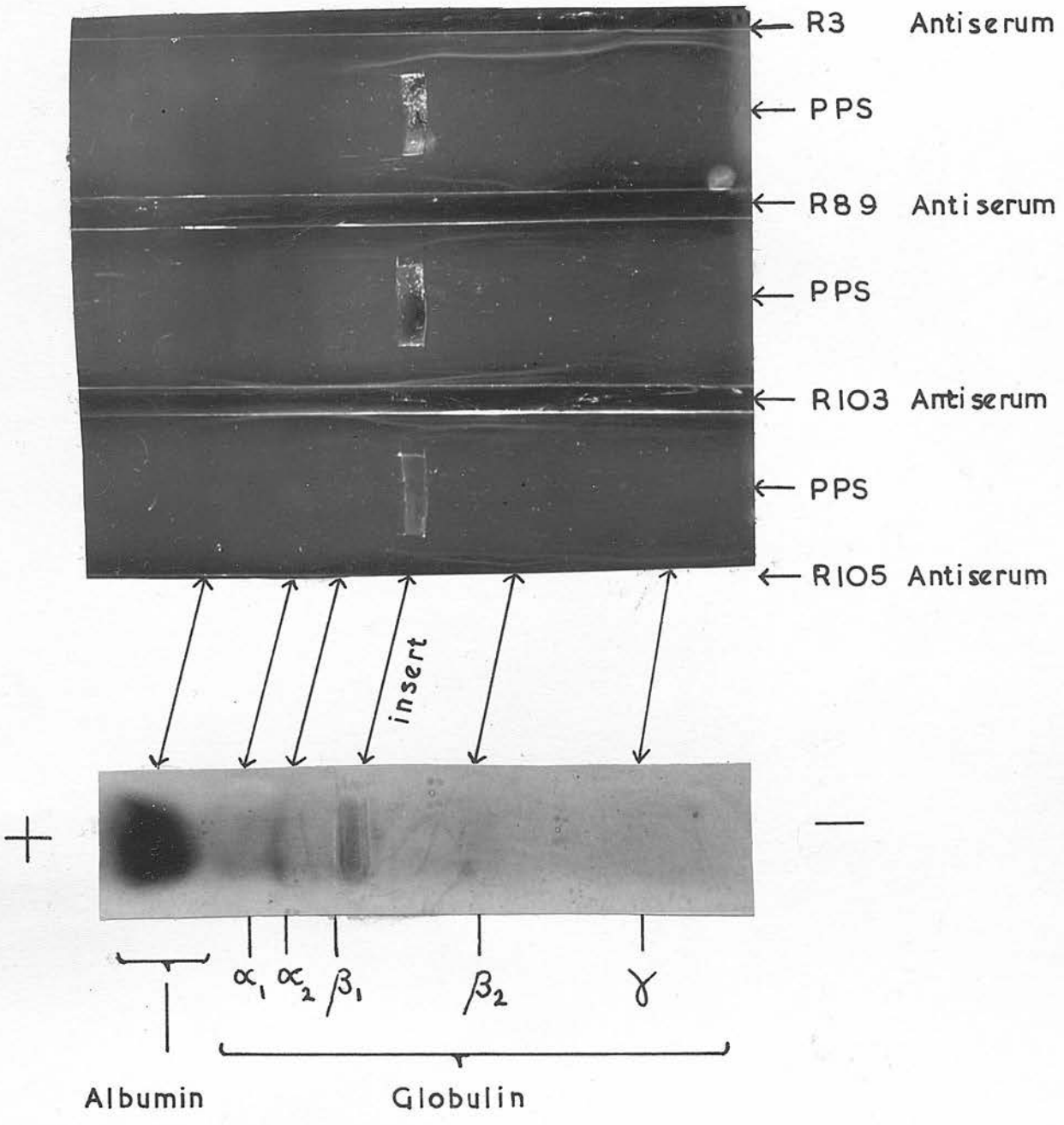
The presence of precipitin bands indicated an antibody response to the pooled pig sera. However, this did not indicate which component(s) of the pooled sera were being precipitated by the rabbit antisera. To establish which components were being precipitated, the immunelectrophoresis technique of Grabar and Williams (1953) was adopted. By electrophoresis, the pooled pig sera was separated into various fractions in the agar gel. The various rabbit antisera were then placed in grooves cut in the gel running 8 mms. apart and parallel to the line the pig sera had followed. As shown in Photograph No. 2, long precipitin bands were present in the region corresponding to the $\beta 1$, $\beta 2$ and γ-globulin zones, as described by Hirschfield (1962).

Rabbits Nos. 3 and 105 appeared to give the best precipitation in the γ-globulin region, and their antisera were used for all the anti-globulin tests. Subsequently, when pig antisera containing incomplete antibodies were obtained both rabbit anti-pig globulin sera showed maximum titres of 1 in 1,024 when tested against sensitised /

Photograph No.1.



Photograph No.2.



Pooled Pig Serum

tised cells.

To prepare the rabbit anti-pig globulin serum for the test, it was first heated to 56°C. for 30 minutes to destroy complement. When cool it was absorbed with a mixture of type A and O cells at a ratio of 1 of cells to 1 serum at room temperature for 30 minutes. This was to remove the species antibody, which was present in the rabbit serum. The absorbing cells were washed thoroughly with fresh saline at least six times before absorption. This was to remove any trace of γ-globulin which might be present in the washed cells. Even a small amount of γ-globulin is sufficient to neutralise the γ-globulin antibody present.

For the test one drop of .03 ml. of a 5% suspension of cells was mixed with .03 ml. of the antiserum dilutions in 6 x 50 mm. test-tubes. The tubes were then one third immersed in a water bath at 37°C., and left for two to three hours. At the end of the incubation time the tubes were spun in a centrifuge, and the supernatant serum carefully removed by suction, so that the cells were left in the bottom of the tube. The cells were then resuspended in a small volume of saline, and then briskly washed by squirting in under pressure a jet of saline from a polythene wash bottle. This suspended the cells in a large volume of saline. They were then spun and the whole process repeated three times. After the final spin the supernatant saline was removed by suction leaving a small button of cells in the bottom of the tube. These cells were resuspended in .015 ml. of saline and then mixed with .015 ml. of suitably diluted, absorbed anti-globulin serum. The tubes were left at room temperature for 30 minutes, and the reaction was read by spreading

and /

and inspecting the contents on a microspope slide under the low power, as for the saline agglutination test. Fine clumping of the cells was observed in a positive reaction. The coarse clumping associated with a direct agglutinin was never seen, and using the scoring system adopted for measuring agglutination, the maximum score rarely exceeded three.

To obtain the optimum dilution at which to use the anti-globulin serum, it was standardised against each individual antibody as recommended by Dunsford and Grant (1959). This is shown in the example for Antibody No. 13 below:-

Standardisation of R3 anti-pig globulin serum against Antibody No. 13

		Dilutions of R3 anti-pig globulin serum 1 in 3	9	27	81	243	729	2187	Cells in saline
Donor B3-255	1 in 3	3	3	3	3	2	1	-	-
cells sensitised	" 9	2	2	2	1	1	0	-	-
with Antibody	" 27	2	2	2	1	0	0	-	-
No. 13 diluted	" 81	1	1	1	-	-	-	-	-
	" 243	-	-	-	-	-	-	-	-
Recipient A4-8457 cells		-	-	-	-	-	-	-	-

For Antibody No. 13 the actual dilution of R3 anti-pig globulin serum selected was 1 in 20.

3. Haemolytic test The standard 'three drop' test was used for detecting haemolysins in isoantiserum. A Pasteur pipette capable of delivering .015 ml. drops was used to put out in each tube one drop of each serial dilution of the antiserum under test. After washing thoroughly in distilled water and saline, the same pipette could be used to add to each tube one drop of the test cell suspension. /

suspension. A weaker suspension of cells estimated at 1.25% concentration was used for this test. To overcome making-up suspensions to this concentration each time, a standard reading was taken in the Eel Colorimeter against a red filter. All subsequent test cell suspensions were diluted to this standard. Tubes of 6 x 50 mm. size were used as before, and held in metal racks capable of holding 600 tubes. The antiserum and the cells were thoroughly mixed by shaking the tubes by hand. After washing the same pipette again, one drop of complement was added to each tube and all tubes shaken thoroughly as before. Complement in the form of fresh guinea-pig and fabbit serum was used, and their complementary effect was compared at different dilutions and temperatures. This is shown in the example below testing donor 254 cells against 1859 antiserum:-

Room temperature (18-20°C.) for 6 hours

		Dilutions of Antiserum										
		1 in 1	3	9	27	81	243	729	2187	C'	S	Score
Dilutions of	1	5	5	5	5	5	3	-	-	-	-	28
Guinea-pig	2	5	5	5	5	5	2	-	-	-	-	27
complement	5	5	5	5	5	5	2	-	-	-	-	27
	10	5	5	5	5	5	2	-	-	-	-	27
	20	5	5	5	5	5	2	-	-	-	-	27
Dilutions of	1	5	5	5	5	5	5	5	-	-	-	35
Rabbit	2	5	5	5	5	5	5	5	-	-	-	35
complement	5	5	5	5	5	5	5	5	-	-	-	35
	10	5	5	5	5	5	5	2	-	-	-	32
	20	5	5	5	5	5	4	1	-	-	-	30

37°C. /

37°C. for 6 hours

		Dilutions of Antiserum 1 in 1	3	9	27	81	243	729	2187	C'	S	Score
Dilutions of	1	5	5	5	5	5	4	2	-	-	-	31
Guinea-pig	2	5	5	5	5	5	4	1	-	-	-	30
complement	5	5	5	5	5	5	3	1	-	-	-	29
	10	5	5	5	5	5	1	-	-	-	-	26
	20	5	5	5	5	5	1	-	-	-	-	26
Dilutions of	1	5	5	5	5	5	5	5	-	-	-	35
Rabbit	2	5	5	5	5	5	5	5	-	-	-	35
complement	5	5	5	5	5	5	5	4	-	-	-	34
	10	5	5	5	5	5	5	3	-	-	-	33
	20	5	5	5	5	5	4	-	-	-	-	29

Increasing the temperature appeared to improve the complementary effect of guinea-pig serum, but this was still not as effective as rabbit serum either at room temperature or 37°C. For routine testing fesh neat rabbit serum was used as a source of complement. It was absorbed with type A pig cells at +4°C. for 30 minutes at a ratio of one volume of cells to four volumes of serum. This was to remove the naturally occurring antibody, which cross reacted with pig type A cells, and the species antibody. The serum was stored in small volumes in tubes at -15 to -20°C. and thawed out rapidly at 56°C. before use. Its potency did not appear to decline after six months' storage, but after this period fresh serum was obtained again, and the old was discarded.

With each haemolytic test, two negative controls were run routinely. The first tube omitted antiserum, but contained two drops of complement to one of cells. This tested the effective removal of natural /

natural and species antibody within the complement. The other tube contained two drops of saline with one of cells, and this acted as a control on the tonicity of the unsensitised cell membrane to withstand the test.

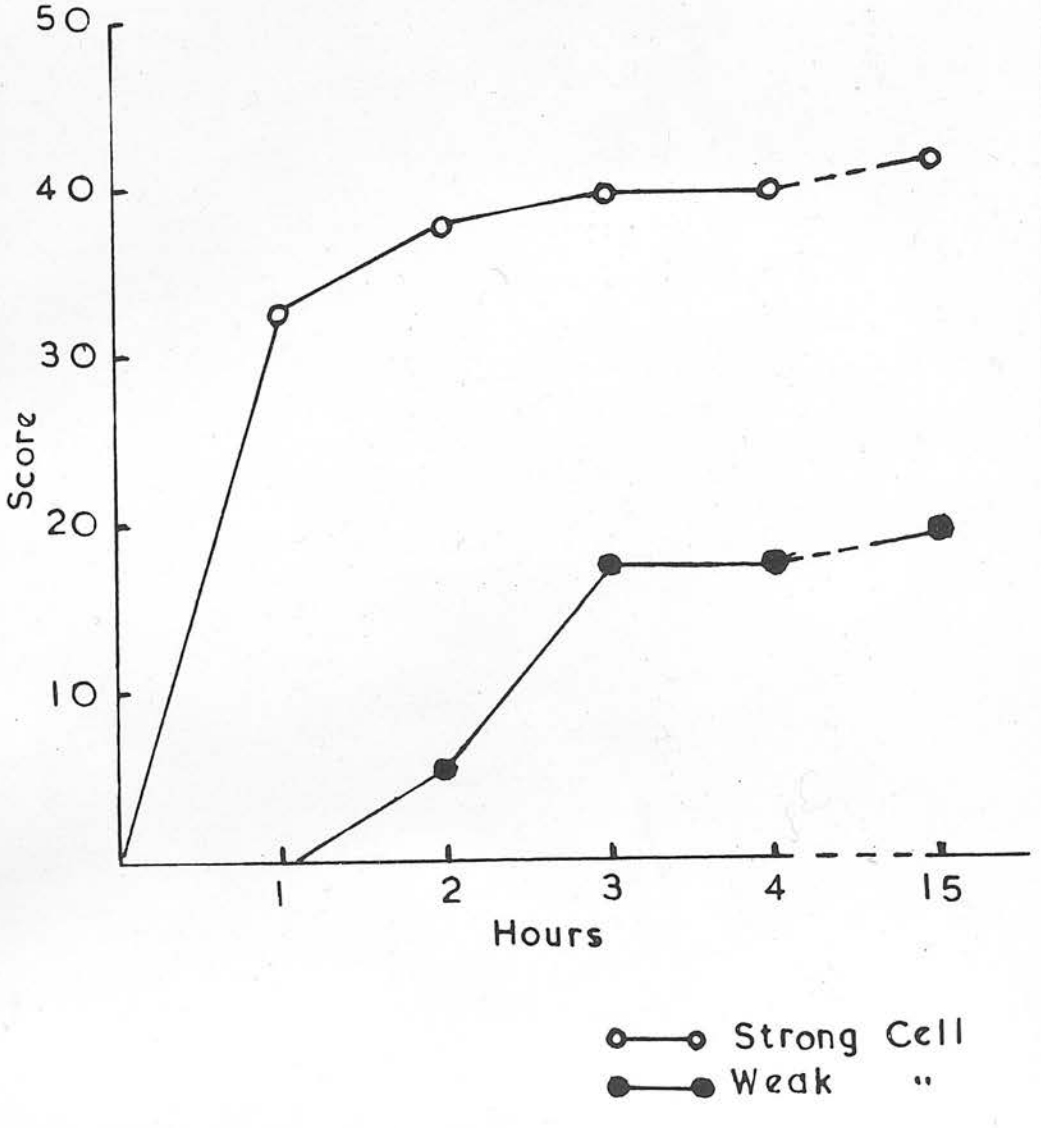
The standard test was conducted at room temperature and the rack carrying the tubes was placed in a mechanical shaker, which automatically shook for one minute at the end of each hour. This allowed the sensitised cells which had settled at the bottom of the tube to come in contact with more complement, which was always present in excess. To find the optimum time for conducting the test, readings were taken every hour for four hours, then the test was left at $+4^{\circ}$C. for 12 hours and read again. The graph in Figure 1 shows the effect of time on the haemolytic reaction obtained with one antiserum against two different reacting cells using neat rabbit serum as complement.

In all the haemolytic tests a scoring system was adopted, which was very similar to that described for the agglutination tests. The degree of haemolysis was scored from 0 to 5, which was assessed by eye and corresponded to a range of haemolysis of 10 to 100%. A guide to the percentage of haemolysis was the amount of unhaemolysed cells left in the bottom of each tube.

6. Analysis of antiserum: If one animal does not have antigenic factors A, B and C on its red cells, then theoretically it should be capable of producing antibody to each of these factors, when cells of another animal carrying factors A, B and C are injected. Ideally, /

Fig. 1

EFFECT OF TIME ON HAEMOLYTIC REACTION



Ideally, this may depend on the antigenic stimulus of each antigen and the antibody response of the recipient. However, in all cases of immunisation if the donor antigens are known, then it can be postulated what antibodies might be present in the antiserum. Sometimes, besides the known factors, there may be several unknown, and in other circumstances, all of the factors injected may be unknown. Before a specific antibody to one factor can be isolated and used for blood grouping purposes, the antiserum from which it originated has to be fractionated by absorption with positive reacting cells.

(a) Antibody absorption If positive reacting cells are mixed with an antiserum, the antigenic factors on these cells will combine with their opposite antibodies in the antiserum. On spinning down the cells and removing them from the antiserum, they will extract the antibodies with which they have combined. This leaves in the antiserum all other antibodies with which the cells did not combine. Thus in the case of the hypothetical antiserum containing antibodies A, B and C, if cells carrying factors A and C are mixed with the antiserum, antibodies A and C will be removed leaving antibody B alone in the antiserum. This is the ideal type of absorption to find, because the fraction of the antiserum which is left will only combine with cells carrying antigenic factor B.

If, however, cells of type A, B and C are used for absorption, then the antiserum will be completely exhausted of all isoantibodies. This type of absorption occurs when donor cells are used to absorb the recipient's serum.

For /

For routine absorptions, cells were washed three times in saline, then packed in the bottom of a tube by spinning at 1,500 G for 30 minutes. The supernatant saline was removed and an equal volume of antiserum added to the cells. They were mixed and left to stand at room temperature for 30 minutes with occasional remixing during this time. After spinning again at 1,500 G for 30 minutes, the serum was carefully removed with a Pasteur pipette and transferred to a clean container. Often the process had to be repeated three or more times using fresh cells each time, before the antiserum was exhausted of all antibodies to the absorbing cells. The final absorption ratio was calculated by dividing the volume of antiserum absorbed by the total volume of cells used during the whole absorption process. Neat serum inactivated at 56°C. for 30 minutes was used for absorption.

(b) Matrix analyses This was the procedure adopted in the isolation of the specific antibodies described in this dissertation. It is based on the method described by Hall (1955). Cells from the reference panel of sows were used in the first analysis. If the donor and recipient animals belonged to this panel, then the hypothetical antibodies could be postulated. However, in instances where either one or both blood types were not known, then the reference panel was used to try and assess what antibodies might be present. As an example of analysis, the fractionation of antiserum 254 is given as an example.

Antigenic factors A, E_f and K_b were the potential antibody stimulators in this instance. Animals from the reference panel having cells which were positive for either one of these factors alone, or in combination, or completely negative were selected. No animals /

animals were available, which were E_f +ve and K_b -ve, but the converse types were present. Cells from the donor and recipient were always included in each analysis.

The selected panel were tested against serial dilution of antiserum 254, and their agglutination scores noted. Aliquots of 254 antiserum were then separately absorbed by each positive acting cell including the donor, also one negative cell and the recipient's own cells. These aliquots were then serially diluted and each tested in turn against fresh cells of all the animals used in the absorption matrix. The results are shown in Table 4.

From this table it is logical to assume that each absorbed aliquot will only contain antibodies against factors not present on the absorbing cells. As non specific absorption may be a potential factor, the absorption with a negative reacting cell was included as a control. In the case of the aliquot absorbed with 1557 cells, all the known antigenic factors A, E_f and K_b were involved, but antibody was still left against cells 9091, 8457 and the donor. This indicated that other unknown factors on the donor's cells had stimulated antibody formation. The absorption with 9091 cells, however, still left antibody for 8457 cells, and vice versa. It was therefore logical to assume as the only known common factor involved for these cells was K_b, that in fact they each had a further unrelated antigenic factor, which must also be present on the donor's cells. These factors may be referred to as X and Y for the meantime. It can also be assumed that antibody to factors A and K_b were present due to the reaction of cells 563 and 262 respectively. Although antibody for factor E_f has also been postulated, /

Table 4

Test Cells	Antiserum 254 absorbed with cells									Antigens on Cells				
	1557	563	255	9091	8457	4731	262	Donor	Recipient					
*1557	-	14	17	14	15	-	12	-	17	A	E_f	K_b	-	-
563	-	-	4	-	-	-	4	-	4	-	-	K_b	-	-
255	-	-	-	-	-	-	-	-	-	-	-	-	-	-
*9091	11	11	18	-	11	12	15	-	18	-	-	K_b	X	-
8457	9	8	15	8	-	9	14	-	15	-	-	K_b	-	Y
*4731	-	13	18	13	14	-	13	-	18	A	E_f	K_b	-	-
262	-	11	10	11	11	-	-	-	11	A	-	-	-	-
*Donor	12	14	18	14	14	16	14	-	18	A	E_f	K_b	X	Y
Recipient	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	A	A	A	A	-	-	-	A					
	-	(E_f)	(E_f)	(E_f)	(E_f)	-	(E_f)	-	(E_f)					
	-	-	K_b	-	-	-	K_b	-	K_b					
	X	X	X	-	X	X	X	-	X					
	Y	Y	Y	Y	-	Y	Y	-	Y					
	Proposed antibodies present													

Table 5

Cells	Antiserum 254 @ 8457 @ 262 absorbed with cells				Antigens	
	1557	9091	4731	Donor		
1557	-	12	-	-	E_f	-
9091	11	-	12	-	-	X
4731	-	13	-	-	E_f	-
Donor	12	14	16	-	E_f	X
	-	E_f	-	-		
	X	-	X	-		
	Proposed antibodies					

postulated, this could not definitely be assumed until further absorptions were carried out. As antibody to factor E_f was required, antiserum 254 was absorbed with cells 8457 and 262 to remove factors A, K_b and Y. The absorbed antiserum left was then divided into aliquots and each absorbed again separately by the three cells carrying factor E_f and 9091 cells carrying factor X, then tested back as before. The results are shown in Table 5.

This matrix confirmed the presence of anti E_f, also a further antibody against factor X. By the same process, absorption with 4731 and 9091 cells would probably have yielded an antibody to factor Y. However, the amount of antiserum limited the extent to which it could be used for yielding adequate amounts of specific antibody. Antiserum 254 absorbed with cells 8457 and 262 was therefore divided into two equal volumes, and each absorbed separately one with 9091 cells and the other with 4731 cells. To further test the unit specificity of these absorbed portions, each was serially diluted and tested against 20 pigs from as varied a population as could be obtained. Slaughterhouse blood had sometimes to be used for this purpose, but it was not completely satisfactory, because of its poor keeping quality, and permanent loss of the source. Aliquots of the absorbed sera were further absorbed with all the positive cells and one negative cell, also the donor, recipient and one other known cell, then tested back as before. The results are shown in Table 6 for one fraction of the antiserum, which was first absorbed with 8457, 262 and 9091 cells.

This /

This table indicates that within the population of cells used, this antiserum could not be fractionated any further, and therefore, was believed to contain an antibody specific for factor E_f. It was called Antiserum No. 11. Although tentatively accepted at this stage as a specific antibody the next section on results will endeavour to establish the specificity of this antibody, and all the other antibodies isolated by similar methods.

3. EXPERIMENTAL RESULTS:-

1. Antiserum No. 1 Sprague (1958) quotes in his paper, that in 1955 (Sprague, 1955) he established that the A, J and R soluble substances of the pig, ox and sheep respectively, cross reacted with the Anti-J found to occur naturally in the serum of cattle. He tested blood samples from 217 pigs with anti-J and found that 94 samples were haemolysed by this antiserum, and the remainder were not. In this way he was able to classify pig cells into type A and type 0 respectively.

To confirm this observation and use anti-J serum as an antibody for the detection of pig type A substance, several cattle were bled to obtain a suitable antiserum. Details of the fractionation of their sera are given in Appendix B (i).

The final antiserum obtained and called Antiserum No. 1, was from cow MZ 206B. This serum was tested against eight cells from the reference panel, of which two cells were A+ve and the remainder were A-ve. The results obtained are compared in a 2 x 2 contingency table:-

		Reference Panel A+ve	Reference Panel A-ve	
MZ 206B Serum	+ve	2	0	2
	-ve	0	6	6
		2	6	8

$$\text{If } P = \frac{2!\ 6!\ 6!\ 2!}{8!} \times \frac{1}{2!\ 6!\ 0!\ 0!}$$

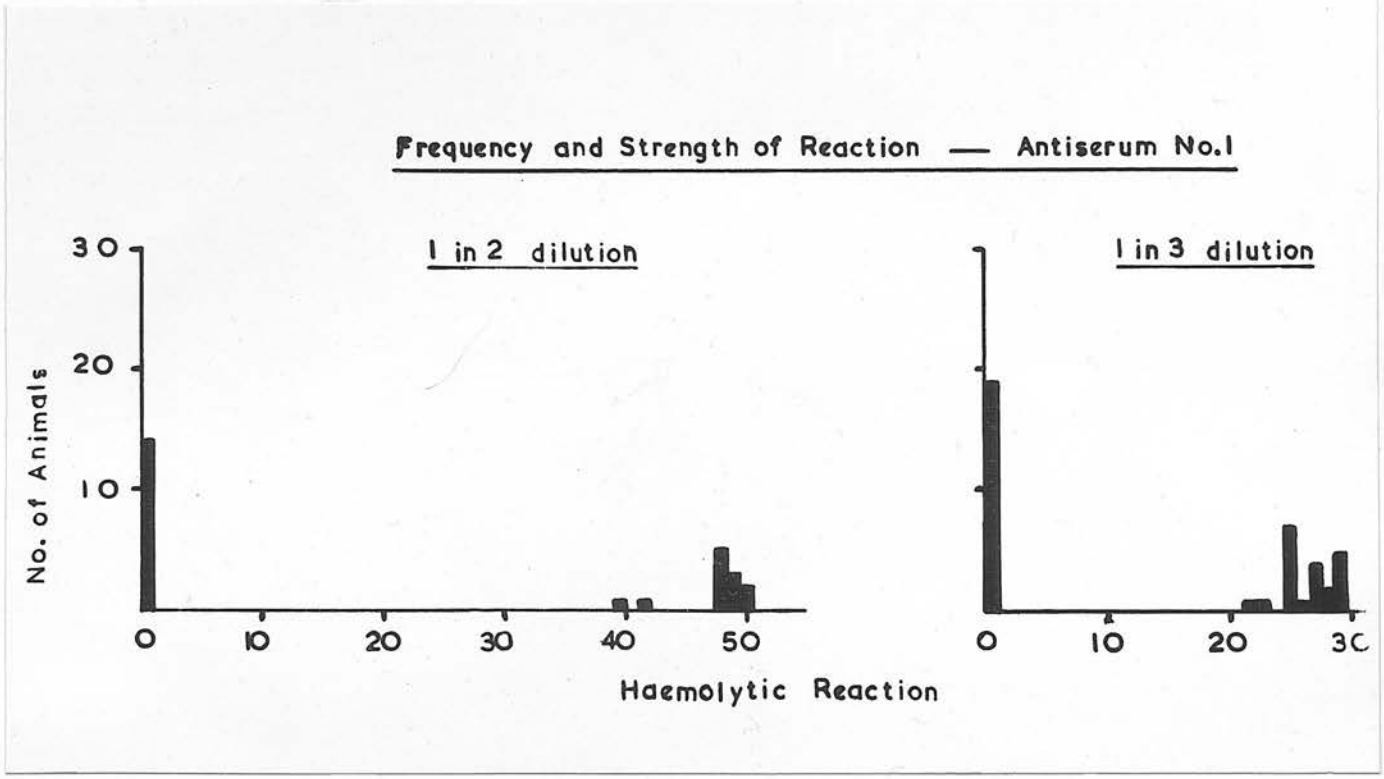
The exact probability of the results being due to chance alone is 1/28.

Qualitative reaction The first step in establishing the specificity of each antibody was to see if it would clearly classify a population of animals into positive and negative groups. Figure 2 shows histograms of the frequency and strength of reaction of Antiserum No. 1 against two different groups of pigs using two different titrations of the antiserum. The actual number of animals tested and the mean reaction score and standard deviations of the antiserum are given below each histogram. The results indicate that the reactions were qualitative.

Inheritance of the A substance Having established that Antiserum No. 1 detected the A substance of pig blood, the hypothesis that it was controlled by a dominant mendelian gene was now tested. A table showing all the possible phenotype matings among families, and the distribution of the character in the offspring according to sex and their reaction is given below:-

Table 7 /

Fig. 2.



Antiserum No. 1	1 in 2 dilutions			1 in 3 dilutions		
	-ve	+ve		-ve	+ve	
Nos. of animals	14	12	= 26	19	21	= 40
Mean haemolytic reaction	= 47.17			= 26.3		
and S.D.	= ±3.6			= ±2.3		

Table 7

Phenotype mating		Parents		Offspring								TOTAL		Combined total
		♂	♀	♂s'				♀s'						
				+		-		+		-				
		No.	No.	Obs.	exp.	Obs.	exp.	Obs.	exp.	Obs.	exp.	+	-	
+	+	5	9	18	15	0	3	29	26	3	6	47	3	50
+	-	5	8	19	14	6	11	18	13	6	11	37	12	49
-	+	4	6	7	6	4	5	8	8	6	6	15	10	25
-	-	10	25	0	0	41	41	0	0	73	73	0	114	114
												TOTAL		238

This table clearly shows that the gene controlling the factor A was dominant, because there were no positive offspring resulting from matings of double negative parents. Only in those instances where one of the parents was positive appeared any positives among the offspring.

Calculation of expected distribution among offspring Assuming that the genotype of the genes controlling the A factor within a population to be AA:Aa:aa, then it is possible to calculate the gene frequencies of the 'A' and 'a' gene having a knowledge of the proportion of 'aa' individuals within a population. In the phenotype distribution the negative class of animals could be assumed to represent the 'aa', or double recessive animals. To calculate the expected distribution of negative and positive offspring from the gene frequencies, one has to assume that the matings were random. The matings in the above table were not completely random in that the Large White breed was represented by several families, and boars were used in more than one mating. However, as approximately half

of /

of the population contained families resulting from a gene pool mixture of four different breeds, it therefore seemed reasonable material on which to test the manner of inheritance of the A factor.

The genotype distribution of the parent population was calculated. A total of 63 parents were involved of which 43 were negative for the A factor:-

	AA	Aa	aa	
Gene frequencies	0.0302	0.287	0.681	= 1.000
Calculated number of parents within population	2	18	43	= 63 parents

The expected frequencies for the offspring resulting from the different genotype matings of the parent population are given beside the observed figures in Table 7.

Backcross matings To complete the inheritance study of the A factor, the classical backcross mating was investigated to establish the dominance of the A gene in the heterozygote. As only one generation of family material was available, the heterozygote genotype had to be assumed on the basis that matings between a positive and negative parent will produce at least one negative offspring:-

i.e.	A+ve		A-ve	Phenotype class
	A^A/A^a	X	A^a/A^a	Parents genotype
	A^A/A^a A^a/A^A		A^a/A^a A^a/A^a	Offspring genotypes
	1.	:	1.	Ratio

If /

The segregation results shown in Table 8 do not fit a one gene hypothesis absolutely, and the possibility of Antiserum No. 1 detecting two factors instead of one should not be overlooked. If a two gene hypothesis is considered, then matings between a positive parent heterozygous for two factors and a parent negative for both factors, where linkage between these factors may or may not exist, the following ratio of positive to negative offspring could be expected:-

Phenotype mating

A+ve;B+ve x A-ve;B-ve.

Genotype mating

$A^A/A^a.B^B/B^b$ x $A^a/A^a.B^b/B^b$

Offspring genotypes

$A^A/B^B.A^a/B^b$ $A^A/B^b.A^a/B^b$ $A^a/B^B.A^a/B^b$ $A^a/B^b.A^a/B^b$

Ratio of +ve to -ve

3 positive : 1 negative

If the deviations from the observed values in Table 8 and the above expected figures are re-calculated in a Chi-squared test, then the level of probability is as follows:-

	No. of offspring +	-
Total observed	33	19
expected	39	13

$$\chi^2 = \frac{(O - E)^2}{E} + \frac{(O - E)^2}{E}$$

$$= 0.92 + 2.77$$

$$p = \text{Between } 0.10 \text{ and } 0.05.$$

If a positive boar was mated to more than one negative sow, then his heterozygosity was based on the evidence within any one family. Proof of his heterozygosity automatically included all other families where the mating was a backcross. Matings between positive sows and negative boars were also included, if at least one of the offspring was negative for the character. The results of all backcross matings are given in Table 8. Deviation from expected is calculated in a Chi-squared test and the level of probability given:-

Table 8

Backcross mating ♂	♀	No. of offspring +	−
+	−		
No. 2	5	18	9
−	+		
No. 4	6	15	10
Total observed		33	19
expected		26	26

$$\chi^2 = \frac{(O - E)^2}{E} + \frac{(O - E)^2}{E}$$

$$= 1.88 + 1.88$$

$$= 3.7 \text{ for } 1 \text{ d.f.}$$

$$P = \underline{\text{Between .10 and .05}}$$

The segregation results indicate that the gene controlling the A factor behaved as a simple mendelian dominant. The chi-squared test applied was taken from Fisher (1950), where $\chi^2 = S\left(\frac{x^2}{m}\right)$.

Subsequent results reported for the other specific antisera are based on a similar interpretation to that presented for Antiserum No. 1.

2. /

2. Antiserum No. 2 Obtained by isoimmunisation of sow AP8914 with whole blood from sow WL9091. Details of the fractionation are given in Appendix B(ii).

The reaction with ten cells from the reference panel of which two cells were E_b +ve and the remainder were E_b negative are compared in a 2 x 2 table.

Reference cells

AP8914	E_b +ve	E_b -ve	
+ve	2	0	2
-ve	0	8	8
	2	8	10

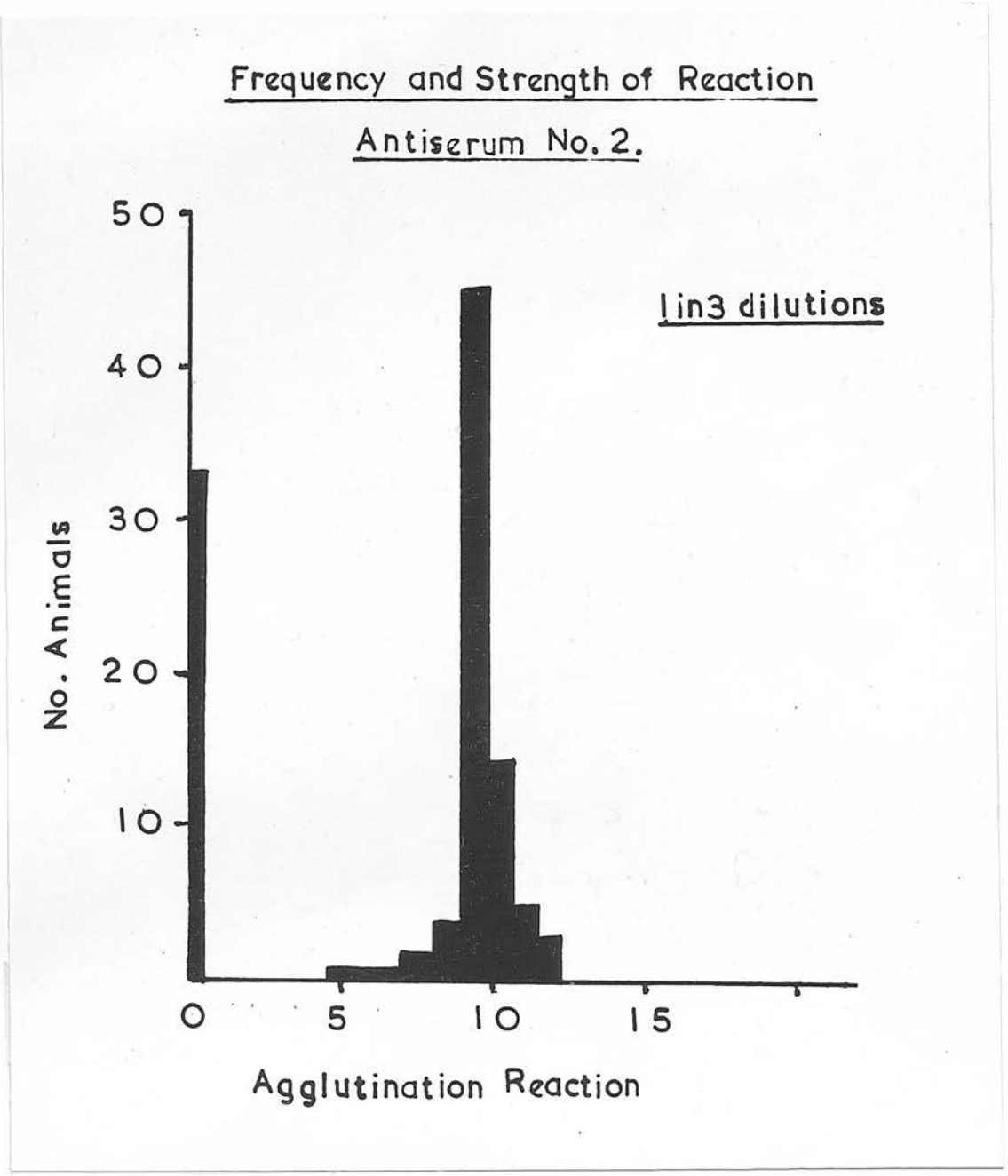
The exact probability of the results being due to chance alone is 1/45. This suggested that Antiserum No. 2 was capable of detecting character E_b.

