UNIVERSITY OF EDINBURGH

BLOOD GROUP SEROLOGY OF THE PIG

A Dissertation presented for
the Degree of
Doctor of Philosophy
of the
University of Edinburgh
by
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SUMMARY

The serology of the pig has been investigated firstly in terms of specific antigen/antibody reactions and secondly on a more general basis of the protein constituents of porcine serum. The antigen/antibody reactions studied have been those involved in red blood cell typing and serum immunoglobulin allotyping. These two approaches have been correlated by using red cell typing reactions and immuno-diffusion in gel, immuno-electrophoretic, specific staining, passive haemagglutination, haemagglutination inhibition, gel filtration, ion exchange and starch gel electrophoretic techniques. In order to achieve this correlation, it has been necessary to partially separate and identify a large number of pig serum components on the basis of molecular size, electrical charge and specific staining characteristics. These components not only include those involved in well established systems of pig serum protein biochemical polymorphism such as haemopexin, transferrin, serum amylase, prealbumin, caeruloplasmin and slow \( \alpha_2 \)-globulin but also additional constituents such as fast and slow lipoprotein, variable \( \alpha \)-globulin, complement components, haptoglobin and the porcine immunoglobulins Ig M, Ig A, Ig G_1 and Ig G_2. A provisional investigation of the porcine A-0 blood group system has also been initiated.

More specifically the following conclusions have been established. Antibodies reacting with pig red blood cell
typing factors by the direct agglutination, haemolytic and indirect sensitisation (Coombs) test have been demonstrated in the 19 S, 7 S and intermediate fractions on Sephadex G200 fractionation. These reactivities have been partially correlated with porcine Ig M, Ig G and only tentatively with Ig A. The heterogeneity of distribution of these reactivities within the 7 S peak of G200 fractionation suggests the possibility of novel sub-classes of Ig G on a molecular size and/or steric hindrance to gel filtration basis.

Iso-immune precipitins against pig immunoglobulin allotypes have been produced using a double emulsion (water-oil-water-WOW) type of adjuvant incorporating whole pig serum. The reactions are demonstrated by double diffusion (Ouchterlony) and immuno-electrophoretic procedures in agar gel. The late hyperimmune precipitin has Ig G₂ characteristics. Antibodies with two specificities anti factor 1 and anti factor 2 have been investigated.

Allotype factor 1 occurs on Ig G molecules with a full range of electrophoretic mobility from α to γ₂. Factor 2 has a more restricted distribution on Ig G₁ molecules. These factors are therefore termed porcine Ig G – allotype factor 1 and porcine Ig G₁ – allotype factor 2 or P. Ig G – A₁ and P. Ig G₁ – A₂.

In the pig A-0 blood type system the failure of neonatal piglets' cells to type as A positive with conventional typing techniques has been shown to be a property of their serum, or
of their cells and serum, but not of their cells alone. It is not caused by a quantitative lack of A blood group substance in the serum.

In adult pigs a provisional investigation has established that all pigs typing as O negative by direct test possess an antibody accessible but non-haemolisable cryptantigenic form of O on their red cells.
I declare that this thesis is a report of my work and is entirely my own composition.

Brian G. Lang.
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INTRODUCTION

Serology means literally "study of serum" and its components, but the term is generally reserved more specifically to refer to the antigen/antibody reactions of serum or plasma. Since in so far as in vitro tests are concerned antibody is largely a component of serum and associated bodily fluids, these two interpretations overlap to a considerable extent. They are therefore linked together.

The antigen/antibody reactions considered in this thesis are restricted to those involved in pig blood grouping. Let me hasten to add that the term "blood grouping" refers to the somewhat broader subject sometimes called immunogenetics, but more of this later. For the moment allow that blood grouping includes serum type systems as well as the classical red cell type systems. This also leads to a fusion of the general and the particular interpretations of the term serology.

REVIEW OF THE LITERATURE

Red Blood Cell Typing Systems in the Pig

Since, with the exception of the A blood group system, the only significance of classical red cell typing for this dissertation is that it provides us with a variety of fully tested and standardised reagents for our serology, this subject will not be exhaustively reviewed.
However, a brief consideration of its historical development, particularly in relation to the types of reagent used and their production, will not be out of place.

Many are aware of the rapid development of human blood group studies during the past three decades, beginning with the recognition of the Rhesus factor in 1939 and 1940 (Levine and Stetson, 1939; Landsteiner and Wiener, 1940). What may not be such common knowledge, however, is that by 1945 there was evidence for the existence of 50 gene-controlled antigentic factors on the erythrocytes of cattle (Bouw et al., 1964), and by 1961 this had expanded to about 100 (Stormont, 1962) factors arranged in 11 independent blood group systems (Bouw et al., 1964). Each blood group system occupied a genetical locus, and one, the B locus, involved at least 300 alleles, each allele said to determine a phenogroup of several blood group factors.

Studies on the pig had not progressed as rapidly since intraspecific agglutination was first observed (Fishbein, 1913; Wescieszky, 1920). Following this, attention was largely concentrated on detection of natural antibodies to red blood cell antigens. Szymanowski, Stetkiewicz and Wachler (1926) described the A antigen and its corresponding antibody anti A (also Kayser, 1929). Similarities between pig A and human A were soon noted (Szymanowski et al., 1926; Kaemffer, 1932a) and also between pig A and sheep A (now called R) (Szymanowski and Wachler, 1927). A number of authors detected "natural" antibodies
other than anti A (Kaemffer, 1932b; Kuhns, 1950; Eyquem, 1953) culminating in a report by Szent-Ivanyi and Szabo (1954) of B, C and D factors as well as A detected by "natural" antibodies. Two years earlier these authors had reported a factor Su, and presented some evidence that it caused a naturally occurring haemolytic disease of the newborn (H.D.N.B.) (Szent-Ivanyi and Szabo, 1952, cited by Szabo et al., 1956). Unfortunately no cross comparisons were carried out by any of these workers, and none of these reagents are now available. As a result of this the letters B, C and D have been re-utilised for recently discovered blood group systems (Baker and Andresen, 1962, 1964; Andresen and Baker, 1963, 1964; Andresen et al., 1965; Rasmusen, 1965b; Andresen, 1966a; factor 7 of Joysey et al., 1959a, renamed Da after Saison et al., 1967).

As early as 1926 Szymanowski and Wachler and later Szymanowski and Frendzel (1936) observed that pigs vaccinated against swine fever occasionally had antibodies against A negative pig cells. Since vaccine is prepared from whole blood they concluded that 0 (non A) pig red cells possessed additional antigenic properties. However, it was not until 1949 that Bruner et al. demonstrated that pregnant sows immunised with blood from the mated boar caused H.D.N.B.

This was followed by a series of reports (1950-57) of clinical cases of H.D.N.B. in piglets (Kershaw, 1950; Szent-Ivanyi and Szabo, 1952, 1953; Buxton and Brooksbank, 1953;
Doll and Brown, 1954; Szabo, Szent-Ivanyi and Szeky, 1956; Newberne et al., 1956). The British cases by Buxton and Brooksbank were referred to the Cambridge school. Dr. R.R.A. Coombs of the Department of Pathology demonstrated antibody coating, and a widespread investigation was started leading to a series of papers between 1955 and 1959 (Buxton et al., 1955; Saison et al., 1955; Goodwin et al., 1955a, 1955b, 1956a, 1956b, 1956c, 1957; Goodwin, 1957a, 1957b; Joysey et al., 1959a, 1959b). This work clearly illuminated the underlying factors in H.D.N.B., reiterated the role of crystal violet vaccine in producing antibodies, demonstrated the detection of anti A in all non A type pigs by use of the indirect anti-globulin test, demonstrated a breed difference in the pig's ability to produce antibody as well as individual differences, showed that the A system was not implicated in H.D.N.B. by virtue of the fact that the A antigen is absent from the cells of newborn pigs, and culminated in the final reports of 11 specific immune grouping reagents detecting red cell factors 1-11 and their distribution in seven breeds of pig.

Unfortunately the Cambridge school was unable to initiate a series of planned matings, and no genetical relationships were established for factors 1-11.

A much more definite approach in this respect was shown by the third line of investigation into pig RBC typing, i.e. deliberate iso-immunisation of a recipient sow with whole blood or washed cells from a donor pig of the same or different breed.
As this approach was initiated by Andresen in Copenhagen largely with a view to parentage studies, the ability to genotype was of paramount importance. Detailed investigation of planned matings by statistical methods to establish serological and genetical relationships was an integral part of the study following production of specific reagents. A series of papers between 1957 and 1961 by Andresen in collaboration with a number of other authors (Andresen, 1957; Andresen and Irwin, 1959a, 1959b; Andresen and Wroblewski, 1959, 1961; Andresen et al., 1959) reported the findings on 16 factors (in addition to A) arranged in 9 blood group systems (Table I).

<table>
<thead>
<tr>
<th>Table I</th>
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<tbody>
<tr>
<td>Pig Red Cell Typing Factors 1961</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>A</th>
<th>Ea</th>
<th>Fa</th>
<th>Ga</th>
<th>Ha</th>
<th>Ia</th>
<th>Ja</th>
<th>Ka</th>
<th>La</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eb</td>
<td>F-</td>
<td>Gb</td>
<td>Hb</td>
<td>I-</td>
<td>J-</td>
<td>Kb</td>
<td>L-</td>
<td>Ed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ef</td>
<td></td>
<td>H-</td>
<td>(Kc)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kd</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>K-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Kc) is a factor common to Ka and Kb red cells. It is detected only by a rabbit hetero-immune reagent, and should not be confused with the factor Kc (reported later) detected by iso-immune reagents.

___ under a system indicates a closed system.

F-, H-, etc., indicates a further factor or factors to be detected, as a blank or negative phenotype has been found.
A further system, the M system, was added by the production of anti Ma in a Landrace pig by injecting blood of a Belgian Pietrain pig (Brauner Nielsen, 1961).

The findings up to 1962 were reviewed (Andresen, 1962) and a summation of Andresen's immunological, serological and statistical methods is to be found in his thesis (Andresen, 1963).

The Cambridge school at this time were fortunate in having an offer from Prof. Moustgaard of Copenhagen of using Andresen's reagents to group their panel of 28 pigs. On the basis of this one-way comparison the antigens 1-11 could be recognised in terms of their genetical relationship as laid down by Andresen (Table II).

<table>
<thead>
<tr>
<th></th>
<th>Total number of animals grouped: 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copenhagen</td>
<td>Cambridge</td>
</tr>
<tr>
<td>A</td>
<td>A</td>
</tr>
<tr>
<td>Ea</td>
<td>2</td>
</tr>
<tr>
<td>Eb</td>
<td>1</td>
</tr>
<tr>
<td>Ef</td>
<td>13 *</td>
</tr>
<tr>
<td>Fa</td>
<td>8</td>
</tr>
<tr>
<td>Gb</td>
<td>6</td>
</tr>
<tr>
<td>Ha</td>
<td>14 *</td>
</tr>
<tr>
<td>Ka</td>
<td>5 ?</td>
</tr>
<tr>
<td>Kd</td>
<td>12 *</td>
</tr>
<tr>
<td>La</td>
<td>10</td>
</tr>
<tr>
<td>Ka</td>
<td>4 + 5 ?</td>
</tr>
<tr>
<td>No equivalent</td>
<td>3, 7, 9, 11 - -</td>
</tr>
<tr>
<td>Be Ga Ta Ja Kb Ma</td>
<td>No equivalent - -</td>
</tr>
</tbody>
</table>
Factors 12-14 were additional factors isolated in Cambridge (Lang - unpublished). A further factor 15 isolated in Cambridge was shown to be identical to a factor Ib reported later.

Antigens 4 and 5 represented a subtyping situation at that time since 4+5- animals were unknown in the British pig population under test (see Ka, Ka₂, Saison and Ingram, 1962). After investigations by other laboratories and following the 1966 comparison test (Hesselholt and Brauner Nielsen, 1966) Ka₁ (4) was renamed Ke, but see also Saison and Ingram (1962), Saison (1967b), Brucks (1966) and Dinklage (1968).

As a result of the first international comparison test (Andresen and Baker, 1962) factor 3 was renamed Le a factor in the L system (Brucks, 1964), and limited mating data made factor 11 eligible as the product of an allele Lᵢ antithetical to Lₑ (Lang - unpublished). At this time it appeared that the L system might consist (like the E system of pigs and the Rhesus system of man) of three pairs of closely linked genes (Lᵃ, Lᵇ and Lᶜ, Lᵈ and Lₑ with Lᵢ) although the option was still open for a single multiple allele locus, each allele producing several factors in different combinations. The latter point of view was substantiated when anti Le from Gottingen was fractionated into Lc, Lg and Li (Brucks - personal communication) and factors La, Lb, Lc, Ld, Lf, Lg, Lh, Li, Lj, Lk and Ll were reported (Hojny et al., 1966). These
appear in a series of complex agglutinogens or phenogroups (Andresen, 1963) governed by the following alleles (the L for the system is left out): adhi, adhjl, adhjk, agi, bcgi, bdfi. In order to clarify the position of the Cambridge factor 3 (the Edinburgh factor Le) and other Cambridge factors 7, 9 and 11 (postulated Lf) a small private one way comparison test was carried out with 12 highly selected pigs from Gottingen (Brucks and Lang - unpublished). This test indicated identity between Gb/Sc La (Cambridge 10) and D/Go La, Gb/Sc Le (Cambridge 3) and D/Go Lg, and Gb/Sc Lf (Cambridge 11) and D/Go Ld. Gb/Sc 7 had no relationship with the L system (and is now known as Da as indicated above) and Gb/Sc 9 remains from the Cambridge factors unidentified. Gb/Sc and D/Go are the code letters for the Edinburgh and Gottingen laboratories when participating in the international comparison tests. A confusing situation has now arisen regarding factor Le since despite the division of the D/Go Le into Lc, Lg and Li the designation Le having been published (Brucks, 1964) should have been retained for the factor now known as Lg (D/Go) to which Gb/Sc Le and Dk Le (Copenhagen) appear to be identical (Hesselholt and Brauner Nielsen, 1966). Unfortunately no great weight can be placed on the 1966 comparison test as the Danish pigs used all had identical phenotypes for L factors b, c, e and g. The distinction between these factors is based on the small test with Brucks and myself, Brucks having substituted c, g and i for D/Go Le. In summary then it appears that Gb/Sc Le and Dk
Le are identical with D/Go Lg and CS Lg (Hojny, Czechoslovakia).

To return momentarily to the factor Gb/Sc Lf (Cambridge 11) equivalent to D/Go Ld (Brucks and Lang - unpublished). In the 1966 comparison test (Hesselholt and Brauner Nielsen, 1966) this factor, Gb/Sc Lf, was reported as equal to D/Go Lh and CS Lh. However, a closer scrutiny of the results will show that this is not incompatible with our results (Brucks and Lang) above, since only one pig could differentiate Lh from Ld amongst the Danish pigs. This pig's blood was not tested with the reagent for Gb/Sc Lf (11) as it arrived from Copenhagen in a haemolised condition. It is interesting to note that, in so far as this batch of 40 Danish pigs was concerned, the only pig differentiating Lh (D/Go and CS) from Ld (D/Go and CS) was also the only pig reacting with anti Lf (D/Go and CS). On this basis also Pl/Po Ld (Poznan, Poland) was equal to Lh (D/Go and CS). This clearly illustrates the difficulties encountered when attempting to establish the specificity of blood typing reagents on a limited breed basis. Unfortunately it is not always possible to test reagents thoroughly by absorption as well as by direct test against an internationally representative panel, as there are practical difficulties in distributing a large enough quantity of blood to a large number of overseas laboratories.

In addition, because of the delay in circulating the results of international comparison tests the opportunity to check doubtful or incorrect results by absorption techniques is lost. Comparison tests, however, serve a useful function in
### Table IIIa

**List of Blood Group Factors in the 1968 Comparison Test**

**Red Blood Cell Typing**

<table>
<thead>
<tr>
<th>System</th>
<th>Factors</th>
<th>Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A0</td>
<td>A, O</td>
<td>A&lt;sup&gt;a&lt;/sup&gt;, A&lt;sup&gt;0&lt;/sup&gt;, S, s</td>
</tr>
<tr>
<td>B</td>
<td>a, b</td>
<td>a, b</td>
</tr>
<tr>
<td>C</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>D</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>E</td>
<td>a, b, d, e, f, g, h, i</td>
<td>dbg, degh, aeg, defh, dbf, aef, -eg, aegi</td>
</tr>
<tr>
<td>F</td>
<td>a, b</td>
<td>a, b</td>
</tr>
<tr>
<td>G</td>
<td>a, b</td>
<td>a, b</td>
</tr>
<tr>
<td>H</td>
<td>a, b, c, d</td>
<td>a, b, c, ab, d</td>
</tr>
<tr>
<td>I</td>
<td>a, b</td>
<td>a, b</td>
</tr>
<tr>
<td>J</td>
<td>a</td>
<td>a</td>
</tr>
<tr>
<td>K</td>
<td>a, b, c, d, e</td>
<td>ac, ace, ad, ade, b, -</td>
</tr>
<tr>
<td>L</td>
<td>a, b, c, d, e, f, g, h, i, j, k, l</td>
<td>adhi, bogi, bdfl, agi, adhjk, adhjl</td>
</tr>
<tr>
<td>M</td>
<td>a, b, c</td>
<td>a, b, c</td>
</tr>
<tr>
<td>N</td>
<td>a, b, c</td>
<td>a, b, bc&lt;sup&gt;+&lt;/sup&gt; (Saison, 1967a)</td>
</tr>
<tr>
<td>O</td>
<td>a, b</td>
<td>a, b</td>
</tr>
</tbody>
</table>

**Note:** To facilitate presentation the capital letter of the system has been omitted from the lists of factors and genes, e.g., the factor Ed is indicated only by its subscript d, and the gene L<sup>bogi</sup> is indicated only by its superscript bogi.

Factors underlined are those in use in Edinburgh at the time of the test.
establishing confidence in the unit specificity of reagents which have been produced and standardised against a panel of limited genotypes and breed. Blood group system relationships are made apparent much more rapidly to a large number of laboratories. A word of warning is necessary here, however, as, due to the delay in publication of data in international journals, conflicting claims for inclusion of new factors in new or old established blood group systems can arise. As long as one individual or laboratory is far in advance of all other workers with results and data of repute, the situation is relatively simple. The complexity of blood group systems, however, increases in direct proportion to the amount of work and number of breeds worked upon, and the 1968 comparison test showed more than one hundred different reaction patterns against only forty pigs. Fifty of these reaction patterns are arranged in fifteen established systems (Table IIIa), but the remainder are non-systematised. Factors should only tentatively be assigned to blood group systems until sufficient breeding data can be accumulated and published.
Classification of Antibodies by Serological Type and Mode of Production

A consideration has been made of the three ways in which pig red blood typing has developed, and this development has provided us with porcine antibodies of well established specificity and different characteristics.

The investigation of "natural" antibodies has frequently been complicated by the use of swine fever vaccine. Many of the earlier investigations utilised only the saline agglutination test and failed to detect many antibodies, e.g. Szymanowski et al. (1926) showed three classes of pig in relation to the A antigen: A positive and A negative, the latter being subdivided according to the presence or absence of anti A (α) in the serum, hence A₀, Oα, and O₀. Later, by the use of the anti-globulin or other more sensitive tests, practically all group O (non A) pigs can be shown to possess anti A (Saison et al., 1955). "Natural" antibodies, other than anti A and anti O (heterologous), are not taken into consideration nowadays, although the discontinuation of swine fever vaccination in the United Kingdom allows a consideration of the "natural" origin of H.D.N.B. Thus recent studies (Linklater, 1968) have shown evidence of transplacental immunisation in the sow to antigens particularly of the E system present in piglets and the boar.

Investigations of H.D.N.B. arising as a result of crystal violet swine fever vaccination gave a large number of high titre antisera, since these were more likely to give rise to
affected piglets. This was the basis of a diagnostic slide test (Goodwin, 1957b). Because of their method of production by repeated injections of whole blood vaccine, however, it was found that these antisera on analysis broke down into reagents of many specificities frequently only detectable by the most sensitive technique. By virtue of the work's location (in Cambridge) this was the indirect sensitisation test (of Coombs) (Saison et al., 1955). A papainised red cell test (Olds, 1961) was introduced at a later date. Unfortunately the antigens $6$ (Gb), $8$ (Fa) and $9$ are totally destroyed by papain (Olds, 1961), and it is likely that other factors within the same blood group systems will suffer similarly. This has been found to be so for the factor Ga (Lang - unpublished results). This phenomenon can be utilised in preparing reagents to high frequency antigens like $6$ (Gb) and $9$, where selection of a suitable absorbing cell negative for such factors, but positive for factors reacting with contaminating antibodies, can be facilitated by destroying the unwanted antigen with papain (Olds, 1961; Lang - unpublished). These facts are of interest in a consideration of red blood cell surface and antigen structure since we can postulate two types - papain susceptible and papain enhanced. This can show some relationship to accessibility to antibody and degree of agglutinability. It has been reported (Hardy, 1968) that incomplete antisera (i.e. antibodies normally requiring the antiglobulin test or enhancement with macromolecular media) to the G system will
react with lightly papainised cells only if antiglobulin is added in the normal way. Where the saline agglutination or antiglobulin technique has been the method of choice the papain treatment of red cells has not been widely used. One reason for this is that in reagents of moderate or weak titre standardised by other techniques, subliminal contaminants are often preferentially enhanced by a papain technique (Lang - unpublished observations). As a result careful control over the degree of enzyme treatment is required, and since different blood group systems vary in this requirement, a uniform method for routine use is difficult to achieve. Similarly where a haemolytic technique is normally used the effects of non-haemolytic contaminants would be revealed. Despite this, however, a recent report (Hardy, 1968) has indicated that light treatment with bromelin or papain used in conjunction with the normal agglutination, haemolytic or antiglobulin technique can enhance titre and shorten incubation time.

If we only consider the more conventional techniques such as saline agglutination, haemolysis and the antiglobulin (Coombs) test then the majority of reagents produced from the H.D.N.B. approach fall into the incomplete class with the exception of antibodies to the E system. Thus out of 51 reagents produced from H.D.N.B. sows' sera only 4 reacted sufficiently well by direct agglutination (Lang - unpublished observation). Two of these were against the Ea factor and two against the Eb factor. Of the remaining 24 reagents
produced by iso-immunisation in Cambridge and Edinburgh only 7 were as suitable or more suitable for use by the direct agglutination test, and all of these were against factors in the E system. Of course this is partly a function of the sensitivity of the antiglobulin test (Marrack, 1963), and it could be argued that use of the antiglobulin test for screening sera for antibodies acts as a selective mechanism for one class of immunoglobulins. However, providing the antiglobulin is a wide spectrum reagent (Dunsford and Grant, 1959) reacting against 19 S and 7 S globulins and components of complement (Polley et al., 1962) the antiglobulin test rarely fails to pick up the weakest reaction.

In many laboratories producing reagents by iso-immunisation the antiglobulin test, although used, is not the system of choice. A prozoning phenomenon has been reported (Andresen, 1963) using rabbit anti pig globulin, and variation in optimum dilution of antiglobulin reagents with different pig blood grouping systems has been shown (Andresen, 1963) (but see later under Methods). In these laboratories a panel of reagents reacting by a wider variety of techniques is usually selected. Andresen lists his panel of reagents (Andresen, 1962). Out of 21 iso-immune reagents only 4 are used as incomplete agglutinins (i.e. requiring antiglobulin or dextran test), 2 are used in the haemolytic test and 15 are agglutinins. Even allowing that 6 agglutinins are for the E system, we still have a much higher frequency of agglutinating antibodies. Of
five normal or hetero-immune sera are lysins, the one exception being a pig normal anti A agglutinin. Two of the lysins are normal cattle sera for the A-0 system of pigs, and two are rabbit hetero-immune reagents for the porcine K system. An almost identical distribution is seen in another small series (Imlah, 1965a). The introduction of the haemolytic test is most common by individuals or laboratories associated with cattle typing. The reason for this is that cattle cells suffer from inagglutinability even by the antiglobulin test, and the haemolytic test is invariably used to overcome this (Gleeson White et al., 1950; Coombs et al., 1951; Seaman and Uhlenbruck, 1963; Uhlenbruck et al., 1967). However, the use of one test only for routine typing is dangerous, particularly where a single tube/single reagent test is operated, as technical error cannot be entirely avoided. In addition, some cells fail to haemolise on a quantitative basis (Franks, 1962) although the papain test and antiglobulin test are positive. Similar results are demonstrated for pig (Lang and Imlah - to be published).

Another technique for use with incomplete antibodies is to use macromolecular media as diluent for the antiserum or red cell suspension or by a "Sheffield replacement" technique (Dunsford and Bowley, 1967). In the latter method used with anti Rhesus reagents of the albumin incomplete type, the red cell suspension and antiserum are incubated in the normal way as for the saline agglutination test. Before reading the
test, however, the supernatant fluid is removed with a flick of the wrist, and one volume of 20% bovine serum albumin is added to the cells remaining. After a further half an hour incubating the results are read in the usual way. Prior to the introduction of the "Coombs" test this "so-called" (Coombs et al., 1961) conglutination test (Wiener, 1945; Wiener et al., 1947) was used to detect coating of infants' cells in H.D.N.B. due to 7 S incomplete antibodies of the Rhesus factor D. Previously the only test available for detection of "incomplete" antibody coating was the blocking test (Wiener, 1944). Other macromolecular substances can be used, however (Dunsford and Bowley, 1967), such as human group AB serum which is used particularly for the Kell-Cellano system in man (Lang and Lodge, 1961).

The only macromolecular medium used in pig blood group serology is dextran. This is a polysaccharide of varying molecular weight used as a plasma substitute in man. Dextrans of different origins (i.e. manufacturing and bacterial origins) appear to have different molecular weight ranges and differ in their clinical effects (Mollison, 1961). These variations may also be reflected in their varying ability to enhance blood group reactions. Thus Andresen (1963) reports that satisfactory results could be obtained for Ga and Ja typing with a 10% solution of Macrodex Pharmacia DRL 204, although a number of false positive results were obtained as compared with the antiglobulin test. These non-specific results could be
prevented by the addition of small quantities of serum (Munk-Andersen, 1959). Dextran was not suitable for reagents to Ha and Ia blood group factors (Andresen, 1963). Hojny and Hradecky (1968), however, report considerable success in reactions with Kb, Kc, Lb, Lc, Ld, Lg, Lf, Lh, Lj, Lk, Ll, Oa and Ob blood factors with a dextran of Czechoslovak manufacture. It would appear, therefore, that different blood systems might benefit from the use of dextrans of different molecular weights or varying mixtures of dextrans with saline or serum, as in the human Rhesus and ABO blood group systems (Munk-Andersen, 1956, 1959). Wiener found that mixtures of human albumin and immune globulins gave the best results in his conglutination test when the ratio equalled the normal albumin/globulin ratio (Wiener et al., 1947). Alternatively in the case of dextran the possibility of some "contaminant" in certain brands cannot be ruled out, as was found to be the case in bovine serum albumin with human Rhesus typing (McCulloch, 1950).

An excellent summary of the various types of antibodies to pig red blood cell factors is given by Hojny and Hradecky (1968). Dividing antisera according to titre and test sera type they find that anti A, Bb, Da, all reagents of the E system (Ea, Eb, Ed, Ee, Ef, Eg, Eh) and Fa, Ka, Kd, Ke, La, Li, more exceptionally Ga, Gb, Kb, and Lg, belong among complete agglutinins. The antibodies giving reactions in the dextran test are given above. Reacting only in the antiglobulin test are numerous reagents against Ca, Ha, Hb, Hc, Ia, Ib, Ja, Ma,
Mc, M(d), Na, Nb, and also Ga and Gb. Least well represented are haemolysins which only appear against A, (Ap) (Hojny and Hala, 1964), Hb, Ka, Kd and Kb. Of course the classification is not so distinct as this list might suggest. For example probably all these reagents would react by the antiglobulin test, but this test is laborious to perform and requires the preparation and standardisation of appropriate antiglobulin reagents. Many of the haemolysins will also agglutinate, but the haemolytic test is very quick and easy to read. The saline agglutination test is a more basic test since it does not require any reagents other than cells and serum, but takes time to read the results especially if this is carried out microscopically on slides.

This type of classification of blood typing reagents is not absolute, therefore, since most antisera are mixtures of different immunoglobulins, and under suitable conditions can be reclassified. However, such information in conjunction with other forms of immunological analysis forms a basis to work from in questions of antigenic configuration and structure, immunoglobulin classes and structure and the general immunological capability of the pig.

Precipitating antibodies in the pig will be dealt with under a later section.

A further technique which has hardly been used in blood typing at all in any species is the direct conglutinating technique and its indirect counterpart the conglutinating
complement consumption technique. Like the antiglobulin test this technique is very sensitive in either of its forms, but requires the use of suitably absorbed and standardised complement and conglutinin or immuno-conglutinin. In addition the antibodies under investigation must be complement consuming in conjunction with their homologous antigen, but not necessarily haemolytic (Soulsby, 1958; Coombs et al., 1961). Theoretically pig serum should be very satisfactory in the indirect conglutinating absorption test, since pig serum has a high titre of conglutinating complement with a bovine antibody/sheep red blood cell indicator system. In the direct test pig antibodies would probably work best with horse complement (Coombs et al., 1961), although this might depend on the antigen/antibody system under consideration.

Hitherto we have been classifying blood typing reagents in terms of their serological reaction and mode of origin, and it is recognised that this type of classification is somewhat arbitrary as most reagents are highly heterogeneous mixtures of various immunoglobin classes. The differentiation of these classes will be dealt with during the consideration of immunoglobulin isotypes and allotypes. In an attempt to link the mode of preparation, the type of serological reaction and the antigen used for injection with some physico-chemical measurement, Podliachouk et al. investigated the action of beta mercaptoethanol on various pig, chicken, duck and cattle reference sera. No relationship was found, however, between
any of the above variables, although it was shown that pig antisera in contrast to chicken reagents were largely resistant to the action of beta mercaptoethanol (Podliachouk et al., 1966). This gives some indication that most of the porcine reagents are probably of a 7 S Ig G type rather than 19 S Ig M.

Serum Type Systems in the Pig

Since the discovery of the human ABO blood groups in 1900 the science of blood grouping, or immunogenetics to give it a modern term, has grown with intensive investigations of human and animal red cell type factors. During the last ten years, however, no "blood grouper's" repertoire has been complete without a knowledge of serum type systems in addition to the classical red cell type approach to blood grouping.

The term "serum type systems" is a broad one, and yet today it fails to cover all the other body fluids, such as non-haemoglobin erythrocyte protein, saliva, seminal plasma, seminal vesicle fluid, milk and colostrum, haemoglobin, gastric juice and cyst fluids which have been investigated for genetical polymorphism (Haut, 1962; Tucker, 1962; Horowitz et al., 1964a, 1964b; Lodge and Usher, 1962; Lodge and Voak, 1968; Lodge et al., 1965; Bednekoff et al., 1963; Goodwin and Coombs, 1956; Hojny and Hala, 1964; Boursnell et al., 1962; Matousek, 1966; Matousek et al., 1966; Wake et al., 1961; Grosclaude et al., 1966; Larsen and Thymann, 1966; King, 1966; Glasnak, 1966; Karlsson, 1966a, 1966b; Evans,
1954; Allison, 1956; Huisman, 1966; Rasmusen and Hall, 1966; Tucker, 1968). In order to encompass this motley of variants the term biochemical polymorphisms has been introduced. This term has the added advantage of having no connotations as regards the technique for demonstrating variation nor does it stress the nature or cause of the polymorphism. Its disadvantage is that it is too general and imprecise and could be taken to include biochemical variation of a purely physiological, nutritional, ontogenic or sexual origin unless we make the proviso that the variation must be inherited. The term immunogenetics not only stresses the inherited nature of variants, but also stresses the method of determination as being as a result of immunization or immunity (the allergic state — Von Pirquet, cited by Gell and Coombs, 1963). Of course this term cuts out serum type systems and indeed non-serum type systems, which are determined by physico-chemical methods except in so far as they are associated with antigenic differences. This distinction is largely an artificial one, however, and illustrates a weakness and a strength of the immunological method now being emphasised by amino acid sequence analysis of immunoglobulins. The weakness is that only differences and not homologies can be demonstrated, and that only by immunization of a suitable animal. The strength is that thereby the intraspecies and interspecies differences are strongly outlined in the sea of homology. The distinction is also largely artificial in that a combination of physico-
chemical and immunological methods are often used to distinguish variant forms (see Immunoglobulins later).

Serum Type Systems determined by Starch Gel Electrophoresis

Until recently most biochemical polymorphisms in the pig other than red cell types have been determined by means of a combination of electrophoresis (usually starch gel electrophoresis) and specific or partially specific staining. A one dimensional continuous buffer starch gel system was first used in 1955 to differentiate human serum groups (Smithies, 1955a, 1955b; Smithies and Walker, 1955, 1956). These serum components were identical with haptoglobins, previously detected by several authors on paper electrophoresis as $\alpha_2$ globulins binding haemoglobin (Jayle et al., 1952; Wieme, 1953; Tuttle, 1955; all cited by Smithies and Walker, 1956) and have since been widely investigated on a genetic, population and preparative basis (e.g. Allison et al., 1958; Connel and Smithies, 1959; Smithies et al., 1962; Nance and Smithies, 1963). From this beginning there followed a rash of investigations on other serum proteins in man, e.g. iron binding proteins or transferrins (Smithies, 1957, 1958; Horsfall and Smithies, 1958; Harris et al., 1958; Smithies and Miller, 1959), and in animals (Ashton, 1957a, b, c, 1958a, b, c, d, e, f; Ashton and McDougall, 1958). Investigations on pigs were a little slow off the mark as Ogden reviewing biochemical polymorphism in 1959 mentions goats, sheep, man, dog and cattle but not pigs.
However, in the following year Ashton (Ashton, 1960) reported thread protein (serum amylase) and transferrin variants in pig. A year later three haptoglobin variants were differentiated (Kristjansson, 1961). Further reports followed on transferrins (Kristjansson, 1960a, 1960b; Imlah, 1963, 1964; Shroffel, 1966; Baker, 1968a), amylases or thread proteins (Imlah, 1963, 1964; Shroffel, 1964; Hesselholt et al., 1966; Hesselholt, 1968), haembinding globulins or haemopexins originally called haptoglobins in error (Hesselholt, 1963; Imlah, 1963, 1964; Shroffel, 1966; Hesselholt and Hristic, 1966; Baker, 1967), ceruloplasmins (Imlah, 1963, 1964), prealbumins (Kristjansson, 1963, 1966) and $S$-alpha$_2$ globulins (Shroffel, 1964). During these years review papers were published on various species (Brummerstedt-Hansen et al., 1962; Graetzer et al., 1964) and on the pig (Imlah, 1964; Baker, 1968b). By varying the operating conditions of the starch gel technique (single dimensional continuous, two dimensional with agar gel, vertical starch gel, cooling, different gel concentrations, voltages or currents) it has been possible to produce good results for almost any system. A notable improvement was the introduction of the discontinuous buffer system (Poulid, 1957), whereby during the run the buffer within the gel is replaced by a different buffer from the electrode tanks. This results in a very sharp potential gradient at the buffer changeover boundary or "brown line" as it is called, and this moves forward in the gel producing a sharp differentiation of
appropriate serum protein components. Probably the most important reason for the success of the starch gel among others is its ability by virtue of its pore size to differentiate serum protein polymorphism on the basis of molecular weight and degree of polymerisation, i.e. molecular filtration as well as by difference in charge. This overlying of the basic charge effect by a molecular and steric hindrance influence results in confusion where proteins have previously been classified after Tiselius on the basis of mobility on free electrophoresis or agar gel electrophoresis. For fundamental work, determination of molecular size can be carried out by ultracentrifugation or gel filtration, and charge or mobility determination can be done on agar gel or cellulose acetate electrophoresis.

For routine determination of biochemical polymorphism in serum proteins or other bodily fluids, however, the starch gel technique is unsurpassed where the variation is a function of molecular size and/or difference in mobility due to electrical charge.

The localisation of particular proteins on starch gel is achieved by specific (e.g. Sudan B, Oil Red 0 for lipoprotein) or partially specific (nigrosin or amido black for protein in general) staining, or more satisfactorily by linking the protein with a biochemical function (digestion of starch by amylase, Fe$^{59}$ binding by transferrins, oxidase activity of caeruloplasmin on paraphenylene-diamine, peroxidase activity of haptoglobin/haemoglobin and haemopexin/haematin complexes on
hydrogen peroxide detected by benzidine). The detection by direct biochemical techniques linked to a possible physiological function is particularly important for enzyme polymorphism (amylases, Hesselholt, 1968; serum alkaline phosphatases, Saison, 1968; lactic dehydrogenases, Hyldgaard-Jensen, 1968; and red cell 6-phosphogluconic dehydrogenase and phosphohexose isomerase, Saison, 1968) since enzymes tend to occur in association with a number of proteins.

Since the normal type of starch gel is opaque it is impossible to use an immunological precipitation technique (immuno-electrophoresis) for localisation of proteins, except by combining a preliminary electrophoresis on starch gel with subsequent embedding of strips of starch gel in agar gel with antibody troughs (Brummerstedt-Hansen et al., 1962). An alternative is to carry out an electrophoresis in starch gel in one direction, and then carry out a further electrophoretic migration at right angles to the first into agar or agarose gel containing antiserum against the specific protein under investigation (Fagerhol and Hauge, 1968).

Generally, however, for biochemical polymorphism manifesting itself by physico-chemical differences (as opposed to antigenic differences) the use of immunological localisation is restricted to proteins where charge differences are sufficient to give adequate separation on agar gel with subsequent formation of precipitation arcs. Thus it is possible to demonstrate an alpha-globulin variation in pigs.
Table IIIb

Biochemical Polymorphism in the Pig

up to the end of 1968

<table>
<thead>
<tr>
<th>Serum</th>
<th>Symbol</th>
<th>Factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transferrins</td>
<td>Tf</td>
<td>A, B, C, E‡, D</td>
</tr>
<tr>
<td>Amylase</td>
<td>Am</td>
<td>1, F2, 2, 3</td>
</tr>
<tr>
<td>Haemopexins</td>
<td>Hx, Hp</td>
<td>0, 4, F1, 1, 2, F3, 3</td>
</tr>
<tr>
<td>Prealbumins</td>
<td>Pa</td>
<td>A, B</td>
</tr>
<tr>
<td>Caeruloplasmins</td>
<td>Cp</td>
<td>A, B</td>
</tr>
<tr>
<td>Slow alpha 2 globulins</td>
<td>Sα2</td>
<td>A, B, C</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>Akp</td>
<td>A, B, C</td>
</tr>
<tr>
<td>Red cell</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-PGD</td>
<td>PGD</td>
<td>A, B</td>
</tr>
</tbody>
</table>

‡ Baker (1968)
(Brummerstedt-Hansen, 1967) similar to Gc types in man (Hirschfeld, 1960).

