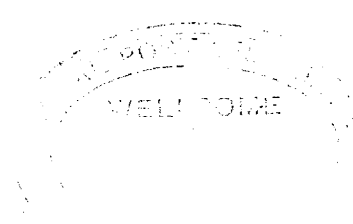

SEROLOGICAL STUDIES

OF

AVIAN INFECTIOUS BRONCHITIS VIRUS

A Thesis
Submitted to the Faculty of Medicine
of the University of London

By

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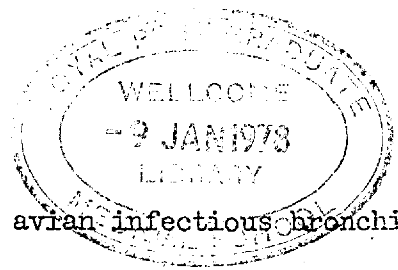

ABSTRACT

10 Reference strains and 15 field strains of avian infectious bronchitis virus were used to examine new methods for antigenic classification. Indirect haemagglutination, complement fixation and plaque reduction methods were studied. Observations on their applications to diagnosis and field assessment of vaccines were included.

Specific-pathogen-free eggs were used to clone 21 of the strains, by 3 successive passages at limiting dilution. Chicks reared from such eggs were used to prepare antisera from 23 of the strains.

Indirect haemagglutination of tanned horse red blood cells occasionally gave high serum titres, but these could not be reproduced consistently.

Direct complement fixation titres of chicken antisera were higher and more reproducible when the sera were heat-inactivated, and small aliquots of a tested batch of unheated normal chicken serum were added. Rapid, economical titrations were possible, of both sera and antigens. Antigenic differences were apparent, but poorly defined. Antigen preparation was difficult for some strains.

Plaque reduction was performed in chicken kidney cell cultures. Unheated foetal calf serum was used in the growth medium. pH Control was by hepes buffer. 75% Reduction was estimated graphically. When compared with the established neutralisation test in eggs, this method was more sensitive and less affected by variations in virulence of test viruses. 7 Reference and 5 field strains were passaged in cell culture to give readable plaques. The titres of the antisera against these strains were highly reproducible, and formed an unexpected, but consistent pattern. The type differences described by previous authors were confirmed, but the heterologous type titres of each antiserum were all close to a particular level, called the "group titre". The level of this titre may be related to certain characteristics of the virus strain that the antiserum was prepared from, possibly to the degree of development of the corona.

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LIST OF ABBREVIATIONS

BEAU	Beaudette
C'1 or C_1	Complement component 1
CEK	Chick embryo kidney
CELO	Chicken embryo lethal orphan
CF	Complement fixation
CH_{50}	50% Haemolytic dose of complement
CK	Chicken kidney
CONN	Connecticut
CPE	Cytopathic effect
EID_{50}	50% Infectious dose for eggs
GAL	Gallus adeno-like
hepes	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IB	Infectious bronchitis
ILT	Infectious laryngotracheitis
M-41	Massachusetts-41
NI	Neutralisation index
PBS	Phosphate-buffered saline
p.s.i.	Pounds per square inch
r.p.m.	Revolutions per minute
SPF	Specific pathogen free
UNCS	Unheated normal chicken serum

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PAPERS ATTACHED

The following publications are attached to the thesis:

(1) Bracewell, C.D. (1973). A direct complement fixation test for infectious bronchitis virus using heat-inactivated chicken sera. Vet. Rec., 92 (17), 452-454.

(2) Bracewell, C.D. (1973). Antigenic relationships between strains of infectious bronchitis virus as shown by the plaque reduction test in chicken kidney cell culture. Vth. int. congr. Wld. vet. Poult. Ass. 1973 Munchen. p.803-818.

(3) Harkness, J. W. and Bracewell, C.D. (1974). Morphological variation among avian infectious bronchitis virus strains. Res. vet. Sci., 16, 128-131.
The candidate was responsible for preparing virus concentrates for electron microscopy, and for preparing antisera and carrying out serological tests. The appropriate sections in the paper were written by the candidate.

(4) Gdovinova, Alzebeta, Bracewell, C.D. and Allan, W.H. (1974). Assessing infectious bronchitis vaccines. Vet. Rec., 95 (23), 533-534.
The senior author was a visiting worker who carried out the serological tests under the candidate's supervision. The candidate prepared and performed the challenge, made the clinical observations, and interpreted the results as a whole. The writing of the paper was by the candidate.

(5) Alexander, D.J., Bracewell, C.D. and Gough, R.E. (1976). Preliminary evaluation of the haemagglutination and haemagglutination inhibition tests for avian infectious bronchitis virus. Avian Path., 5, 125-134.
These studies included titrations of the stock antisera prepared by the candidate, by means of a haemagglutination inhibition test, and comparison of these results with their titres by the plaque reduction test, which were extracted from this thesis.

GENERAL INTRODUCTION

The economic importance of IB virus as a cause of disease in intensively housed chickens is now fully recognised. It is responsible not only for the acute respiratory disease with which it was first associated, but also for serious losses of egg production, due to the damage it can cause to the oviduct, and for outbreaks of nephritis, which may result in as much as 20% mortality. In broilers, an attack of IB often leads to such a depression of growth rates that the whole crop becomes unprofitable. In birds of this age, the main damage is usually brought about by secondary infections, which give rise to the syndromes known as chronic respiratory disease (CRD), and E. coli septicaemia.

Use has been made of the specific immunity that develops after infection. Serum antibody tests are useful in diagnosis, and the serological approach is often more successful than virus isolation, which tends to be laborious and lengthy. Live vaccines are in common use, administered in the drinking water or as aerosols.

Antigenic differences between virus strains were first reported in 1956, and their existence has since been confirmed by workers in many countries. However, no system of type classification has so far emerged. An unsatisfactory situation is apparent, in which virus strains are being used extensively for vaccine production and serological testing without adequate knowledge of their antigenic relationships with the field strains. How far this problem is due to inherent difficulties with IB virus, and how far to the use of unsuitable methods, is not known.

The serological method that has been most frequently applied to the demonstration of antigenic differences is the neutralisation test in eggs. The historical importance of the egg assay system in the growth of knowledge of IB virus made it inevitable that this method should have received the most attention. The method certainly can be very sensitive to antigenic

differences. Indeed, it was the main means by which such differences were first recognised. However, the results of cross-neutralisation tests between different strains show uncontrolled variations, especially when the antisera are prepared, and the tests performed, in different laboratories. Even when the tests are performed entirely at the same laboratory, under carefully controlled conditions, and the titres obtained are reproducible at that laboratory, as for example in the studies on 21 strains by Dawson and Gough (1971), a type classification is still not feasible, because of the complexity of the pattern of cross-reactions.

This project consists of the examination of alternative serological methods for IB virus. The primary objective was to find a method that would be more satisfactory for defining antigenic relationships between virus strains. Since the project would involve trying out techniques that were relatively new for IB virus, it was considered possible that other potential applications would emerge during the course of the work, so the development of these other applications was made the secondary objective.

Chapter I: SURVEY OF LITERATURE AND SELECTION OF METHODS

(1) IB Virus Strains And Antigenic Differences

The discovery by Beaudette and Hudson (1937) that the newly-recognised virus of infectious bronchitis could be cultivated in the embryonated egg opened the way for rapid advances in knowledge. Passaging in eggs was found to make the virus progressively easier to handle. The lethal effect on the embryo became more marked, producing more well-defined end-points in titrations, and the release of virus into the allantoic fluid was greatly increased, enabling the harvest of high-titred stocks, suitable for a variety of studies. The Beaudette strain became very widely used as a standard virus for serological tests, since it was found to be strongly neutralised by convalescent sera.

Highly passaged strains like the Beaudette were soon found to be attenuated in virulence for chickens (Delaplane & Stuart, 1941) and were quickly tried out as live vaccines. Unfortunately, many of the strains tried in the early years were lacking in immunogenicity, and useless for this purpose. The Beaudette strain itself is a perfect example of this phenomenon, being completely avirulent for chickens (although highly virulent for embryos) and having very low immunogenicity in chickens. Although widely used as a standard virus strain, these properties made it useless as a challenge strain, or for preparing standard antisera. [Antisera against Beaudette could only be prepared by using special inoculation schedules. (Chomiak et al, 1963.)] The need for a standard virulent strain was met by the Massachusetts-41 strain, isolated by Van Roekel et al (1950) at the University of Massachusetts, which was maintained by numerous passages in chickens. This strain became generally accepted as the standard reference strain for IB, used to prepare antisera that had high neutralisation titres against the Beaudette strain and most field strains.

Asplin (1948) confirmed that the virus occurred in Britain, by isolating several strains which he showed to be antigenically similar to the American reference strains. One of these British strains, the Allen strain, has since been maintained at Weybridge as a standard virus for work in both eggs and chickens. This strain has proved particularly useful because it has retained its virulence for chickens although well enough adapted to eggs for easy handling in the laboratory.

Until about 1955, the virus was generally considered to be uniform antigenically. The disease had been recognised by then in several parts of the U.S.A. and Canada outside the New England and Delmarva states where it first gained prominence. As many more isolates were made and studied, several workers began to suspect that not all strains were like Massachusetts-41. Jungherr et al (1956) of the University of Connecticut reported the results of cross-neutralisation and cross-protection tests on four strains. Three of these strains, which included Beaudette, Massachusetts-41, and one other, were similar, while the fourth was different, by both kinds of test. This "different" strain was studied extensively at other laboratories, and became known as the "Connecticut strain".

After this discovery it became acceptable to regard IB virus as existing in two "types": the Massachusetts type, to which the great majority of isolates seemed to belong, and the much rarer Connecticut type. This concept of two types lingered on in some quarters for a long time. Strictly speaking it became untenable only two years later when Hofstad (1958) of the University of Iowa found three more isolates that differed from the Massachusetts-like strains, and also from each other. Two of these isolates, Iowa 97 and Iowa 609, were used as reference strains by many later workers.

Hofstad (1961) went on to cast further doubt on the significance of the antigenic differences shown by the neutralisation test in eggs when he reported a lack of correlation with the results of cross-immunity tests in

chickens. He found that there was a considerable degree of cross-immunity between all of the 19 isolates studied, which included Connecticut, Iowa 97 and Iowa 609 in addition to the Massachusetts-like strains. Cross-immunity seemed to be more affected by the dates of isolation of strains than by their antigenic relationships. The more recent isolates tended to be more effective at both inducing cross-immunity against, and breaking the immunity given by, the older isolates.

Further new "types" of IB virus were discovered during investigations of the viruses causing nephritis in chickens. After a period of confusion with the nephritis caused by the agent of "Gumboro disease" (later renamed infectious bursal disease), the ability of some strains of IB virus to cause nephritis was finally demonstrated by Winterfield and Hitchner (1962). The virus strains Gray and Holte, which had been handled in the laboratory for some time, and were known to induce nephritis in chicks, were identified as IB virus. However, they differed antigenically from the existing reference strains, and from each other. These antigenic differences were not clearly defined. For instance, the antisera prepared with Gray and Holte did have high neutralisation titres against Beaudette. Also, the cross-immunity tests did not entirely agree with the serological tests. The authors commented that a complex immunologic picture was arising for IB.

In Australia, IB was unrecognised until Cumming (1962 and 1963) showed that the disease known as "uraemia", characterised by severe nephritis, was caused by strains of a virus closely similar to that of IB. The antigenic relationships between the Australian and American strains were studied by Winterfield, Cumming and Hitchner (1964). Sera from flocks in Australia recovering from uraemia were able to neutralise all six of the American type reference strains, but antisera prepared with the American strains failed to neutralise the Australian strains. Strains isolated in different regions of Australia showed minor, but significant, antigenic differences.

For example, convalescent sera from Armidale did not neutralise Gray and Holte as effectively as did sera from Sydney. The authors expressed doubts on the validity of "types" of IB virus.

Reports of the isolation of new types continued to appear from all over the world. (Table 1.). Some of these isolates were reported to represent significant, new, epidemic strains, that were breaking existing vaccines. The strains Florida, Clark 333 and Ark 99, which were reported after these studies had commenced, were in this category. Other isolates seem to have been the only ones of their type to have been found. The four types isolated in Italy by Zanella (1968) all consisted of single isolates, recovered from cases of nephritis over a short period of time, and not encountered again. (Zanella, 1971).