Frequency and strength of reaction The histogram in Figure 3 indicates that the reaction was qualitative.

Inheritance of Factor E_b - Table 9 gives the observed distribution of offspring resulting from the different matings:-

Table 9 /

Fig.3.



Antiserum No. 2	1 in 3 dilutions		
	-ve	+ve	
Nos. of animals	33	75	= 108
Mean agglutination reaction	= 9.2		
and S.D.	= ±1.13		

Table 9

Phenotype mating		Parents		Offspring						Combined total
		♂	♀	♂s'		♀s'		Total		
				+	-	+	-	+	-	
		No.	No.	Obs.	Obs.	Obs.	Obs.	Obs.	Obs.	
+	+	11	32	63	3	72	4	135	7	142
+	-	5	7	5	5	21	15	26	20	46
-	+	3	5	6	4	12	3	18	7	25
-	-	2	3	0	6	0	12	0	18	18
									TOTAL	220

Because of seven positive offspring appearing in one family, where both parents were negative for the character, complete dominance of the gene controlling the E_b factor could not be accepted on this family data. Unfortunately, both parents had been slaughtered before the offspring were bled and typed, and therefore could not be re-typed. On further enquiry it was found, that during the time of mating there had been a breakout of boars, and the possibility of another sire being involved could not be dismissed. The guilty family has been omitted from Table 9. If the results for this one family are overlooked, then dominance must be present.

Backcross matings Results are given in Table 10.

Table 10 /

Table 10

Backcross mating			No. of offspring	
	♂	♀		
	+	-	+	-
No.	2	4	9	20
	-	+		
No.	1	2	0	7
Total observed			9	27
expected			18	18

$$\chi^2 = \frac{(O - E)^2}{E} + \frac{(O - E)^2}{E}$$

$$= 4.5 + 4.5$$

$$= 9.0$$

P = Between .01 and .001

The segregation results shown indicate that further backcross data is necessary before it can be established that this factor is controlled by a dominant gene. A certain amount of bias was introduced in the mating data given in this table, because the offspring from two sows mated to one heterozygous boar were all negative. Also, one of these families was represented by only one viable offspring. The section on discussion on results will show that E_b was a contrasting factor to E_e, and the distribution of segregants within families for these two factors will be given.

3. Antiserum No. 3 Andresen (1959c) has shown that rabbits will produce specific antibody for the antigens of the K system. To confirm this work, six rabbits were immunised with washed cells from sow TH 203, which had antigen K_b. The antisera they produced were absorbed with cells from sow TH 262, which was recessive for the K system. The serum from rabbit No. 114 was believed to contain

antibody /

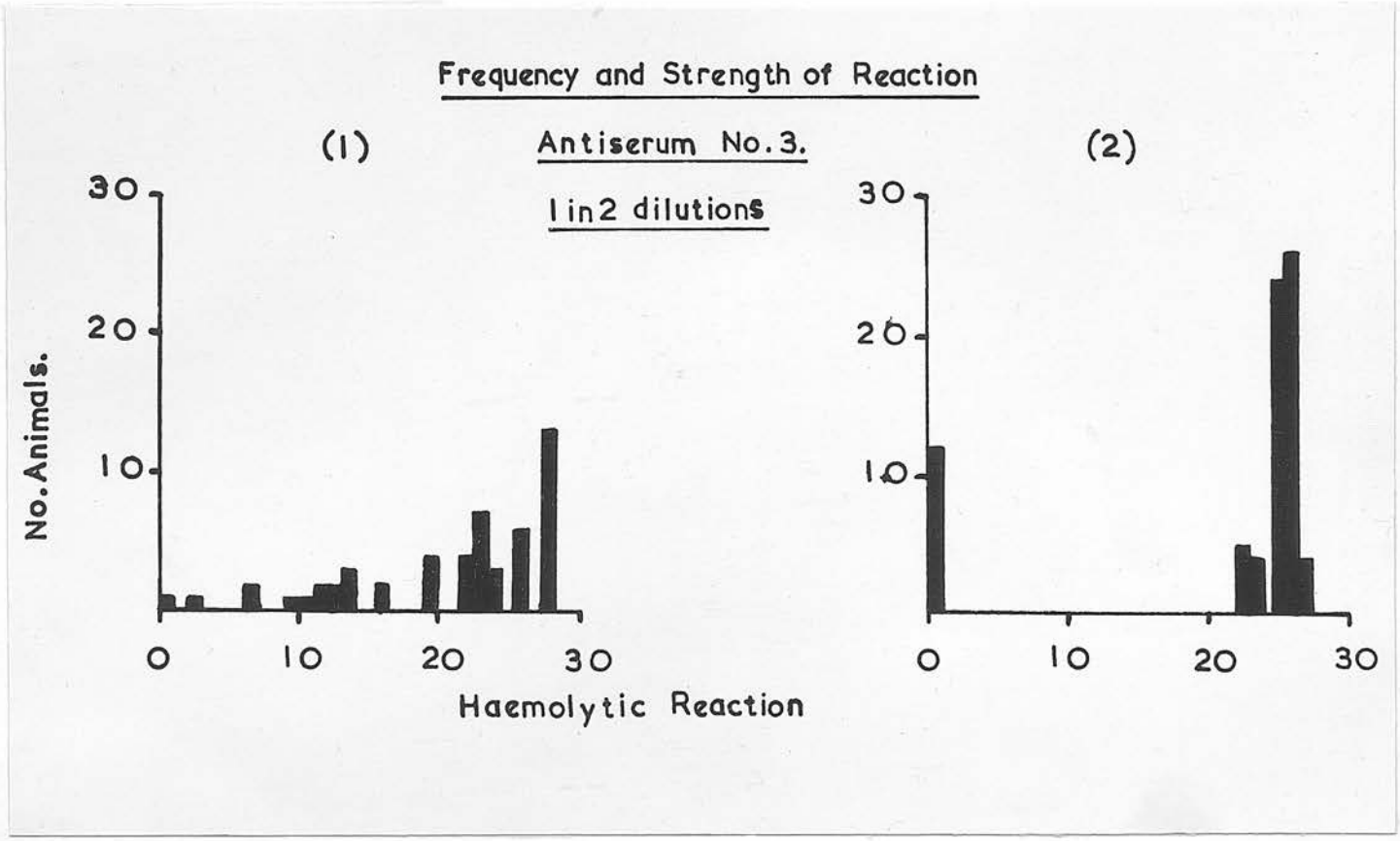
antibody for K_b, and was called Antiserum No. 3. This assumption was based on the fact that all the animals in the reference panel, except TH 262, were K_b positive, and reacted with this serum. Therefore, the possibility of anti K_b being present in the serum could not be excluded. It will be shown later in the section on the Ames Comparison test, that this antiserum was in fact detecting factor K_b. Details of the fractionation are given in Appendix B (iii).

Qualitative reaction Two histograms are shown in Figure 4. No. 1 histogram illustrates that for the first group of pigs studied the reactions were not qualitative, and further fractionation of the antiserum was found to be necessary. This point is explained in Appendix B(iii). After absorption and test in a further matrix analysis, the new fraction was able to classify animals clearly into negative and positive groups as shown in histogram No. 2.

Inheritance of Factor K_b Table 11 shows the observed and expected figures for the distribution of offspring:-

Table 11 /

Fig.4.



Antiserum No. 3	Before @ 1 in 2 dilutions			After @		
	-ve	+ve		-ve	+ve	
Nos. of animals	1	55	= 56	12	63	= 75
Mean agglutination reaction and S.D.	= 21.3 ± 6.8			= 25.3 ± 0.91		

Table 11

Phenotype mating	Parents ♂	Parents ♀	Offspring ♂s' + Obs.	♂s' + exp.	♂s' − Obs.	♂s' − exp.	♀s' + Obs.	♀s' + exp.	♀s' − Obs.	♀s' − exp.	TOTAL +	TOTAL −	Combined total
+ +	10	29	60	58	0	2	78	76	0	2	138	0	138
+ −	4	6	7	9	2	0	13	20	8	1	20	10	30
− +	3	11	18	24	7	1	37	40	4	1	55	11	66
− −	1	1			1	1			3	3		4	4
											TOTAL		238

	AA	Aa	aa	
Gene frequencies	0.681	0.288	.031	
Calculated No. within parent population =	43	18	2	= 63

Backcross matings

Table 12

Backcross mating ♂	Backcross mating ♀	No. of offspring +	No. of offspring −
+	−		
No. 2	3	4	7
−	+		
No. 2	4	20	11
Total observed		24	18
expected		21	21

$$\chi^2 = 0.43 + 0.43$$

$$= 0.86 \text{ for } 1 \text{ d.f.}$$

P = Between 0.5 to 0.3

The segregation patterns in Table 11 and 12 support the opinion that the gene controlling K_b behaved as a mendelian dominant.

4. /

4. Antiserum No. 4 Like the previous antiserum, this was a haemolytic heteroimmune antiserum. Six rabbits were injected with cells from boar B3 - 1443 carrying antigens K_a and K_b. Absorption with boar B3 - 1523 cells, which were K_a negative and K_b positive left additional antibody in three rabbit sera. The additional antibody in each case appeared to be anti-K_a, which reacted with two out of six cells from the reference panel. The two positive cells carried antigen K_a:-

Reference cells

			K_a +ve	K_a -ve	
R 92)		+ve	2	0	2
R 108)	Sera	-ve	0	4	4
R 128)			2	4	6

The probability was equal to 1/16.

Although all three sera appeared equally effective in detecting the K_a antigen, serum R 108 was selected and called Antiserum No. 4. Fractionation details are given in Appendix B (iv).

Frequency and strength of reaction Figure 5 confirms a qualitative reaction.

Phenotype matings and inheritance of factor K_a Observed and expected distribution of offspring are recorded in Table 13:-

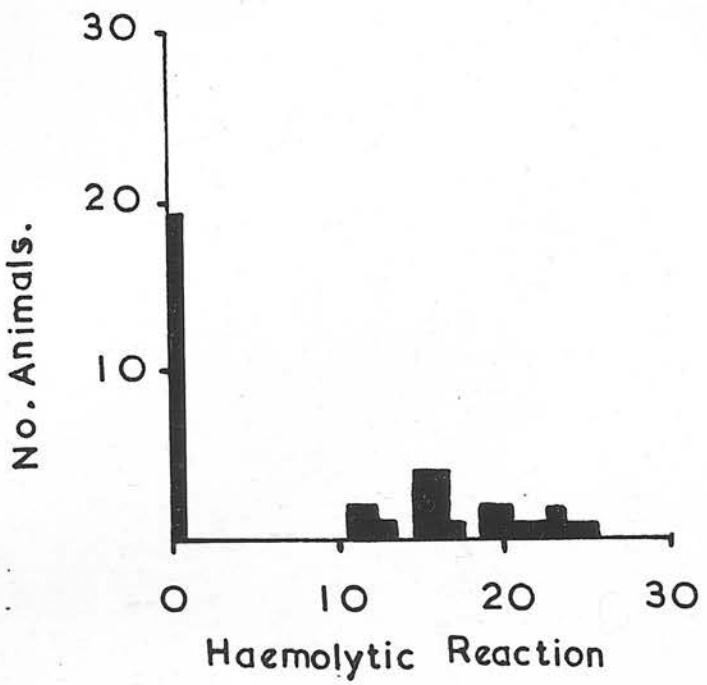
Table 13 /

Fig.5.

Frequency and Strength of Reaction

Antiserum No.4.

1 in 2 dilutions



Antiserum No. 4	1 in 2 dilutions		
	-ve	+ve	
Nos. of animals	33	33	= 66
Mean agglutination reaction	= 16.6		
and S.D.	= ±4.24		

Table 13

Phenotype mating	Parents		Offspring										Combined total
	♂	♀	♂s'				♀s'				TOTAL		
			+		-		+		-				
	No.	No.	Obs.	exp.	Obs.	exp.	Obs.	exp.	Obs.	exp.	+	-	
+ +	6	9	12	14	4	1	18	20	5	3	30	9	39
+ -	5	11	7	17	13	3	21	33	17	5	28	30	58
- +	6	11	13	17	7	3	17	27	15	5	30	22	52
- -	6	16			39	39			50	50		89	89
											TOTAL		238

	AA	Aa	aa	
Gene frequencies	0.158	0.478	0.364	
Calculated No. within parent population	= 10	30	23	= 63

Backcross matings

Table 14

Backcross mating			No. of offspring	
	♂	♀		
	+	-	+	-
No.	4	8	12	30
	-	+		
No.	5	7	20	18
Total observed			32	48
expected			40	40

χ^2 = 1.6 + 1.6

= 3.2 for 1 d.f.

P = Between 0.10 and 0.05

The gene for the factor K_a was dominant in the homozygote and heterozygote.

5. /

5. Antiserum No. 5 A saline agglutinin obtained by isoimmunisation of sow B3 - 1554 with whole blood from sow PL 8927. Details of the fractionation are given in Appendix B(v).

The reaction of Antiserum No. 5 with seven cells from the reference panel of which three cells were E_a +ve and the remainder E_a negative are compared in a contingency table:-

		Reference panel: E_a +ve	Reference panel: E_a -ve	
B3-1554	+ve	3	0	3
	-ve	0	4	4
		3	4	7

The exact probability of these results occurring by chance alone is 1/36. This appeared to indicate that Antiserum No. 5 could detect antigen E_a, and subsequently the comparison test confirmed this opinion.

Frequency and strength of reaction The histogram in Figure 6 shows a qualitative reaction.

Phenotype matings and inheritance of E_a Table 15 gives the observed and expected distribution of offspring from the different matings.

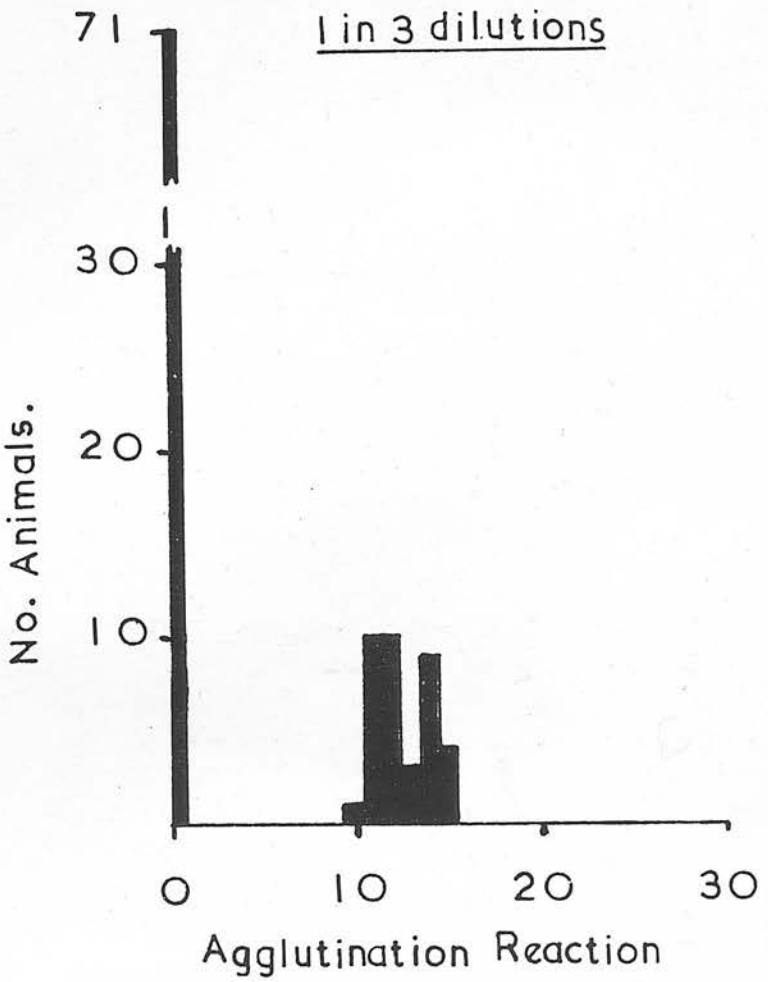
Table 15 /

Fig.6.

Frequency and Strength of Reaction

Antiserum No.5.

1 in 3 dilutions



Antiserum No. 5	1 in 3 dilutions		
	-ve	+ve	
Nos. of animals	71	37	= 108
Mean agglutination reaction = 12.5			
and S.D.	= ±1.46		

Table 15

Phenotype mating	Parents ♂	Parents ♀	Offspring ♂s' + Obs.	♂s' + exp.	♂s' − Obs.	♂s' − exp.	♀s' + Obs.	♀s' + exp.	♀s' − Obs.	♀s' − exp.	TOTAL +	TOTAL −	Combined total
+ +	3	5	14	12	0	2	16	13	0	3	30	0	30
+ −	4	6	6	12	9	3	7	20	17	4	13	26	39
− +	6	11	13	20	11	4	20	31	17	7	33	28	61
− −	9	26			42	42			66	66	108		108
											TOTAL		238

	AA	Aa	aa	
Gene frequencies	0.091	0.422	0.487	
Calculated No. within parent population	6	26	31	= 63

Backcross matings

Table 16

Backcross mating ♂	Backcross mating ♀	No. of offspring +	No. of offspring −
+	−		
No. 3	4	8	20
−	+		
No. 5	8	22	28
Total observed		30	48
expected		39	39

χ^2 = 2.08 + 2.08

= 4.16 for 1 d.f.

P = Between 0.05 and 0.02

The /

The probability of the results in Table 16 occurring by chance are greater than 1/20, which could indicate that the disproportion is slightly greater than expected. However, one must again consider the family material from which the backcross matings were selected. Two completely negative families involving two negative boars mated to positive sows were included in this data. This again biased the segregation observed in that the positive sows were not expressed in any other family.

The distribution of offspring from the different phenotype matings indicated that the gene controlling factor E_a behaved as a simple mendelian dominant.

6. Antiserum No. 6 This antiserum was obtained from sow W 1488. It was one of several sera from Wessex sows investigated for antibody content approximately fourteen days after the sows were injected with their second or third annual dose of crystal violet vaccine.

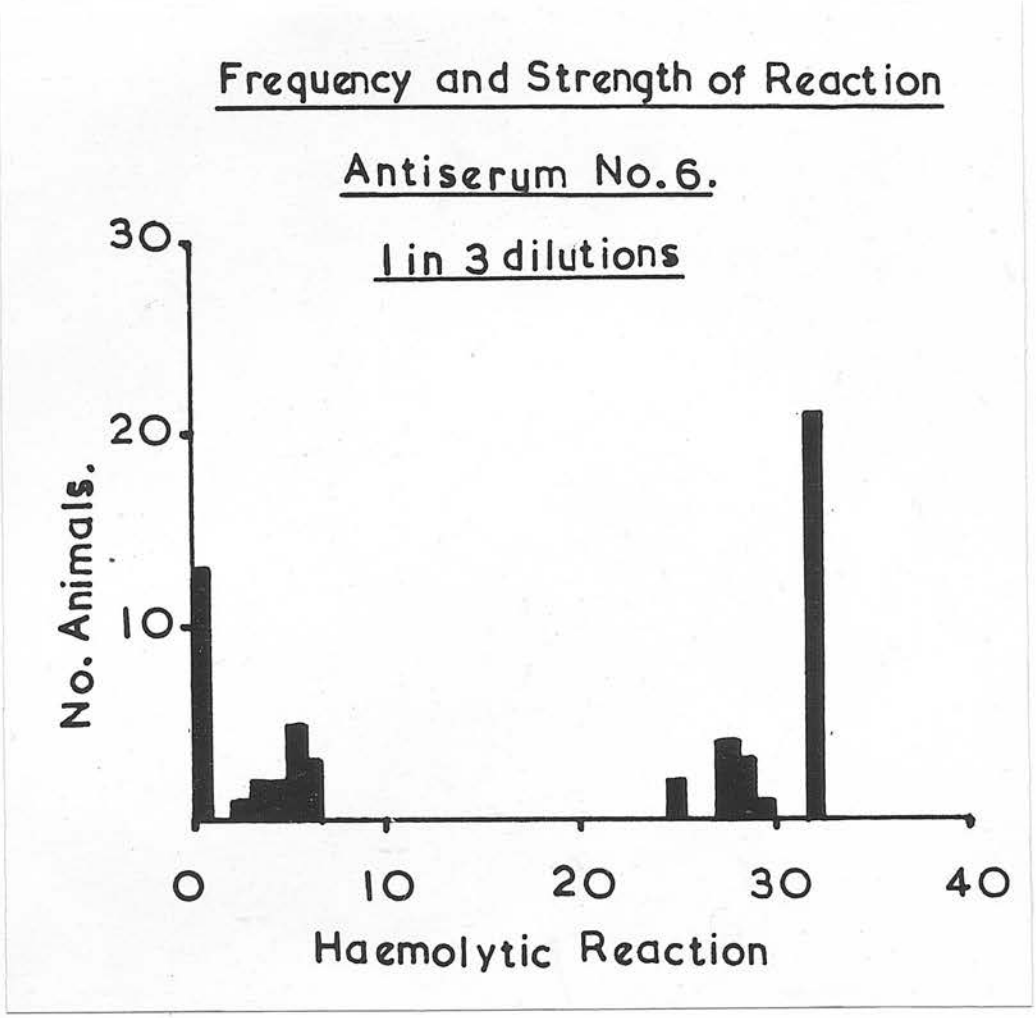
A haemolysin giving positive results completely in parallel with Antiserum No. 4 was isolated. It was called Antiserum No. 6, and like No. 4, it appeared to be specific for antigen K_a. Details of the fractionation are given in Appendix B(vi).

The histogram in Figure 7 showed that it classified pigs qualitatively.

Inheritance studies on families and segregation ratios are exactly similar to Antiserum No. 4.

7. Antiserum No. 8 One of two antibodies isolated from Wessex sow W 989 after immunisation with cells from S 7881. The donor cells /

Fig.7.



Antiserum No. 6	1 in 3 dilutions		
	-ve	+ve	
Nos. of animals	27(14)	31	= 58
Mean agglutination reaction	= 30.4		
and S.D.	= ±2.4		

cells carried antigen F_a, and this was the only member of the panel to have the antigen. After fractionation, details of which are given in Appendix B(vii), there was an antibody left which still reacted with S 7881 cells, but with none of the other cells from the reference panel. This fraction was believed to contain anti-F_a, and was called Antiserum No. 8.

Qualitative reaction The histogram in Figure 8 shows that the reaction was qualitative.

Inheritance of Factor F_a Table 17 gives the observed and expected distribution of offspring from the different matings:-

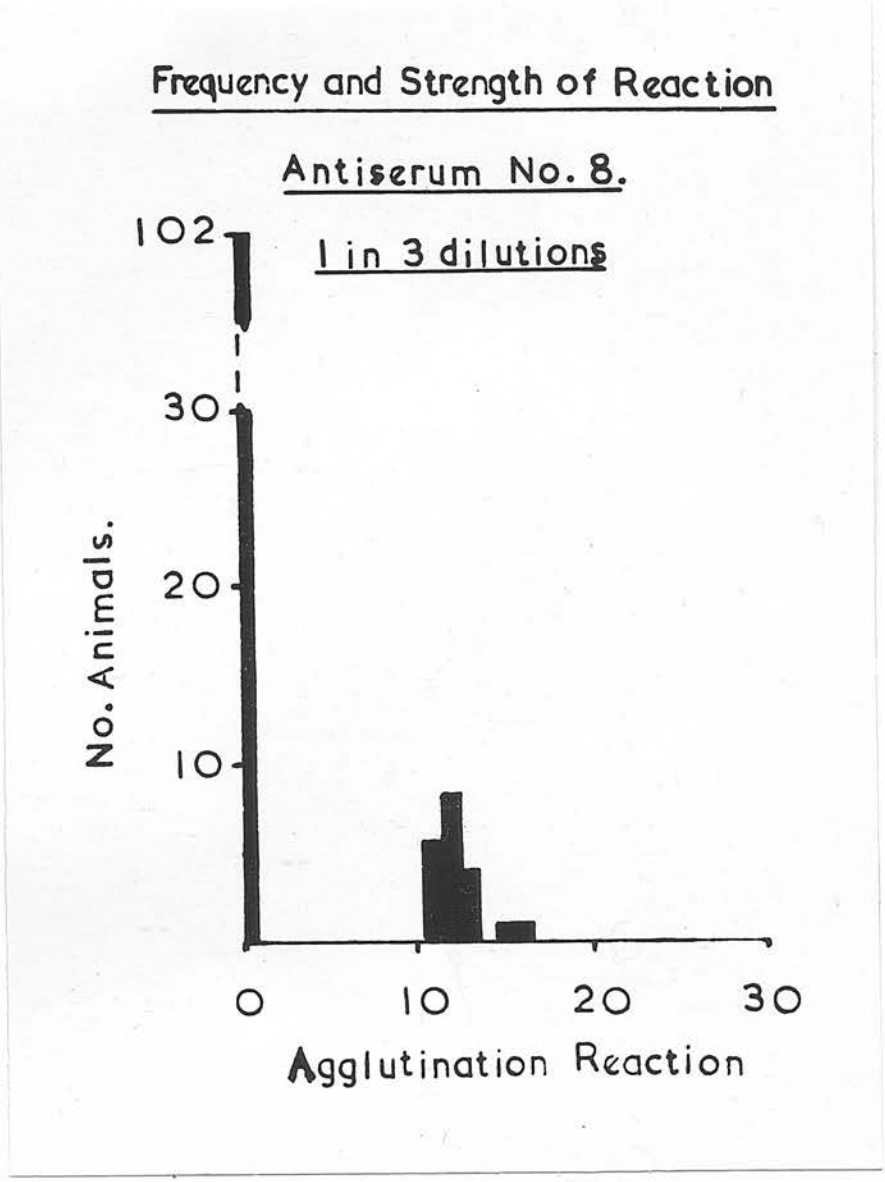
Table 17

Phenotype mating	Parents ♂	Parents ♀	Offspring ♂s' + Obs.	♂s' + exp.	♂s' − Obs.	♂s' − exp.	♀s' + Obs.	♀s' + exp.	♀s' − Obs.	♀s' − exp.	TOTAL +	TOTAL −	Combined total
+ +	1	1	0	0	0	0	5	5	2	2	5	2	7
+ −	1	2	4	7	5	2	3	6	5	2	7	10	17
− +	3	4	5	7	4	2	7	12	8	3	12	12	24
− −	14	41			77	77			113	113	190		190
											TOTAL		238

	AA	Aa	aa	
Gene frequencies	0.009	0.172	0.819	
Calculated number of parents within population =	1	11	51	= 63

Table 18 /

Fig. 8.



Antiserum No. 8

	-ve	+ve	
Nos. of animals	102	20	= 122

Mean agglutination reaction = 12.25

and S.D. = ±1.4

Backcross matings

Table 18

	Backcross mating ♂	♀	No. of offspring	
	+	-	+	-
No.	1	2	7	10
	-	+		
No.	3	4	12	12
	Total observed		19	22
	expected		20.5	20.5

χ^2 = 0.11 + 0.11

= 0.22 for 1 d.f.

P = Between 0.70 to 0.50

The segregation ratios show that the gene for factor F_a behaved as a mendelian dominant.

8. Antiserum No. 9 This was the other unit isolated from W 989 antiserum. The details of fractionation are given in Appendix B (viii).

A weak saline agglutinin of titre less than 1 in 8, but giving exactly similar reactions to Antiserum No. 5. In over 400 animals grouped with these two antisera the reactions have been completely in parallel without exception. Consequently it is believed to be another antibody capable of detecting factor E_a.

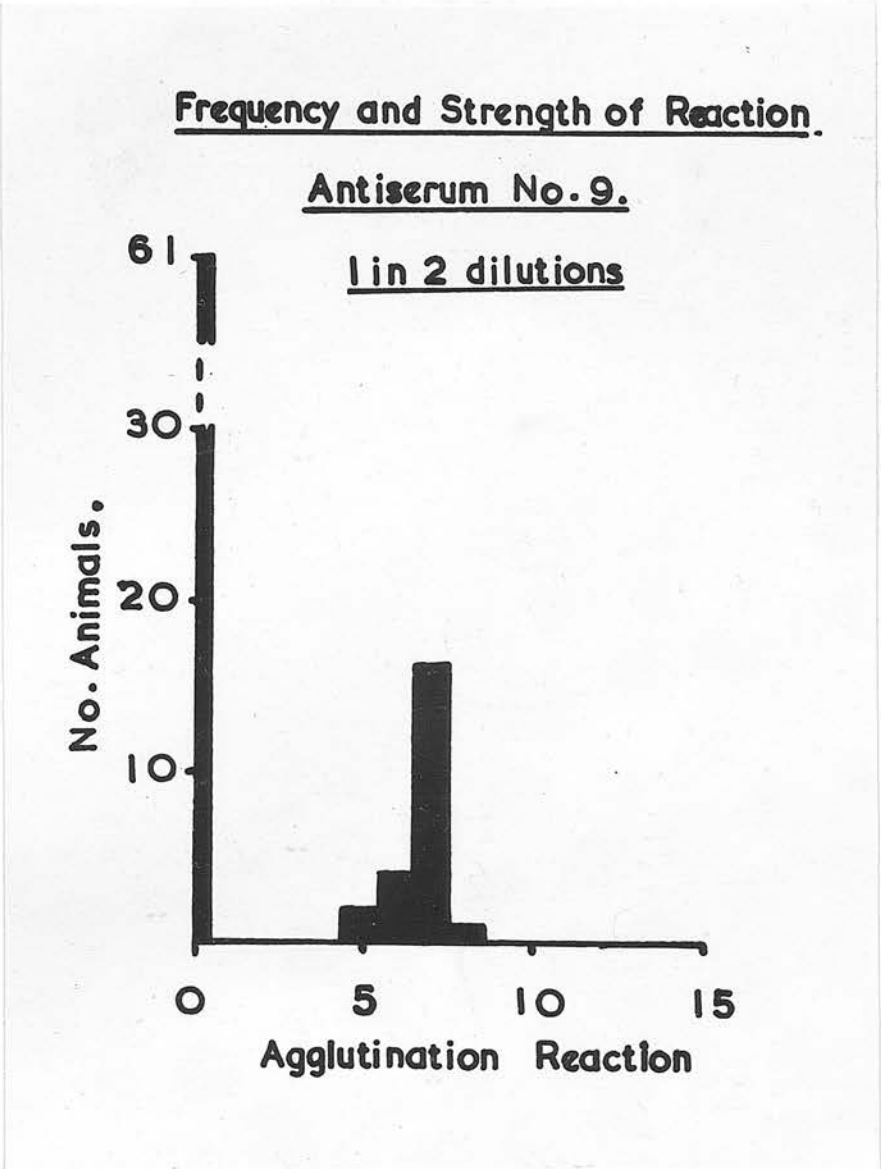
The histogram in Figure 9 shows that the reactions were qualitative.

All inheritance studies are exactly similar to Antiserum No. 5.

9. Antiserum No. 10 Obtained from WL 9091 serum after immunisation with B3-254 cells. Fractionation details are given in Appendix B(ix).

On /

Fig.9.



Antiserum No. 9

	-ve	+ve	
Nos. of animals	61	23	= 84
Mean agglutination reaction =	6.7		
and S.D. =	0.71		

On testing against the reference panel, seven cells with antigen E_e were positive, and one cell which did not have E_e was negative. The exact probability of these reactions recurring by chance is given in a 2 x 2 table.

		Reference cells		
		E_e +ve	E_e -ve	
WL 9091 Antiserum	+ve	7	0	7
	-ve	0	1	1
		7	1	8

The probability was equivalent to 1/80. This meant that Antiserum No. 10 appeared to have a specificity for antigen E_e.

Frequency and strength of reaction The histogram in Figure 10 shows clear negative and positive groups.

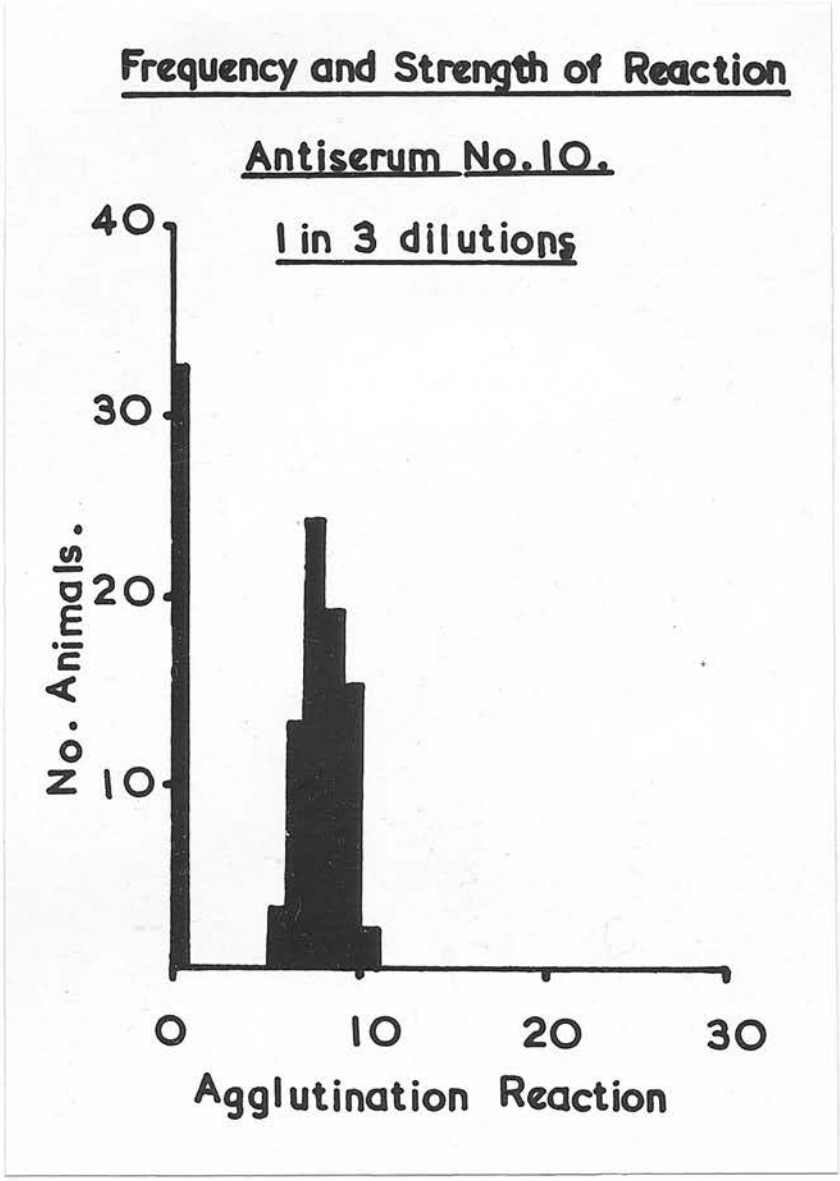
Inheritance of Factor E_e The observed and expected distribution of offspring are shown in Table 19.