By the end of 1968 the well established systems of serum polymorphism in the pig, briefly reviewed by Baker (1968b), were expanded by the inclusion of serum alkaline phosphatase variants (Saison, 1968). In addition, investigation of pig red cell enzyme systems revealed variation in 6-phosphogluconic dehydrogenase (6-PGD) and phosphohexose isomerase (Saison, 1968). An analysis of 6-PGD inheritance has been made (Saison and Giblett, 1969) revealing the existence of two alleles (PGD^A and PGD^B) and three phenotypes.

The systems under investigation in the 1968 comparison test (Dinklage, 1968) are summarised in Table IIIb, although not all the variants were represented in the limited panel available.
Serum Immunogenetics

In the previous section I have dealt with serum groups detected as a result of differences in molecular size and/or electrical charge, and the localisation of proteins by means of specific precipitating antisera was only secondary to this.

The starch gel electrophoretic technique is not the method of choice for all serum groups, either because an earlier or easier method already exists of a more sharply specific type, or because some proteins by virtue of their continuous size and charge distribution or invariant distribution do not show banding or show no variation in that banding.

In these situations resort is frequently made to an immunological method of demonstrating antigenic variation by using hetero- or iso-immune reagents, "natural" antibodies or other biological reagents, e.g. extracts of plant seeds (Datta, 1968; Hossaini, 1968; Dechary, 1968; Boyd, 1963), snails (Gold and Thompson, 1969) or marine sponges (Dodd et al., 1968).

In the pig only two systems have yielded to this form of investigation: porcine gamma globulin allotyping (Rasmusen, 1965a) and the soluble A0 blood group substances (Rasmusen, 1964).

The Immunoglobulins of the Pig

The proteins with the slowest electrophoretic mobility, that is the gamma globulins, do not resolve themselves into discrete bands even on starch gel electrophoresis. On the
contrary they consist of an extremely heterogeneous mixture of globulins whose iso-electric points show a continuous distribution over 1-2 pH units. However, since this group of globulins, the immunoglobulins, is associated with antibody activity and the allergic state (after von Pirquet, cited by Gell and Coombs, 1963), they are of great interest to investigators from many fields. As a consequence contributions to the elucidation of their biological properties, classification, isolation, chemical structure, synthesis and mode of inheritance have been derived from many disciplines and involved a tremendous collection of techniques and combinations of techniques.

This has resulted in a great deal of information and knowledge about immunoglobulins particularly in man. A detailed review of this knowledge and its methods of collection is beyond the scope of this thesis, and I shall draw freely on various review articles (Cohen and Milstein, 1967; Lennox and Cohn, 1967; Oudin, 1966; Webb and Goodman, 1967) to present a summary of the findings up to the beginning of 1968. With the exception of a limited number of experimental animals such as rabbit, guinea pig, mouse and the horse, the sera of animals is less well served. A start has been made, however, on investigations of the pig, sheep, goat, dog, ox, chicken, baboon and rat, and with the increasing interest in the evolution of the immunological response and the genetics of immunoglobulins even in the body fluids of lower vertebrates such as the bullfrog
Early investigations were largely dependent upon rather crude salting out or precipitation procedures involving the differential solubility of different proteins in distilled water, salt solutions, organic solvents or at different pH values, and also upon simple electrophoresis either in free solution or on paper (Sehon, 1963; Tiselius and Kabat, 1939; Tiselius, 1937). Using these techniques and others, such as ultracentrifugation and isolation of antibody from specific immune antigen/antibody aggregates, a limited amount of physico-chemical data on the gamma globulins of pigs was obtained, and the percentage of the various serum proteins (albumin, alpha, beta and gamma globulins) could be ascertained in different pigs of various ages, pre- and post-natal, pre- and post-colostral.

The isoelectric point of pig gamma globulin was shown to be at pH 5.86 (Tiselius and Kabat, 1939) but isolated antibody against pneumococcal polysaccharide had an isoelectric point of 5.1. This antibody had a molecular weight of 990,000 and a sedimentation coefficient of 19.8 (Kabat, 1939). Koenig (1949), however, found a sedimentation coefficient of 7.25 - 7.28 for isolated porcine gamma globulin. The gamma globulin area on free electrophoresis was divided into two areas, gamma 1 and gamma 2, and these could be separated by alcohol fractionation. Both these fractions had a molecular weight of 168,000 and a sedimentation coefficient of 7.1.
The iso-electric points of gamma 1 and gamma 2 were 6.1 and 7.2 respectively (Cammarata and Deutsch, 1950). Other investigators have found 6.7 S and 18 S proteins among the gamma globulins of pigs (Franek et al., 1961).

There appears to be evidence above for at least two and probably three different classes of gamma globulin on physicochemical grounds. Most of the work done on quantitative and qualitative analysis of pig serum components with particular relation to antibody activity, however, has been done without reference to any particular types of antibody. Activity has either been crudely equated with the gamma globulin area on paper or free electrophoresis, sulphate precipitation turbidity or calculated by serological activity of various types against specific antigens such as bacteria. Thus twenty-nine papers have been reviewed (Brummerstedt-Hansen, 1967) describing the percentage occurrence of albumin, alpha, beta and gamma globulins in pig serum as determined by free electrophoresis or on paper or cellulose acetate.

A particularly active area for investigation has been the qualitative and quantitative differences between the serum constituents of pre-natal, post-natal and adult pigs. These researches have been concerned with production of antibody by the piglet, passage of antibody across the placenta, and the acquisition of passive immunity through the colostrum. Since the serum of foetuses and newborn piglets differ qualitatively as well as quantitatively from that of mature pigs,
investigators utilising only electrophoresis found it difficult to correlate the various fractions obtained at different ages. Conflicting results particularly in relation to the presence of gamma globulins in pig foetuses and immediate post-natal piglets have been found (Moore et al., 1945-present; Rook et al., 1951 - absent; Waddill et al., 1962 - present up to 10%; Earle, 1935 - absent; Jakobsen and Moustgaard, 1950 - absent; Rook et al., 1951 - absent; Barrick et al., 1954 - absent; Bauriedel et al., 1954 - absent; Moustgaard, 1956 - absent; Kohler, 1956 - absent; Gwatkin and Annau, 1959 - absent; Lecce and Matrone, 1960 - absent; Lecce et al., 1961 - absent; Asplund et al., 1962 - absent; Foster et al., 1951 - 2.5 - 8.7% present; Miller et al., 1961 - present; Owen et al., 1961 - present; Pirtle and Deyoe, 1963 - present; all cited by Brummerstedt-Hansen, 1967). With such a large number of conflicting views it is obvious that a number of causes must be operating, and the question also arises as to whether antibody crosses the placenta. Here again the position is not clear. A so-called normal non-antibody gamma globulin is found by concentrating sera of foetuses or newborn animals up to 100 times. This protein is found in amounts of 10-40 μg per ml of original serum (Sterzl et al., 1960; Franek et al., 1961; Franek and Riha, 1964, cited by Brummerstedt-Hansen, 1967). These authors consider that antibody does not pass the placenta in swine, and pigs along with the horse, ox, sheep and goat are generally thought to obtain their passive immunity via the
colostrum (Brambell, 1958; Olsson, 1959a,b,c). Evidence has been shown of transplacental passage by the finding of specific antibody in colostrum-deprived piglets from a passively immunised sow (Myers and Segre, 1963). However, Kim et al. in a whole sequence of papers (1966a,b,c, 1967a,b, 1968) consider that germ-free, colostrum-deprived piglets are immunologically virgin, and are suitable for investigation of the true primary immune response. Basic to this concept is the establishment that, if adequate care is taken in separating piglets aseptically from the sow in such a way that no contamination with maternal serum occurs, there is no antibody present in piglets' serum. High titre antibodies were produced in the sow to known specific antigens, which were used in a very sensitive assay for antibody on several times concentrated piglets' serum. They concluded that germ-free, colostrum-deprived piglets' serum did not contain antibody with one or two exceptions. One is where a piglet was allowed to become contaminated with maternal body fluids, and the other is where bacterial toxins were thought to have produced placental damage. The question of antibody production and/or transplacental transfer in foetuses in the field is still largely open to doubt.

There is no question, however, that the greatest change in piglet serum constituents including the acquisition of passive immunity occurs as a result of colostrum uptake, and absorption of antibody from colostrum and/or serum has been the subject of
many papers. Here again the method of identification and/or quantitation of antibody has depended to a large extent on salting out experiments (Earle, 1935; Jakobsen and Moustgaard, 1950), on paper electrophoresis (Miller et al., 1962; Asplund et al., 1962; Waddill et al., 1962; Rook et al., 1951; Nordbring and Olsson, 1957) or on activity against bacterial antigens and toxoid (Speer et al., 1959 - antibody against Escherichia coli; Perry and Watson, 1967a, b - antibody to Salmonella pullorum; Olsson, 1959b - bovine antibody to S. paratyphi A). Use has also been made of heterologous serum proteins in assessing globulin uptake (Locke et al., 1964; Payne and Marsh, 1962; Olsson, 1959b). These methods have also been used in assessing the ability of piglets to respond to immunological stimulus in relation to a number of factors such as age, whether colostrum fed or deprived at different times, and also the effect of the existing presence of antibody to the antigen used. The results of this type of investigation were reviewed up to 1959 by Olsson (1959a, b, c) and later investigations have been reported (Segre, 1966). Reports are rather sharply divided between authors who consider that a) transplacental passage of gamma globulin occurs and therefore piglets tend to have antibody at birth, b) colostrum-deprived piglets are relatively immunologically incompetent, c) that this can be overcome by feeding colostrum or by administration of small amounts of specific antibody and d) that these results support Jerne's concept that antibodies are required for
antibody formation; and those authors who take more or less the opposite view. The former views are typified by the work of Segre, Myers, Kaeberle and others (Myers and Segre, 1963; Hoerlein, 1957; Jerne, 1955, 1960; Eisen and Karush, 1964; Segre and Kaeberle, 1962a,b; Kaeberle and Segre, 1964a,b; Locke et al., 1964; Dawe et al., 1965; all cited by Segre, 1966). The opposite view is taken, however, by Kim and co-workers, and they consider that the serum of piglets derived by hysterectomy is completely devoid of immunoglobulin if adequate precautions are taken and there is no placental damage. Colostrum is rich in all the known immunoglobulins and these are all rapidly absorbed into the blood through the gastro-intestinal tract. Immunologically virgin colostrum-deprived germ-free piglets are immunologically competent when obtained by hysterectomy 3 to 5 days before full term. These piglets give a better immunological response to single antigens than piglets already exposed to the ubiquitous antigens found in food and the environment. This can be explained on a basis of antigenic competition (Kim in Discussion to Segre, 1966; Kim et al., 1966a). Kim et al. used an extremely sensitive actinophage neutralising technique with 20 times concentrated piglets' serum or fractions of serum, and in addition characterized the immunoglobulins formed at different stages of the immune response both in colostrum-deprived germ-free newborn piglets and older pigs by IE, Ouchterlony, ultracentrifugation, gel filtration, ion exchange analysis and mercapto-
ethanol susceptibility techniques. Their opinion was that the sensitivity of Segre's method for detecting antibody is inadequate for the early (19 S) immune response, and that the soluble toxoid antigens used are of low antigenicity which might, however, benefit from the adjuvant action of complexing with the appropriate amount and type of immunoglobulin (Kim et al., Discussion to Segre, 1966; Kim et al., 1966a). Since the situation created by Kim et al. is an artificial one, and the two opposing views are somewhat extreme, the position in the field is probably intermediate and variable in nature.

Characterization, Classification and Structure of Immunoglobulins in Man and Other Species

The greatest problem in regard to characterizing immunoglobulins has been and still is their great heterogeneity. It appears that whichever technique is used to isolate or characterize a particular molecular species, it will still be heterogeneous by other criteria. Monoclonal proteins of myelomas and lymphoproliferative disorders are a partial exception to this in being considerably more homogeneous than normal proteins, but even proteins produced by single clones of cells assume heterogeneity on secretion (Awdeh et al., 1967; Kolsch, 1967) into serum or incubation in vitro with serum. The occurrence of such proteins in man and the mouse, however, has been of enormous value in providing single molecular species for investigation particularly in recent years for the purely
biochemical amino acid sequence analyses. Other difficulties such as the size and lability of immunoglobulin molecules have been avoided by the use of physical methods such as ultracentrifugation, gel filtration (molecular sieving), electrophoretic separation and combinations of these with chemical methods such as enzymatic digestion, reduction, peptide mapping and amino acid sequence analysis. Despite the use of these relatively mild techniques the lability and variability of the biological properties of immunoglobulin proteins and physico-chemical orientation of immunoglobulin proteins makes any investigation a complex but fascinating exercise in physico-chemical and immunological techniques.

Because of this extreme complexity and mixing of different molecular populations the most important contribution to the characterization and classification of immunoglobulin (Ig) molecules has been to use them as antigens. By discovering or preparing various types of anti-antibodies it has been possible to arrange Igs into a series of classes, subclasses, allotypes and idiotypes, which in many cases can be partially correlated with specific physico-chemical and biological characteristics.

The greatest volume of work has been on man, and of course the Igs of other species have their own peculiar species specificities (although, because of homologies in primary structure, there is also cross reactivity). Because of this the relatively limited work carried out on animals has been by analogy and homology with the physico-chemical and immunological
classification in man. Since investigations in the pig are still in a fairly rudimentary stage it is necessary to review the position in man in order to lay a basis for comparison.

Anti-antibodies fall into several categories: heterologous, isologous and autologous, and using these it has been possible to demonstrate that in general the Igs of different animals have antigenic determinants specific to and present throughout all individuals of the species (isotypes), antigenic determinants varying from individual to individual within the species (allotypes), antigenic determinants associated with antibody molecules of a particular antibody specificity within certain individuals only (idiotypes) and finally some only existing or detectable after Ig molecules have been modified or changed in certain ways.

**Heterologous Antibodies and Isotyping**

Since Ig molecules or any other sort of protein molecules possessing isotypic determinants are normally present in all individuals of a species, isotypes are normally detected by hetero-immune reagents produced in a convenient animal such as rabbit, goat or horse. Occasionally iso-immune reagents can be produced in an animal where a particular protein is abnormally absent. Thus Gahne reported the production of antibodies against slow $\alpha_2$-globulins of cattle by one of three cattle found without the slow $\alpha_2$ or 19 S-$\alpha_2$-glycoprotein zone on starch gel electrophoresis (Gahne, 1964). This antigenic
determinant was incorrectly reported as an allotype since in this instance the protein in question was missing, and by definition (Dray et al., 1962) the presence of the isotype must be established in the animal producing a postulated allotyping reagent. In this case the protein carrying the isotype determinant was absent, and the reagent produced reacted with all of forty cattle sera tested by double diffusion precipitation technique in immuno-electrophoresis. This cattle serum unlike a number of reagents produced in rabbits (against human $\alpha_2$-macroglobulin and rat slow $\alpha_2$-globulin - Heim, 1968; against human $\alpha_2$-macroglobulin and pig $\alpha_2$ macroglobulin - Brummerstedt-Hansen, 1967) did not cross react with the $\alpha_2$-macroglobulin of a number of species tested (man, sheep, pigs, reindeer, horse, mink and rabbit) so that the antiserum appeared specific for cattle slow $\alpha_2$-globulin.

Almost 25 years ago R.R.A. Coombs developed the anti-globulin test (Coombs et al., 1945, 1946) previously used by Moreschi (Moreschi, 1908a,b) for detecting incomplete antibodies on the surface of bacteria. Heterologous antisera to globulins (Coombs' reagent) are used to agglutinate red cells (or bacteria, inert particles or any suitable cell) which have previously been coated or sensitised in vitro or in vivo with the appropriate globulin. Coombs' reagent is normally prepared by injecting whole serum or particular fractions of whole serum into a rabbit, goat or a horse (Dunsford and Grant, 1959) or by injecting antigen/antibody aggregates (Gold and

In the normal immuno-haematological use of the Coombs test first proposed for the detection of predominantly 7 S Ig G of anti-Rhesus specificity in blood typing or in H.D.N.B. the specific part of the reaction is in the in vivo (for the D/c) or vitro (for the I/c) sensitising of antigen with specific antibody. Any other serum proteins are then washed off and only the specific coating presenting the appropriate configuration is detected by what is otherwise a multispecific reagent (Stratton and Jones, 1955; Pirofsky et al., 1962a,b). The detection of an Ig G coating on red cells in H.D.N.B. or acquired haemolytic anaemia can soon be prevented by adding purified gamma globulin (7 S Ig G) to the antiglobulin serum (Coombs' reagent) (Coombs and Mourant, 1947). If the Coombs' reagent is a broad spectrum one (Dunsford and Grant, 1959) produced against whole serum, it will still give a positive antiglobulin test against cells from a cold antibody type of haemolytic anaemia (Dacie, 1951), cells sensitised with normal incomplete cold antibody in the presence of complement (Crawford and Mollison, 1951; Dacie, 1950; Polley and Mollison, 1961) or with anti Le^a with or without complement, and even against immature red cells (for example in sickle cell anaemia) which take up transferrin (Nelken, 1961; Jandl and Katz, 1963) or reticulocytes of persons suffering from lead poisoning (Sutherland and Eisentraut, 1956). The explanation
of these reactions is that broad spectrum Coombs' reagent contains antibody against species specific isotypic determinants of many serum proteins (Crawford and Mollison, 1951) including Ig M antibody (e.g. anti Le^a - Stratton, 1961; Polley et al., 1962), β-globulin components of complement (Polley et al., 1962; Stratton, 1961; Mollison, 1961) and transferrin (Nelken, 1961; Jandl and Katz, 1963). Thus in so far as immuno-haematological and blood grouping reactions are concerned it is possible to have a wide spectrum anti-globulin (Coombs') reagent reacting against all serum proteins including albumin, or by selecting and purifying either the initial antigen or the resulting antibody to have specific anti 19 S Ig M, anti 7 S Ig G or anti β complement components (Polley et al., 1962; Gold and Gillespie, 1961; Gold and Lockyer, 1964).

The most significant advance in the use of heterologous reagents for detection of different serum protein classes was the development of antigen/antibody precipitation in gel either by the single diffusion in tube method (Oudin, 1946, 1949, 1955) or double diffusion technique (Elek, 1948; Ouchterlony, 1948, 1949, 1961, 1962). Prior to this, precipitation or flocculation tests were carried out as an interfacial ring test between two liquid reactants. Although reactions could be timed and optimal ratios determined the reactants in a multispecific reaction could not be separated. In diffusion-in-gel techniques the individual reactions are separated in time and space.
# Table IV
## Nomenclature of Immunoglobulins and their Subunits

<table>
<thead>
<tr>
<th>Substance</th>
<th>Recommended nomenclature</th>
<th>Previous nomenclature</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Immunoglobulins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ig G or γ G</td>
<td>γ, γ ss, 7 S γ, 6.6 S γ</td>
<td></td>
</tr>
<tr>
<td>Ig A or γ A</td>
<td>γ₁ A, β₂ A</td>
<td></td>
</tr>
<tr>
<td>Ig M or γ M</td>
<td>γ₂ M, β₂ M, 19 S γ</td>
<td></td>
</tr>
<tr>
<td>Ig D or γ D</td>
<td>γ₂ M, 19 S γ</td>
<td></td>
</tr>
<tr>
<td>Ig E or γ E</td>
<td>γ₂ M, 19 S γ</td>
<td></td>
</tr>
<tr>
<td><strong>Papain fragments</strong></td>
<td>Fab</td>
<td>I, II, A, C, S</td>
</tr>
<tr>
<td></td>
<td>Fc</td>
<td>III, B, F</td>
</tr>
<tr>
<td></td>
<td>Fd</td>
<td>A piece</td>
</tr>
<tr>
<td><strong>Peptic fragments</strong></td>
<td>F(ab')²</td>
<td>5 S divalent fragment</td>
</tr>
<tr>
<td></td>
<td>Fab'</td>
<td>Univalent fragment</td>
</tr>
<tr>
<td></td>
<td>Fc'</td>
<td></td>
</tr>
<tr>
<td><strong>Chains</strong></td>
<td>Heavy chain</td>
<td>H, A</td>
</tr>
<tr>
<td></td>
<td>Light chain</td>
<td>L, B</td>
</tr>
<tr>
<td><strong>Classes of heavy chain</strong></td>
<td>Ig G</td>
<td>γ</td>
</tr>
<tr>
<td></td>
<td>Ig A</td>
<td>α</td>
</tr>
<tr>
<td></td>
<td>Ig M</td>
<td>μ</td>
</tr>
<tr>
<td></td>
<td>Ig D</td>
<td>δ</td>
</tr>
<tr>
<td></td>
<td>Ig E</td>
<td>ε</td>
</tr>
<tr>
<td><strong>Subclasses of heavy chain</strong></td>
<td>γ 1 Fc</td>
<td>We γ₂b C 70-80</td>
</tr>
<tr>
<td>with location of type specificity</td>
<td>γ 2 Fc</td>
<td>Ne γ₂a 13-18</td>
</tr>
<tr>
<td></td>
<td>γ 3 Fc or Fd</td>
<td>Vi γ₂c Z 6-8</td>
</tr>
<tr>
<td></td>
<td>γ 4 Fc or Fd</td>
<td>Ge γ₂d 3</td>
</tr>
<tr>
<td><strong>Types of light chain</strong></td>
<td>K or κ chains</td>
<td>I B</td>
</tr>
<tr>
<td></td>
<td>L or λ chains</td>
<td>II A</td>
</tr>
<tr>
<td><strong>Subclasses of heavy chain</strong></td>
<td>α a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>α b</td>
<td></td>
</tr>
</tbody>
</table>

Each immunoglobulin molecule is a 4 chain structure (or 10 chain for Ig M) consisting of multiples of LH monomers.
according to the concentration and diffusion constants of the individual components. A further refinement consists of utilizing double diffusion in agar gel after prior separation of antigen or antibody by electrophoresis in the same gel (Gordon et al., 1949) thus achieving a further separation in space between individual components of a complex system in this immuno-electrophoresis (Grabar and Williams, 1953; Grabar, 1954, 1959; Scheidegger, 1955; Wieme, 1959; Hirschfeld, 1960, 1962; Peetoom, 1963). Since agar gel can be made with a very high water content and the pore size is large, electrophoresis in this medium is virtually identical to free electrophoresis as practised by Tiselius, and unlike other media such as starch gel or paper the basic classification of proteins according to mobility is simultaneously determined. Largely by the use of immunological methods including immuno-electrophoresis (I.E.), but in conjunction with physico-chemical methods, it has been shown that Igs in man exist in a minimum of five major classes: Ig G, Ig M, Ig A, Ig D, and Ig E (WHO Committee, 1964, 1965, 1966, 1968; Rowe and Fahey, 1965a,b; Rowe et al., 1968; Ishizaka et al., 1966) (Tables IV and V).

Each of these classes in addition to having their own specific isotypic determinants, also have common isotypic determinants which label them as members of the Ig group in general. Thus heterologous reagents against immunoglobulins in general or even against one class in particular will not only contain antibodies against each of the classes or class,
<table>
<thead>
<tr>
<th>Properties</th>
<th>Ig G</th>
<th>Ig A</th>
<th>Ig M</th>
<th>Ig D</th>
<th>Ig E</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Biological</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum Conc. mg %</td>
<td>800-1680</td>
<td>140-420</td>
<td>50-190</td>
<td>0.3 - 40</td>
<td>low</td>
</tr>
<tr>
<td>Synthesis rate mg/kg/day</td>
<td>20-40</td>
<td>2.7-55</td>
<td>3.2-16.9</td>
<td>0.03-1.49</td>
<td></td>
</tr>
<tr>
<td>Catabolic rate % I.V. pool/d</td>
<td>4-7</td>
<td>14-34</td>
<td>14-25</td>
<td>18-60</td>
<td></td>
</tr>
<tr>
<td>Distribution % in I.V. pool</td>
<td>48-62</td>
<td>40</td>
<td>65-100</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Antibody activity</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complement fixation</td>
<td>+</td>
<td>0</td>
<td>+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placental passage</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Presence in cerebrospinal fluid</td>
<td>+</td>
<td>+</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selective seromucous secretion</td>
<td>0</td>
<td>+</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin sensitisation:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>heterologous</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>homologous</td>
<td>0</td>
<td>?</td>
<td>0</td>
<td></td>
<td>+</td>
</tr>
<tr>
<td><strong>Immunological</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light chain types</td>
<td>K\lambda</td>
<td>K\lambda</td>
<td>K\lambda</td>
<td>K\lambda</td>
<td>K\lambda</td>
</tr>
<tr>
<td>Heavy chain class</td>
<td>\gamma</td>
<td>\gamma</td>
<td>\gamma</td>
<td>\delta</td>
<td>\epsilon</td>
</tr>
<tr>
<td>subclasses</td>
<td>4</td>
<td>2</td>
<td>2?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allotypes Gm</td>
<td>+</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inv</td>
<td>+</td>
<td>+</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Physico-chemical</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S20, w</td>
<td>6.5 -7.0</td>
<td>7,10,13,15,17</td>
<td>18-20, &gt;30</td>
<td>6.2-6.8</td>
<td>7.7</td>
</tr>
<tr>
<td>Ammonium sulphate ppttion. (molar c.)</td>
<td>1.49-1.64</td>
<td>1.64-2.05</td>
<td>11.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total CBH %</td>
<td>2.9</td>
<td>7.5</td>
<td>11.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexose %</td>
<td>1.10</td>
<td>3.2</td>
<td>5.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetyl hexosamine %</td>
<td>1.30</td>
<td>2.3</td>
<td>4.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sialic acid %</td>
<td>0.30</td>
<td>1.8</td>
<td>1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fucose %</td>
<td>0.20</td>
<td>0.22</td>
<td>0.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Note:* (a) Myeloma Ig Ds are predominantly type L and so are normal Ig D molecules (Pernis et al., 1969). (From Cohen and Milstein, 1967)
but will have cross reacting antibody against all classes as well. This fact has been utilised in demonstrating the presence of six immunoglobulin classes in the dog (Johnson and Vaughan, 1967; Johnson et al., 1967). Reagents produced against any one class (e.g. anti Ig M) have therefore usually to be absorbed with molecules of another class (e.g. Ig G) before they will be specific for the class used as antigen.

The use of heterologous antisera is not limited to demonstrating the five major classes of Igs. Four sub-classes of Ig G have been detected as normal constituents of human serum Ig G1 - Ig G4 (WHO Committee, 1966), and other classes have also been sub-divided. It will be convenient to leave consideration of this until the structure of Igs is given (Table VII).

In characterizing Igs as a class it is fortunate that in the first instance Ig G has such a characteristic range of electrophoretic mobility. From this and by comparison of the immunological cross-reactivities together with correlation of all the biological and physico-chemical properties (Table V), particularly the antibody reactivities, it has been possible to draw up the present day classification of Igs in man and animals.

**Isologous Antibodies and Allotyping**

Since by definition (Dray et al., 1962) allotypes are demonstrated by antigenic determinants present on the serum Igs
<table>
<thead>
<tr>
<th>Immunoglobulin</th>
<th>Rabbit</th>
<th>Bullfrog</th>
<th>Dogfish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ig G</td>
<td>140,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>γ Chain</td>
<td>53,000</td>
<td></td>
<td>54,000</td>
</tr>
<tr>
<td>Ig A (milk)</td>
<td>370,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α Chain</td>
<td>64,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ig M</td>
<td>850-900,000</td>
<td></td>
<td>980,000</td>
</tr>
<tr>
<td>7 S units of Ig M</td>
<td>180,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>μ Chain</td>
<td>70,000</td>
<td></td>
<td>72,000</td>
</tr>
<tr>
<td>Light chain</td>
<td>22-23,000</td>
<td></td>
<td>20-22,000</td>
</tr>
<tr>
<td>Ig D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>δ Chain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ig E</td>
<td>200,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ε Chain</td>
<td>75,500</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

From Cohen and Milstein, 1967
of only some individuals in a species, it follows that individuals suitably negative for any one allotype factor can make an isologous antibody against it. Of course an individual of any other species, lacking the particular configuration involved, would be able to produce a heterologous antibody against an allotype, but such a reagent would be complicated by reactions against isotypic determinants common to the same Ig molecules used as antigen. Thus human allotypes can be determined by reagents prepared in rabbits (Litwin and Kunkel, 1966; Oudin, 1962b) and rabbit allotyping reagents can be produced in guinea pigs.

As with other immunological systems iso-immune reagents can be produced without worrying about xenogeneic variation.

Allotyping by Haemagglutination-Inhibition Technique

In 1956 Grubb first demonstrated that certain human sera agglutinated Rhesus (D) positive red cells sensitised (coated) with a non-agglutinating (incomplete) anti Rhesus (D) reagent. Some of these sera causing agglutination were from rheumatoid arthritic persons who possess a macromolecular factor known as rheumatoid factor RF. The nature of RF is not entirely clear since rheumatoid sera appear to contain activity against rabbit gamma globulin (attached to sheep erythrocytes in the Waaler/Rose diagnostic test - Waaler, 1940; Rose et al., 1948), against human gamma globulin (attached to red cells, latex and polystyrene particles - Heller et al., 1949), against gamma
### Table VII

#### Subclasses of Ig G in Man

<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>% occur.</th>
<th>% Type K</th>
<th>% Type L</th>
<th>Skin sensit.</th>
<th>C' fixation</th>
<th>Location of type specific</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \gamma_2 ) Ne  ( \gamma_2^{2a} )</td>
<td>11</td>
<td>5.8</td>
<td>5.2</td>
<td>-</td>
<td>+</td>
<td>Fc (2)</td>
</tr>
<tr>
<td>( \gamma_1 ) We  ( \gamma_2^{2b} ) C</td>
<td>77</td>
<td>54.5</td>
<td>22.5</td>
<td>+</td>
<td>+</td>
<td>Fc (2)</td>
</tr>
<tr>
<td>( \gamma_3 ) Vi  ( \gamma_2^{2c} ) Z</td>
<td>9</td>
<td>4.7</td>
<td>4.2</td>
<td>+</td>
<td>+</td>
<td>Fc or Fd (1)</td>
</tr>
<tr>
<td>( \gamma_4 ) Ge  ( \gamma_2^{2d} )</td>
<td>3</td>
<td>2.6</td>
<td>0.5</td>
<td>+</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td><strong>100%</strong></td>
<td><strong>67.6%</strong></td>
<td><strong>32.4%</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(6)(1) (2) (3) (4) (4) (4) (5)

**References**

4. Terry et al., 1965 - analysis of 191 Ig G myeloma proteins.

See Notation for Human Ig Subclasses, WHO Bull. 1966.
globulin of many species (Glynn et al., 1957), against gamma globulin aggregated by heating (Franklin et al., 1957; Edelman et al., 1958) and even against autologous gamma globulin with which an aggregate is formed in vivo (Harboe, 1960; Hannestad, 1968). Overall, therefore, R.F. can be regarded as heterologous, isologous and autologous as well as reacting with modified serum proteins. The impression gained is that R.F. has a number of reactivities some of which can be separated (Fudenberg, 1967). For the purpose of allotyping, however, R.F. is acting as an isologous antiglobulin.

Some of Grubb's antiglobulin sera could be inhibited by certain human sera. These human sera, capable of inhibiting the agglutination of the D positive red cell/anti-D/anti-globulin indicator system, were classed as Gm (a). Sera incapable of inhibiting this reaction are Gm (a) negative. The anti-D serum used for coating the red cells must carry the Gm (a) factor, since only then can the anti Gm (a) reactivity in the R.F. serum produce agglutination capable of inhibition. Because of the number of variables the haemagglutination inhibition system is not the technique of choice, and in addition the possibility that any of the variables may break down or some other factor may inhibit involves the use of a multitude of controls. Despite this more than twenty allotypic factors in two genetic systems have been detected. Rheumatoid arthritis agglutinating (Ragg) systems are less frequently used now, since they are usually multispecific.
Investigation has revealed the presence of antiglobulins in normal sera (SNagg) with a frequency of \( \frac{1}{500} \) (Steinbuch et al., 1965). Antibodies to Gm factors have also resulted from multiple transfusions (Allen and Kunkel, 1963) and maternal/foetal incompatibility (Fudenberg and Fudenberg, 1964).

The largest number of factors has been found in the Gm system of man (at least 20 by 1966 - Klemperer et al., 1966). They have been useful in population genetics, forensic and paternity investigations, analyses of gamma globulin production and absorption by the foetus and formulation of concepts regarding quantitative defects in immunoglobulin production (Fudenberg, 1967). The study of allotypes has also contributed significantly to the elucidation of immunoglobulin structure. The occurrence of the Gm factors is limited to Ig G molecules only, while the factors of the other human system Inv are found on some molecules of all Ig classes. This will be dealt with more fully in a later section on Ig structure.

Although techniques involving haemagglutination are extremely sensitive (Marrack, 1963), the haemagglutination-inhibition (HI) technique has found little favour for Ig allotyping in other species because of its complexity and the presence of an alternative system.

Rasmussen (1965a) briefly reported on two gamma globulin iso-antigens which were products of two co-dominant autosomal alleles Gl\(^a\) and Gl\(^b\) in pigs.

Initial attempts to produce antibodies suitable for the
HI test were prolonged and although successful the final HI activity was difficult to interpret. The antiglobulins eventually used for allotyping were discovered by screening young pigs of about 4 months old. This was based on a suggestion by Prof. Steinberg following the discovery of high frequencies of anti Gm and anti Inv reagents in young children (Wilson and Steinberg, 1965). Although the inheritance of Gla and Glb factors appeared clear cut in the original American population, following a transatlantic crossing the "natural" allotyping reagents did not differentiate British pigs so clearly, and in some instances typing was at odds with the mating data. It is not known whether extra specificities were clouding the issue, the reagents had suffered during importation, or the red cells used for coating were unsatisfactory.

It is well known that in the haemagglutination test the reactivity is dependent not only on the antiglobulin and globulin coat but also on the red cell used (Gold, 1965).

A new Ig allotype A 11 in rabbits (A eleven) has recently been demonstrated as an agglutinating specificity (Mandy and Todd, 1968a,b) inhibitable by sera positive for the factor as in the HI for Gm and Inv typing.

It may well be that allotypic determinants reacting by HI only are univalent, whereas allotypes detected by precipitation techniques are bivalent and fail to react with univalent fragments. Gell and Kelus (1964, cited by Kelus and Gell, 1967) report the use of an HI technique using rabbit anti sheep
(R.B.C.s) for the detection of rabbit allotypes.

**Allotyping by Precipitation in Gel Techniques**

The detection of allotypes in animals particularly in rabbits has largely been by precipitation in gel techniques. This technique being a direct reaction between antibody and test antigen eliminates many of the variables and possible causes of error inherent in the complexity of the HI test. Its sensitivity is not so great, however (Marrack, 1963), and its dependence on production of a suitable precipitating antibody against the necessary bivalent antigen is a disadvantage. Judging by the reports of HI techniques for man, pig and rabbit it seems that some allotypes may not be detected by precipitation techniques, and others may be ineffective by HI techniques. The double diffusion Ouchterlony or single diffusion Oudin techniques have the advantage of giving an immediate indication of multispecific reactions by the appearance of many lines (Kelus and Gell, 1967).

The precipitation technique has been used almost exclusively for Ig allotyping in rabbits (Oudin, 1966; Kelus and Gell, 1967), mice (Potter and Lieberman, 1966, 1967) and chickens (Skalba, 1964, 1966; McDermid, 1968). Allotypes have also been demonstrated in Igs of guinea pig, baboon, Rhesus monkeys, duck and rat and in the lipoproteins and $\alpha_2$ globulin of the Rhesus monkey (Benacerraf and Gell, 1961; Kelus and Moor-Jankowski, 1962; Moor-Jankowski, 1962;
Table VIII
Currently Recognizable Rabbit Allotype Factors

<table>
<thead>
<tr>
<th>Specificity and Locus</th>
<th>Ig Class</th>
<th>Molec. Loc.</th>
<th>Associated factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>b E Aa 1 L a )</td>
<td>Ig G</td>
<td></td>
<td>Al' Al&quot;</td>
</tr>
<tr>
<td>c B Aa 2 J a )</td>
<td>Ig M</td>
<td>Fd</td>
<td>A2' A2&quot;</td>
</tr>
<tr>
<td>d D Aa 3 K a )</td>
<td>Ig A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a F Ab 4 II b )</td>
<td>Ig G</td>
<td></td>
<td>A4' A4&quot;</td>
</tr>
<tr>
<td>g A Ab 5 I b )</td>
<td>Ig M</td>
<td>L chain</td>
<td>A5' A5&quot; A5</td>
</tr>
<tr>
<td>f C Ab 6 b )</td>
<td>Ig A</td>
<td></td>
<td>A6' A6&quot;</td>
</tr>
<tr>
<td>/ Ac 7 P c</td>
<td>Ig G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A 8 ?d</td>
<td>Ig G</td>
<td>Fc</td>
<td></td>
</tr>
<tr>
<td>Ab 9 b</td>
<td>Ig G</td>
<td>L chain</td>
<td></td>
</tr>
<tr>
<td>A 10 ?d</td>
<td>Ig G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A 11 ?</td>
<td>Ig G</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ms 1</td>
<td>Ig M</td>
<td></td>
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<td>Ms 2</td>
<td>Ig M</td>
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<td>e e</td>
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<tr>
<td>T T</td>
<td>Ig G</td>
<td></td>
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<tr>
<td>*</td>
<td>/</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Oudin / Dubiski and Kelus / Dray et al.

Table modified from Cohen and Milstein, 1967.