The majority of isolates found in all countries continued to be of the Massachusetts type. (Fritzche et al, 1969). However, there have been some recent reports from the U.S.A. that in certain regions the prevailing type is no longer Massachusetts. Johnson et al (1972) found that 24 out of 26 isolates, taken from flocks in the Delmarva region where vaccine breaks had occurred, were of an antigenic type similar to the JMK strain which was originally isolated in the same area by Winterfield, Hitchner and Appleton (1964)

Hitchner et al (1964) commented on the state of confusion regarding the distribution and significance of IB virus types, with a plea for a systematic approach to classification.

A collection of 15 British field strains was used by Dawson and Gough (1971) at Weybridge for a systematic study of antigenic relationships as shown by the neutralisation test in eggs. Antisera were prepared in chickens against these strains, and also the seven type reference strains known up to 1964, and a complete chequer-board of cross-neutralisation tests was constructed. Instead of the commonly used "alpha" method of performing the test, (the virus dilution method - see Materials and Methods, Section (2)),

TABLE 1. DESCRIPTIONS OF NEW "TYPES" OF IB VIRUS

Reference Strain	Country of Origin	Authors and Year
Connecticut	U.S.A.	Jungherr et al (1956)
Iowa 97		Hofstad (1958)
Iowa 609		
Gray		Winterfield & Hitchner (1962)
Holte		
T (Tweed-Heads)	Australia	Winterfield, Cumming and Hitchner (1964)
JMK	U.S.A.	Winterfield, Hitchner and Appleton (1964)
RPL-IBV		Purchase et al (1966a)
IBV-10 (Cuxhaven)	Germany	Von Bülow (1967a)
Massey	New Zealand	Pohl (1967)
446/66	Italy	Zanella et al (1968)
693/66		
429/67		
455/67		
0353	Japan	Kawamura et al (1968)
KH		
IBV-K4 (Koblenz)	Germany	Von Bülow (1969)
SE-17	U.S.A.	Hopkins (1969)
Florida		Winterfield et al (1971)
Clark 333		Cowen et al (1971)
Ark 99		Fields (1973). Johnson et al (1973)

the "beta" method was selected for this work. This method was considered more suitable for making exact comparisons of serum titres. In spite of careful standardisation of all procedures, a very complex pattern of cross-reactions emerged. The authors concluded: "It is considered that it was not feasible at this time to describe accurately a serotype classification for IBV similar to that described for other virus groups." It was however clear that considerable antigenic variation did occur between the British strains, although the degree of variation was relatively less than that occurring among the type reference strains. This work led logically to the examination of alternative serological methods, as undertaken in these studies.

(2) IB Antisera

The choice of methods for preparing antisera to IB virus is severely limited by the nature of the disease and the virus. In contrast to the wide host ranges of some other respiratory viruses, such as Newcastle disease and avian influenza, IB virus is known to cause disease only in the chicken, and there is no strong evidence that the virus can undergo sub-clinical replication in any other species. (Miller and Yates (1968) did however find a higher incidence of neutralising antibody to IB virus in the sera of persons whose occupations brought them more in contact with chickens.) The chicken is therefore in practice the only experimental animal that can be used for preparing IB antisera by means of infection.

Preparation of antisera by means of inoculation with non-replicating viral antigens is also very limited in practice. There have been few reports of the use of this method for serological studies. Chomiak et al (1963) succeeded in preparing an antiserum against the Beaudette strain by repeated inoculations of chickens with a virus preparation including aluminium hydroxide as adjuvant, but they used this method only because of the lack of immunogenicity of this strain when given as a live virus, and their antisera to other strains were prepared by infection. Steele and Luginbuhl (1964) prepared antisera in guinea-

pigs against Beaudette by a schedule of inoculations with chicken kidney cell harvest, which were effective in direct and indirect complement fixation tests. (See Section 7 of this chapter.) Berry and Stokes (1968) prepared antisera against a variety of strains by repeated inoculations of chickens with virus preparations containing aluminium hydroxide. They reported finding higher levels of cross-reaction on the neturalisation test in eggs than they obtained from antisera prepared by the usual infection method. They concluded that the antigenic differences between strains were of a minor, sub-type nature.

Since the main object was to study antigenic relationships between strains, it was considered better to avoid methods of preparing antisera that tended to give high levels of cross-reaction. Repeated inoculations of large quantities of virus, and the use of adjuvants, were therefore decided against. The preparation of antisera in species other than the chicken was thought to be especially liable to produce cross-reaction, because Berry and Almeida (1968) showed that the IB virus envelope contains antigens derived from the host cell. When the virus has been grown in the allantoic cavity, this antigen is derived from chicken cells, and will stimulate rabbits or guinea-pigs to form antibodies that will react with all virus strains having an envelope of this kind, perhaps even giving cross-reactions with completely different virus species, that have been grown in chicken cells.

The secondary objective, to develop new serological methods for practical use in the control of IB, was also likely to be better served if the stock antisera were prepared by infection of chickens, since the methods found to be suitable for these antisera would be likely to be immediately applicable to chicken sera from the field or from vaccine trials.

The difficulties in preparing antisera in chickens lie in ensuring that no extraneous antibodies are present. The chickens must not have been exposed to IB or any other infection that could possibly be present in the virus harvests used as test antigens, in order to avoid confusing results. In

practice, this means that the birds should be hatched from eggs produced from specific-pathogen-free (SPF) stock, and reared in isolation from unspecified stock. Before use, they should be tested for absence of IB antibody, and during and after infection they should be housed in effective isolation. Because of the great ability of IB virus to spread through the air, the risk of cross-infection between groups of birds inoculated with different virus strains is particularly high, and the isolation facilities must be especially well designed and maintained. However, the buildings of the Respiratory Disease Unit were considered adequate for this purpose.

The decision was made to prepare the stock of antisera against the virus strains to be studied by means of infection of chickens.

(3) Selection Of Serological Methods

Passaging and titration of the virus in eggs has been an essential basis for all kinds of work in IB, from a few years after its discovery up to the present day, and the neutralisation test in eggs has always been the most widely used serological method. Since virtually all strains of virus have been isolated, and subsequently passaged, in eggs, preliminary adaptation of the virus is not often needed before it can be used for titrating sera. A steady supply of eggs suitable for assays is fairly simple to establish and maintain. However, the technique is very laborious, and requires considerable skill and experience if reproducible titres are to be obtained. Sharp end-points are obtained with the faster-replicating, more rapidly lethal strains, but the slower strains give vague end-points which depend on the experience of the operator in recognising the signs of infection in embryos that are still alive 7 or 8 days after inoculation. Consistency of results between laboratories is difficult to obtain. Following the discovery of serological differences between IB virus strains (Jungherr et al, 1956), the application of the method to studies of antigenic relationships has produced the confusing situation described in Section (1) of this chapter. The method is also too slow and

laborious to be ideal for use in diagnosis and vaccine trials, although it has given better service in these areas of work than in the serological classification of strains.

The search for alternative serological methods has been going on since the earliest years of work with IB. After the spectacular success of haemagglutination inhibition with Newcastle disease, this method was naturally one of the first to be tried. It is quantitative, reproducible, rapid and economical, and quite widely applicable among the viruses. One can even say that most viruses will haemagglutinate, given the right red blood cells and the right conditions for the reaction. The early workers (Hofstad, 1945; Fabricant, 1949) soon found that IB virus failed to haemagglutinate chick cells in saline, unlike the viruses of Newcastle disease and fowl plague. That failure itself became widely used in identifying the virus of IB. The subsequent literature on the method is reviewed more fully in Section (6), where also the reasons for selecting the indirect (tannic acid) method for study are given.

Another serological method that has proved of immense value in a wide range of viruses is the complement fixation test. However, the failure of chicken antibody to fix guinea-pig complement after the normal routine heat-treatment of the sera (Bushnell and Hudson, 1927; Rice, 1947) presented a major difficulty. As described in Section (7), various ways have been found of overcoming this difficulty, but at the time of planning these studies, the use of the method was still very limited. Because of its many inherent virtues, which are the same as those of the haemagglutination inhibition test, the method was considered worthy of study for our purposes. A survey of the literature on the CF test as applied to IB, and a more detailed explanation of our approach, is given in Section (7).

The use of cell cultures as an alternative assay system for the neutralisation test became possible during the nineteen-fifties because of the great advances at that time in the techniques of trypsinisation and growth of

cells on glass. Primary kidney cell cultures from chick embryos or young chickens were found suitable for assay of IB virus. The difficulties here were: firstly, in producing adequate quantities of satisfactory cultures; and secondly, in adapting virus strains to the point where they caused visible CPE. No cell line of avian kidney cells was available, and the technique of preparing primary cultures required much labour and skill, and some luck. In practice, only those virus strains which were already well adapted to eggs would grow in cell culture, and primary isolation of typical field strains in cell culture was not possible. Cell culture methods are reviewed in Section (4).

Neutralisation of IB virus in cell culture has been shown to be a practical method of serum assay, within the limitations mentioned in the paragraph above, and the literature on this subject is reviewed in Section (5). It was a little puzzling to find that there were so few reports of the application of cell culture methods to the problem of antigenic variation, but we concluded that this was due to the technical difficulties. A plaque reduction test had been applied to various special studies by several authors, but, at the time of planning, the only account of the effect of antigenic difference on its results was by Cunningham and Spring (1965), and they used only one test virus and two antisera.

In planning these studies, it was decided at the outset to put the main effort into cell culture. The entire programme was built around the expectation that this would prove to be the most suitable method for studying antigenic relationships. It could not be known how feasible a plaque reduction test would be until several different strains had been successfully adapted for use as test viruses, and cross-neutralisation tests carried out, but it was decided to plan on the assumption that plaque reduction would be the best method. A regular, weekly supply of chicken kidney cells was arranged. While waiting for the programme for preparing cloned virus strains in SPF eggs to be

completed, experience in handling IB virus in these cultures was obtained, using uncloned Beaudette strain, so that as soon as the first few cloned strains became available, plaque reduction tests could be begun immediately. The main programme for preparing antisera in SPF chickens using cloned virus strains was monitored by means of a homologous neutralisation test in eggs. That is, serum was collected only when a satisfactory neutralisation titre had been produced, on the assumption that the plaque reduction titre would then also be satisfactory. This policy was necessary in order to avoid having to wait for each strain to be adapted as a test virus before preparing an antiserum to it. Because of the difficulty in adapting some strains, long delays would have followed from a waiting policy.

While the main programmes of strain cloning, antiserum preparation and cell culture were progressing, it was decided to make use of interim periods to study the haemagglutination and CF tests. Because of limitations of staff, it was thought to be better not to tackle both methods simultaneously. It was realised that whichever method was studied later would have the advantages of a much larger stock of virus preparations and antisera to work on, and the use of an ultracentrifuge, which was to be purchased for the Department but was not available at the commencement. It was decided to give this advantage to the CF test. Accordingly, studies on the haemagglutination test were started first. The plan was to study this method, judged to be less promising, for a limited period, and then to decide, on the basis of the results obtained, whether to continue or to drop it in order to allow studies to be started on the CF test. If, after a more exhaustive study, the CF test proved equally unpromising, the intention was to allow enough time for a re-examination of the haemagglutination test at a later stage when better materials and equipment would be available.

In the event, work on the haemagglutination test ceased after 5 months, and for the rest of the time work continued on the CF test, in parallel with

the main, cell culture programme.

It is worth mentioning briefly the other serological methods which were considered for study. The gel diffusion test, which was well established for IB, and had proved to be useful in diagnosis and certain specialised laboratory studies, was considered unsuitable, because it is basically a qualitative, not a quantitative, method, and it had been shown to give complete cross-reaction between IB virus strains (Dawson, 1970). The fluorescent antibody test was reported by Lukert (1969) and Lucio and Hitchner (1970) to be affected by antigenic difference, but was also unsuitable because of its qualitative character.