Table 19

Phenotype mating	Parents		Offspring								TOTAL		Combined total
	♂	♀	♂s'				♀s'						
			+		-		+		-				
	No.	No.	Obs.	exp.	Obs.	exp.	Obs.	exp.	Obs.	exp.	+	-	
+ +	7	15	29	32	6	3	48	49	5	4	77	11	88
+ -	3	30	6	7	2	1	5	6	2	1	11	4	15
- +	8	17	18	25	9	2	38	47	13	4	56	22	78
- -	7	12			25	25			32	32		57	57
											TOTAL		238

Gene /

Fig.10.



Antiserum No. 10

	-ve	+ve	
Nos. of animals	32	76	= 108
Mean agglutination reaction	= 8.5		
and S.D.	= ±1.09		

	AA	Aa	aa	
Gene frequencies	0.383	0.472	0.145	
Calculated number of parents within population =	24	30	9	= 63

Backcross matings

Table 20

	Backcross mating ♂	Backcross mating ♀	No. of offspring +	No. of offspring -
	+	-		
No.	1	1	3	4
	-	+		
No.	6	10	24	22
	Total observed		27	26
	expected		26.5	26.5

χ^2 = .009 + .009

= .018 for 1 d.f.

P = Between 0.9 and 0.8

The facts are in agreement with the opinion that the gene for factor E_e is a dominant mendelian gene.

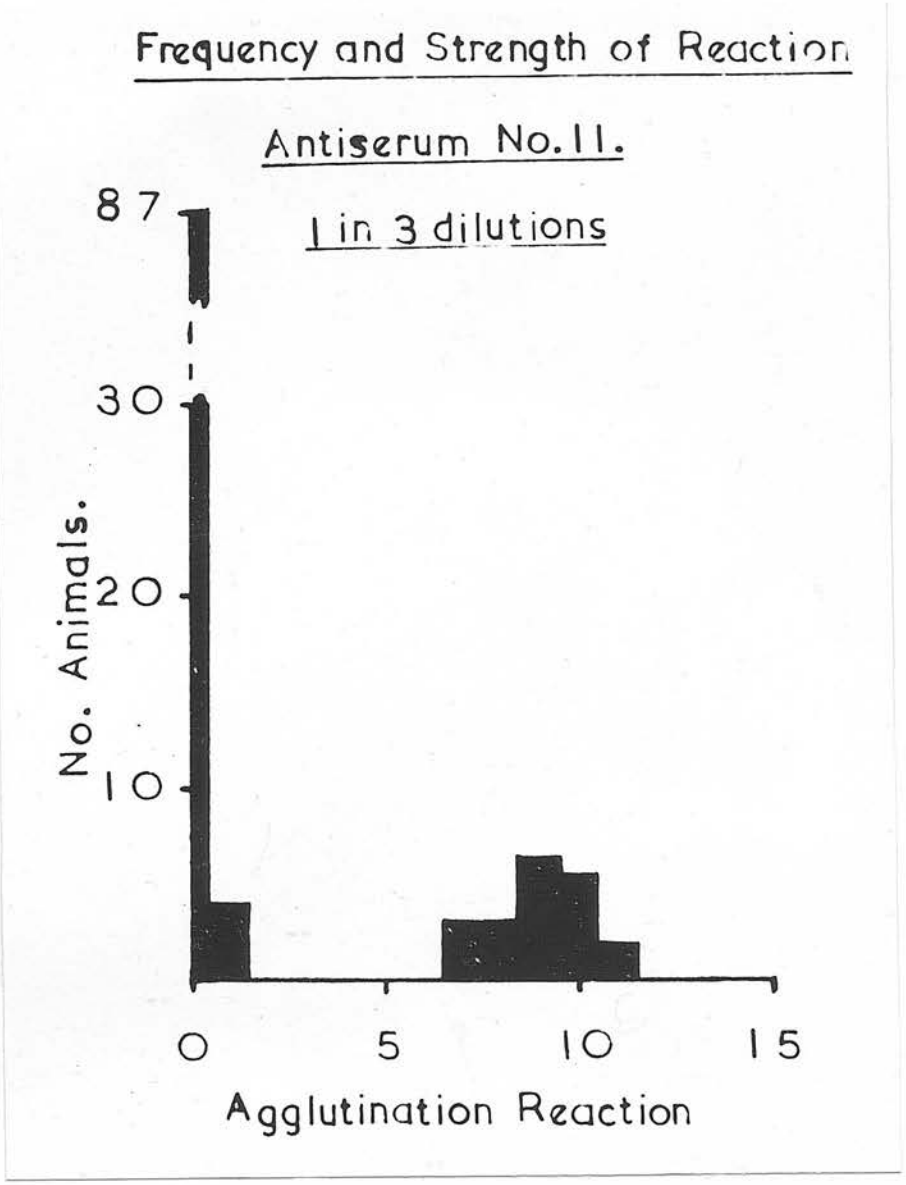
10. Antiserum No. 11 The details of fractionation of this serum were described in the section on Matrix Analysis. It was proposed in this section, that the antiserum was capable of detecting factor E_f. This specificity was confirmed by the Ames Comparison test.

Qualitative reaction The histogram in Figure 11 shows clear division of positive and negative classes.

Inheritance of Factor E_f The observed and expected distribution of offspring are shown in Table 21:-

Table 21 /

Fig.11.



Antiserum No. 11

	-ve	+ve	
Nos. of animals	91(4)	21	= 112
Mean agglutination reaction =	9		
and S.D.	= ±1.14		

Table 21

Phenotype mating	Parents ♂	Parents ♀	Offspring ♂s' + Obs.	♂s' + exp.	♂s' − Obs.	♂s' − exp.	♀s' + Obs.	♀s' + exp.	♀s' − Obs.	♀s' − exp.	TOTAL +	TOTAL −	Combined total
+ +	1	1	2	2	0	0	3	2	0	0	5	0	5
+ −	0	0	0	0	0	0	0	0	0	0	0	0	0
− +	6	6	6	7	3	2	9	18	14	5	15	17	32
− −	14	41			84	84			117	117	201		201
												TOTAL	238

	AA	Aa	aa	
Gene frequencies	0.016	0.222	0.762	
Calculated number of parents within population	= 1	14	48	= 63

Backcross matings

Table 22

Backcross mating ♂	Backcross mating ♀	No. of offspring +	No. of offspring −
+	−		
No. 0	0	0	0
−	+		
No. 4	5	12	13
Total observed		12	13
expected		12.5	12.5

χ^2 = 0.02 + 0.02

= 0.04 for 1 d.f.

P = Between 0.9 to 0.8

Tables 21 and 22 indicate that factor E_f is controlled by a dominant mendelian gene.

11. /

11. Antiserum No. 12 This antiserum was obtained from sow 254, and is the second fraction mentioned in the section on Matrix Analysis. It is believed that it detected an unknown factor referred to as factor X. On testing against the reference panel, this antiserum only reacted with the donor cells, and therefore appeared to be a new factor, that is, it was additional to any of the known antigens. Details of its final analysis are given in Appendix B(x).

Qualitative reaction Like the other antisera, the histogram in Figure 12 shows clear division of positive and negative classes.

Inheritance of Factor X Table 23 gives the observed and expected distribution of offspring from the phenotype matings:-

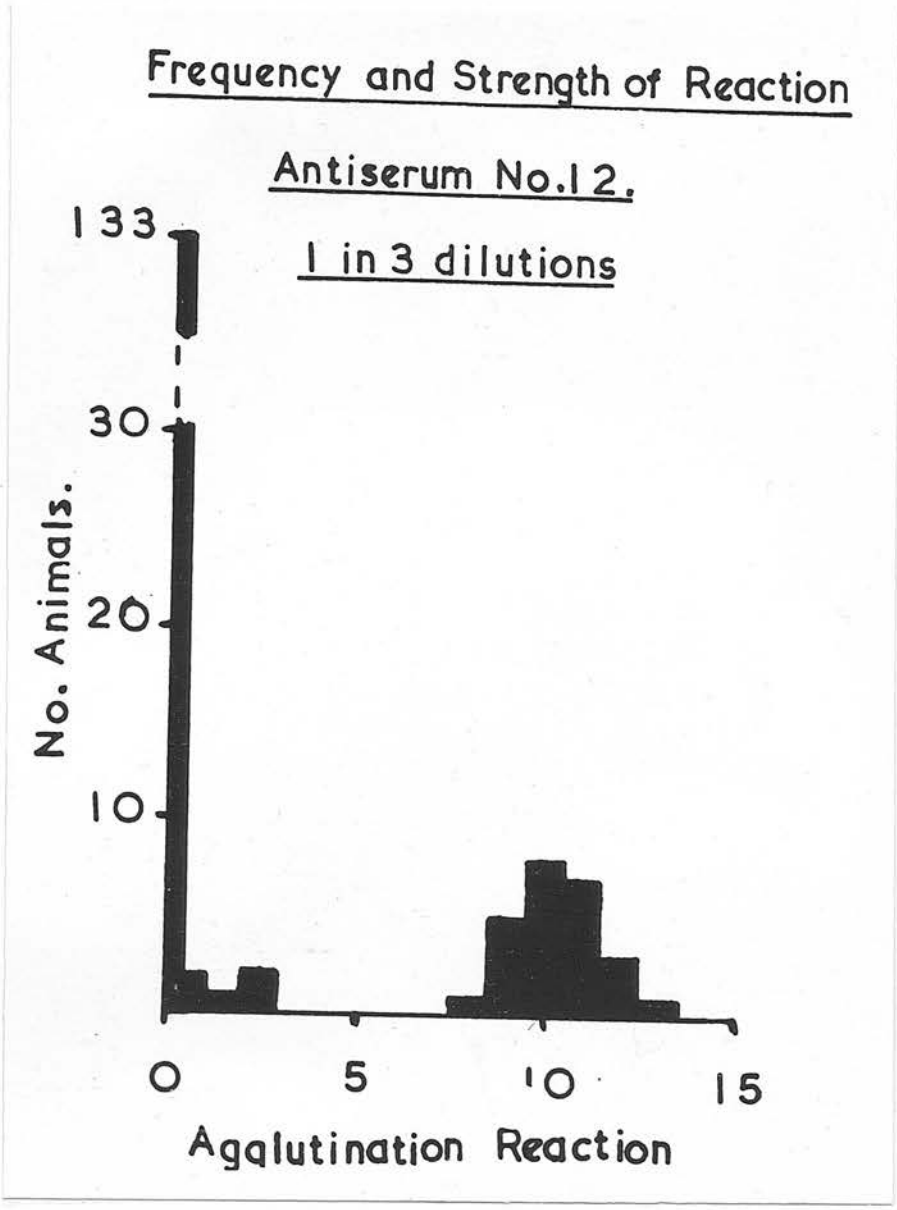
Table 23

Phenotype mating	Parents ♂	Parents ♀	Offspring ♂s' + Obs.	♂s' + exp.	♂s' − Obs.	♂s' − exp.	♀s' + Obs.	♀s' + exp.	♀s' − Obs.	♀s' − exp.	TOTAL +	TOTAL −	Combined total
+ +	1	2	1	1	0	0	5	5	2	2	6	2	8
+ −	1	3	5	4	0	1	4	6	4	2	9	4	13
− +	2	4	5	8	2	5	5	6	3	2	10	8	18
− −	13	38			77	77			117	117	194		194
											TOTAL		233

	AA	Aa	aa	
Gene frequencies	0.032	0.185	0.783	
Calculated number of parents within population	2	11	48	= 61

Backcross matings /

Fig.12.



Antiserum No. 12

	-ve	+ve	
Nos. of animals	139(6)	25	= 164
Mean agglutination reaction	= 10.4		
and S.D.	= ±1.19		

Backcross matings

Table 24

Backcross matings		No. of offspring	
♂	♀		
+	-	+	-
No. 1	2	6	2
-	+		
No. 2	3	6	8
Total observed		12	10
expected		11	11

χ^2 = 0.09 + 0.09

= 0.18 for 1 d.f.

P = Between 0.7 to 0.5

The new factor X appears to be controlled by a mendelian dominant gene.

12. Antiserum No. 13 An incomplete agglutinin obtained from the serum of sow A4 - 8457 after immunisation with cells from sow B3 - 255. Fractionation details are given in Appendix B(xi). On testing against eight cells of the Reference panel, four G_a +ve animals showed a positive reaction, and the other four G_a -ve cells were negative. The likelihood of this occurring by chance is obtained from the 2 x 2 table:-

	Reference cells		
	G_a +ve	G_a -ve	
Antiserum No. 13 +ve	4	0	4
Antiserum No. 13 -ve	0	4	4
	4	4	8

The /

The exact probability is equivalent to 1/70. Antiserum No. 13 was therefore believed to detect antigen G_a, a fact confirmed by the Ames Comparison test.

Qualitative reaction The histogram in Figure 13 shows clear division of positive and negative classes.

Inheritance of Factor G_a Observed and expected distribution of offspring is shown in Table 25.

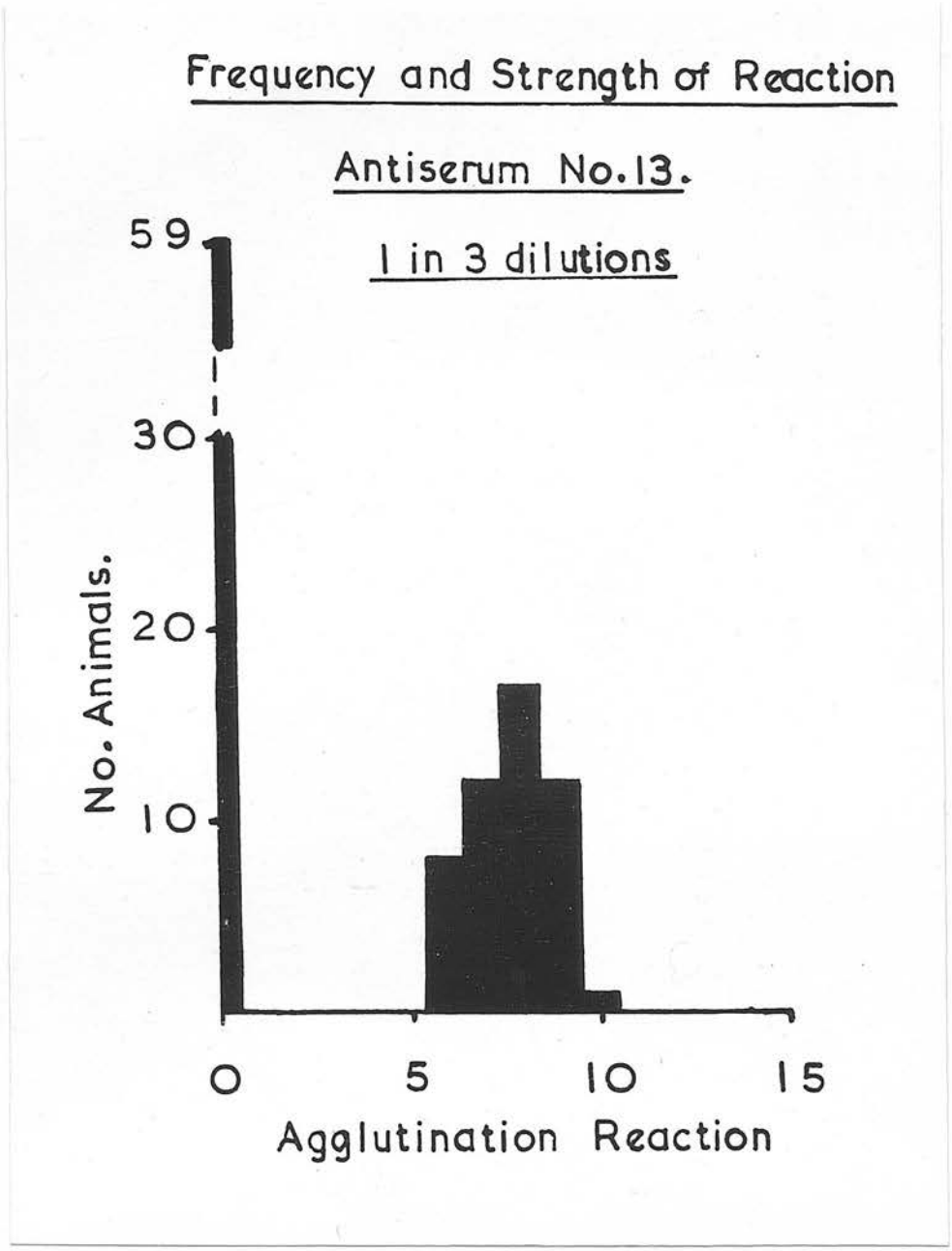
Table 25

Phenotype mating	Parents ♂	Parents ♀	Offspring ♂s' + Obs.	♂s' + exp.	♂s' - Obs.	♂s' - exp.	♀s' + Obs.	♀s' + exp.	♀s' - Obs.	♀s' - exp.	TOTAL +	TOTAL -	Combined total
+ +	5	9	12	14	5	3	15	20	9	4	27	14	41
+ -	5	14	17	29	18	6	28	36	16	8	45	34	79
- +	4	7	7	14	10	3	8	19	15	4	15	25	40
- -	8	17			26	26			52	52	78		78
											TOTAL		238

	AA	Aa	aa	
Gene frequencies	0.101	0.432	0.467	
Calculated number of parents within population	= 6	27	30	= 63

Backcross matings /

Fig.13.



Antiserum No. 13

	-ve	+ve	
Nos. of animals	59	50	= 109
Mean agglutination reaction	= 7.7		
and S.D.	= ±1.07		

Backcross matings

Table 26

	Backcross matings ♂	♀	No. of offspring +	−
	+	−		
No.	4	13	41	34
	−	+		
No.	4	7	15	25
	Total observed		56	59
	expected		57.5	57.5

χ^2 = 0.039 + 0.039

= 0.078 for 1 d.f.

P = Between 0.8 to 0.7

Antiserum No. 13 appears to detect character G_a, which is controlled by a mendelian dominant gene.

13. Antiserum No. 14 An incomplete agglutinin obtained from Sow TH 262 after immunisation with sow P 3760 cells. Details are given in Appendix B(xii). It was believed that an antibody to antigen G_b had been produced, because all the reference cells were G_b positive and reacted positively. However, there were no G_b negative cells available other than the recipients to confirm this observation. Its ability to detect G_b was confirmed by the AMES Comparison test.

Qualitative reaction The distribution of the groups in the histogram in Figure 14 is qualitative.

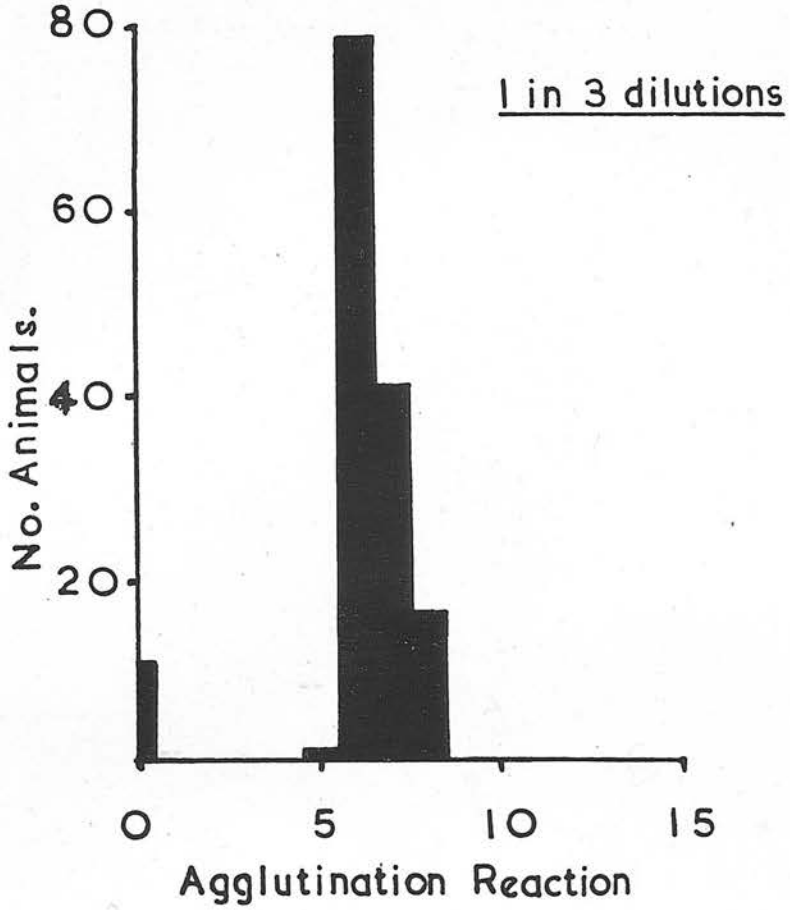
Inheritance of G_b No segregation details can be given, because Antiserum No. 14 was isolated after completion of the typing of the parents involved in other inheritance studies.

4. /

Fig.14.

Frequency and Strength of Reaction

Antiserum No. 14.



Antiserum No. 14

	-ve	+ve
Nos. of animals	12	137
Mean agglutination reaction	= 6.5	
and S.D.	= ±0.71	

4. TESTS FOR ASSOCIATION AND LINKAGE BETWEEN ANTIGENS:-

The family studies confirmed that the various antigenic factors were heritable, and therefore must be controlled by genes. Some of these genes may be linked on the same chromosome. To investigate this possibility, two different methods can be applied.

The first method is statistical, and involves testing the independence of THE OCCURRENCE OF each antigen against one another in 2 x 2 contingency tables. A null hypothesis is adopted by assuming that the distribution of the numbers making-up each of the four classes in such a table is not different from THAT expected BY CHANCE ALONE. If there is a disproportion in any class, then the probability of this occurring by chance alone can be assessed. This probability only gives an indication of how many times one would have to repeat such a test before getting the same result again. If the probability is less than 0.05, however, then it can be assumed, that some bias OTHER THAN CHANCE must be present to upset the independence of either factor. By measuring the frequency of occurrence of one factor against another in a random population, it is possible to see where these two factors occur more frequently together, that is positively, or where they occur infrequently together, that is negatively. Either type of association may mean some form of linkage.

By the second method, either formof association can be explained genetically. In the positive association, the reason may be that two antigens are controlled by two genes on the same chromosome. Alternatively, in the negative association, one gene may be on one chromosome, and the other on it's homologue.

The /

The ideal type of mating to establish this genetic linkage is the double backcross mating. As explained before for the single backcross, a heterozygous type mated to a recessive for the character should give an expected segregation ratio of 1 : 1 in the offspring. In the double backcross, animals heterozygous for two antigens mated to double recessives will give a segregation ratio of 1 : 1 : 1 : 1 in the offspring. Referring back to the positive type of association, where two genes are positively linked on the same chromosome, this may appear in a double backcross mating as follows:-

Assuming one gene to be A and the other B, their allelomorphs are called a and b.

e.g. Genotype matings

Aa/Bb x aa/bb

Expected offspring genotypes	Aa/Bb	Aa/bb (RECOMBINANTS)	aa/Bb	aa/bb
Ratio	1.	1.	1.	1.

If the segregation ratios conform as above, then the genes A and B cannot be linked, but if the ~~proportion~~ NUMBER of Aa/Bb and aa/bb genotypes, or non-recombinants is significantly ~~greater~~ DIFFERENT than Aa/bb and aa/Bb genotypes, or recombinants, then they ~~can be~~ ARE linked. Where it is found that the A and B genes occur together in the heterozygote, they are said to be in coupling. Their genotype is expressed as AB/ab showing that they are on the same chromosome.

The converse holds true of the negative type of association, where the two genes A and B never occur together on the same chromosome, /

chromosome, except when crossing over occurs. In this case the heterozygote is said to be in repulsion for these two genes. This genctype is expressed as Ab/aB. In this instance the ratio of recombinant type offspring will be significantly greater than non-recombinants in a double backcross mating.

A knowledge of the proportion of recombinant to non-recombinant classes in the offspring in cases where two genes are known either to be in coupling, or repulsion will give an estimate of the percentage crossing over of the chromosomes carrying these genes. This can be expressed as a linkage value referred to as the recombination fraction. The closer two genes are together on a chromosome, then the less chance there is of them becoming divided due to crossing over of the chromosomes. The classifying of closely linked genes or alleles for antigenic factors establishes these factors in different systems.

1. Tests of independences: Chi-squared values and exact probabilities were calculated where stated on all the following 2 x 2 tables:-

		Factor 2 or E_b +	Factor 2 or E_b -	
Factor 1 or A	+	29	12	41
	-	44	19	63
		73	31	104

χ^2 for 1 d.f. = .009

		Factor 3 or K_b +	Factor 3 or K_b -	
Factor 1 or A	+	32	9	41
	-	52	11	63
		84	20	104

χ^2 for 1 d.f. = .032

Factor 4 or K_a

		+	-	
Factor 1	+	16	25	41
or A	-	30	33	63
		46	58	104

χ^2 for 1 d.f. = .074

Factor 5 or E_a

		+	-	
Factor 1	+	16	25	41
or A	-	20	43	63
		36	68	104

χ^2 for 1 d.f. = .058

Factor 6 or K_a

Factor 1 or A — Similar to Factor 4

Factor 8 or F_a

		+	-	
Factor 1	+	9	32	41
or A	-	11	52	63
		20	84	104

χ^2 for 1 d.f. = .032

Factor 9 or E_a

Factor 1 or A — Similar to Factor 5

Factor 10 or E_e

		+	-	
Factor 1	+	31	10	41
or A	-	44	19	63
		75	29	104

χ^2 for 1 d.f. = .041

Factor 11 or E_f

		+	-	
Factor 1	+	8	33	41
or A	-	13	50	63
		21	83	104

χ^2 for 1 d.f. = .0019

Factor 12 or X

		+	-	
Factor 1	+	3	38	41
or A	-	8	55	63
		11	93	104

χ^2 for 1 d.f. = .076

Factor 13 or G_a

		+	-	
Factor 1	+	21	20	41
or A	-	27	36	63
		48	56	104

χ^2 for 1 d.f. = .069

Factor 3 or K_b

		+	-	
Factor 2	+	57	16	73
or E_b	-	27	4	31
		84	20	104

χ^2 for 1 d.f. = 1.14

Factor 4 or K_a

		+	-	
Factor 2	+	32	41	73
or E_b	-	14	17	31
		46	58	104

χ^2 for 1 d.f. = .0015

Factor 5 or E_a

		+	-	
Factor 2	+	23	50	73
or E_b	-	13	18	31
		36	68	104

χ^2 for 1 d.f. = 1.04

Factor 6 or K_a

Factor 2 or E_b	Similar to Factor 4

Factor 8 or F_a

		+	-	
Factor 2	+	12	61	73
or E_b	-	8	23	31
		20	84	104

χ^2 for 1 d.f. = 1.23

Factor 9 or E_a

Factor 2 or E_b	Similar to Factor 5

Factor 10 or E_e

		+	-	
Factor 2	+	44	29	73
or E_b	-	31	0	31
		75	29	104

χ^2 of 1 d.f. = 15.45

Factor 11 or E_f

		+	-	
Factor 2	+	5	68	73
or E_b	-	16	15	31
		21	83	104

χ^2 for 1 d.f. = 27.06

Factor 12 or X

		+	-	
Factor 2	+	6	67	73
or E_b	-	5	26	31
		11	93	104

χ^2 for 1 d.f. = 1.42

Factor 13 or G_a

		+	-	
Factor 2	+	32	41	73
or E_b	-	16	15	31
		48	56	104

χ^2 for 1 d.f. = .052

Factor 4 or K_a

		+	-	
Factor 3	+	27	57	84
or K_b	-	19	1	20
		46	58	104

χ^2 for 1 d.f. = 25.87

Factor 5 or E_a

		+	-	
Factor 3	+	35	49	84
or K_b	-	1	19	20
		36	68	104

χ^2 for 1 d.f. = 9.59

Factor 6 or K_a

Factor 3 or K_b — Similar to Factor 4

Factor 8 or F_a

		+	-	
Factor 3	+	16	68	84
or K_b	-	4	16	20
		20	84	104

χ^2 for 1 d.f. = .009

Factor 9 or E_a

Factor 3 or K_b — Similar to Factor 5

Factor 10 or E_e

		+	-	
Factor 3	+	63	21	84
or K_b	-	12	8	20
		75	29	104

χ^2 for 1 d.f. = 1.81

Factor 11 or E_f

		+	-	
Factor 3	+	18	66	84
or K_b	-	3	17	20
		21	83	104

χ^2 for 1 d.f. = .04

Factor 12 or X

		+	-	
Factor 3	+	10	74	84
or K_b	-	1	19	20
		11	93	104

χ^2 for 1 d.f. = ~~.08~~ 0.8

Factor 13 or G_a

		+	-	
Factor 3	+	37	47	84
or K_b	-	11	9	20
		48	56	104

χ^2 for 1 d.f. = .078

Factor 5 or E_a

		+	-	
Factor 4	+	9	37	46
or K_a	-	27	31	58
		36	68	104

χ^2 for 1 d.f. = 8.25

Factor 6 or K_a

		+	-	
Factor 4	+	46	0	46
or K_a	-	0	58	58
		46	58	104

χ^2 for 1 d.f. = 104.0

Factor 8 or F_a

		+	-	
Factor 4	+	9	37	46
or K_a	-	11	47	58
		20	84	104

χ^2 for 1 d.f. = .0059

Factor 9 or E_a

Factor 4 or K_a	Similar to Factor 5

Factor 10 or E_e

		+	-	
Factor 4	+	31	15	46
or K_a	-	44	14	58
		75	29	104

χ^2 for 1 d.f. = .09

Factor 11 or E_f

		+	-	
Factor 4	+	9	37	46
or K_a	-	12	46	58
		21	83	104

χ^2 for 1 d.f. = .002

Factor 12 or X

		+	-	
Factor 4	+	1	45	46
or K_a	-	10	48	58
		11	93	104

Exact probability = .012

Factor 13 or G_a

		+	-	
Factor 4	+	23	23	46
or K_a	-	25	33	58
		48	56	104

χ^2 for 1 d.f. = .05

Factor 6 or K_a

		+	-	
Factor 5	+	9	27	36
or E_a	-	37	31	68
		46	58	104

χ^2 for 1 d.f. = 8.25

Factor 8 or F_a

		+	-	
Factor 5	+	11	25	36
or E_a	-	9	59	68
		20	84	104

χ^2 for 1 d.f. = 4.55

Factor 9 or E_a

		+	-	
Factor 5	+	36	0	36
or E_a	-	0	68	68
		36	68	104

χ^2 for 1 d.f. = 100.7

Factor 10 or E_e

		+	-	
Factor 5	+	36	0	36
or E_a	-	39	29	68
		75	29	104

χ^2 for 1 d.f. = 19.73

Factor 11 or E_f

		+	-	
Factor 5	+	2	34	36
or E_a	-	19	49	68
		21	83	104

Exact probability = .0057

Factor 12 or X

		+	-	
Factor 5	+	3	33	36
or E_a	-	8	60	68
		11	93	104

χ^2 for 1 d.f. = 0.29

Factor 13 or G_a

		+	-	
Factor 5	+	20	16	36
or E_a	-	28	40	68
		48	56	104

χ^2 for 1 d.f. = 1.96

Associations for Factor 6 exactly similar to Factor 4

Factor 9 or E_a

		+	-	
Factor 8	+	11	9	20
or F_a	-	25	59	84
		36	68	104

χ^2 for 1 d.f. = 4.55

Factor 10 or E_e

		+	-	
Factor 8	+	16	4	20
or F_a	-	59	25	84
		75	29	104

χ^2 for 1 d.f. = .076

Factor 11 or E_f

		+	-	
Factor 8	+	6	14	20
or F_a	-	15	69	84
		21	83	104

χ^2 for 1 d.f. = 1.48

Factor 12 or X

		+	-	
Factor 8	+	0	20	20
or F_a	-	11	73	84
		11	93	104

χ^2 for 1 d.f. = 1.31

Factor 13 or G_a

		+	-	
Factor 8	+	12	8	20
or F_a	-	36	48	84
		48	56	104

χ^2 for 1 d.f. = 1.9

Associations for Factor 9 exactly similar to Factor 5

Factor 11 or E_f

		+	-	
Factor 10	+	21	54	75
or E_e	-	0	29	29
		21	83	104

χ^2 for 1 d.f. = 8.45

Factor 12 or X

		+	-	
Factor 10	+	10	65	75
or E_e	-	1	28	29
		11	93	104

Exact probability = .112

Factor 13 or G_a

		+	-	
Factor 10	+	34	41	75
or E_e	-	14	15	29
		48	56	104

χ^2 for 1 d.f. = .073

	Factor 12 or X +	Factor 12 or X -	
Factor 11 +	3	18	21
or E_f -	8	75	83
	11	93	104

χ^2 for 1 d.f. = 1.16

	Factor 13 or G_a +	Factor 13 or G_a -	
Factor 11 +	9	12	21
or E_f -	39	44	83
	48	56	104

χ^2 for 1 d.f. = .115

	Factor 13 or G_a +	Factor 13 or G_a -	
Factor 12 +	2	9	11
or X -	46	47	93
	48	56	104

Exact probability = .0467

A summary of the probabilities for association between antigens is given in Table 27. Positive associations are given in red and negative associations are given in blue.

2. Association between antigens: The information obtained from Table 27 indicates that the following categories exist:-

(a) No association Antigen A shows no association with any other antigen.

(b) Positive association

(i) Antigen E_b Associated with E_e. All animals tested appear either E_b and E_e positive, or E_b +ve and E_e -ve, or E_b -ve and E_e +ve. No animals were negative for both antigens. On investigating all animals tested for these two antigens, a double negative has not been obtained. This type of association may be explained genetically as two antithetical alleles at the same locus. Segregation studies are necessary to confirm this hypothesis.

(ii) Antigen K_b Associated with K_a. Only one double negative animal among 104 pigs.

All /

Table 27

Summary of probabilities of association between antigens

	Antigens										
	A 1	E_b 2	K_b 3	K_a 4	E_a 5	K_a 6	F_a 8	E_a 9	E_e 10	E_f 11	X 12
G_a 13	0.8 - 0.7	0.9 - 0.8	0.8 - 0.7	0.9 - 0.8	0.2 - 0.1	0.9 - 0.8	0.2 - 0.1	0.2 - 0.1	0.8 - 0.7	0.8 - 0.7	.05 -.02
X 12	0.8 - 0.7	0.3 - 0.2	0.9 - 0.8	.0118 exact	0.7 - 0.5	.0118 exact	0.3 - 0.2	0.7 - 0.5	.112 exact	0.3 - 0.2	
E_f 11	0.98- 0.95	.001	0.9 - 0.8	0.98 - 0.95	.0057 exact	0.98 - 0.95	0.3 - 0.2	.0057 exact	.01 -.001		
E_e 10	0.9 - 0.8	.001	0.2 - 0.1	0.8 - 0.7	.001	0.8 - 0.7	0.8 - 0.7	.001			
E_a 9	0.9 - 0.8	0.5 - 0.3	.01 -.001	.01 -.001	.001	.01 -.001	.05 -.02				
F_a 8	0.9 - 0.8	0.3 - 0.2	0.98 - 0.95	0.95 - 0.9	.05 -.02	0.95 - 0.9					
K_a 6	0.8 - 0.7	0.98 - 0.95	.001	.001	.01 -.001						
E_a 5	0.9 - 0.8	0.5 - 0.3	.01 -.001	.01 -.001							
K_a 4	0.8 - 0.7	0.98 - 0.95	.001								
K_b 3	0.9 - 0.8	0.3 - 0.2									
E_b 2	0.98 - 0.95										

Values for p taken from Table IV - Statistical Tables - Fisher & Yates (1953)

All but one animal out of 36 positive for E_a were also positive for K_b. This might have suggested a mixed antibody effect, or a sub-group, that is, antigen E_a could appear alone, but antigen K_b when present only occurred when E_a was present. The presence of one K_b negative animal with an E_a positive, overrules this theory.