2. Oudin, 1960a,b.
3. Dray et al., 1963a.
Kaminski and Ligouzat, 1964; Barabas and Kelus, 1967; Greuter and Butler, 1963; all cited by Kelus and Gell, 1967). A considerable amount of work has also been carried out on at least two systems of beta lipoprotein allotypes in man known as Lp and Ag systems (Allison and Blumberg, 1961, 1965; Berg, 1963, 1965; Blumberg et al., 1962; Butler, 1965, 1967) detected by heterologous and isologous reagents respectively. Lipoprotein variants also occur in rabbits (Albers, 1968; Cohen et al., 1968). The genetics and structure of these low density lipoprotein (L.D.L.) variants has, however, not been so adequately established as those for Ig allotypes in man, rabbit and the mouse.

In the rabbit thirteen different Ig G variants have been demonstrated (Table VIII) although only the original six factors have been thoroughly characterised. Of these six, factors 1-3 are determined at the a. locus and factors 5-6 at the b. locus. The allotypic specificity is usually denoted by a Roman A followed by the numerical factor, e.g. A1, A2, or, if it is understood that rabbit allotypes are under discussion, the Roman A is missed out, and the symbol for the locus may be substituted, e.g. a3, b5, etc., or a combination of both, e.g. Aa1, Ab6, etc. Other authors preface the factor number with As, e.g. As 7, etc. The factor A7 was originally reported as P (Dray et al., 1963a) but is now known as c7 at a third locus c (Nisonoff and Thorbecke, 1964; Mage et al., 1968). Two further factors, A8 and A10, are found in association with AI
when certain antisera show the double line phenomenon (Hamers and Hamers-Casterman, 1965, 1967; Hamers et al., 1965, 1966). It was suggested in the case of A8 that two different Ig G classes existed, one carrying A1 and the other carrying a closely linked allele A8 possibly at a locus d (Hamers and Hamers-Casterman, 1966). The double line phenomenon was also shown by Oudin (1960a) and by Dray et al. (1963a) who showed factors A4' and A5' which did not segregate from A4 and A5 respectively. A9 is an additional factor at the b locus (Dubiski and Muller, 1967; Gell and Hughes, 1968) and All is a factor determined by the HI technique in rabbits which are homozygous or heterozygous at the a and b loci (Mandy and Todd, 1968a,b).

Unlike the situation in man with Gm and Inv factors, the factors of both the a and b loci are found in association with Igs other than Ig G such as Ig M (Todd, 1963; Gell and Kelus, 1964; Feinstein and Kelus, in press, cited by Gell and Kelus, 1967; Hoyer et al., 1967; Stemke and Fischer, 1965) or Ig A (Lichter, 1967; Conway et al., 1968; Feinstein, 1963). Additional factors are also found specific to Ig M (Ms 1 - Kelus and Gell, 1965; Ms 2 - Gell, 1966; Ms 3 - Stemke and Fischer, 1965; Kelus, in preparation, cited by Kelus and Gell, 1967) and Ig A (Conway et al., 1968).

Large scale genetic analyses have been carried out but mating data is comprehensive only for factors A1 - A6. The two loci a and b are not linked to one another or to sex (Dray
et al., 1963a; Kelus and Gell, 1963; Dubiski et al., 1962; Kelus, in preparation, cited by Kelus and Gell, 1967); although Dubiski et al. (1962) found some correlation between factor E (As 1) and sex, this has not been substantiated in other colonies by other workers (Kelus and Gell, 1967).

Litters are not normally typed until 10-12 weeks of age as early typings are contaminated with maternal globulins. In addition the young animal does not develop Igs to the fullest extent until 8 weeks of age. Some factors (As 1 and As 4) have been found at 20 weeks even though not detected at 12 weeks (Kelus, in preparation, cited by Kelus and Gell, 1967). In litters from mothers with allotyping antibody against the paternal type there is a likelihood that the development of the paternal type will be restricted for many months despite the absence of antibody in the serum (Dray, 1962; Mage and Dray, 1965). A similar suppression occurs if newborn rabbits are immunised in early neonatal life to factors they would normally develop later (Dubiski, cited by Kelus and Gell, 1967). Suppression by maternal antibody can be prevented if offspring are given paternal Ig intraperitoneally or through colostrum of fostermothers (Young and Mage, 1968).

Preparation of Rabbit Allotyping Reagents

In 1902 Schutze immunised rabbits with whole rabbit serum subcutaneously, and produced reagents precipitating the serum of some rabbits but not others. Little use was made of this
finding until Oudin produced precipitins against some rabbit sera by injecting ovalbumin/anti-ovalbumin complexes into rabbits, and was able to define allotypy (Oudin, 1956a,b). The variety of reactions obtained enabled him to distinguish at least seven factors (a, b, c, d, e, f and g) although one of them was later discontinued (e) (Table VIII). Many of these factors existed as families of reactivities (Oudin, 1960a,b,c) giving multiple lines on single or double diffusion. The antibodies to certain factors (g and f, later termed A5 and A6) crossreact so that, as Kelus and Gell (1967) point out, it is necessary to raise an anti As 5 in an As -/-4, 6 animal rather than an As -/-4, as even if one injects an As -/-5 serum the reagent produced in an As -/-4 will crossreact with A6.

The use of whole serum only with adjuvants is favoured by some (Dray and Young, 1958a,b), and using this technique a similar range of factors with the same specificity but different designations (RGG1, RGG2, J, K, L, P and T) were eventually classified as gamma globulin allotypes. Other reactivities concerned alpha and beta globulins (Dray and Young, 1959, 1960, 1961; Dray et al., 1965a). Another widely used technique is to prepare antibodies in a group of rabbits against bacteria such as Proteus vulgaris, Salmonella typhi- murium, Streptococcus pyogenes (Dubiski et al., 1959a,b; Kelus and Gell, 1967), and then inject washed aggregates of these antibodies and bacteria into another group of rabbits. Allo-typing reagents detecting factors Da (later called A), B, C, D
and E were produced (Dubiski and Kelus, 1960; Dubiski et al., 1961).

As with allotypes of man (Cumley and Irwin, 1943; Oudin, 1962b; Litwin and Kunkel, 1966) reagents can also be produced recognising differences between individuals by hetero-immunization. Thus goats (Bornstein and Oudin, 1964; Leskowitz, 1963), chickens (Bornstein and Oudin, 1964), as well as guinea pigs (Gell, 1965 - unpublished data cited by Kelus and Gell, 1967) have been used. As previously indicated, the goat and chicken reagents required careful absorption, but the guinea pig produces specific anti-As 6 after injection with purified rabbit As -/6 Ig G (10 μg in complete adjuvant) simultaneously with serial dosage of milligram amounts of non-As 6 Ig G intraperitoneally.
Other Miscellaneous Antiglobulins or "Autologous" Antibodies

This class of antibodies is found apparently "naturally" occurring in man and rabbits particularly. Their origin and classification is doubtful, but they can be classified as auto-antiglobulins in the sense that there is no known external stimulus and they are formed against determinants not normally available to the immunological mechanism but arising from reaction within the body.

Rheumatoid factor has already been dealt with in a limited way, and a comprehensive consideration of this mixture of anti-globulins and its relationship with rheumatoid arthritis and related diseases in man is beyond the scope of this review. However, it is probable that, in addition to acting as an isologous reagent in allotyping, R.F. also contains an auto-logous factor known as anti-antibody.

Anti-antibody

Anti-antibody (A-A) has been described in the human (Milgrom et al., 1956) and in rabbits (Milgrom, 1962) as a 19 S serum globulin, which reacts with 7 S gamma G antibody only when it is combined with antigen. It was demonstrated against anti-D coated D+ red cells in man and against anti-G coated G+ red cells in rabbits (Fudenberg et al., 1964). Unlike Ragg and Snagg agglutinating systems it is not inhibitable by whole serum or gamma globulin fractions thereof, but can be absorbed
by immune antibody/antigen aggregates (BSA/anti BSA) or by antigen aggregates with F(ab')\textsubscript{2} or intact Ig G, but not by non-precipitating anti DNP/DNP soluble complexes (Fudenberg et al., 1964).

The classic A-A (Milgrom type) is crossreactive and quite unselective as regards the antigen/antibody complex it reacts with as long as it forms a lattice (Fudenberg, 1967). Other types are more selective.

**Andresen type A-A.** This type found in man only reacts with antigen/antibody complexes where the Ig G used bears the appropriate Gm allotype. Like A-A, and unlike anti Gm reagents of Ragg or Snagg types it cannot be inhibited by native Ig G even of the appropriate Gm type unless specifically combined with antigen (Andresen, 1963; Gold and Lockyer, 1964).

**Oudin idiotypic type.** This type produced in man and rabbit has a highly selective affinity for determinants on antibody molecules produced against an antigen (Oudin, 1966) of one single specificity, e.g. Salmonella/anti Salmonella, and even to the one antibody formed to that antigen in one individual. As we shall see when discussing the relationship of Ig primary structure with isotypes and allotypes the occurrence of idiotypes has interesting implications if a similar relationship with primary structure can be shown.

**Pepsin determinants.** A high frequency of rheumatoid sera and also many normal sera of man and rabbits contain an anti-globulin reacting with the 5 S fragment produced by pepsin
digestion (i.e. Fab'). The specificity of this antigen appears to be associated with hidden determinants on the Fd portion of the H chain, or sometimes on the L chain while in others it depends on the integrity of union between Fd and L chain after pepsin treatment of native Ig G. These pepsin fragment agglutinators can be inhibited by the appropriate fragments, but not by whole Ig G (Fudenberg, 1967).

**Homoreactant or Papain (Fab) Determinant**

Rabbit sera in addition to having crossreactive anti-antibody (A-A) and anti-pepsin determinant also contain in almost every case an antibody against the univalent Fab fragment produced by papain digestion of native Ig G (Mandy et al., 1965; Mandy, 1966). Homoreactant causes agglutination of univalent rabbit Ig G Fab coated human red blood cells, and this agglutination is inhibited by Fab of normal rabbit Ig G, but not by whole Ig G nor by peptic fragments. Homoreactant is a 7 S Ig G non-dialysable mercapto-ethanol resistant factor.
Structure and Properties of Immunoglobulins

The unique feature about the complexity and heterogeneity of the immunoglobulins is the way in which different structural aspects must be varying independently of one another. These structural variables express themselves in the various characteristics and properties of the immunoglobulins, so that, as I have already stressed, classification by any one criterion leaves each category heterogeneous by any other criteria.

Early attempts to biochemically analyse Igs, especially their finer structure, amino acid sequences, N terminal amino acids and peptide patterns, were not entirely successful in distinguishing differences related to any other properties or characteristics such as antibody activity (Porter, 1963). It has been necessary to attempt a very gradual strip down of the gross structure of the Ig molecule retaining the biological properties and characteristics intact so that each one could be ascribed to distinct structural entities. Two distinct methods have proved most useful in this respect: digestion by papain (Porter, 1959) or pepsin, and reduction of disulphide bonds (by mercapto-ethanol in 6 M urea – Edelman, 1959; Edelman and Poulik, 1961; mercapto-ethanol in aqueous solution followed by iodoacetamide and dissociation and fractionation in acidic solution – Fleischman et al., 1962). Using these techniques and others (electrophoresis, double diffusion Ouchterlony precipitation tests, immuno-electrophoresis, ion exchange chromatography, molecular sieving, etc.) it has been shown that
Igs of man, rabbit, horse, mouse and mammals in general consist of two sorts of polypeptide chains, heavy (H) and light (L), and these chains are linked by interchain disulphide (SS) bonds to form a monomer (LH) which dimerises (by linking the H chains with more SS bonds) to form a basic 4-chain structure (LH)\textsubscript{2} characteristic of most Ig classes (Figs. 1 and 2). Other classes form polymers (LH)\textsubscript{2n} (e.g. for 19 S Ig M n = 5).

Ig A forms a 9-11 S polymer which in secretions (colostrum, saliva, tears) has an extra piece called the T or transport piece. This has extra antigenic specificities and also may confer antitryptic properties in colostral Ig A to allow intestinal absorption of intact molecules (Shim et al., 1969).

There are several classes of each type of chain, particularly the heavy chain (see Tables V and VI), and each class has a number of members or subclasses, so that the availability of at least ten different H chains and at least 2 or 3 L chains in different combinations in higher vertebrates gives rise to the heterogeneity of Ig molecules found in a normal serum. Thus the five major classes - Ig G, Ig M, Ig A, Ig D and Ig E - consist of combinations of \(\gamma\), \(\mu\), \(\alpha\), \(\delta\) and \(\varepsilon\) chains respectively with \(\kappa\) and \(\lambda\) light chains giving ten major types of monomers. The division of H chains into further subclasses \(\gamma_{1}, \gamma_{2}, \gamma_{3}, \gamma_{4}, \alpha_{1}, \alpha_{2}, \mu_{1}, \mu_{2}\) etc.) increases the total number of combinations proportionately. In vivo any particular molecular species does not contain a mixture of LH monomers so that, for example, a molecule of
Figure 1. Generalised structure of diagrammatic Ig G (LM) dimer. Broken part of A and B chains indicates variable (V) portion. Continuous part of A and B chains indicates invariant or constant (C) portion.
Fig. 1

CHO

S–S

CHO

150

N

34

S–S

CHO

N

A chain

A chain

B chain
Ig G_2 will be \((K \gamma_2)^2\) or \((\lambda \gamma_2)^2\) but not \((K \gamma_2\lambda \gamma_2)\) or \((K \gamma_2 K \gamma_2)\). Artificial mixtures of H and L chains of different classes and subclasses will associate in a partially random fashion producing hybrid molecules of dissimilar univalent fragments. A selective effect is found, however, as the percentage of bivalent antibody activity recovered in such mixtures is higher than would be expected from random association. This selective effect is also shown by myeloma protein subunits in a mixture of subunits from normal proteins.

The dissociation of Igs into individual polypeptide chains is achieved by reduction of disulphide bonds. These individual chains, although retaining many of the isotypic antigenic determinants which allow the classes and subclasses of the chains, and therefore of the original Igs, to be determined, lose many of the biological properties including antibody activity. These properties depend upon the integrity of the union between parts of the two sorts of chain.

Digestion of Ig molecules with proteolytic enzymes such as papain or pepsin splits the protein in a different direction to that produced by disulphide bond reduction. The use of papain produces three fragments. Two of these fragments (Fab) are identical, carry the antibody activity of the Ig and consist of an L chain coupled to the amino-terminal half of the H chain known as the Fd part. The third fragment (Fc) is crystallizable, and depending upon the Ig class carries a number of biological properties and antigenic determinants.
Figure 2. Diagrammatic representation of Ig subunits produced by reduction and enzymatic cleavage.

For nomenclature see text and Table IV.
Fig. 2

$F(ab')_2$

$Fc$

$F'_c$

$pF_c'$

$Fab$

$Fd$
Pepsin splits the molecule across the heavy chains at a point just to the carboxy-terminal side of the interchain disulphide bonds. This leaves the two antibody active fragments (Fab') still coupled in a 5 S peptic fragment F(ab')$_2$ together with a number of small peptide fragments and a fragment pFc'. After gel filtration on G200 and recycling gel filtration on G50 pFc' can be separated from pepsin digests of human Ig G. A smaller fragment Fc' is then produced by papain digestion of pFc' (Turner et al., 1969) (Fig. 2).
Figure 3a. Relationship between biological properties and location within Ig molecule.
Fig 3a

Fc Fragment

Allotypic SNagg antigens

Allotypic Ragg antigens

Milgrom type A-A antigens

Skin attachment sites

Complement fixation sites

Most isotypic antigen sites

Rhf. and RhF.-like antigens

Placental transfer site

Fab Fragment

Allotypic

Snagg

Ab. comb. site

Allotypic

Snagg

Fd

Ab. comb. site

Homoreactant

Fab Fragment
Relationship between Isotypes, Allotypes, other Biological Properties and the Structure of Immunoglobulins

As we have seen, the basic classification of Igs in mammals is dependent upon the presence of individual isotypic antigenic determinants on the heavy and light chains. This has resulted in identification of at least ten types of heavy chains, and two types of light chains (K and L or kappa and lambda). Kappa and lambda chains occur with a frequency of about 60% and 30% in Ig G, Ig A and Ig M of man. Ig D, however, has a higher frequency of L chains - 87% (Pernis et al., 1969). Within Ig G subclasses, however, this ratio of 2:1 is not always maintained as Ig G1 shows an excess of type K and Ig G2 an excess of type L (Table VII). Ten per cent of light chains do not show either K or L specificity. Light chains occur as low molecular weight proteins and also light chain fragments in urine of patients with Bence-Jones proteinuria. Since these are invariably of one type (K or L) it has resulted in the early elucidation of light chain amino acid sequence. Some monoclonal myeloma proteins fail to show reaction with antisera to K or L type light chains until after reduction when their isotypic determinants are apparently revealed and similarly with normal human Ig (Nachman and Engle, 1964).

Heavy chains too are characterised by individual isotypes for each chain, and although these are specific to each chain and also species specific, additional determinants show cross
Figure 3b. Relationship between allotype factors and amino acid sequence analysis in the four Ig G subclasses of man.

Numbering indicates position of half cystine residues and distance between intrachain SS loops.

Light chains numbered from C and N terminal ends do not agree in the variable half because they are derived from different analyses.
Fig. 3b

**Fab Fragment**

K Chain: 23 → 65 → 135 → 195 → 214

γ Chain: S → S → S → S

**Fc Fragment**

Inv 1, 1+2, 3.

K Chain: 23 → 88 → 135 → 195 → 214

γ Chain: S → S → S → S

Gm a, y, x: γ1

Gm n: γ2

Gm z, f: γ1

Gm b^0, b^1, g, b^3, b^4, c, s, t: γ3
reactivity with reagents to other chains and to chains of other species. Thus antisera produced against pig Ig G will cross react with Ig G of many other animals (cow, goat, horse, dog, etc.). As we shall see, the reason for this is that heavy chains are probably all derived from a basic ancestral unit and therefore have certain structural similarities as well as dissimilarities. In addition, study of individual myeloma proteins has shown that both types of chain have a relatively invariant or constant part in which there is a repeating unit of about 100 amino acid residues, and at the amino terminal end of each chain a highly variable portion (Figs. 1, 2 and 3). It is likely that these variants will contribute to cross reactivity.

In so far as the variant portion of the chains is concerned no two myeloma proteins are alike, and it is probable that more than $10^2$ and possibly $10^4$ structural variations occur in each class of chain at this point. Since this part of the Ig molecule also contains the antibody combining site, some insight into the possible range of antibody heterogeneity is hereby revealed. The remainder of each of the chains is relatively invariant, however, and it is not at all clear whether cistrons are present in the germ line coding for the variable part or whether clones arise by somatic mutation to produce each individual variant possibly as part of the immunological mechanism. The alternative possibility is that immunisation is a selective process only triggering those lines
of cells producing antibody with a reasonably close fit.

The constant portion of the chain is so relatively invariant that the few variations of primary structure which do occur (isotypes and allotypes) enable us to clearly delineate classes and subclasses, and hardly disturb the considerable degree of homology which exists between species such as man, rabbit and the mouse. Most of the clearly demonstrated biological properties of Ig molecules other than antibody activity are associated with the invariant portion of their chains (see Figs. 2 and 3 and Tables VII and VIII) and particularly the Fc part of the heavy chains. Thus the latter contains most of the heavy chain isotype antigens, the complement fixation sites, skin attachment sites, placental transfer site and the antigenic determinants for Rh. F, Rh. F like and Milgrom type A-A activities as well as the bulk of human allotypic antigens detected by Ragg and SNagg systems. The human Inv allotypes are only found on the constant region of K light chains.

In the rabbit the a and b locus allotypes are found on the invariant portion of the H and L chains respectively, the a locus factors being mainly on Fd apart from A8 on Fc (Table VIII) (Kelus et al., 1961; Marrack et al., 1962; Feinstein et al., 1963; Stemke, 1964; Hamers and Hamers-Casterman, 1965; Wilhelm and Lamm, 1966). Koshland located the a locus factors a1, a2 and a3 between residues 8 to 39 of their respective Fd fragments (Koshland, 1967). In so far as factors A1 - A6 are
concerned in artificial mixtures recombination of half molecules to form hybrid molecules has been demonstrated (Seth et al., 1965). Naturally occurring molecules, however, show internal symmetry, and in a double heterozygote at the a and b locus for any factors A1 - A6 only one factor for each locus occurs on any one molecule. From molecule to molecule, however, either factor at the a locus may combine with any factor at the b locus. Thus combination of, for example, Aa 3/Aa 1 and Ab 4/Ab 5 in a double heterozygote is random giving molecules with Aa 3/Ab 4, Aa 3/Ab 5, Aa 1/Ab 4, Aa 1/Ab 5 in proportions consistent with the percentage occurrence of each type in the serum (Dray et al., 1963b; Gilman et al., 1964). In addition, molecules occur which are negative for either a or b locus factors or both (Dray et al., 1963b; Stemke, 1965; Bornstein and Oudin, 1964). The explanation for this might partly lie with the fact that rabbit immunoglobulin synthesis is controlled at other additional loci, e.g. factor c 7 (Mage et al., 1968). There is no segregation of parental combinations. Hence it was found in one rabbit that about 66% of molecules with Aa 1 also had Ab 4 even though the genes came from different parents (Dray et al., 1963b). In two heterozygotes examined Ab 4 molecules were 64% and 66% and Ab 5 molecules 27% and 24% while 10-22% failed to react with b locus determinant antisera (Gilman et al., 1964). An excess of Ab 4 type molecules was found in an isolated antibody to type III pneumococcus polysaccharide, but this is probably
related to preferential production of Ab 4 as indicated above (Catty et al., 1969), and not to any selective ability of Ab 4 molecules with respect to this antigen.

The observations given above suggest that individual Ig molecules are produced by an individual clone of cells with H and L chains coded by one cistron at the a and b locus respectively. Since the genotype may be doubly heterozygous at the a and b loci, this indicates either a segregation of individual parental chromosomes in a random manner in Ig producing cells, or else a suppressive control mechanism in the same cells. Although the bulk of the evidence supports a one antibody/one cell concept (Vazquez, 1961; White, 1958; Coons, 1958) the evidence from localisation of single molecular species by fluorescent allotyping reagents in rabbits is conflicting. The cells in rabbit lymphoid tissue in general only show Ig of one allotype of the a or b loci even in heterozygotes (Pernis et al., 1965), but certain cells in the germinal centres of secondary lymphoid follicles show the presence of two allotypic specificities at any one locus (Colberg and Dray, 1964; Pernis et al., 1965).

In man the situation is quite complex. Early realisation that Gm factors are associated with the H chains of Ig G only, while Inv factors are found on light chains (Kappa) in all classes, brought forward the idea that individual H and L chains were encoded by allotypic genes at the Gm and Inv loci respectively on a one gene/one polypeptide chain basis
(Franklin et al., 1962; Fudenberg et al., 1963). This also started a search for allotyping reagents for factors at a locus encoding for Ig M and Ig A. Isologous antibodies to Ig M (Mackenzie et al., 1967) and Ig A (Fudenberg et al., 1968) determinants have recently been demonstrated by agglutination of Waldonstrom macroglobulin or Ig A myeloma globulin coated tanned red cells. It is not known at the moment at which part of the heavy chains these reactivities are directed. The anti Ig M reagents have only limited specificity and could be reacting with a determinant in the variable sequence peculiar to the macroglobulin used.

With the increase in the number of Gm factors detectable, however, it soon became clear that the situation was extremely complex and that some genes for Gm factors were not strictly allelic but related to different mutational sites or cistrons. Thus the Gm factors are inherited in certain fixed combinations which differ from race to race. Each combination behaves as a unit of inheritance in family studies in the same way that blood group factor phenogroups determined by multiple alleles behave. Despite the fact that the whole Ig G content of a normal serum might have two, three or even eight Gm factors expressed in the phenotype (depending on the number of reagents used) any one myeloma protein would only have one or two or even none (Martensson, 1966, with modifications to allow for increased number of factors now demonstrable). Since myeloma proteins are regarded as monoclonal developments of normal
serum Ig constituents it follows that any one Gm factor is only expressed in a small proportion of a normal person's Ig G molecules. Thus myeloma proteins have a much higher Gm factor activity per unit weight than the same weight of normal serum Ig G. For Gm b\textsuperscript{1} the factor is about 8-16 fold so that only 1 out of 8-16 gamma G molecules carry Gm b\textsuperscript{1} in normal serum. The occurrence of particular factors on myeloma proteins reflect their frequency among normal Ig G molecules in an individual as well as their frequency in the population in general. Thus Gm a and Gm f occur in a much greater proportion of molecules (both normal Ig G and myeloma) than Gm b\textsuperscript{1}, but only one of these factors occurs on any one myeloma protein in Caucasians. Each one must be a product on a separate polypeptide chain, and if we have a one gene/one polypeptide chain hypothesis then the three genes cannot all be allelic particularly as some proteins are negative for all three indicating another "allele". As in rabbits only one gene is active in each clone of cells producing H chains.

Following the detection of Ig G subclasses by using rabbit antisera to human myeloma proteins (Ne, We, Vi and Ge - Grey and Kunkel, 1964) or fragments of heavy chains (C and Z - Ballieux et al., 1964) or by using monkey antisera to pooled human Ig G (\(\gamma_2a\), \(\gamma_2b\), \(\gamma_2c\), and \(\gamma_2d\) - Terry and Fahey, 1964) it soon became clear that certain allotype factors of the Gm system were associated with each subtype. Since we must assume that each type of H polypeptide chain in all Igs is the
product of a separate locus (as all classes of Ig are present in the serum of normal individuals), it would seem that the 4 Ig G subclasses with their associated allotypic factors are the products of 4 different genetic loci each with a number of alleles. Investigation of Ig G myeloma proteins has shown the following combinations:

<table>
<thead>
<tr>
<th>Gamma subclass</th>
<th>Caucasians</th>
<th>Negroes</th>
<th>Chinese</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gm(n +), Gm -</td>
<td>Gm -</td>
<td>Gm(a + f + y +)</td>
</tr>
<tr>
<td></td>
<td>Gm(a + x +), Gm(f + y +), Gm(a + z +)</td>
<td>Gm(a + z +), Gm(f +)</td>
<td>Gm(b3 +)</td>
</tr>
<tr>
<td></td>
<td>Gm(b1 + b3 + b4 +), Gm -</td>
<td>Gm(b1 + c +), Gm(b1 + c + b4 +)</td>
<td>Gm(b3 +)</td>
</tr>
</tbody>
</table>

By comparing the relationships between these various antigens in different races in conjunction with their molecular locations it has been possible to build up a chromosome map of four closely linked loci for the Ig G H chain inheritance (Fig. 4).
Figure 4. Possible chromosome map for cistrons controlling the synthesis of some gamma globulins in man.
Kunkel et al. (1966) cited by Cohen and Milstein (1967) and modified after Mårtensson (1966)
This map is based on the fundamental concepts of one gene (cistron)/one chain and a linear correspondence between gene and polypeptide, although the order of the genes is to a certain extent arbitrary.

Although various human allotypic factors have been localised in relation to particular parts of H and L subunits, and these play a major part in their specificity, many factors show a considerable reduction in activity on reduction or digestion of Ig G molecules. Inv (b +) and Inv (a +) light chains have been shown to differ by substitution of valine for leucine at residue 191. Despite this association with primary structure in kappa chains it has been reported that Inv activity is only present when K chains are associated with gamma 2b or gamma 2c heavy chains. Gm f activity is dependent on the quaternary structure of Ig since it cannot be detected after reduction or digestion. Activity is restored, however, after recombination of a Gm f positive H chain with a K or L type light chain even if the latter is from a Gm f negative molecule. There is some evidence that the specificity Gm a requires the close proximity of a tetrapeptide sequence (Arg-Asp-Glu-Leu) near residue 90 of the Fc portion of the gamma 1 (gamma 2b) chain (numbering from the COOH end) with an amino acid in residues 1-13, since Gm a activity and the homologous Gm "non a" activity remains intact in pepsin fragment p Fc', but is impaired after papain and cysteine treatment which splits off residues 1-13 in producing Fc' (Turner et al.,
1969) (Fig. 2). Some rabbit allotyping antisera where donor and recipient differ in both heavy and light chain determinants contain peculiar antibodies which only react with heavy chain determinants when an appropriate light chain antigen is also present. Anti-Al antibodies produced by injecting Aa 1/1 b 4/4 Ig into an Aa 2/3 b 9/9 rabbit precipitated Aa 1/1 b 4/4, Aa 1/1 b 5/5 and Aa 1/1 b 6/6 but not Aa 1/1 b 9/9 rabbit sera. Reactions by HI test were specific for Aa 1 irrespective of the L chain type.

In the H chains of man, however, as for human kappa chains, it has been possible to relate allotypic variants either to variation in peptide maps or in amino acid sequences. Originally it was thought that peptide differences in Fc fragments were correlated with the presence of Gm (a) and Gm (b) (Meltzer et al., 1964; Fudenberg et al., 1964; Frangione and Franklin, 1965, all cited by Cohen and Milstein, 1967). Subsequently, however, the peptide apparently characteristic for Gm (b) was found in all Gm (a -) myeloma proteins of gamma 2a, gamma 2b and gamma 2c type, so that it appears that the distinction is between Gm (a +) and Gm (a -). The following sequences were obtained for peptides from two gamma 2b proteins:

\[
\text{Gm (a +) - Thr-Leu-Pro-Pro-Ser-Asp-Glu-Leu-Thr-Lys} \\
\text{Gm a - - Thr-Leu-Pro-Pro-Ser-Arg-Met-Glu-Glu-Thr-Lys}
\]

A single amino acid substitution (Phe for Tyr) has also been implicated in the Gm (b)/Gm (g) allelic differentiation.

In summary, therefore, it would seem that although some
isotypic and allotypic specificities are only detectable in the intact molecule or in intact sections of the molecule, while others (like isotypes of myeloma kappa chains) can only be detected after reduction, fundamentally these specificities (and also the secondary, tertiary and quaternary structure) are a product of primary amino acid sequence and therefore of gene action.

Attempts to link allotypic factors with primary structure in the rabbit have been hindered by the difficulty, in the absence of myeloma and Bence-Jones proteins, of obtaining individual populations of one class of Ig. By isolating H and L chains of rabbit Ig G homozygous at the a and b loci respectively, it has been possible to show that differences occur involving various numbers of residues (6-15) and amino acids (5-9) between the various factors on the H (Inman and Reisfeld, 1968; Koshland et al., 1968) and L (Reisfeld et al., 1965; Reisfeld and Inman, 1968) chains. Peptide maps of similar isolated chains show practically identical fingerprinting, although the presence of a yellow spot and absence of a brown spot has been correlated with the factor Aa 2 on heavy chains (Small et al., 1966) and a certain amount of heterogeneity in peptide fingerprinting and electrophoretic mobility of light chains has been associated with the b locus (Small et al., 1965). Reisfeld and Inman (1968) found very large numbers of residues (up to 28) differing in light chains of various b locus allotypes. It is difficult to see how much
of the variation occurs in the constant part of the chains, and how much is a result of differences in the variable portion associated with different antibodies. This would indicate a correlation between antibody and allotype determination, however, and it has been reported that variations in primary structure associated with isolated antibodies to various haptens are independent of variations produced by allotypic coding (Koshland, 1966; Koshland et al., 1968). Other authors, however, have shown that among isolated antibodies to simple haptens or carbohydrates the percentage occurrence of molecules carrying particular allotypes deviates from the average in the Ig G population as a whole (Gell and Kelus, 1962; Rieder and Oudin, 1963). Such deviations, however, could be linked to possible subclasses of Ig G and the occurrence of particular factors in each subclass and their relationship with the stage of immunisation reached with regard to that particular antibody.
The A-0 System

The earliest example of a serum type system which has not yielded to starch gel analysis is closely related to classical red cell typing in that it is that group of closely related systems in man and animals involving soluble blood group substances. These include the J system of cattle, the R system of sheep, the A system of pigs, the J(A) system of goats and the ABOH(Ii) Lewis complex of man. Although all these systems were originally determined by red cell typing, it has become increasingly clear that they are a soluble property of serum and other bodily fluids, particularly saliva, in addition to occurring at the red cell surface (Lodge and Usher, 1962; Lodge et al., 1965; Lodge and Voak, 1968; Sneath and Sneath, 1959; Fried et al., 1968; Stone, 1962; Horowitz et al., 1964a,b; Tucker, 1962; Andresen, 1963; Rasmusen, 1964; Goodwin and Coombs, 1956; Hayashi et al., 1958; Stone and Irwin, 1954).

Red Cell Antigen/Antibody Test Systems

The method of detecting soluble blood group substances is haemagglutination inhibition using a red cell antigen/antibody indicator system. A consideration of this system is a necessary prerequisite to understanding the soluble blood group properties.

Mention has already been made of the early history of the A blood group system of pigs and its corresponding "natural"
antibody anti-A. Similarly in sheep, goats and cattle and in man a similar antigenic specificity occurs, usually detected on the surface of the red cell by means of a "naturally" occurring antibody such as anti-R from non R sheep (Yeas, 1949), anti-J from non J cattle (Stone, 1962) and anti-A from non A persons (Race and Sanger, 1962, 1968). The detection of antigen on the red cell is usually by agglutination (human A and some pig A), haemolysis (cattle J, sheep R, goat J(A) and sometimes pig A) or by use of the indirect antiglobulin test or dextran test for pig A when the homologous antibody is being used.

In man a factor B antithetical to A occurs, and is detected by anti-B from the serum of non B persons. B like specificities have been shown in rabbits, guinea pigs (Friedenreich and With, 1933), opossum, hamsters and rats (Owen, 1954). Pig gastric mucosa is used commercially as a source of B reactivity as well as A. The presence of a B or B like antigen has also been noted on pig red cells in connection with their use for diagnosis of human ABO H.D.N.B. (Konugres and Coombs, 1958; Voak - personal communication; Lockyer and Tovey, 1960; Tovey et al., 1962).

The absence of the A or B antigen results in a group 0 person without any apparent antigen. When Schiff discovered in 1927 that certain bovine sera react preferentially with 0 and A human cells after absorption with A or A B blood, the antibody concerned was called anti 0 since it was believed to
be detecting a product of the O gene. However, this reagent failed to differentiate AA from AO and BB from BO genotypes. Similarly anti O found in the sera of some A\textsubscript{1}B and A\textsubscript{1} persons reacts strongly with O cells and to a lesser extent with A\textsubscript{2} and some B cells. Anti O is found in certain humans, oxen, goats, chickens, eels, cats, pigs, sheep, rabbits and in extracts from many seeds (Race and Sanger, 1962). Some clarification was arrived at when anti O reagents were divided into those that were inhibited by human secretor saliva and others that were not inhibited (Morgan and Watkins, 1948). The former are known as anti H and the latter retain the title anti O. However, since anti O is a misnomer some authors prefer the terms anti H\textsuperscript{S} and anti H\textsuperscript{C} respectively (Voak et al., 1968; Wiener et al., 1966).

A factor O antithetical to R of sheep, J (A) of goats, A of pigs and J of cattle can be detected on red cells and/or in serum by use of "natural" cattle, pig or goat anti O (Stormont, 1951; Sprague, 1958a,b; Rasmusen, 1964). J, R and pig A are generally regarded as being dominant or epistatic to O at least at the cellular level. The alleles for A and O in animals are thought to be at a single locus or cistron A\textsuperscript{a}/A\textsuperscript{o} in pigs (Andresen, 1963; Rasmusen, 1964), J/J\textsuperscript{a} (O) incattle (Stone, 1962) and R/r (O) in sheep (Rendel et al., 1953). However, since some animals are found which show neither J (A or R) or O reactivity on cells, or in serum or both, the activity of a further locus (I/i in sheep - Rendel et al., 1953; Ss in pigs
- Rasmusen, 1964) has been postulated. In the homozygote recessive situation (ii or ss) the production of blood group substance is prevented in i sheep, or its acquisition by red cells is blocked in "-" pigs.

**Inter-species Relationships**

Since A like antigens and anti A like reagents are so widely found in man, animals, plants, fungi and bacteria the A-O and ABO H (Ii) Lewis blood group systems are a fertile field for study of inter-species relationships. There are many similarities. We have already seen how heterologous anti O (H) from various species can be used for typing red cells in animals and man. Bovine anti J after suitable absorption or appropriately diluted can be used to type for sheep R, goat J (A), pig A and human A. Anti-R from r (O) sheep and anti-A from non A (O) pigs can also be used for human A (Tucker, 1962; Neimann-Sorensen et al., 1954; Andresen, 1963; Rasmusen, 1964; Gold, 1967). The A antigen seems to stand out strongly in all species as a relatively unified specificity.

However, there are many differences at cellular level, both quantitative and qualitative. Hence cattle anti J reacts more strongly with R sheep cells than with J cattle cells, and this may partially be due to an extra specificity reactive with sheep R and human A after absorption with cattle J cells (Neimann-Sorensen et al., 1954). Anti R, on the other hand,
does not lyse cattle cells. "Natural" anti A from man does not react with pig A cells, although immune anti-A haemolysins do react with pig A (Winstanley et al., 1957; Konugres and Coombs, 1958).

Pig saliva or gastric mucin containing soluble A substance in common with human A secretor saliva will inhibit the reaction between "natural" anti A and human A cells, as well as the reaction between pig anti A and pig cells or human A cells. Winstanley et al. interpreted this to mean that pig A saliva and gastric mucin possessed an additional specificity to that on pig cells. However, pig anti A reacting against A pig cells (A^P of Winstanley et al.) by the antiglobulin test only, readily agglutinates human A cells in saline. Saison et al. (1955) put this down to the peculiar nature or situation of the antigen on the pig red cell membrane. The reactivity of pig A cells both in the direct test and in absorbing anti-A reagents is increased by treatment with papain (Olds, 1961).

Hojny and Hala (1964), using a bovine anti J, two porcine anti A sera, and a pool of rabbit anti human A_1 sera, found a wide variation in activity against A pig cells by the haemolytic, saline agglutination, indirect antiglobulin sensitisation and dextran techniques. A class of pigs designated Ac reacted to varying extents with all the reagents, but an additional class of pigs (Ap) only reacted with one particular porcine anti A by the dextran test. This class (Ap) had a subtype relationship to Ac in that it only absorbed reaction
against itself, whereas Ac cells removed reaction against Ac and Ap cells. This appears to indicate a separate but related specificity. A further related specificity is Pl8 of Rasmusen (1964). Anti Pl8 reacts preferentially with "-" pigs and juvenile pigs, i.e. pigs lacking A and 0 or only just acquiring it, and the frequency of reaction falls off in adult A and 0 pigs. Possibly Pl8 is competing for basic blood group substance or alternatively it may be basic BGS.