A method based on the ability of IB virus to interfere with the growth of Newcastle disease virus in chicken kidney cell culture was described by Beard (1967, 1968). He suggested that the method could be used to titrate sera with IB virus strains that were not well enough adapted to cell culture to enable a normal neutralisation test, based on suppression of CPE, to be performed. This method was considered unsuitable for our purposes, because, in addition to the inherent difficulty in standardising the test dose of IB virus, and the complications derived from involving the poorly-understood phenomenon of interference (which apparently does not involve interferon), it appeared to be insensitive, and uneconomical in its use of cells. Our policy regarding the less well adapted IB strains was to give them further egg passages and then try again in cell culture.

The use of tracheal organ cultures as an assay system for the neutralisation test had certain attractions. The human coronaviruses had been discovered by means of primary isolation in tracheal cultures (Tyrrell and Bynoe, 1965; McIntosh et al, 1967), and it was later found that IB virus could also be isolated in this way. The method was used by Johnson et al (1969) in an attempt to demonstrate local immunity to IB, by collecting tracheal rings from convalescent chickens and testing their susceptibility to infection. This work

showed the feasibility of the technique as an assay system. Colwell and Lukert (1969) confirmed replication of IB virus in tracheal cultures, by means of immunofluroescence. Preliminary studies on the method at Weybridge (Dawson, 1970) had, however, shown that it was not suitable for sustaining the virus through successive passages, or for maintaining high-titred virus stocks, because of the very low infectivity titres of the harvests. Since our programme demanded fluency in handling a large number of existing virus strains over a protracted period of time, it was considered better to apply our efforts to the use of eggs and cell cultures.

Later, Johnson and Newman (1971) succeeded in passaging 5 reference strains of IB virus in tracheal cultures, by preparing the inoculum for each passage by a 100-fold ultracentrifuge concentration of the harvest from a large number of tracheal rings. By carrying out neutralisation tests in tracheal cultures, they showed that the antigenic differences between these strains had not been affected by their passages in tracheal culture. Still later, Johnson et al (1972) used tracheal cultures to study the antigenic relationships of 26 isolates from apparent "vaccine breaks" in the Delmarva area of the U.S.A. However, by this time we were committed to cell culture work, and the advantages of tracheal culture did not seem to be great enough to justify expanding our programme to include this technique.

(4) <u>The Use Of Cell Culture For IB Virus</u>

The earliest successes in growing IB virus in cell cultures were achieved with the highly egg-adapted Beaudette strain. Fahey and Crawley (1956) reported virus growth, in chick embryo liver and heart cell cultures, but no CPE. The first attempts to use chick embryo kidney (CEK) (Buthala and Mathews, 1957) were unsuccessful, but soon afterwards Chomiak et al (1958) reported both growth and CPE in these cells, and Wright and Sagik (1958) showed that plaque formation was possible with the Beaudette strain in CEK cells. Virus assays and neutralisation tests in tubes were described by Pette (1960). This author

used chick embryo fibroblasts, but the superiority of kidney cells began to be more generally asserted after this time.

During the nineteen-sixties, the rival merits of kidney cells from chicks a few weeks old, as compared with chick embryos, were advocated by different authors: Kawamura et al (1961) used kidney cells from adult or young chickens (CK), and successfully adapted 3 Japanese isolates to the level where titrations could be perfomed, based on CPE. These authors made the very important general observation that adaptation to cell culture was only possible after a considerable, preliminary adaptation to eggs. For their 3 virus strains, 6 to 10 egg passages were required. They also remarked on the greater sensitivity of eggs as an assay system. At the beginning of adaptation of a strain to cell culture, the cell culture harvests commonly gave titres 1000-fold higher in eggs than in cell culture. With successive cell culture passages, the titre in cell culture usually rose, while the titre in eggs often remained fairly constant, so that the difference in sensitivity of the two systems became progressively reduced. However, even after 20 passages in cell culture, the harvests still gave slightly higher titres in eggs.

In the U.S.A., Mohanty and Chang (1963) confirmed that CK cell cultures were practicable, but Cunningham (1963) advocated CEK cells, on grounds of higher susceptibility and cell growth rate. CEK cells were used by Cunningham and Spring (1965) for the earliest reported studies on well-controlled plaque assay with IB virus. Lukert (1965) compared CEK and chick embryo liver cells for use in plaque assay, and showed that CEK were more suitable. Following this observation, he developed a plaque reduction test in CEK (Lukert, 1966).

Meanwhile in Britain, Churchill (1965) confirmed the findings of Kawamura (1961) that CK cells were a practical system for IB virus. He preferred to use chicks of 4 to 5 weeks old as the source of the cells, and emphasised the importance of precise control over the trypsinisation. A series of very short periods of digestion, terminated abruptly by cooling and addition

of serum to the decanted cell suspensions, was considered vital to success. Butler (1965) noted the greater susceptibility to IB virus of epithelial-like cells, from whatever source, as compared with fibroblast-like cells, and considered this to be an important factor in deciding the degree of success of cell cultures.

CEK cells continued to be much used in America for a variety of plaque assay studies in which small numbers of highly accurate titrations of IB virus were desired. Coria (1969) used this system for measuring the low levels of infectivity released from cells to which the virus was poorly adapted. Stinski (1969) used it for studies on the kinetics of virus penetration, and Lukert (1972) used it for studies on the chemical nature of the cell receptor sites. Adaptation of 9 strains of IB virus for plaque assay in CEK cells was described by Gillette (1973).

CK cells were used by Beard (1967, 1968) for his Newcastle disease interference test, as a monolayer in plastic petri-dishes. The generalised CPE which developed over the whole monolayer, as a result of growth of Newcastle disease virus, was demonstrated by staining it with 0.5% crystal violet.

At the time of commencing these studies, there had been no description of the use of CK cells for plaque assay of IB virus. However, plaque assay of influenza virus, in CK cells from chicks of 5 or 6 days of age, had been described by Babiker and Rott (1968); of infectious laryngotracheitis virus by Sharma and Raggi (1969), and of Marek's disease virus by Churchill (1968). (Marek's disease virus plaques differ from those of the other viruses named, in their very small size and lack of requirement for an agar overlay. These features are due to the inability of the virus to spread from cell to cell unless they are in direct contact.)

Plaque assay of CELO virus in CEK cells was described by Anderson et al (1971).

The techniques employed in these studies were gleaned from the

descriptions of many of these previous authors, combined with the practical experience of growing CK cells, that was already in existence in the Department, and was based on the methods of Churchill (1965).

The growth of IB virus in cells derived from animal species other than the chicken has met with relatively little success.

Primary monkey kidney cells were found to release virus in high titre over a long period, but without CPE (Fahey and Crawley, 1956; Steele and Luginbuhl, 1964). VERO cells (an African green monkey cell line) were shown by Cunningham et al (1972) to be capable of giving CPE, which could be utilised for plaque assay, when infected with a specialised line of Beaudette that had been previously adapted to grow in suckling mouse brain. Coria and Ritchie (1973) succeeded in adapting the Beaudette, Massachusetts -41 and Iowa 97 strains to VERO cells after preliminary adaptation to CEK cells. About 21 passages were required for the appearance of typical IB virus CPE (syncytium formation) in VERO cells, and the plaques formed very slowly in comparison with their speed in CEK or CK.

A variety of avian and mammalian cells have been shown to support replication of IB virus within their cytoplasm, as demonstrated by fluorescent antibody. (Lukert, 1966; Coria, 1969). However, release of virus into the medium is a less common ability, and Coria (1969) remarked that cells of avian origin were generally better than mammalian cells in this respect. Earle's L cells (mouse fibroblast cell line) were reported to be completely resistant to IB virus, including Beaudette, by Fahey and Crawley (1956), as were HeLa cells (human cell line) by Davis (cited by Coria, 1969). Coria and Peterson (1971) reported propagation of IB virus in turkey embryo kidney cells, a finding that could be valuable for special applications where the specific avoidance of chicken cells was necessary.

(5) Neutralisation Test Methods For IB Virus

This section consists of an examination of the basic methods that have been used for the neutralisation test for IB virus, and a consideration of how they may have contributed to the confusion which now exists over antigenic relationships.

The neutralisation test is performed in two distinct stages: firstly, the in vitro reaction between virus and antibody, and secondly, the in vivo assay of infectivity. However, to imagine that what actually happens is divided simply in the same way is to ignore the complexities caused by the continuing, three-sided interaction between cell, virus and antibody. Antibody-virus complexes may be pinocytosed by some kinds of cell, and reversal of neutralisation might occur in some circumstances. These effects mean that the nature of the in vivo assay system used can have profound effects on the results observed. When egg embryos are used an additional dimension of complexity arises because the results are read from gross changes in the whole organism, which are much affected by variations in virulence between virus strains, and in susceptibility between embryos.

There is evidence that IB virus is particularly strongly affected by genetic variation in embryos, in its ability to cause disease and death. (Purchase et al, 1966b.) It has also been shown that small variations in test conditions, such as temperature of incubation, may lead to striking alterations in observed effects (Simpson and Groupé, 1958).

The wide variation in virulence between IB virus strains is evident within a very few passages after isolation, so that any attempt to standardise the virulence by restricting the number of passages is doomed to failure. It would be more feasible to try to bring all strains up to the same, high level of virulence, by means of repeated passaging of the less virulent strains. Unfortunately, many strains remain slow even after the expenditure of much time and labour in repeated passaging.

Von Bülow (1967b) studied the neutralisation regression line for IB, and showed that, both in eggs and in cell cultures, the degree of adaptation of the virus strain to the assay system markedly affected the slope. From these findings he concluded that no exact antigenic comparison of strains could be made unless their regression lines were parallel.

Von Bülow's cell culture assays were carried out in tubes, read by microscopic observation of CPE. In this system he observed that the slope obtained for poorly adapted strains could be greatly improved (i.e. brought nearer to $^1/_1$) if the cultures were washed immediately after the absorption period. One could postulate that a poorly adapted strain contains particles of varying affinity for the cell. Those of higher affinity adsorb within the standard time, and those of lower affinity remain in the medium in an unwashed culture but are removed from a washed culture. Thus washing has the effect of selecting that part of the virus population more likely to escape from the binding of the antibody, giving a reduction in the proportion of virus/antibody needed to initiate infection. This explanation implies that virus and antibody remain in a dynamic, reversible union for a considerable time after inoculation.

IB virus strains can be used for tube assay at a lower level of adaptation than that required for plaque assay. This is because there are, broadly speaking, two kinds of CPE observed with IB virus in cell culture : the formation of groups of rounded cells, which is seen early in adaptation, and the syncytium which is seen when adaptation is complete. Plaque assay requires syncytium formation, which is associated with macroscopic changes in the cell sheet. Thus the determination to employ plaque assay does have the effect of imposing a uniformly high level of adaptation on the test viruses. The additional passaging of strains required for plaque assay may seem to be a lot of labour, but this process may well legislate for uniformity of slopes in regression lines.

It is also worth remarking that plaque assay resembles a washed tube

culture, in that the presence of agar in the medium will tend to prevent infection of the cells by slow-adsorbing virus. Lukert (1966) found a slope of $^1/_1$ for his plaque reduction test, compared with $^1/_{2.1}$ for the neutralisation test in eggs using a fairly well adapted strain. (Slopes of less than $^1/_3$ were found by Von Bülow (1967b)for poorly adapted strains in eggs.)

It is also probably true that individual variation in susceptibility is less between cell cultures from the same batch of cells than between the eggs from a flock of hens. Variation between batches may be largely offset by the use of control virus titrations, especially if a method of neutralisation test is chosen which depends on a comparison of the readings obtained within a single test, such as the "alpha" (constant serum, varying virus) method or percentage plaque reduction.

The in vitro stage of the neutralisation of IB virus was studied by Cunningham (1951), Crawley (1951), Fabricant (1951) and Page and Cunningham (1962), using eggs as the in vivo assay system. The fundamental characteristics of the reaction were shown to be similar to those found in the typical animal virus-antibody reaction, as for example those defined by Burnet et al (1937). The very rapid initial rate of reaction was confirmed by Page and Cunningham (1962), who found a survival rate of less than 10^{-5} after 15 minutes' reaction with undiluted serum. Proportionality between the fraction surviving and the serum dilution was also demonstrated by these authors.