(iii) Antigen E_a All E_a positive animals were also positive for E_e, but E_e animals can be independent of E_a.

(iv) Antigen E_e Like E_a above, E_f positive animals were also positive for E_e, but E_e animals can be independent of E_f.

(v) Antigens K_a and E_a The comparable specificities of the Antibodies 4 and 6 and 5 and 9 respectively is fully illustrated by the 2 x 2 tables.

(c) Negative associations

(i) Antigen E_b Very few E_b positive animals are positive for E_f.

(ii) Antigen K_a Very few E_a positive animals are positive for K_a, and vice versa. The same type of picture is presented with Antigen X.

(iii) Antigen E_a Slight negative association with F_a, but this may not be significant. A larger sample may be necessary to confirm this trend.

Also, negatively associated with E_f. Most animals negative for both factors, or positive for one and negative for the other.

(iv) Antigen 12 or X A slight negative association with G_a.

2. /

3. Gene linkage: To establish linkage between any two genes controlling antigenic factors where association is believed to be present, it is necessary to consider all backcross matings involving these genes. Unfortunately, within the scope of the family data investigated, there were only a few matings of this type. As in the instance of the single backcross, the heterozygous genotype had to be assumed on the basis of matings between a positive and negative parent, will produce at least one negative offspring. For the double backcross, matings had to be found where one parent was positive for two antigens and the other parent was negative for both, and at least one of the offspring in either case was negative.

All backcross matings relevant to the associations given above have been extracted from the family material where possible, and are given below; pairs of antigens showing positive association are considered first:-

(a) Positive association: (i) Antigens K_b and E_a In all the available backcross matings involving these two antigens, there were no heterozygous boars, so that all the data is based on matings between double negative boars and heterozygous sows. The segregation of offspring is given separately for each family in Table 28. To simplify writing the genotype, the gene for antigen K_b is called K and its allelomorph k, whereas E represents E_a and its allelomorph is e.

Table 28 /

Table 28

Mating ♂ kkee ♀ KkEe	Segregation of offspring			
	(a_1) KkEe	(a_2) Kkee	(a_3) kkEe	(a_4) kkee
1st Family	6	2	2	1
2nd "	4	2	2	1
Total observed	10	4	4	2
expected	5	5	5	5

The deviation of K,k segregation from 1 : 1, and E,e from 1 : 1 may be calculated from the formulae given by Mather (1951).

$$\chi^2_K = \frac{(a_1 + a_2 - a_3 - a_4)^2}{n}$$

$$= \frac{(10 + 4 - 4 - 2)^2}{20}$$

$$= \underline{3.2 \text{ for } 1 \text{ d.f.}} \qquad p = \text{Between } 0.1 \text{ and } .05$$

$$\chi^2_E = \frac{(a_1 - a_2 + a_3 - a_4)^2}{n}$$

$$= \frac{(10 - 4 + 4 - 2)^2}{20}$$

$$= \underline{3.2 \text{ for } 1 \text{ d.f.}} \qquad p = \text{Between } 0.1 \text{ and } .05$$

The joint segregation or linkage between K and E can be calculated from the following formula:-

$$\chi^2_L = \frac{(a_1 - a_2 - a_3 + a_4)^2}{n}$$

$$= \frac{(10 - 4 - 4 + 2)^2}{20}$$

$$= \underline{0.8 \text{ for } 1 \text{ d.f.}} \qquad p = \text{Between } 0.5 \text{ and } 0.3$$

There appears to be evidence that the genes for antigens K_b and E_a are segregating independently.

(ii) /

(ii) Antigens E_a and E_e Only one family from one heterozygous boar for these two antigens is available. The remaining families are compiled from matings between heterozygous sows and double negative boars. Let genes A,a represent E_a and E,e represent E_e in Table 29.

Table 29

Mating ♂ AaEe ♀ aaee	Segregation of offspring			
	AaEe	Aaee	aaEe	aaee
1st Family	3	0	0	3
expected	1.5	1.5	1.5	1.5
♂ aaee ♀ AaEe				
1st Family	2	0	0	2
2nd "	1	0	0	3
3rd "	6	0	0	3
4th "	8	0	0	3
Total observed	17	0	0	11
expected	7	7	7	7

The recombination fraction does not appear to differ between the male and female side, and the product of the two fractions are considered. Although there is no additional information on individual sows as to whether the genes for E_a and E_e are in coupling or repulsion, the evidence seems clear that they are in coupling. The deviations from expected are calculated as follows:-

$$\chi^2_A = \frac{(20 + 0 - 0 - 14)^2}{34}$$

$$= \underline{1.06 \text{ for } 1 \text{ d.f.}} \qquad p = \text{Between } 0.5 \text{ and } 0.3$$

$$\chi^2_E = \frac{(20 - 0 + 0 - 14)^2}{34}$$

$$= 1.06 \text{ for } 1 \text{ d.f.} \qquad p = \text{Between } 0.5 \text{ and } 0.3$$

$$\chi^2_L = \frac{(20 - 0 - 0 + 14)^2}{34}$$

$$= \underline{34 \text{ for } 1 \text{ d.f.}} \qquad p = .001$$

There is good evidence to show that the individual segregation of genes controlling factors E_a and E_e is good, but they are not segregating independently of one another. This appears to indicate the existence of linkage in the coupling phase.

(b) Negative association: (i) Antigens E_a and F_a Only families from heterozygous sows and double negative boars were available. Let E,e genes represent E_a and F,f genes represent F_a in Table 30.

Table 30

Mating	Segregation of offspring			
♂ eeff × ♀ EeFf	EeFf	Eeff	eeFf	eeff
1st Family	1	2	3	0
2nd "	1	5	2	1
Observed total	2	7	5	1
Expected	3.75	3.75	3.75	3.75

The probability values for deviation from expected are calculated as follows:-

$$\chi^2_E = \frac{(2 + 7 - 5 - 1)^2}{15}$$

$$= \underline{0.6 \text{ for } 1 \text{ d.f.}} \quad p = \text{Between } 0.5 \text{ to } 0.3$$

$$\chi^2_F = \frac{(2 - 7 + 5 - 1)^2}{15}$$

$$= \underline{0.067 \text{ for } 1 \text{ d.f.}} \quad p = \text{Between } 0.8 \text{ to } 0.7$$

$$\chi^2_L = \frac{(2 - 7 - 5 + 1)^2}{15}$$

χ^2_L CORRECTED BY YATES ADJUSTMENT = 3.2 FOR 1 d.f. ($p = 0.1$ TO 0.05)

$$= \underline{5.4 \text{ for } 1 \text{ d.f.}} \quad p = 0.02$$

There appears to be evidence of linkage between the genes controlling factors E_a and F_a in the repulsion phase.

The intensity of the linkage can be measured by calculating the recombination fraction, where p is this fraction. (Mather, 1951).

$$p = \frac{a_1 + a_4}{n} \text{ in repulsion}$$

$$= \frac{3}{15} = \underline{0.2 \text{ or } 20\%}$$

The standard error for this value may be calculated as follows:-

$$S_p = \sqrt{\frac{P(1 - P)}{n}} = \underline{0.103 \text{ or } 10\%}$$

It would appear that the factors E_a and F_a are controlled by linked genes with a recombination value of 20%. The precision of this estimate has a variance of 10%.

(ii) Antigens E_a and E_f Only one family was available to show backcross segregation. In this family the sow was heterozygous for the two antigens. Let genes A,a and F,f represent the two antigens respectively in Table 31:-

Table 31 /

Table 31

Mating	Segregation of offspring			
♂ aaff × ♀ AaFf	AaFf	Aaff	aaFf	aaff
1st Family	0	3	3	0
expected	1.5	1.5	1.5	1.5

The numbers do not appear to be adequate to apply a statistical test.

5. AMES INTERNATIONAL COMPARISON TEST:-

In November, 1962, the first international comparison test of its kind for pig blood groups was organised by Dr. Andresen from Ames. The object of the test was to compare the specificities of antibodies obtained and isolated by different laboratories independently of one another. Those laboratories where work on pig blood groups was developing were invited to participate.

Aliquots of the same samples of blood from 20 Duroc and 20 Hampshire pigs were sent to seven laboratories. Each laboratory blood typed these samples with the antibodies they had isolated, and sent their report back to the Ames Laboratory, who also participated. On obtaining the results from all the laboratories, they were compiled and compared. Only those tests relevant to the antibodies described in this dissertation are given in the following Tables 32 to 35.

The eight laboratories participating were located as follows:-

1. /

1. Department of Veterinary Clinical Studies,
School of Veterinary Medicine,
University of Cambridge.

2. Animal Breeding Research Organisation,
6, South Oswald Road,
Edinburgh, 9.

3. Veterinary Institute of Slovenia,
Ljubljana,
Yugoslavia.

4. Faculty of Agriculture,
University of Novi Sad,
Yugoslavia.

5. Tierärztliches Institut der Universität Göttingen,
West Germany.

6. Antigenic Laboratory,
Iowa State University,
Ames,
U.S.A.

7. Afd. for Fysiologi,
Endokrinologi og Blodtypeforskning,
Den kgl. Veterinaer-og Landbohøjskole,
Copenhagen,
Denmark.

8. Laborator Pro Biologii,
Prague,
Czechoslovakia.

DISCUSSION ON RESULTS:-

The object of this part of the dissertation has been to demonstrate the existence of different blood group factors on the erythrocytes of pig blood. The results show that natural, isoimmune and heteroimmune antibodies can be used to identify these factors. Evidence is also presented to show that they are controlled by mendelian genes. These genes express themselves in a dominant manner, in that, the antigenic factors they control are detectable when present, providing their particular antibody is used.

No /

Table 32

Results of International Comparison Test

Laboratory No.	1	2	2	3	4	4	5	6	7	8	1	2	3	5	6	7	8	1	2	4	4	5	6	7	7	8
Antibody	A	No. A	1 No. A	15	J	A 22	A 22	A	A	A	A	E_b =1	No. E_b	2	E_b	E_b	E_b	E_b	E_b	K_b	No. K_b	3	K_b 11	K_b 37	K_b	K_b	K_b	K_b	K_b	K_b
Animal																													
D 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+
D 2	+	+	.	+	.	.	.	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
D 3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D 4	.	.	.	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H 5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D 6	+	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+
D 7	+	+	+	+	+	.	.	+	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	.	.
D 8	+	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+
H 9	.	.	.	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H 10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	.	+	+	+	+
H 11	.	.	.	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+	+	+	.	+	+	+	+
H 12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H 13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H 14	.	.	.	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H 15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H 16	.	.	.	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H 17	+	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+
D 18	.	.	.	.	+	.	.	.	.	.	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+
H 19	.	.	.	.	.	.	.	.	.	.	+	+	+	+	+	+	+	.	.	.	.	.	.	.	.	.
D 20	+	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+
D 21	+	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
D 22	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+
D 23	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+
D 24	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+
D 25	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+
D 26	+	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H 27	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H 28	+	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+
H 29	.	.	.	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+	+	SL	.	+	+	+	+
D 30	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+
H 31	.	.	.	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H 32	.	.	.	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D 33	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H 34	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D 35	.	.	.	.	.	.	.	.	.	.	+	+	+	+	+	+	+	.	.	.	.	.	.	.	.	.
D 36	+	+	+	+	+	SL	+	+	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	.	.
H 37	+	+	+	+	+	+	+	+	+	+	.	.	.	.	.	.	.	+	+	+	+	+	+	+	+	+
H 38	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H 39	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D 40	.	.	.	.	.	.	.	.	.	.	+	+	+	+	+	+	+	.	.	.	.	.	.	.	.	.

Table 33

Laboratory No.	1	1	2	2	3	3	4	4	5	6	7	8	1	2	2	3	3	5	6	7	8
			No.	No.										No.	No.						
Antibody	K_a	K_a	4	6	K_a	K_a	K_a	K_a	K_a	K_a	K_a	K_a	E_a	5	9	E_a	E_a	E_a	E_a	E_a	E_a
	H	H15	K_a	K_a	150	114	377	14					2	E_a	E_a						
Animal																					
D 1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D 2	+	+	+	+	+	+	+	+	+	+	+	+	•	•	•	•	•	•	•	•	•
D 3	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
D 4	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
H 5	•	•	•	•	•	•	•	•	•	•	•	•	+	+	+	+	+	+	+	+	+
D 6	+	+	+	+	+	+	+	+	+	+	+	+	•	•	•	•	•	•	•	•	•
D 7	+	+	+	+	+	+	+	+	+	+	+	+	•	•	•	•	•	•	•	•	•
D 8	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
H 9	•	•	•	•	•	•	•	•	•	•	•	•	+	+	+	+	+	+	+	+	+
H 10	+	+	+	+	+	+	SL	+	+	+	+	+	+	+	+	+	+	+	+	+	+
H 11	+	+	+	+	+	+	•	+	+	+	+	+	•	•	•	•	•	•	•	•	•
H 12	•	•	•	•	•	•	•	•	•	•	•	•	+	+	+	+	+	+	+	+	+
H 13	•	•	•	•	•	•	•	•	•	•	•	•	+	+	+	+	+	+	+	+	+
H 14	•	•	•	•	•	•	•	•	•	•	•	•	+	+	+	+	+	+	+	+	+
H 15	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
H 16	•	•	•	•	•	•	•	•	•	•	•	•	+	+	+	+	+	+	+	+	+
H 17	•	•	•	•	•	•	•	•	•	•	•	•	+	+	+	+	+	+	+	+	+
D 18	+	+	+	+	+	+	•	•	+	+	+	+	•	•	•	•	•	•	•	•	•
H 19	+	+	+	+	+	+	+	+	+	+	+	+	•	•	•	•	•	•	•	•	•
D 20	+	+	+	+	+	+	+	+	+	+	+	+	•	•	•	•	•	•	•	•	•
D 21	+	+	+	+	+	+	+	+	+	+	+	+	•	•	•	•	•	•	•	•	•
D 22	+	+	+	+	+	+	+	+	+	+	+	+	•	•	•	•	•	•	•	•	•
D 23	+	+	+	+	+	+	+	+	+	+	+	+	•	•	•	•	•	•	•	•	•
D 24	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
D 25	•	•	•	•	•	•	•	•	•	•	•	•	+	+	+	+	+	+	+	+	+
D 26	+	+	+	+	+	+	+	+	+	+	+	+	•	•	•	•	•	•	•	•	•
H 27	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
H 28	•	•	•	•	•	•	•	•	•	•	•	•	+	+	+	+	+	+	+	+	+
H 29	+	+	+	+	+	+	+	+	+	+	+	+	•	•	•	•	•	•	•	•	•
D 30	+	+	+	+	+	+	+	+	+	+	+	+	•	•	•	•	•	•	•	•	•
H 31	•	•	•	•	•	•	•	•	•	•	•	•	+	+	+	+	+	+	+	+	+
H 32	+	+	+	+	+	+	+	+	•	+	+	+	+	+	+	+	+	+	+	+	+
D 33	+	+	+	+	+	+	+	+	•	+	+	+	•	•	•	•	•	•	•	•	•
H 34	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
D 35	+	+	+	+	+	+	+	+	SL	+	+	+	+	+	+	+	+	+	+	+	+
D 36	+	+	+	+	+	+	+	+	SL	+	+	+	•	•	•	•	•	•	•	•	•
H 37	•	•	•	•	•	•	•	•	•	•	•	•	+	+	+	+	+	+	+	+	+
H 38	•	•	•	•	•	•	•	•	•	•	•	•	+	+	+	+	+	+	+	+	+
H 39	+	+	+	+	+	+	+	+	•	+	+	+	•	•	•	•	•	•	•	•	•
D 40	+	+	+	+	+	+	+	+	•	+	+	+	•	•	•	•	•	•	•	•	•

Table 34

Laboratory No.	1	2	3	6	7	8	1	2	4	6	7	8	2	5	6	7	1	2	1	2	5	6	7	8
Antibody	F_a	8	F_a	F_a	F_a	F_a	E_e	10	E_e	E_e	E_e	E_e	11	E_f	E_f	E_f	7	12	G_a	13	G_a	G_a	G_a	G_a
	8	F_a						E_e	K/L				E_f							G_a				
Animal																								
D 1	·	·	·	·	·	·	+	+	+	+	+	+	·	·	·	·	·	·	·	·	·	·	·	+
D 2	·	·	·	·	·	·	+	+	+	+	+	+	+	+	+	+	·	·	+	+	+	+	+	+
D 3	·	·	·	·	·	·	+	+	+	+	+	+	+	+	+	+	·	·	·	·	·	·	·	·
D 4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	·	·	·	·	·	·	·	·
H 5	+	+	+	+	+	+	+	+	+	+	+	+	·	·	·	·	·	·	·	·	·	·	·	·
D 6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	·	·	+	+	+	+	+	+
D 7	·	·	·	·	·	·	+	+	+	+	+	+	·	·	·	·	·	·	·	·	·	·	·	·
D 8	·	·	·	·	·	+	+	+	+	+	+	+	+	+	+	+	·	·	·	·	·	·	·	+
H 9	+	+	+	+	+	+	+	+	+	+	+	+	·	·	·	·	·	·	·	·	·	·	·	·
H 10	·	·	·	·	·	·	+	+	+	+	+	+	·	·	·	·	·	·	+	+	+	+	+	+
H 11	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	+	+	+	+	+	+
H 12	·	·	·	·	·	·	+	+	+	+	+	+	·	·	·	·	·	·	+	+	+	+	+	+
H 13	·	·	·	·	·	·	+	+	+	+	+	+	·	·	·	·	·	·	+	+	+	+	+	+
H 14	+	+	+	+	+	+	+	+	+	+	+	+	·	·	·	·	·	·	+	+	+	+	+	+
H 15	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	+	+	+	+	+	+
H 16	+	+	+	+	+	+	+	+	+	+	+	+	·	·	·	·	·	·	+	+	+	+	+	+
H 17	+	+	+	+	+	+	+	+	+	+	+	+	·	·	·	·	·	·	+	+	+	+	+	+
D 18	·	·	·	·	·	·	+	+	+	+	+	+	·	·	·	·	·	·	·	·	·	·	·	·
H 19	+	+	+	+	+	+	·	·	·	·	·	·	·	·	·	·	·	·	+	+	+	+	+	+
D 20	?	·	·	·	·	·	+	+	+	+	+	+	+	+	+	+	·	·	+	+	+	+	+	+
D 21	·	·	·	·	·	·	+	+	+	+	+	+	·	·	·	·	+	+	+	+	+	+	+	+
D 22	·	·	·	·	·	·	+	+	+	+	+	+	+	+	+	+	·	·	+	+	+	+	+	+
D 23	·	·	·	·	·	·	+	+	+	+	+	+	+	+	+	+	·	·	+	+	+	+	+	+
D 24	?	·	·	·	·	·	+	+	+	+	+	+	+	+	+	+	·	·	+	+	+	+	+	+
D 25	+	·	·	·	·	·	+	+	+	+	+	+	+	+	+	+	·	·	+	+	+	+	+	+
D 26	+	·	·	·	·	·	+	+	+	+	+	+	·	·	·	·	·	·	+	+	+	+	+	+
H 27	+	+	+	+	+	+	·	·	·	·	·	·	·	·	·	·	·	·	+	+	+	+	+	+
H 28	+	+	+	+	+	+	+	+	+	+	+	+	·	·	·	·	·	·	+	+	+	+	+	+
H 29	·	·	·	·	·	·	+	+	+	+	+	+	·	·	·	·	·	·	·	·	·	·	·	·
D 30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	·	·	·	·	·	·	·	+
H 31	+	+	+	+	+	+	+	+	+	+	+	+	·	·	·	·	?	·	·	·	·	·	·	·
H 32	+	+	+	+	+	+	+	+	+	+	+	+	·	·	·	·	·	·	+	+	+	+	+	+
D 33	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	·	·	+	+	+	+	+	+
H 34	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
D 35	·	·	·	·	·	·	+	+	+	+	+	+	·	·	·	·	·	·	·	·	·	·	·	+
D 36	·	·	·	·	·	·	+	+	+	+	+	+	·	·	·	·	·	·	·	·	·	·	·	·
H 37	·	·	·	·	·	·	+	+	+	+	+	+	·	·	·	·	·	·	+	+	+	+	+	+
H 38	·	·	·	·	·	·	+	+	+	+	+	+	·	·	·	·	·	·	+	+	+	+	+	+
H 39	·	·	·	·	·	·	+	+	+	+	+	+	·	·	·	·	·	·	·	·	·	·	·	·
D 40	·	·	·	·	·	·	+	+	+	+	+	+	·	·	·	·	·	·	·	·	·	·	·	·

Table 35

Laboratory No.	1	2	3	5	6	7	8
Antibody	G_b =b	No. 14 G_b	G_b	G_b	G_b	G_b	G_b
Animal							
D 1	+	+	+	+	+	+	+
D 2	+	+	+	+	+	+	+
D 3	+	+	+	+	+	+	+
D 4	+	+	+	+	+	+	+
H 5	+	+	+	+	+	+	+
D 6	•	•	•	•	•	•	•
D 7	+	+	+	+	+	+	+
D 8	+	+	+	+	+	+	+
H 9	+	+	+	+	+	+	+
H 10	+	+	+	+	+	+	+
H 11	+	+	+	+	+	+	+
H 12	+	+	+	+	+	+	+
H 13	•	•	•	•	•	•	•
H 14	+	+	+	+	+	+	+
H 15	+	+	+	+	+	+	+
H 16	+	+	+	+	+	+	+
H 17	•	•	•	•	•	•	•
D 18	+	+	+	+	+	+	+
H 19	+	+	+	+	+	+	+
D 20	•	•	•	•	•	•	•
D 21	+	+	+	+	+	+	+
D 22	+	+	+	+	+	+	+
D 23	+	+	+	+	+	+	+
D 24	+	+	+	+	+	+	+
D 25	+	+	+	+	+	+	+
D 26	+	+	+	+	+	+	+
H 27	+	+	+	+	+	+	+
H 28	+	+	+	+	+	+	+
H 29	+	+	+	+	+	+	+
D 30	+	+	+	+	+	+	+
H 31	+	+	+	+	+	+	+
H 32	•	•	•	•	•	•	•
D 33	+	+	+	+	+	+	+
H 34	+	+	+	+	+	+	+
D 35	+	+	+	+	+	+	+
D 36	+	+	+	+	+	+	+
H 37	•	•	•	•	•	•	•
H 38	•	•	•	•	•	•	•
H 39	+	+	+	+	+	+	+
D 40	+	+	+	+	+	+	+

No attempt has been made to classify individual pigs on the basis of natural antibodies occurring in sera, other than the detection of the A substance. Previous reports on this subject (c.f. Review of literature) have not been very satisfactory, and circumstances were such that a reliable investigation could not be made, because of the widespread use of crystal violet vaccine amongst the experimental herds. The most satisfactory approach to the whole subject of detection of natural isoagglutinins and soluble blood group substances, would appear to be along the lines of Springer et al. (1958), who in chickens used 'disease free' birds. Also, in this instance it would be necessary to maintain an unvaccinated herd of pigs. It is hoped that investigations along these lines will be attempted in the future.

The detection of the A substance on pig cells by the use of natural anti-J serum from cattle was practicable. This was demonstrated by the comparison test. In this test, another antibody called No. 15 was used, which was isolated from a pre-injection sample of serum from a sow. This appeared to be a natural haemolytic antibody, which reacted with type A cells. It has not been discussed in this dissertation, because it was only discovered at the end of the family studies. In the comparison test it can be seen that this antibody failed to detect the A substance in animal No. 2, whereas the anti-J serum was positive. This result is not in agreement with Saison (1962), who used pig anti A, sheep anti-R, cattle anti-J and cattle anti-sheep O in a comparison test. Of 312 abattoir samples tested, 195 reacted with the cattle anti-sheep O; 87 reacted with the pig anti-A and sheep anti-R; whereas only 82 of these 87 were positive to the anti-J. The remaining 30 samples were /

were negative to all four antisera. This discrepancy could be explained in terms of potency of the different anti-J sera used, because it is known that there is a marked seasonal variation in the potency of J antiserum, with a peak in the Autumn months (Jamieson, 1963). No. 1 antiserum was obtained in the month of October.

It is also claimed by Andresen, that he has detected exceptions to the theory of dominant inheritance for the cellular A substance (1962). He states that, "Family data has been confirmed, that the cellular A substance can be present in offspring from parents which do not possess this substance on their red cells as detected in a conventional haemolytic test with a potent reagent". None of the 25 families examined and listed in this treatise were exceptions to the theory of dominance. It is not stated by Andresen, however, if he attempted to detect the A substance in the sera of the respective parents by means of a haemolytic inhibition test.

The use of isoimmune antisera as a source of identifying antibodies for the demonstration of antigenic individuality of pig red cells has been confirmed in this study. It has also been shown that certain antigenic factors appear to be associated with one another, and may therefore be controlled by allelic genes at the same locus on a chromosome. All the E factors show a positive association with one another, and allelism has been proved in the case of the E^a and E^e genes. The latter is an interesting association, because the E_a factor does not exist by itself, and only appears in combination with E_e. This is consistent with the

mosaic /

mosaic structure of red cell agglutinogens, or multiple allelism as proposed by Wiener and Wexler (1952). The E system in pigs as defined by Andresen (1962) appears to be confirmed to a certain extent by this study. He has proposed that the following phenogroups (i.e. groups of agglutinogens) are controlled by five allelic genes:-

Andresen's E system of pigs

Genes	Phenogroups	Reaction with six reagents					
		E_a	E_d	E_b	E_e	E_f	E_g
E^1	E_{bdg}	•	+	+	•	•	+
E^2	E_{edg}	•	+	•	+	•	+
E^3	E_{aeg}	+	•	•	+	•	+
E^4	E_{efd}	•	+	•	+	+	•
E^5	E_{bfd}	•	+	+	•	+	•

The contrasting factors E_d and E_g to E_a and E_f respectively were not found in this investigation. However, the emergence of E_b and E_e as contrasting factors was shown by the 2 x 2 contingency table. No individuals were found lacking both characters. On the basis of the existence of the following genotypes: E^b/E^b; E^b/E^e and E^e/E^e the random distribution of these types was calculated and compared with expected within the Large White parent population. This population was chosen, because all the possible phenotype matings were present, and the distribution of the offspring for these different matings could be shown and compared with expected. The results are given overleaf:-

Genotype distribution of E^b and E^e within Large White parents

Genotype	E^b/E^b	E^b/E^e	E^e/E^e
No. observed	9	14	5
Gene frequencies calculated	.326	.496	.184
No. expected	9	14	5

This population of Large Whites appeared to be in a state of genetic equilibrium for these two factors.

Distribution of E_b and E_e phenotypes among 108 offspring from 21 Large White families

Mating type	No. of families	Offspring			χ^2 values
		E_b	E_bE_e	E_e	
$E_b \times E_b$	6	29			
$E_b \times E_bE_e$	7	13(10.5)	8(10.5)		0.6 for 1 d.f.
$E_b \times E_e$	1		4		
$E_bE_e \times E_bE_e$	4	7(4.25)	4(8.5)	6(4.25)	4.9 for 2 d.f.
$E_bE_e \times E_e$	2		2(6)	10(6)	5.4 for 1 d.f.
$E_e \times E_e$	1			5	

(expected values are given in parenthesis)

The evidence given above supports the theory that E_b and E_e behave as contrasting characters, and therefore form a closed "system". One advantage of this type of system is that the phenotype reflect the genotype, and homozygotes may be distinguished from heterozygotes on blood type alone at this locus.

A /

A positive association has also been established in the 2 x 2 tables for K_a and K_b. This observation supports the claim by Andresen (1959c), that these characters are produced by allelic genes in the K system. From the present data, it appeared at first that these factors might be contrasting, but the existence of a double negative animal amongst the parent population and six amongst the offspring overruled this explanation. Further evidence in several families involving the segregation of these two factors suggested that at least one other allele must be assumed to explain the results. For example, in three families of mating type K(a - b+) x K(a + b+) six offspring appeared of type K(a + b-), also in eleven matings of type K(a + b-) x K(a - b+), 32 offspring were type K(a - b+) and five were K(a + b-).

To test the genetic equilibrium of these two factors within the parent population, then the existence of a third allele K^- was assumed. The existence of three alleles in this system makes it similar to the human ABO system, and the same method for calculation of the gene frequencies may be adopted. Bernstein's improved method (1930) was applied to the data, and the following gene frequencies for the three alleles were obtained:-

gene frequencies K^a = .264; K^b = .586; K^- = .148

As the number of genotypes = $\frac{1}{2}$ m(m + 1) = 6, where m represents the number of alleles, then the number of genotypes exceeds the number of phenotypes. According to Cotterman (1954), therefore, a state of genetic equilibrium is expressed by the expansion of the polynome $(pK^a + qK^b + rK^- .....)^2$. Each parameter in the expansion give the relative frequency of the corresponding genotype. /

genotype. This means that the expected frequencies of the different phenotypes within the population can be calculated from the formulae given below, and the deviation from observed calculated as a χ^2 value:-

Phenotype	No. observed	No. expected	χ^2
K(a+b-)	19	$n(P^2 + 2p.r) = 15.392$	0.85
K(a-b+)	57	$n(q^2 + 2q.r) = 53.664$	0.21
K(a+b+)	27	$2n.P.q. = 32.178$	0.83
K(a-b-)	1	$n.r = 2.278$	0.72

Total = 104 = n Total = 2.61 for 3 d.f.

The observed and expected figures show good agreement, and it may be concluded that the parent population investigated was in genetic equilibrium for the genes controlling blood group factors K_a and K_b.

One important feature of the comparison test was the confirmation that Antiserum No. 14 had a specificity for character G_b. Andresen (1961) claims that factors G_a and G_b are contrasting characters controlled by two allelic genes within the G system. The possibility of association between these two factors was not investigated in the parent population. However, in all the animals where red cells were typed for both G_a and G_b, the independence of these two factors was tested in the following 2 x 2 contengency table:-

		G_b +ve	G_b -ve	
G_a	+ve	76	7	83
	-ve	145	0	145
		221	7	228

$$\chi^2 = \frac{(145 \times 7 - 76)^2 \times 228}{221 \times 7 \times 83 \times 145} = 10.8 \text{ for 1 d.f.}$$

The likelihood is so great that there appears to be considerable evidence to support the claim that these factors are contrasting.

As a result of statistical analyses, there is a certain amount of evidence to suggest that linkage exists between some of the factors. For example, the factors K_a and K_b may be linked to the E system. The positive association between K_b and E_a, however, could not be proved on a genetic basis from backcross data on two families. Obviously, more family data is required to test this association. Linkage appeared to be established between factors F_a and E_a, but again the evidence is based on a limited number of families. Andresen (1959) has suggested that each system may represent a pair of autosomal chromosomes, but does not mention the possibility of linkage, or that several systems may perhaps belong on the same pair of chromosomes. The evidence presented in this thesis throws considerable doubt on the suggestion of one system, one chromosome, but further proof in the form of suitable backcross data is required.

The remaining factor, called X appears to be a new antigen, possibly linked to the K system. The frequency of this antigen within the parent population was equivalent to 0.172, which is low. However, it only appeared in the Large White breed, and for the number of animals bled within this breed, the antigen frequency was equivalent to 0.407. It also appears from the comparison test, that its Antibody No. 12 may have a specificity very close to the No. 7 antibody used by Cambridge.

PART II - BIOCHEMICAL STUDY

1. INTRODUCTION:-

The amount of literature on the subject of genetically determined biochemical individuality within different species has grown in momentum during the past decade. Any comprehensive review of the subject would be beyond the scope of this dissertation, and inadequate compared to the excellent review by Ogden (1961), in which all the information on the subject of biochemical polymorphism in farm animals has been collated.

In this section on a biochemical study, the investigation has been confined to measurement of electrolyte constituents of the blood, and electrophoresis of serum proteins by starch gel electrophoresis. In the latter, the identifications of variants within specific protein components, and the control of these variants by genes segregating in a simple mendelian manner has also been studied. Reference to specific work has been quoted where relevant.

2. STUDY OF BLOOD POTASSIUM AND SODIUM LEVELS IN DIFFERENT BREEDS OF PIGS:-

The first indication of electrolyte variation within a species was shown by Kerr (1937). In a study in Syria, he was able to classify sheep into three groups with respect to the amounts of potassium and sodium in their erythrocytes. In 1954, Evans showed that sheep could be classified into two groups on the potassium levels of their blood. These groups were called High potassium (mean K value of 36 m. Eq./litre of whole blood) and Low potassium (mean K value of 13 m. Eq./litre of whole blood) types. It was also found that the whole blood sodium concentration varied inversely /

inversely with that of the potassium. Later, Evans and King (1955) established that these two types are genetically determined in a simple mendelian manner, and the HK type is recessive.

The only reference found giving sodium and potassium levels in pig blood was that by McCance and Widdowson (1956a) and Widdowson and McCance (1956b). They made estimations on 13 adult pigs for sodium levels in the serum, and nine adult pigs for sodium and potassium levels in the erythrocytes. In view of the lack of knowledge of electrolyte levels within the blood of pigs, it was decided to investigate potassium and sodium levels in a large number of pigs of different breeds.

1. Animals investigated: Blood was obtained from pigs at the National Pig Progeny Testing Stations. This material was chosen, because it enabled one to compare levels in animals of different breeds and families, also of similar age groups, and under similar environmental conditions. Facts about the animals sampled are as follows:-

Breed	No. sampled	No. of boar families	Av. age in days
Large White	193	56	78.54
Landrace	118	37	78.68
Wessex	46	17	-
Gloucester Old Spots	9	1	131

2. Methods: Approximately 5 ml. of blood was collected in a clean polystyrene bottle containing 50 i u. of heparin.

(a) Estimation of Sodium and Potassium A volume of 0.25 ml. of each sample of well mixed whole blood was suspended in

50 /

50 ml. of glass distilled water in a volumetric flask giving a final dilution of 1 in 200. Estimations on the diluted solutions of blood were made in an Eel Flame Photometer. Readings were taken and plotted on a known standard graph, and estimations calculated in milliequivalents per litre. Prior to each run the machine was calibrated and set for two known standards for both Sodium and Potassium. These standards were made-up from stock solutions of spectrographically pure KCl and NaCl in glass distilled water. The two NaCl working standards used were equivalent to 120 and 240 milliequivalents of sodium per litre, and the KCl standards were equivalent to 20 and 40 milliequivalent of potassium per litre at 1 in 250 and 1 in 100 dilutions respectively.

In calculating the potassium levels, these were adjusted according to an interference curve which was calibrated to correct for sodium interference, as shown by Wynn et al. (1950). At a ratio of 1 : 2 of potassium to sodium, this interference could be as much as 5%. Ratios from 1 : 2 to 1 : 12 of mgms. % sodium added to a standard solution of 0.5 mgms. % potassium were calibrated.

(b) Haematocrit values These values were estimated on whole blood samples by the micro-haematocrit method using capillary tubes sealed and spun at 12,000 g. for five minutes.

(c) Plasma Sodium and Potassium values Estimations were similar to the whole blood method. Blood samples were spun and 1 in 200 dilutions of plasma in glass distilled water made in volumetric flasks.

(d) Calculation of Potassium content of Red Cells By estimating /

estimating the whole blood and plasma content, and knowing the haematocrit value, it was possible to calculate what quantity of potassium was present in a known volume of erythrocytes. The calculated value for a known volume of cells was adjusted to give a value equivalent to an erythrocyte volume of 100%, or unit volume of cells.