The variations in the reactions of different anti A reagents against pig cells are consistent not only with the ability of different animals to produce antibodies against different subfactors of the A mosaic, but also with the proportions of different immunoglobulin classes produced by different animals capable of agglutination and haemolysis of different parts of the antigen or the same antigen at different levels of accessibility. Alternatively, mating experiments with Ap, Ac and A negative pigs suggest complex interactions of a qualitative and/or quantitative type at genetic level since Ap X A negative matings gave 58 Ac pigs, 23 Ap and 60 A negative offspring. The interesting point here, of course, is that, using the haemolytic or antiglobulin test reagents alone (and not the dextran reagent), some A negative parents will have A positive offspring. This is the basis for postulating AO suppression by genes at the Ii locus in sheep and the Ss locus in pigs. Unfortunately Hojny and Hala did not use anti 0 to determine the relationship of Ac and Ap with 0.
In the 1968 comparison test (Dinklage, 1968) only two Ap were supplied. These two pigs were 0 negative with three anti-0 reagents but reacted with a fourth (anti-0 Gb/Sc) from this laboratory. After incubating the reaction for 6 hours (Imlah, 1963) this reagent, a heterologous goat serum, cross reacts with A positive pigs, and gives a higher frequency of positive reactions overall. In fact most cattle anti-0 reagents cross react with A pigs if the test is left long enough (Saison - personal communication; Tucker - personal communication), and this is perhaps not surprising. Heterologous reagents are unlikely to give such a clear cut differentiation as isologous reagents, which, being produced by animals with the antithetical antigen, do not normally cross react with it.

Thus although some selected cattle antisera largely from "-" type cattle (Sprague, 1958b; Tucker, 1962) react more strongly with certain non A type pigs it is still possible that pig A and possibly "-" pigs are in fact 0 positive to a lesser extent or less accessible to detection by haemolytic antibody.

**Soluble Blood Group Substances**

Animal A-0 systems, although serologically similar to human ABH, are in other ways like the Lewis blood group system of man. Thus, like Lewis, the A and 0 properties are acquired by taking up soluble blood group substance from the plasma
either in \textit{vivo} or \textit{vitro} (Andresen, 1962; Ycas, 1949; Rendel et al., 1954; Stormont, 1949; Saison and Ingram, 1962; Rasmusen, 1964; Sneath and Sneath, 1959). In fact the blood type of these animals can be changed by incubating red cells of one group in plasma or serum of the antithetical type. Pig red cells can carry both A and 0 specificities (Saison - personal communication), human cells can carry both Le\textsuperscript{a} and Le\textsuperscript{b} substances (Sneath and Sneath, 1955), and sheep cells can be made to react as R and r (0) positive (Rendel et al., 1954).

It has already been pointed out, however, that A (J and R) are dominant or epistatic to 0 at least at the cellular level, and this enables an analogy to be drawn between 0 of animals and H since A and H show a quantitative reciprocity in man (with certain exceptions in infants and A intermediates - Voak and Lodge, 1968). The common origin of anti-0c and anti-H in bovine sera supports this, as does the inhibition of the bovine anti 0/pig 0 reaction by human secretor saliva (Rasmusen, 1964).

Le\textsuperscript{b} is apparently dominant or epistatic to Le\textsuperscript{a} at cellular and serum levels in humans, although Le\textsuperscript{a} is almost invariably present in saliva except in Le (a- b-) persons. The Le\textsuperscript{b} character determined by certain anti Le\textsuperscript{b} reagents shows a marked epistasis in the presence of the A\textsubscript{1} gene in man. Other reagents react equally well with A\textsubscript{1} Le (b+) persons and people of other ABO groups.

All these conflicting views and analogies between animal A-0 and human ABH Lewis soluble blood group substances can be
partially reconciled by a consideration of the human soluble substance biosynthetic pathways (Morgan and Watkins, 1969). Unidentified genes cause the production of a precursor glycoprotein with a number of polysaccharide side chains. Under the influence of the A, B, H and Le genes various additions are made to these chains by particular linkages. H, Le^a and Le^b will arise by the addition of L-fucose residues to the precursor. The H gene alone causes the enzymatic transfer and attachment of an L-fucose to D-galactose by an alpha 1,2 linkage on side chains 1 and 2, and this produces H substance. The Le gene alone attaches on L-fucose to N-acetylglucosamine by an alpha 1,4 linkage on side chain 1 and produces Le^a substance. In the presence of both H and Le genes two L-fucose units are attached to side chain 1 by their respective linkages and in close proximity result in Le^b substance. There is therefore no gene for Le^b as it is a hybrid substance produced at sero-morphological level from Le^a and H substances. H substance is still produced by the attachment of L-fucose to side chain 2. Hence Le^b and H are linked (Marr et al., 1967) on the same molecule in a similar way to allotype specificities on Ig molecules. Here, however, since the specificity is carbohydrate and therefore not a primary gene product, the opportunities for interaction at the sero-morphological level is increased. Although interaction products can be traced back to gene action this can arise at several independent loci.

The A and B genes in man act on H active substance adding
N-acetylgalactosamine and/or galactose respectively. This explains the suppression of H by A (particularly A\(_1\)) and B.

The occurrence of BGS in saliva in man is governed by the Se/se locus, and in addition the serum and therefore cellular Le\(_b\) only results from the action of the Se gene as well as the Le and H genes. The cellular A, B and H arise directly from the A, B and H genes irrespective of secretor status, and in this man differs from animals which only take up A and O when it is secreted in serum.

It can be seen from this consideration of human biosynthetic pathways that Le\(_a\) and H are basic to the formation of Le\(_b\) and that all three are biochemically similar. There are also many serological similarities. For example, Kornstad (1969) recently demonstrated by titration and absorption that anti Le\(_b\) reagents from A\(_1\) or A\(_1\)B Le (a+ b−) donors have a large anti H reactivity, whereas similar reagents from Le (a− b−) donors also have an anti H component, but it is significantly smaller. These two types are regarded as anti H (Le\(_b\)) and anti (H) Le\(_b\) respectively. Both types of sera gave better reactions where greater amounts of H are present on the red cells, i.e. in O, A\(_2\) and some group B persons, and all reactivity could eventually be absorbed with O Le (a− b−) cells. It appears likely that in general all these reagents are anti-L-fucose immuno determinants, and the differences arise from the different sero-morphological configurations brought about by the different linkages.
A-O-H Soluble Blood Group Substances of Domestic Animals

Although at the cellular level the A (J and R) factors are regarded as dominant over O there is some variation in this particularly when we consider serum and saliva. Thus Tucker (1962, 1962a) investigated the cells, serum and saliva of sheep, goats, pigs and cattle using a bovine anti J and anti O. In addition she tested the cells and saliva for the presence of the H factor using a plant lectin Ulex europaeus. None of the animals' cells reacted with Ulex europaeus extract, and the particular anti O used failed to react with pig 0 cells. Other authors have reported reactions between pig 0 cells and bovine, goat and porcine anti O reagents (Sprague, 1958a; Saison and Ingram, 1962; Rasmusen, 1964). With the exception of the H/anti H results and the failure of bovine anti O to haemolise pig 0 cells, the results shown in the following table have been confirmed by the following workers: sheep (Rendel, 1957; Tucker, 1961), goats (Suzuki and Stormont, 1961, cited by Tucker, 1962), pigs (Sprague, 1958a; Rasmusen, 1964). Tucker, however, has demonstrated a number of intermediate categories, e.g. goat type J, the pig with J, 0 and H in its saliva. Sprague (1958a) found cattle with J and 0 in their serum.
<table>
<thead>
<tr>
<th>Species</th>
<th>Type</th>
<th>Cells</th>
<th>Sera</th>
<th>Saliva</th>
<th>No. of animals</th>
<th>Antibody in serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheep</td>
<td>J</td>
<td>J</td>
<td>OH</td>
<td>7</td>
<td>10</td>
<td>Anti-R (7)</td>
</tr>
<tr>
<td>Pigs</td>
<td>J</td>
<td>J</td>
<td>OH</td>
<td>15</td>
<td>15</td>
<td>Anti-A (15)</td>
</tr>
<tr>
<td>Goats</td>
<td>J</td>
<td>J</td>
<td>OH</td>
<td>8</td>
<td>8</td>
<td>Anti-J (7)</td>
</tr>
</tbody>
</table>
From the table it can be seen that sheep are the most straightforward in that the dominance of R (J) over O is complete except in saliva. Sheep of type i have no detectable BGS, and in saliva of other sheep O is synonymous in its distribution with H. Goats are similar to sheep but soluble O substance is present with J in serum in type JO. The pig, however, is unique among the four species in that the serum of all pigs contains O substance (even "-" pigs - Rasmusen, 1964), or at least some substance inhibiting the reaction between anti O and sheep O cells (Tucker, 1962) or pig O cells (Rasmusen, 1964).

In sheep and goats, therefore, O and H are possibly identical, and in serum and on red cells of R sheep the O (H) substance is converted to R, which is thus dominant to O (H).

There is, therefore, no reason to stipulate separate O and H genes in sheep, although my analogy with human biosynthetic pathways necessitates that some gene (presumably H or a precursor gene) produces basic carbohydrate for R to act upon in the R/R homozygote. This condition would be satisfied if the O gene is an amorph as in man and the I gene produces O (H) or allows its expression or secretion after production by the H gene. In the i sheep the homozygote recessive genotype ii either fails to produce O (H) or fails to allow its expression or secretion after production by a separate Hh locus. The analogous situations in man are the hh and sese genotypes respectively (Race and Sanger, 1968).
The occurrence of O substance in parallel with J in the JO goats indicates that the dominance of J over O is not absolute at the serum level. This can be explained on a quantitative basis.

In pigs not only is O substance invariably in serum and saliva irrespective of AO type, but O occurs in saliva in the absence of H in the J (A) type of pig and in the fourth category of pig. It is possible that the latter is in fact a "-" pig, but this could not be confirmed since the anti O failed to react even with genuine O pigs' cells. H appears to be predominantly associated with the saliva of the cellular type O pig, and since, unlike sheep O and H, porcine O deviates from H at least at the saliva level, an alternative hypothesis for porcine AO is required. Rasmusen suggested (1964) that an independent locus Ss not only interacted with the A^a/A^o locus to produce A and O, but also controlled their acquisition by the red cells. This is an ingenious suggestion, but ignores the fact that the point of application of the S/s genes for A differs from that for O. Thus in the "-" pig of ss genotype no A substance whatsoever is found even though some "-" pigs must be homozygote A^a/A^a. For O, however, even "-" pigs of presumed genotype A^a/A^a ss still possess O substance in serum and saliva. A further locus may have to be advanced to explain the presence of O substance, leaving the A^o gene once again as an amorph. The ss genotype would then prevent the production of A and H at the cellular, serum and saliva level.
just as in sheep.

The intermediate type of A pig lacking cellular J may in actual fact be A positive since anti J varies in its ability to react with A (Saison and Ingram, 1962). The quantity of A substance in pigs' serum varies considerably, and it is likely that a series of A alleles exist as in cattle. Cattle fall into three groups with regard to J:

\[ J^{cs} \text{ - J on cells and in serum} \]
\[ J^s \text{ - J in serum} \]
\[ j^a \text{ - J negative} \]

The situation between \( J^{cs} \) and \( J^s \) is largely quantitative, and a quantitative analysis indicates that there are at least four subclasses within each class governed by a series of 8 graded alleles. Above a certain threshold value J is acquired by the red cells giving \( J^{cs} \) cattle. Some animals, however, fall outside the class distribution on the basis of the threshold value, and it may be that some other factor is operative in addition to quantity of J substance (review by Stone, 1962).

In cattle it appears that an even greater variety of combinations of 0, or H and 0, occur with different quantities of J. How far these can be explained on the basis of quantitative or qualitative interaction at genetic or sero-morphological level is largely unknown.
Ontogenic Variation in A and O Coating Ability of Porcine Serum

Blood group substances A and O are absent from the cells of newborn pigs, but appear on the cells at varying times during the first month of life (Goodwin and Coombs, 1956; Saison and Ingram, 1962; Andresen, 1963). A similar situation exists for J of cattle (Stone and Irwin, 1954), R of sheep (Ycas, 1949) and Lewis blood group factors of man (Sneath and Sneath, 1959). Substances with A inhibiting activity are found, however, in saliva, gastric juice (Goodwin and Coombs, 1956) and serum (Andresen, 1963; Lang – personal observations), and the serum of newborn calves, lambs and babies of the appropriate group (to be) contains J, R and Lewis substances (Stone and Irwin, 1954; Ycas, 1949; Lawler and Marshall, 1961a,b). It seems likely that O substance will also be present at birth in pigs since all adult pigs have O substance, although as we know only group 0 pig red cells become fully reactive with anti O reagents. In A and "-" pigs the juvenile condition of non-reactivity with anti-O continues, although the reason is not necessarily the same in each case. In cattle the non-reactivity of calves' cells with anti-J continues into adult life in the case of J^s animals.

Various reasons have been advanced for the non-reactivity of neonatal piglets. Some authors (Goodwin and Coombs, 1956; Andresen, 1963) have suggested that piglets' cells differ in their ability to take up BGS, because of some inherent differences in early cells (Goodwin, 1954, cited by Goodwin and
Coombs, 1956). Alternatively a quantitative hypothesis has been advanced that insufficient A substance is present in piglets' serum (Andresen, 1962).

Small quantities of blood for tests purposes were obtained from the ear vein of both piglets and piglets using sterile needles. In reality the only way to get a blood sample was with a quick clip with a needle and placing it directly into the test tube. Care should be taken not to contaminate the sample with test solutions or other reagents. For large volumes of blood the whole method needs to be used for eg to test for vitamin deficiency providing the subject felt secure and comfortable. For subjects maintained by gentle pressure on the side of the ear, and it was occasionally necessary to pin down the ear of a sheep.

After bleeding the ears were washed.

Piglets were restrained in a modified nest made by wrapping a thin walled plastic tube around the wall, and tying it in place with a rope. This was placed under the upper jaw and around the nose. Blunt tips were inserted into the ears and a 7 ml evacuated tubes connected. After filling with saline a 10 ml evacuated tube was attached to the 7 ml evacuated tube. A T-shaped system (Becton, Dickinson and Co, Falmouth MA). The ear veins were raised up and placed next to the ear and pinched. Piglets could also be laid between a sheet of open plastic (6 x 25 x 0.25 mm) with a piece of adjacent transparent P.V.C. tubing (1.8 x 2 mm) being affixed along with the vein. The closed vacuum system was a convenient improvement particularly as the tube did not need to be connected
MATERIALS AND METHODS

Red Cell Serology

Blood Sampling

Small quantities of blood for test purposes were obtained from the ear veins of both pigs and rabbits by aseptic techniques. In rabbits the outer ear vein was nicked longitudinally with a scalpel blade, and blood taken into 5 ml "bijou" or 1 oz wide-necked "Universal" vials containing A.C.D. (for cells) or dry (for serum). For large quantities of serum the same method could be used for up to 70-80 ml of whole blood, providing the rabbit felt secure and relaxed. Flow was maintained by gentle pressure on the base of the ear, and it was occasionally necessary to moisten the ear with xylol. After bleeding the ears were washed.

Pigs were restrained in a tubular steel crate hinged to the wall, and they were attached to this by a rope noose round the upper jaw and nose. Blood was withdrawn from an ear vein into a 7 ml evacuated tube containing A.C.D. (for cells) or into a 10 ml evacuated tube dry (for serum) by the "Vacutainer" system (Becton, Dickinson and Co., Rutherford, New Jersey, U.S.A.). The ear veins were raised by an elastic band over the ear base. Pigs could also be bled through a sterile open needle (1½ ins 16 G) with a piece of attached translucent P.V.C. tubing (1½ - 2 ins long) by gravity flow into open vials. The closed Vacutainer system is a considerable improvement particularly as the fine (20 G) needle used creates
less damage.

Large volumes of blood for serum or absorptions were taken by courtesy of Dr. Imlah from the anterior vena cava of pigs restrained in the standing position. This was done through a closed sterile P.V.C. bleeding set (based on that on p. 206, Dunsford and Bowley, 1955) with a 6 in. 15-16 G needle and a vacuum (10-25 mm of Hg) applied to the bottle (1 pint B.S.S. Blood Transfusion Service bottle incorporating 120 ml of A.C.D. solution for cells or dry for serum) via the filtered airway.

Vacutainer tubes are supplied sterile. All other containers and the bleeding sets (wrapped in autoclavable Portland Plastics nylon sleeving in tins) were steam sterilised at 15 lbs/sq.in. for 20 mins. The bleeding sets were dried overnight in a hot air oven at 80° - 90°C before closing and taping.

Red Cell Suspensions

Blood taken into A.C.D. was immediately gently mixed with anticoagulant, and stored at +4°C. Cells for test purposes were generally used as a 2% suspension in saline (0.9% NaCl w/v aq. dist. with 10 ml of \(
\frac{M}{5}
\) phosphate buffer pH 7.2 added per litre). Red cells were washed four times in isotonic saline before being standardised by comparison with a standard 2% suspension prepared from packed cells. The comparison was made by eye before an illuminated opalescent glass sheet or in an Eel Portable Colorimeter. Suspensions were prepared daily as required, and samples were not used beyond 5 days old.
Serum Samples and Antisera

Samples taken for serum were incubated at 37°C without shaking for up to 1 hour to allow satisfactory clot formation to take place. The clot was then separated from the glass by gentle shaking, and the sample was stored overnight at +4°C. This facilitates the removal of cold autoagglutinating antibodies by adsorption on to the red cells, and allows satisfactory clot retraction. Small quantities of serum for preliminary tests were merely poured off the clots, centrifuged, decanted, recentrifuged (M.S.E. Minor Bench Centrifuge - 3,500 r.p.m.) and tubed or bottled. Larger quantities of serum bled aseptically for antisera or for injection were separated by sterile separating sets (Dunsford and Bowley, 1955, p. 119) using a water vacuum pump (Speedivac Stainless Steel Water Jet Pump, Edwards High Vacuum Ltd., Manor Royal, Crawley, Sussex). After centrifugation of the separated serum for 30 mins. (M.S.E. refrigerated Magnum 1,800 r.p.m. Special M.S.E. buckets with a protective collar for 1 pint B.S.S. transfusion bottles) in sterile 1 pint bottles, it was transferred aseptically using a separating set into a sterile bottle or 1 oz vials.

Only serum shortly to be absorbed with red cells in preparation of reagents (red cell typing or rabbit antiglobulin) was heat inactivated at 56°C for 30 minutes. Bulk sera originating before 1960 and transferred from Cambridge to Edinburgh had all been heat inactivated after separation from the clot. Serum taken specifically for serum protein
investigations was not heat inactivated unless specifically stated in the text.

**Red Cell Antigen/Antibody Techniques**

In this context, the term "sensitisation" refers to the specific attachment of antibody to red cells, and has no other immunological connotations. As indicated earlier, all tests were generally carried out using a 2% suspension of red cells. The measurement of quantities in tests is on a 1 drop:1 drop basis with a Pasteur pipette dropping 0.025 ml. All tests were carried out in tubes (glass precipitin tubes 50 x 7 mm). Antiserum dilutions and cell suspensions are prepared in larger tubes (50 x 10 mm and 75 x 10 mm respectively). The advantages of small tubes are many. Small quantities of reagent can be incubated for long periods without evaporating. Even with small quantities of reagent the column of reactants is quite high, and the red cells settle slowly through the entire volume of antiserum. This takes time but results in improved sensitisation of the cells. Primary incubation is for a minimum of 1½ hours. This can be extended up to periods of 3-4 hours without deleterious effect, and even 6 hours in tests at room temperature (R.T.). Tests are carried out at 37°C except where indicated, apart from haemolytic tests which are carried out in a refrigerated incubator/shaker maintained at 20°C.

Titrations are carried out in doubling dilutions of anti-
serum or other reactant. Neat serum is indicated by $\frac{1}{1}$ as opposed to 1:1, and thus takes no account of dilution by antigen or complement. For red cell typing the final dilution for use of typing reagents does take into account the addition of human group A secretor saliva, where this is necessary to neutralise unwanted anti A.

**Direct Agglutination Technique (D.A.)**

This technique is a one-stage test and depends on the presence of antibody capable of producing agglutination directly following sensitisation of one drop of red cell suspension by one drop of suitably diluted antiserum for 1$\frac{1}{2}$ hours at 37°C. The results are read microscopically on slides and scored descriptively ($V =$ visual, $\dagger =$ double positive, etc.) or numerically ($V = 10, \dagger = 8,$ etc.) after Dunsford and Bowley (1955). In general D.A. produces a clumping type of reaction and is best read microscopically (Gold, 1968). In describing the test the details are abbreviated as follows: D.A. 2% 37°C 1$\frac{1}{2}$ hrs.

**Indirect Sensitisation (Coombs) Test (I.S.T.)**

This test is used with incomplete antibodies which fail to agglutinate directly, and was carried out according to Coombs et al. (1945). This is a two-stage test. The first stage is identical with the D.A. technique. The contents of the tubes are then washed four times, the final supernatant is removed
using a Pasteur pipette and water vacuum pump, and rabbit anti-pig globulin is added at a suitable dilution. The preparation and standardisation of rabbit anti-pig globulin is described later under reagent production.

The details for the test are abbreviated as follows:

I.S.T. 2% 2% 37°C 37°C 1½ hrs 1 hr Bacon 24/27 $\frac{1}{35}$

"Bacon" is the Department of Pathology, Cambridge, code for rabbit anti-pig globulin Coombs reagent, and this is followed by the batch number and dilution.

This test largely produces sedimentation pattern types 3-5 inclusive (Gold, 1968) but rarely types 1 and 2, although type 2 occurs with particularly strong reactions where the clumping component is stronger. The I.S.T. is therefore read macroscopically over a concave mirror, and microscopic readings invariably give a lower score. Scoring is as for the D.A. test, but scores C = complete = 12 and V = visual = 10 are reserved for the rare type 1 and 2 patterns which are confirmed microscopically. When the Coombs reagent is added the reactants must be thoroughly mixed.

During the course of investigating pig sera for anti-globulin activity it was shown that the addition of normal pig serum to washed sensitised cells produced agglutination. This is regarded as being due to the macromolecular media effect of the pig serum and this type of test is termed the M.M.M.I.S.T. to distinguish it from the M.M.M.R.T. to be described next.
Macromolecular Media Replacement Technique (M.M.M.R.T.)

This technique is set up as for the one-stage D.A. test, but following incubation of cells and serum the supernatant fluid is removed by flicking or pipetting off, and is replaced by some macromolecular medium, in this case normal pig serum. After a further 30 mins incubation the results are read microscopically on slides and scored as for D.A. The M.M.M.R.T. differs from the M.M.M.I.S.T. in that there is no washing of cells, and using pig serum the M.M.M.R.T. gave lower titration scores. The basis of the test is the Sheffield albumin replacement technique (Dunsford and Bowley, 1955) in which diluted bovine serum albumin facilitates Rhesus typing in man with certain incomplete type anti-sera.

Haemolytic Technique (H.T.)

This is a normal two-stage test after Imlah (1963). The concentration of the red cell suspension used, however, is kept at 2%. In order to ensure adequate mixing during shaking the Pasteur pipette drop size is reduced to 0.015 ml. This size is also more economical on reagents. After mixing one drop of antiserum and one drop of 2% cell suspension the tubes are left for 30 mins at R.T. One drop of fresh rabbit complement (RC') previously absorbed at 4°C with washed packed A and O pig cells is then added, and the rack shaken for 2 mins every hour for 6 hours in a refrigerated/incubator shaker maintained at 20°C. The tests then remain at 20°C until read without shaking or are
transferred to a +4°C refrigerator overnight and read the following morning over a concave mirror. Scores were read on a numerical scale 0-5 which corresponds to a range of haemolysis 10-100% (Imlah, 1963). Smaller degrees of haemolysis were recorded as trace (tr.) amounts.

**Haemagglutination Inhibition Technique (H.I.)**

This is an extremely sensitive method of detecting and quantitating a soluble antigen where a suitable haemagglutination system incorporating that antigen is available or can be made up. It has been used to detect and measure soluble A blood group substance in the serum and saliva of pigs and rabbits and in human secretor saliva.

The fluid under test is titrated in doubling dilutions. To each dilution is added 8-25 minimal agglutinating (+) doses (as appropriate) of anti A. After mixing and standing at R.T. for 30 minutes to allow neutralisation to occur, group A pig cells are added, and tested by the normal technique (I.S.T.) to detect residual anti A. This method gives a prozone of negative results proportional to the degree of inhibition. The result is expressed as the reciprocal inhibition titre of one M.A.D.

Where prior tests indicate an antigen/antibody reaction between the test fluid and the A pig cells used, the test fluid must be absorbed free of antibody preferably using the test cells (see absorption later). A minimal agglutinating dose
(M.A.D.) is determined by prior titration of anti A against the test cell, and the number of M.A.D.s used in the test should be adequate to give a positive result before reaching the end of the test fluid titration.

The H.I. test is also used in the determination of serum allotypes. Since these are antigenic properties of serum components some method is required to attach the particular component to the red cells. In Ig allotyping use is made of the antigen/antibody reaction between a red cell typing reagent and appropriate red cells. The red cell typing reagent must carry the allotype to be determined on a sufficient proportion of its anti red cell antigen Ig molecules, and these must be presented on the surface of the red cell in such a way that they are capable of agglutination by the anti-allotype reagent. Even among coating reagents bearing the appropriate allotype and coating the cell satisfactorily, only a certain limited number of cell/antibody combinations are suitable for the allo­typing H.I. test (Steinbuch et al., 1965; Gold and Lockyer, 1964; Rasmusen - personal communication; Lawler, 1967). An investigation of the H.I. test for allotyping is dealt with under the appropriate heading and details of the technique after Rasmusen (1965a) will be found there.

Where the occurrence of allotypes of other serum components are suspected or anticipated the antigenic components can be attached by means of the tanned cell technique.
Tanned Red Cell Technique

Pig red cells were tanned and coated with whole pig serum after a technique by Herbert subsequently published (Herbert, 1967).

The use of this technique in the demonstration of anticipated anti-antibody and anti-allotype reaction is discussed more fully under a later heading.

Absorption

Absorption is the process of rendering an antiserum more specific by removing or neutralising unwanted antibodies to additional antigen(s).

In the case of antibodies against red cell antigenic factors the serum is mixed with packed washed cells carrying the antigen whose homologous antibody is to be removed. After incubation at the optimum temperature for combination of antigen and antibody for 30 mins, the serum is recovered by centrifugation. The red cells should, of course, be negative for the antigen whose antibody is to be retained. Absorption is repeated until no reaction occurs with the absorbing cell. The use of the term "absorbing cell" or "positive cell" is to avoid repetition of the phrase "cells of the positively reacting animal".

Production of Red Cell Typing Reagents

The bulk antisera for the production of red cell typing
Reagents were drawn from a number of sources:

1. Supplies of sera from sows vaccinated with crystal violet vaccine whose litters had suffered from H.D.N.B. were donated by courtesy of Dr R.F.W. Goodwin and the Agricultural Research Council.

2. Sera containing antibodies produced as a result of deliberate iso-immunisation of selected recipients with donor blood selected for one or more antigenic differences.

3. Screening of normal pig sera for natural anti A (Goodwin and Coombs, 1956; Saison et al., 1955) and normal goat sera for natural anti O (Rasmusen, 1964).

The mixtures of antibodies produced by crystal violet vaccine and even by planned immunisations were analysed and quantitated by the normal checkerboard, absorption and titration methods using mainly the D.A. and I.S.T. techniques after Joysey et al. (1959a). Instead of testing initially at a dilution of $\frac{1}{10}$, however, serum was invariably tested at two dilutions $\frac{1}{2}$ and $\frac{1}{4}$ or neat and $\frac{1}{2}$. As a result the primary checkerboard rarely gave recognisable patterns of reaction.

Progressive removal of antibody was accomplished by selecting one or more columns from the primary checkerboard, and after absorption of some serum with the primary absorbing cell, subjecting the absorbed serum to a secondary checkerboard with the remaining positive reactors in that primary column. This process of breakdown and analysis continues sometimes through
numerous secondary and tertiary checkerboards. Eventually a pattern of reaction of unit specificity remains, as demonstrated by the fact that absorption by any positively reacting cell removes all activity against all reacting cells. The identity of antibodies in mixtures is established not only by the patterns of reaction remaining after absorption, but also by reactions removed by absorption.

Reagents are prepared by absorption of raw antiserum using the sequence of cells indicated by the checkerboards. Where this sequence is drawn out and where all the antibodies can be identified, however, it is often possible and preferable to select cells which will absorb reagents in one or two steps only. Whichever procedure is adopted the unit specificity of the reagent prepared should be established by:

1. Testing at the dilutions used in the checkerboard against a large number of positive and negative cells by the technique(s) of choice.

2. Absorption by additional positive reactors other than those used in the checkerboard.

3. Using the reagent at a dilution selected by titration in parallel with a reagent of established specificity in a programme of routine blood typing. This function was performed by using the reagents produced in the Blood Group Research Unit's progeny blood typing programme.
Serum Protein Analytical Techniques

Gel filtration techniques

a) Sephadex G200. Gel filtration was performed using Sephadex G200 in Pharmacia columns K25/45 (2.5 x 45 cms) and K25/100 (2.5 x 100 cms) with upward flow adaptors after Flodin and Killander (1962) and Killander (1964). The columns were packed according to the manufacturer's booklet (Sephadex - gel filtration in theory and practice), and packing pressure did not exceed 6” hydrostatic head. Upward flow was used, and optimum separation was found with flow rates down to 10 ml/hr. Since this gives a rather prolonged run on the large column a flow rate of 16-17 ml/hour was operated by a delta peristaltic pump. Loading with serum was 5-10 ml.

Fractionations gave the usual triple peak (19 S, 7 S and 4.5 S) distribution of protein followed after some time by the 4th peak containing small molecular weight material with a Kd = 1.0.

b) Sephadex G25 Fine was used for buffer exchange or desalting experiments after Flodin (1961) in the Sephadex K25/45 and K15/30 (1.5 cms x 30 cms) columns by downward or upward flow. Since substances in excess of 5,000 M.W. are excluded from G25 this gel effectively separates proteins as a single peak distinct from substances of low molecular weight (less than 5,000), which normally appear in the 4th peak on G200 runs.

c) Recycling chromatography (Porath and Bennich, 1962). Unless a very long column is used, the three peaks of a
Sephadex G200 fractionation still show considerable overlap. One solution to this is to pump the fractions round the column again thus increasing the effective column length. Unless loading in relation to column length and diameter is very carefully calculated, this can lead to mixing of fractions. On G200, macromolecules in excess of 800,000 M.W. (for globular proteins) are excluded from the gel, appearing in the first (19 S) peak immediately after the void volume (i.e. the volume of buffer lying between the gel particles = Vo) has been pumped off and replaced by effluent buffer. Since the remaining proteins (of less than 800,000 M.W.) enter the gel particles and are retarded to varying extents depending on molecular size (molecular weight and shape), a 19 S macroglobulin peak pumped immediately back on to the column quickly reappears in the effluent (after one further void volume has been pumped) while the third (4.5 S) peak is still emerging from the column on primary separation. In order to avoid this, two steps were taken. The primary fractionation was carried out on the K25/100 column as if for a single pass, and half the macroglobulin peak (Peak A) was pumped off for collection. This is not deleterious since the macroglobulins being excluded fall outside the fractionation range of G200 (5,000 - 800,000 for globular proteins), and are not being materially separated from one another, but only from the 7 S peak. The remaining half of the macroglobulin peak, being contaminated by still overlapping the 7 S peak (Peak B), and the rest of the primary
eluant is then pumped on to the K25/45 column before returning through the K25/100 (second pass). The introduction of the small column delays the return of the 19 S peak by an amount equal to the Vo of the K25/45, thereby allowing the recycled peak A (A') to fall between the 4.5 S peak (Peak C) and the 4th peak (Peak D) still emerging on primary separation. As soon as peak C has been passed on for recycling, the line connecting the K25/100 to the K25/45 is disconnected, pump on is continued with eluting buffer, and peaks A', D (primary peak to waste), B' and C' are pumped off for collection. If necessary, peaks B' and C' can be recycled once more to improve separation. Two factors intervene, however, to make this undesirable. Firstly, operating the column at 17 ml/hr gives a run time of 72 hours, and, secondly, peak spreading due to diffusion of the proteins in the eluting buffer produces an increasingly dilute eluant.

Protein Quantitation

Protein was measured semi-quantitatively using UV absorption at 2537A on an LKB Uvicord absorptiometer with a quartz measuring cell (initially a standard 0.1 ml circular cell with 3 mm path, but later a 0.1 ml rectangular cell with 2 mm optical path). The absorption was recorded on an LKB Chopper Bar Recorder mainly at a chart speed of 10 mm/1 hour. Collection was made into 7 ml tubes on an LKB Radirac automatic fraction collector controlled to give constant volume either by
a siphon tube and balance or by using a synchronous motor timer in conjunction with a synchronous motor pump. With a pump speed of 16-17 ml/hour the latter method gave samples of 5-6 ml in 20 minutes.

**Ion Exchange Chromatography**

Ion exchange chromatography was carried out using D.E.A.E.-Sephadex A-50 and C.M.-Sephadex C-50 either to further fractionate the peaks from Sephadex G200 or as a preparative method from whole serum. D.E.A.E.-Sephadex was used by a column elution technique using starting buffer conditions (Levy and Sober, 1960) and elution gradients (Aalund et al., 1965) based on the original method (Peterson and Sober, 1956) using D.E.A.E.-cellulose as anion exchanger. The Sephadex anion exchanger was also used in a batch process using various starting buffers to isolate porcine Ig G2 on a preparative scale after Baumstark et al. (1964). These preparations were further fractionated by column chromatography on CM-Sephadex C-50 using starting buffer, gradient and stepwise elution for analysis by I.D. and I.E.

**Pressure Dialysis and Protein Concentrates**

Individual fractions or pools were concentrated to their original volumes or smaller by pressure dialysis in Visking tubing against a 5-10% solution of Carbowax 4,000. The resultant concentrates were freed of Carbowax monomers by
pressure (8-9 ft of water) dialysis against isotonic buffered saline (pH 7.7) which also made them suitable for red cell serology.

**Buffer Solutions**

Initially gel filtration experiments were eluted using 0.1 M Tris/HCl pH 8.0 + 1 M NaCl after Killander (1964). Since this buffer is not isotonic for red cell serology parallel fractionations with 0.9% NaCl w/v aq. dist. buffered with \( \frac{M}{2} \) Na/K phosphate pH 7.2 (1800 ml NaCl sol. + 200 ml buffer) were carried out and analysed by I.E. No substantial differences could be noted and the use of Tris/HCl + NaCl was discontinued.

Ion exchangers were equilibrated with large volumes of starting buffer after swelling according to the Procedure B as laid down in the manufacturer's booklet "Sephadex Ion Exchangers - an outstanding aid in biochemistry". Since D.E.A.E.-Sephadex is an anion exchanger early column fractions were carried out with a Tris/HCl pH 8.0 buffer in a gradient 0.015 M \( \rightarrow \) 0.03 M. This procedure was used to fractionate 19 S and 7 S peaks from G200 fractionation runs. Following the changeover to phosphate buffered solutions for molecular sieving, and coincident with the combined use of D.E.A.E.- and C.M.-Sephadex Na/K phosphate (Sorensen) buffer was used exclusively for batch and column work to avoid the necessity of frequent buffer exchanges on Sephadex G25. Sorensen buffer
was used in various starting molarities from 0.01 to 0.025 M and in the pH range 6.5 - 7.2, and gradients were operated from 0.01 M phosphate buffer pH 6.5 → 0.9% NaCl w/v aq. dist. phosphate buffered at pH 7.2. Combinations of gradients and step-wise elutions were used from 0.025 M phosphate pH 6.6 to 0.05 M phosphate pH 7.8 with a final clearance of the column with 1 M NaCl. Regeneration of the ion exchangers was carried out as per manufacturer's instructions using 0.5 N NaOH and 0.5 N HCl.

**Electrophoresis and Immuno-electrophoresis on Agar Gel**

A micro technique on microscope slides was carried out on an LKB apparatus with 1% Difco (Special Agar Noble) in a Veronal/Calcium Lactate buffer pH 8.6 after Hirschfeld (1960). The migration of albumin was checked at 20 mm from the origin by using Bromophenol Blue. Whole serum or fractions were run on microscope slides arranged in six groups of three slides making eighteen slides per run. Current flow across each slide was 3-4 mA at a potential of approximately 6V/cm. There was no cooling, and a run lasted approximately 1½ - 1¾ hrs for an albumin migration of 20 mm. Antigen was applied by micro pipette. Amounts varying from 3-10 lambda were used depending upon the protein concentration and optimal proportions of the reaction in hand. Antiserum was applied in a longitudinal trough using a 0.1 ml graduated pipette in amounts from 50-200 lambda according to requirements. Precipitation reactions were examined twice daily for 48 hours on average, but
occasionally for longer. They were then washed in two changes of isotonic saline and finally in distilled water over a period of two days. After drying under lint free filter paper the slides bearing the dried agar film could then be used in staining procedures as for ordinary microscope sections (Photographs 1, 2a, b).

Where samples were electrophoresed only and no antiserum added the proteins were fixed by rinsing the wet slides in 2% acetic acid for 15 mins. The slides were then put through normal washing and drying procedures.

Precipitation reactions were carried out at R.T. in a moist chamber.

**Antiseptic Agents**

Sodium azide $\frac{1}{5,000}$ or Thiomersal $\frac{1}{10,000}$ was added to buffer solutions for gel filtration, I.D. and I.E., pressure dialysis. N-butanol was used as a preservative for ion-exchange slurry.