The preliminary heat-treatment of sera at 56°C for 30 minutes has for long been a customary practice for IB, as for most other virus-antibody systems, in order to reduce non-specific neutralisation. This undesirable effect is particularly strong for IB, as was shown clearly by Berry and Almeida (1968). Since the main object of these studies was to seek better methods of defining antigenic differences, maximum specificity was desirable, and so it was decided at the outset to follow this traditional practice.

The method of combining the dilutions of virus and antibody has a profound effect on the characteristics of the neutralisation test, since it determines not only the manner of reading the end-point and expressing the serum titre, but whether the failure of infection observed is due always to neutralisation or may at times be due to extreme virus dilution, or a combination of these effects. Two schools of thought continue to argue on the rival merits of the "alpha" (constant serum-varying virus) and "beta" (constant virus-varying serum) methods. The alpha method is easier to perform satisfactorily, because it does not require a preliminary virus titration, and it is unaffected by small differences in susceptibility between batches of eggs or cells, which tend to spoil many beta method tests. However, the beta method is preferred on theoretical grounds because it is a better principle to vary the reagent under study (the antibody), and the method results in failures of infection being due entirely to genuine neutralisation. The advocates of the beta method claim that although the number of invalid tests is higher, the results of valid tests are more reproducible and give a better assessment of the amount of antibody present in serum samples.

When cell cultures are used as the assay system, a decision to employ tubes immediately brings up the question of whether to use the alpha or beta method, since tubes and eggs are essentially similar in being all-or-nothing, or quantal, assay systems. In practice, variation in susceptibility between batches of CK cells is fairly high, so that the practical argument for using the alpha method is strong. If however the decision is made to employ plaque assay, this old argument can be transcended. There is no need to use a traditional alpha method, as described by Lukert (1966), for the plaque reduction test. The percentage plaque reduction test combines the best features of both alpha and beta methods with the fundamental advantages in accuracy of an enumerative method. As with the beta method, varying serum dilutions are made to react with a constant virus dilution, which is determined

by a preliminary virus titration as that dilution which will give the optimum number of readable plaques. In the test proper, serum end-points are read as that dilution estimated to give a selected percentage reduction in number of plaques, in relation to the controls, which consist of virus + diluent only. By these means, the variations in cell susceptibility between batches which do occur, do not affect the serum titres obtained, unless they are gross enough to make the plaque counts unreliable, i.e. either confluent or in such low number as to be inaccurate. The well-known "percentage law" (described fully and expanded by Burnet et al, 1937) states that the serum titre is independent of the absolute concentration of virus present, but is inversely related to the percentage survival of virus. There thus appears to be good theoretical as well as practical justification for the percentage plaque reduction test.

Two practical problems that remained to be settled were the selection of suitable times for the in vitro reaction and the adsorption of virus on to the cells before adding the overlay.

The selection of an appropriate reaction time is necessarily governed mainly by convenience in performing the work. Whatever time is selected, if a serum dilution method is used there will be an area of the titration around the end-point where insufficient time has been allowed for completion of neutralisation, since the time required becomes longer as the serum is diluted out. There is a danger in extending the time of reaction that undesirable effects may begin to affect the results, such as thermal inactivation of the virus, which probably varies between different virus strains. Burnet et al (1937) showed that the failure of the more dilute serum mixtures to reach equilibrium causes a deviation of the curve of serum concentration/survivor from the straight line which is more marked in the area of low neutralisation. In other words, the curve tends to become more linear as it approaches complete neutralisation. From this it follows that it is better to choose to read the end-point at greater than 50% neutralisation, and the choice of about 75%

plaque reduction should help considerably to reduce the effect of failure to achieve completion. The choice of the optimum percentage can also be determined experimentally by analysis of the results of replicate serum titrations, and it was planned to do this at an early stage. At the time of carrying out this work, we were unaware that it is possible to calculate theoretically the optimum percentage. For a foot-and-mouth disease virus system, McVicar et al (1974) showed that this figure was 73%, a figure reassuringly close to the one we had derived for IB virus by experiment.

The selection of the adsorption period is a similar compromise between theoretical considerations and expediency. Cunningham and Spring (1965) showed that for IB plaques there was a rapid initial rate of adsorption, leading to attainment of greater than 50% of the maximum count within 10 minutes. This was followed by a progressively falling rate, and they obtained the maximum count after 90 minutes. It would, from these results, perhaps be logical to use an adsorption period of 90 minutes, and this time was indeed used by Lukert (1966) for the plaque reduction test. However, we decided to use 30 minutes because it fitted more easily into the work routine, and gave less danger of damaging the cells by lack of nutrients and evaporation. It was considered that the possibility of slightly sub-maximal counts being obtained was unimportant because of the comparative nature of the work, provided that the shorter period was uniformly applied. In any case, it was expected that this effect would be completely overshadowed by factors such as variation in cell susceptibility.

(6) The Haemagglutination Test

In a review of the basic properties of IB virus, Cunningham (1957) described the lack of haemagglutination found by the earlier workers. Subsequently, this author was concerned in attempts to obtain haemagglutination by a variety of means, including the use of several species of mammalian red blood cells (Corbo and Cunningham, 1959), trypsinisation of virus harvests

(Corbo and Cunningham, 1959), and separation of a haemagglutinating fraction from virus harvests by means of DEAE-cellulose chromatography (Biswal et al, 1966). Although haemagglutination of chick cells was demonstrated with the two latter preparations, inhibition by antibody could not be shown, and the specificity of the reactions remained in doubt.

Berry (1966) obtained haemagglutination of chick cells with an ether-treated virus harvest, and succeeded in demonstrating the appearance of antibody capable of inhibiting the reaction at an appropriate time (2-3 weeks) after experimental infection of chicks. Unfortunately, he then encountered difficulty in replicating results, and found no correlation between serum titres by the neutralisation and haemagglutination inhibition tests.

Doherty (1967) found no haemagglutination of the cells of 16 widely different species of animals and birds, in straightforward, direct tests with unconcentrated allantoic fluid harvests. The prospect of finding a suitable species by a lucky chance therefore seemed too dim to encourage a further, more extensive search.

In the meantime, several authors in the U.S.A. had reported success with an indirect test, in which red cells of the horse or sheep were treated with tannic acid, and then made to react first with IB virus and then with IB antiserum. The result was to demonstrate haemagglutination patterns of the spread-out type up to titres of about $^1/_{1024}$ with known IB antisera, and significantly lower titres with control sera (Brown et al, 1962; Vasington et al, 1963; Mohanty et al, 1964). The method was based on the modifications of Stavitsky (1954) of the original discovery of Boyden (1951) that a wide variety of antigens could be made to adhere to tanned red blood cells, which would then agglutinate when mixed with their specific antibodies. Scott et al (1957) had shown that the method would work for Herpes simplex, and Eidson and Schmittle (1969) later reported success with Marek's disease virus. At the time of planning these studies, the tannic acid method appeared from the

literature to be the most promising of those described, being the only method for which specific antibody reactions had been reported consistently by more than one author.

Difficulty in reproducing the reactions of the tannic acid test were reported by Szedo and Romvary (1967), and, at the time of planning in 1970, the method had not been accepted for routine use.

Kaye and Dowdle (1969) reported direct agglutination of rat and mouse red blood cells by the two similar human coronavirus strains OC38 and OC43, the specificity of which was proved by its inhibition by known antisera. However, broad application of the method was hindered by the failure to haemagglutinate of all other human coronavirus strains. The authors commented on the general lack of haemagglutination with coronaviruses as a group.

(7) The Complement Fixation Test

At the time of planning these studies, the CF test was well established for ornithosis (Benedict and McFarland, 1956; Neal and Davis, 1958; Boulanger and Bannister, 1961) but was very little used for other avian antibody systems, beacuse of the difficulty in fixing complement, described in Section (3). This situation was mentioned by Estola (1970) in a review of the properties of IB virus and other coronaviruses.

The failure of avian antibody to fix guinea-pig complement can be circumvented by preparing antisera in mammals, such as rabbits or guinea-pigs. This antiserum can then be used either in direct tests, or in indirect tests in which it serves to demonstrate whether a reaction has occurred between the antigen and dilutions of the avian serum under test. Steele and Luginbuhl (1964) prepared antiserum in guinea-pigs against chicken kidney cell harvests of the Beaudette strain, and with it obtained specific fixation in the reaction with an ultracentrifuge concentrate of an allantoic fluid harvest of the same strain. An antiserum prepared in chickens against Beaudette was titrated by an indirect test, using this mammalian antiserum. No reaction was obtained

with chicken antisera prepared against Massachusets -41 and Connecticut.

The indirect test was further developed by Sayed (1968) (seen only in abstract). Rabbit antisera were prepared against Beaudette and Massachusetts -41 antigens which consisted of specially prepared extracts. Results were recorded in percentage lysis. When applied to field sera, this method gave titres running parallel to those given by the neutralisation test.

Meanwhile, another school of thought was working on the alternative approach to the problem. Nitzchke (1954, 1956) reported that chicken antisera to Newcastle disease virus would fix guinea-pig complement if used unheated. The difficulties in avoiding or controlling pro- and anti-complementary effects when attempting to obtain reproducible results with a direct test on unheated chicken sera were partly overcome by Brumfield and Pomeroy (1957). One of the 3 systems studied by these authors was IB. Anti-complementary effects were reduced by separating sera from the clots as soon as possible, and storing them immediately at -20°C. These sera frequently showed strong lytic effects on sheep red blood cells, unsensitised cells being lysed to a dilution of $^1/_4$, and sensitised cells to $^1/_8$. However, this potentially confusing effect was found to disappear in the presence of a specific, complement-fixing reaction between antibody in the serum and antigen. Allowance was made for the anti-complementary effect by selecting the dose of complement on the basis of preliminary titrations carried out in the presence of $^1/_8$ dilutions of 10 unheated avian sera. A considerably higher dose of complement was thereby used than would have been required with a heated mammalian antiserum system. A fixation period of 1 hour at 37°C was employed, and a reaction period of 30 minutes at 37°C. The IB virus antigen was an ultracentrifuge concentrate of allantoic fluid harvest, and was used at 4 units. The authors reported that some batches of antigen were discarded as unsuitable, because their antigen content was too low, or their anti-complementary effect was too high.

There appears to have much conflict of opinion at this time regarding the

interpretation of the confusing behaviour of avian sera in the CF test. Later work by the same group of authors (Brumfield and Pomeroy, 1959; Benson et al, 1961; Brumfield et al, 1961) put forward the concept that fresh, unheated chicken serum contains a factor, essential for the fixation of guinea-pig complement by avian antibody, which is destroyed by heating the serum at 56°C for 30 minutes. In this later work, these authors developed a method of performing a direct test of heat-treated avian sera, in which fixation was at first implemented by the addition of small amounts of unheated normal chicken serum, and later by the addition of a globulin fraction of such serum. The IB system was not included in these studies.

The essential factor in unheated chicken serum was found by these authors to be insoluble in water, and removable by adsorption on kaolin. On the basis of these properties, and its thermal lability, they considered it to be identical with the C_1 component of chicken complement. Their method of preparing the factor for use in the test was to precipitate the globulins from unheated chicken serum, at pH 5.2, and redissolve them in heat-inactivated chicken serum to one third of the original volume. This material was approximately 3 times as effective in implementing fixation as the original unheated serum. Any lytic effect was destroyed during the preparation. The heat-inactivated serum was added because it was found that it prevented the manifestation of a strong anti-complementary effect by the preparation, which made it unusable if the precipitate was redissolved, for example, in saline.

Following the development of the method by these authors, it was applied to the IB system by Jeon (1962), in his Ph.D thesis. Owing to the lack of appearance of this work in accessible literature, and the lack of citation by subsequent workers with the CF test for IB, it was unknown to us at the time of planning these studies. When it eventually came to light, through a citation in a new edition of a textbook, it was too late for it to have any influence on the development of our own method.

The direct test of Brumfield and Pomeroy (1957) was applied by Tsubahara et al (1958) to test unheated chicken sera for antibodies to Japanese encephalitis virus. They used acetone treatment for removing the lytic effect. They found that anti-complementary effects were very marked when long fixation periods were tried, and settled on a period of 1 hour at 37°C.