3. Results: The whole blood levels for sodium and potassium are summarised in Table 36, for four different breeds:-

Table 36

1. Whole blood levels of Sodium (m. Eq./litre)

Breed	Av. Na level	S.D.	Range
Large White	90.9	± 7.96	74 - 107.5
Landrace	89.7	± 6.3	78.5 - 111
Wessex	87.5	± 6.1	71 - 104
Gloucester Old Spot	84.2	± 7.7	73 - 92

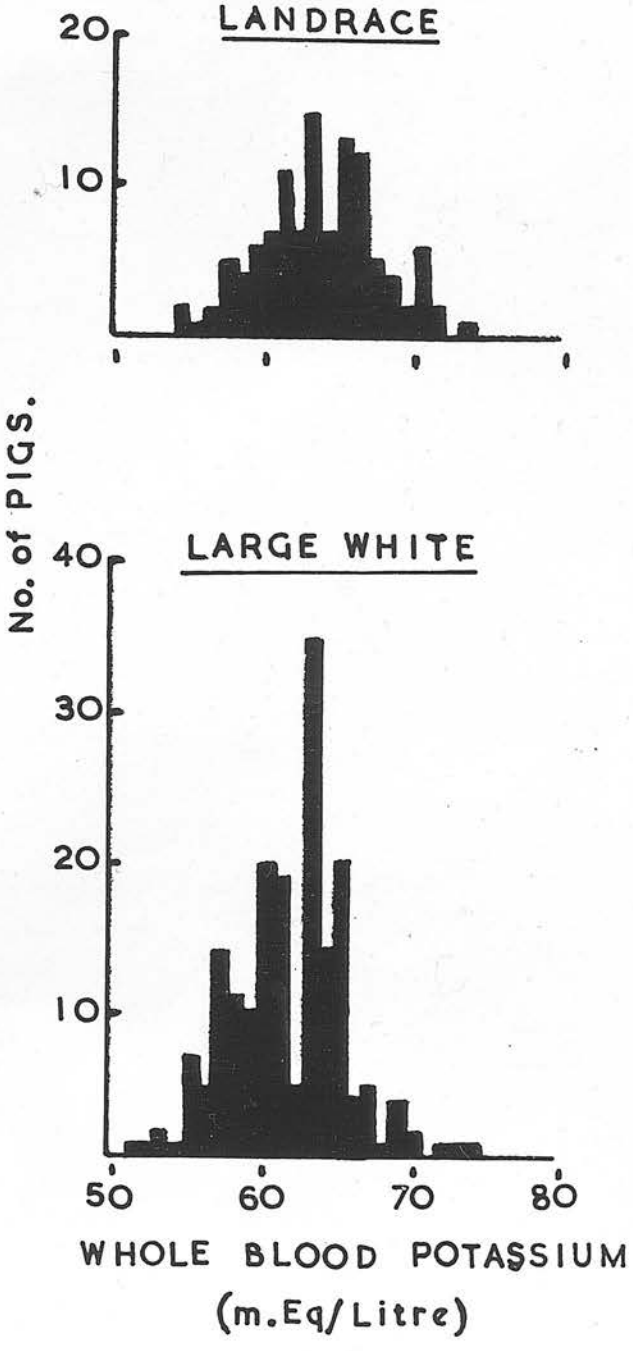
2. Whole blood levels of Potassium (m. Eq./litre)

Breed	Av. K level	S.D.	Range
Large White	61.4	± 3.7	52 - 73.5
Landrace	63.0	± 3.8	53.5 - 72.6
Wessex	61.1	± 4.7	51 - 73.5
Gloucester Old Spot	61.1	± 2.07	57.5 - 65.2

A comparison of the distribution of potassium levels within the Large White and Landrace breed is shown in histogram form in Figure 15. The distribution is unimodal in each case.

An /

Fig.15.


LANDRACE
20
10
LARGE WHITE
40
30
20
10
No. of PIGS.
50
60
70
80
WHOLE BLOOD POTASSIUM
(m.Eq/Litre)

An Analysis of Variance was made on the whole blood potassium levels between individuals and families within the Large White, Landrace and Wessex breeds. The results are given in Table 37:-

Table 37

Analysis of Variance within Breeds:-

1. Large White breed

Source of Variation	Sums of squares	Degrees of freedom	Variance estimate (Mean square)	Significance
Between (~~samples~~ FAMILIES)	1,477	55	26.85	$N^1 = 55$; $N^2 = 125$
Within (~~samples~~ FAMILIES) (BETWEEN INDIVIDUALS)	1,133	125	9.06	F = 2.96
Total	2,610	180	14.5	Significant at 1%

2. Landrace breed

Source of Variation	Sums of Squares	Degrees of Freedom	Variance estimate (Mean square)	Significance
Between ~~samples~~ FAMILIES	762.43	36	21.18	$N^1 = 36$; $N^2 = 75$
Within ~~samples~~ FAMILIES (BETWEEN INDIVIDUALS)	981.52	75	13.09	F = 1.62
Total	1,743.95	111	15.7	Significant at 5%

3. /

3. Wessex breed

Source of Variation	Sums of Squares	Degrees of Freedom	Variance estimate (Mean square)	Significance
Between ~~samples~~ FAMILIES	533.6	16	33.35	$N^1 = 16$; $N^2 = 28$
Within ~~samples~~ FAMILIES (BETWEEN INDIVIDUALS)	448.18	28	16.01	F = 2.08
Total	981.78	44	16.36	Significant at 5%

An analysis was also carried out to see if there was any variance between the breeds for whole blood potassium levels. The results of this analysis are given in Table 38:-

Table 38

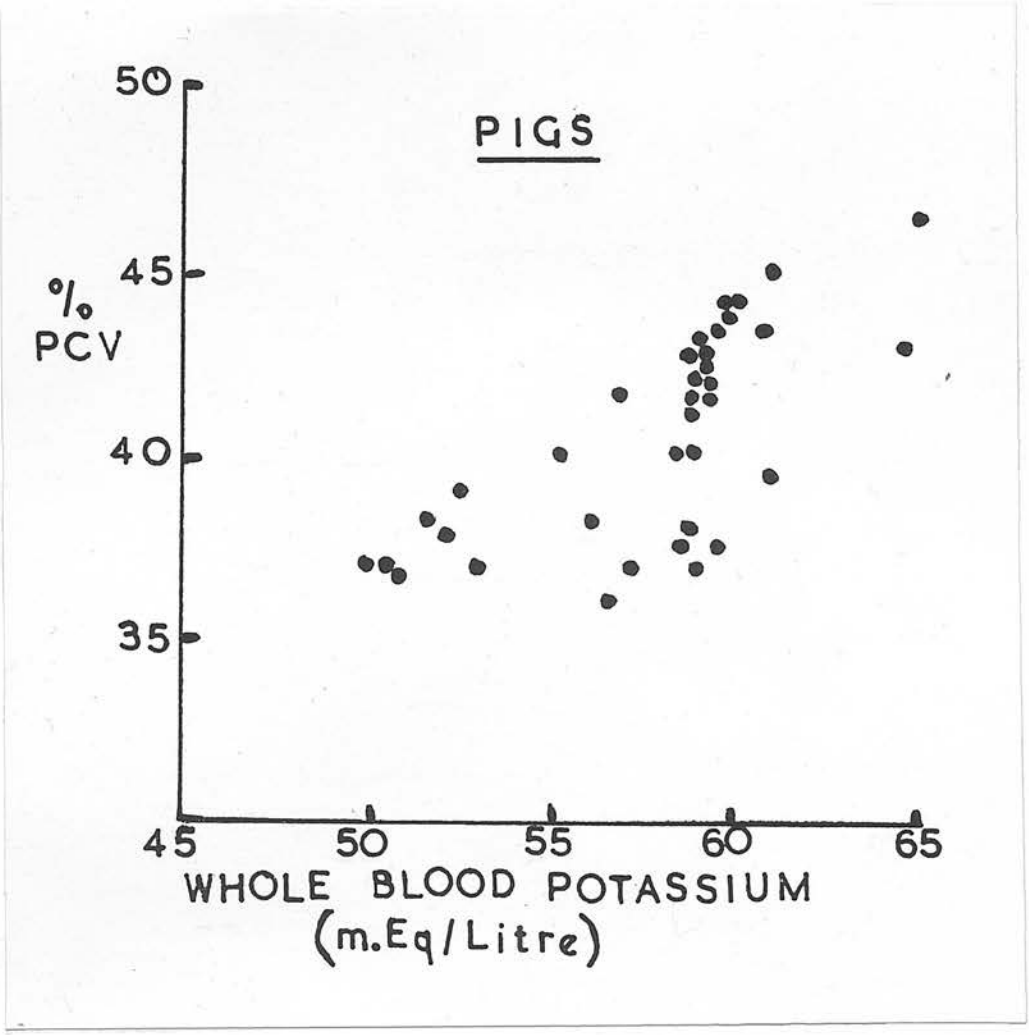
Analysis of Variance between Breeds

Source of Variation	Sums of Squares	Degrees of Freedom	Variance estimate (Mean square)	Significance
Between ~~samples~~ BREEDS	201.25	3	67.08	$N^1 = 3$; $N^2 = 335$
Within ~~samples~~ BREEDS (BETWEEN INDIVIDUALS)	9,589.56	335	28.63	F = 2.34
Total	9,790.81	338	28.97	Not significant

The estimation of packed cell volumes in correlation with the potassium values revealed that the latter varied in a linear manner and showed a positive correlation with the haematocrit values. All breeds showed a wide scatter without forming distinct groups. A few samples have been selected at random and are illustrated in Figure 16.

It was also found that the potassium values showed an inverse /

Fig.16.


PIGS
50
45
40
35
% PCV
45
50
55
60
65
WHOLE BLOOD POTASSIUM
(m.Eq/Litre)

inverse relationship to the sodium, and this is emphasised on plotting values against each other as in Figure 17. The distribution again was diverse.

By obtaining the packed cell volume and estimating the plasma potassium, which gave an average value of 7.37 m. Eq./litre (± 0.75), it was possible to calculate the actual potassium content of the red cells. The values obtained are shown in Table 39 below:-

Table 39

Potassium content of erythrocytes

Breed	No. of samples	Av. P.C.V. percentage, S.D. and range	Av. K content of red cells in m. Eq./litre per unit volume of cells, S.D. and range
Large White	120	40.14 ± 2.88 (33 - 51)	141.2 ± 5.4 (131 - 156)
Landrace	67	41.02 ± 3.05 (33 - 49)	140.3 ± 4.8 (133 - 157)

A comparison of the distribution of the erythrocyte values for both breeds is shown in the histograms in Figure 18. In both instances, the distribution is positively skew. The true values are plotted instead of logarithmic values.

The plasma sodium was also estimated in the Large White breed. An average value of 144 m. Eq./litre (± 8.86) was obtained.

In order to assess the repeatability of individual potassium levels in whole blood, 22 animals were resampled after an interval of one week. The mean values for the respective weeks were 61.6 and 61.2 m. Eq./litre. An analysis of variance was calculated on these values to assess whether the variance within individual animals was greater than between. The results are shown in Table 40.

Table 40 /

Fig.17.

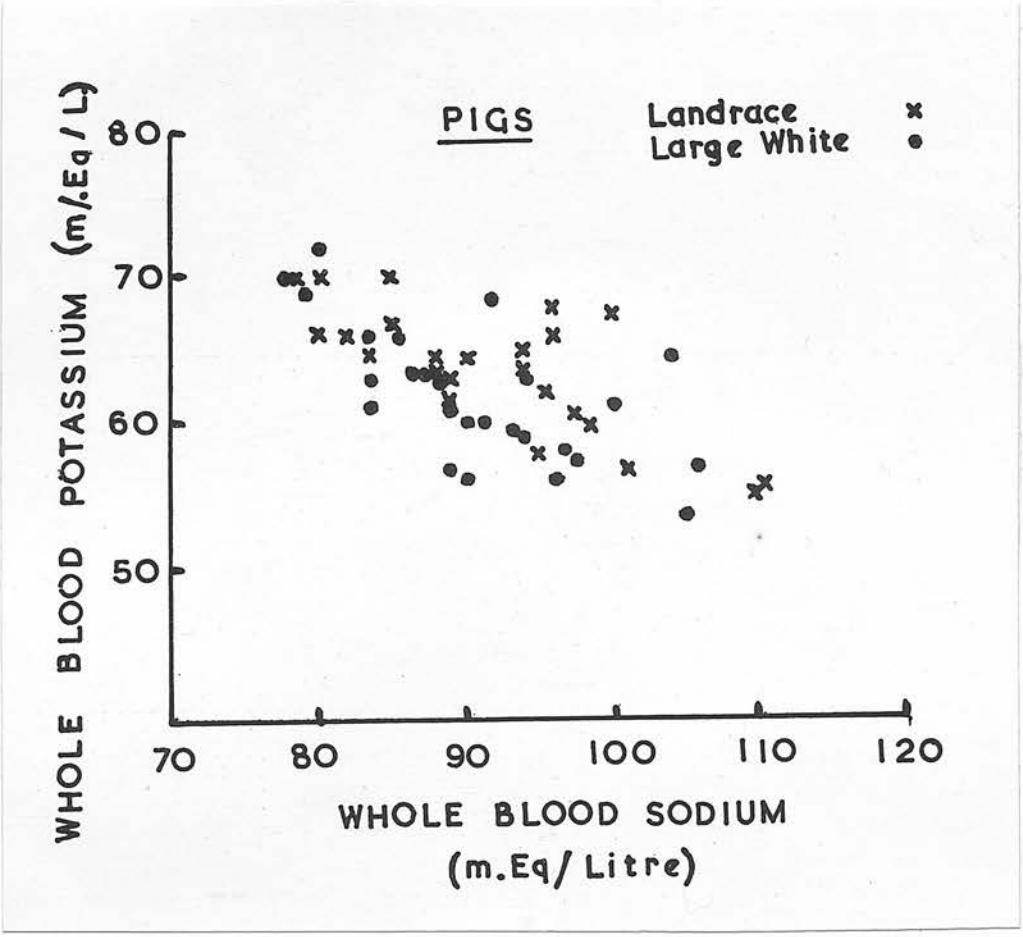

PIGS
Landrace
Large White
WHOLE BLOOD POTASSIUM (m/.Eq/L)
80
70
60
50
70
80
90
100
110
120
WHOLE BLOOD SODIUM
(m.Eq/Litre)

Fig.18.

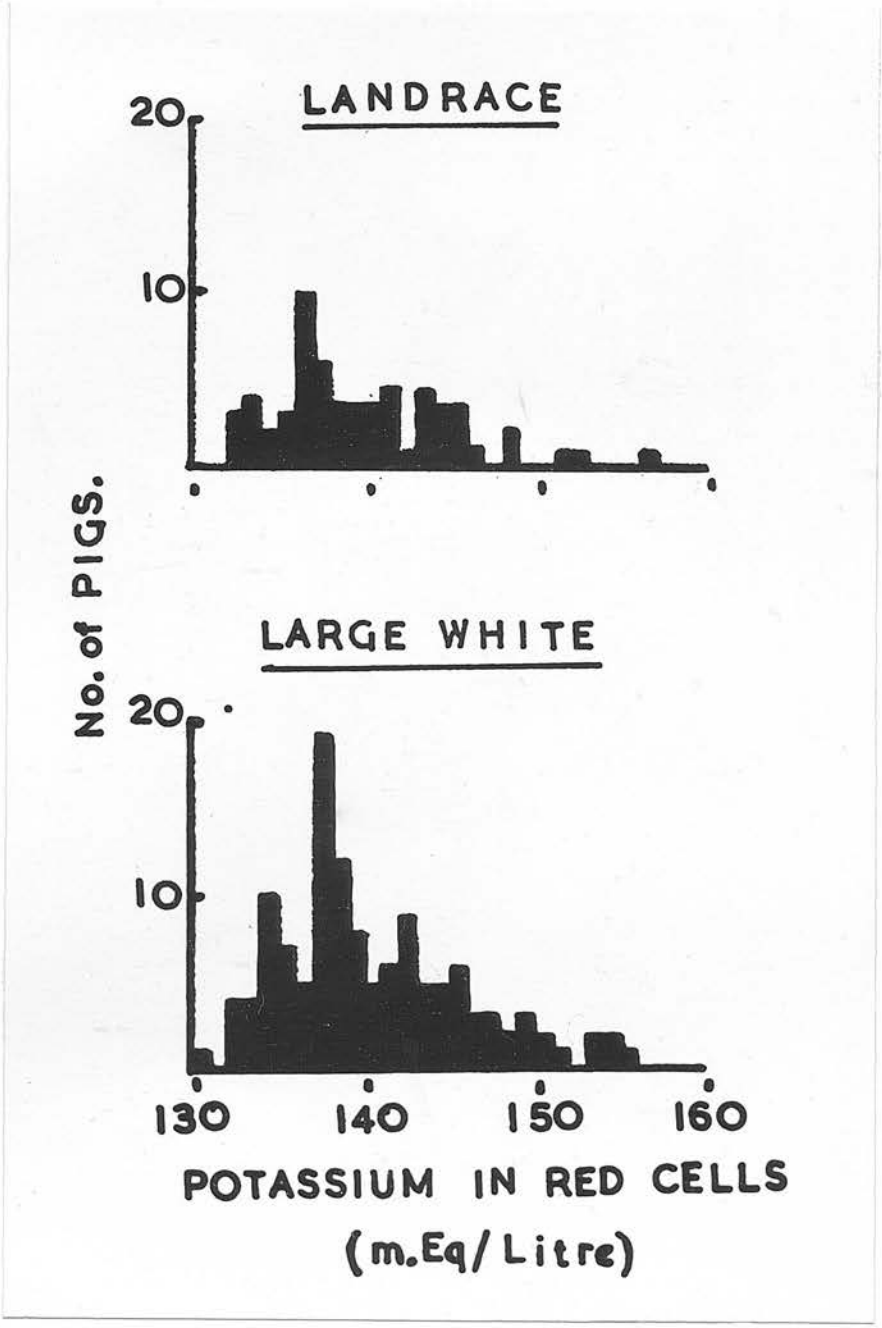

LANDRACE
20
10
LARGE WHITE
20
10
No. of PIGS.
130
140
150
160
POTASSIUM IN RED CELLS
(m.Eq/Litre)

Table 40

Analysis of Variance on repeat Potassium estimations

Source of Variation	Sums of Squares	Degrees of Freedom	Variance estimate (Mean square)	Significance
Between ~~samples~~ INDIVIDUALS	235.04	21	11.19	$N^1 = 21$; $N^2 = 22$
~~Within~~ BETWEEN samples WITHIN INDIVIDUALS	123.58	22	5.62	F = 1.99
Total	358.62	43	8.34	Not significant at 5% but significant at 10%

It appears from Table 40, that the variance was greater between individuals, than within, but this variance was not quite significant.

3. STARCH GEL ELECTROPHORESIS TECHNIQUES TO DEMONSTRATE SERUM PROTEIN VARIANTS:-

1. Pig Serum Transferrins: The technique of starch gel electrophoresis first introduced by Smithies (1955) has been applied to this study of serum proteins in pigs. Smithies (1957 and 1958) demonstrated that three beta-globulin or transferrin phenotypes existed in human sera, and were controlled by a pair of autosomal alleles with no dominance. Variations within the beta-globulins of cattle have also been demonstrated by Hickman and Smithies (1957) and Ashton (1957), also in sheep (Ashton, 1958a and b) and pigs (Ashton, 1960).

The methods used to demonstrate the serum proteins to be described in this section are based on one dimensional, horizontal, starch gel electrophoresis using a discontinuous buffer system. Basically /

Basically the procedure adopted was similar for all fractions investigated, but techniques had to be varied for certain protein components, and the methods adopted will be described where appropriate. For the demonstration of transferrins, the following methods were used:-

(a) Materials and methods: (i) Preparation of starch gel

A 12% suspension of commercially produced hydrolysed starch (Connaught Laboratories, Toronto) in 500 ml. of dilute buffer solution was heated with shaking in a conical flask over a naked flame. The buffer used was similar to that described by Poulik (1957), and contained 0.005 M citric acid and 0.076 M tris (hydroxymethyl) aminomethane. Poulik recommended a pH of 9 for serum protein separations, but in this study of pig transferrins, it was found necessary to adjust the pH to 7.6 by varying the proportions of these two salts. A mixture of 812.5 ml. of citric acid with 187.5 ml. of tris gave the desired pH.

The starch was heated in this buffer until a clear homogenous mixture was obtained, then air bubbles were removed from the solution by boiling under reduced pressure for 20 to 30 seconds. This mixture was then poured into pre-heated glass trays with removable side pieces. The trays measured 12 x 25 cms. internally and were 6 mms. deep. On pouring the starch into the tray, a thin polythene sheet was rolled over the surface of the starch, so that all air bubbles were excluded. This sheet was then held firmly in position by a glass plate placed on top of the tray. Gels were allowed to set and stand overnight for approximately 15 hours, but could be used within 5 hours of pouring provided they were placed in a refrigerator at $+4^{\circ}C$. for two hours prior to use.

(ii) /

(ii) Insertion of serum samples

Serum samples of 0.06 ml. volume were absorbed on to small pieces of No. 17 Whatman filter paper measuring 0.6 x 1 cms., then inserted into the gel by first cutting across the breadth of the gel at a point 5 cms. from one edge. On separating the two pieces of gel slightly, the filter paper inserts were then placed separately into the channel formed, so that each was evenly spaced leaving a gap of 3 mms. between each insert. A total of nine inserts could be placed in a gel in this manner. The two divided sections of gel were then pushed firmly together on either side of the pieces of filter paper.

(iii) Tank buffer and running conditions Electrical contact was then made to either end of the gel by means of several thicknesses of gauze soaked in a different buffer solution to that in the gel. The buffer used was similar to that recommended by Poulik (1957) as modified by Lush (1961). Two separate tanks contained 0.3 M boric acid and 0.1 M sodium hydroxide at a pH of 8.6. The glass plate carrying the gel was supported between these two tanks, so that the edges of the tray hung over the buffer in the tanks. One gauze wick was placed 2.5 cms. from and parallel to the insert line, and the other placed at the other end of the gel 20 cms. from and parallel to the inner edge of the first wick. The whole gel was then covered with a thin sheet of polythene, and a voltage gradient of 6 volts/cm. was applied along the gel at a current of 16 milliamps. Current was applied by means of stainless steel electrodes, which were connected to a D.C. power supply pack, and inserted in either tank solution.

The /

The cathode being at the insert end of the gel. After 30 minutes, the serum had moved out of the filter paper inserts into the substance of the gel. The inserts were then removed, and the cut edges of the gel pushed firmly together again, so that no air gaps were present. Electrophoresis was continued under an increased voltage gradient of 8 - 9 volts./cm. Increasing the voltage meant an increase in wattage, and this resulted in an ambient of 40 to 45°C. within the gel. As denaturation does not occur at this temperature, it was possible to run the electrophoresis at room temperature.

Being a discontinuous system, it was possible to follow the front of the tank buffer as it moved through the gel. This is referred to as the "brown line". As the albumin zone follows very close behind this line, it acts as a very good guide as to how far the serum proteins move through the gel. For the pig transferrins, electrophoresis was stopped, when the brown line had gone 9 cms. beyond the insert line.

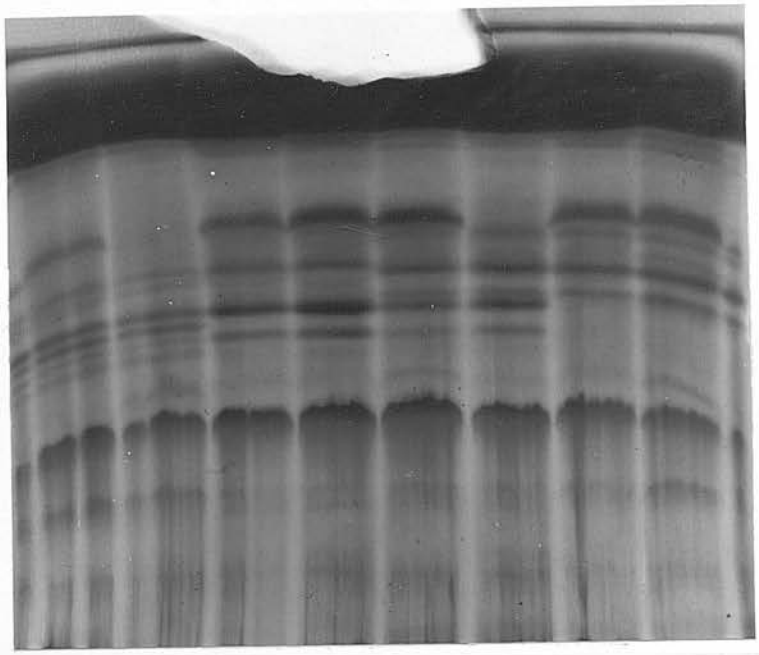
(iv) Cutting and staining of gels The side pieces of the tray were removed and the gel sliced across approximately 2 cms. in front of the brown line. Two pieces of perspex, half the thickness of the gel, were laid on either side of the gel, and with a fine wire it was sliced on a horizontal plane into two halves of equal thickness. The top half was immersed for one minute in a 1% nigrosin solution made-up in a 50 : 50 : 10 methanol, water and GLACIAL acetic acid mixture. The gel was then washed in tap water and left in a washing solution mixture of methanol, water and acetic acid until the background had destained.

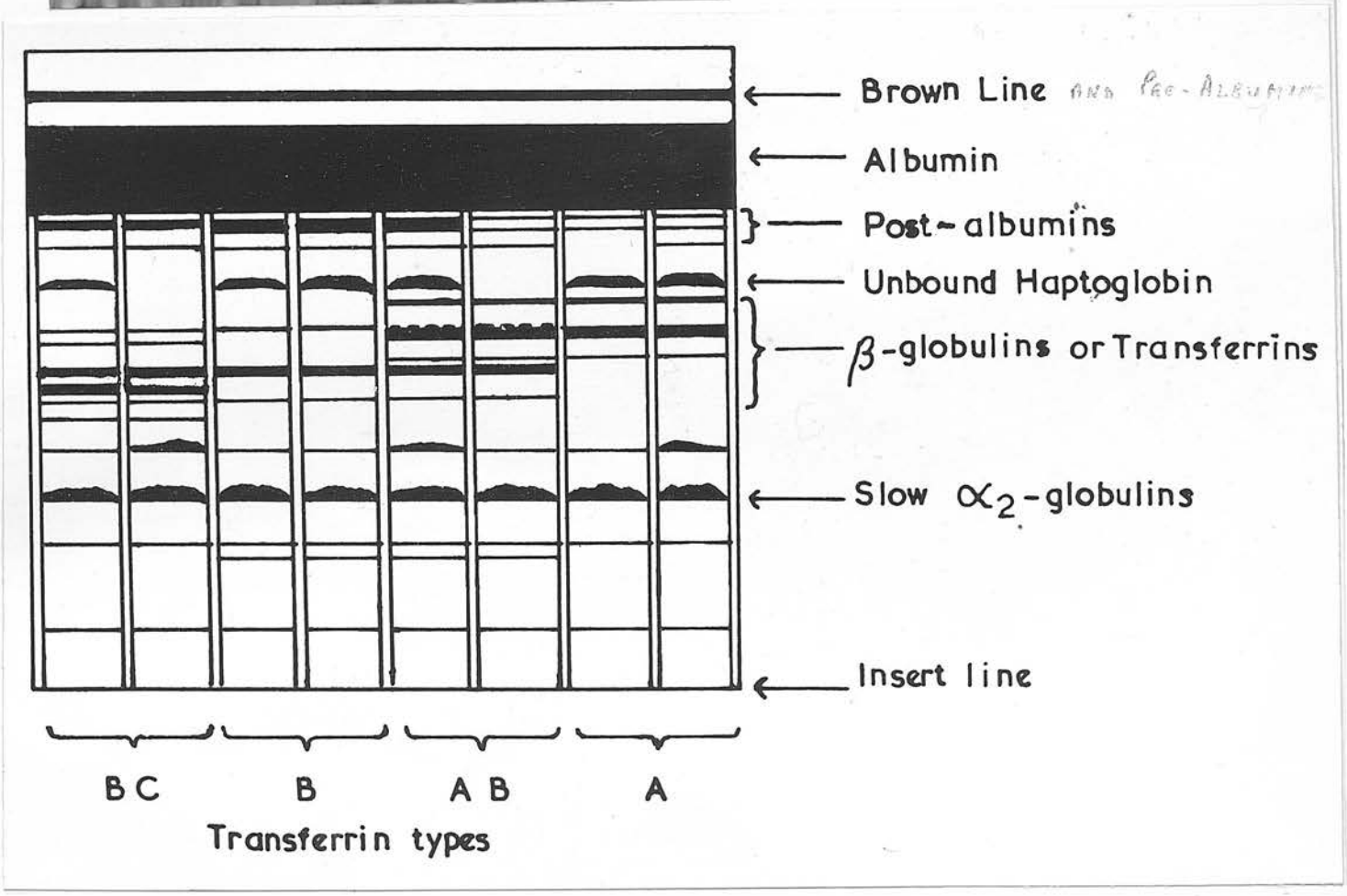
The /

The bottom half of the gel was kept for a specific staining technique to be described later. On destaining of the other half, the separate protein components stained with nigrosin were clearly visible and ready for interpretation. The gel could either be photographed, or kept in the washing solution for over a year without deterioration.

(b) Results: In photograph No. 3 and the schematic drawing of this photograph, the distribution of the separate fractions are shown and labelled. This photograph shows the serum proteins of eight unrelated pigs on one gel. Within the beta-globulin region, in particular, several distinct components are apparent, and similar types have been paired-up to show their existence among unrelated animals. It appears from the patterns presented in the beta-globulin region, that three distinct bands are present in some animals, and five or six in others. In the next paragraph, it will be shown, that each of these bands is in fact a transferrin (i.e. iron-binding protein), and in the inheritance studies, it can be shown that three bands represent the homozygous expression of the genes, and five or six bands, the heterozygous condition. In the homozygote there are two thin bands on either side of one major band, and in the heterozygote, there is a combination of three bands from either homozygote forming six bands, with the two major bands in the centre. In photograph No. 3, the different types have been given a notation, and it can be seen, that the AB type does not completely show all six bands, because the major band of the A almost merges with the leading band of the B. However, in the other heterozygote BC, where a further combination of types occur, all six bands /

Photograph No.3.





bands are present. No animals of type C in the homozygous form have been found to date. This thesis proposes the existence of three distinct transferrin types, called A, B and C with combinations of these types in the various heterozygous forms. If these protein bands are phenotypic representations of the genes controlling their protein synthesis, (then dominance and recessiveness are absent,) because either product is equally represented in all heterozygotes. This means that the phenotypic representation is a direct expression of the gene and is therefore a genotypic representation. The family studies to be given later will substantiate this point.

(c) Labelling serum beta-globulins with Fe^{59}: To establish that the beta-globulin bands were in fact iron-binding proteins, an experiment was conducted with the co-operation of the Haematology and the Medical Physics Research Departments of Edinburgh Royal Infirmary using Fe^{59}. The following procedure was adopted:-

1. Method A 1.4 ml. solution of ferric chloride containing 10 µ curies of Fe^{59} was diluted to 3.4 mls. with 2 ml. of normal saline. A 0.5 ml. aliquot of this solution was added to each one ml. sample of three sera representing three different beta-globulin types. The three types were A, B and BC. This meant that each sample contained an equivalent of 1,470 millimicro-curies of Fe^{59}. The sera were incubated at 37°C. for 30 minutes to facilitate binding of the iron, then each sample was dialysed against distilled water for 24 hours to allow free Fe^{59} to dialyse out. A 0.05 ml. volume of each sample was then absorbed on to filter paper inserts and placed one cm. apart in a starch gel. Each sample subjected to electrophoresis, therefore, contained approximately

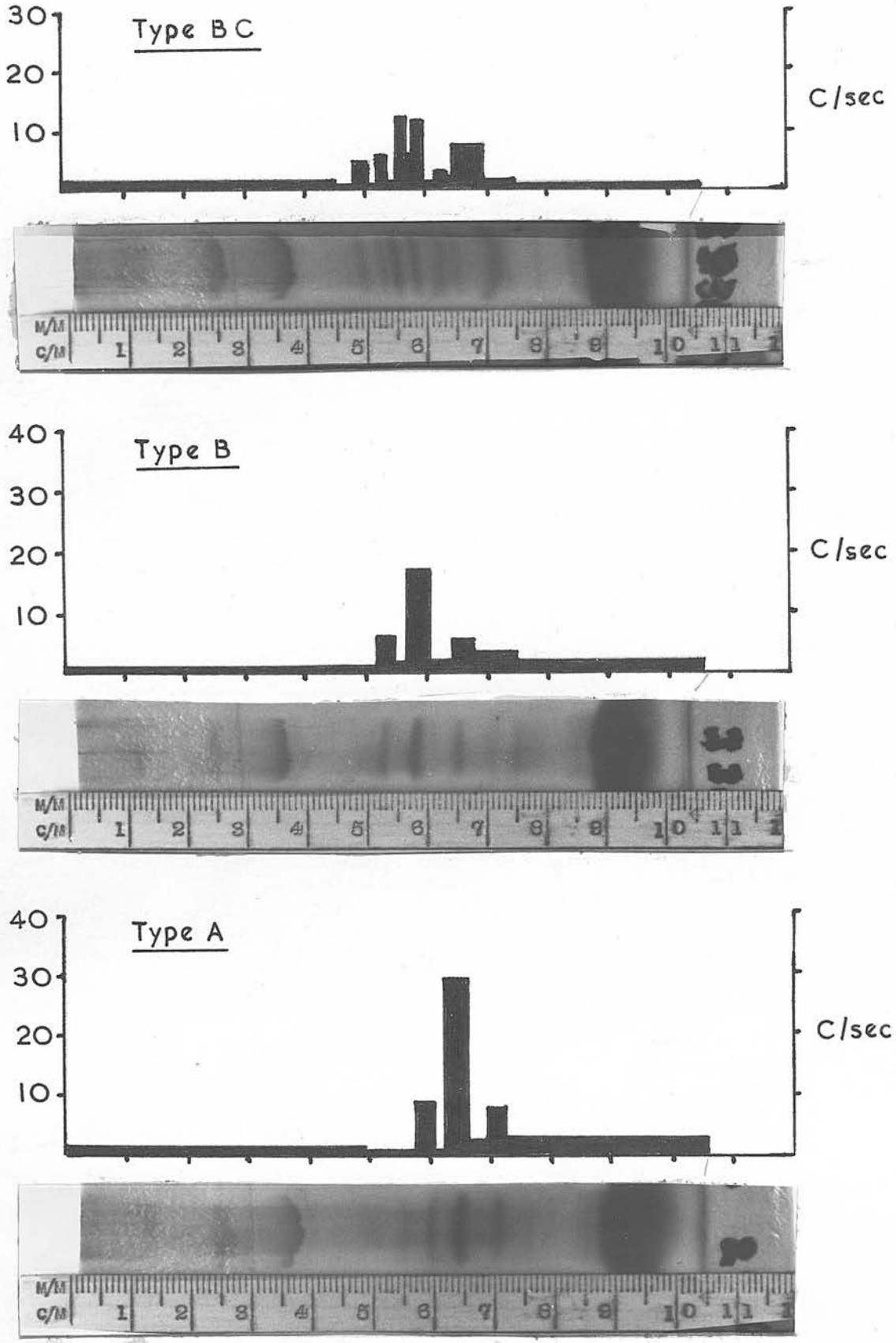
50 /

50 millimicrocuries of Fe^{59}. Electrophoretic conditions were as described previously, only the brown line was allowed to travel 10.5 cms. from the insert line to allow greater separation of the bands. The gel was split, stained and allowed to clear in washing solution. The top half of the gel was left in washing solution, and the bottom half taken out and divided into longitudinal strips, so that each serum electropherogram was separated from the other. Each strip was then placed alongside the millimetre scale of a ruler, and very carefully divided up into sections, the exact distance from the insert line being noted for each section. Then each section was placed in a small test-tube and placed in the well chamber of a sodium iodide crystal scintillation counter. Background counts were taken at the beginning and end of each run. Radioactivity counts for 100 seconds were taken for each piece of gel. The counts per second were then calculated and background counts deducted from each gel value. The remaining half of the gel was then taken out of the washing solution, and a millimetre scale placed alongside each electropherogram and photographed in situ. In the illustration in Figure 19, the radioactivity for each section of the top half of the gel is plotted against the bottom half enlarged to scale. Due to the washing solution, the gels have shrunk slightly, and are not quite to scale, but alignment is sufficiently accurate to establish, that each beta-globulin band within the different types is a transferrin. The intensity of the staining of the major bands, also indicates the point of greatest radioactivity, or binding of the Fe^{59}.