**Immuno Double Diffusion in Gel (Ouchterlony) Technique**

Apart from some early provisional investigations carried out with large wells in petri dishes which are described in the text, immuno diffusion (I.D.) runs were carried out as a micro technique on microscope slides. Ten ml of agar (1.5% Difco Special Agar Noble in isotonic buffered saline) was poured on to rows of three slides in plastic L.K.B. racks as supplied for
I.E., and held on a levelling table until set. After maturing usually overnight a honeycomb pattern of 31 3 mm holes was cut using a special die with 31 cutters. This die was held in an L.K.B. Gel Punch set with a spring recoil action, and 31 wells could be punched simultaneously. Agar plugs were removed using a wide bore (2.5 mm O.D.) Pasteur pipette and a water vacuum pump. This pattern could be adapted to give various arrangements (see Photographs 15a, 16, 17), but the most usual was for screening two precipitin reagents each at four dilutions (neat – $\frac{1}{2}$) against one or two antigens each at four dilutions (neat – $\frac{1}{6}$), giving 16 reactions for each reagent, i.e. 32 per slide. Additional control reactions could also be accommodated, e.g. rabbit anti pig globulin or saline controls. The diffusion distance between all adjacent wells was 2 mm, and reactions were left in a moist chamber at R.T. for an average of 48 hrs, but occasionally for 3-4 days. At the end of the reaction period the slides were washed, dried and stained as in the I.E. procedure.

This micro technique is extremely economical using only 5 lambda of antigen and antibody for each reaction, and since 40 reactions at least could be arranged on one slide it was economical on space and other supplies. Experience has shown that pig iso-precipitins in particular suffer from prozoning in antibody excess as well as antigen excess. It was therefore essential to screen for possible antigen and antibody at a number of dilutions of each, and this slide technique was ideal.
With up to twenty antisera to screen against one or more antigens, sometimes with daily bleeds it was impractical to test at dilutions beyond $\frac{1}{6}$. As the titre of antibodies developed some difficulties arose. This took the form of pseudo-spurring at right angles to the normal precipitation lines, and was caused by antigen diffusing across from the next adjacent set of dilutions (Photograph 15b). There was a strong indication from this that contrary to some workers' expectations antigen or antibody can pass through lines of precipitation and form a further line with extra antibody or antigen diffusing from the well. This occurs most readily when there is a lack of optimal proportions, and throws some doubt on the rule that multiple lines must necessarily mean multiple antigen-antibody systems. This will be dealt with later when discussing the "double line" phenomenon.

Scoring of Ouchterlony Reactions in Allotyping

As already indicated, the occurrence of prozoning both in Ab. as well as Ag. excess necessitated titrating both reactants. Not only did the titre of antibody against the standard antigen (donor serum) vary during immunisation but the range over which optimal proportions existed also varied. Considering this in terms of the normal plot of varying antigen versus quantity of precipitate for a standard quantity of antiserum (Fig. 5), it is apparent that the entire unimodal curve can move to left or right, become shorter or taller and broad or narrow. In these
Figure 5. Hypothetical Ag/Ab precipitation plot.
Fig. 5

Ab. constant.

Ab. constant but less.
circumstances specifying the titre of either antigen or antibody only gives a horizontal dimension across part or all the curve depending on its position, i.e. the degree of prozoning. In order to introduce the vertical dimension and hence the area under the curve \(1\) plus the log (to the power of two) of the reciprocal titre was multiplied by an avidity factor based on an estimate of precipitation at optimum proportions. Thus

\[ + \rightarrow \frac{1}{4} = 6 \times (1 + 2) = 18 \]

\[ (+) \rightarrow \frac{1}{2} = 4 \times (1 + 1) = 8 \]

Two or three reagents gave the appearance of going beyond \(\frac{1}{4}\) and were then merely classed as \(\geq \frac{1}{4}\). In these circumstances avidity is plotted as a separate curve.

### Production of Hetero-immune Antiglobulin Reagents

Heterologous reagents were produced in rabbits and fell into three main types:

1. **Coombs Reagent.** In order to produce a wide spectrum reagent against all immunoglobulins and components of complement, rabbits were injected with a pool of several pigs' sera. However, extremely potent antisera also resulted from injections of individual pig's serum. Sera were titred and standardised against cells sensitised with various types of red cell typing reagents to find a common optimum dilution for use (see Dunsford and Grant, 1959). Where several reagents had widely differing optima a satisfactory reagent was produced by pooling two reagents in inverse ratio to the reciprocal of their individual
optimum titres. Antisera were also tested against non-sensitised cells for anti red cell antibody, and where necessary this was absorbed out using packed six times washed red cells from A and 0 pigs after heat inactivation of the serum (56°C for 30 mins).

2. Immuno-electrophoresis reagents. Although many Coombs reagents were suitable for use in identifying pig serum components on I.E. this was not necessarily the case for non-immunoglobulin components. Consequently specific I.E. reagents were produced by injecting individual whole sera or A', B' and C' concentrates from G200 recycle chromatography runs. The purpose of the latter was to confirm the identification and distribution of different pig serum components in the various peaks of a G200 fractionation as demonstrated by I.E. using rabbit anti whole serum reagents.

3. Reagents produced by injecting rabbits with their own washed cells after incubation with certain individual pig's sera. This was part of an attempt to:
   a) produce antibody against pig A0 serum components by coupling these to rabbit cells; and
   b) produce antibody against immunoglobulins selected by combination with rabbit species specific red cell antigens (Gold and Lockyer, 1961; Hamilton-Fairley and Harris, 1962; Yokoyama and Resnik, 1964).

Both these attempts proved abortive, and these rabbits
eventually produced Coombs and I.E. reagents after injection with whole serum of individual pigs.

**Immunisation Procedure**

The basic procedure was to give 0.5 - 1.5 ml of washed sensitised rabbit cells, whole serum or concentrated fractions subcutaneously on 3-4 occasions at 3-4 day intervals as a primary immunisation course. After a rest period which was prolonged as long as possible, but was usually only 2-3 months, a booster course was given of 1.0 ml of antigen on 4-5 occasions at 3-5 day intervals, the last injection or two being intramuscular. Frequent pilot samples were taken during the course and the number and timing of injections varied according to the progress of antibody titre and avidity. After the final injection samples were taken at 2 or 3 day intervals, and a large sample bled for reagent between 7-10 days after the final injection. No adjuvant was used.

**Staining Techniques**

To facilitate the localisation and identification of certain serum components on I.E. and I.D. a number of specific or semi-specific stains were utilised, and these are similar to those reviewed by Imlah (1963) for starch gel electrophoresis or by Peetoom (1963) for I.E.

**General protein staining** was by Amido Black (0.1% in a 50:50:10 methanol, water and acetic acid mixture, which was
also used as a wash solution) or Azocarmine (0.1% in acetate buffer). The latter was washed in 2% acetic acid in water. Since the agar was not being removed from the slides, glycerin (as recommended by Uriel and Grabar (1957) for macro plates) was omitted from staining and wash solution. Slides were stained from the dry state for 5-10 mins in Amido Black but for up to an hour or more in Azocarmine. Amido Black was given four 15 minute washes, but Azocarmine was rinsed fairly quickly in 2-3 changes until the background was clear.

Lipoprotein staining was by Sudan B in 60% ethanol (saturated solution) without the addition of NaOH. Depending upon the quantity of fat present the staining time varied, but at least 1 hour was desirable. Differentiation was rapid in two changes of 50% ethanol and was by inspection.

Glycoprotein staining was by a Periodic acid/Schiff technique. Although pretty it was of little diagnostic use since the rabbit and pig immunoglobulin molecules contained appreciable carbohydrate so that all precipitin lines were stained. The technique was after Bodman (1957) as cited in the Oxoid booklet - "Electrophoresis with Oxoid Cellulose Acetate Strips".

Caeruloplasmin staining was achieved by its oxidase ability on \(5 \times 10^{-3}\) M paraphenylene diamine in 0.1 M acetate buffer pH 5.7 (Brummerstedt-Hansen, 1967, after Uriel, 1957). Both these authors are mistaken in the quantity of P.P.D. required to make up a \(5 \times 10^{-3}\) M solution! The solution is
poured over the dried slides sufficient to soak the agar thoroughly, but not to cover it to any depth as access to air is required. After 1-2 hours at 37°C the slides are washed in 2% acetic acid solution. The P.P.D. is oxidised to form a dark brown compound, which shows up clearly against azocarmine counterstaining.

**Haptoglobin and haem-binding globulin (haemopexin)** staining was demonstrated by prior combination of these proteins with haemoglobin and its breakdown product haematin respectively, and using the peroxidase activity of this group to bring about a bright blue colour with 0.2% benzidine in the presence of hydrogen peroxide (Imlah, 1963).

**Combined Staining on One Slide**

In starch gel electrophoretic investigations gels can be sliced horizontally into two or even three sections to allow individual investigations of the one run by specific staining techniques.

Using I.E. to investigate the many fractions from molecular sieving or ion exchange runs it became impractical to duplicate I.E. runs for each staining technique. To overcome this difficulty the possibility was investigated of staining with each of four staining techniques consecutively. It was found that P.P.D. staining would only function if carried out first. After the acetic acid wash to remove P.P.D. the slides were washed in distilled water to remove the acid which other-
wise inhibited staining by benzidine for haptoglobin and haemopexin. This light blue staining, although occurring rapidly, faded equally rapidly (within 15-30 mins). The position of these arcs could be noted in the protocol, marked on the back of the slide using a diamond cutter, or pricked out with a mounted needle. After washing again in distilled water the slides were then stained as normally with Sudan B followed by Azocarmine.
EXPERIMENTAL WORK AND RESULTS

Immunoglobulin Allotyping and Anti-antibody Investigations

The primary aim of these experiments was to find allotyping reagents for immunoglobulins or other pig serum components with a view to assisting in their classification from a serological point of view as much as from the genetic and progeny testing angle. Eventually of course such reagents would also help in the localisation of structural variation in proteins and its relation to function, as well as helping to form concepts about the mode of production and transport (across membranes, in colostrum, etc.) of different serum components. There is little need to elaborate on the excellent examples set by man, rabbit, mouse and other species in this and other respects. A second aim was to look out for the possibility of finding or producing anti-antibody and rheumatoid factor (R.F.) types of reagent with the further thought that some immunological effect might thereby be mediated.

Initially, therefore, an experiment was designed based on the use by Dubiski et al. (1959a, b) of bacteria coated with antibacterial immunoglobulin. Instead of antibodies against bacteria, however, red cell typing reagents were used to coat the recipients' own red cells for injection. This gave an
opportunity to produce allotype and idiotype reagents as well as antibody against immunoglobulins modified by combining with antigen (Experiment 1).

Following the reported finding of gamma globulin allotypes Gl a and Gl b in pigs (Rasmusen, 1965a), it appeared that this approach might bring success, since these factors were found by a haemagglutination-inhibition system. Gamma allotyping of the animals used in Experiment 1, however, indicated that Gl b+ animals had received Gl b+ serum.

Experiment 2 was set up specifically to produce anti Gl a and anti Gl b. Only one definite strong Gl a+ pig could be demonstrated among the panel animals and all the recipients appeared to be Gl b+. This situation was utilised in setting up a controlled experiment to compare the use of a double emulsion adjuvant WOW (water in mineral oil in water) containing whole pig serum with the use of pig serum alone without adjuvant. Whole serum was used in preference to fractions since it was easier, gave the possibility of detecting allotypes of other proteins in addition to Ig G and took into account the difficulty found by other investigators (Pickup - personal communication) in preparing immuno-electrophoretically pure fractions.

Since Experiment 1 had not given the coated cell technique an adequate opportunity, a small number of recipients again received their own cells coated with antibody or serum proteins passively attached by the tanned cell technique.
After the initial use of whole serum and WOW in a primary immunisation course the recipient pigs were boosted with whole serum (Courses 2a, 2b and 2c).

Following on the typing of the panel with the precipitins produced in Experiment 2, an extension of this experiment was set up to produce precipitins against a possible allelic product to the factors being detected (Experiment 3). A double emulsion (WOW) incorporating non-reacting serum was injected into pigs whose serum reacted with the precipitins including nine pigs of a double positive mating. Also included in Experiment 3 for purposes of administration and presentation was a repeat of Experiment 2 using the surviving members of both schedules as recipients for WOW.

**Antibody Coated Erythrocytes Technique**

**EXPERIMENT 1**

Nine pigs were injected with their own washed red blood cells coated with iso-immune antibody to red cell antigens, and three pigs were given their own red cells only as a control. A volume of cells equal to 10 ml of whole blood was washed four times, packed and the packed cells incubated at 37°C for 1½ hours with a volume of neat antibody equal to the packed cells (i.e. about 4–5 ml). The cells were then washed four times in isotonic buffered saline, reconstituted to a 50% suspension in saline and injected intramuscularly (i/m) in the neck. A 2% suspension of the washed cells gave a maximum positive direct Coombs test with a rabbit anti-pig globulin reagent at the
routine dilution for detection of red cell typing reactions.

Injections were continued weekly for 6 weeks i/m between 26/4/65 - 31/5/65 incl. and serum samples were taken immediately prior to injection from a convenient ear vein. All samples are given the code $\gamma$ and numbered consecutively $\gamma_1 - \gamma_6$ ($\gamma_1$ is the preimmunisation sample).

**Pigs and Coating Sera Used**

Five of the pigs were Large White, and the remaining seven were from litters of Gene Pool dams crossed with Large White or Landrace boars. At the start of the experiment they were mature gilts of 6-12 months of age, and were selected by red cell typing to be positive for red cell factors Eb, Gb, 3 and 9. These are high frequency antigens to which antibodies were known to be present in the coating sera. The schedule of recipients was as follows:

<table>
<thead>
<tr>
<th>Control pigs</th>
<th>1 12</th>
<th>5 42A</th>
<th>9 9325</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pigs receiving</td>
<td>2 16</td>
<td>6 42</td>
<td>10 18</td>
</tr>
<tr>
<td>own cells</td>
<td>3 22</td>
<td>7 38</td>
<td>11 23</td>
</tr>
<tr>
<td>coated with</td>
<td>4 29</td>
<td>8 9328</td>
<td>12 9326</td>
</tr>
<tr>
<td>antibody:</td>
<td>Bates Sow 3 serum</td>
<td>Dean 41 serum</td>
<td>Kate serum</td>
</tr>
</tbody>
</table>

All pigs were positive for the factors indicated above with the exception of pig 12 which did not have factor 3.
The coating reagents were obtained from sows vaccinated with crystal violet vaccine which had produced haemolytic disease of the newborn in their litters. On slaughter or before they were bled for a large volume of serum, which had been stored for about 10 years at -20°C after heat inactivation at 56°C for 30 mins.

These sera were investigated for antibodies against red cell antigens by the direct agglutination and indirect antiglobulin sensitisation (Coombs or I.S.T.) techniques using the standard checkerboard, absorption and titration techniques. The principal antibodies found in each serum were prepared and standardised as specific reagents by absorption and titration.

<table>
<thead>
<tr>
<th>Serum</th>
<th>Absorbed by pig No.</th>
<th>Antibody to factor</th>
<th>Titre</th>
<th>DA</th>
<th>IST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bates</td>
<td>Htp 1 W5525</td>
<td>Gb</td>
<td>tr</td>
<td>1/16</td>
<td></td>
</tr>
<tr>
<td>Sow 3 bled</td>
<td></td>
<td>Ka</td>
<td>1/2</td>
<td>1/8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>9</td>
<td>1/2</td>
<td>1/8</td>
<td></td>
</tr>
<tr>
<td>-/5/54</td>
<td></td>
<td>A, Eb, W</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Dean 41 bled</td>
<td>4581 6914</td>
<td>Eb</td>
<td>1/32</td>
<td>1/32</td>
<td></td>
</tr>
<tr>
<td>29/9/55</td>
<td>Htp 1 W3184</td>
<td>Gb</td>
<td>nil</td>
<td>1/20</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>A, Ea, 9</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ka, ? La</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Kate bled</td>
<td>4731</td>
<td>Eb</td>
<td>1/4</td>
<td>1/16</td>
<td></td>
</tr>
<tr>
<td>16/2/56</td>
<td>T38p 12</td>
<td>3</td>
<td>1/2</td>
<td>1/32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Gb</td>
<td>tr</td>
<td>1/4</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ib or 9</td>
<td>1/2</td>
<td>1/16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
No specific reagent was prepared in bulk against factors given in the table where absorbing animals are not indicated. These additional specificities were present, however, in the raw sera used for coating cells, although because of the recipients' blood type some, like anti-Ka or -A, would not react in every case.

Following upon the weekly i/m injections the same quantity of antigen was given intravenously at less frequent intervals (16.7.65, 13.8.65, 26.8.65 and 6.9.65) and serum samples \( \gamma_7 \) to \( \gamma_{10} \) were taken (except in the case of pig 29 where no injection was carried out and no sample taken corresponding to \( \gamma_{10} \)).

**Tests and Results for Experiment 1 - by Haemagglutination Technique**

Since the intention was to demonstrate the possible presence and/or production of isologous anti-globulins the serum samples \( (\gamma_1 - \gamma_{10}) \) were to be used in an I.S.T. in the same way as for normal red cell typing, but using raw (un-absorbed) Bates Sow 3 (B3), Dean 41 (D41) and Kate (K) serum as sensitising reagents and the gamma serum samples in lieu of rabbit anti-pig globulin reagent. There were two points to bear in mind. Firstly, the sensitising reagents reacted strongly with the cells by direct agglutination in saline (DA) as well as by I.S.T., and, secondly, the addition of macromolecular media (in the form of neat autologous pre-immune serum) to sensitised cells even after washing several times had
a strong enhancing effect on agglutination. The titration scores for DA (normal gravity sedimentation for 1½ hrs with microscope reading), for M.M.M.I.S.T. (using each recipient's neat autologous \( \frac{1}{1} \) pre-immune serum in lieu of Coombs reagent) and for some macromolecular medium replacement technique titrations are compared in the following table. In each case the sensitising serum as indicated (B3, D41 and K) was titred in doubling dilutions to the extent indicated. After sensitisation the tests for M.M.M.I.S.T. were washed 4 times as for the normal I.S.T. before adding neat \( \frac{1}{1} \), appropriate to each recipient under test. Saline and \( \frac{1}{1} \) serum auto-agglutinin controls were put up with sensitised and non-sensitised cells respectively. At this time a slight serum auto-agglutinin manifested itself against the non-sensitised cells giving a very weak agglutination in a negative field with neat autologous serum for most of the recipients.

<table>
<thead>
<tr>
<th>Anti serum</th>
<th>Recipients' cells</th>
<th>DA ( \frac{1}{1} - \frac{1}{32} )</th>
<th>MMMIST ( \frac{1}{1} - \frac{1}{128} )</th>
<th>MMMRT ( \frac{1}{1} - \frac{1}{512} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>B3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>13</td>
<td>62</td>
<td></td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>21</td>
<td>&gt;78</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>20</td>
<td>&gt;65</td>
<td></td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>24</td>
<td>68</td>
<td></td>
</tr>
<tr>
<td>D41</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>42A</td>
<td>22</td>
<td>&gt;80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>37</td>
<td>&gt;80</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>39</td>
<td>&gt;80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9328</td>
<td>35</td>
<td>&gt;78</td>
<td></td>
</tr>
<tr>
<td>K</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9325</td>
<td>25</td>
<td>&gt;78</td>
<td></td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>38</td>
<td>&gt;78</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>41</td>
<td>&gt;78</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9326</td>
<td>33</td>
<td>66</td>
<td></td>
</tr>
</tbody>
</table>

All tests were carried out at 37°C.
In order to avoid the D.A. effect of the sensitising antiserum a dilution of \( \frac{1}{16} \) in isotonic saline was used in testing the antiglobulin properties of the gamma serum samples. Since the enhancing effect of the \( \gamma_1 \) autologous serum was shown against unwashed cells and coating serum in the M.M.M.R.T. as well as against washed sensitised cells in the M.M.M.I.S.T., there was a possibility that this represented an autologous anti antibody of the Milgrom type. Preliminary investigations indicated that using a standard dilution (\( \frac{1}{4} \)) of an incomplete reagent (Kate abs. T38p 12 anti 3 D.A. titre \( \frac{1}{2} \) I.S.T. titre \( \frac{1}{32} \)) as a sensitising agent the enhancing effect on titration of \( \gamma_1 \) from pigs 18, 23 and 9326 did not extend beyond a dilution of \( \frac{1}{8} \). The titration scores of B3, D41 and K by the M.M.M.I.S.T. were not reduced by absorbing \( \gamma_1 \) serum aliquots with three consecutive volumes of packed washed autologous red cells previously sensitised with B3, D41 or K as appropriate.

The red cell antigen/antibody aggregates were prepared by incubating at 37\(^\circ\)C packed washed recipient pigs' red cells with equal volumes of a \( \frac{1}{4} \) dilution of appropriate antiserum for 1\( \frac{1}{2} \) hours before washing 4 times and repacking prior to absorption. Equal volumes of each \( \gamma_1 \) serum and packed cells were mixed, and left for 15 mins with periodic agitation before being packed and the supernatant removed. This absorption was carried out three times (twice at 37\(^\circ\)C and once at R.T.). In addition to not being removed by absorption with antigen/antibody aggregates in one case at least (pig 42A \( \gamma_1 \)
and 5 serum samples) the enhancement effect was not destroyed by inactivation at 56°C for 30 mins.

In testing for isologous antiglobulins the recipients' red cells (2% suspensions) were coated with the appropriate reagent (B3, D41 or K) at a dilution of \( \frac{1}{16} \), and the gamma samples were titred from \( \frac{1}{2} \) to \( \frac{1}{64} \) in doubling dilutions to allow for a possible prozoning effect at the dilution of sensitising reagent used. The sensitised cells were checked with a rabbit anti pig globulin reagent and a serum auto-agglutinin test against unsensitised cells was included. Reading of the tests was by microscope except for the D/c check which was read macroscopically. The type of agglutination pattern given by pig serum in the D.A. and M.M.M.I.S.T. or M.M.M.R.T. is quite different from that given by rabbit serum in the normal I.S.T. The latter corresponds to sedimentation pattern types 3-5 as defined by Gold (1968), whereas the former are much more likely to give combinations of types 1 and 2. If type 3 sedimentation patterns are expected, type 1 and to a lesser extent type 2 patterns can be read as negative by macroscopic reading although they may give strong clumping as read by microscope on a slide. Type 3 patterns, however, regularly give stronger reactions read macroscopically. It is always important to consider which patterns and modes of reading shall be considered significant: macroscopic without shaking (sedimentation pattern), macroscopic without shaking followed by shaking (sedimentation pattern and clumping but the latter partially destroyed),
macroscopic (sedimentation pattern) and microscopic on slides (clumping but also a tendency for break up due to handling) and finally microscopic reading on slides alone (clumping).

The titration of $\gamma_1 - \gamma_6$ against washed sensitised cells gave the following scores by microscope reading on slides:

<table>
<thead>
<tr>
<th>Gamma No.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>B3</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>1/16</td>
<td>22</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>D41</td>
<td>42</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>42A</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>1/16</td>
<td>38</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>9328</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>K</td>
<td>18</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1/16</td>
<td>23</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>9326</td>
<td>0</td>
<td>0</td>
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Each score is for a doubling dilution titration of $\frac{1}{2} - \frac{1}{64}$.

Over a period of six weeks and after five i/m injections there was no change in the ability of the gamma serum samples...
to produce agglutination of cells sensitised with subagglutinating doses of antiserum. The average score for an antiglobulin reagent (hetero-immune) under the same conditions would be 70 although the scoring would not be strictly comparable.

After one more i/m injection (the sixth) and three i/v injections $\gamma_{10}$ was titred ($\frac{1}{4} - \frac{1}{64}$) against cells sensitised with purified specific reagents (diluted $\frac{1}{4}$).

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</tr>
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<table>
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<th>$\gamma_{10}$</th>
<th>Bacon</th>
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<td>$\frac{1}{64}$</td>
<td>$\frac{1}{40}$</td>
<td>$\frac{1}{4}$</td>
</tr>
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</table>
Bacon is the code word for rabbit anti pig globulin reagent. Pig 12 is negative with K anti 3 because its red cells are 3 negative. In this test \( \sqrt[9]{9} \) was used for pig 29 since no serum sample or injection corresponding to \( \sqrt[10]{10} \) were made in this pig. The titration scores are larger than in the previous table against certain cells, because the titre was started with neat gamma serum. There is, therefore, still no indication of an anti-globulin reaction in these gamma sera.

Tests by Double-diffusion Ouchterlony Technique

As a preliminary investigation into production of precipitins in pigs, certain selected gamma serum samples were tested against their appropriate whole serum in double diffusion in agar in plastic petri dishes. Agar (1% Difco Ion agar) was prepared both in distilled water and in phosphate buffered (pH 7.2) saline, and both normal serum and serum heat aggregated at 63°C for 30 mins were used as antigens at dilutions of \( \frac{1}{20}, \frac{1}{40}, \frac{1}{80} \) and \( \frac{1}{160} \). Neat \( \sqrt[1]{1} \) or \( \sqrt[6]{6} \) serum was placed in a 6 mm centre well, and the antigen in 4 surrounding wells of the same size giving a diffusion distance of 4 mm. Each antigen and antibody was tested against saline as negative control, and the antigen serum dilutions both normal and aggregated were put up against rabbit anti whole pig serum precipitins as positive control. The latter gave strong multiple lines of precipitation, but all other tests were clear negative except for tests in distilled water agar which gave concentric white halos around certain
wells. These were proportional to the quantity of fat in the serum, and as indicated subsequently by staining with Sudan B were probably caused by aggregation between the agar and low density lipoprotein. Fatty serum could be improved by centrifugation in a refrigerated M.S.E. Magnum for upwards of 4-5 hours at 3,000 r.p.m. at 4°C followed by removal of the surface layer of fat by gentle suction through a Pasteur pipette.

\[ Y_1 \text{ and } Y_6 \text{ serum samples of 42, 38 and 9328 were tested against dilutions of D41 both normal and heat aggregated in distilled water and saline agar. Serum samples (} Y_1 \text{ and } Y_6 \text{) of 16, 22 and 29 were similarly tested against B3, and 18, 23 and 9326 against K serum. All these tests were negative apart from the aforementioned lipid aggregation in distilled water agar. Plates were inspected twice daily for three days. All tests were kept at R.T.} \]

Conclusion

In conclusion the results of Experiment 1 gave no evidence for the production of isologous or autologous antiglobulins of allotypic, anti-antibody, idiootypic or Andresen type as determined by haemagglutination or precipitation techniques.
Investigation of the Haemagglutination-Inhibition System of Allotyping Pigs

Dr B.A. Rasmusen at the termination of his visit to Scotland kindly left a small quantity of reagents for his system of gamma allotyping in pigs. This system consisted of four reagents:

1. Serum P5* (\(\frac{1}{16}\)) a Gla+ anti-Kb coating reagent for sensitising a Kb+ red cell with a sub-agglutinating dose of Gla+ gamma globulin.

2. Serum 420 (\(\frac{1}{16}\)) an isologous anti-Gla to agglutinate Gla+ coated red cells.

3. Serum P64 (\(\frac{1}{4}\)) a Glb+ anti-Lc coating reagent for sensitising an Lc+ red cell with a sub-agglutinating dose of Glb+ gamma globulin.

4. Serum 266 (\(\frac{1}{16}\)) an isologous anti-Glb to agglutinate Glb+ coated cells.

An animal whose red cells were Kb+ and Lc+ and which gave fairly satisfactory reactions had been selected by Dr Rasmusen from the pig panel.

In view of the fact that results with these reagents at the dilutions normally used by Dr Rasmusen (given in brackets after each serum above) were difficult to interpret, an initial restandardisation was attempted. Pig BWR red cells were sensitised for 1\(\frac{1}{2}\) hours at 37°C with the normal dilutions of P5* and P64 plus a doubling dilution above and below (i.e. P5* \(\frac{1}{8}\), \(\frac{1}{16}\), \(\frac{1}{32}\); P64 \(\frac{1}{2}\), \(\frac{1}{4}\), \(\frac{1}{8}\)), and after washing 4 times in isotonic
saline each dilution was tested with the normal dilution of the appropriate isologous anti-globulin plus a doubling dilution above and below (i.e. 420 anti Gla $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$; 266 anti Glb $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$). After mixing and incubation at 37°C the results were read microscopically on slides. The Gla/anti-Gla system reacted satisfactorily at all dilutions both of sensitizing anti-serum and anti-globulin. The results for the Glb/anti-Glb system were barely satisfactory at the lowest dilutions (i.e. $\frac{1}{8}$ and $\frac{1}{6}$ respectively). The coating as determined by rabbit anti-pig globulin was satisfactory at all dilutions of sensitizing anti-sera.

The haemagglutination test systems for the two factors Gla and Glb were used at the following dilutions:

1. P5 Gla+ anti-Kb $\frac{1}{16}$  
2. 420 Anti-Gla $\frac{1}{10}$  
3. P64 Glb+ anti-Lc $\frac{1}{2}$  
4. 266 Anti-Glb $\frac{1}{8}$

Some improvement was found if the secondary incubation (i.e. after washing the coated cells and adding antiglobulin) was carried out for 2 hours at R.T. Using a titration inhibition system a well established Gla+ serum (L8729) inhibited anti Gla ($\frac{1}{16}$) completely at a dilution of $\frac{1}{4}$ and partially at a dilution of $\frac{1}{64}$. A well established Glb+ serum (gamma pig 12) only partially inhibited anti Gla when added neat, but completely inhibited anti Glb ($\frac{1}{3}$) at up to a dilution of $\frac{1}{64}$. Serum L8729 (Gla+ Glb ?) also inhibited anti Glb ($\frac{1}{8}$) partially at a dilution of $\frac{1}{4}$. Using the sera under test at a dilution of $\frac{1}{4}$
and §, and mixing a volume of these dilutions with an equal volume of each appropriately diluted antiglobulin before adding the sensitized cell indicator system, gave clear results in agreement with Dr Rasmusen's for definite Gla+ and Glb+ sera. On using this system with the serum of Minnesota Miniature pigs, however, the occurrence of varying degrees of partial inhibition was difficult to interpret. The Gla indicator system was less easily inhibited than the Glb system, so that although some sera seemed definitely Glb+ the frequency of the Gla factor in the sera depended on the degree of inhibition selected as significant. A large scale quantitative assessment of inhibition by titration inhibition techniques would be necessary to ascertain statistically the number of classes of variance. Unfortunately, there was insufficient coating reagent for this.

Another complication was the appearance of agglutination in the control tests where saline was substituted for the antiglobulin reagent. In addition in the titration inhibition system the red cells were agglutinated when the antiglobulin reactivity was strongly inhibited by neat test serum, and this was presumably due to antibodies against the test cell present in the sera under test. Even at a dilution of $\frac{1}{4}$ or $\frac{1}{3}$ this subliminal activity together with the sub-agglutinating dose of sensitising antibody would be enough to give some agglutination in the test and confuse the result as indicated in the control tests above.
In summary, therefore, it appears that in agreement with Dr Rasmusen's own findings the sera of pigs under test in Edinburgh did not all fall into clearly delineated positive and negative classes in regard to the factors Gla and Glb. It is not known how far this represents the heterozygous class since the distribution of variation between positive and negative classes was heterogeneous.

Pig Serum Allotyping and the Passive Haemagglutination Technique

One of the disadvantages of the haemagglutination inhibition system is the need for a suitable coating serum. In Gm and Inv typing in man only a small proportion of anti D reagents, even amongst those carrying the appropriate allotype, are capable of providing the configuration detectable by allotyping reagents. Red cells may also vary in their ability to be coated satisfactorily. In addition a system dependent on coating by an antibody is clearly not suitable for showing allotypy of other serum proteins.

Following upon the apparent failure of Experiment 1 to produce iso-immune antibodies against immunoglobulin coated red cells, Experiment 2 was initiated with a view not only to producing antiglobulins for Gla and Glb gamma globulin allotyping factors, but also to demonstrate any possible allotypy of other serum proteins. Since it appeared that pigs might not produce precipitins to allotypic factors (Rasmusen - personal communication) an attempt was made with the remaining
antiglobulin reagents, anti Gla and anti Glb, to set up a passive haemagglutination system using tanned red cells as used in human Gma typing (Steinbuch et al., 1965).

The advantages of such a system are many. It is very sensitive (Herbert, 1967) and eliminates the need for a coating antibody. In addition any protein or carbohydrate antigen can be attached directly to the red cell. For preliminary screening of pig anti whole pig serum reagents it is only necessary in theory to compare the titration score with uncoated and pig serum coated cells.

Accordingly pig BWR red cells were tanned and coated with Gla+ (L8729) $\frac{1}{10}$ and Glb+ (gamma p 12) $\frac{1}{10}$ whole serum before being tested with doubling dilutions of rabbit anti whole pig serum, anti Gla serum 420 and anti Glb serum 266. Uncoated tanned BWR cells were similarly tested. In accordance with the method heat inactivated rabbit serum previously absorbed with pig A and O red cells was added to the saline for all washing, dilutions and suspensions to a final concentration of 1%.

Although the rabbit anti whole pig serum reacted only with the pig serum coated cells (to a dilution of at least $\frac{1}{2000}$, the anti Gla and anti Glb reacted equally well with tanned cells both coated and uncoated to a dilution of $\frac{1}{64}$. In addition the two antiglobulins made no distinction between tanned cells coated with L8729 or gamma p 12. Saline controls (containing 1% rabbit serum) were negative indicating that coated and
uncoated tanned cells were not spontaneously agglutinated.

Attempts to absorb out activity with tanned uncoated BWR red cells failed to give consistent results, and it seemed that one or more unknown variables were operative. Repeated attempts at absorption brought no clarification but introduced a number of general observations. Systems involving the use of normal rabbit serum as a stabilising medium consistently gave higher titres in comparison with normal isotonic saline. The titration scores for results read macroscopically (sedimentation patterns) were consistently higher than those read by microscope on slides (clumping). Systems involving normal rabbit serum still gave agglutination to a high titre with anti Gla and anti Glb previously absorbed with tanned cells provided that the tanned cells used for absorbing had not been prepared in a system involving normal rabbit serum. Anti Gla and anti Glb absorbed with tanned cells prepared in saline containing normal rabbit serum were devoid of activity against the same cells, and almost devoid of activity against tanned cells coated with pig serum. Unabsorbed anti Gla and anti Glb agglutinated uncoated tanned red cells in the absence of normal rabbit serum (1%), but did so to a higher titre when rabbit serum was present.
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**NR** - Not read.

A - Bw Runt tanned red cells coated with L8729 serum.
B - Bw Runt tanned red cells coated with gamma p 12 serum.
C - Bw Runt tanned red cells.
Ars - As above but prepared in a system incorporating normal rabbit serum.

Tests were carried out at 37°C.

Although, with the limited amount of serum available, these results were far from conclusive, there appeared to be evidence for the following conclusions:
1. Anti Gla and anti Glb had absorbable activity against tanned pig red cells uncoated and coated with pig serum.

2. The activity against pig serum coated cells bears some relation to the reactivity against tanned cells inadvertently coated with normal heat inactivated rabbit serum components.

3. There is no evidence that anti Gla and anti Glb could differentiate between L8729 (Gla+) and gamma p 12 (Glb+) coating on tanned red cells.

Therefore if the activity shown against coated cells is a true antiglobulin reactivity then it is directed against a factor common to L8729 and gamma p 12 and possibly to normal rabbit serum.
EXPERIMENT 2

Since all the animals used in Experiment 1 appeared to be G1 (a- b+), i.e. G1^b/b and only one animal L8729 typed as strongly Gla+, the possibility of producing anti Glb as well as anti Gla seemed fairly remote. The opportunity was therefore taken to try out Dr W.J. Herbert's double emulsion type of adjuvant. This type of adjuvant has low toxicity, low viscosity, high stability in vitro and disperses readily on injection producing many small depots from which antigen is slowly released. Unlike Freund's complete adjuvant it does not contain Mycobacterium, does not have such a tendency to produce localised lesions, but still has a considerable adjuvant activity (Herbert, 1965, 1966a,b,c,d; Herbert et al., 1965).

Eight animals were available for this part of the experiment in comparing the injection of whole pig serum in water in mineral oil in water (WOW) adjuvant with the injection of whole serum alone. In preliminary experiments Dr Herbert ascertained that a satisfactory double emulsion could be obtained using whole pig serum in saline at a dilution of \( \frac{1}{64} \) giving a final dilution in adjuvant of approximately \( \frac{1}{200} \). Control pigs received whole serum diluted \( \frac{1}{64} \) with sterile isotonic buffered saline. Since some pigs were kept inside a permanent building on concrete, while others were in wooden arks in paved enclosures outside, and since i/m and s/c injections were also to be compared, the injections were given according to the Schedule for Experiment 2.
Since the coated red cell method of immunisation had little opportunity to justify itself in Experiment 1 an Auxiliary Schedule for Experiment 2 was also prepared:
Pigs receiving WOW were injected with 10 ml of adjuvant containing L8729 at a final dilution of \( \frac{1}{200} \) on one occasion only (4/3/66) in Experiment 2 primary immunisation programme. On the other hand, pigs receiving serum only were given 10 ml of L8729 serum diluted \( \frac{1}{64} \) at weekly intervals for a total of six injections. Serum samples were taken weekly on the dates indicated in the table of sampling and injection dates and given gamma numbers 20-26 including pre-immunisation and post-immunisation samples.

It is perhaps appropriate at this point to make an explanation of the gamma numbering system. The code gamma was to distinguish samples meant for allotyping investigation from serum samples taken from the panel animals for other purposes. Since a number of auxiliary immunisation and booster courses followed the primary course of Experiment 2 and these started and finished at different times, all contemporaneous samples were given the same gamma number in order to avoid confusion on any one day's bleeding and injections. Although there were 47 gamma bleeding sessions (1-10, 20-56) any one animal need not necessarily start at gamma 1 and would only be sampled at a proportion of them. Thus sampling and injection dates fall into blocks (see Table IX), and any gamma number is not used for two different dates with the exception of two blocks: 27-33 and 28-32. This is because the serum injection booster course given to the Experiment 2 recipients after a rest period of approximately 12 months was split into two groups which were
boosted during two consecutive but separate periods of time (2a and 2b, Table IX).