The modified direct test of Brumfield and Pomeroy (1959) was applied by Rice et al (1960) to the Salmonella system.

A series of papers on the properties of fowl serum (for example, Orlans et al, 1962) described the variety of complex interactions given by mixtures of heated and unheated chicken and guinea-pig sera, as shown by haemolysis of red blood cells of sheep and chicken, and fixation of complement of guinea-pig and chicken. They pointed out that by making certain mixtures it was possible to mimic the effect of an antibody titre, although no known antigen-antibody system was present. This work served as a warning of the dangers of being deceived when working in this rather confusing field, and emphasised the need for self-criticism, especially with regard to the specificity of any reactions observed.

The method of Brumfield et al (1961) was applied by Okazaki et al (1962) to the estimation of antibodies to Rous sarcoma virus in sera of turkeys and chickens. These authors confirmed Brumfield's observation of the value of including some heated normal chicken serum along with the C'_1 preparation, in order to avoid anti-complementary effects, which would otherwise appear as non-specific reactions at the higher dilutions of the antiserum beyond its true end-point. They included the C'_1 fraction in the complement aliquot, as did the previous authors, but disagreed with their observation that the order of adding the reagents was important. The antigen was an ultracentrifuge concentrate. With their virus system, it was found necessary to allow 4 hours at 4°C for fixation, since only traces of fixation were observed after 1 hour at 37°C or 4°C, and only low titres after 2 hours at 4°C. Comparisons with

neutralisation titres tended to confirm the specificity of the titres, but there was a lack of close correlation, especially with the chicken antisera, in which, out of 10 positive sera, only 6 were positive on both tests, 3 being positive on the neutralisation test only, and 1 on the CF test only.

A thesis by Goedbloed (1966) described a direct CF test on unheated chicken serum, using various antigen systems, including IB virus. This work was seen only in a short abstract.

Uppal (1970) applied the method of Brumfield and Pomeroy (1957) to the IB system. This work included titrations of individual bird sera at intervals after experimental inoculation with IB virus, which convincingly demonstrated the specificity of the method. It was selected as the basis for starting work on the CF test, and is described in more detail in the Introduction to Chapter IV.

Chapter II: PREPARATION OF BASIC MATERIALS

INTRODUCTION

The basic materials for these serological studies consisted of a collection of strains of IB virus, and a stock of antisera prepared against them. This chapter describes how these strains were selected, obtained, adapted by passage, and purified by cloning; and how the antisera were prepared, collected, stored and tested. It also described the basic facilities needed for this work; namely, the supplies of suitable chick embryos and chickens, and the provision of isolation units suitable for work with air-borne infections of birds.

MATERIALS AND METHODS

(1) Virus Strains

The virus strains used are listed in Table 2. Some were freshly obtained from original sources. (See Acknowledgements). H120 was obtained from a commercial vaccine vial. Massachusetts-41 was obtained from Lasswade, where it had been maintained by bird passage and used as a challenge strain for several years. The remainder were available from stocks held at Weybridge.

(2) Eggs

SPF ("Specific Pathogen Free") eggs were purchased from commercial suppliers. (Initially from Spafas Inc., Norwich, Connecticut. Later from Pharmazeutisches Werk GMBH & Co., Department Valo, Cuxhaven, W. Germany; and from Dalton Laboratories, Salisbury, Wiltshire). The flocks from which these eggs came were guaranteed by the suppliers to have been free of a range of common pathogens, on the basis of frequest serological tests. The pathogens tested for included: the viruses of Newcastle disease, infectious bronchitis, infectious laryngotracheitis, infectious encephalomyelitis, fowl pox, leucosis, Rous sarcoma and chicken-embryo-lethal-orphan; *Mycoplasma gallisepticum* and

TABLE 2 VIRUS STRAINS USED

Virus Strain	Authors	No. of Egg Passages (Where Known)
Beaudette	Beaudette & Hudson (1937)	>250
Massachusetts-41	Van Roekel et al (1950)	(402 Bird Passes) 4
Connecticut	Jungherr et al (1956)	9
Iowa 97	Hofstad (1958)	
Iowa 609		
Gray	Winterfield & Hitchner (1962)	7
Holte		18
T	Winterfield et al (1964a)	3
SE-17	Hopkins (1969)	7
H120	Hoekstra & Rispens (1960)	120
Houghton 116	McDougall (1971)	
Houghton 140		3
Allen	Asplin (1948)	14
183	Dawson & Gough (1971)	5
225		6
227		8
265		10
317		8
551		10
591		6
604		6
690		10
860		6
918		5
927		6

Salmonella pullorum. Tests for avian influenza virus were carried out irregularly. Some of the flocks were also guaranteed free of Marek's disease virus, but others were recognised to be possibly infected with this pathogen.

In 1972, eggs began to be supplied to us from Lasswade, from a flock known to be free of the same common pathogens.

All passaging of virus strains was carried out in commercial SPF eggs or Lasswade eggs. Some difficulty was experienced in obtaining regular supplies, and poor fertility and hatchability were problems at times. The commercial eggs were also very expensive. Because of these disadvantages, maximum use was made of eggs from the Coombelands flock at Weybridge, for all purposes other than passaging or preparation of standard virus stocks. For example, Coombelands eggs were used for virus titrations, serum neutralisation tests, and the preparation of antigens for the complement fixation test. The supply was very reliable, and convenient in that virtually any number of eggs could be obtained on any day of the week, at the required age of incubation. The flock was known to be infected with IB and avian adenovirus, but nevertheless these eggs were consistently susceptible to the IB virus strains used.

Eggs from the closed flock at Compton, which had been shown to be free of IB, were tried in comparative assays with Coombelands eggs. They showed no advantages over the Coombelands eggs in the height or accuracy of the virus titres

(3) Chickens

Chicks were regularly hatched from a proportion of the commercial SPF and Lasswade eggs, for use in tissue culture and antiserum preparation. The SPF chicks were a White Leghorn type, and the Lasswade were Brown Leghorn. The hatching and rearing of these birds was carried out in premises well away from other chickens.

Initially it was planned to keep the chicks in the hatching premises until two weeks of age, and then to transfer them to the isolation units for rearing and holding before use. This had the advantage of relieving the pressure on space in the hatching premises, and making fuller use of the accommodation in

the isolation building, which was not yet at full stretch because of the early stage of the programme. This system was soon discontinued, because IB occurred spontaneously among the rearing stock. It is very probable that the infection spread from the other side of the building, where experimental infection of chicks with the Massachusetts-41 strain was being carried out. Blockage of the outlet air filter of the infected unit, leading to the development of positive pressure, had been observed to happen on one occasion. Although routines were then introduced to prevent this happening again, the rearing of clean stock in such close proximity to infection was clearly too hazardous.

When reared in the hatching premises, the stock remained free of IB, and in good general health. Later on a mild form of Marek's disease occurred in this stock. A very small proportion of the birds were affected with symptoms that varied in severity between mild leg weakness, and the typical fowl paralysis syndrome. The diagnosis was confirmed by histopathology of the lumbar plexus, isolation and identification of the virus, and gel diffusion tests on sera. Widespread sub-clinical infection was shown by the gel diffusion tests. The disease was eradicated by slaughter of all stock and disinfection of the premises. A routine of fumigation of SPF and Lasswade eggs with formalin on arrival was set up, because it was possible for the virus to be carried in the dust on the egg shells. No further cases occurred.

(4) Isolation Units

One of the four standard isolation buildings used by the Respiratory Diseases Unit of the Poultry Department was made available for this project (Fig. 1). The plan of this building is shown diagrammatically in Fig. 2. The six separately ventilated isolation units each consisted of two or three rooms opening into a short corridor. Each unit had an independent ducting system, operated by its own inlet and outlet motors which were balanced so as to maintain negative pressure in the unit relative to the air outside. The inlet air went through a coarse filter to remove dust and insects. The outlet air went through a similar coarse pre-filter and then a virus-proof filter. The drainage

Fig.1 VIEW OF ISOLATION BUILDING FROM PLANT ROOM END

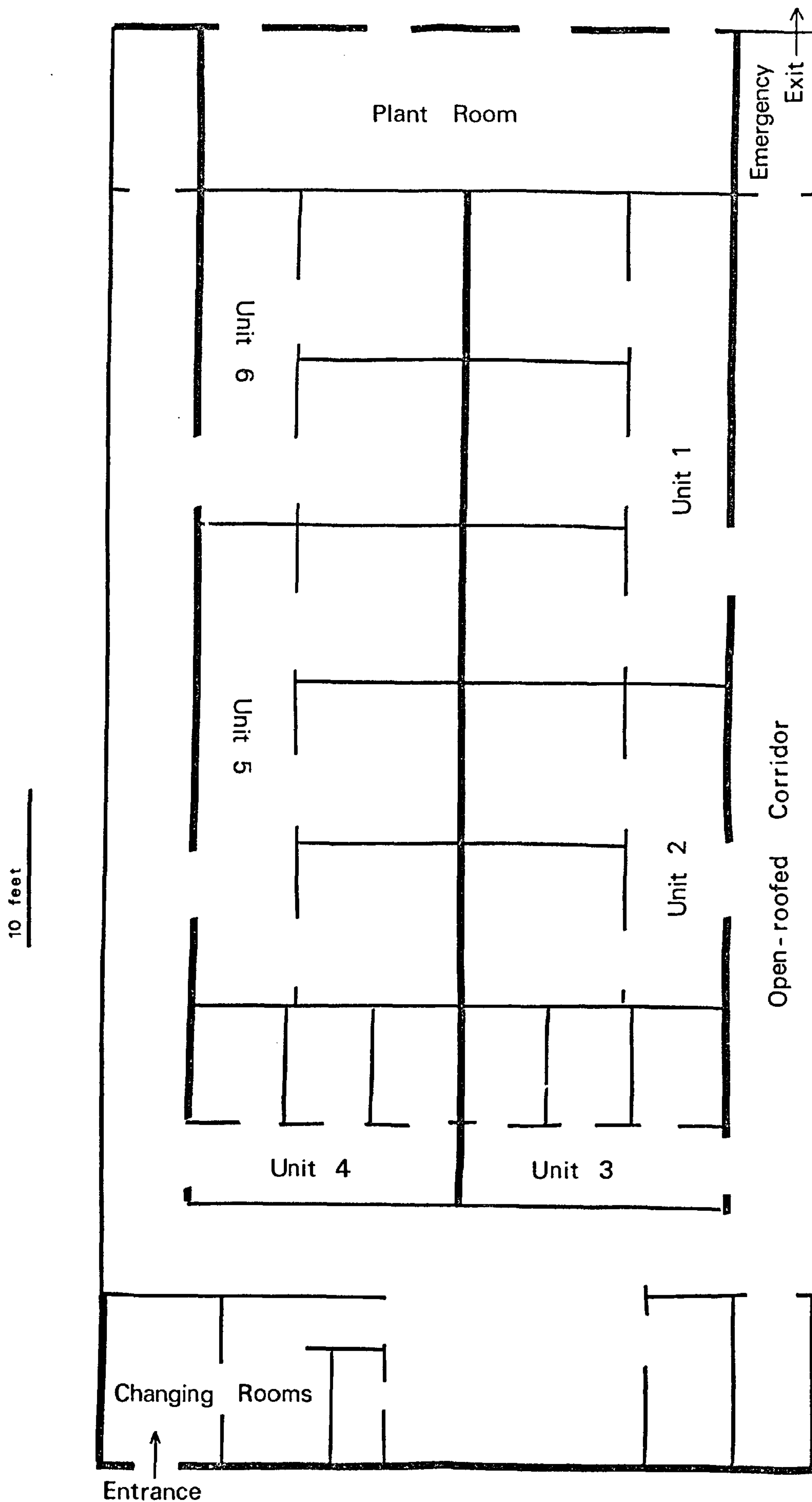


Fig.2 PLAN OF ISOLATION BUILDING

outlets in each room were kept sealed during the course of each experiment in order to prevent air communication between the units via the drains.

Foot-baths and showers were provided at the entrances to each unit, for washing down the waterproof coats, hats and boots that were always worn, and whenever practical separate kits were reserved for each unit.