(d) Inheritance of pig transferrins: In Table 41 the distribution /

Fig. 19.

Labelling serum beta-globulins with Fe^{59}



distribution of offspring from all the different matings collected within the same families studied for red cell antigens are shown. Matings between all types were not available within this population, especially any involving type C transferrin. This type has a low frequency, and did not appear within these families.

Table 41

Segregation of transferrin types among offspring of different matings

Mating type		Segregation of offspring			Total	d.f.	χ^2
♂	♀	A	AB	B			
AB	A	3	6		9	1	0.1
AB	AB	3	3	3	9	2	0.55
AB	B		2	3	5	1	0.2
B	A		4		4		
B	AB		18	22	40	1	0.4
B	B			172	172		

The segregation of offspring are in accord with the theory of simple mendelian inheritance, and the genetic hypothesis is that types A and B represent the products of alleles at a single locus. Their genes may therefore be designated T_f^A and T_f^B. Although the C-type was not involved in the mating data, there can be little doubt that it is the product of a third allele T_f^C at the same locus.

(e) Gene frequencies The frequency of these alleles were calculated in the Large White and Landrace breeds from samples obtained from pigs entering the National Pig Progeny Testing Station. The relative frequencies are compared in Table 42.

Table 42 /

Table 42

Frequency of three alleles at the T_f locus in two breeds of pigs

T_f allele	Breed and No. tested	
	Large White (113)	Landrace (83)
A	0.389	0
B	0.611	0.976
C	0	0.024

The A transferrin was completely absent in the Landrace breed, also the C was absent in the Large Whites. It is also of interest that the C transferrin was found in two animals of the Wessex breed, and the BC types shown in Photograph No. 3 were from these two animals.

2. Pig Serum Haptoglobins Proteins migrating with the α_2-globulins of human sera and capable of binding haemoglobin were first seen on paper electrophoresis by Polonovski and Jayle (1940). They called these proteins haptoglobins. In 1955, Smithies using his starch gel electrophoresis method observed three different types occurring in normal human sera. The results of family studies indicated that these differences were under the simple genetic control, of two autosomal alleles (Smithies and Walker, 1955). Later, Connell and Smithies (1959) described a fourth phenotype called haptoglobin type 2-1 (modified), and its inheritance was later described in one family by Smithies and Hiller (1959).

In 1961, Kristjansson using the starch gel technique presented data to show that six haptoglobin phenotypes occurred in pig sera, and were under the control of alleles called Hp^1, Hp^2 and Hp^3 /

Hp^3. To establish this report and investigate the possibility of further differences in pig haptoglobins, the starch gel method described earlier was adapted for the following investigation.

(a) Method: The electrophoresis technique is as described, only the bottom half of the gel was stained with a benzidine stain. This stain consisted of 0.2 gms. of benzidine in a mixture of 0.5 ml. of glacial acetic acid in 100 mls. of water. On dissolving the benzidine, 0.2 mls. of 30% hydrogen peroxide was added (Hermans et al., 1960). As the haemoglobin-haptoglobin complex has a high peroxidase activity, this acted on the hydrogen peroxide and oxidised the benzidine to give a blue staining reaction, when the gel was immersed in the stain. Staining developed within seconds, but required at least 30 minutes for full development.

The procedure adopted was to stain several samples of serum obtained at different times from the same animal. This meant that some were haemolysed and others were not. Samples completely free of haemoglobin did not stain with benzidine. This procedure was repeated for several animals until a wide range of types could be selected. Then solutions of haemoglobin in water were made-up in varying concentrations from the washed cells of one ml. of fresh whole blood. The concentration of haemoglobin in these solutions ranged fromapproximately 6 to 200 mgms. per ml. A volume of 0.05 ml. of each haemoglobin solution was mixed with 0.1 ml. samples of a serum. These samples were left for a minimum period of 30 minutes to allow binding to take place, then each was placed in a starch gel. The top half of the gel was stained with nigrosin and the bottom with benzidine as described.

(b) /

(b) Results: At first it appeared from the random selection of serum, that many haptoglobin types were present, which were difficult to interpret. It was also observed, however, that several distinct variations occurred within the serum of some individuals. In an attempt to clarify the picture, the transferrin region was taken as a point of reference. By doing this it was possible to divide the occurrence of types into three zones on the gel. The types within the first zone occurred between the pre-albumin and the transferrins, and only appeared when a haemolysed sample of serum was used. No variation occurred within samples from an individual for this zone. The second zone occurred immediately in the region of the transferrins, and because of the width of bands and depth of staining with nigrosin, these types often masked some of the transferrin bands. Variations within samples from one individual were very distinct for this region. Finally, the third zone was taken between the transferrins and the insert line. The types occurring within this region were often very faint, and within samples from any individual pig could be haphazard in occurrence.

As the heaviest staining bands were in the second zone, this area was investigated first. Within this zone it appeared at first, that there were three phenotypes, that is a two band system, one band and no bands. The latter being comparable to the anhaptoglobinaemic type found in man, Smithies (1955); Allison et al. (1958) and Blumberg (1960). Further investigation showed that by adding varying amounts of a pig's own fresh haemoglobin to an unhaemolysed sample of it's own serum, that the binding capacity of /

of the haptoglobin moved through three phases as follows:-

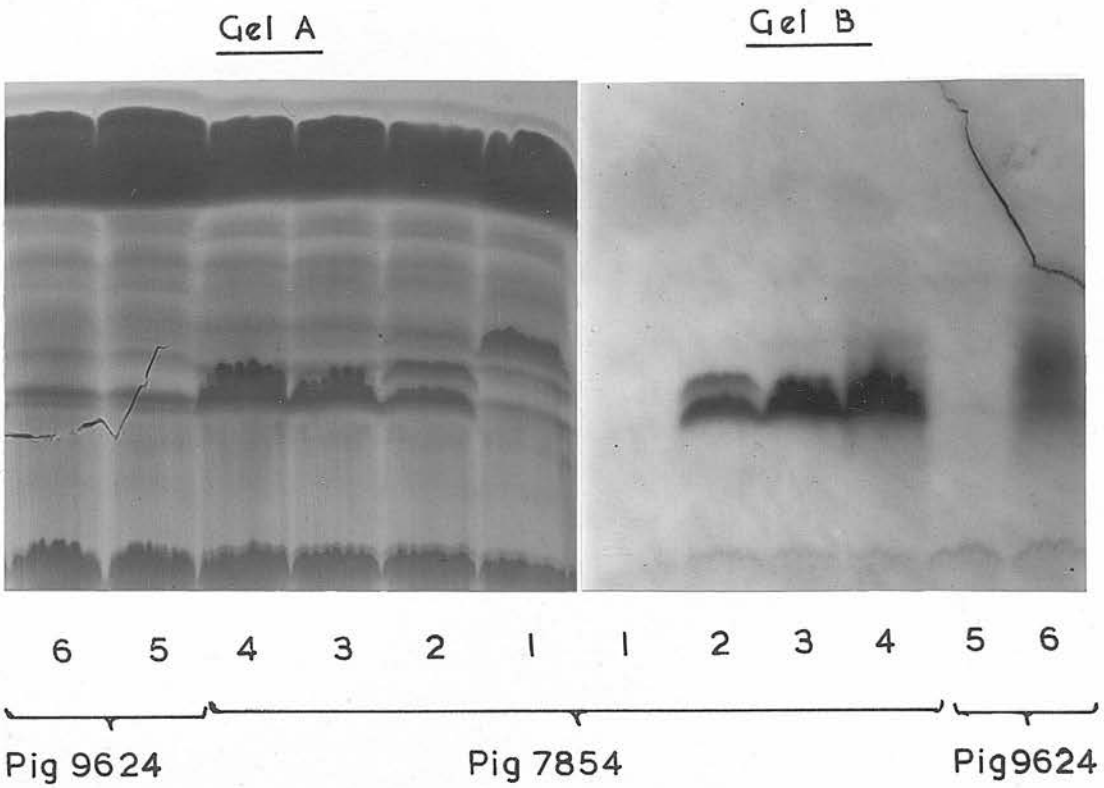
1. Phase I: The unbound haptoglobin in an unhaemolysed sample appeared as a thick band in an advanced position in front of the transferrin bands. It was stainable with nigrosin but not with benzidine.

2. Phase II: A partially bound haptoglobin/haemoglobin complex appeared in the middle of the transferrin bands. It could appear with 1 above, or 3 below, or all three could occur together, all of which were stainable with nigrosin. This band was stainable with benzidine.

3. Phase III: A completely bound haptoglobin/haemoglobin complex which appeared as a very thick, dark staining band in a backward position at the end of the transferrin region. This band could appear with 1 and 2 above, or with 2, or completely alone when fully saturated with haemoglobin. It was stainable with nigrosin and benzidine.

These three phases are illustrated in Photograph No. 4, which shows two halves of the same gel. Gel A is stained for proteins with nigrosin, and gel B stained with benzidine for haemoglobin. The No. 1 position on Gel A is comparable to the No. 1 position in Gel B. In this position, pig No. 7854 had no haemoglobin present in the serum sample, and the unbound fraction of haptoglobin is shown in Phase I in Gel A. In positions 2, 3 and 4, increasing amounts of haemoglobin from pig 7854 were added to it's own serum. With the addition of haemoglobin, the mobility of the haptoglobin changed and is shown as a two band system becoming a one band system in Gel B, and a three band system becoming a one band system in /

Photograph No.4.



in gel A.

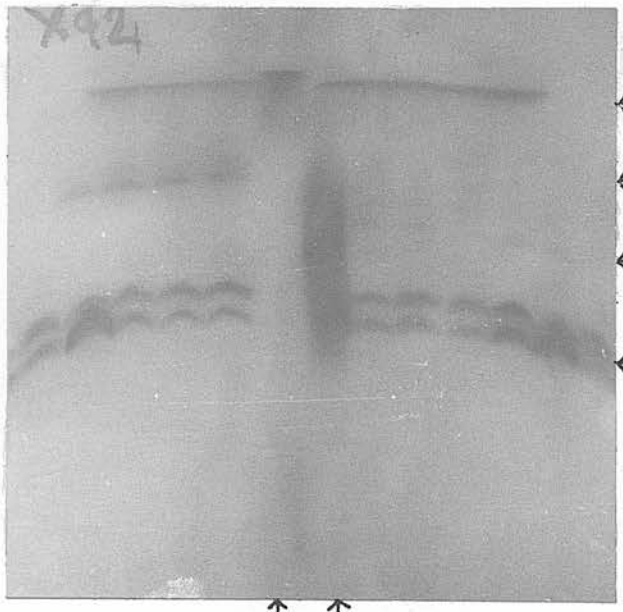
In position No. 5, pig 9624 is an example of an anhaptoglobinaemic type with no bands present in either gel. On adding the pig's own fresh haemoglobin in excess to the same serum sample, it is shown in position No. 6, that there is still no formation of bands. Therefore no binding of the haemoglobin took place. Several pigs showed only a very faint haptoglobin band even after complete saturation with haemoglobin. The location of the haemoglobin stained with benzidine as shown in position No. 6 is identical to that occupied by free haemoglobin, when subjected to starch gel electrophoresis. This is illustrated in photograph No. 5 mentioned later in the text. The hypothesis is therefore advanced, that all the phases mentioned above involve one protein, which appears to be a true haptoglobin in that it binds haemoglobin. The various phases are stages in the binding of this protein to the haemoglobin molecule.

It will also be seen from Gel B, that despite the addition of fresh haemoglobin, no lines appeared in front of zone No. 2. But in other haemolysed serum samples from these same pigs, it was observed that definite staining occurred with benzidine in the albumin region, also bands appeared between the albumin and zone No. 2. This implied that some breakdown had occurred in the haemoglobin contained in the haemolysed samples during storage. To investigate further, fresh haemoglobin was converted to alkaline haematin, and added to the unhaemolysed samples of the same sera. Electrophoresis revealed bands identical to those shown by the haemolysed samples. Also, the bands between the albumin and the true /

true haptoglobins in zone No. 2 appeared to resemble the haptoglobin phenotypes mentioned by Kristjansson (1961), and corresponded to the haem-protein bands of the fast α_2-globulin in human sera mentioned by Allison (1957). The occurrence of methaemalbumin has also been reported in human sera by Hensley and Blackburn (1952) and Allison (1957), and would appear similar to the albumin staining observed when haematin is added to pig serum. In photograph No. 5, the position of methaemalbumin, haem-binding proteins, free haematin and free haemoglobin are shown in relation to the true haptoglobin of zone No. 2. A further experiment with pig 7854 serum is shown in photograph No. 6, which also indicates the independence of haptoglobins from haem-binding proteins. In position 1 on this photograph, there is the normal unhaemolysed sample of this serum, and in positions 2, 3 and 4, increasing concentrations of fresh haemoglobin have been added to the same sample. The position of the haptoglobin protein is altered, but no methaemalbumin or haem-binding protein is visible. On adding alkaline haematin in increasing concentration to the unhaemolysed sample in position 6 and 7, no alteration is apparent in the haptoglobin protein, but methaemalbumin and haem-binding proteins become visible. The adding of fresh haemoglobin to a sample already containing haematin in position 8, immediately affects the haptoglobin bands, but there is no change in the other fractions. This illustrates that only haptoglobins bind fresh haemoglobin, and only haem-binding proteins and albumin bind haematin.

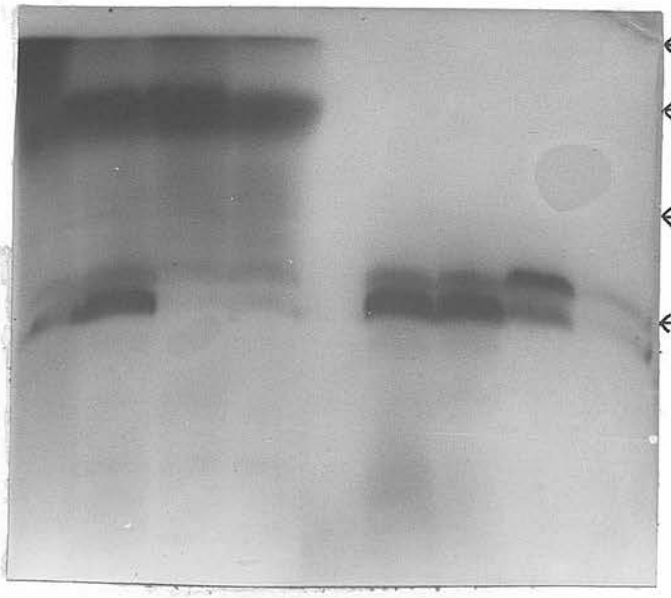
It should be emphasised that this experiment can only be executed successfully, if the haemoglobin is taken from fresh whole blood. /

Photograph No.5.



Photograph No.6.

Benzidine stain



Pig 7854

blood. Even storage in a refrigerator for 24 hours will not prevent breakdown in the haemoglobin, and binding to albumin and haem-binding proteins will occur, if 24 hours old haemoglobin is added to an unhaemolysed sample of serum.

It is proposed to discuss the system of haem-binding proteins, or globulins in the next section, as a separate entity from haptoglobins.

The final zone No. 3 has still to be fully investigated, although photograph No. 6 presents some explanation for some of the bands which occur in this area. It will be noted, that in position No. 4 and slightly in 3, the appearance of a dark band approximately 1.5 cms. from the insert line. This is absent in the samples containing haematin, but reappears in position No. 8, when fresh haemoglobin is present. Possibly this is another haptoglobin, but it only appears when the haptoglobin of zone No. 2 is nearly saturated, because there is no suggestion of this band in positions 1 and 2, where the haptoglobin was not saturated. Slightly ahead of this other haptoglobin, there is a band appearing only in positions 6, 7 and 8, and again this may be another haematin-binding protein, because it does not appear in positions 1 to 4. Ahead of this band there is another faint band, which is slightly in front of the slow α_2-globulins. This is common to both haemoglobin and haematin samples. A further band is often seen just behind the main saturated haptoglobin. Although not present in this gel, it appears to be associated with complete saturation of this haptoglobin.

(c) /

(c) Inheritance of haptoglobins: In an attempt to find if the genes controlling the presence or absence of haptoglobin segregated in a mendelian manner, the distribution of offspring from matings involving these types were calculated. The results are shown in Table 43.

Table 43

Mating type		Segregation of offspring		Total
♂	♀	+	-	
-	-	6	0	6
-	+	58	5	63
+	+	171	0	171

The segregation is not mendelian (See 'Discussion')

3. Haem-binding proteins (globulins) in pig sera The method adopted to clearly identify the different types of these proteins was different from that described for transferrins. In attempting to demonstrate polymorphism in the post-albumins of pig sera, the method used was found to give very clear haem-binding protein bands. The following method was therefore adopted routinely for their demonstration.

(a) Methods: The electrode buffer was similar to that described for transferrins, but the gel buffer was composed of 0.103 M. tris and 0.007 citric acid. A final pH of 8.2 was obtained by mixing 125 mls. of tris solution with 375 mls. of citric acid solution. At this pH, the albumin was separated further from the transferrins, and was concentrated into a smaller band, thus leaving a clear field for the haem-binding proteins. However, the increase in molarity resulted in an increase in the temperature within /

within the gel, and at room temperature under increased voltage, the ambient was as high as 55 to 60°C. To obviate the possibility of denaturation, the complete electrophoresis run was carried out in a refrigerator at +4°C. This kept the ambient within the gel down to 30 to 35°C.

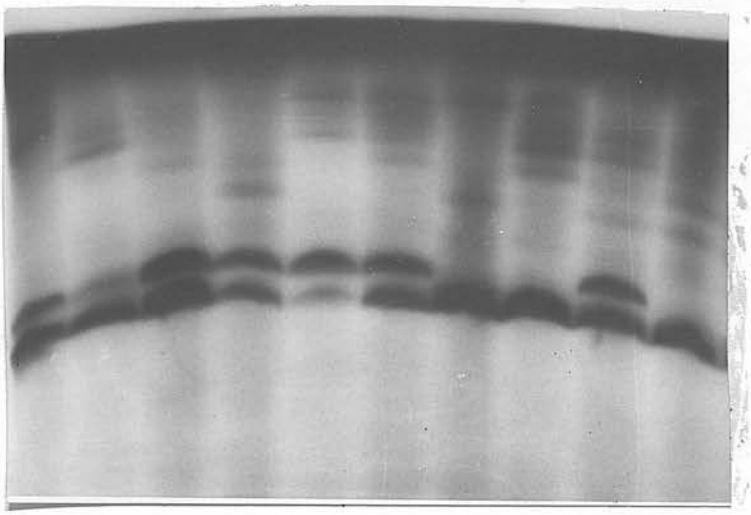
All serum samples which were not already haemolysed were mixed with alkaline haematin. The haematin was obtained by boiling the equivalent of 200 mgms. of haemoglobin solution in 1 ml. of water with 5 ml. of 0.1 N sodium hydroxide for five minutes. When cool, 0.05 ml. of this solution was mixed with 0.1 ml. of serum and left for 24 hours at +4°C.

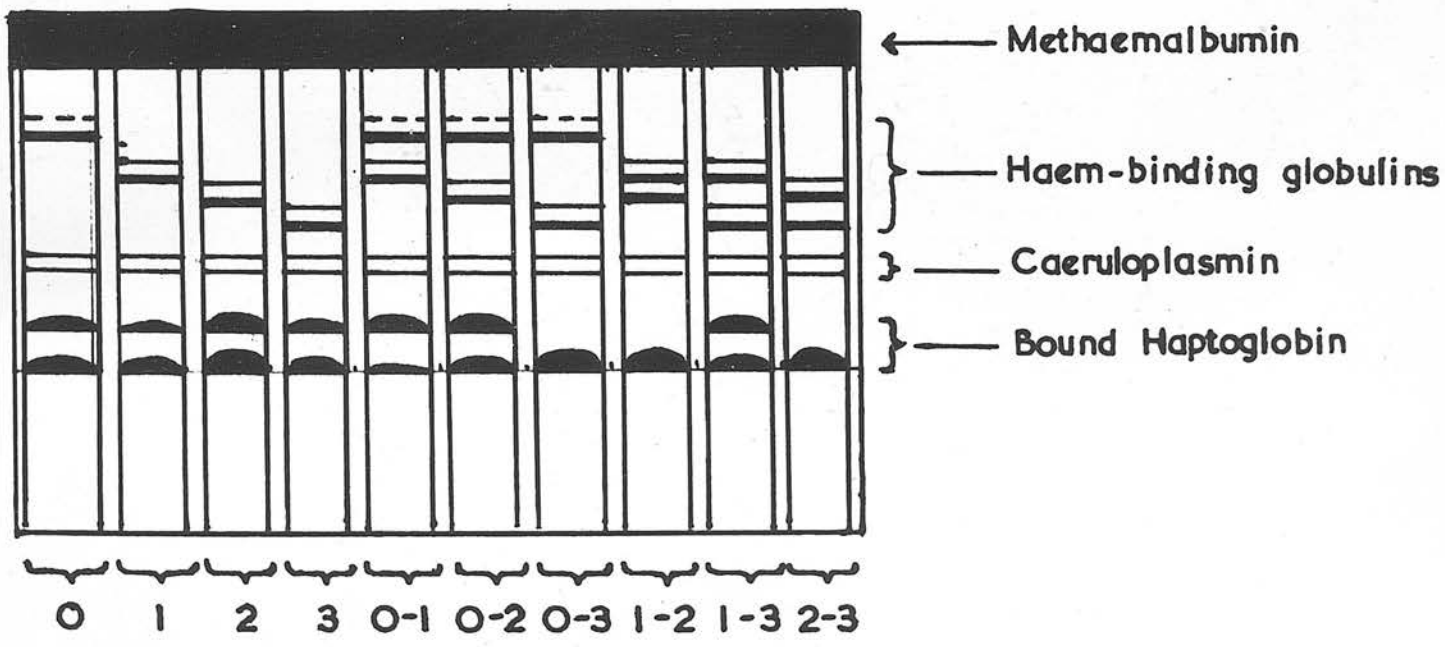
Electrophoresis was similar to that described for transferrins, except that after removal of the inserts, the voltage gradient was increased from six volts per centimetre to 13 to 15 volts per centimetre. The brown line was allowed to move 11 cms. from the insert line before electrophoresis was stopped.

The staining procedure for the gel was similar to that described previously.

(b) Results: As the methaemalbumin zone remained constant, only the other haem-binding proteins showed variations, and as they occur behind the albumin with the globulins, it is proposed that they be referred to as haem-binding globulins. A total of ten phenotypes were detectable and each type isillustrated in photograph No. 7, with the aid of a schematic drawing of this photograph. The homozygote types appeared as two bands and are referred to as 0, 1, 2 and 3 haem-binding globulins. The various combinations of these types /

Photograph No.7.





Haem-binding globulin types

types in the heterozygote form, as demonstrated in the photograph, appear as three or four bands depending on the combination. With benzidine stain, it is not completely satisfactory from a photographic point of view, but all the bands shown in the schematic drawing have been observed at one time or other during the course of this investigation. All the bands cannot be shown completely on one gel, because their demonstration depends on the relative haemolysed state of the serum of the different types.

It is proposed in this thesis, that the above phenotypes are controlled by four alleles at the same locus. These alleles are referred to as H_g^0, H_g^1, H_g^2 and H_g^3. Their existence is demonstrated in the segregation results shown for the different phenotype matings within the parent population described elsewhere. These results with chi-squared values for deviations from expected are shown in Table 44.

Table 44 /

Table 44

Segregation of haem-binding globulins among offspring of different matings

Mating type		Distribution of offspring							Total	d.f.	χ^2
♂	♀	0	03	1	12	13	23	3			
03	03	2	3						5		
03	33		3					4	7	1	0.14
1	1			116					116		
1	13			8		8			16	1	0
1	3					9			9		
13	1			10		7			17	1	0.52
13	13			4		1		2	7	2	4.3
23	1				2				2		
23	13				3	3	1	0	7	3	3.85
23	3						1	3	4		
3	1					10			10		
3	13					17		11	28	1	1.28
3	23						8	3	11	1	2.2

None of the deviations from expected are significant, and the segregation results are in agreement with the existence of four alleles at a single locus, referred to as the haem-binding globulin locus.

4. Pig serum caeruloplasmin The electrophoretic analysis by Uriel (1958) first indicated that human plasma copper protein is heterogenous with respect to its molecular composition. Morell and Scheinberg (1960) then purified by chromotography human caeruloplasmin subfractions from the plasma of 9109 donors. Electrophoresis of these subfractions showed at least four different caeruloplasmins. Then McAlister et al. (1961) presented evidence for multiple caeruloplasmin components in human sera. Using vertical starch gel electrophoresis, /

electrophoresis, five electrophoretically distinct proteins having one or more characteristic properties of caeruloplasmin were isolated.

It was first shown by Holmberg and Laurell (1951), that caeruloplasmin could oxidise a number of substances, the best substrate being p-phenylenediamine (PPD). Using the PPD oxidase acitivity as a measure of the caeruloplasmin in sera of different species, it was shown by McCosker (1961), that pig sera has twice the activity of human sera, and four times that of other domestic species.

To investigate the possibility of caeruloplasmin variants within the sera from different pigs, the following method was applied:-

(a) Method: Electrophoresis was exactly similar to that described for transferrins, except that the brown line was allowed to move 11 cms. from the insert line before electrophoresis was stopped. Two techniques for the detection of caeruloplasmin were tried. In the first, the bottom layer of the cut gel was stained according to the method described by Morell and Scheinberg (1960) with modifications. In their method, paper strips saturated in a solution of 1.0 M sodium acetate buffer (pH 5) containing 0.5% p-phenylenediamine dihydrochloride were pressed on the cut surface of the gel for five minutes, and then removed. The gel was then placed in a closed, moist chamber and allowed to stand for one to three hours at room temperature until dark purple bands developed.

In the modified technique for pig sera, the same solution of acetate buffer and PPD was used. Instead of using paper strips, however, /

however, it was found that total immersion of the cut gel in the buffer at 37°C. gave better results. The optimum time for clearly distinguishable caeruloplasmin proteins was approximately 45 to 60 minutes after incubation. At this stage the gel was photographed, and the different types classified. After one hour of incubation the oxidase activity of the copper proteins began to give a diffuse purple staining reaction with the PPD, and obliterated any bands which were previously discernible.

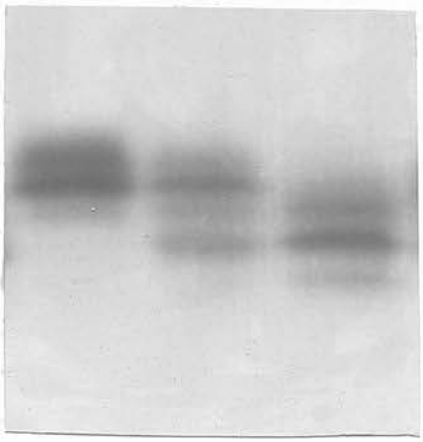
In the second method, an attempt was made to precipitate the bound copper as copper rubeanate within the starch gel using a solution of 0.1% rubeanic acid in 70% ethanol as described by Uzman (1956). This method was not successful.

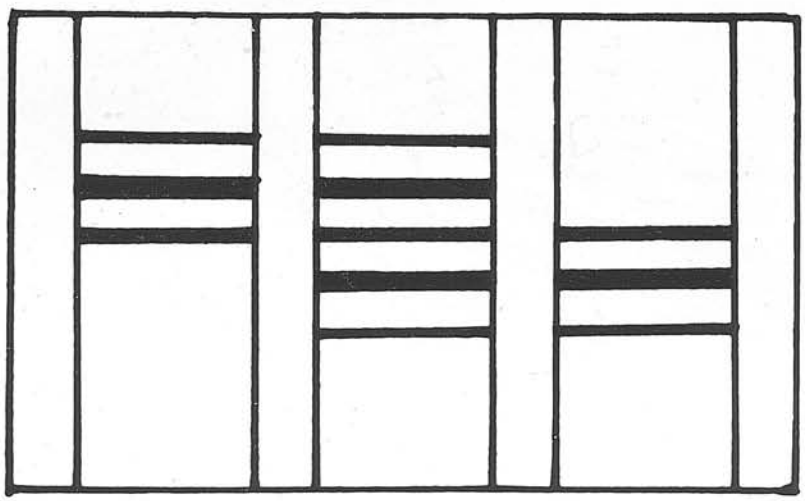
(b) Results: A total of three phenotypes were detected during the investigation, and each type is illustrated in photograph No. 8. The schematic drawing of this photograph is also shown to indicate the interpretation of these types. The homozygotes appear as three bands with the middle band showing the darker staining reaction. Two homozygous types are recognised and classified as type A and B with the A-type being the faster migrating protein. The combination of these two types in the heterozygote gives a phenotype showing five bands, which is also illustrated in the above photograph.

In relation to the other proteins, the caeruloplasmins are slightly slower in mobility, than the transferrins. At pH 7.6 they appear as three bands in the homozygote form as described above, and are in the same zone as the transferrins. At pH 8.2, however, they appear as two bands and are behind the transferrins.

It /

Photograph No.8.





It should be noted, that because of their oxidising ability they are stained slightly with benzidine, and therefore should not be confused with haem-binding globulins. At a gel pH of 8.2, they appear as faint lines between type-3 haem-binding globulin and the saturated haptoglobin.

It is proposed that the three phenotypes described above are controlled by two allelic genes at a single locus. Their alleles have been called Cu^A and Cu^B. To test the simple genetic hypothesis of two alleles, the results of tests of sera from offspring from different matings of parents are shown in Table 45. All mating types were not available, because of the low frequency of type A caeruloplasmin in the parent population. The latter is the same population as described in other studies.

Table 45

Distribution of caeruloplasmin types among offspring of different matings

Mating type ♂ × ♀	Distribution of offspring A	AB	B	Total	d.f.	χ^2
AB × AB	2	3	0	5	2	0.2
AB × B		6	1	7	1	3.6
B × AB		3	3	6	1	0
B × B			223	223		

The results in Table 45 are in accord with the genetic hypothesis of two alleles at a single locus.

(c) Gene frequencies: The frequency of these alleles were calculated in the Large White and Landrace breeds from the same samples used for the transferrin gene frequency study. The results are /

are shown in Table 46.

Table 46

Frequency of two alleles at the Cu-locus in two breeds of pigs

Cu alleles	Breed and No. tested	
	Large White (113)	Landrace (83)
A	0	0.054
B	1.0	0.946

The A-type caeruloplasmin was completely absent in the Large White breed, and of relatively low frequency in the Landrace breed.

5. Pig serum amylases During the process evolved for the staining of caeruloplasmin with p-phenylenediamine, the gel was incubated at 37°C. for nearly one hour. This appeared to activate an enzyme present in the serum, which digested the starch. The areas digested appeared as collapsed areas in gels which had been incubated and then left to dry at room temperature for 24 hours. These digested areas showed variation between individuals. As the enzyme involved, digested starch, it was believed that it might be amylase. The following methods were used to investigate this possibility.

(a) Methods: The electrophoresis technique described for the transferrins was used to separate serum proteins. One layer of the cut gel was incubated at 37°C. in acetate buffer (pH 5) without PPD being added. It was left for 24 hours in the buffer at this temperature. Then it was removed from the buffer, washed with distilled /

distilled water and heated very gently in a solution of Benedict's reagent in a tray. As amylase is capable of hydrolysing starch to maltose, which is a disaccharide, this in turn can reduce an alkaline copper solution (i.e. Benedict's reagent) to cuprous oxide. On boiling the gel, a green colouration appeared in the gel from the insert line to around the areas occupied by the enzyme, and within these areas a light orange colour developed. Beyond the zone occupied by the enzyme, the gel did not change colour. The appearance of precipitates of cuprous oxide indicated the presence of a reducing sugar. To demonstrate the hydrolysis of the starch, a gel was sliced transversely into sections immediately after electrophoresis, then each section of gel was incubated at 37°C. in test tubes containing normal saline. A drop of dilute iodine solution was then added to each tube after five hours. The presence of starch was indicated by a blue colouration. In those sections of the gel believed to contain amylase, there was no colour reaction, but other sections of the gel beyond the amylase zone gave a blue colouration. This confirmed that the starch in the areas occupied by the enzyme was hydrolysed.

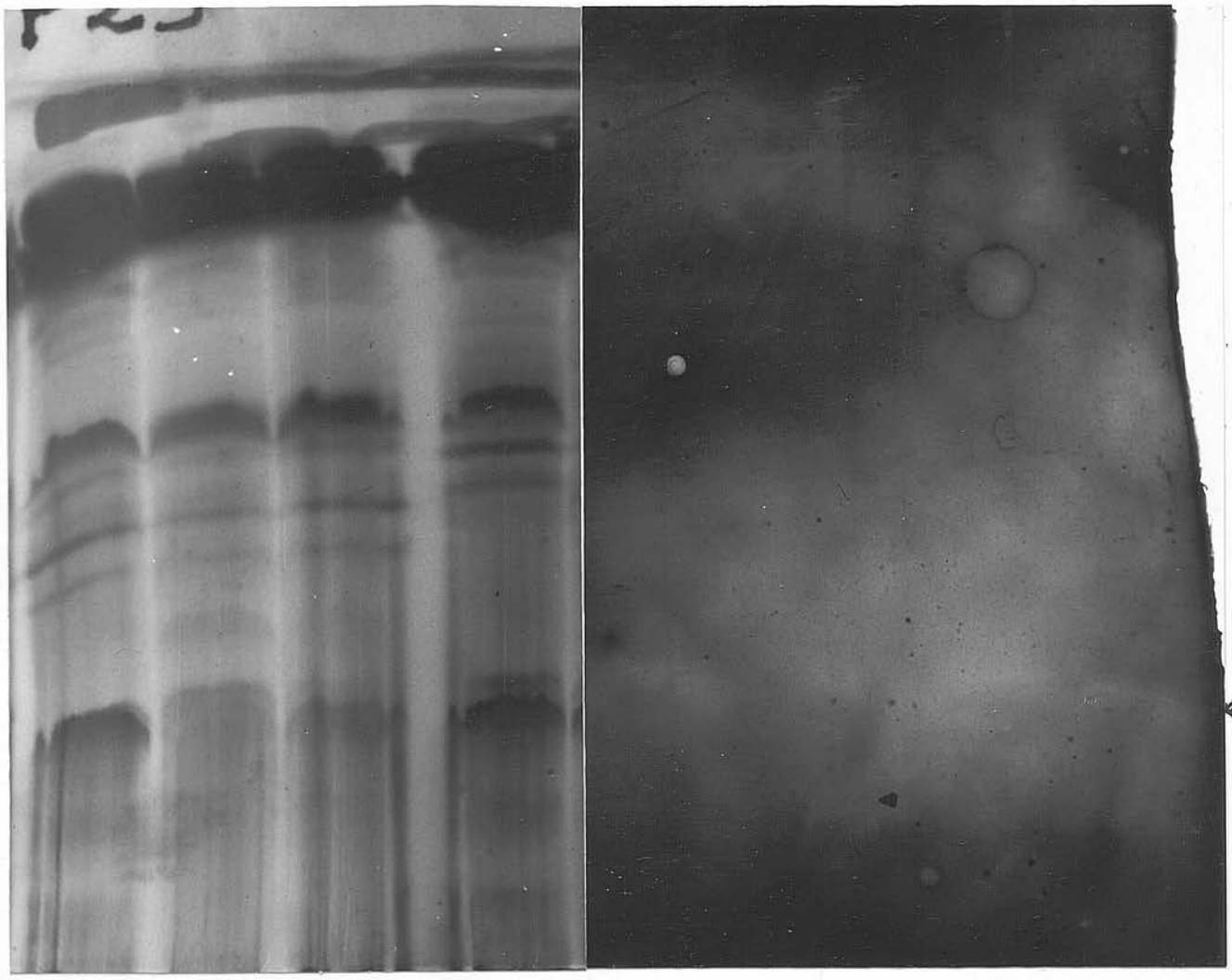
It is also known, that the activity of amylase is inhibited by heavy metals and urea (Long, 1961). To investigate this fact, 0.1 ml. serum samples from one animal were incubated with 0.05 ml. of a 1% and 5% solution of urea at room temperature for 15 hours. These serum samples along with a normal sample from the same animal were run under standard electrophoresis conditions. One layer of the cut gel was stained with nigrosin, and the other incubated in acetate buffer at 37°C. for 24 hours. The latter was then /

then boiled for five minutes in a 10% mixture of glacial acetic acid and glycerin in distilled water until the gel cleared. It was allowed to cool and then dry out on a glass plate at room temperature. In photograph No. 9 it can be seen that a definite digested, collapsed area appeared in the gel for the normal sample, and a less definite area also appeared for the sample containing 1% urea. No collapsed area could be seen for the sample incubated with 5% urea. The other gel stained with nigrosin did not reveal any absence of protein fractions through precipitation by the urea. Therefore, it appeared that the presence of urea in sufficient concentration inhibited the activity of the enzyme. The enzyme in question therefore had properties similar to amylase.