Generally speaking the first gamma sample in any block of numbers is the pre-immunisation sample for the injection associated with it. Occasionally, however, where courses are following on fairly rapidly the pre-immunisation sample for a new block of samples will be the late post-immunisation sample from the last course, in which case the first injection of a course may apparently occur before the first sample is taken.

After a rest of approximately eleven months pigs 22 and 42 were given a booster injection of 10 ml of whole serum from L8729, followed six days later by two 2 ml injections of whole serum with a two-day interval. Serum samples were taken at 2-3 day intervals (see Course 2a, Table IX) and numbered 27-33 inclusive. Gamma 33 was a large quantity bled as a reagent.

Pigs 42A, 38, 9328, 23 and 9326 were boosted after approximately twelve months' rest with 10 ml of whole serum from L8729 followed six days later by three 2 ml injections of whole serum at two-day intervals (samples and injections 28-32, Course 2b, Table IX).

The pigs in the auxiliary schedule to Experiment 2 (15, 18, 16 and L8729) were only bled and/or injected with 10 ml of their respective antigens on gamma 22-26 (L8729) and gamma 23-26 (15, 18 and 16). After a twelve months' rest pigs 15, 16 and L8729 were boosted with whole serum injections as per course 2b above but using CO07, CO02 and gamma p 12 as donors of whole serum.
Table IX
Sampling and Injection Dates
for Gamma Allotyping Investigations

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<th>Injection</th>
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<td></td>
</tr>
<tr>
<td>28</td>
<td>3/3/67</td>
<td></td>
<td>49</td>
<td>18/1/68</td>
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<td>8/3/67</td>
<td>50</td>
<td>19/1/68</td>
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</tr>
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<td>30</td>
<td>10/3/67</td>
<td>10/3/67</td>
<td>51</td>
<td>22/1/68</td>
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<td>31</td>
<td>13/3/67</td>
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<td>52</td>
<td>2/2/68</td>
<td>29/1/68</td>
</tr>
<tr>
<td>32</td>
<td>15/3/67</td>
<td></td>
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<td>5/2/68</td>
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</tr>
<tr>
<td>33</td>
<td>16/3/67</td>
<td></td>
<td>54</td>
<td>7/2/68</td>
<td>3c</td>
</tr>
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<td>28</td>
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<td>22/3/67</td>
<td>55</td>
<td>9/2/68</td>
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</tr>
<tr>
<td>29</td>
<td>28/3/67</td>
<td>28/3/67</td>
<td>56</td>
<td>12/2/68</td>
<td></td>
</tr>
</tbody>
</table>
Table X

Distribution of Sampling and Injection Dates amongst Individual Pigs

<table>
<thead>
<tr>
<th></th>
<th>Dates</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>1 - 10, 20 - 26</td>
</tr>
<tr>
<td>22</td>
<td>(1 - 10, 20 - 26, 27 - 33, 34 - 37, 38 - 41, 42, 46 - 51)</td>
</tr>
<tr>
<td>29</td>
<td>1 - 9</td>
</tr>
<tr>
<td>15</td>
<td>23 - 26, 28 - 32</td>
</tr>
<tr>
<td>18</td>
<td>1 - 10, 23 - 26</td>
</tr>
<tr>
<td>16</td>
<td>1 - 10, 23 - 26, 28 - 32, 34 - 36</td>
</tr>
<tr>
<td>12p22, 7p6</td>
<td>38 - 41, 42 - 45, 52 - 56</td>
</tr>
<tr>
<td>4683, I413</td>
<td></td>
</tr>
<tr>
<td>412 x boar 11</td>
<td>38 - 41, 42 - 44</td>
</tr>
<tr>
<td>litter</td>
<td></td>
</tr>
<tr>
<td>4684, boar 11</td>
<td>38 - 41, 42, 46 - 51</td>
</tr>
<tr>
<td>71pl6</td>
<td>38 - 41, 42 - 45</td>
</tr>
</tbody>
</table>

Note: Blocks of sample numbers underlined are those devoted to whole serum booster courses. The remaining blocks of sample numbers are as indicated in the schedules to each experiment. Injections were only given as indicated in Table IX and not on every sampling date.
Pigs 42A, 22, 42, 38, 9328, 23 and 9326 were given a further course of four whole serum injections (L8729) at two to three day intervals and samples were taken on gamma 34-37 (Course 2c, Table IX). Pigs L8729 and 16 were similarly treated with gamma p 12 and C002 serum respectively.

Tests and Results for Experiment 2

Passive Haemagglutination Technique

The primary immunisation course for Experiment 2 gave rise to serum samples gamma 20-26 for animals in the major schedule, and gamma 22-26 for L8729 and gamma 23-26 for pl5, 16 and 18 in the auxiliary schedule. Samples 20, 22 and 23 were the pre-immunisation samples for the respective groups.

A preliminary titration of gamma 20 against

A - tanned cells of pig BWR in normal saline;
B - tanned cells of pig BWR in saline containing 1% normal rabbit serum; and
C - normal washed cells of pig BWR in normal saline

gave the following scores after 1½ hours incubation at R.T., reading macroscopically:
The scores in brackets are for the same tests but scored from $\frac{1}{4} - \frac{1}{128}$ only.

None of the sera reacted at $\frac{1}{4}$ with either normal BWR or tanned BWR cells providing that normal rabbit serum was not incorporated.

The primary immunisation samples gamma 23 - gamma 25 inclusive were tested by titration $\frac{1}{4} - \frac{1}{32}$ against:

A - pig BWR tanned cells coated with L8729 serum (diluted $\frac{1}{10}$);
B - pig BWR tanned cells coated with gamma p 12 serum (diluted $\frac{1}{10}$); and
C - pig BWR tanned cells uncoated.

<table>
<thead>
<tr>
<th>Serum Samples</th>
<th>$\frac{1}{1} - \frac{1}{128}$ versus</th>
<th>Titration Scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gamma 20</td>
<td></td>
<td>A</td>
</tr>
<tr>
<td>from p 12</td>
<td>8 (0)</td>
<td>18 (3)</td>
</tr>
<tr>
<td>p 22</td>
<td>13 (0)</td>
<td>27 (7)</td>
</tr>
<tr>
<td>p 23</td>
<td>0 (0)</td>
<td>9 (0)</td>
</tr>
<tr>
<td>p 42</td>
<td>7 (0)</td>
<td>26 (6)</td>
</tr>
<tr>
<td>p 9326</td>
<td>1 (0)</td>
<td>8 (0)</td>
</tr>
<tr>
<td>p 9328</td>
<td>9 (0)</td>
<td>31 (11)</td>
</tr>
<tr>
<td>p 42A</td>
<td>6 (0)</td>
<td>8 (0)</td>
</tr>
<tr>
<td>p 38</td>
<td>5 (0)</td>
<td>13 (4)</td>
</tr>
</tbody>
</table>
No rabbit serum was incorporated in any of the tests, but the coating was tested with rabbit anti whole pig serum proteins reagent.

The titration scores for the post-immunisation samples tested at R.T. show no increase in activity against coated or uncoated cells (Table XI), and there was no marked difference between the reactions against cells coated with Gla+ or Glb+ serum and tanned cells uncoated.

Controls using L8729 and gamma p 12 pre-immunisation samples and saline were negative. Rabbit anti porcine whole serum (Bacon) showed satisfactory coating of the tanned cells.
### Table XI

**Titration Scores for Gamma Serum Samples against Coated and Uncoated Cells**

<table>
<thead>
<tr>
<th>Serum samples from</th>
<th>Gamma 23 ( \frac{1}{4} - \frac{1}{32} ) versus</th>
<th>Gamma 24 ( \frac{1}{4} - \frac{1}{32} ) versus</th>
<th>Gamma 25 ( \frac{1}{4} - \frac{1}{32} ) versus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>p 12</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>p 22</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>p 23</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>p 42</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>p 9326</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>p 9328</td>
<td>3</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>p 42A</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>p 38</td>
<td>1</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>p L8729</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>p 15 preimm.</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>p 18 preimm.</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>p 16 preimm.</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>p L8729 preimm.</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>p 12 preimm.</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bacon 1/35</td>
<td>++</td>
<td>++</td>
<td>0</td>
</tr>
<tr>
<td>Saline</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
</tbody>
</table>

**Note:** The presence of agglutination in the saline control versus uncoated tanned cells is caused by the instability of the tanned cells in the absence of a serum protein stabiliser.

For identification of A, B and C see text.

NT = not tested or no test because not applicable.
Figures 6 - 18. Precipitin production by pigs 22, 42 and 9328.

Note: The symbols for score and avidity are as given on Fig. 6. Figs. 6 - 14 refer to production of anti 2 only. The symbols for distinguishing anti 1 and anti 2 in pig 9328 are as given on Fig. 15. Each graph overlaps the previous and the succeeding one by one plotted value except between courses 2c and 3 where they overlap by two values. The slope of the broken line joining courses is presumptive, and is only put in for continuity.
Experiment 2  Pig22  Recipient
L8729  Donor

Fig. 6

- (Log reciprocal titre +1) (Avidity)
- Avidity

Titration Score

15
10
5

 Weeks

0 2 4 6

11

10 ml WOW i/m

Months
Experiment 2a  Pig 22  Whole Serum  Boost 1

Fig. 7

Score

Weeks

ml serum

> \frac{1}{8}

10

2

2

16
Experiment 2c  Pig 22  Whole Serum  Boost 2

Fig. 8

Score

Weeks

ml serum

ml WOW

>1/8

30

20

10

0

0

2

2

2

2

3

4

7

8
Experiment 3b  Pig22  Re-immunisation
Whole Serum  Boost

Fig. 10

- Score
- Weeks
- ml serum

- 0 10 11 12
- 10 4
Experiment 2a Pig42 Whole Serum Boost 1

Fig. 11

Score

Weeks

ml serum
Fig. 12

Experiment 2c Pig 42 Whole Serum Boost 2

Score

Weeks  ml serum  ml WOW
Experiment 3 Pig42 WOW Re-immunisation

Fig. 13
Experiment 3b  Pig 42  Re-immunisation Whole Serum Boost

Fig. 14

Score

Weeks

ml serum
Experiment 2b Pig 9328 Whole Serum Boost 1

Fig. 15

Anti 1

Anti 2
Fig. 6

Experiment 2c  Pig 9328  Whole Serum Boost 2

Score

Weeks  ml serum  ml WOW
Fig. 17

Experiment 3  Pig 9328  WOW  Re-immunisation

Score

Weeks

ml WOW
Experiment 3b Pig 9328 Re-immunisation Whole Serum Boost

Fig. 18

Score

Weeks ml serum
Double Diffusion in Gel (Ouchterlony) Technique

The passive haemagglutination technique having given no indication of antibody to serum proteins in the immediate post-immunisation samples, the final post-immunisation sample (Gamma 26) was tested against the appropriate antigens by precipitation in gel on microscope slides (Immuno-diffusion Run No. 1, i.e. I.D. 1 - 5 mm diffusion distance, 5 microlitre quantities of serum antigen at $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, tested against each dilution of antiserum at $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$ for 4 days at R.T.). The serum of only one pig (p 22) showed any reaction under these conditions. The test was repeated (I.D. 2) with 2 mm diffusion distance and washed, dried and stained after two days. Pig 22 reacted more strongly and a further pig (p 42) reacted weakly. Since p 22 showed the strongest reaction p 22 serum samples Gamma 1-10 and 20-27, previously tested by the sensitised red cell and passive haemagglutination techniques respectively and found negative, were tested (I.D. 3 - 2 mm diff. dist., 5 lambda, serum antigen N, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$ versus antibody serum N, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, washed, dried and stained with Sudan B and Azocarmine at 2 days, rabbit anti whole pig serum positive control, antibody versus saline as negative control). Precipitation reaction first appeared in gamma 23, i.e. three weeks after receiving WOW, reached a peak at gamma 25 but was falling off by gamma 26 (6 weeks after receiving WOW). Antibody was still present in gamma 27 eleven months after injection (trace only). These results are plotted in Figure 6.

Following the whole serum booster course 2a, both 22 and
42 showed a marked increase in titre and avidity against L8729 serum. These reactions showed an optimum score at gamma 29-31 for p 22 and at gamma 31-32 inclusive for p 42 (Figs. 7, 11). The single values plotted at the end of each graph usually after a gap give the spacing between courses. The slope of the curve at this point is speculative, but is put in to give continuity between courses. In most instances a reasonable inference can be drawn from events in the subsequent course.

Since the serum boost had been so successful with 22 and 42, the remaining animals from both schedules to Experiment 2 were given a whole serum course in Experiment 2b. Despite their negative results in gamma 25 and gamma 26 of Experiment 2, three further pigs produced precipitins against L8729 serum making a total of five pigs with antibody. Four of these (22, 42, 9328 and 38) had received WOW as primary injection and only one (23) was immunised solely on whole serum (diluted at \( \frac{1}{64} \) for primary immunisation). Pigs 12, 9326, 42A, 15, 16 and L8729 failed to produce antibody against their respective antigens (L8729, C007, C002 and gamma p 12).

**Evaluation of the Precipitation Reaction by Immunodiffusion and Immuno-electrophoretic Analysis**

**Antisera.** All five antibodies had a single predominant reactivity as indicated by a line of identity linking their respective precipitin arcs on immunodiffusion (Ph. 17). Under conditions of non-optimal proportions, which occurred at
different dilutions for each antiserum, there was a marked tendency for two lines to appear. As an approach was made to optimal proportions, however, these two merged (Ph. 17). It is not clear how far this represents an additional minor cross reactive component or some idiosyncrasy of the I.D. arrangement, as it could not be reproduced on I.E. An additional complication already mentioned under Methods was the occurrence of pseudo-spurring by cross over reaction at high concentrations of antibody between adjacent sets of wells (Ph. 15b).

The predominantly unit specificity of the five reagents was substantiated by the all or none reactivity obtained when typing the blood group panel. Out of 38 animals typed, 23 were reactive with all reagents. Of the 15 negative sera 5 reacted very weakly with an extra antibody present in pig 22, and one (boar 11) reacted only with reagent 9328. The extra antibody in p 22 would appear to be a distinct specificity since 2 of the 5 reactors were the pre-immune samples of 9328 and 38. However, as the reaction was very weak and could not be reproduced on I.E., no further work was carried out with it.

As part of a programme of characterizing porcine immunoglobulins (see later) late hyperimmune samples of 22 (gamma 49), 42 (gamma 50) and 9328 (gamma 49) reagents were run on a reversed I.E. Each antiserum (10 lambda) was electrophoresed and 50 lambda of various dilutions of the antigen (L8729 serum) were placed in the antibody troughs. In this way the mobility of the porcine precipitin was shown to be gamma 2, and to
151.

coincide with Ig G2 (Cammarata and Deutsch, 1950; Metzger and Fougereau, 1967) (Ph. 9a,b).

Although all five reagents reacted with one antigen in L8729 there was considerable quantitative variation so that optimal proportions varied. Since the quantity of antigen present in the sera of the panel animals tested also varied considerably the two weakest reagents (38 and 23) occasionally barely managed to react due to antigen excess. At the other end of the scale the stronger reagents (p 22 and 9328), particularly when antigen was lacking in quantity, showed prozoning or lack of reaction due to antibody excess. It follows from this that using either reagents or test antigens at one dilution only (e.g. neat) would result in the five reagents apparently having different specificities.

Reagent 9328 in addition to having a reactivity in common with the other four reagents, which I shall call reactivity 2, also had a further reactivity (reactivity 1), which on I.D. appeared to be a minor reactivity. Investigation on I.E. showed it to be the major reaction, however, and the reason it appeared to show little avidity on I.D. was that the optimal proportions were eventually shown to be \( \frac{1}{32} \) antigen versus neat antiserum, i.e. there was a marked antigen excess (Ph. 16a). Reactivity 2, on the other hand, although evenly balanced on I.D., frequently did not appear on I.E. until the antibody had been diluted \( \frac{1}{4} \), i.e. there was a relative lack of antigen (Ph. 4c, 8c, 16b).
At certain dilutions on I.E., reagent 22 also showed reactivity 1 in addition to reactivity 2. Unlike reagent 9328, however, it only rarely showed this reaction on I.D. as a faint line when neat antiserum reacted with antigen diluted \( \frac{1}{2} \).

**Antigen.** Since whole serum had been used as antigen there was a theoretical possibility of the precipitation reaction being directed against any of the serum components. In anticipation of this a programme of partial separation and identification of all the major proteins was undertaken. This will be dealt with later. Initially, however, I.D. and I.E. studies gave some indication of the nature and development of the antigen.

Firstly, as mentioned above, the pig blood group panel sera were tested and found to fall into definite positive or negative classes. This typing was carried out not only with freshly taken sera, but also with samples stored for up to 3-4 years at \(-20^\circ C\). There were no marked differences indicating that the antigen was not of a temporary physiological or pathological nature. The distribution of positive and negative results was not synonymous with any of the established red cell or serum polymorphisms in Tables IIIa and IIIb. The precipitation arcs on I.D. failed to stain with specific stains for lipoprotein, haptoglobin, haemopexin or caeruloplasmin.

A small number of litters was tested at various ages from two weeks upwards. Using reagent 22 (gamma 24) only, three
litters from positive sow times negative boar matings gave no reaction at two, three and four weeks of age respectively. Thirteen of the original 16 piglets were retyped at 9 months. Nine now reacted roughly to the same degree as their Minnesota Miniature mothers.

Using all five reagents (gamma 31) two positive sow/positive boar matings gave five and nine positive piglets at two months and seven months respectively. Piglets show considerable quantitative variation, presumably due to different rates of development and absorption from colostrum. This was not closely followed from birth at this stage.

Immuno-electrophoretic analysis of L8729 serum using the porcine iso-immune and rabbit hetero-immune reagents showed that factor 2 has a $\gamma_1$ mobility (Ph. 4c, 5, 8). Suitable samples of all five reagents gave this reaction.

Using reagent 9328 (gamma 31) and to a certain extent 22 (gamma 31) the precipitation arc of factor 1 on the other hand extended through from $\gamma_2$ to $\alpha_1$, and was therefore characteristic of Ig G in general. The two sections of this long arc, i.e. the $\alpha_1 - \alpha_2$ and $\beta_1 - \gamma_2$ sections had different optimal proportions, and at extreme proportions almost appear as separate reactivities. At intermediate proportions, however, they can be seen to fuse quite clearly (Ph. 4).

During the typing of over 80 adult pig sera there was no fully established example of factors 1 and 2 segregating
independently. It is not quite clear at this juncture whether the linkage is serological or genetical, and this problem will be raised again in the Discussion under the "double line phenomenon".

Further Booster Immunisation Course

The pigs from whole serum booster courses 2a (p 22 and p 42) and 2b (p 42A, 38, 9328, 23, 9326, L8729 and 16) after a rest of three months and two months respectively were given a further whole serum booster course 2c. This consisted of four 2 ml injections of their appropriate antigens at 3, 2 and 3 days intervals. These time intervals were kept short as the earlier serum boosts had, in contrast to injections of WOW, been short-lived, moving to a maximum within about 5 days and then falling off (Fig. 12) in titre or avidity 5-6 days after the final injection.

Although in some instances titres went beyond $\frac{1}{2}$ (Figs. 8, 16), the avidity of the antisera resulting from Experiment 2c (gamma 34-37) fell short of previous donations (Figs. 7, 8, 11, 12, 15, 16).

Pigs from this course were therefore incorporated in Experiment 3, utilising WOW containing whole serum.
EXPEDIMENT 3

The pig panel sera showed a discrete distribution of positive and negative reactors with the five precipitating antisera produced in Experiment 2, and there was every probability of the antigen(s) being heritable. This was confirmed by the limited number of litters examined by the precipitation reaction.

More than half the panel tested was positive, and it seemed equally likely that some animals would be homozygote at one or more loci particularly if they were taken from a double positive mating. Six strongly positive adult pigs and nine piglets from a positive sow x positive boar mating were chosen to receive WOW containing the serum of a negative reactor (42A). The piglets were 3 months of age.

Schedule to Experiment 3

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Type</th>
<th>Antigen</th>
<th>Inj. Site</th>
<th>Donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 p 22</td>
<td>+</td>
<td>WOW</td>
<td>i/m</td>
<td>42A</td>
</tr>
<tr>
<td>7 p 6</td>
<td>+</td>
<td>WOW</td>
<td>i/m</td>
<td>42A</td>
</tr>
<tr>
<td>4683</td>
<td>+</td>
<td>WOW</td>
<td>i/m</td>
<td>42A</td>
</tr>
<tr>
<td>L8729</td>
<td>+</td>
<td>WOW</td>
<td>i/m</td>
<td>42A</td>
</tr>
<tr>
<td>I413</td>
<td>+</td>
<td>WOW</td>
<td>i/m</td>
<td>42A</td>
</tr>
<tr>
<td>71 p 16</td>
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<td>p 170</td>
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<td>- 173</td>
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<td>- 179</td>
<td>+</td>
<td>WOW</td>
<td>i/m</td>
<td>42A</td>
</tr>
</tbody>
</table>
The surviving pigs from both schedules for Experiment 2 were re-immunised with 10 ml of WOW since those animals which failed to produce precipitating antibodies had not previously received adjuvant. Two additional animals were also included to replace dead sows. One (4684) was a negative reactor and therefore received WOW containing L8729 in anticipation of a similar response to that already found. Boar 11, however, was peculiar in that, although reacting strongly with only one out of the five reagents, when mated with the pig (9328) supplying this reagent, produced a litter which eventually reacted weakly with all five reagents. It was therefore of special interest to examine the result of immunising with WOW containing L8729.

All pigs in both schedules to Experiment 3 received one injection of WOW (8-10 ml) i/m, and then after an initial period of three weeks were bled at weekly intervals for gamma 38-41 inclusive (see Tables IX and X).

A little over four months after being injected with WOW the pigs in the major schedule to Experiment 3 were boosted with 6 ml of 42A serum, but also received 10 ml of WOW (containing 42A serum). A further 4 ml of 42A serum was administered a week later. Samples were taken at approximately weekly intervals (gamma 42-45), the last sample being a fortnight after the first booster, and a week after the second booster (Course 3a, Table IX).

After two months' rest adult pigs 12 p 22, 7 p 6, 4683, L413 and L8729 were given a further booster course of two
injections of whole serum (10 ml and 6 ml) with an interval of one week. Serum samples were taken on gamma 52-56 at 2-3 day intervals (Course 3c, Table IX).

Pigs in the auxiliary schedule to Experiment 3 were rested for six months following initial stimulus with WOW, and were then given a booster course of 10 ml and 4 ml of L8729 serum at a one week interval. Samples were taken at 2-3 intervals on gamma 46-51 (Course 3b, Table IX).

<table>
<thead>
<tr>
<th>Recipient</th>
<th>Type</th>
<th>Antigen</th>
<th>Inj. Site</th>
<th>Donor</th>
</tr>
</thead>
<tbody>
<tr>
<td>42A</td>
<td>-</td>
<td>WOW</td>
<td>i/m</td>
<td>L8729</td>
</tr>
<tr>
<td>22</td>
<td>-</td>
<td>WOW</td>
<td>i/m</td>
<td>L8729</td>
</tr>
<tr>
<td>42</td>
<td>-</td>
<td>WOW</td>
<td>i/m</td>
<td>L8729</td>
</tr>
<tr>
<td>38</td>
<td>-</td>
<td>WOW</td>
<td>i/m</td>
<td>L8729</td>
</tr>
<tr>
<td>9328</td>
<td>-</td>
<td>WOW</td>
<td>i/m</td>
<td>L8729</td>
</tr>
<tr>
<td>23</td>
<td>-</td>
<td>WOW</td>
<td>i/m</td>
<td>L8729</td>
</tr>
<tr>
<td>9326</td>
<td>-</td>
<td>WOW</td>
<td>i/m</td>
<td>L8729</td>
</tr>
<tr>
<td>4684</td>
<td>-</td>
<td>WOW</td>
<td>i/m</td>
<td>L8729</td>
</tr>
<tr>
<td>Boar 11</td>
<td>(+)</td>
<td>WOW</td>
<td>i/m</td>
<td>L8729</td>
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* A single line of precipitation has been observed between these pigs' sera and one reagent (p 22). They are therefore not entirely negative.
Results for Experiment 3
Double Diffusion Ouchterlony Precipitation Tests

Of the five adult pigs and nine piglets in the main schedule none produced antibody reacting by precipitation technique after receiving WOW, and only one adult pig (12 p 22) produced a trace of precipitin (maximum score of 4 in gamma 44 and 45) following booster course 3a. The adult pigs were re-boosted and sampled (gamma 52-56) 2½ months later (Course 3c, Tables IX and X) when 12 p 22 and one other pig (7 p 6) produced a trace of precipitin. The response was short-lived, however, giving scores of 4 and 2 (12 p 22 and 7 p 6 respectively) on gamma 53 only.

The administration of WOW to the pigs in the Auxiliary Schedule to Experiment 3 failed to produce any substantial rise in titre or avidity in the five pigs already possessing antibody. In fact, the indications are that, in pigs already boosted to a high titre by booster course 2a, the titre and avidity fell a number of weeks following injection with WOW before rising slightly and stabilising at a new level (Figs. 8, 9, 12, 13, 16, 17). Pigs 38 and 23, always the weakest reactors, and also 42, failed to produce a substantial reagent. Only pigs 22 and 9328 were bled for a large amount (gamma 49) and reserved for further investigation following booster course 3b. Pig 9326, having failed to produce precipitation through courses 1, 2, 2b, 2c and 3, finally made a weak precipitin (maximum score 9 on gamma 47) against L8729 during booster
course 3b. Of the eight pigs originally included in the main schedule to Experiment 2 only two (42A and 12) had failed to produce antibody (p 12 died after gamma 26). The four strongest reactors (22, 9328, 42 and 38) were originally immunised with WOW containing L8729 serum. Of the pigs originally injected with serum diluted \( \frac{1}{64} \) 23 produced only a weak reaction and 9326 was reactive only after being given WOW in Experiment 3.
Photograph 1. Normal electrophoresis of pig serum on agar gel.

Upper well:  pig 8729 serum - 3 lambda
Lower well:  pig 7881 serum - 3 lambda
Antibody trough:  nil
Stain:  Sudan B and Azocarmine (S.B. and A.)

Photograph 3. I.E. of Sephadex G200 Peak A non-concentrated and whole serum.

Upper well:  recycle run II Pk A - 5 lambda
Lower well:  pig 7881 serum - 3 lambda
Antibody trough:  rabbit anti whole pig serum - 100 lambda
Stain:  S.B. and A.

Note: In all I.E. photographs the anode is to the left and cathode to the right.
Ph. 1

Albumin 
\[ \alpha_1 \alpha_2 \] 
\[ \beta_1 \beta_2 \] 
\( \gamma \) globulin

Lipalbumin

Prealb. \( \alpha_1 \) lipoprotein

Ph. 3

chilomicron

chilomicron

\( \alpha_1 \) lipoprotein
Photographs 4a, b and c. Pig allotyping reagent 9328 v. L8729 serum showing factor 1 reaction with alpha, beta and gamma mobility. Factor 2 reaction is only visible in Ph. 4c with $\gamma_1$ mobility.

Upper well: L8729 serum. Neat - 5 lambda
Lower well: L8729 serum. $\frac{1}{2}$ - 5 lambda
Antibody trough: 9328 serum - 100 lambda
Ph. 4a: Neat
Ph. 4b: $\frac{1}{3}$
Ph. 4c: $\frac{1}{4}$
Staining: Amido black (A.B.)

Photograph 4d. Normal electrophoresis of L8729 serum for comparison of mobility.

Photograph 5. Pig allotyping reagent 22 v. 411 serum showing factor 2 reaction with $\gamma_1$ mobility.

Upper well: 411 serum. Neat - 3 lambda
Lower well: 411 serum. $\frac{1}{2}$ - 3 lambda
Antibody trough: 22 serum. $\frac{1}{3}$ - 100 lambda
Staining: A. and weak A.B.
Photographs 6a, b, c and d. Rabbit anti pig serum (R9) v. pigs 20, 411, 12 p. 22 and 18729 with added chicken Hb. These slides demonstrate the difficulty of distinguishing the variable Hg from Hp which also varies in its mobility on combining with chicken Hb. The staining with Benzidine and Hydrogen Peroxide (B. and P.) has been pricked out and the slides counterstained with A.B.
Photograph 7. R9 v. pig serum and stained with paraphenylenediamine to demonstrate caeruloplasmin.


Cp - BB


Cp - AB


Staining: P.F.D. counterstained with A.
Ph. 7

Cp.BB

Cp.AB

? X component
Photographs 8a, b, c, d. R9, p. 22 and p. 9328 reagents v. 15% Sodium Sulphate precipitated, crude gamma globulin preparations.

Lower well: gamma globulin prep. $\frac{1}{2}$ - 5 lambda.
Antibody trough: Ph. 8a. R9 serum. $\frac{1}{3}$ - 100 lambda. 
Ph. 8b. p. 22 serum. $\frac{1}{3}$ - 100 lambda.
Ph. 8c. p. 22 serum.  $\frac{1}{4}$ - 100 lambda.
Ph. 8d. p. 9328 serum. N - 100 lambda.

Staining: A.B.
Allotype factor 2 reactivity shows with $\gamma_1$ mobility.
Allotype factor 1 reactivity shows with $\gamma$ mobility in Ph. 8d only.
Ph. 8a non fusing unid. $\beta_i$ comp.

Ph. 8b

Ph. 8c

Ph. 8d
Photographs 9a, b. Reversed I.E. to demonstrate $\sqrt{2}$ mobility of allotyping anti-factor 2.

Upper well: p 22 gamma 49. Neat - 10 lambda.
Middle well: p 42 gamma 50. Neat - 10 lambda.
Lower well: p 9328 gamma 49. Neat - 10 lambda.
Antibody troughs: Ph. 9a. pig L8729 serum.

$\frac{1}{3}$ - 50 lambda in both troughs.
Ph. 9b. R9 serum. $\frac{1}{3}$ - 50 lambda.

Pig 42 failed to react at the dilutions used.
Ph. 9a,b.
Photographs 10a, b, c. R9 v. concentrated Sephadex G200 fractionation pools from Fr. 53, to show separation of serum components.

Upper and lower well: pool concentrate. N and \( \frac{1}{2} \) respectively - 5 lambda.

Antibody trough: R9 serum. N or \( \frac{1}{2} \) as appropriate - 100 lambda.

Staining: P.P.D., B and P, S.B. and A.

Ph. 10a: 53 c. 2 - Pk. A.
Ph. 10b: 53 c. 5 - Pk. B - early.
Ph. 10c: 53 c. 6 - Pk. B - middle.

Note: Certain components have been marked in using a diamond stylus on the back of the slide.
Photographs 10d, e.  As for 10a, b, c.

Ph. 10d: 53 c 7 - Pk. B - late.

Ph. 10e: 53 c 8 - Pk. C - early.
Ph. 1Od

Prealbumin

Fast lipoprotein

Ph. 1Oe

Prealbumin

Labile β comp.

Hg  Tf

var. Λ comp.

Albumin

Cp

IgG
Photograph 11. R9 v. Fr. 55 c 6 to demonstrate the unidentified $\beta_1$ component with a strong fusion with Ig G and also the non-fusing unidentified $\beta_2$ component. The quantity of antigen has been doubled compared with previous slides.

Photograph 12a. R9 v. Ig G concentrate from D.E.A.E.-Sephadex Batch Process 1 to illustrate contamination with Tf and Hg.

Photograph 13a.  R9 v. concentrated protein eluted from C.M.-Sephadex Fr. 62 by phosphate buffered saline to illustrate contamination of Ig G2 with beta globulin.

Photograph 13b.  Normal electrophoresis of same fraction.
Photographs 14a, b, c. R9 and p. 9328 serum v. concentrated Ig G₂ protein eluted from C.M.-Sephadex Fr. 65 by 0.1 M phosphate buffer pH 6.5 to illustrate the lack of contamination with other protein.

Upper and lower wells: Fr. 65 c. 4 - Neat and $\frac{1}{2}$ respectively - 15 lambda.

Antibody trough:

Ph. 14b: R9 serum. $\frac{1}{2}$ - 100 lambda.
Ph. 14c: p. 9328 serum. $\frac{1}{2}$ - 100 lambda.
Photograph 15a. Pig 42 reagent (anti-2) and p. 9328 reagent (anti-1 and anti-2) v. pig L8729 serum.

Lower wells: L8729 serum. 1/8 and 1/4 alternately - 5 lambda.

Middle wells: First well empty. Pig 42 serum. N, 1/2, 1/4, 1/8 - 5 lambda.
Rabbit anti pig serum positive control - 5 lambda. and in last well Pig 9328 serum. N, 1/2, 1/4, 1/8 - 5 lambda.

Note: With p. 9328 serum the inner ring of reactivity is factor 1.
Photograph 15b. Pig 22 (anti-2) and p. 42 (anti-2) reagents v. Ht p. 1 serum to show cross-over spurring from adjacent wells and double line phenomenon at non-optimal proportions.

Photograph 16a. Titration of p. 9328 reagent v. L8729 serum. Centre wells: p. 9328 serum. N, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$ - 5 lambda. Outer wells: p. L8729 serum. N, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$ - 5 lambda.

Factor 1 reactivity is the inner ring.
Neat antigen starts at 11 o'clock and dilutions proceed clockwise.

Photograph 16b. Titration of p. 22 reagent v. crude gamma globulin preparation.
Proportions as for 16a.
Photograph 17. Line of identity test between six pig anti-2 reagents.

Centre wells: Pig L8729 serum. N, \( \frac{1}{2}, \frac{1}{4}, \frac{1}{8} \) - 5 lambda.

Outer wells: Pigs 22, 42, 9328, 38 and 23 all gamma 31 and p. 22 gamma 33. All neat - 5 lambda.

Pig 22 gamma 31 at 11 o'clock in each group and reagents proceed clockwise in above order.

Note 1: Line doubling at non-optimal proportions.

2: Anti 1 reactivity at 3 o'clock in each group between p. 9328 and centre well.

3: Extra reactivity between each group between p. 22 gamma 33 and p. 9328 gamma 31.
Photograph 18. I.E. of R9 v. every fourth fraction of D.E.A.E.-Sephadex Fr. 46 and Fr. 47 to show correlation between Ig G of $\gamma_1$ mobility and allotype factor reactivity.

Upper well of each slide: Fr. 46 samples.
Lower well of each slide: Fr. 47 samples.

The factor 2 reactivity score is derived from immuno-diffusion tests with p. 22 reagent.
Photograph 19. I.E. of R9 v. the final six concentrated fractionation pools from C.M.-Sephadex Fr. 69, to show correlation of Ig G₁ and Ig G with allotype factors 2 and 1 respectively.

Upper well of each slide: protein concentrate.
Neat - 5 lambda.

Lower well of each slide: protein concentrate.
½ - 5 lambda.

Note: The last fraction eluted is at the top.
Factor 1 and 2 score derived from immunodiffusion tests with p. 22 and p. 9328 reagents.
Ph. 19

Eluant

1 M NaCl

0.05 M / 7.8

Gradient

0.05 M / 7.4

Phosphate

Score

Factor

1 2

12 0

12 0

8 tr

12 1

9 tr

12 8
Photograph 20. Starch gel electrophoresis of Sephadex G200 concentrated protein fractions as indicated.

Staining: Nigrosin.
Photograph 22. As for Ph. 21 but staining with nigrosin instead of B and F.
Photograph 23. Starch gel electrophoresis of concentrated Peaks A, B and C from a Sephadex G200 recycling chromatography run to illustrate major components.

The five serum samples to the right of the gel are part of another investigation.
At an early stage in my research programme it became essential to develop the means of identifying a comprehensive spectrum of pig serum protein constituents. Although the possibility existed for antigenic variation in many serum proteins, the porcine immunoglobulins were of particular interest because of their specific connection with antigen/antibody reaction and their resistance to analysis by starch gel electrophoresis.

The approach to this analysis was twofold. The distribution of pig serum components was ascertained on a molecular size and electrical charge basis using molecular sieving, ion exchange, electrophoresis, immuno-diffusion and immuno-electrophoresis with hetero- and iso-immune reagents to partially separate and identify a spectrum of components. Then the distribution of porcine antibodies and protein iso-antigens established on the same basis was partially correlated with various types of serological reaction such as agglutination, haemolysis and precipitation.

The specific identification of general serum components by immuno-electrophoresis and specific staining was based on criteria reviewed by Brummerstedt-Hansen (1967). Prior to that, however, considerable assistance was obtained from comparable investigations into human serology, particularly in relation to molecular sieving (Flodin and Killander, 1962;
Gelotte et al., 1962; Killander, 1963, 1964; Killander and Flodin, 1962) and ion exchange chromatography (Sober et al., 1956; Fahey et al., 1958; Levy and Sober, 1960). Confirmation of the identity of proteins in chromatographic fractions was obtained on starch gel electrophoresis by courtesy of Dr Imlah based on criteria reviewed by him (Imlah, 1963, 1964a, b).

Sephadex G200 Fractionations

The following fractionations were included in this part of the investigations:

Fractionations 49 and 50. These were identical runs of Anti 3 Reagent 30 except that 49 was carried out in 0.1 M Tris/HCl + 1 M NaCl whereas 50 was eluted with isotonic phosphate buffered saline. Immuno-electrophoretic analysis of every fourth sample from both runs gave no indication of major differences. Subsequent runs were operated using phosphate buffered saline (pH 7.2 - see buffer solutions under Methods).

Reagent 30 was a largely incomplete red cell typing antibody of H.D.N.B. and C.V.V. origins reacting to a titre of \( \frac{1}{512} \) and a score of 69 by I.S.T. The haemolytic titre was nil, and the serum agglutinated only when undiluted (score 8). The reaction of additional antibodies anti Da (titre \( \frac{1}{4} \) score 17) and anti Kd (trace only) could be avoided by using appropriately negative cells. After fractionating, the fractions were pooled in pools of four (20 ml for FR 49, 22 ml for FR 50) which were concentrated down to 4.5 ml (FR 49) and 4.1 ml (FR 50). Since
8 ml of serum was originally applied the volume of each concen­
trate is approximately half. There were 14 and 13 concentrates
respectively for FR 49 and FR 50 since FR 49 was pumped slightly
slower.

All these fractionations were carried out on a combined
column length of 140 cms (K 25/100 + K 25/45) with a pump speed
of 15-18 ml/hr.