Steps were taken to minimise the risks of cross-infection between the IB virus strains used. Only one strain was used in each unit, even though this policy meant that usually only one room was in use in each unit. Whenever possible, adjacent rooms were not used at the same time, so that infected rooms were liberally interspersed with clean, empty rooms. The number of birds in each group was kept down to below 12, so reducing the rate of blocking of the pre-filters. Units were well cleaned and fumigated with formaldehyde vapour between experiments. Clean birds were not introduced until the day of inoculation. At the same time a fresh pre-filter was fitted, thus avoiding the possibility of blockage occurring during the first 4 or 5 days after infection, when the rate of virus excretion would be highest.

(5) Virus Work In Eggs

Traditional techniques were employed in the inoculation of the allantoic cavity, "candling" of eggs to observe changes in the embryo, and harvesting of the allantoic fluid. The use of these methods for IB virus has been fully described by many authors (for example Cunningham (1966)) and they have been in constant use at Weybridge since the work of Asplin (1948).

Passaging of virus strains was carried out in one of two ways: at low dilution, for the purpose of adaptation, or at high (limiting) dilution, for the purpose of cloning.

Low dilution passage was performed by inoculation of the allantoic cavity of 5-15 eggs of 9-11 days' incubation with 0.1 or 0.2 ml. of a $^1/_5$ or $^1/_{10}$ dilution of allantoic fluid harvest in nutrient broth containing 200 units per ml. of penicillin and 200 ug. per ml. of streptomycin. ("Antibiotic broth"). Strains that were very slow to adapt were given one or two passages with

undiluted inocula before continuing at a low dilution. Eggs that died within 24 hours of inoculation were discarded as non-specific, then harvests of allantoic fluid were taken from the next group of eggs to die. Usually 3 or 4 eggs were pooled for harvesting. Eggs that died later or survived were discarded.

High dilution passage was performed in a similar manner except that 10 eggs were inoculated with each of 3 or 4 ten-fold dilutions, chosen to cover the titration end-point. A single egg was harvested from the highest dilution to give a positive. Three successive high dilution passages were given, and then the strain was regarded as cloned. Occasionally during these passages a strain was lost. This happened only with strains that were poorly adapted. The difficulty was overcome by returning to the uncloned stocks and carrying out several more low dilution passages before trying high dilution passages again. Passages after cloning were usually limited to one low-dilution passage.

In describing the history of virus strains, cloning passages were signified by the abbreviation $\overline{\text{pp}}$ (standing for "purification passage") followed by the passage number. For example, $\overline{\text{pp2}}$ = the harvest of the second cloning passage. Low dilution passages were abbreviated to E, followed by the passage number. When a strain had been cloned it was described as Clone (1) and the previous history was usually omitted, but subsequent low-dilution passages were added on. For example, Clone (1) E3 = the harvest of the third low-dilution passage after cloning. Names of strains were enclosed in brackets to avoid confusion with passage histories.

Allantoic fluid harvests were clarified by centrifugation at 1,500-2,000 r.p.m. for 15 minutes on an M.S.E. "Minor", and stored at -60°C in a Cliffco cabinet. The storage system consisted of bijou bottles held in aluminium containers with partitioned drawers. Easy location was assisted by a stock card system.

All the operations involved in passaging were carried out in a clean,

quiet room set aside for this purpose. Only one strain was handled in the room at a time. The handling of different strains was separated by as much time as possible, and their harvesting was nearly always performed on different days.

For virus titrations deaths were recorded daily; and on the 7th day after inoculation the eggs were opened and examined for signs of infection. (Stunting and curling of the embryo). End-points were calculated from the total number of positive infections by the Kärber method (Finney, 1964) and expressed as log EID_{50} per ml. Virus titrations always employed a ten-fold dilution series, and 10 eggs were inoculated with each of at least 4 successive dilutions. Antibiotic broth was used as diluent.

Serum neutralisation tests were performed by the alpha (virus dilution constant serum) method when making controls and checks during the preparation of antisera (See Section (6), below), and by the beta (serum dulution constant virus) method when titrating the antisera for comparison with the plaque reduction test. Sera were always treated at 56°C for 30 minutes before test.

For the alpha method, ten-fold dilutions of the test virus were mixed with equal volumes of a $^1/5$ dilution of the serum, and allowed to react for 45 minutes at room temperature (20-25°C). The virus control titration consisted of the same dilutions of virus mixed with diluent only and left under the same conditions. The serum titre was given by the difference between the two titrations, and expressed in log form as the neutralisation index. (N.I.).

For the beta method, two-fold serum dilutions were mixed with equal volumes of a constant virus dilution, calculated from preliminary titrations to provide a standard virus concentration of 2.0 log EID_{50} per 0.1 ml. The mixtures were allowed to react for 45 minutes at room temperature. 5 or 10 eggs were inoculated with each serum dilution, the dose per egg being 0.2 ml. End-points were calculated by the Kärber method, as that initial serum

dilution estimated to prevent lesions in 50% of eggs inoculated. The serum titres were expressed as $\log_2$ reciprocals, to the first decimal place, as for the plaque reduction test. The virus control titration, carried out under the same conditions, was required to show that the standard virus dose used in the test contained not less than 1.5 or more than 2.5 log EID_{50}. Tests in which the dose was shown to be outside this range were regarded as invalid.

(6) Preparation of Antisera

Serum batches were prepared in groups of between 6 and 15 chicks, aged 6 to 14 weeks at the time of inoculation. Before inoculation, a pooled serum sample from the chicks was tested by the alpha method neutralisation test in eggs, using Beaudette as the test virus. The N.I. was required to be less than 1.5, the level recognised to be the limit for non-specific neutralisation. (Cunningham, 1951).

Each bird was given 1 ml. of undiluted allantoic fluid harvest, as a combined intra-nasal and intra-tracheal inoculation. This dose was divided as follows: 0.25 ml. was run into each nostril, to be inhaled into the nasal cavity, and 0.5 ml. was injected into the trachea. The trachea was protected from damage by means of a short length of plastic tubing that covered the needle of the syringe.

The group as a whole was observed for clinical signs during the two weeks after inoculation. Between 3 and 4 weeks, a pooled serum sample was tested against the homologous virus strain by the alpha method. If the N.I. was 3.5 or greater, the titre was considered satisfactory. No further inoculations were given, and the birds were killed for serum collection. Such collections were usually made between 4½ and 7 weeks after inoculation. If the N.I. was less than 3.5, a second inoculation was given, identical to the first, and two weeks later the birds were killed for serum collection.

The birds were killed by administering pentobarbitone intravenously,

then withdrawing blood from the heart. The blood was transferred to polystyrene or glass containers, where clotting occurred. These containers were incubated at 37°C for 3 hours. The serum could be removed then, or after a further 18 hours at room temperature. The yield was greater after the longer period, so this was the method normally used. However, the shorter period was used when the antiserum was required for the complement fixation test, by Uppal's method, which required preservation of certain properties. (See Chapter IV). The serum was poured through muslin to remove the clots, then centrifuged to remove all traces of red blood cells. Storage was at -20°C.

Special inoculation schedules were used for the Beaudette strain and the British strains 317 and 927. For the first inoculation, in addition to the usual 1 ml. dose given intranasally and intratracheally, another 1 ml. was given into the abdomen. These doses were repeated 8 weeks later, and the serum was collected 2 weeks after the second inoculation. Two batches of Beaudette antiserum were prepared in this way, in order to confirm that the surprisingly high titre obtained with the first batch was repeatable.

Intravenous booster doses were used for two batches of antiserum prepared with Massachusetts-41, in an attempt to produce higher titres for the complement fixation test by Uppal's method. (See Chapter IV).

The antiserum for the Allen strain (Batch 10.9.70) was prepared for use in other studies, and the inoculation schedule consisted of an initial exposure by aerosol, followed by 3 intravenous injections.

The studies on the haemagglutination test were made before the programme of antiserum preparation from cloned virus strains had got under way, so use was made of other antisera that were available at the time. These are described in Chapter III.

For the initial work on the complement fixation test, batches of antiserum collected between 2 and 3 weeks after infection were used. These are described in Chapter IV.

TABLE 3 RECORDS OF EGG PASSAGES PERFORMED AND ANTISERA PREPARED

Virus Strain	Pre-cloning Passages (SPF Eggs)	Cloned *	Antisera Prepared		
			Batch No.	No. of Inoculations and Routes ≠	Clinical Signs
Beaudette	0	*	As 25	2 I/N I/T I/P	-
"			As 43	2 I/N I/T I/P	-
Massachusetts-41	1	*	As 32	1 I/N I/T	+
"			As 14	2 I/N I/T I/V	+
"			As 15	2 I/N I/T I/V	+
Connecticut	10	*	As 16	1 I/N I/T	-
Iowa 97	8	*	As 39	1 I/N I/T	-
Iowa 609	9	*	As 26	2 I/N I/T	-
Gray	7	*	As 21	1 I/N I/T	-
Holte	5	*	As 18	1 I/N I/T	-
T	3	*	As 37	1 I/N I/T	+
SE-17	9	*	As 36	1 I/N I/T	-
H120	3		As 27	1 I/N I/T	+
Houghton 116	1		As 29	1 I/N I/T	+
Houghton 140	1		As 28	1 I/N I/T	+
Allen			(10.9.70)	4 Aerosol & I/V	+
183	3	*	As 51	1 I/N I/T	-
225	4	*	As 45	2 I/N I/T	-
227	8	*	As 40	2 I/N I/T	-
265	3	*			
317	11	*	As 42	1 I/N I/T	+
"			As 49	2 I/N I/T I/P	+
551	1	*	As 46	1 I/N I/T	+
591	6	*	As 44	1 I/N I/T	+
604	7	*	As 41	1 I/N I/T	+
690	1	*	As 48	1 I/N I/T	-
860	2	*	As 47	1 I/N I/T	-
918	5	*			
927	2	*	As 22	2 I/N I/T I/P	-
"			As 50	2 I/N I/T I/P	-

≠ Routes: I/N = Intranasal
I/T = Intratracheal
I/P = Intraperitoneal
I/V = Intravenous

RESULTS

(1) Passaging of Virus Strains and Preparation of Antisera

The detailed records of passaging and antiserum preparation are given in Table 3. Cloning was carried out successfully on 9 reference strains and 12 British field strains. Satisfactory antisera were prepared from 19 cloned strains, and from 4 uncloned strains. (The vaccine strain H 120 and the British field strains Allen, Houghton 116, and Houghton 140).

The number of pre-cloning passages can be taken to indicate the degree of difficulty in adapting a strain to eggs to the level at which it could be cloned.

The clinical signs are simply recorded as the presence or absence of respiratory symptoms in the group of birds as a whole.

(2) Serum Neutralisation Tests

A limited number of titrations were carried out on the antisera by the beta method for comparison with the results of the plaque reduction test. These titres are shown in Tables 4 and 5. Table 4 consists of homologous titres, which were required either for direct comparison with the corresponding value obtained by the plaque reduction test, or, where the corresponding value was unobtainable (due to lack of a tissue-culture-adapted homologous virus strain), in order to obtain some measure of the homologous titre, to assist in interpreting the heterologous titres. Table 5 consists of the titres of 6 antisera titrated against two virus strains that are antigenically similar, but very different in their degree of adaptation to eggs: Beaudette and Massachusetts-41. The object was to study how the titres given by a representative range of antisera would be affected by the two variables: assay system and test virus.

These results are discussed in Chapter V.