In the routine investigation of pig sera for amylase variants, the slice of gel used for caeruloplasmin identification was also used for amylases, and left in the buffer at 37°C. for several more hours. After approximately three hours the gel and buffer were taken from the incubator and left to stand at room temperature overnight. After 15 hours at room temperature, the gel was removed from the buffer, and boiled for five minutes until clear in a mixture of acetic acid, glycerin and water. When cool the gel was placed on a glass plate and allowed to dry at room temperature. The areas occupied by amylase appeared as discrete indentations in the substance of the gel, and were easily identified.

(b) Results: The most frequent amylase band identified appeared in the region of the slow α_2-globulins. It is referred to as type-2 amylase. In some pigs, however, another band appeared between /

Photograph No.9.



between this type and the saturated haptoglobin, and it is called type-1 amylase. A further band appeared in other individuals between the No. 2 type and the insert line. This is referred to as type-3 amylase. These bands could appear either alone or in pairs in any individual serum. Only four phenotypes have been identified so far, and they are illustrated in photograph No. 10, and the schematic drawing of this photograph. The four phenotypes are types 1, 1-2, 2 and 2-3. No individuals of type 1-3 and 3 have been found, but are postulated.

In the distribution of offspring from different phenotype matings within the parent population, the existence of three alleles at a single locus is proposed. The results are shown in Table 47.

Table 47

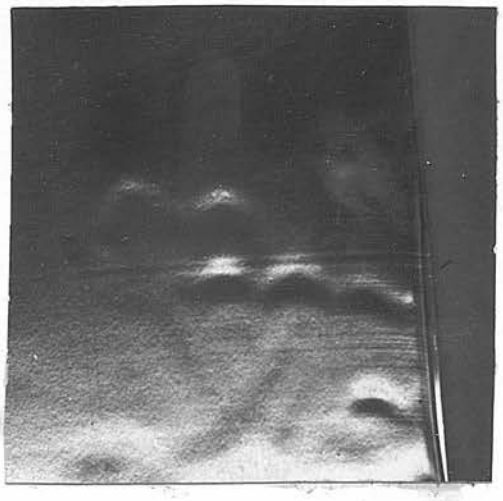
Distribution of amylase types among offspring of different phenotype matings

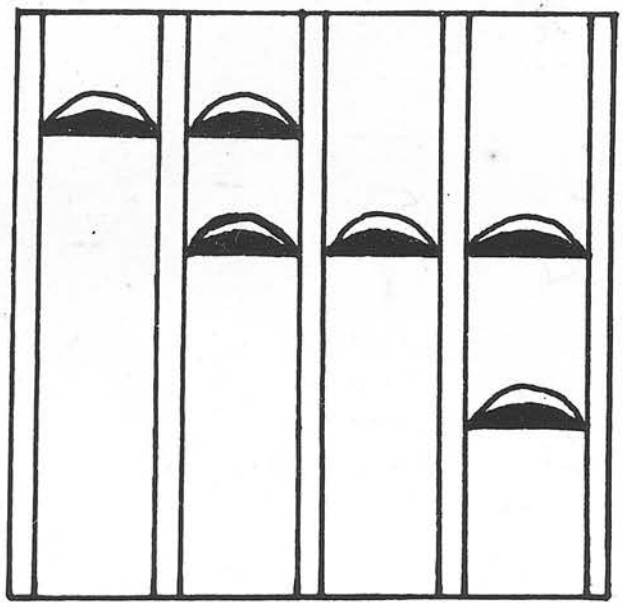
Mating type	Distribution of offspring			Total	d.f.	χ^2
♂ ♀	12	2	23			
2 × 12	3	4	0	7	1	1.42
2 × 2	0	204	0	204		
2 × 23	0	15	14	29	1	0.04

Unlike the other protein fractions discussed in this section, the phenotype representations of the gene controlling their synthesis is a single band in the homozygote and a double band in the heterozygote. The segregation results are in agreement with the existence of three allelic genes at a single locus. It is proposed that these genes be called Am^1, Am^2 and Am^3.

(c) /

Photograph No.10.





1 1-2 2 2-3

Amylase types

(c) Gene frequencies: Frequencies of these alleles were calculated in the Large White and Landrace breeds. The results are given in Table 48.

Table 48

Frequency of three alleles at the Am-locus in two breeds of pigs

Am alleles	Breed and No. tested	
	Large White (113)	Landrace (83)
1	0.07	0.2
2	0.93	0.8
3	0	0

The No. 1 amylase appeared more frequently in the Landrace breed, but No. 3 was absent in both breeds. In the gene pool group of animals in the parent population, No. 3-type amylase was identified, therefore it seems probable that it was introduced by either the Wessex or Tamworth breeds.

TESTS FOR ASSOCIATION BETWEEN DIFFERENT SERUM TYPES:-

To test the independence of the genes controlling the various types within the transferrin, haptoglobin, haem-binding globulin, caeruloplasmin and amylase loci, 2 x n contingency tables were calculated according to Fisher (1950). The parent population was again treated as random for the following tests. Figures for expected values are in parenthesis. The chi-squared test has not been applied, because many of the values are less than five in number, but it will be observed that agreement between observed and expected is extremely good.

		2. Haptoglobins		Totals
		+	-	
1. Transferrins	AA	3(2.8)	-	3
	AB	12(11.2)	-	12
	BB	43(44)	4(3)	47
Totals		58	4	62

		3. Haem-binding globulins					Totals
		03	11	13	23	33	
	AA		3(1.89)				3
1. Transferrins	AB		9(7.5)	2(2.3)		1(1.4)	12
	BB	2(1.5)	27(29.6)	10(9.1)	2(1.5)	6(5.3)	47
Totals		2	39	12	2	7	62

		4. Caeruloplasmins		Totals
		AB	BB	
	AA		3(2.9)	3
1. Transferrins	AB		12(11.2)	12
	BB	3(2.3)	44(44.7)	47
Totals		3	59	62

		5. Amylases			Totals
		12	22	23	
	AA		3(2.8)		3
1. Transferrins	AB		12(11)		12
	BB	1(0.76)	42(43.2)	4(3)	47
Totals		1	57	4	62

		3. Haem-binding globulins					Totals
		03	11	13	23	33	
2. Haptoglobins	+	2(1.9)	37(36.5)	11(11.2)	2(1.9)	6(6.5)	58
	-		2(2.5)	1(0.8)		1(0.5)	4
Totals		2	39	12	2	7	62

		4. Caeruloplasmins		Totals
		12	22	
2. Haptoglobins	+	3	55	58
	-		4	4
Totals		3	59	62

$\chi^2 = 2.8$ for 1 d.f.
p = Between 0.1 and 0.05

		5. Amylases			Totals
		12	22	23	
2. Haptoglobins	+	1(0.9)	53(53.3)	4(3.7)	58
	-		4(3.7)		4
Totals		1	57	4	62

		3. Haem-binding globulins					Totals
		03	11	13	23	33	
4. Caeruloplasmins	AB	1(0.1)	1(1.9)			1(0.3)	3
	BB	1(1.9)	38(37.1)	12(11.4)	2(1.9)	6(6.7)	59
Totals		2	39	12	2	7	62

		3. Haem-binding globulins					Totals
		03	11	13	23	33	
5. Amylases	12		1(0.6)				1
	22	2(1.8)	36(36)	10(11)	2(1.8)	7(6.4)	57
	23		2(2.5)	2(0.8)			4
Totals		2	39	12	2	7	62

		5. Amylases			Totals
		12	22	23	
4. Caeruloplasmins	AB		3(2.8)		3
	BB	1(0.9)	54(54.2)	4(3.8)	59
Totals		1	57	4	62

In none of the tables considered is there a deviation from expected, which is significant. It can therefore be assumed that the various genes within the five serum protein loci are statistically independent of one another for this population.

DISCUSSION ON RESULTS:-

The observations in this study of biochemical variation within pig blood, as confined to the electrolyte constituents and serum proteins, supports and extends the knowledge previously found in other species. Like man and the other domestic animals, the pig is no exception to the new biology of biochemical individuality. In this study alone, the pig has shown more individuality among serum proteins, than has been determined in many other species hitherto. Present observations indicate that this variance may be extended to other serum components, especially pre- and post-albumins, and other enzyme systems. In considering the results of the present investigation, several facts have emerged, which form the basis of this discussion.

It is apparent that pigs cannot be divided into distinct genetically determined groups on the basis of their blood potassium and sodium content. Statistical evidence, however, indicates that variation does occur, and this variation may be familial. Whether this means that it is heritable in the sense that the expression of the genes controlling potassium, for instance, could be qualitative, or it is a maternal effect is speculative. One factor which could have a mediating effect is environment, and this can be dismissed considering that all the samples were obtained from animals kept in a constant environment. No family studies were made involving animals of particular potassium levels, because the variance within individuals, although not as high as that between, was not significantly different to give a clear distinction. It was noticeable, however, that the average red cell potassium value for two families could differ by as much as 10 mEq./litre, which is twice the standard /

standard deviation for the mean value in any breed. Also, individuals from two different families could differ by as much as 20 mEq./litre, whereas the deviation within each family was never as high. Careful selection of potassium types and interbreeding within types at two extreme levels of the normal distribution curve might create two distinct classes of pig for potassium values. Obviously the genetic basis for control of these different levels of potassium in the blood of pigs is more diverse than the single locus control found in sheep.

Some of the serum values in this study show good agreement with those published for thirteen adult pigs by Widdowson and McCance (1956b). For serum potassium, they quote a value of 6 m Eq./litre $\pm$ 0.6, and for serum sodium, a value of 144 m Eq./litre $\pm$ 4.7 is given. No whole blood values were calculated. In their other communication, McCance and Widdowson (1956a) give potassium values of 471 mgms./100 gms. of packed cells, and 182 m Eq./litre for cell water for nine adult pigs. The two values given were calculated by first weighing packed cells devoid of plasma, then lysing these cells in an equal volume of water and then estimating the potassium content in this cell water. As the volume of cells weighed is not stipulated in their data, there is no way of relating their estimations to those given in this dissertation.

The electrophoresis technique applied to starch gel as a supporting medium for the separation of serum proteins, has proved as in other species, to be a most rewarding technique for the demonstration of variants in pig sera. In the transferrin fraction studied three allelic genes, called T_f^A, T_f^B and T_f^C appear to be responsible /

responsible for six transferrin phenotypes called A, AB, AC, B, BC and C. The existence of two alleles at this locus has been reported independently by Ashton (1960) and Kristjansson (1960). It would appear from the description given in both papers, that these alleles are similar to the T_f^A and T_f^B reported here. Ashton reported that each allele gave rise to three zones in starch gel using phosphate buffer. These zones were divided into a faintly staining zone, followed by a moderately staining zone, and then by a more intensely staining zone. The pH of the gel buffer was exactly similar to that described for transferrins in this thesis. Kristjansson, on the other hand, using tris and dilute hydrochloric acid as a gel buffer was able to demonstrate two transferrin zones in starch representing one allele. Of these two zones one band was more intensely stained than the other. He did not give the pH of the gel buffer, but this picture is very similar to the change in the transferrin zones observed in the present study, when the gel buffer pH was increased to 8.2 for the haem-binding globulins. Is is therefore obvious that the gel buffer and ionic strength of this buffer can affect the change on the transferrin protein molecule quite considerably. Although molecular size plays an important role in separation by starch gel, any observation on differences within a component should be carefully related to the composition of the buffer solution used. The range of techniques in the application of electrophoresis to starch gel is almost as diverse as the serological techniques applied to red cell antigens. Without exact standardisation of techniques, the only alternative in assessing new variants reported by other workers, would be the exchange /

exchange of sera of known types. It is inevitable, however, that the techniques reported by some workers may have to be adopted by others before these variants become apparent.

The C transferrin described in this dissertation appears to be an additional type to those reported previously at the transferrin locus. The labelling of the three different types A, B and C with F_e^{59} substantiates the claim that these proteins are transferrins, as suggested for types A and B by Kristjansson (1960) after staining gels with Nitroso R salt reagent.

It may also be claimed that haptoglobin, that is the haemoglobin-binding protein does not show genetic variations within pig sera. That is, with the possible exception of some pigs, which do not appear to have this protein, or only have it present to a limited extent. It is suggested in this dissertation, that the haptoglobin types controlled by three allelic genes as reported by Kristjansson (1961), and an additional type recently reported by Hesselholt (1963) are in fact haem-binding globulins. In this report, Hesselholt describes four haptoglobin types called 0, 1, 2 and 3, which appear identical to Kristjansson's three types, with the exception of type 0. These types also appear similar to the 0, 1, 2 and 3 haem-binding globulins described in this dissertation. It has now been established with the co-operation of Hesselholt, that the four haptoglobin types described in his paper are in fact haem-binding globulins, and not true haptoglobins.

It is also interesting to note a passage from Kristjansson's paper on haptoglobins (1961) in which he states, "the amount of haptoglobins /

haptoglobins 4 and 5 present, varied considerably from sample to sample, but no attempt was made to study the variation." A photograph illustrating haptoglobins of type 1 to 10 in the above communication indicates that haptoglobins 4 and 5 are similar to the phases II and III described for the true haptoglobins in this investigation.

The complete absence of haptoglobin in the sera of some pigs has not been referred to by other workers, but has been reported in man (Allison et al., 1958). They found in Europeans that the proportion of individuals without haptoglobin is less than 1%, but in Nigerians was found to be as much as 33%. It must be remembered, however, that Laurell and Nyman (1957) demonstrated in humans, that the intravenous administration of haemoglobin solution resulted in no haemoglobinurea occurring until the plasma haptoglobin was fully saturated. Once fully saturated, however, it was rapidly eliminated from the body, and if sufficient haemoglobin was present the plasma could be completely depleted of haptoglobin within 24 hours. On stopping the haemolytic process, haptoglobins reappeared in the plasma within a few days. Those pigs showing an absence of haptoglobin were not resampled, so it is not known if the absence was transitionary and perhaps due to a temporary intravascular haemolysis. As this condition is very unlikely in the adult pig, and several other animals were observed to have only minute quantities of haptoglobin in their sera, this could indicate that some other genes may have a suppressor effect on the synthesis of haptoglobin. The evidence from the family studies showed that the /

the inheritance was not of a simple mendelian pattern. It would appear that further matings involving consanguinous animals are essential before any genetic interpretation can be established for the presence or absence of haptoglobin.

The picture presented by the other haptoglobins and haem-binding proteins in zone No. 3 is not clear as yet. Results were not consistent, and further experimentation is necessary. There was some indication, however, that variation might occur in the haem-binding protein in this zone.

The evidence for two alleles called A and B controlling three caeruloplasmin phenotypes A, AB and B is believed to be the first report of variation for this component in pig sera. Proof that these components are caeruloplasmins is based on the oxidation of paraphenylenediamine and benzidine only, and not of other substrates, as has been shown by Laurell (1960). Experiments using specific inhibitors are being investigated to substantiate the claim that these fractions are copper-binding proteins. As far as can be determined, this is the first report for any species presenting evidence for genetic control of caeruloplasmin by allelic genes at a single locus.

The variation in an enzyme capable of splitting starch and having properties similar to amylase is believed to be an original observation for any species. However, a report by Ashton (1960) on thread protein polymorphism in the pig may bear some relation to this enzyme. Ashton was not clear about the nature of the thread proteins he observed, but was able to show, that they were controlled by two alleles called T^A and T^B, and were represented by three phenotypes called T1, T1/T2 and T2. In a recent communication with Ashton /

Ashton it was agreed, that reference sera should be exchanged to establish the possible connection between these fractions. As it is believed they may be similar, the alleles Am^1 and Am^2 may correspond to the T^A and T^B alleles above. The Am^3 allele reported here will therefore form a new allele within this system, and the possibility of six phenotypes is suggested, of which, four have already been found.

In the tests for association, no correlation could be found between the different serum types. To confirm these findings at the level of the gene, all relevant double backcross matings were examined. The number of families giving this type of mating were very few. In those families which did, however, the following tables indicate that genes at the transferrin and haem-binding globulin loci were segregating independently, also no linkage could be found between the haem-binding globulin and amylase genes.

1. Test for linkage between transferrins and haem-binding globulins:

Mating	Segregation of offspring			
♂ T_f^{BB}/H_g^{11} x ♀ T_f^{AB}/H_g^{13}	a_1 T_f^{AB}/H_g^{13}	a_2 T_f^{AB}/H_g^{11}	a_3 T_f^{BB}/H_g^{13}	a_4 T_f^{BB}/H_g^{11}
1st Family	0	1	2	2
2nd "	1	0	2	1
Total observed	1	1	4	3
expected	2.25	2.25	2.25	2.25

This gives a ratio of five non-parental to four parental types, which does not deviate significantly from expected.

2. Test for linkage between haem-binding globulins and amylase:

Mating	Segregation of offspring			
♂ ♀	a_1	a_2	a_3	a_4
$H_g^{33}/A_m^{22} \times H_g^{13}/A_m^{23}$	H_g^{13}/A_m^{23}	H_g^{13}/A_m^{22}	H_g^{33}/A_m^{23}	H_g^{33}/A_m^{22}
1st Family	3	2	1	0
2nd "	4	2	1	2
Total observed	7	4	2	2
expected	3.75	3.75	3.75	3.75

The ratio of non-parental to parental types is 6 : 9, which again is not significant.

It is interesting to note that Smithies and Hiller (1959) observed independent segregation of genes at the transferrin locus, and the genes controlling the haptoglobin types, also some red cell blood groups in man. They did not exclude linkage, however, but believed it to be very unlikely. The question of association and linkage between the red cell antigens and the serum types of pigs is considered in the next and final section.

TESTS FOR ASSOCIATION AND LINKAGE BETWEEN RED CELL ANTIGENS AND SERUM TYPES:-

Independence of the genes controlling serum types and red cell antigens within the parent population were again tested in 2 x 2 and 2 x n contingency tables (Fisher, 1950). Exact probabilities were calculated for all the 2 x 2 tables, because they contained values less than five. In the 2 x n tables, expected values are given in parenthesis beside the observed figures. In those instances where deviations are apparent chi-squared values were not calculated, because they also contained values less than five. The agreement between observed and expected can be observed by inspection, however, where chi-values were not calculated.

1. Test for association between red cell antigens and transferrin types:

		Tf AA	Tf AB	Tf BB	
Antigen A	+	2(0.9)	3(4)	15(15)	20
	-	1(2)	10(9)	32(32)	43
		3	13	47	63

		Tf AA	Tf AB	Tf BB	
E_b	+	3(2.3)	10(10)	36(36.5)	49
	-	0(0.7)	3(3)	11(10.4)	14
		3	13	47	63

		AA	AB	BB	
K_b	+	3(2.5)	13(10.6)	36(38.8)	52
	-	0(0.5)	0(2.2)	11(8.2)	11
		3	13	47	63

		AA	AB	BB	
K_a	+	1(1.2)	2(5)	22(18.6)	25
	-	2(1.8)	11(8)	25(28.4)	38
		3	13	47	63

		AA	AB	BB	
E_a	+	1(1)	3(4)	16(15)	20
	-	2(2)	10(9)	31(32)	43
		3	13	47	63

		AA	AB	BB	
F_a	+	0	0(1.2)	6(4.5)	6
	-	3(3)	13(11.8)	41(42.5)	57
		3	13	47	62

		AA	AB	BB	
E_e	+	2(2)	9(8.2)	29(30)	40
	-	1(1)	4(4.8)	18(17)	23
		3	13	47	63

		AA	AB	BB	
E_f	+	0(0.4)	2(1.9)	7(6.7)	9
	-	3(2.6)	11(11.1)	40(40.3)	54
		3	13	47	63

		AA	AB	BB	
X	+	1(0.5)	5(2)	4(7.4)	10
	-	2(2.5)	8(11)	43(39.6)	53
		3	13	47	63

		AA	AB	BB	
G_a	+	0(1)	4(4.7)	19(17)	23
	-	3(2)	9(8.3)	28(30)	40
		3	13	47	63

The values given in the above table do not show a significant deviation from expected, and therefore no association can be found between the red cell antigens and transferrins.

2. /

2. **Test for association between haptoglobin and red cell antigens:**

Antigen A

H_g	+	−	
+	18	40	58
−	2	2	4
	20	42	62

P = 0.39

E_b

H_g	+	−	
+	45	13	58
−	3	1	4
	48	14	62

P = ~~0.78~~ 0.55

K_b

H_g	+	−	
+	48	10	58
−	3	1	4
	51	11	62

P = ~~0.86~~ 0.53

K_a

H_g	+	−	
+	23	35	58
−	2	2	4
	25	37	62

P = 0.53

E_b

H_g	+	−	
+	18	40	58
−	2	2	4
	20	42	62

P = 0.39

F_a

H_g	+	−	
+	5	53	58
−	1	3	4
	6	56	62

P = ~~0.96~~ 0.42

E_e

H_g	+	−	
+	37	21	58
−	2	2	4
	39	23	62

P = ~~0.86~~ 0.48

E_f

H_g	+	−	
+	8	50	58
−	0	4	4
	8	54	62

P = 0.57

X

H_g	+	−	
+	9	49	58
−	0	4	4
	9	53	62

P = 0.52

G_a

H_g	+	−	
+	21	37	58
−	2	2	4
	23	39	62

P = 0.48

The probabilities indicate that haptoglobin is independent of the red cell antigens.

3. Test for association between haem-binding globulins and red cell antigens

		Haem-binding globulins					Totals
		03	11	13	23	33	
Antigen A	+	0(0.6)	13(12.6)	5(4)	0(0.6)	2(2)	20
	-	2(1.4)	26(26.4)	7(8)	2(1.4)	5(5)	42
Totals		2	39	12	2	7	62

		03	11	13	23	33	Totals
E_b	+	2(1.5)	29(30)	9(9)	1(1.5)	7(5.4)	48
	-	0(0.5)	10(9)	3(3)	1(0.5)	0(1.6)	14
Totals		2	39	12	2	7	62

		03	11	13	23	33	Totals
K_b	+	2(1.6)	35(32)	11(9.9)	2(1.6)	1(5.7)	51
	-	0(0.4)	4(7)	1(2.1)	0(0.4)	6(1.3)	11
Totals		2	39	12	2	7	62

		03	11	13	23	33	Totals
K_a	+	0(0.8)	14(15.7)	4(4.8)	1(0.8)	6(2.8)	25
	-	2(1.2)	25(23.3)	8(7.2)	1(1.2)	1(4.2)	37
Totals		2	39	12	2	7	62

		03	11	13	23	33	Totals
E_a	+	0(0.6)	13(12.6)	5(4)	2(0.6)	0(2)	20
	-	2(1.4)	26(26.4)	7(8)	0(1.4)	7(5)	42
Totals		2	39	12	2	7	62

		Haem-binding globulins					Totals
		03	11	13	23	33	
F_a	+	0(0.2)	2(3.8)	2(1.2)	0(0.2)	2(0.7)	6
	-	2(1.8)	37(35.3)	10(10.8)	2(1.8)	5(6.3)	56
Totals		2	39	12	2	7	62
		03	11	13	23	33	Totals
E_e	+	0(1.3)	26(24.5)	10(7.5)	2(1.3)	1(4.4)	39
	-	2(0.7)	13(14.5)	2(4.5)	0(0.7)	6(2.6)	23
Totals		2	39	12	2	7	62
		03	11	13	23	33	Totals
E_f	+	0(0.3)	4(5)	4(1.5)	0(0.3)	0(1)	8
	-	2(1.7)	35(34)	8(10.5)	2(1.7)	7(6)	54
Totals		2	39	12	2	7	62
		03	11	13	23	33	Totals
K	+	0(0.3)	6(5.6)	3(1.7)	0(0.3)	0(1)	9
	-	2(1.7)	33(33.4)	9(10.3)	2(1.7)	7(6)	53
Totals		2	39	12	2	7	62
		03	11	13	23	33	Totals
G_a	+	2(0.7)	11(14)	5(4)	1(0.7)	4(2.6)	23
	-	0(1.3)	28(25)	7(8)	1(1.3)	3(4.4)	39
Totals		2	39	12	2	7	62

On inspection of the above tables an obvious discrepancy exists between the observed and expected figures in the cells for antigen K_b and haem-binding globulins, types 1 and 3. A discrepancy is also apparent between these types and antigens K_a and E_e. The possibility of the genes for these types being linked will be considered in the next section.

4. /

4. **Test for association between caeruloplasmins and red cell antigens:**

Antigen A

	C_u AB	C_u BB	
+	0	20	20
-	3	39	42
	3	59	62

P = 0.3

E_b

	C_u AB	C_u BB	
+	2	46	48
-	1	13	14
	3	59	62

P = ~~0.08~~ 0.54

K_b

	C_u AB	C_u BB	
+	2	49	51
-	1	10	11
	3	59	62

P = ~~0.9~~ 0.45

K_a

	C_u AB	C_u BB	
+	0	25	25
-	3	34	37
	3	59	62

P = 0.2

E_a

	C_u AB	C_u BB	
+	1	19	20
-	2	40	42
	3	59	62

P = 0.7~~4~~

F_a

	C_u AB	C_u BB	
+	1	5	6
-	2	54	56
	3	59	62

P = 0.27

E_e

	C_u AB	C_u BB	
+	2	37	39
-	1	22	23
	3	59	62

P = 0.69

E_f

	C_u AB	C_u BB	
+	1	7	8
-	2	52	54
	3	59	62

P = ~~0.96~~ 0.35

X

	C_u AB	C_u BB	
+	0	9	9
-	3	50	53
	3	59	62

P = 0.6

G_a

	C_u AB	C_u BB	
+	2	21	23
-	1	38	39
	3	59	62

P = 0.3

The probabilities indicate that there is no correlation between caeruloplasmins and the red cell antigens.

5. Test for association between amylases and red cell antigens:

		Amylases			Totals
		12	22	23	
Antigen A	+	1(0.3)	17(18)	2(1.3)	20
	-	0(0.7)	40(39)	2(2.7)	42
Totals		1	57	4	62

		Amylases			Totals
		12	22	23	
E_b	+	1(0.8)	46(44)	1(3)	48
	-	0(0.2)	11(13)	3(1)	14
Totals		1	57	4	62

		Amylases			Totals
		12	22	23	
K_b	+	1(1)	46(47)	4(3.3)	51
	-	0(0)	11(10)	0(0.7)	11
Totals		1	57	4	62

		Amylases			Totals
		12	22	23	
K_a	+	0(0.4)	22(23)	3(1.6)	25
	-	1(0.6)	35(34)	1(2.4)	37
Totals		1	57	4	62

		Amylases			Totals
		12	22	23	
E_a	+	0(0.3)	18(18)	2(1.3)	20
	-	1(0.7)	39(39)	2(2.7)	42
Totals		1	57	4	62

		Amylases			Totals
		12	22	23	
F_a	+	0(0)	3(5.5)	3(0.4)	6
	-	1(1)	54(51.5)	1(3.6)	56
Totals		1	57	4	62

		Amylases			Totals
		12	22	23	
E_e	+	0(0.6)	35(36)	4(2.5)	39
	-	1(0.4)	22(21)	0(1.5)	23
Totals		1	57	4	62

		Amylases			Totals
		12	22	23	
E_f	+	0(0)	6(7)	2(0.5)	8
	-	1(1)	51(50)	2(3.5)	54
Totals		1	57	4	62

		Amylases			Totals
		12	22	23	
X	+	0(0)	9(8)	0(0.6)	9
	-	1(1)	48(49)	4(3.4)	53
Totals		1	57	4	62

		Amylases			Totals
		12	22	23	
G_a	+	0(0.4)	21(21)	2(1.5)	23
	-	1(0.6)	36(36)	2(2.5)	39
Totals		1	57	4	62

The observed and expected figures in the above tables show good agreement indicating that no association could be found between amylases and red cell antigens.

6. Test for linkage between genes for red cell antigens and serum proteins:

(a) The only serum protein component which showed association with some red cell antigens was the haem-binding globulin fraction. In the 2 x n tables, antigenic factor K_b showed a negative association with type 3 haem-binding globulin. To establish the possibility of linkage between the genes for haem-binding globulins and antigenic factor K_b, all the available double backcross matings involving these genes were investigated. The various families showing backcross matings involving the different haem-binding globulin alleles and K^b are grouped together in Table 49 (i to iii):-

Table 49

(i)

Mating	Segregation of offspring			
δ K^{bb}/H_g^{33} x ♀ K^{Bb}/H_g^{23}	(a_1) K^{Bb}/H_g^{23}	(a_2) K^{Bb}/H_g^{33}	(a_3) K^{bb}/H_g^{23}	(a_4) K^{bb}/H_g^{33}
1st Family	8	0	0	3
2nd "	1	0	0	3
Total observed	9	0	0	6
No. expected	3.75	3.75	3.75	3.75

χ^2_L = 15 for 1 d.f. $\therefore$ p = .001

(ii)

Mating	Segregation of offspring			
♂ ♀ $K^{bb}/H_g^{33} \times K^{Bb}/H_g^{13}$	(a_1) K^{Bb}/H_g^{13}	(a_2) K^{Bb}/H_g^{33}	(a_3) K^{bb}/H_g^{13}	(a_4) K^{bb}/H_g^{33}
1st Family	3	1	0	2
2nd "	6	0	0	3
3rd "	2	0	0	3
Total observed	11	1	0	8
No. expected	5	5	5	5

$$\chi^2_L = \frac{(11 - 1 - 0 + 8)^2}{20}$$

$$= 16.4 \text{ for 1 d.f.} \quad \therefore p = .001$$

(iii)

Mating	Segregation of offspring			
♂ ♀ $K^{Bb}/H_g^{03} \times K^{bb}/H_g^{33}$	(a_1) K^{Bb}/H_g^{03}	(a_2) K^{Bb}/H_g^{33}	(a_3) K^{bb}/H_g^{03}	(a_4) K^{bb}/H_g^{33}
1st Family	1	0	0	1
2nd "	2	0	0	3
Total observed	3	0	0	4
No. expected	1.75	1.75	1.75	1.75

$\chi^2_L = 7$ for 1 d.f. $\therefore p$ = Between .01 and .001

There is significant evidence of linkage in the coupling phase between haem-binding globulin alleles H_g^0, H_g^1 and H_g^2 and antigenic factor K^B. The intensity of this linkage, where p is the recombination fraction and Sp the variance may be calculated as follows:-

$$p = \frac{a_2 + a_3}{n} \text{ in coupling}$$

$$= \frac{1}{42} = \underline{0.024 \text{ or } 2.4\%}$$

$$Sp = \sqrt{\frac{p(1-p)}{n}} = \underline{0.024 \text{ or } 2.4\%}$$

Unfortunately /

Unfortunately no family data was available to confirm that the H_g^3 allele was in the repulsion phase with the K^B allele. The statistical evidence for negative association, and the above data indicate that this is probably the case. In calculating the frequency of these types within the parent population on the basis of their phenotype frequencies, the expected number of animals appearing as K^b and H_g^3 positive were calculated:-

	K^b	x	H_g^3	x	No. of animals	
frequencies	0.59	x	0.24	x	63	= 9 animals

On this basis, therefore, 9 animals were expected to be type K^b and H_g^3 positive, whereas in fact there was only one animal of this type.

(b) The possibility of association between factor K^a and the haem-binding globulin alleles was suggested by the above data and the 2 x n' table. Only one double backcross mating was available, and the segregation obtained within this family is shown in Table 50:-

Table 50

Mating	Segregation of offspring			
♂ ♀ $K^{aa}/H_g^{11} \times K^{Aa}/H_g^{13}$	K^{Ae}/H_g^{13}	K^{Aa}/H_g^{11}	K^{aa}/H_g^{13}	K^{aa}/H_g^{11}
No. observed	1	1	1	1
No. expected	1	1	1	1

The segregation of genes within this small family was normal.

(c) The only other antigenic factor showing deviation from expected within the 2 x n' table was factor E^e. Families giving backcross /

backcross matings for three haem-binding globulin alleles and the E^e gene are given in Table 51 (i to iii):-

Table 51

(i)

Mating	Segregation of offspring			
♂ ♀ $E^{Ee}/H_g^{13} \times E^{ee}/H_g^{11}$	(a_1) E^{Ee}/H_g^{13}	(a_2) E^{Ee}/H_g^{11}	(a_3) E^{ee}/H_g^{13}	(a_4) E^{ee}/H_g^{11}
1st Family	0	3	2	2
2nd "	0	1	1	1
No. observed	0	4	3	3
No. expected	2.5	2.5	2.5	2.5

$$\chi^2_L = \frac{(0 - 4 - 3 + 3)^2}{10}$$

$$= 1.6 \text{ for } 1 \text{ d.f.} \qquad p = \underline{\text{Between 0.3 to 0.2}}$$

(ii)

Mating	Segregation of offspring			
♂ ♀ $E^{ee}/H_g^{33} \times E^{Ee}/H_g^{23}$	(a_1) E^{Ee}/H_g^{23}	(a_2) E^{Ee}/H_g^{33}	(a_3) E^{ee}/H_g^{23}	(a_4) E^{ee}/H_g^{33}
1st Family	6	2	2	1
No. expected	2.75	2.75	2.75	2.75

$$\chi^2_L = \frac{(6 - 2 - 2 + 1)^2}{11}$$

$$= 0.8 \text{ for } 1 \text{ d.f.} \qquad p = \underline{\text{Between 0.5 and 0.3}}$$

(iii)

Mating	Segregation of offspring			
♂ ♀ $E^{ee}/H_g{}^{33} \times E^{Ee}/H_g{}^{13}$	(a_1) $E^{Ee}/H_g{}^{13}$	(a_2) $E^{Ee}/H_g{}^{33}$	(a_3) $E^{ee}/H_g{}^{13}$	(a_4) $E^{ee}/H_g{}^{33}$
1st Family	4	2	2	1
No. expected	2.25	2.25	2.25	2.25

$$\chi^2_L = \frac{(4 - 2 - 2 + 1)^2}{9}$$

$$= 0.1 \text{ for } 1 \text{ d.f.} \qquad p = \underline{\text{Between 0.8 to 0.7}}$$

The distribution of offspring from backcross matings involving haem-binding globulin genes $H_g{}^1$, $H_g{}^2$ and $H_g{}^3$ with the gene for antigenic factor E^e indicate that no linkage was detectable.

DISCUSSION ON RESULTS:-

Autosomal linkage between genes for red cell groups and other marker genes has been widely investigated in man. Race and Sanger (1962) give a list of publications by various authors on this subject. None of these investigations have revealed linkage between serum protein markers and the red cell groups. Linnet-Jepson et al. (1958) showed independent segregation of the genes controlling haptoglobin, G_m groups and red cell blood groups: ABO., MNS., Rh., P, Lewis, Lutheran and Duffy. As quoted previously, Smithies and Hiller (1959) also found independent segregation of genes at the transferrin and haptoglobin loci, and the red cell blood groups: ABO., MNS., P and Rh.