**Fractionation 52.** A direct agglutinating reagent, R4 - anti
Ed D.A. $\frac{1}{512}$ (score 90) I.S.T. $\geq \frac{1}{512}$ ( $\geq 80$) haemolytic titre
$\frac{1}{512}$ (46), was fractionated, and 15 pools of four tubes each
were concentrated from 22 ml to 4.5 ml. The original volume
of serum was 8 ml. The extra pool concentrate compared with
other fractions resulted from a slight extension of the uv
absorption trace beyond the 4.5 S peak. The haemolytic
reaction of this serum was rendered non-specific by the
presence of a non-absorbable pan-haemolysin.

**Fractionations 53, 54 and 55.** These fractionations were of
8 ml of two porcine late hyper-immune iso-precipitins, 9328
gamma 49 and 22 gamma 49, and their homologous iso-antigen
serum L8729 respectively. Fr. 53 and Fr. 54, each pooled in
pools of 5 fraction tubes, produced 11 and 12 pools respectively
of 27.5 - 28.5 ml. These were concentrated down to 4-5 ml.
Fr. 55, on the other hand, was pooled in 13 pools of four tubes
(23 ml) each pool, and these were reduced to approximately 5 ml.

**Fractionation 56.** This fractionation, the last in this series,
was of a porcine iso-immune reagent R6, kindly donated by Dr
Figures 19 - 21. Distribution of pig serum components and serological activities in relation to gel filtration fractions.
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**Fig 20**

**Fr. 55**

**Pooled Fracts. Concentr. No.**

1 2 3 4 5 6 7 8 9 10 11 12 13
### Serological Activity

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<th>Anti Ig G factor 2 Precipitin</th>
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<th>P Ig G factor 2 Iso-antigen</th>
<th>Ig M</th>
<th>Ig G</th>
<th>Unid. ( \beta_1 ) comp.</th>
<th>Anti-3 I.S.T. only</th>
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<td>tr 32 32 24 tr 8729 Fract. 55</td>
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<td>(+) + ++ + (+)</td>
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### Fig. 21
- Fr. 49
- Fr. 56

### Pooled Fracts. Concentr. No.

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Imlah. Used by the haemolytic technique this was a specific anti Ka \( \frac{1}{256} \) (42), but it contained additional antibodies by other techniques. This was not disadvantageous at this juncture since only a relationship was sought between immunoglobulins and mode of serological activity. The much more specific relationship of the distribution of immunoglobulins and serological activity against a specific antigen in relation to different stages of immunisation was not yet under investigation. In fact the distribution of somewhat weaker activity against a Ka negative cell by D.A. and I.S.T. closely paralleled that against a Ka positive cell. The values against a Ka positive cell by D.A. and I.S.T. were therefore still plotted, but it must be borne in mind that additional activities other than anti Ka are probably involved.

Results and Analysis

Numerical scores derived by different techniques are not strictly comparable.

In order to facilitate comparison in the distribution of the various serum components including antibody and antigenic properties the combined or average results from several fractionations have been plotted against a typical three peaked trace of uv absorption. Fortunately, as can be seen from Figure 19, the traces for Fr. 53, 54 and 55 have an almost identical distribution, which is intermediate in horizontal length between Fr. 49 and Fr. 52. The trace of Fr. 49 is
marginally more widely spaced, and that of Fr. 52 is somewhat more compressed. This variation only becomes noticeable by the third peak, and since only antibody activity is plotted from Fr. 49 and Fr. 52 this is not significant. Fractionation 55 (Fig. 20) is plotted separately from Fr. 53 and 54, and it must be noted that the pool size of the latter (5 tubes) differs from that of the former (4 tubes). Despite the larger pool size the anti Ig G factor 1 activity of reagent 9328 gamma 49 failed to react and cannot be plotted.

The final figure (Fig. 21), giving a summation of serological activity in all the sera, is plotted on the basis of a four tube pool concentrate. The precipitin activity of Fr. 53 and Fr. 54 had to be obtained by interpolation from a five tube pool concentrate plot, and the values differ from those in Figure 19.

The trace for Fr. 56 assumed an unsatisfactory asymmetrical or saw toothed appearance. This is a result of a disturbed column packing or pump on. Serum R6 was in short supply and the run could therefore not be repeated. Anti Ka results in Figure 21 should be viewed in relation to the second somewhat skewed trace with badly trailing peaks.

Peak A or 19 S Peak

This peak contains the macroglobulins, but the term "19 S peak" is a bit of a misnomer since it also contains low density lipoproteins of 2.9 - 7.0 S in man and 3.15 S in sheep (Aalund
Lipoprotein and Chilomicron

In order to prevent clogging of the column with particles the serum was centrifuged before fractionating. After spinning at +4°C for 3 hrs and before decanting the clear serum, the low density lipids were removed by suction. As a consequence $\alpha_1$ lipoprotein and the low density lipoprotein described by Brummerstedt-Hansen as chilomicron (Brummerstedt-Hansen, 1967) are not satisfactorily demonstrated in these fractionations (however see Ph. 1, 3, 10a).

$\alpha_2$ Macroglobulin or $S\alpha_2$ globulin

Although this protein is usually reported as a single substance showing as a strong asymmetrical arc on I.E., an extra, more symmetrical arc with $\alpha_2$ mobility is demonstrated from the macroglobulin peak (Ph. 10a). To distinguish between these two arcs the major component is regarded as $\alpha_2$ macro-1 and the other as $\alpha_2$ macro-2.

$\beta_2$ Macroglobulin or Ig M

This immunoglobulin appears as an elongated arc on I.E. extending from the $\beta_2$ to the $\alpha_2$ position. It almost invariably shows as a double line of precipitation both in G200 fraction A and in whole serum where it partially fuses with the
most anodic part of the Ig G arc (Ph. 10a, 2b). The appearance of double lines has been mentioned previously and is open to many interpretations. These will be discussed later.

Starch Gel Electrophoresis

On starch gel electrophoresis the macroglobulin peak shows three bands of protein. These appear in an area between the insert line and the trailing band of the transferrins. The leading and heaviest band is symmetrically distributed in Peak A, and is undoubtedly equivalent in quantity and distribution to the $\alpha_2$ macro-1 arc on I.E. The protein in this band, the S-$\alpha_2$ band (Imlah, 1963), is antigenically identical with $\alpha_2$ macro-1 (Brummerstedt-Hansen, 1967), and variations in mobility on I.E. can be correlated with the known S-$\alpha_2$ polymorphic type (Table IIIb). The second and third bands from Peak A on starch gel electrophoresis are asymmetrically distributed (Ph. 20). Despite this, they cannot be satisfactorily correlated with the remaining arcs on I.E. It can be assumed, however, that the $\alpha_1$ lipoprotein does not appear on the starch gel as it remains adsorbed to the paper insert (Imlah - personal communication).

Peak B or 7 S Peak

This peak contains serum components whose molecular size is equivalent to 7 S Ig G, and the latter forms a predominant part of its I.E. analysis.
Ig G

As indicated in the review and subsequently there is some evidence for two subclasses of Ig G in the pig - Ig G₁ and Ig G₂. Since these are of the same molecular weight and sedimentation coefficient, however, molecular sieving through Sephadex G200 fails to differentiate between these subclasses. I.E., therefore, shows the characteristic elongated arc of Ig G in general with γ₁ and γ₂ mobility (Ph. 2, 4, 8a, 11). Kim et al. are of the opinion that the first formed Ig G of γ₁ mobility in mouse and pig is a 19 S immunoglobulin distinct from Ig M, and that 7 S Ig G has only γ₂ mobility (Kim et al., 1968).

Haptoglobin (Hp)

Another predominant arc widely distributed in the middle peak is the haptoglobin arc (Ph. 10b, c, 11). This can be identified as a strong arc migrating in the α₂ position on I.E. Maximum staining with benzidine is only obtained after adding either pig or chicken haemoglobin to the fractions or original serum. Most sera, however, contain sufficient haemoglobin to give a detectable amount of staining. The mobility of free Hb being slower than α₂ it might be expected that attachment of Hb to Hp would alter the mobility of the latter. On agar this only occurs to a marked extent after adding an excess of chicken Hb so that the Hp became saturated. It then assumed a β₁ mobility and became difficult to distinguish from
some haem-binding globulins or haemopexins (Ph. 6).

The combination of Hb with Hp prior to fractionation might be expected to cause a change in its distribution on a molecular size basis. Thus fractionations 53 and 54, which were deliberately carried out with moderately haemolised serum, show a somewhat more advanced occurrence of Hp than Fr. 55 (Figs. 19, 20). This is not marked, however, and the situation will obviously vary according to the degree of saturation with Hb (Killander, 1964).

Fast Lipoprotein

The principal lipoprotein in pig serum is the $\alpha_1$ lipoprotein. Other pig proteins stain with lipid stain, however, and these include lipo-albumin and a further lipoprotein which migrates slightly in advance of albumin. Since on paper electrophoresis this protein migrates with $\alpha$ mobility and the $\alpha_1$ lipoprotein becomes a $\beta$ globulin, Brummerstedt-Hansen (1967) uses the terms fast and slow lipoprotein (after Grabar and Burtin, 1960). The mobility of the fast lipoprotein increases with storage or incubation at $37^\circ C$ becoming as fast as prealbumin (Ph. 1, 2, 10b,c,d). Its distribution in peak B is more or less symmetrical (Figs. 19, 20) although Fr. 54 showed a broader based distribution with a bias to the trailing edge of the trace.
Unidentified Beta Components

The beta area on I.E. shows a number of unidentified components which only appear at more extreme antigen/antibody ratios. One minor component consistently appears as a slightly asymmetrical arc after fractionation of serum. This arc, with a slow $\beta_2$ mobility at the cathodic end of the transferrin arc's normal position, is near to the Ig G line, but does not fuse with it. It is plotted as the unidentified $\beta_2$ component, and fractionates in the leading part of the 7 S peak (Ph. 10b,c, 11, Fig. 20).

Fractionating in a similar position is a $\beta_1$ component with a markedly asymmetrical arc and a definite tendency to fuse with the $\beta_1$ section of the Ig G line of precipitation (Ph. 11). This arc only appears on I.E. when at least double quantities of antigen are electrophoresed. In view of its antigenic affinity with Ig G and its position on fractionating, there is some probability of this being Ig A. This has not, however, been confirmed. Porcine Ig A has not been adequately characterised, although numerous authors have indicated an Ig A arc on I.E. on a hypothetical basis (Karlsson, 1966a,b; Kim et al., 1966a). Recent provisional reports (Porter and Allen, 1969; Bourne, 1969), however, suggest that porcine Ig A exists in colostrum as a "secretory" form aggregated with a "T piece" as well as a "non-secretory" form. In serum, as in the human, Ig A fractionates over a range from 7 S - 11 S, thus lying asymmetrically between the 19 S and 7 S peaks (Porter et al.,
Caeruloplasmin (Cp)

This copper-binding protein which shows marked polymorphism on starch gel (Imlah, 1964, and see Table IIIb) also shows the same variations in mobility on agar I.E. (Ph. 7, 10c,d). Its basic mobility on agar gel is $\beta_1$, and it appears on fractionation on the trailing edge of the middle peak overlapping into peak C (Fig. 19, 20). This is in agreement with its molecular weight of 151,000 compared with 168,000 for Ig G and 88,000 for transferrin (Holmberg and Laurell, 1948; Cammarata and Deutsch, 1950; Laurell and Ingelman, 1947; all cited by Brummerstedt-Hansen, 1967).

Prealbumin

Also occurring in an intermediate position between peaks B and C is a protein with a fast prealbumin mobility. A definite relationship has not been established between this protein and the prealbumin showing polymorphism on I.E. (Brummerstedt-Hansen, 1967) and starch gel electrophoresis (Kristjansson, 1963, 1966; Table IIIb). In the serum fractionations examined in detail to date it always shows as a short, rather weak arc crossing the anodic end of the albumin arc some distance from the antibody trough. In this position it would be equivalent to the fast component of the variable prealbumin or to an additional non-variable prealbumin reported by Brummerstedt-
Starch Gel Electrophoresis

Of the proteins occurring in or near the middle peak of Sephadex G200 the presence of Ig G, Hp and Cp has been confirmed. Haptoglobin can be seen with an alpha mobility in the unbound state after staining with nigrosin (Ph. 20). After adding chicken Hb, however, it shows a slower but variable mobility (Ph. 21). This depends on the relative amounts of Hp and Hb, i.e. on the degree of binding or saturation (Imlah, 1963). Ig G migrates cathodically from the insert line appearing as a smooth unbroken area of protein without definite banding (Ph. 20).

A satisfactory photograph of caeruloplasmin oxidase activity could not be obtained as the staining of the fractions although visible to the eye was diffuse and weak.

A similar situation exists for serum alkaline phosphatase and variable serum amylase, which are also distributed in the 7 S peak.

Also appearing on starch gel electrophoresis in the 7 S peak is a fine two banded beta protein normally concealed by the transferrin bands. It is clearly seen in Fr. 55 c. 5, 6 and 7, and thus has a similar distribution to the unidentified \( B_2 \) component on I.E. (Ph. 20, 23).

A little more symmetrically placed in peak B is one further protein which only leaves the insert line by a short
distance on starch gel. Its slow mobility and its distribution in the G200 fractions suggest either some association with the gamma immunoglobulins or some molecular aggregate unable to migrate far in the gel because of shape or size (Ph. 20, 23).

*Peak C or Albumin (Alb.) Peak*

As the name suggests the predominant feature of this peak is serum albumin, and this is very strongly and symmetrically distributed in these fractions. On I.E. it appears as a strong precipitate with a characteristic fast mobility at the most anodic end of the slide exceeded only by prealbumin and fast lipoprotein. This protein frequently shows double precipitation lines with identical mobility (Ph. 1, 2, 10e) (Figs. 19, 20).

**Transferrin (Tf)**

Another protein showing double lines and a strong precipitate is transferrin or iron-binding globulin. Its asymmetrical distribution at the leading side of the third peak is in accordance with its molecular weight of 88,000. It moves with a slow $\beta_2$ mobility which shows no discernible variation in accordance with the known polymorphism visible on starch gel (Ph. 2, 10e). With the exception of Ig $G_2$ the Tf arc is the most cathodic in position, and this, together with its prominent appearance, renders it easily recognizable.
Labile Beta Component

Very close within the curvature of the transferrin arc on I.E. is a markedly asymmetrical arc with a curved cathodic portion but a straight tail moving anodically. This is the labile beta component and it is thought to be related to the human Muller-Eberhard factor $\beta_1 C$ (Brummerstedt-Hansen, 1967). It shows a marked tendency to fuse with other unidentified components in the $\alpha_2$ area, and is distributed symmetrically in the albumin peak (Figs. 19, 20; Ph. 2, 10e).

Haemopexin or Haem-binding Globulins (Hx or Hg)

These proteins were originally confused with haptoglobins (Kristjansson, 1961) until it was shown that they do not combine with entirely fresh haemoglobin but only with the haem or alkaline haematin portion of the Hb molecule (Imlah, 1963). After adding aged chicken Hb to pig serum both Hp and Hg stain strongly with benzidine in the presence of peroxide. Depending on the degree of saturation of Hp with Hb, it becomes difficult to differentiate the Hp arc from the 1-1 or 2-2 homozygote Hg arc. The remaining Hg homozygote variants (Table IIIb) show a slower or faster mobility and the heterozygotes show an elongated or even two peaked arc. They can thus be easily distinguished from Hp (Ph. 6).

Gel filtration on G200 gives additional evidence that Hp and Hg are dissimilar in that they occur in separate peaks (Fig. 19).
Variable Alpha Component

The variable alpha component (Brummerstedt-Hansen and Hirschfeld, 1961) occurs as a fairly strong symmetrical arc either in the $\alpha_1$ position (type A) or $\alpha_2$ position (type B) or as an elongated two peaked arc, the presumed heterozygote. However, intermediate types occur and a definite classification is often difficult. However, it is usually possible to identify this arc by its strength and by its appearance close to the antibody trough cutting the cathodic end of the albumin arc (Ph. 2, 10e).

Starch Gel Electrophoresis

The occurrence of albumin, transferrin and some additional protein in the post-albumin position can readily be confirmed in the albumin peak by starch gel stained with nigrosin (Ph. 20, 23). Haemopexin can be shown by addition of aged chicken Hb prior to electrophoresis and staining by benzidine after electrophoresis (Ph. 21).

An additional alpha component migrating between unbound Hp and Tf is only clearly visible in a concentrated pool of a whole peak C' after recycling chromatography (Ph. 23).
Antigen/Antibody Components and Gel Filtration

Antibodies

The distribution of serological activities is summarised in Figure 21. The differences in the distribution of antibody activity are immediately apparent even in this small series. There is a marked absence of macroglobulin activity in serum R6 compared with R4 which shows reactivity by all techniques in Peak A. Reagent 30 occupies an intermediate position in showing no marked peak of macroglobulin activity, but having a wider spread of 7 S activity extending into the first peak. Despite this, R30 in company with R6 is a predominantly 7 S reagent since its peak of reactivity coincides approximately with Peak B. Even within the middle peak of G200 fractionations, however, there is considerable variation in the distribution of maximum activity according to antigen, technique or donor of the reagent. Thus the peak of I.S.T. activity in R30, despite a possible augmentation by Ig A, is to the right of Peak B, whereas elsewhere it either coincides exactly (R6) or correlates with the leading edge (R4). Again with the haemolytic technique there is an approximate correlation with the 7 S peak (R6) or a marked deviation to the left (R4). If we can assume that the unidentified $\beta_1$ component is Ig A or that Ig A has a similar distribution, then such deviations to the left can be attributed to Ig A. Deviations to the right are found in some cases (R6 - D.A., R30 - I.S.T.), and the most noticeable example is in the precipitin activity of 9328 $\gamma_1$ 49
compared with 22 49 against the same antigen. In this example the peak of reactivity almost coincides with that of caeruloplasmin with its molecular weight of 151,000, whereas more centrally distributed Igs are in agreement with values of 168,000 (Cammarata and Deutsch, 1950) and 170,000 (Kim et al., 1968).

Despite this evidence of heterogeneity on a molecular size or steric hindrance to gel filtration basis, the reversed I.E. technique has shown both precipitins to have 2 mobility and therefore to be classed as Ig G2.

The occurrence of haemolysis in 19 S, intermediate and 7 S positions is an indication that at least one class or subclass of Ig in each molecular size is complement consuming, and similarly with direct agglutinating ability.

The pan-haemolysin against all cells tested was detected in R4 during initial standardisation. In order to break down this apparent mixture of haemolysins a primary checkerboard analysis was performed using the limited number of Ed negative pigs available (5 pigs) and one Ed positive pig. Absorption of heat inactivated aliquots of serum with each of these animals' cells failed to remove the haemolytic activity against the same cells as used for absorption. No haemolysis occurred in the complement plus saline control, although a very slight haemolysis was found in the serum plus saline control for each aliquot tested against its own absorbing cells. The reaction is complement dependent since no haemolysis occurs in the D.A.
and I.S.T. reactions, and the factor apparently assists in the lysis of red cells in the presence of rabbit serum and complement. If we consider that rabbit complement having been absorbed with pig cells at +4°C may still contain subliminal quantities of haemolysin, this lytic factor becomes very similar to a heat stable factor – auxilysin – occurring in association with conglutinin in heated bovine serum and also in guinea pig serum (Bordet and Gay, 1906; Bordet and Streng, 1909; Gengou, 1909; all cited by Coombs et al., 1961).

The haemolytic reaction of the macroglobulin peak of R4 is partially masked by this phenomenon, although the markedly asymmetrical distribution of this auxilysic factor against an Ed-ve cell allows an assumption that genuine macroglobulin activity is also present against an Ed +ve cell. In fact, the absorption of antibody by an Ed +ve cell from concentrate 1 at the leading edge of Peak A appears to give some protection against the auxilysin since the titration score for the "auxilysin" against an Ed -ve cell is larger (Fig. 21).

Alternatively, Killander (1963), investigating anti-streptolysin in human serum, found, in addition to a specific 7 S fraction, a factor fractionating in the first peak on Sephadex G200. This non-specific macroglobulin, unlike Ig M, migrated with the alpha globulins on electrophoresis and on ultracentrifugation appeared with the low density lipoproteins.

The serum R4 has been stored for a number of years, and it is equally possible that some product of decomposition or
aggregation has accumulated rendering it pan-haemolytic.

Iso-antigen

Although both iso-antigenic factors 1 and 2 are found in the 7 S peak on Sephadex G200 fractionation their distribution in the peak is not identical nor symmetrical (Figs. 20, 21). This appears to be characteristic of Ig Gs as indicated by their antibody activity. There is no doubt that allotype factor 1 is a property of Ig G molecules if we take into account its I.E. mobility and distribution in Peak B. All other symmetrically distributed components in Peak B (fast lipoprotein and haptoglobin) are of the wrong electrophoretic mobility and are specifically stained. The unidentified $\beta_2$ component, although of similar mobility to allotype factor 2, does not have an identical molecular size distribution, and the mobility of the unidentified $\beta_1$ component is too rapid (Ph. 11).

Ion Exchange Chromatography

Ion exchange chromatography was carried out on a qualitative and a preparative basis. The former was an aid to the further identification of the porcine iso-antigens 1 and 2, and was carried out using a column process. The latter was an attempt to produce Ig G$_2$ at least in sufficient quantity for immuno-chemical analysis after reduction and digestion (Fleishmann et al., 1962; Porter, 1959), and was carried out using a batch process.
Column Chromatography

Two 5 ml quantities of pig 7881 serum were fractionated on Sephadex G200 (Fr. 36 and 37 - column K25/40, eluant P.B.S. pH 7.2, pump speed 10 ml/hr). The 7 S peaks after equilibration with 0.015 M Tris/HCl pH 8.0 by buffer exchange on G25 Fine (Fr. 42 and 43 - column K25/40, eluant 0.015 M Tris/HCl pH 8.0, pump speed 12 ml/hr) were pooled to give 80 ml of dilute protein. Half of this was pumped on to each of two DEAE-Sephadex A50 fractionations (Fr. 46 and 47 - DEAE-Sephadex A50, column K25/40, starting buffer 0.015 M Tris/HCl pH 8.0, pump speed 16-17 ml/hr) and was adsorbed in the uppermost layer of exchanger. The protein was then eluted with a buffer gradient produced by pumping 0.3 M Tris/HCl pH 8.0 into a mixing chamber containing 100 ml of 0.015 M buffer at 16-17 ml/hr. Every third fraction was examined on I.E. (I.E. 34) and I.D. (I.D. 45) using rabbit antiserum and porcine allotyping reagents respectively. Photography of I.E. 34 (Ph. 18) shows the progressive elution of Ig G of increasing mobility particularly from Fr. 46 (shown on the upper part of each slide). Fr. 47 at this point is more dilute (see lower part of each slide) and Ig G of faster mobility only appears on later slides. The reaction scores for allotype factor 2 only occur when the mobility of Ig G approaches \( \sqrt{1} \). At the concentrations used in this comparison anti-factor 1 failed to function satisfactorily except where protein concentrations were highest.

DEAE-Sephadex equilibrated with low ionic strength buffer
has, unfortunately, a pronounced tendency to shrink to a fraction of its original volume in the column as gradient elution proceeds. This makes the fractionations less consistent as uncontrolled mixing of buffers takes place in the column. The use of DEAE-cellulose is preferable in this particular respect although its anion equivalent capacity is less than the Sephadex derivative.

The 19 S macroglobulin peaks of Sephadex H200 fractionations 36 and 37 were also fractionated on anion exchanger in a similar way, but the opportunity for a detailed I.E. and I.D. analysis did not arise. In respect of the Ig allotyping this was not important as allotyping activity (antigenic and antibody) only occurred in Peak B on gel filtration.

**Batch Process**

Molecular sieving on Sephadex G200 proved an ideal method for the initial separation of 7 S material prior to ion exchange column chromatography. However, the amount of serum processed was a limiting factor. In order to get over this problem a batch process originally designed for human serum (Baumstark et al., 1964) was used in an attempt to prepare pure Ig G fractions showing allotype antigenic reactivity 1 and 2. Success in this aim would have facilitated absorption of multiple allotyping reagents, quantitative precipitation experiments, immuno-chemical analysis of the purified fractions, and enabled preparation of pure heterologous reagents for use
in differential precipitation experiments.

**Batch Process I**

After contacting 80 ml of pig High House serum at +4°C with two consecutive batches of 200 gms each of wet DEAE-Sephadex slurry equilibrated with 0.01 M phosphate buffer pH 6.5, the resultant 600 ml of filtrate and washing was concentrated at +4°C against P.B.S. pH 7.7 down to a volume of 40 ml. Although containing Ig G this material was heavily contaminated with transferrin, a $\beta_1$ component and a trace of alpha globulin (Ph. 12a), and in addition it failed to react with anti-2 reagent 22 $\gamma$ 49 although reacting moderately with anti-1 reagent 9328 $\gamma$ 49 (Ph. 12b).

A disadvantage of this method if maximum recovery is required is the large volume of dilute protein obtained as a result of washing the slurry and its consequent deterioration during concentration.

The eluant from subsequent batch processes, which were carried out at different molar strengths, pHs and proportions of serum to exchanger, was adsorbed on to a column (K25/40) of C.M.-Sephadex equilibrated with 0.01 M phosphate pH 6.5 in order to concentrate it. The protein can then be sharply eluted as a single peak by pumping saline solution (Levy and Sober, 1960).

Since the initial filtrate from the anion exchanger could not be obtained free of contaminants and give a reasonable
yield of wide spectrum Ig G simultaneously, this method of elution from the cation exchanger still gave a contaminated product (C.M.-Sephadex fractionations 60 and 62) (Ph. 13). In addition the affinity for ion exchangers and the quantity of several beta globulins, particularly Tf and Hg, is very similar to that of Ig G with \( \gamma_1 \) mobility, and a single step fractionation was not adequate to separate these components on a molecular charge basis. Following Batch Processes II - V, therefore, C.M.-Sephadex fractionations (Fr.64, 65, 69 and 70) were used as an extra fractionation step by eluting with various buffer gradients (see Buffer Solutions under Methods).

Equilibrating the C.M.-Sephadex at the same molarity and pH as the anion exchanger used in the batch process, allowed many uncharged marginal contaminants (near to their isoelectric points) to pass through the cation exchanger. Depending on the molarity, pH, loading and, therefore, retention of the batch process, the first eluant under starting buffer conditions from the C.M.-Sephadex was more or less heavily contaminated with alpha and beta globulins. In addition a good deal of \( \gamma_1 \) Ig G was lost in this way. The remaining Ig G1, Ig G2, some Tf and Hg were taken up and subsequently eluted.

A representative result is given by fractionation 69, a C.M.-Sephadex fractionation equilibrated with 0.025 M Na/K phosphate pH 6.6 buffer in the K25/40. Under starting buffer conditions 247 ml of filtrate from Batch Process IV (55 ml
I8729 serum contacted with four 20 gm batches of wet DEAE-Sephadex slurry equilibrated with 0.025 M Na/K phosphate pH 6.6) was pumped on to this column at 16 ml/hr, and the bulk of the protein came straight through containing Ig G and a number of \( \alpha_2^\prime, \beta_1 \) and \( \beta_2 \) contaminants including Tf, Hg and possibly labile beta component. Starting buffer conditions were operated until no further protein emerged. Molarity and pH were then increased but no further protein emerged until 0.05 M phosphate pH 7.4 was reached. Photograph 19 shows the final elution concentrates (six 6 tube pools of 31 ml each concentrated to 2 ml against P.B.S. pH 7.7) after pumping 0.05 M pH 7.4 phosphate (for 5 hours) followed by a 0.05 M pH 7.4/0.05 M pH 7.8 gradient. The final concentrate consists of a \( \gamma_1 \) component cleared from the column using 1 M NaCl.

As found by other workers (Metzger and Fougereau, 1967), it seems relatively easy to obtain Ig \( G_2 \) uncontaminated with other protein by careful selection of concentrated fractions from either anion or cation exchange fractionations. These have factor 1 reactivity as expected (Ph. 14a,b,c) from its wider mobility range on I.E. The separation of Ig \( G_1 \) free of contaminants has proved more difficult. Metzger and Fougereau (1967) report that Ig \( G_1 \) (porcine) is relatively insoluble in low ionic strength buffer, and it might have been thought that the final protein with \( \gamma_1 \) mobility eluted from Fr. 69 by 1 M NaCl would be pure Ig \( G_1 \). This fraction, however, failed to react with anti-2 although giving a moderate score with allo-
typing reagent anti 1 in 9328 \sqrt{49} (Ph. 19). It seems likely that a combination of ion exchange with either gel filtration or salt precipitation might facilitate further purification of Ig G_{1}.

The red blood cell antigen has been examined in the Blood Group Research Unit by both a positive antij and a positive anti-\beta. The latter, a strong haemolytic reagent \( AB 368 \) previously reported by Kallab (1963), almost invariably paralleled the reactions of the positive anti-\beta (Evans pig II or PP II). Dr. Kallab attributed this to its reactivity during autumn at a time of unusual "natural" antibody. Occasionally, however, this anti-\beta failed to react with exceptionally weak \( \alpha \) reagents, and in these circumstances PP II was the reagent of choice providing it was used at its optimum dilution \( \frac{1}{9} \). With a normal pig PP II has a titre of \( 10^{7} \) (score 56-64) by the antiglobulin test. This reagent has no C.H. activity and little haemolytic activity, although after prolonged incubation with hourly shaking for 6 hrs a haemolytic score of 20 could be produced using heat rabbit complement.

PP II was also used to estimate soluble \( \alpha \) haemolysin by a haemagglutination inhibition technique. The result is given as the reciprocal inhibition titre of one animal (\( + \) agglutinating dose (MAD)). The \( \alpha \) haemolysins of a number of panel animals are given in the following table.

**Note:** The reagent PP II was supplied by Dr. R.W. Goodwin, Dept. of Veterinary Causal Studies, University of Cambridge.
Typing pigs for the red blood cell antigen A has been carried out in the Blood Group Research Unit by using a porcine anti A and a bovine anti J. The latter, a strong haemolytic reagent MZ 206 B previously reported by Imlah (1963), almost invariably paralleled the reactions of the porcine anti A (Feltwell pig 11 or FP 11). Dr Imlah attributes this to its collection during autumn at a time of maximum "natural" antibody. Occasionally, however, this anti J failed to react with exceptionally weak A reactors, and in these circumstances FP 11 was the reagent of choice providing it was used at its optimum dilution $\frac{1}{3}$. With a normal A pig FP 11 has a titre of $\frac{1}{256}$ (score 56-64) by the antiglobulin test. This reagent has no D.A. activity and little haemolytic activity, although after prolonged incubation with hourly shaking for 6 hrs a haemolytic score of 20 could be produced using neat rabbit complement.

FP 11 was also used to estimate soluble A substance by a hemagglutination inhibition technique. The result is given as the reciprocal inhibition titre of one minimal (+) agglutinating dose (MAD). The A reactivities of a number of panel animals are given in the following table.

Note: The reagent FP 11 was supplied by Dr. R. F. W. Goodwin, Dept. of Veterinary Clinical Studies, University of Cambridge.
### A Activity Score in Adult Pigs of Various Breeds

<table>
<thead>
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<th>Pig No.</th>
<th>A type score</th>
<th>Pig No.</th>
<th>A type score</th>
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<td>Serum</td>
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<td>72</td>
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<tr>
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</tr>
<tr>
<td>6</td>
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<tr>
<td>7</td>
<td>56</td>
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</tr>
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</table>

* Cells not available.

G3, 28 and 22 are group O pigs.

Figures in brackets after cells score are scores with cattle anti J by haemolytic technique.

In order to further characterise and establish the specificity of FP 11 anti A, titration and absorption studies were carried out with human A₁, A₂, B and O cells as well as standard pig A and O cells. The doubling dilution titration scores by D.A. and I.S.T. are given in the following columns. Human blood cells were obtained from the Blood Transfusion Service as freshly bled or glycerinated frozen blood for absorption purposes. Cells for test purposes were usually bled fresh from the departmental staff.
Titration Scores of FP II before and after Absorption

Titration $\frac{1}{1} - \frac{1}{2048}$ IST 2% 2% RT 37°C Bacon 14 (abs. human and pig cells) diluted $\frac{1}{10}$, 1½ hrs, 1 hr macro reading

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<th>3xA₂</th>
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<th>6xA₂</th>
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<tr>
<td>B</td>
<td>70</td>
<td>65</td>
<td>0</td>
<td>61</td>
<td>58</td>
<td>50</td>
<td>46</td>
<td>50</td>
<td>51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pig</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>61</td>
<td>55</td>
<td>44</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Titration $\frac{1}{1} - \frac{1}{64}$ DA RT 2% 1½ hrs micro reading

| human      |        |     |     |      |     |      |      |     |     |       |      |
| A₂         | 54     | 46  | 43  | 0    | 31  | 0    |      |     |     |       |      |
| A₁         | 56     | 54  | 44  | 18   | 45  | 0    |      |     |     |       |      |
| A₁ (glyc.) | 54     | 54  | 41  | 16   | 41  | 0    |      |     |     |       |      |
| 0          | 34     | 0   | 0   | 0    | 0   | 0    |      |     |     |       |      |
| B          | 48     | 33  | 0   | 21   | 26  | 16   |      |     |     |       |      |

Note: - indicates not tested.
Analysis of the preceding results is indicative of a number of specificities in FP 11 in relation to human and pig red blood cells. These are as follows:

1. **Anti human species**

   This is readily absorbed by human A₁, A₂, B and 0 cells as tested against 0 cells (human), and the titration score after its removal must be the level against which further interpretations are made. The reduction in score by I.S.T. as a result of absorbing with human 0 cells is extremely uniform. By D.A., however, there is some indication of an anti 0 (H) specificity in that the reduction of titre against different cells is inversely proportional to the quantity of A or B reactivity.

2. **Anti pig A/human A**

   Although readily taken up on human A₁ and A₂ and on pig A cells when testing against pig A cells, this reactivity is absorbed only slightly by pig A cells when tested against human A blood. Human A cells remove activity against themselves distinguishing between A₁ and A₂ on a quantitative basis. Depending on the nature of the distinction between human and pig A in regard to testing and absorbing (quantitative, qualitative or quantitative/qualitative), it is possible to consider a further specificity.

3. **Anti human A**

   This is that part of FP 11 anti A absorbed by A₁ cells, more slowly by A₂ cells and not at all by pig A cells. It
only exists as distinct from the previous category if human A has an extra qualitatively different specificity in addition to pig A.

4. Anti pig A/human A/B

This is a component absorbed fairly readily by group B human cells as measured versus human group B and pig A, but leaving an additional component against pig A. Since human A₁ and A₂ remove all activity against pig A, they must also be reactive with this anti B component. Despite this, little reduction is found in the I.S.T. score against A cells after absorption with B cells and vice versa, unless four to six volumes of absorbing cells are used. By D.A. the reduction in score is more marked, but even after many absorptions with A₁, A₂, 0 and pA a component remains against human B cells.

5. Anti human B

This residual component is apparently only completely removed by human B cells.

This provisional investigation of FP 11 anti A is merely the equivalent of a primary checkerboard as applied to iso-immune reagents. Although provision of a titration score gives an extra indication of the correct interpretation, further checkerboard analysis is required to establish how far the above specificities cross react, are one and the same or are quite distinct.
Ontogenic Variation in A-coating Ability of Porcine Serum

Anti A frequently appears in pig serum with a titre in excess of $\frac{1}{1000}$ (Saison et al., 1955) particularly during infection with *Ascaris lumbricoides* (Soulsby and Coombs, 1956). In fact anti A titres can fluctuate wildly between $\frac{1}{32}$ and $\frac{1}{32,000}$ over a period of months or weeks. Since anti A also appears in colostrum there was a theoretical possibility of H.D.N.B. due to A incompatibility. However, during investigations into this possibility (Goodwin and Coombs, 1956), it was found to the authors' surprise that all supposedly group A piglets had a negative direct Coombs test despite considerable titres of maternal anti A. In fact, piglets only become A positive at varying times during the first month of life, although A substance was present in saliva and gastric mucin. It was subsequently shown that A inhibiting substance is present in serum of newborn type A piglets (to be) (Andresen, 1962; Lang - unpublished observation). It has been postulated that newborn pigs differ in their ability to take up A BGS (Goodwin and Coombs, 1956), or alternatively a quantitative hypothesis has been advanced that insufficient A substance is present in piglets' serum (Andresen, 1963). An investigation of some of these possibilities has been attempted.

The mating used as a source of piglets' blood cells, serum and saliva was a sow to son backcross (Sow 7 x Boar 50) of two abnormal A phenotypes (see table of A activity above). The pigs showing weak A phenotypes in the blood group panel all
Table XII
Sow 7 x 50 litter - farrowed 30/10

<table>
<thead>
<tr>
<th>Piglet No.</th>
<th>Date sampled:</th>
<th>Circulating antibody</th>
<th>D/C test cells</th>
<th>A type score cells</th>
<th>Inhibition Scores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30/10 3/12</td>
<td>30/10 3/12</td>
<td>30/10 12/11</td>
<td>20/11 3/12</td>
<td>30/10 3/12 30/10</td>
</tr>
<tr>
<td>1</td>
<td>++ +</td>
<td>- -</td>
<td>0 0</td>
<td>0 0</td>
<td>17 x 170</td>
</tr>
<tr>
<td>2</td>
<td>- -</td>
<td>++ -</td>
<td>x x x</td>
<td>54</td>
<td>170 169 272</td>
</tr>
<tr>
<td>3</td>
<td>w -</td>
<td>++ -</td>
<td>x x x</td>
<td>50</td>
<td>170 39 272</td>
</tr>
<tr>
<td>4</td>
<td>- -</td>
<td>- -</td>
<td>0 0 21 45</td>
<td>9 10</td>
<td>272</td>
</tr>
<tr>
<td>5</td>
<td>- -</td>
<td>+ -</td>
<td>x x x</td>
<td>0</td>
<td>15 x 272</td>
</tr>
<tr>
<td>6</td>
<td>++ +</td>
<td>- -</td>
<td>0 0 0</td>
<td>0</td>
<td>17 x 136</td>
</tr>
<tr>
<td>7</td>
<td>w x</td>
<td>++ x</td>
<td>x x x x x</td>
<td>25 x</td>
<td>x</td>
</tr>
<tr>
<td>8</td>
<td>w -</td>
<td>+ -</td>
<td>x x x x 55</td>
<td>136 364 272</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>- x</td>
<td>++ x</td>
<td>x x x</td>
<td>340 x</td>
<td>x</td>
</tr>
<tr>
<td>10</td>
<td>w -</td>
<td>++ -</td>
<td>x x x</td>
<td>136 130 272</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>++ +</td>
<td>- -</td>
<td>0 0 30 46</td>
<td>102 39 272</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>- -</td>
<td>++ -</td>
<td>x x x</td>
<td>136 104 272</td>
<td></td>
</tr>
</tbody>
</table>

x - not sampled or dead or result indeterminate because of positive D/C.
came from two limited breeding herds. One herd of the rare Gloucester Old Spot breed was maintained for show purposes, and the other was an experimental herd produced from imported Pitman-Moore Minnesota Miniature pigs. This has probable significance in relation to abnormal A alleles, gene interaction and the possibility of homozygous recessive suppressor genes as demonstrated in man (Race and Sanger, 1968). In order to investigate the A-coating ability of piglet and adult sera, two in vitro transformation experiments were carried out with adult and piglet non-A cells.