TABLE 4. HOMOLOGOUS NEUTRALISATION TITRES IN EGGS OF 17 ANTISERA

Virus Strain	Antiserum Batch No.	Serum Titre (Log_2 Reciprocal)
Beaudette	As 43	7.3
Massachusetts-	As 32	7.5
Connecticut	As 16	5.7
Iowa 97	As 39	9.7
Iowa 609	As 26	8.7
Gray	As 21	9.3
Holte	As 18	8.1
T	As 37	10.9
SE-17	As 36	9.5
H120	As 27	6.7
Houghto	As 29	7.8
225	As 45	8.1
227	As 40	8.9
317	As 42	8.3
	As 49	9.3
591	As 44	8.7
604	As 41	8.5

TABLE 5. NEUTRALISATION TITRES IN EGGS OF SIX ANTISERA, TITRATED AGAINST BEAUDETTE AND MASSACHUSETTS-41

Antiserum		Neutralisation Titres (Log_2 Reciprocals) Against Test Viruses:	
Batch No.	Virus Strain	Beaudette	Massachusetts-41
As 43	Beaudette	7.3	4.7
As 32	Massachusetts-41	4.7	7.5
As 37	T	4.3	5.5
As 28	Houghton 140	7.5	6.9
As 40	227	6.3	6.5
As 41	604	3.9	5.7

DISCUSSION

The main aims of the programme of work with virus strains and antisera were achieved. A suitable selection of IB virus strains were obtained, cloned by means of three successive passages at limiting dilution in SPF eggs, and used to prepare antisera in SPF chickens. However, the course of the work, as might be expected, was not without incidents and minor crises, some of which will be reported here, chiefly in the hope that they may be of interest to other workers in this field.

With only one exception, there was no reason to doubt the identity of the virus strains obtained. We did receive one reference strain (not among those listed) which was found to contain Newcastle disease virus, verified by haemagglutination inhibition tests and electron microscopy. This strain was excluded from the studies. The policy was to accept strains to be as they were labelled, and commence work with them immediately, relying on the results obtained in the course of the work to give warning of wrong identity. The contaminated strain referred to was detected at an early stage, suspicions being aroused by its extraordinarily high titre in eggs. 12 strains were examined under the electron microscope at the passage level at which they were used for preparing antisera. Apart from the variations in morphology described by Harkness and Bracewell (1974), the virus particles seen were all typical of IB. The titres of the antisera by the plaque reduction test gave added confidence on the identity of the virus strains. The homologous titres were nearly always the highest obtained, and the behaviour of the reference strains agreed well with what was expected from the literature.

The use of SPF eggs for passaging and laying down stocks of virus strains was expensive, and inconvenient because of the difficulty of obtaining regular supplies with good fertility. However, it was considered to be necessary because of the very real danger of contaminating strains with extraneous agents. Berry and Stokes (1968) found that out of 12 strains

thought to be IB virus, 6 were mixtures of IB virus with another agent. In 5 of these strains the other agent was an adenovirus. Avian adenoviruses have been shown to be particularly insidious and common as contaminants of virus strains passaged in eggs. An example of what can happen is the virus strain B1209 . Originally isolated as infectious laryngotracheitis virus, this strain, as it now exists at Weybridge, is a typical avian adenovirus. Adenoviruses are not only commonly carried in the embryo, but when passaged they may give rise to lesions which are indistinguishable from those given by IB virus, especially the newly isolated field strain. The embryo-lethal effect of a highly egg-adapted IB strain may be simulated by *Mycoplasma gallisepticum*, Newcastle disease virus, infectious laryngotracheitis virus, and avian infectious encephalomyelitis virus, so it is important that these agents should also be excluded. Marek's disease virus is not a danger in this respect, since it is not normally carried in the embryo (Solomon et al, 1970), and even if it did happen to be there it would be most unlikely to cause lesions that could be mistaken for those of IB, or to persist during passaging in competition with an IB strain.

The occurrence of Marek's disease in the SPF chicks intended to be used for antiserum preparation was more serious, because of its possible effects on antibody responses, in addition to the loss of birds from clinical disease. Its eradication was considered to be essential for the continuation of the programme.

The degree of isolation provided by the building in which the chickens were housed for the preparation of antisera was probably inferior to that which might have been provided by flexible or rigid isolators. Although fully accepted at Weybridge now, such isolators were not yet established at the time the work was begun. By the time they became available, most of the programme had already been completed, and it seemed pointless to prepare the last few batches of antiserum in this way. The difficulties in designing a

building to give completely reliable isolation for work with respiratory diseases are enormous. It would be not only unfair but incorrect to criticise the building deeply. Without it the work could not have begun. Provided that the number of birds in the units was kept right down, and the outlet pre-filters were changed or cleaned frequently, the isolation provided was probably good enough for our purposes. We obviously ran into trouble when we infected about 30 chickens simultaneously with Massachusetts-41 in Unit 1, while attempting to keep clean SPF stock in Units 5 and 6. (See Fig. 1). The large amount of dust released into the air by these birds in Unit 1 blocked the outlet pre-filters on one occasion and created positive pressure in this unit. It is possible that virus was then forced out into the air outside the building, and from there was taken into the inlets of the other units. The inlet air is not taken through a virus-proof filter, so there would have been no exclusion of virus when this happened.

The causes of variation in the results of the serum neutralisation test in eggs are manifold. During the comparative assays performed in eggs from Compton and Coombelands, it was observed that 2 out of the 6 virus strains titrated differed very markedly in their lethality for the embryos from the two flocks, although their final titres were not very different. These two virus strains were much more lethal for the Coombelands eggs. They also happened to be the two Weybridge strains, which had a history of passage in Coombelands eggs. The deduction was that the performance of a virus strain in eggs may be affected by changes in the source of the eggs used. The few comparative serum neutralisation tests carried out did not show any marked differences, but only homologous reactions were studied, and it is possible that heterologous reactions would have been more affected.

The possible effect of the embryo lethality of the test virus on serum neutralisation titres will be further discussed in Chapter V.

Many of the virus strains failed to give clinical signs when used for

antiserum preparation. This suggests that loss of virulence can occur after relatively few passages in eggs, for many virus strains. However, this does not happen with some other virus strains. For example, the live vaccine strain H120 gave very marked respiratory symptoms, in spite of its large number of egg passages. When used as a vaccine, the virus dose would of course be much lower than that used in this experiment.

SUMMARY

10 Reference strains and 15 British strains of IB virus were selected for serological studies. 9 of the reference strains and 12 of the British strains were cloned by means of 3 successive passages at limiting dilution in SPF embryos. Antisera were prepared, from all but 2 of the strains, by inoculation of chickens hatched from SPF eggs, reared in premises well away from other chickens, and maintained after inoculation in isolation units. Batches of antiserum were collected when a good antibody response against the homologous virus strain had been shown to have occurred, by means of a neutralisation test in embryos. For the majority of batches a single, combined intra-nasal/intratracheal inoculation was sufficient, and for the others 2 such inoculations were used. Intra-abdominal inoculations were used for 3 virus strains of unusually low immunogenicity.

A small number of neutralisation tests in embryos were carried out on the antisera, for comparison with plaque reduction tests, and for additional information on batches for which homologous plaque reduction test titres were not available.

Chapter III: INDIRECT HAEMAGGLUTINATION TEST

INTRODUCTION

The reasons for selecting the indirect haemagglutination test for study have been given in Chapter I. The difficulty in reproducing the reactions consistently, reported by Szedo and Romvary (1967), was taken as a warning that all techniques should be particularly precise and well controlled. It was decided to begin by attempting to reproduce the first method described for IB, that of Brown et al (1962), as closely as possible. Only when it was certain that the same kind of positive reactions were being obtained would any modifications be tried.

MATERIALS AND METHODS

Red Blood Cells

Regular weekly bleedings were made from one of two horses. The blood was allowed to flow from an open needle inserted in the jugular vein straight into 100 ml. of Alsever's solution (Appendix 1), and approximately 100 ml. of blood was collected. After a period of 1 to 5 days at 4°C, 80 ml. of the mixture was centrifuged, and the deposited cells were washed 3 times in normal saline, and stored as packed cells at 4°C.

On the day of the test, a 2.5% suspension was prepared by mixing 0.25 ml. of packed cells with 10 ml. of PBS at pH 7.1. The packed cells were also used for absorption treatment of the chicken antisera to be tested and the rabbit serum used in the diluent.

Phosphate-Buffered Saline (PBS)

PBS of 2 different pH values was required : pH 6.4 and pH 7.1. These were prepared on the day of the test by adding 10 ml. of 0.2 M phosphate buffer of the required pH to 190 ml. of sterile normal saline. The 0.2 M phosphate buffers were prepared by mixing the required proportions of stock 0.2 M

solutions of mono- and di-hydrogen phosphates. All these solutions were stored at 4°C.

Rabbit Serum

20 ml. of blood was collected from the marginal ear vein of the same normal rabbit, on 4 occasions at intervals of 1 to 3 weeks. The serum was stored at 4°C. Some time before the day of the test, it was treated at 56°C for 30 minutes, and later mixed with an equal volume of packed horse red cells, retained at room temperature for 30 minutes, and then separated from the cells by centrifugation.

Test Diluent

The test diluent consisted of 0.5% rabbit serum in PBS pH 7.1, made up on the day of the test.

Tannic Acid

BP grade tannic acid was used, at 1/20,000 in normal saline. On the day of the test, 0.1 gramme of the tannic acid powder was dissolved in 20 ml. of sterile normal saline, and 1 ml. of this was added to 100 ml. of sterile normal saline.

Antigens

2 Allantoic fluid harvests of the Beaudette strain (E7 of 14.10.68 and E10 of 19.3.70) were used successfully as antigens. 6 other materials were used unsuccessfully. The details of these will be given under Results.

Antisera

Because these studies were carried out in early 1970, at a time when our own programme of serum preparation from cloned virus strains had not yet got under way, use had to be made of some large batches of chicken antiserum prepared against the British field strain Allen and the Massachusetts -41 reference strain. These were hyperimmune sera, selected for pooling on their high neutralisation titres, and intended primarily for fluorescent antibody development work. The batches were described as : Allen batch C, Allen batch B, and M-41 batch I-L. The sera were prepared in chickens from

Coombelands farm, and therefore not SPF.

Equipment

76 x 9 m.m. Glass tubes were used initially, following Brown's method. Once positive reactions had been obtained, the test was transferred to standard "WHO" perspex haemagglutination trays with 8 x 10 wells, because of easier handling and clearer readings.

The serum dilutions were carried out by means of 1 ml. graduated pipettes, in separate sets of bijou bottles or tubes.

Tanning of Red Blood Cells

The treatment of the red blood cells with tannic acid was carried out on the day of the test, using freshly prepared tannic acid. 10 ml. of 2.5% red cells in PBS pH 7.1 was mixed with 10 ml. of the tannic acid, and incubated at 37°C in a water bath for 10 minutes. The cells were then centrifuged and resuspended twice in normal saline, the final resuspension being in a volume of 10 ml. The final concentration of red cells was therefore 2.5%.

Sensitisation Of Tanned Cells

2 ml. of antigen (usually undiluted allantoic fluid harvest) was mixed with 2 ml. of sterile normal saline, and this was mixed with 16 ml. of PBS pH 6.4, followed by 4 ml. of tanned cells. The mixture was left at room temperature for 15 minutes. It was then centrifuged, the supernate was discarded, and the cells were resuspended in test diluent. This process was repeated twice more, the final suspension being in 20 ml. of diluent, making the final concentration of sensitised cells 0.5%. This was kept at 4°C until required, which was always within 3 hours.

Controls

Every titration was performed in duplicate (i.e. 2 rows of wells on the tray). Every serum being titrated with sensitised cells was also titrated in parallel with tanned cells. In several tests it was also titrated with cells treated with normal allantoic fluid instead of virus-infected allantoic fluid.

The tanned cells for use as control were washed and resuspended at 0.5% in test diluent.

Autohaemagglutination controls were set up, consisting of at least 4 wells of : (1) sensitised cells + diluent instead of serum dilution; (2) tanned cells + diluent instead of serum dilution.

Test Procedure

The heat-inactivation and absorption with horse red cells of the rabbit and chicken sera were carried out before the day of the test. During the morning the various reagents described above were prepared ready for use and held at 4°C. Early in the afternoon, doubling dilutions of the antisera were made in tubes or bijou bottles, and the selected dilutions were transferred to the perspex trays in volumes of 0.3 ml. Similar volumes of diluent were used for the controls omitting antiserum. Then 0.3 ml. of sensitised cells was added to each well, except for the control wells where tanned cells were substituted. The final volume in each well was 0.6 ml. The reagents were mixed by side-to-side agitation of the trays, and then the trays were placed at 4°C, on a vibration-free shelf of the cold room, where they were left overnight.

The next morning, the trays were removed from the cold room and placed over a white background for reading. The wells were scored as +, - or ± by the degree of spread shown by the deposited red cells. The reading was completed within 1 hour of removal from the cold room.