The findings in this investigation of close linkage between the haem-binding globulin locus and the K red cell antigen locus, therefore appears to be unique for any species. At one stage of the study of the different backcross matings, it was thought that K^b and H_g genes were alleles at a single locus. However, /

However, the appearance of one offspring type (K^B/K^b. H_g^3/H_g^3) in the recombinant class dismisses this possibility. In the parent and offspring populations, four animals of similar type, that is K_b and H_g^3 positive were found. The distribution of gametes in these animals which were either K(a + b +) or K (a - b +) and H_g^3/H_g^3 means that the H_g^3 gene must appear in both cases on the same chromosome with K^b. These animals indicate that some crossing over must occur between the two loci, and confirms the appearance of the one recombinant in the double backcross matings.

The question of whether the haem-binding globulins may be serum antigens which can coat the erythrocytes and thereby be related to the K antigens is also raised. It is known that the Lewis substance in man is absorbed from the serum on to the red cells (Sneath and Sneath, 1955), also that the J substance in cattle (Stormont, 1949); the R substance in sheep (Rendel, 1957) and the A substance in pigs are water soluble substances absorbed on to the cells from the serum. None of these substances are antigenic however.

Oudin (1960) has demonstrated serum allotypes in rabbits by injecting rabbit antibody-antigen complexes into other rabbits to produce several detectable precipitins, thereby demonstrating individual differences in gamma globulin antigens in rabbits.

There is also evidence by the immunoelectrophoresis technique for α_2-globulin (G_c) antigenic differences in human serum as shown by heteroimmunisation (Hirschfeld, 1959), and by isoimmunisation (Allison and Blumberg, 1961). It has also been demonstrated by Bo Gahne (1962), that in two Guernsey cattle lacking the slow α_2-globulin, when injected with cattle serum containing slow α_2-globulin produced precipitating antibodies against this glycoprotein. /

glycoprotein.

The fact that K_a and K_b antigens stimulate antibody by heteroimmunisation and isoimmunisation has been demonstrated in this dissertation and by Andresen (1962). It is also known from a recent communication with Hesselholt, that in combined work with Brummerstedt-Hansen, the four haptoglobins (i.e. haem-binding globulins 0, 1, 2 and 3) he described have also been distinguished by immunoelectrophoresis against rabbit anti-pig serum. Therefore the haem-binding globulins probably have antigenic properties, which are distinguishable. Whether a linkage of $2\frac{1}{2}\%$ between genes responsible for the synthesis of a serum component and a red cell antigen can have any bearing on a possible cross specificity between these factors is a challenging question. The present answer is equivocal, but the challenge is unequivocal.

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ACKNOWLEDGEMENTS

I would like to record my appreciation of the financial support provided for this work by the Pig Industry Development Authority.

I wish to convey my thanks to Dr. H. P. Donald, who has provided laboratory space, services and animals for experimental purposes within the Animal Breeding Research Organisation. This tribute also extends to his staff on the farms, who provided valuable and willing assistance at all time.

To my supervisor, Professor A. Robertson, I am especially indebted for his advice, encouragement and help during the past three years.

I hope that in this dissertation I can pay tribute to my other supervisor, Dr. J. G. Hall, by reflecting the high standards of direction he has constantly and patiently given. I am indebted to his guidance and richer for his advice.

I also wish to thank Dr. A. Jamieson for the help and advice he has so freely given.

I would like to acknowledge the facilities provided by Edinburgh Corporation, and the help of the staff at the City Abattoir, Gorgie. Also, the manager and staff of the National Pig Progeny Station, Bridge of Allan, for providing samples so willingly.

Most of the photography shown in this dissertation has been carried out by Mr. R. H. Hood and his staff, to whom I am very grateful.

Finally, and perhaps appropriately so, my appreciation and thanks extends to Mrs. T. Hamilton for her efficient typing and cheerful execution of this work.

APPENDIX A AND B

Appendix 1A

List of animals giving an antibody response after immunisation

Key:- WB = Whole blood
WC = Washed cells
NK = Not known
NT = Not typed

No.	No. of donor and breed	No. of recipient and breed	Pre-injection titre (agg.)	Transfusate and route	Antibody titre response			Antigen difference if known	Antibody isolated
					Agg.	Haem.	Antiglob.		
1	LW 9011	LW 9091	-	WB i/m	1 in 16	-	NT	E_e	-
2	LW 9493	LW 9280	-	" "	1 in 128	-	NT	J_a	-
3	LW 9091	LW 8914	-	" "	1 in 64	-	NT	E_b	No. 2
4	LW 8927	LW 1554	1 in 8	" "	1 in 256	-	NT	E_a + unknown	No. 5
5	LW 9011	LW 9091	1 in 1	" "	1 in 4	-	NT	E_e	-
6	W 3217	LW 8438	-	" "	<1 in 32	-	NT	Not known	-
7	W 3217	LW 8402	-	" "	1 in 1	-	NT	" "	-
8	W 3217	LW 8447	-	" "	<1 in 32	-	NT	" "	-
9	-	W 1487	NK	2nd C.V.V.	<1 in 8	-	1 in 16>	" "	-
10	-	W 1488	NK	2nd C.V.V.	1 in 2	1 in 512>	<1 in 64	" "	No. 6
11	LW 5048	W 5806	-	WB i/m	<1 in 16	-	1 in 16	" "	-
12	"	W 5807	-	" "	1 in 8	-	<1 in 64	" "	-
13	"	W 6101	-	" "	<1 in 2	-	<1 in 2	" "	-
14	"	W 5871	-	" "	<1 in 2	NT	NT	" "	-
15	"	W 5861	-	" "	1 in 2>	"	1 in 27	" "	-
16	"	W 5862	-	" "	1 in 2>	"	1 in 64>	" "	-
17	"	W 5863	-	" "	<1 in 128	"	"	" "	-
18	LW 7881	W 989	-	WB & WC i/m	<1 in 81	1 in 32>	<1 in 128	" "	No. 8&9
19	"	W 3220	-	WB & WC	1 in 64	1 in 4	1 in 64	" "	-
20	"	W 3338	-	WB & WC	<1 in 128	1 in 128	NT	" "	-

Appendix 1A (contd.)

No.	No. of donor and breed	No. of recipient and breed	Pre-injection titre (agg.)	Transfusate and route	Antibody titre response			Antigen difference if known	Antibody isolated
					Agg.	Haem.	Antiglob.		
21	W 989	W 6157	-	WB i/m	$\frac{1}{2}>$	NT	NT	NK	-
22	"	W 6181	-	" "	$\frac{1}{2}>$	NT	NT	NK	-
23	"	W 6182	-	" "	$<\frac{1}{2}$	$\frac{1}{2}>$	NT	NK	-
24	"	W 6197	-	" "	$\frac{1}{2}>$	NT	NT	NK	-
25	"	W 6262	-	" "	$\frac{1}{2}>$	NT	NT	NK	-
26	"	W 6658	-	" "	$\frac{1}{2}>$	NT	NT	NK	-
27	"	W 6715	-	" "	$<\frac{1}{2}$	$\frac{1}{2}>$	NT	NK	-
28	LW 254	LW 9091	<1/3	WB+Crill+WC	1/27	NT	NT	E_e, G_a, H_a, J_a, K_a	No. 10
29	LW 255	LW 8457	-	" " "	-	<1/3	1/81	E_e, G_a & K_a	No. 13
30	LW 1859	LW 254	<1/9	" " "	1/81>	1 in 81	NT	A, E_f & K_b	No. 11 & 12
31	LW 1557	LW 563	1/3	" " "	1/27>	NT	NT	E_f	-
32	LW 4731	LW 255	-	" " "	1/27	NT	NT	A, E_f, K_b	-
33	LW 3760	LW 262	-	WB+Crill+WC	-	<1/9	<1/27	G_b, K_b	No. 14
34	LW 1859	LW 254	<1/27	WB & WC	-	<1 in 2187	NT	A, E_f & K_b	No. 15

Appendix 2A

List of animals giving no antibody response after immunisation

No.	No. of donor and breed	No. of recipient and breed	Pre-injection titre (agg.)	Transfusate and route	Antibody response	Antigen difference if known
1	LW 8927	LW 8914	-	WB i/m	-	E_f
2	LW 203	LW 262	-	"	-	K_b
3	LW 262	LW 203	-	"	-	H_a
4	LW 1443	LW 1523	-	"	-	K_b
5	LW 3754	LW 3727	-	"	-	J_a
6	LW 3760	LW 3980	-	"	-	H_a
7	LW 6291	LW 6629	-	"	-	$H_{a/a}$
8	LW 1859	LW 3760	-	"	-	E_f
9	LW 564	LW 3754	-	"	-	G_a K_a J_a
10	LW 5048	W 5870	-	"	-	NK
11	"	W 5852	-	"	-	NK
12	"	W 6008	-	"	-	NK
13	"	W 6138	-	"	-	NK
14	"	W 6139	-	"	-	NK
15	LW 4907	LW 4731	-	WB+'Crill'	-	J_a

Appendix B

(i) Fractionation of Antiserum No. 1: The sera from three pairs of monozygous cattle twins known to contain J antibody were tested against known J positive cells. The results of a haemolytic test are shown below:-

J +ve cells	Dilutions of sera									Sera
	1 in 1	2	4	8	16	32	64	SC	C'C	
MZ 111B	5	5	2	-	-	-	-	-	-	MZ 252 A
"	5	5	5	5	2	-	-	-	-	" B
"	5	5	5	-	-	-	-	-	-	MZ 243 A
"	5	5	5	-	-	-	-	-	-	" B
"	5	5	5	5	3	-	-	-	-	MZ ~~252~~ 206 A
"	5	5	5	5	5	4	3	-	-	" B
~~MZ~~ U17A	5	5	5	5	5	3	-	-	-	" B

As MZ 206B serum had the highest titre, it was selected for testing against pig cells.

Six unknown pig cells were tested against this serum in a haemolytic test:-

Pig cells	Dilutions of MZ 206B serum											
	1 in 1	2	4	8	16	32	64	128	256	512	SC	C'C
No. 1	4	4	3	1	1	-	-	-	-	-	-	-
" 2	4	4	3	2	1	1	-	-	-	-	-	-
" 3	5	5	5	5	5	5	5	4	3	1	-	-
" 4	5	5	5	5	5	5	5	4	2	1	-	-
" 5	4	4	3	2	1	1	-	-	-	-	-	-
" 6	4	4	3	2	1	-	-	-	-	-	-	-

A matrix analysis was then carried out with these cells:-

Test cells	MZ 206B absorbed with cells						Proposed antigens
	P1	P2	P3	P4	P5	P6	
P1	-	-	-	-	-	-	-
P2	-	-	-	-	-	-	-
P3	43	43	-	-	44	42	A
P4	41	42	-	-	42	41	A
P5	-	-	-	-	-	-	-
P6	-	-	-	-	-	-	-
	A	A	-	-	A	A	
	Proposed antibodies present						

Cell No. 5 was selected to remove the species antibody at an absorption ratio of 1 to 1.

MZ 206B serum absorbed pig 5 cells was then tested against a further 6 pig cells:-

Pig cells	Dilutions of MZ 206B absorbed pig 5 cells											
	1 in 1	2	4	8	16	32	64	128	256	512	SC	C'C
Pig 7	5	5	5	5	5	5	5	4	2	-	-	-
" 8	5	5	5	5	5	5	5	4	3	-	-	-
" 9	-	-	-	-	-	-	-	-	-	-	-	-
" 10	-	-	-	-	-	-	-	-	-	-	-	-
" 11	-	-	-	-	-	-	-	-	-	-	-	-
" 12	-	-	-	-	-	-	-	-	-	-	-	-

As it was believed that all the negative reacting pigs did not have A antigen on their cells, then it was possible that they had anti A in their serum. To test this hypothesis, then all twelve cells were tested against each serum in a direct agglutination test. The individual scores are given:-

Test Cells	Serum from animals											
	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12
P1	•	•	•	•	•	•	•	•	•	4	•	•
P2	•	•	•	•	•	•	•	•	•	•	•	•
(A) P3	7	4	•	•	3	3	•	•	4	4	3	2
(A) P4	6	1	•	•	1	1	•	•	3	5	1	1
P5	•	•	•	•	•	•	•	•	•	5	•	•
P6	•	•	•	•	•	•	•	•	•	•	•	•
(A) P7	4	3	•	•	3	3	•	•	3	5	3	3
(A) P8	3	2	•	•	3	3	•	•	3	5	3	2
P9	•	•	•	•	•	•	•	•	•	•	•	•
P10	•		•	•	•	•	•	•	•	•	•	•
P11	•		•	•	•	•	•	•	•	•	•	•
P12	•		•	•	•	•	•	•	•	•	•	•

This confirmed the findings with MZ 206B serum, in that only the negative pigs showed antibody against the A positive pigs. The serum from pig No. 10 showed an additional antibody to anti-A, because it also reacted with two A negative cells. The titre of anti-A found in the pig sera did not compare favourably with that of MZ 206B, and consequently the latter was selected for detecting the A antigen on pig cells.

The results of testing against cells from the known reference panel are given on page 41.

To further test the unity of MZ 206B serum absorbed pig 5 cells, a further panel of ten positive cells were tested in a matrix analysis along with a known A positive cell. All the cells showed a negative reaction with the absorbed aliquots, thus confirming the specificity of the antibody.

(ii) /

(ii) Fractionation of Antiserum No. 2: The first matrix analysis includes four unknown positive cells with the donor cells and one negative cell:-

(Scores for 1 in 2 dilutions of antiserum)

Test cells	AP 8914 antiserum absorbed with cells							Proposed antigens on cells
	7304	7215	7310	6907	7225	Donor	Recipient	
7304	-	-	-	20	-	-	21	E^b
7215	-	-	-	22	-	-	22	E^b
7310	-	-	-	22	-	-	22	E^b
6907	-	-	-	-	-	-	-	-
7225	-	-	-	22	-	-	22	E^b
Donor	-	-	-	23	-	-	24	E^b
Recipient	-	-	-	-	-	-	-	-
	-	-	-	E^b	-	-	E^b	
	Proposed antibodies in serum							

This analysis indicated that the antiserum was homogenous. To confirm the antibody content another matrix analysis using ten cells of the reference panel including the donor and the recipient was completed:-

(Scores for 1 in 2 dilutions of antiserum)

Test cells	AP 8914 antiserum absorbed with cells										Antigens
	8927	262	9011	203	3727	3754	3760	3980	Donor	Recipient	
8927	.	.	.	.	.	.	.	.	.	.	-
262	.	.	.	.	.	.	.	.	.	.	-
9011	19	19	.	20	20	19	21	20	.	21	E_b
203	.	.	.	.	.	.	.	.	.	.	-
3727	.	.	.	.	.	.	.	.	.	.	-
3754	.	.	.	.	.	.	.	.	.	.	-
3760	.	.	.	.	.	.	.	.	.	.	-
3980	.	.	.	.	.	.	.	.	.	.	-
Donor	22	20	.	20	22	23	20	21	.	22	E_b
Recipient	.	.	.	.	.	.	.	.	.	.	-
	E_b	E_b	-	E_b	E_b	E_b	E_b	E_b	-	E_b	
	Antibodies										

(iii) Fractionation of Antiserum No. 3: As explained in the text, this antiserum was produced by immunisation of six rabbits with washed cells from sow 203, which carried antigen K_b. Each rabbit antiserum was then absorbed with sow 262 cells, at different absorption ratios. The absorbing cells were K_b negative. Each absorbed aliquot was then tested back against these two cells as shown overleaf:-

(Score for 1 in 2 dilutions of antiserum)

Haemolytic score after four hours

Test cells	Absorbing ratio @ 262 1:1	1:4	1:6	1:8	1:10	Rabbit Antiserum
203 (D)	13	13	12	13	12	R 112
262	11	8	3	1	-	
203 (D)	27	27	28	26	26	R 114
262	2	-	-	-	-	
203 (D)	3	-	-	-	-	R 90
262	3	-	-	-	-	
203 (D)	9	3	1	-	-	R 130
262	5	2	-	-	-	
203 (D)	21	20	21	20	19	R 132
262	5	2	-	-	-	
203 (D)	5	2	-	-	-	R 134
262	5	2	-	-	-	

On the basis of the above test, rabbit antiserum 114 was selected and absorbed with cells 262 at an absorption ratio of four volumes of cells to one volume of serum. The absorbed antiserum was then tested against four cells of the reference panel, of which cells 262 were included as a control, because this was the only source of K_b negative cells at that time.

(1 in 2 dilutions of antiserum)

Test cells	R 114 absorbed with cells						Antigens
	8914	9091	9011	8927	Donor	262	
8914	-	-	-	-	-	24	K_b
9091	-	-	-	-	-	22	K_b
9011	-	-	-	-	-	20	K_b
8927	-	-	-	-	-	24	K_b
Donor	-	-	-	-	-	23	K_b
262	-	-	-	-	-	-	-
	-	-	-	-	-	K_b	
	Antibodies						

It was later shown that testing R 114 antiserum @ 262 cells against random cells did not give a clear qualitative reaction, and it was subsequently found that this antiserum required further fractionation. The antiserum was tested against twenty unknown cells, seventeen of which gave haemolytic scores ranging from 23 to 25, and three cells gave haemolytic scores of 11 to 13. One of the low scoring cells was selected for absorbing the R 114 @ 262 antiserum. On testing the absorbed aliquots against all other cells used previously, antibodies were removed completely for the low scoring cells and left for the seventeen high scoring cells. Further absorption with each of the seventeen cells showed that the absorbed antiserum could not be fractionated any further.

(iv) Fractionation of Antiserum No. 4: This antiserum was obtained as a result of immunisation of six rabbits with 1443 cells carrying antigens K_a and K_b. Each rabbit antiserum was then absorbed with 1523 cells at different absorption ratios. The absorbing cells were /

were K_b positive and K_a negative. Each absorbed aliquot was then tested back against 1443 and 1523 cells as shown in the table below:-

(Scores for 1 in 2 dilutions of antiserum)

Haemolytic score after four hours

Test cells	Absorbing ratio @ 1523 1:2	1:4	1:6	1:8	Rabbit Antiserum
1443 (D)	22	23	23	20	R 92
1523	3	-	-	-	
1443 (D)	-	-	-	-	R 94
1523	-	-	-	-	
1443 (D)	16	17	18	15	R 96
1523	11	5	-	-	
1443 (D)	40	39	41	40	R 98
1523	15	7	-	-	
1443 (D)	22	25	23	22	R 108
1523	5	-	-	-	
1443 (D)	25	25	22	23	R 128
1523	5	3	-	-	

Several rabbits' antisera appeared equally effective for the possible detection of K_a. Antiserum R 108 was selected for further fractionation after absorption with cells 1523 at a 1 in 4 ratio. An absorbed aliquot was then tested against six cells of the reference panel including the donor:-

Cells	R 108 @ 1523 cells in dilutions of							K Antigens present
	1 in 2	4	8	16	32	64	SC	
203	-	-	-	-	-	-	-	K_b
262	-	-	-	-	-	-	-	-
9091	-	-	-	-	-	-	-	K_b
8914	5	5	5	5	5	-	-	K_a, K_b
1443 (D)	5	5	5	5	2	-	-	K_a, K_b
1523	-	-	-	-	-	-	-	K_b

Aliquots of R 108 @ 1523 antiserum were then absorbed with each cell of the reference panel including the donor and tested back in a matrix analysis:-

Test cells	R 108 @ 1523 absorbed with						Antigens
	Cells 203	262	9091	8914	1443	1523	
203	-	-	-	-	-	-	K_b
262	-	-	-	-	-	-	-
9091	-	-	-	-	-	-	K_b
8914	23	25	25	-	-	25	K_a, K_b
1443 (D)	22	24	23	-	-	23	K_a, K_b
1523	-	-	-	-	-	-	K_b
	K_a	K_a	K_a	-	-	K_a	
	Antibodies						

(v) <u>Fractionation of Antiserum No. 5</u>: Obtained from pig 1554 after immunisation with whole blood from pig 8927. In the first matrix analysis, ten unknown positive cells plus the donor and one known cell are included in the analysis:-

(Scores for 1 in 2 dilutions)

Agglutination reaction - two hours at room temperature

Test cells	B3-1554 antiserum @ with cells												Proposed antigens	
	Pig 6	" 7	" 8	" 9	" 10	" 11	" 12	" 14	" 16	6291	Donor	Recipient		
Pig 6	-	-	-	28	29	29	-	30	28	29	-	28	E_a	-
" 7	12	-	13	27	26	28	11	27	27	26	13	26	E_a	E_b
" 8	-	-	-	28	28	25	-	28	29	29	-	28	E_a	-
" 9	10	-	11	-	-	-	10	-	-	-	10	11	-	E_b
" 10	10	-	9	-	-	-	10	-	-	-	11	11	-	E_b
" 11	10	-	9	-	-	-	9	-	-	-	9	10	-	E_b
" 12	-	-	-	27	26	27	-	27	26	28	-	27	E_a	-
" 14	12	-	12	-	-	-	11	-	-	-	13	13	-	E_b
" 16	11	-	10	-	-	-	11	-	-	-	12	13	-	E_b
6291	11	-	11	-	-	-	11	-	-	-	11	11	-	E_b
Donor	-	-	-	30	28	31	-	29	30	29	-	30	E_a	-
Recipient	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	a	a	a	-	a	a	a		a		
	b	-	b	-	-	-	b	-	-	-	b	b		
	Proposed antibodies													

It was apparent from this matrix analysis that serum 1554 must have contained an antibody prior to immunisation with 8927 cells. The pre-immunisation reaction between 8927 cells and 1554 serum was negative. To test the possibility of this other antibody being anti-E_b, all the cells in the above matrix were tested against No. 2 antiserum (i.e. Anti E_b). Cells Nos. 7, 9, 10, 11, 14, 16 and 6291 were all positive, and the remainder were negative. Anti-serum B3-1554 was therefore absorbed with A4-6291 cells to remove the E_b antibody.

On /

On testing against seven cells in the reference panel, this absorbed antiserum reacted with three E_a positive cells and was negative to four E_a negative cells. Absorption and matrix analysis appeared to confirm the unity of this reaction. Subsequently, however, on typing cells from three Wessex sows, No. 989 gave a partial reaction, which suggested a further antibody. Absorption with these cells removed this antibody and still left antibody for type E_a cells. A further matrix analysis involving eight cells of the reference panel indicated that antiserum B3-1554 @ 6291 @ 989 cells could not be broken down any further.

(vi) Fractionation of Antiserum No. 6: This antiserum was obtained from one of several Wessex sows investigated for presence of antibody 14 days after the second [illegible]third injection of crystal violet vaccine. Antiserum 1488 contained a strong acting haemolysin giving titres beyond 1 in 1024 using rabbit complement. This haemolysin appeared to be specific for one antigenic factor as can be shown from the matrix below. Ten positive unknown cells and one known reference cell were used in this analysis:-

(Scores for 1 in 2 dilutions)

Haemolytic reaction - four hours at room temperature

Test cells	W 1488 antiserum absorbed with cells												Proposed antigens	
	Cells 8914	Pig 1	" 2	" 3	" 7	" 8	" 12	" 13	" 14	" 16	" 19	1488		
8914	-	-	-	-	5	-	-	-	-	-	-	47	a	b
Pig 1	-	-	-	-	5	-	-	-	-	-	-	47	a	b
" 2	-	-	-	-	5	-	-	-	-	-	-	41	a	b
" 3	-	-	-	-	2	-	-	-	-	-	-	50	a	b
" 7	-	-	-	-	-	-	-	-	-	-	-	52	a	-
" 8	-	-	-	-	3	-	-	-	-	-	-	48	a	b
" 12	-	-	-	-	5	-	-	-	-	-	-	48	a	b
" 13	-	-	-	-	6	-	-	-	-	-	-	48	a	b
" 14	-	-	-	-	4	-	-	-	-	-	-	44	a	b
" 16	-	-	-	-	2	-	-	-	-	-	-	47	a	b
" 19	-	-	-	-	4	-	-	-	-	-	-	49	a	b
1488	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	-	-	-	-	-	-	a		
	-	-	-	-	b	-	-	-	-	-	-	b		
	Proposed antibodies													

The titre of the weak antibody did not extent beyond a dilution of 1 in 4. Therefore it was decided to use antiserum W 1488 at titres starting above this dilution, so that the minor reaction would be avoided. On testing W 1488 antiserum against known cells of the reference panel only, four cells out of thirteen different bloods gave a positive reaction. It was apparent that these four cells had the factor K_a in common. As Antiserum No. 4 was used to identify antigenic factor K_a, the two antisera were used in parallel against a considerable number of cells from different animals. In every case without exception in testing over 400 bloods, the positive and negative reactions to these cells were exactly /

exactly similar for both antisera. It was therefore concluded that antiserum W 1488 was capable of detecting antigenic factor K_a.

(vii) Fractionation of Antiserum No. 8: Wessex sow 989 was immunised with cells from sow S 7881, the only animal in the reference panel to have factor F_a. As the blood type of Wessex sow 989 was not known, there was no way of forecasting what the antiserum would contain apart from all the known antigens on S 7881 cells, which was unlikely. In the first matrix analysis, eleven unknown positive reacting cells plus six from the reference panel including the donor and recipient were involved. Two of the reference panel cells were included, because they were negative to the antiserum, and were used as a check on the potential antibodies present:-

(Agglutination score for 1 in 3 dilutions)

Test cells	W 989 antiserum absorbed with cells:-																		Proposed antigens					
	Pig 1	Pig 2	Pig 3	Pig 4	Pig 6	Pig 7	Pig 8	Pig 9	Pig 10	Pig 11	Pig 12	3760	8457	1859	8914	Donor	Recipient							
Pig 1	-	-	3	3	2	-	-	-	-	-	-	2	-	1	2	-	3		-	-	-	X	Y	
" 2	-	-	3	3	1	-	-	-	-	-	-	2	-	1	2	-	3		-	-	-	X	Y	
" 3	10	10	-	-	10	-	-	-	-	-	-	10	-	11	9	-	11		-	E_b	-	X	-	
" 4	11	10	10	-	10	10	10	10	10	11	10	11	10	11	10	10	11		E_a	E_b	-	X	-	
" 6	-	-	-	-	-	-	-	-	-	-	-	2	-	2	2	-	2		-	-	-	X	-	
" 7	10	10	2	2	10	-	-	-	-	-	-	10	-	10	10	-	10		-	E_b	-	X	Y	
" 8	10	10	2	2	10	-	-	-	-	-	-	10	-	10	10	-	10		-	E_b	-	X	Y	
" 9	10	10	2	2	10	-	-	-	-	-	-	11	-	11	10	-	10		-	E_b	-	X	Y	
" 10	10	10	2	2	10	-	-	-	-	-	-	11	-	11	10	-	11		-	E_b	-	X	Y	
" 11	10	10	2	2	10	-	-	-	-	-	-	10	-	10	10	-	11		-	E_b	-	X	Y	
" 12	10	10	2	2	10	-	-	-	-	-	-	10	-	10	9	-	10		-	E_b	-	X	Y	
3760	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	E_e
8457	11	10	2	2	10	-	-	-	-	-	-	10	-	10	10	-	10		-	E_b	-	X	Y	
1859	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	E_e E_f
8914	9	9	11	-	10	9	10	10	11	10	10	11	11	11	-	10	11		E_a	-	-	-	-	
Donor	17	17	17	17	17	17	17	17	17	17	17	18	17	18	15	-	18		-	E_b	F_a	X	Y	E_e E_f
Recipient	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	
	a	a	a	-	a	a	a	a	a	a	a	a	a	a	a	-	a	a						
	b	b	-	-	b	-	-	-	-	-	-	b	-	b	b	-	b							
	F_a	F_a	F_a	F_a	F_a	F_a	F_a	F_a	F_a	F_a	F_a	F_a	F_a	F_a	F_a	F_a	-	F_a						
	-	-	-	-	-	-	-	-	-	-	-	X	-	X	X	-	X							
	-	-	Y	Y	Y	-	-	-	-	-	-	Y	-	Y	Y	-	Y							
Proposed antibodies																								

It appeared from this matrix that W 989 antiserum had an antibody present, which was not induced by S 7881 cells. By absorbing with a mixture of pigs 9 and 11 cells, antibody was still left exclusively for S 7881 cells, also for the unknown antibody. Another matrix analysis was carried out involving four unknown positive cells, also the donor and 8914 cells against W 989 antiserum absorbed pigs 9 and 11 cells:-

(Agglutination score for 1 in 3 dilutions)

Test cells	W 989 @ 9 and 11 @ with cells							Antigens
	Donor	Pig 14	Pig 20	Pig 21	Pig 22	8914	Recip.	
Donor	-	18	18	20	-	18	18	- F_a
Pig 14	5	-	-	-	7	-	6	E_a -
" 20	6	-	-	-	8	-	6	E_a -
" 21	5	-	-	-	8	-	7	E_a -
" 22	-	19	20	21	-	20	19	- F_a
8914	5	-	-	-	8	-	10	E_a -
Recipient	-	-	-	-	-	-	-	- -
	a	-	-	-	a	-	a	
	-	F_a	F_a	F_a	-	F_a	F_a	
	Antibodies							

From this matrix analysis W 989 antiserum absorbed 9 and 11 cells, also absorbed pig 21 cells was used for detecting antigenic factor F_a.

(viii) Fractionation of Antiserum No. 9: The last matrix analysis, also indicated that an antibody was present for character E_a. Therefore W 989 antiserum absorbed cells 9 and 11, also absorbed pig 22 cells was used as an additional antibody for the detection of factor E_a. It is shown in the text, that this antibody gave identical results to Antiserum No. 5.

(ix) Fractionation of Antiserum No. 10: In the first matrix analysis, eight positive reacting cells from the reference panel including the donor, also the recipient were involved. From this analysis it appeared that three antibodies were present:-

(Agglutination score for 1 in 3 dilutions)

Test cells	WL 9091 antiserum absorbed with cells									Antigens proposed		
	Donor	4731	1859	255	262	8457	1557	563	Recipient			
Donor	-	11	11	-	11	12	-	-	13	E	G	H
4731	-	-	-	-	-	12	-	-	14	E	-	H
1859	-	-	-	-	-	12	-	-	13	E	-	H
255	-	12	12	-	11	14	-	-	13	E	G	H
262	-	-	-	-	-	12	-	-	14	E	-	H
8457	-	-	-	-	-	-	-	-	3	-	-	H
1557	-	11	11	-	11	14	-	-	13	E	G	H
563	-	11	10	-	11	14	-	-	13	E	G	H
Recipient	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	-	E	-	-	E			
	-	G	G	-	G	G	-	-	G			
	-	-	-	-	-	-	-	-	H			
Antibodies proposed												

As anti E and G could not be divided by any member of this panel, the 9091 antiserum was absorbed with 8457 cells to remove the partial reacting antibody to H. The absorbed serum was then fractionated further by four positive reacting cells plus two members of the above panel and the donor and recipient:-

Test cells	WL 9091 antiserum @ 8457 @ with								Antigens
	7582	7704	7707	7892	1557	563	Donor	Recipient	
7582	-	6	-	7	-	-	-	6	- G
7704	9	-	9	-	-	-	-	11	E -
7707	-	6	-	6	-	-	-	7	- G
7892	9	-	10	-	-	-	-	8	E -
1557	10	7	10	8	-	-	-	11	E G
563	10	7	9	8	-	-	-	11	E G
Donor	12	8	11	9	-	-	-	12	E G
Recipient	-	-	-	-	-	-	-	-	- -
	E	-	E	-	-	-	-	E	
	-	G	-	G	-	-	-	G	
	Antibodies								

From this analysis, cells from pig 7707 were selected to absorb antiserum WL 9091 absorbed 8457 cells. This left an antibody which reacted with seven positive E_e cells from the reference panel. Absorption with these cells demonstrated that the antibody isolated was specific and unit. This antibody was called Antiserum No. 10.

(x) Fractionation of Antiserum No. 11: The breakdown of the antiserum from which this antibody was derived is fully explained in the text.

(xi) Fractionation of Antiserum No. 12: During the fractionation of Antiserum No. 11 as explained in the text, another antibody was isolated. This was obtained by absorbing antiserum 254 with cells from pig Nos. 8457 and 262, then with 4731. Aliquots of the absorbed serum were further absorbed with six positive acting cells, also the donor and recipient, and tested back in another matrix analysis:-

Test cells	254 @ 8457 262 and 4731 @								Antigens		
	Donor	Pig 2	Pig 3	Pig 5	Pig 6	Pig 9	Pig 10	Recip.			
Donor	-	2	10	11	2	-	11	12	X	Y	Z
Pig 2	-	-	8	9	-	-	10	10	X	-	Z
" 3	-	-	-	-	1	-	-	2	-	Y	Z
" 5	-	1	-	-	-	-	-	3	-	Y	Z
" 6	-	-	8	9	-	-	10	10	X	Y	-
" 9	-	2	9	11	2	-	10	11	X	Y	Z
" 10	-	1	-	-	2	-	-	3	-	Y	Z
Recipient	-	-	-	-	-	-	-	-	-	-	-
	-	-	X	X	-	-	X	X			
	-	Y	-	-	-	-	-	Y			
	-	-	-	-	Z	-	-	Z			
	Antibodies										

Antiserum 254 absorbed 8457, 262 and 4731 cells was therefore absorbed further with a mixture of cells from pigs 3, 5 and 10, thus leaving an antibody for factor X.

(xii) Fractionation of Antiserum No. 13: In the first matrix analysis of A4-8457 antiserum, seven positive cells plus the donor, and one negative cell plus the recipient were included. All cells were taken from the reference panel:-

(Score for agglutination at 1 in 3 dilutions)

Test cells	A4-8457 antiserum absorbed with cells									Antigens		
	Donor	1557	563	254	9091	1859	4731	262	Recipient			
Donor	-	9	-	-	11	10	10	-	11	E	G	-
1557	-	-	-	-	11	-	-	-	11	E	-	-
563	-	9	-	-	11	9	9	-	12	E	G	-
254	-	9	-	-	10	8	9	-	11	E	G	-
9091	-	-	-	-	-	-	-	-	-	-	-	-
1859	-	5	5	4	6	-	6	5	7	E	-	X
4731	-	-	-	-	9	-	-	-	9	E	-	-
262	-	9	-	-	10	10	10	-	11	E	G	-
Recipient	-	-	-	-	-	-	-	-	-	-	-	-
	-	-	-	-	E	-	-	-	E			
	-	G	-	-	G	G	G	-	G			
	-	X	X	X	X	-	X	X	X			
	Antibodies											

From this analysis it appeared that absorption with 1859 cells would leave an antibody against antigen G_a. On doing this and further absorption analysis within the reference panel, a specific antibody for G_a appeared to be left. To complete the analysis, twenty unknown cells from a wide selection of animals were tested against 8457 antiserum absorbed 1859 cells. Only four positive animals were found and on absorbing the antiserum with these cells, also the four positive G_a cells from the reference panel and testing back in a matrix analysis, confirmed the unit specificity of this antibody.

(xiii) Fractionation of Antiserum No. 14: Like Antiserum No. 13, the antibody isolated was an incomplete agglutinin. In the initial fractionation, six animals from the reference panel including the donor and recipient were included in the matrix analysis:-

(Agglutinin score for 1 in 3 dilutions)

Test cells	TH 262 antiserum absorbed						Anti-gens
	Cells Donor	254	255	8250	3184	Recip.	
Donor	-	3	2	-	8	11	G K
254	-	-	-	-	7	10	G -
255	-	-	-	-	8	11	G -
8250	-	2	2	-	8	9	G K
3184	-	3	3	-	-	2	- K
Recipient	-	-	-	-	-	-	
	-	-	-	-	G	G	
	-	K	K	-	-	K	
	Antibodies						

Antiserum TH 262 was absorbed with W 3184 cells, and this absorbed antiserum was tested against six positive cells selected from twenty unknown cells plus the donor in a further matrix analysis. The analysis showed that antiserum TH 262 absorbed W 3184 cells appeared to contain only one antibody, which could not be fractionated by further absorption.