Preparation of Piglet and Adult Coating Serum

The use of serum in coating and inhibition tests was complicated by the presence of normal anti A in the serum of adult non-A control pigs, and by the presence in the piglets' post-colostral serum samples of iso-immune antibodies (for red cell typing systems other than A) derived from the maternal circulation via the colostrum. Direct Coombs test positive animals had less circulating antibody, presumably because it was adsorbed on to their cells (Table XII). Attempts were made to elute antibody off the piglets' cells, but this was not completely successful. Hence only the cells of piglets 1, 4, 6 and 11 were suitable for coating. It can be seen (Table XII) that, with the exception of piglets eventually remaining non-A, the piglets' serum A inhibition scores were at least as high as adult pigs (piglet 4 is an exception which may be
connected with its failure to suckle adequately in the first 24 hours of life).

The serum of piglets 3 (group A) and 9 (group A), sow 6 (group A) and sow G3 (non-A) were selected as suitable for coating reagents. Antibodies in the coating sera against the cells to be coated and against the A inhibition indicator cells were absorbed out with equal volumes of packed four times washed cells.

Transformation Experiments

Experiment 1

In the first experiment piglet 11 (non-A at birth, but becoming A later) and adult sow HT (non-A) cells were incubated at 37°C with frequent (every 30 minutes) agitation for 4 hours in the ratio of one volume of packed washed cells to ten volumes of newborn piglet 9 (group A), sow 6 (group A) and sow G3 (non-A) serum. The cells were then washed four times, re-suspended in saline and tested for A coating by a doubling dilution titration of anti A as follows:
<table>
<thead>
<tr>
<th>Sow 6 (A) serum</th>
<th>1/1</th>
<th>1/2</th>
<th>1/4</th>
<th>1/8</th>
<th>1/16</th>
<th>Saline</th>
<th>Score</th>
<th>1 volume of following added</th>
</tr>
</thead>
<tbody>
<tr>
<td>piglet 6</td>
<td>++</td>
<td>++</td>
<td>(+)</td>
<td>gw</td>
<td>vw</td>
<td>-</td>
<td>21</td>
<td>Saline</td>
</tr>
<tr>
<td>sow 12</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>w</td>
<td>vw</td>
<td>-</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td><strong>2% R.B.C.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>piglet 6</td>
<td>+</td>
<td>+</td>
<td>vw</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10</td>
<td>Piglet 9 (A) serum</td>
</tr>
<tr>
<td>sow 12</td>
<td>++</td>
<td>+</td>
<td>w</td>
<td>vw</td>
<td>-</td>
<td>-</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td><strong>suspension added</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>piglet 6</td>
<td>++</td>
<td>+</td>
<td>vw</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>13</td>
<td>Sow G3 (non-A) serum</td>
</tr>
<tr>
<td>sow 12</td>
<td>++</td>
<td>+</td>
<td>vw</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>
From this it can be seen that Sow 6 serum coated both adult and piglet non-A type cells. Piglet 9 serum, despite having a greater A inhibition titre than sow 6, failed to coat any cells.

Experiment 2

A further experiment was then carried out to determine whether piglet A serum and adult non-A serum had any effect on the A coating ability of adult A serum or piglet A serum respectively. The transformations were set up and carried out as indicated in Tables XIII and XIV in precipitin tubes. A separate saline control (D/C) was set up with fresh washed cells of piglet 6 (non-A) and sow 12 (adult non-A) to check on any unsuspected globulin coating on the native cells.
Table XIV

<table>
<thead>
<tr>
<th>Piglet 3 (A) serum</th>
<th>$\frac{1}{1}$</th>
<th>$\frac{1}{2}$</th>
<th>$\frac{1}{4}$</th>
<th>$\frac{1}{8}$</th>
<th>$\frac{1}{16}$</th>
<th>Saline</th>
<th>Score</th>
<th>1 volume of following added</th>
</tr>
</thead>
<tbody>
<tr>
<td>piglet 6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>Saline</td>
</tr>
<tr>
<td>sow 12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>Sow 6 (A) serum</td>
</tr>
<tr>
<td>2% R.B.C.</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>piglet 6</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>#</td>
<td>Sow 6 (A) serum</td>
</tr>
<tr>
<td>suspension</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>#</td>
<td></td>
</tr>
<tr>
<td>added</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>piglet 6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td>Sow G3 (non-A) serum</td>
</tr>
<tr>
<td>sow 12</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

After incubation at $37^\circ$C with agitation and storage overnight at $4^\circ$C tubes were washed four times; 64 MAD of Anti A was added to each tube and the normal antiglobulin test completed.

* Score has no significance in these rows.
Results

Experiment 1 demonstrated that piglets' and adult cells can be transformed by adult A containing serum, but not by newborn piglet A containing serum. The results of Experiment 2 are not so clear cut (Tables XIII and XIV). They confirm the results of Experiment 1 in that adult serum in the presence of saline converts piglet and adult cells into A cells, while newborn piglet 3 serum fails to do so. Doubling dilutions of piglet 3 serum fail to prevent the acquisition of A substance from sow 6 serum by piglet and adult cells (Table XIV). This is not surprising as sow 6 serum was added undiluted to every tube in these rows. In Table XIII, however, the addition of neat A containing piglets' serum and adult non-A serum has marginally reduced the A coating ability of sow 6 serum in relation to the saline control. This also militates against the possibility that a substance present in adult A serum could facilitate the coating by piglet A serum as suggested by Andresen (1962). At higher dilutions of adult A serum in the presence of A piglet and adult non-A serum coating is less than in the saline control.

Anti O and the 0 Antigen

Satisfactory sources of anti O are few and far between compared with sources of anti A. This is perhaps not surprising if we accept that 0 is basic to A in the biosynthetic pathway. Sprague (1958b) found that bovine anti O_0
invariably occurred in "-" type cattle. Rasmusen (1964) has, however, reported two satisfactory anti O reagents from group A pigs. These two pigs seem to be an exception to his finding that all pigs have soluble O substance in their serum (Rasmusen - personal communication; Rasmusen, 1964). One further reagent reported by Rasmusen was from a "-" French Alpine goat, and this gave very clear cut results in line with the hypothesis that A cells should be O negative.

In an attempt to find a suitable anti O reagent 58 goat sera were screened against an A, an O and a "-" cell by D.A. and haemolytic technique. The sera were obtained from the Animal Breeding Research Organisation in Edinburgh by courtesy of Dr J.G. Hall, and they had been deep frozen for some time. By the D.A. technique three goat sera showed anti O specificity with a score of 10-23 at the four dilutions used, and one serum showed an apparent anti "-" reactivity. The remaining sera either gave no reaction, a nondescript weak reaction or a stronger non-specific reaction. By haemolytic technique the reactions were totally different against the same three cells, except in the case of G6 serum, which still retained anti O specificity with a score of 17. Of the remainder of the four sera two anti O by D.A. became anti A by haemolytic technique, and the anti "-" lost all reactivity. By haemolytic technique three out of 58 sera gave anti O reactivity with a score in the range 13-17 for the four dilutions, and six gave a predominantly anti A reactivity with
scores of 14-20. These nine sera were rescreened against a further 3 adult A pigs, 3 adult 0 pigs, and a further "-" pig, and the specificity held up for three anti 0 and four anti A sera. Of these seven reagents only the donor goats for four of them (three anti 0 and one anti A) were available for resampling. On resampling only one anti 0 (G6) still gave a specific reaction. One serum previously anti 0 gave no reaction, one anti 0 gave anti-A and -0 reactivity, and the anti A gave non-specific reactivity! It is apparent from this that the "natural" antibody reactivity of these goats is not consistent but varies, possibly on an ontogenic or seasonal basis in response to varying environmental stimuli. The anti 0 specificity of the three stored sera of that reactivity was partially confirmed by inhibition tests with serum of A and 0 pigs. These did not confirm Rasmusen's findings of equal amounts of 0 substance in A and 0 pigs, since the serum of an 0 pig inhibited anti 0 about eight times more strongly than that of an A pig.

G6, being the only satisfactory anti 0 donor, was bled for a large quantity in August of 1966. On titration from $\frac{1}{2} - \frac{1}{128}$ this reagent gave a titre of $\frac{1}{16}$ and a score of 17-19 against known adult group 0 pigs by the haemolytic technique. It is used routinely at a dilution of $\frac{1}{6}$ by the haemolytic technique (haem. 1.5% ½ hr R.T. RC' 6 hrs 20°C incubator/shaker) giving 4-5 haemolysis with group 0 pigs' cells. Under these conditions, however, considerable cross-reaction occurs with
Table XV
G6 Serum Primary Checkerboard Analysis

<table>
<thead>
<tr>
<th>Test Cells</th>
<th>G6 serum absorbed twice with equal volumes of the following cells:</th>
<th>G6 unabs.</th>
<th>FP 11 unabs.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>72</td>
<td>C002</td>
<td>3</td>
</tr>
<tr>
<td>Sow 72 A</td>
<td>$\frac{1}{2}$</td>
<td>$\frac{1}{4}$</td>
<td>$\frac{1}{2}$</td>
</tr>
<tr>
<td>Sow C002 Aw</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>Piglet 3 &quot;-&quot;</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>&quot; 12 A</td>
<td>.</td>
<td>.</td>
<td>4</td>
</tr>
<tr>
<td>&quot; 16 O</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>&quot; 15 A</td>
<td>.</td>
<td>.</td>
<td>3</td>
</tr>
<tr>
<td>&quot; 10 A</td>
<td>.</td>
<td>.</td>
<td>3</td>
</tr>
<tr>
<td>&quot; 13 A</td>
<td>.</td>
<td>.</td>
<td>2</td>
</tr>
</tbody>
</table>
group A cells, and this appears on a litter basis, i.e. certain litters reacting predominantly as AO pigs. G6 reagent was therefore subjected to a primary checkerboard analysis involving adult A, O and "-" cells as well as cross-reacting AO pigs. Also included in the checkerboard as a matter of interest was a weak A reactor C002 (see table of A activities in adult pigs in previous section).

The results of G6 serum primary checkerboard analysis are given in Table XV. This clearly indicates that all pigs absorb out anti O haemolytic activity as tested against group O piglet 16 except for pig C002. There is therefore apparently a hidden O reactivity even in "-" pigs' cells, and this reacts with the anti O without producing haemolysis. The cells of the pig C002 are not only short of A substance but also of hidden O substance as well. This points to a basic shortage of blood group substance as opposed to a weak A gene or to a partial "-" (ss) pig, since even "-" pigs' cells absorb out anti O. Continued absorption with C002 does reduce the score against group O cells and also against the strongly cross-reacting A cells. The amount of cross-reaction against A cells is not proportional to the A type score, and at first sight it might appear that three distinct specificities are involved in this checkerboard as follows:

1. Hypothetical anti O against piglet 16. This is removed by the cells of all pigs, but to a limited extent by C002.
2. The reaction against sow 72 which is also removed by the cells of all pigs including C002.

3. A reactivity left in against piglets 10, 13 and 15, which is not removed by non-A pigs.

Continued absorption by C002 or any other animal's cells progressively removes residual activity, however, and produces results which suggest that the distinction between the three reactivities above is largely quantitative. However, the failure of piglet 16, a "genuine group 0" with a high score of 27, to remove activity against piglets 10, 13 and 15 is also indicative of an anti A type of reactivity distinct from anti O. As Professor R.D. Owen pointed out in his investigation into the B like antigens of animals (Owen, 1954), it is difficult to distinguish sharply defined groups or sub-groups in terms of heterologous reagents because of the complex range of cross-reactivities involved. This produces a "take a little bit out and a leave a little bit in" situation in which the distinction between qualitative and quantitative functions becomes blurred.
Iso-immunisation

The use of coated cells as an antigen in producing Ig allotyping reagents was apparently unsatisfactory in so far as detection by a haemagglutination system was concerned. The sera used for coating the pigs' cells were subsequently typed for factors 1 and 2. Dean 41 serum reacted normally for both factors, Bates sow 3 only reacted weakly at the higher dilutions of antibody for factor 2, and Kate serum had an unusually high concentration of factor 2 by I.D. and I.E. but did not show any factor 1 reactivity. This apparent segregation of factors 1 and 2 could be caused by the age of the Kate serum sample, by the high concentration of Ig in Kate serum coupled with the poor avidity of anti 1 in 9328 γ 49, or because factors 1 and 2 had in fact segregated. The opportunity to investigate this segregation did not arise. At any rate at least a 50% chance existed that Experiment 1 could theoretically have produced something. It may not be entirely coincidental that, in subsequent courses with WOW, only animals previously injected with coated cells in Experiment 1 gave a really satisfactory response. Judging by the human and rabbit models it would be entirely fortuitous how big a percentage of the coating molecules carried the required antigen and whether the correct configuration was presented for antibody production and subsequent detection. Hence the precipitins which were
present in at least two pigs during Experiment 2 failed to agglutinate passively sensitised tanned cells although heterologous anti porcine reagent reacted strongly.

Rasmusen reports that his first anti Gla was produced by deliberate iso-immunisation of pigs using a coated bacteria technique after Dubiski et al. (1959a), but that many injections were required and that the H.I. tests were difficult to interpret (Rasmusen, 1965a). The anti Gla and anti Glb reagents eventually used routinely and tested in Edinburgh were assumed to have arisen as a result of maternal/neonatal or maternal/foetal incompatibility. The limited tests I have carried out with these two reagents using coated tanned cells suggest that they are more akin to the human Ragg system in having a generalised activity against pig and possibly rabbit serum components in addition to reacting by the H.I. technique. Presumably the specific anti Gla and Glb reactivity only functions when the homologous antigen is correctly presented by attachment to a red cell by the antibody reactive site present on Fab. Pickup (1968) failed to produce allotyping reagents in pig using antibody coated bacteria as antigen, although the same pigs eventually produced precipitins after injection with globulin preparations in Freund's complete adjuvant. The H.I. test was not used by Pickup, and Rasmusen's reagents do not react by precipitation in gel technique (personal observation). It is possible that Pickup's use of Dubiski's method might have shown a result by H.I. technique. Dubiski et al. (1959a)
report that rabbit allotyping reagents could be used either by H.I. test or as precipitins. Mandy and Todd (1968a,b), however, find that a reagent detecting rabbit A 11 only reacts by H.I., and suggest the possibility that some allotype factors are monovalent and fail to form a precipitation lattice.

Pickup's eventual success with Freund's complete adjuvant clearly points to the need for a strong adjuvant action in producing porcine precipitins. This has been supported by the clearly demonstrated superiority of WOW containing whole serum over the repeated use of whole serum alone. Double emulsion adjuvant proved convenient to prepare and use in its highly liquid form compared with single water-in-oil adjuvant. No visible lesions occurred in pigs following its use. In the one pig whose primary response was examined (p. 22) the response was relatively sustained (Fig. 6) in comparison with subsequent whole serum booster courses (Figs. 7, 8), although the level of the primary response was fairly low. The avidity and titre of the secondary response elicited by the first serum booster course after almost one year's rest was much better, as might be expected. In pigs showing a detectable primary response in gamma 25 and gamma 26 (p 22 and p 42) the secondary response was rapid rising to a peak height within 5-6 days. The primary response with WOW was comparatively slow with a peak at 4 weeks, although the response to serum booster injections was also slow in pigs failing to show a detectable precipitin in gamma 25-26 of the primary course. Thus p 9328, p 38 and p 23 reached a
peak score in gamma 31 and gamma 32 ten days to a fortnight after the first serum booster injection compared with the 5-6 days of p 22 and p 42. The use of WOW (Figs. 9, 13, 17) ten days after the final serum injections of booster course 2c (Figs. 8, 12, 16) failed to reverse the rapid fall in titration score which takes place 5-7 days after a final whole serum injection (Figs. 8, 11, 12, 16). Instead, the score and avidity stabilised at a new lower level about 3-4 weeks after injection with WOW and was maintained at this level for some weeks (Figs. 9, 13, 17). This is consistent with the slow release mechanism of water-in-oil emulsions in general (Herbert, 1966a) and the work reported by Herbert (1966d) on the value of booster injections of soluble antigen alone in producing a sharp rise in titre followed by a sharp fall.

In summary therefore it can be seen that the use of WOW adjuvant is more advantageous in producing a sustained primary and secondary response than the use of serum alone. The level of the response in this porcine isologous system is neither as high nor as sustained as those reported in heterologous systems for WOW adjuvant (Herbert, 1965). Now that an allotyping system has been developed and comparative estimates of antigen can be worked out a greater attention can be paid to the question of dose and frequency of injection. The use of a whole serum boost appears satisfactory in producing a sharp rise in titre and avidity to a peak value at from 5 days onward depending on the degree of primary sensitisation. This rise
is not sustained, however, and test bleeding must be at frequent intervals to obtain a reagent of maximum titre and avidity.

In regard to secondary immunisation courses it is noteworthy that, in animals showing a rapid maximum response to the first serum booster course, the response to subsequent courses although rising in titre never gave the same avidity. This was particularly noticeable for anti 2 reactivity, and in fact having missed a major bleed at gamma 31 by testing en retard the opportunity for such a satisfactory reagent from all donors never arose again. Some improvement followed from the use (after three months' rest) of WOW in Experiment 3 particularly in the anti 2 reactivity of p 9328 γ 39-41, but unfortunately the anti 1 reactivity remained poor (Fig. 17). The gamma 49 sampling of p 22 and p 9328 eventually selected for major bleed following course 3b were inferior in titre and avidity. An increase in titre without a similar increase in avidity (contrast Figs. 7 and 8, 15 and 16) would indicate continued production of precipitin, but with increased production of a non-precipitating component.

In rabbits continued injection of antigen in Freund's adjuvant leads to the production of this type of antibody which blocks the activity of an existing precipitin (Feinberg, 1958). In a similar way Coombs reagent from frequently injected rabbits when used in haemagglutination tests often show a marked prozone (Dunsford and Grant, 1959). One explanation proffered for this is that incomplete antibody has a greater association constant
than saline agglutinating antibody and attaches more quickly to the available antigenic sites. In these porcine immunisations the frequency of courses has probably contributed to this change in the immunological response. The late hyperimmune precipitin has $\gamma_2$ mobility and on gel filtration behaved as a 7 S globulin so it is presumably Ig $G_2$. The property of precipitin blocking could possibly be ascribed to another Ig sub-class by further investigating Igs separated on a molecular size and charge basis.

Porcine Immunoglobulins, Allotypes and other Serum Components

Early immunological response is usually characterised by 19 S $\beta_2$ M (Ig M) antibody of a 2-mercaptoethanol (2-ME) susceptible type, and later hyperimmune sera contain an increased proportion of Ig G and Ig A antibody. In many animals studied (mouse, guinea pig, rat, rabbit and horse) Ig G is divided into $\gamma_1$ and $\gamma_2$ on the basis of antigenic specificity, biological properties and electrophoretic mobility (Stanworth and Pardoe in Weir, 1967, p. 139). In addition $\gamma_2$ molecules are further subdivided into subclasses in the mouse, horse and rat. As I have already indicated in the review, Ig G in man is subdivided into four subclasses, and particular Gm allotypes are associated with each of the four different gamma chains involved together with certain biological properties (Table VII, Figs. 3a, 3b). In the pig evidence has been presented of two subclasses of Ig G $\gamma_1$ and $\gamma_2$ separated
either by ethanol fractionation (Cammarata and Deutsch, 1950) or by salt precipitation and ion exchange chromatography (Metzger and Fougereau, 1967; Bourne, 1969). Both these subclasses are 7 S molecules of approximately 168,000 - 170,000 molecular weight. Distinct biological properties have not been characterised. Kim et al., on the other hand, are of the opinion that the \( \gamma_1 \) portion of the long continuous Ig G arc in the pig, mouse and rabbit consists of a 19 S \( \gamma_1 \) M component antigenically identical with \( \gamma_2 \) Ig G, but distinct from 19 S \( \beta_2 \) M (Ig M). They consider that the true primary immune response of piglets obtained by hysterectomy consists solely of this \( \gamma_1 \) M (19 S) immunoglobulin, and that \( \beta_2 \) M appears considerably later and is unrelated to antigenic stimulus. According to these authors the secondary immune response consists of 7 S \( \gamma_2 \) G which resists the activity of 2-ME (Kim and Watson, 1965; Kim et al., 1964, 1966a,b,c, 1967a,b, 1968). Kim et al. also report the characterisation of an X component in colostrum deprived, germ free piglets devoid of Igs. This component has a \( \gamma_2 \) electrophoretic mobility (Ph. 7) and ion exchange characteristic, but is antigenically distinct from Ig G, Ig A and Ig M. It is susceptible to the action of 2-ME which modifies its electrophoretic mobility, but has no antibody activity and is not increased by antigenic stimulus. In mice a component with similar mobility arises as a result of 2-ME activity on Ig M, the Ig M arc on I.E. disappearing. The reported 19 S \( \gamma_1 \) M I.E. arc in the mouse is unaltered by 2-ME
activity despite the authors' claim that the phage neutralisation ability of early response serum is largely 19 S and 2-ME susceptible (Kim and Watson, 1965). Contrary to their claim this would associate the neutralisation activity with Ig M. In germ free piglets, however, they claim that no Ig M is formed for many weeks. Thus 19 S \( \gamma_1 \) M can be separated from true early immune response sera by ion exchange and characterised by ultra-centrifugation and I.E. as 19 S \( \gamma_1 \) antigenically identical with \( \gamma G \), without confusion with Ig M which has the same mobility and ion exchange characteristics and also cross reacts with anti Ig G sera. In adult pigs whole serum on I.E. shows a continuous \( \gamma_2 \) and \( \gamma_1 \) (M) arc, an Ig A arc and a single Ig M arc near to the electrophoretic mid line. After Sephadex filtration (G200) I.E. shows double parallel arcs in the characteristic Ig M position in Peak A, and Kim et al. (1968) put this down to the presence of Ig M and 19 S \( \gamma_1 \) M in the macroglobulin peak. As we have seen, sets of double parallel lines with identical mobility on I.E. appear frequently (e.g. \( \beta_2 \) M, Ph. 10a; fast lipoprotein, Ph. 10b; albumin, Ph. 10e; albumin, \( \beta_2 \) M and Tf, Ph. 2 and 3). It is open to doubt how far these represent separate antigenic classes. Although theoretically under ideal conditions of performance and arrangement each precipitation arc should represent a separate antigenic specificity, in practice a number of factors can contribute to changing this. These include marked lack of optimal proportions, big differences in
diffusion constant between antigen and antibody, changes in
temperature during the reaction, any changes which alter the
diffusion constants of the reactants such as aggregation with
other proteins in soluble or insoluble complexes or polymerisa-
tion. Even if we allow that two specificities are present a
diagnosis of two Ig classes might be premature. In rabbit
allotyping anti-As 1 reagents often show the double line
phenomenon in I.D. in gel reactions. The extra line was
regarded as a further factor As 8 associated with As 1, and on
this basis the occurrence of two Ig G subclasses was reported
in rabbits (Hamers et al., 1965):- 
Ig G_1 determined by genes
for factors Asa 1, 2 and 3 and Ig G_2 determined by the gene
for factor 8 at locus d. After separating the two antibody
specificities and after papain digestion of rabbit Ig G it
became clear that factor As 1 is present on Fd and As 8 on Fc
of the same Ig molecules, and the linkage was genetical. Yet
double lines were formed and it seems that anti 8 and anti 1
must interact with the antigen in such a way as to produce two
lines of precipitation of the same antigen molecules. It
would be preferable for Kim et al. to investigate the immuno-
structural basis for any distinction between Ig M and 19 S γ_1 M
since the antigenic identity of 19 S γ_1 M and 7 S γ_2 was only
demonstrated using an antibody prepared against purified γ_2
Ig G. On the normal model for Igs this could contain anti-
body against common light chain factors present in Ig M as well
as Ig G. In my I.E. experiments Ig M shows double lines in
whole serum (when the Ig G precipitation arc intrudes between antibody trough and Ig M depot) as well as in Sephadex G200 fractionations Peak A. Unlike rabbit allotyping factors As 1 and As 8 porcine Ig G factors 1 and 2 show different mobility on I.E., and the separation of factor 1 reactivity from factor 2 reactivity by ion exchange chromatography clearly indicates that at least for some molecules factors 1 and 2 are not present together. The distribution of factor 1 reactivity on I.E. from $\gamma_2$ to $\alpha_2$ mobility and its fractionation in Peak B on G200 leads to the conclusion that 7 S Ig G also extends its I.E. arc over the same distribution. No factor 1 reactivity was found in the 19 S macroglobulin Peak A. Factor 2, on the other hand, has a much more restricted distribution in the $\gamma_1$ position (Ph. 4a, b, c, d and 5) and on balance is associated with a $\gamma_1$ 7 S class of immunoglobulin Ig G$_1$. I find no evidence to support the idea of two 19 S components of distinct antigenic class with one antigenically identical with Ig G as put forward by Kim et al.

The association between factors 1 and 2 in the same animals does not appear to be serological since at no time was cross reaction indicated by lines of identity. However, now that Ig G$_2$ has been separated in an I.E. pure form this could be verified by absorption experiments. The association is presumably due to a genetic linkage, the degree of which can only be estimated by progeny testing. Investigations of three selected litters of two positive and one negative sow
matings with a negative boar indicated that precolostral serum samples were negative and post-colostral samples had received the maternal type (1+ 2+ or negative) through the colostrum. There were indications from these and earlier litter investigations (mentioned earlier in the text) that the maternal type has disappeared by 3-4 weeks, but that the piglets' allotype is not fully developed until 8-9 weeks or even later. The usefulness of Ig allotyping in progeny testing is therefore much reduced, and litter samples arriving at the Blood Group Research Unit were too immature for consistent results. The genetic basis for factor 1 and 2 association could be partially clarified by immunochemical analysis of subunits of Ig G\textsubscript{1} and Ig G\textsubscript{2} after reduction and digestion respectively. The continued difficulty in separating Ig G\textsubscript{1} has restricted progress in this direction, although Ig G\textsubscript{2} could now be investigated.

Other Serological Activities

In human blood typing 19 S Ig M antibodies are associated with saline agglutinating reagents of the so-called "natural" type with cold thermal optima between 4°C and 20°C (anti-M, -N, -P\textsubscript{1}, -I\textsubscript{e}\textsuperscript{a}, -P+P\textsubscript{1}, -H, -I, -A and -B) and also with certain saline agglutinating antibodies with warm 37°C optima (saline agglutinating anti-C, -C + D, -D and -E) (Mollison, 1961, p. 219). Early immune responses are also usually of the saline agglutinating 2-ME susceptible type and are then followed by
7 S antibodies of incomplete or haemolytic type (Mollison, 1961, p. 219 et seq.). However, the above relationships are not invariable, and 19 S antibodies can be complement consuming and haemolise and 7 S antibodies can agglutinate.

The separation of porcine blood typing reagents by Sephadex G200 fractionation has demonstrated saline agglutinating, haemolytic complement dependent and incomplete agglutinating ability in 19 S, 7 S and intermediate fractions. Not all of these serological characteristics invariably occur in the one serum. No doubt this has some connection with the method of immunisation and the stage of immunisation reached for each reagent. Thus both R30 and R6 have little or no macroglobulin activity being strongly hyperimmune reagents. R4, however, is also a product like R30 of repeated secondary immunisations with crystal violet vaccine, and yet this serum contains considerable 19 S agglutinating activity against the Ed factor. Analysis of further E system reagents would possibly confirm the possibility already introduced in the review that some peculiarity of structure in the E system (which is very similar to the Rhesus system of man - Andresen, 1963) agglutinogen favours the continued production of saline agglutinating classes of Ig. Alternatively the E system agglutinogens may have factors peculiarly well disposed for agglutination in saline suspension by more classes of Ig than other porcine blood group factors.

The separation of different classes of immunoglobulin and
serological activity on the basis of molecular size gives only a partial solution to the question of the relationship between porcine immunoglobulins and their biological properties. Further information could be obtained by using a combination of gel filtration and ion exchange chromatography or preparative electrophoresis to achieve a more complete separation of the expected immunoglobulins (see Porter and Allen, 1969; Porter et al., 1969; Bourne, 1969). However, the demonstration of a heterogeneous distribution within gel filtration peaks of different serological reactivities or the same serological activities from different donors or against different antigens has raised the interesting possibility of separating novel classes of immunoglobulin on the basis of gel filtration alone.

The A–O System

Although the ABO (HIi Lewis) blood group system of man was the first to be characterised it is by no means fully understood today and still excites considerable interest and investigation. The patient and brilliant work of Morgan and Watkins (reviewed recently - Morgan and Watkins, 1969) in clarifying the biochemical synthetic pathways for formation of blood factors on soluble substances and red cells has given us a clear insight into the way in which blood group specificities can be built up and occlude one another. In what is considered this unique system not only do several chromosomal loci interact at genetic level in making available basic
mucopolysaccharide and lipopolysaccharide on which additional polysaccharide residues are attached by enzymatic action, but interaction at sero-morphological level also creates secondary or compound antigens such as Le\(^b\) (Marr et al., 1967). At its inception the idea of more than one specificity on a macromolecule was a novel one. Because of this finding, however, it has been possible to explain many of the quantitative, qualitative and quantitative/qualitative distinctions made in the human ABO HII Lewis system on the basis of cross reaction with these various specificities interacting at different levels in the molecule.

The work I have described on the porcine A-O system is only a provisional investigation into the added complexity which a consideration of interspecies relationships brings. It has been included, however, because it continues this theme of the production of serological specificity at different levels, the protein equivalent of which has already been seen in the antigenic factors of Igs. Thus certain allotypic factors are associated with individual amino-acid substitutions or peptides, and are much more immediate products of gene action at one mutational site or cistron. Other factors depend on the integrity of the whole structure or part of it so that although they are still finally products of gene action, this is more remote and opportunities for interaction at genetic and/or sero-morphological level are more frequent. Since carbohydrates are not primary products of gene action the
opportunities for interaction and interference with antigen production at various levels is more manifest in systems involving carbohydrates. In man this results in a variety of abnormal phenotypes in the ABO HII Lewis system (see Bombay bloods, Am, Ah, A₂h, Ahm, Ohm, Ainter - Race and Sanger, 1968; Voak et al., 1968; Lodge et al., 1965). In animals too we have odd scraps of evidence suggesting abnormal types in the A-O system. Thus Stone (1962) reports that there are certain exceptions to the threshold quantity rule for the occurrence of J on the cells of J CS cattle compared with its absence from cells of J S cattle. Some cattle fall outside the class expected from the quantity of J in their serum, and can have cellular J with a low serum level or lack cellular J with a high serum level. Recent research has suggested the presence of a lipid blocking factor (Stone - personal communication). In pigs too the strength of cellular A is roughly in accordance with the H.I. score of their serum and saliva. Exceptions occur, however, such as sow 7 and piglet 4 (see table of A activities of adult pigs and Table XII) where the cellular level is moderately high despite a low serum level. Newborn piglets remain A negative (and O negative - Saison and Ingram, 1962) despite high levels of soluble A substance. My finding that this is a property of the piglet's serum (or a property of the cell in the environment of piglet's serum) unrelated to the quantity of A substance is also found in cattle (Stone, 1962). This result still leaves several
options open. It is possible that the piglet lacks some enzyme responsible for attaching A substance, although piglet's serum did not coat any better in the presence of adult serum. The A substance in adult serum is possibly more available to the red cell, while piglet's serum, which has a different constitution, locks up its A substance in an inhibitable but non-available form. Another alternative is that piglets' cells are A and/or O positive but are non-agglutinable or non-haemolisable by available reagents.

It has been known for some years that ox cells fall into at least two categories - agglutinable and inagglutinable - with regard to certain antibodies, depending on how extensive a distribution of sialic acid residues occurred on their carbohydrate chains (Gleeson White et al., 1950; Coombs et al., 1951; Seaman and Uhlenbruck, 1963; Uhlenbruck et al., 1967). This concept has been considerably expanded in recent years to investigations into many species including man by the use of reagents derived from such unlikely sources as the albumin gland of Helix pomatia and the eggs and/or roe of salmon, perch and carp. These reagents agglutinate red cells of various origins before and/or after treatment with several enzymes (pronase, neuraminidase), and are specific for certain immunochromatic groupings (Prokop et al., 1968). Thus anti A<sub>Hp</sub> (Helix pomatia albumin gland) is specific for N-acetyl-D-D-galactosamine-non-reducing-alpha or beta linked, i.e. for the grouping causing group A (Morgan and Watkins, 1969). Before
enzyme treatment of human cells anti \( A_{Hp} \) only reacts with \( A_1 \) and \( A_2 \) cells. After treatment with neuraminidase, however, which merely removes neuraminidase susceptible neuraminic acid this reagent agglutinates B and O cells. In so far as this agglutinin is concerned the A specificity is dependent not on the presence of N-acetyl-D-galactosamine, but on the presence of certain neuraminidase susceptible neuraminic acids. Anti \( A_{Hp} \) also reacts with various animal cells including pigs of the group \( A_{HeJ} \) (Schmid and Buschmann, 1966). The distribution of \( A_{HeJ} \) in pigs unlike \( A_{Hp} \) in man does not coincide with pig A as demonstrated by isologous or heterologous anti-A (J) reagents. After neuraminidase treatment all pigs react with anti \( A_{Hp} \) so that \( A_{HeJ} \) negative pigs possess the specificity in a cryptantigenic form. Cattle do not react with anti \( A_{Hp} \) not even after enzyme treatment. It is clear from this and other reactions with these peculiar agglutinins that antigenic specificity exists at more levels on the red cells than is suggested even by Morgan and Watkins' work on soluble substances. Thus for various systems the following levels exist:

1. Normal agglutination, e.g. by anti \( A_{Hp} \) (like a saline agglutinating anti-A, -B, -D or porcine anti-\( \text{Ed} \), etc.), directed against surface glycolipid of A specificity in, for example, human A, sheep cells or pig \( A_{HeJ} \) cells.

2. Incomplete agglutination, e.g. by anti-B\(_{ss}\) or -B\(_{si}\) (from eggs of *Salmo salar* and *S. irideus* respectively), which react in some cases only after pronase treatment
for one of the following reasons:

a) the agglutinin reaches the receptor, but cannot agglutinate the red cells, due either to "incompleteness" of the agglutinin or the thickness of the red cell membrane: pseudocryptantigen of first order;

b) the receptor is not accessible to the agglutinin until it is exposed by pronase or protease treatment: pseudocryptantigen of second order.

Subtilisin A also reveals antigen like pronase.

3. Agglutinins (like anti $A_{Hp}$) which react with cryptogenic structures (previously known as Friedenreich antigens) which only become accessible after neuraminidase treatment.

It has long been realised that blood grouping was not simply a matter of $X + \text{anti-}X = \text{agglutination}$, and that the original idea of direct correspondence between gene and simple antigenic factor would have to give way to a complex mosaic of factors governed by a number of cistrons or mutational sites. Now, however, it can be seen that in addition to complex interaction between linked and unlinked genetic sites and interaction at sero-morphological level between parts of antigenic mosaics and other unsuspected factors (such as neuraminic acid), the constitution of reagents, not only in terms of their specific combining sites (which might in actual fact be rather limited in variability - Lang - to be published) but also in terms of
proportions of different immunoglobulins with the spatial ability to reach antigenic sites, will affect the specificity of serological reaction.

Thus we have the distinction between Ac and Ap where Ap cells are only satisfactorily demonstrated using a porcine reagent by the dextran technique. Anti A reagents (and other reagents) which are normally incomplete become saline agglutinating and more readily absorbed after treatment of pig A cells with papain (Olds, 1961). Human saline agglutinating "natural" anti A reagents fail to agglutinate Ap cells, but immune anti A haemolysins readily do so (Winstanley et al., 1957). On the other hand, soluble A substance of any origin (sheep R, cattle J, pig A, human A, etc.) will readily inhibit all anti A or anti A type reactions, e.g.

- pig anti A x pig A cells
- pig anti A x human cells
- human anti A x human A cells, etc.

(Winstanley et al., 1957; Saison et al., 1955; Lang - personal observations). The differences only arise when the A or A like antigen is tested on the cell particularly in interspecies comparisons (Neimann-Sorensen et al., 1954; and with the B antigen - Owen, 1954) where distinctions are attempted on a qualitative or quantitative basis.

It is not surprising that analysis by simple titration and absorption will fail to find a final solution to the complex crossreactions which will result from the use of heterologous
reagents in combination with the complex variations in serological specificity reviewed above.

In the anti-0/0 system differences also arise between species at the serum/saliva level so that, for example, O substance is present in all pigs. This is presumably a consequence of the fact that A (J or R) is added to the macromolecule after 0 (H) so that according to the attachment of A (J or R) both quantitatively and qualitatively 0 is variously occluded. Hence the dominance of A which is biochemical rather than genetical. My demonstration that 0 actually exists even on "-" cells as an antibody accessible but non-haemolisable cryptantigen gives some indication of the relevance of sero-morphological studies.

It would appear that, as for immunoglobulins, the most advantageous course to pursue is to combine the abilities of the serologist or immunologist with those of the biochemist in breaking down the complexity of the porcine cell sero-morphology in relation to the A-0 and other blood group systems.
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