Use of "WHO" Perspex Trays

As soon as good positive reactions had been obtained in tubes, the test was transferred to perspex trays, maintaining the same procedure and volumes of reagents. The reactions were wider in the trays and easier to read, and the trays were easier to handle than the tubes. The ability of tubes to be centrifuged, was found to be no advantage because centrifugation prevented the development of positive reactions, so could not be used.

The trays were cleaned by immersion in 4% sodium hydroxide for 30 minutes, followed by a rinse under the tap and then immersion in 2% hydrochloric acid for 30 minutes; and finally rinsed in distilled water.

Use Of Sheep Cells

Brown claimed that sheep cells could also be used successfully in the test, although usually giving lower titres than horse cells. Sheep cells were tried in a few tests. The specific titre appeared to be the same as with horse cells, but the negatives were less clear, and non-specific titres were higher. It was decided to continue using horse cells.

Inhibition Test

Specific inhibition of haemagglutination by the presence of an excess of free antigen, was not investigated by Brown et al (1962) or Vasington et al (1963), but was described by Mohanty et al (1964). The latter authors reported successful demonstration of specific inhibition by a method in which serial two-fold dilutions of partially purified and concentrated virus of the Beaudette strain was added in 0.15 ml. volumes to 0.15 ml. volumes of antiserum at a dilution calculated to contain 8 haemagglutinating units. After 30 minutes at room temperature, 0.3 ml. of the sensitised cell suspension was added.

The inhibition test method used by us was similar in broad principle to that described by Mohanty et al (1964). However, the volume of antiserum was 0.3 ml., and the antigen was 0.1 ml. Unconcentrated allantoic fluid harvests were used as antigens. There was no deliberate delay before adding the sensitised cells. Because of lack of precision in the titre of the antiserum, the dilution selected for the test was the lowest dilution that just avoided non-specific haemagglutination as shown by the tanned cell control, instead of 8 units, which was too difficult to define.

Control antigens used included normal allantoic fluid and an allantoic fluid harvest of the avian adenovirus CELO (Phelps). Further tests to

establish the specificity of the reactions included the use of cells sensitised with this CELO material.

A considerable variety of IB and adenovirus materials, including some chicken kidney cell culture harvests, were tested undiluted as inhibiting antigens, in order to establish the specificity by checking the identity of a wide range of different preparations.

Concentration And Purification Of Antigens

An ultracentrifuge was not available at the time this work was carried out. Concentration by precipitation with 50% saturated ammonium sulphate was carried out as follows:

100 ml. of pooled allantoic fluid harvest was put into each of 4 x 250 ml. centrifuge pots. To each pot was added a mixture of 106 ml. of saturated ammonium sulphate and 6 ml. of N/10 sodium hydroxide, this being the amount needed to bring the pH to 7.4. All liquids were at 4°C. After 2 hours at 4°C, a floccular precipitate had formed. This was centrifuged at 2,200 r.p.m. for 30 minutes on an M.S.E. "Major", and the precipitate then resuspended in 20 ml. of PBS pH 7.4. After centrifugation of this material, the supernate was retained, and dialysed against 2 litres of the same buffer, for 42 hours at 4°C. The volume was only very slightly increased.

9 ml. of this concentrate was used for purification by successive shakings with equal volumes of Arcton 113. The shaking consisted of 2 minutes of vigorous hand shaking, carried out at 4°C. This was followed by centrifugation, also at 4°C, and removal of the upper (aqueous) phase for the next treatment. The treatments continued until the opacity disappeared from the solvent phase.

A similar purification by Arcton treatment was carried out on an unconcentrated allantoic fluid harvest. The opacity in the solvent disappeared more quickly than with the concentrate, so that only 3 treatments were necessary. The treatment was shown to have had little or no effect on the

infectivity of this preparation, by means of titrations in egg-embryos of the material before and after treatment.

These methods of concentration and purification were based on routine methods for foot-and-mouth disease virus used at the Animal Virus Research Institute, Pirbright. (Brown and Cartwright, 1960).

Expression of Dilutions and Titres of Serum and Antigen

Since two-fold dilutions were always used for the titration of sera and antigens, it was decided to express their dilutions as negative powers of 2, and their titres as $\log_2$ reciprocals. For example, an end-point at a dilution of 1/4 was expressed as a dilution of 2^{-2}, or a titre of 2.

In order to avoid confusion, only the initial dilution of the reagent was considered, and the dilutions inherent in the test itself, caused by the addition of other reagents, were ignored.

RESULTS

Positive reactions, of the kind described by Brown et al (1962), were obtained when the serum batch "C", prepared against the Allen strain, was titrated with an allantoic fluid harvest of Beaudette strain as the sensitising antigen. The titre ranged between 6 and 11, and usually exceeded by at least 3 dilutions the titres given by the controls. These consisted of:

(1) Dilutions of the antiserum with tanned, but non-sensitised cells.

(2) Dilutions of antiserum with cells sensitised with normal allantoic fluid instead of virus harvest.

(3) Dilutions of a known IB-negative chicken serum with cells sensitised with Beaudette allantoic fluid harvest.

The titrations did not give clear end-points. Their effect was graded, so that the eye was not drawn to any particular regions of marked change which could be easily selected for reading the titres. Positive patterns were formed by a weak adhesion of the red blood cells to the surface of the wall, which was easily disturbed or lost. For example, when the trays were taken out of the cold room for reading on the laboratory bench, it was necessary to read them immediately, because if they were left out for about 1 hour the cells would often slip down, making the readings even more difficult. If the trays were left to settle overnight at room temperature, instead of in the cold room, no positive readings were obtained. There was never any really strong clumping of the cells, such as is observed with strongly haemagglutinating viruses like Newcastle disease and influenza. No definite signs of clumping were seen when the cells from positive wells were examined microscopically.

Seeking confirmation of the specificity of the positive readings that were being made, the same batch of Allen antiserum was titrated with cells sensitised with CELO virus allantoic fluid harvest. This batch of serum had been shown to be negative on the neutralisation test in eggs against CELO virus.

Surprisingly, it gave positive readings on the indirect haemagglutination test against CELO virus, and moreover the titre was closely similar to that shown against IB (Beaudette).

In order to clarify this unexpected and confusing situation, a series of inhibition tests were performed. IB and CELO virus harvests were used as inhibiting antigens, which were titrated in doubling dilutions, in presence of a constant dilution of the Allen antiserum (selected to be just beyond the level of non-specific reactions), and cells sensitised with either IB or CELO virus harvests. Remarkably, the results of these inhibition tests were very clear and conclusive: the reaction with IB-sensitised cells was inhibited by IB but not by CELO: and the reaction with CELO-sensitised cells was inhibited by CELO but not by IB. The inhibiting effects of the harvests could be diluted out, the IB having a titre of 3 ($^1/_8$) and the CELO having a titre of 5 ($^1/_{32}$).

Further inhibition tests were performed, in which 10 different virus harvests, 5 of which were known to be IB and 5 known to be avian adenovirus (CELO or GAL), were tested, undiluted, for their ability to inhibit the reactions given by the Allen antiserum with both IB- and CELO-sensitised cells. Again the results were remarkably clear: IB harvests would inhibit IB reactions but not CELO; and vice versa.

Following these encouraging demonstrations of the specificity of the method, the studies were extended, firstly to the use of a variety of virus materials as sensitising antigens, still titrating the same Allen batch C antiserum, and secondly to titrating 2 other known IB antisera. The results are shown in Table 6. Satisfactory positive reactions could be obtained with only a few of the combinations. The harvests concentrated by ammonium sulphate and/or purified by Arcton 113 were not successful as antigens.

TABLE 6. TANNIC ACID HAEMAGGLUTINATION TEST: REACTIONS OBTAINED WITH VARIOUS ANTISERA AND ANTIGENS

Antiserum		Sensitising Antigen	Reaction Obtained
Batch	Neutralisation Titres in Eggs		
Allen Batch C	Against IB (Allen) = 9	IB (Beaudette) E7 of 14.10.68	+
		CELO (Phelps) E4 of 10.11.69	+
		IB (Beaudette) E10 of 19.3.70	+
	Against CELO = 0	IB (Beaudette) E6 of 10.6.70	-
		IB (Allen) E11 of 2.5.69	-
		IB (Beaudette) E8 CK2 of 11.3.70	-
		IB (Allen) E13 of 6.3.70: Dialysed Ammonium Sulphate Concentrate	-
		IB (Allen) E13 of 6.3.70: Dialysed Ammonium Sulphate Concentrate Treated 9 Times with Arcton 113:	-
		IB (Beaudette) E10 of 19.3.70, Treated 3 Times with Arcton 113:	-
Allen Batch B	Against IB (Allen) = >7	IB (Beaudette) E6 of 10.6.70	-
		IB (927) E7 of 2.6.70	-
M-41 Batch I-L	Against IB (M-41) = 10 Against IB (Beaudette) = 7	IB (Beaudette) E7 of 14.10.68	-

DISCUSSION

The serum titres which were given by the Allen batch C antiserum against horse red blood cells sensitised by Beaudette allantoic fluid harvests were similar to those reported for IB antisera by Brown et al (1962), Vasington et al (1963), and Mohanty et al (1964). Their specificity was confirmed by inhibition tests, as described by Mohanty et al (1964), and a full range of controls, including titrations of normal chicken serum, and the use of cells "sensitised" with normal allantoic fluid. Therefore, the method described in this literature must be assumed to have been successfully reproduced.

Difficulty in reproducing the reactions consistently with a variety of antigens and antisera was reported by Szedo and Romvary (1967), and was also encountered in these studies. It seems likely that this inconsistency explains why the method has not been widely adopted for routine use, in spite of the great need for an alternative serological test for IB.

In order to be of use in studying antigenic relationships, it was necessary that the method should be applicable to a wide range of materials. To discover the reasons for its inconsistencies was therefore an essential first step. It was suspected that the antigen required concentration and purification. An ultracentrifuge was not available, so concentration with ammonium sulphate and purification by Arcton 113 was carried out. When these methods also failed, it was decided to suspend work on the indirect haemagglutination test, in order to allow studies to commence on the complement fixation test. The plan was that if the CF test should prove equally unpromising, by the time that that had been established an ultracentrifuge would be available, and also the large stocks of antisera that were planned would have been prepared, and so it would then be possible to examine the indirect haemagglutination test more thoroughly. In the event, it proved more rewarding to continue work on the CF test.

Kaye et al (1972) reported success with an indirect (tannic acid) test for

human coronavirus 229E, one of the strains for which a direct test is not available. The antigen was prepared from growth in the RU-1 strain of diploid human foetal lung fibroblasts. At an early stage of CPE, most of the medium was discarded, and the monolayer was removed from the glass by freezing and thawing and this material was then given 3 further cycles of freezing and thawing, then it was centrifuged, and the supernatant was stored at -70°C. The test appears to have detected higher and more persistent titres than the CF test, and also was highly specific in that it gave no reaction with antisera against OC43, another human coronavirus strain.

This work suggests than an antigen of very high titre is necessary for application of this method to coronaviruses, and that lack of a good antigen may have been the main reason for the difficulties found with IB virus. The high strain-specificity of the test may also have contributed to the difficulties, for example the lack of a reaction between Beaudette antigen and Massachusetts-41 antiserum.

Greig et al (1971) reported direct haemagglutination by the coronavirus of pigs associated with encephalomyelitis, but that associated with transmissible gastro-enteritis appears to be completely non-haemagglutinating in untreated harvests, like IB virus.

Some time after the completion of these studies, Bingham et al (1975) succeeded in obtaining direct haemagglutination of red blood cells of various species by the Connecticut strain of IB virus after sucrose gradient purification, and by the Massachusetts-41 strain after sucrose gradient purification followed by treatment with phospholipase C. The Beaudette strain could not be made to haemagglutinate in these ways. The reaction was inhibited by known IB antisera, but the titre of the serum varied greatly according to the virus strain and type of preparation used, and the species of red blood cell. Treatments with trypsin, neuraminidase and bromelain were found to destroy the haemagglutinin.

This report was followed by studies on haemagglutination inhibition by the stock antisera prepared during these studies, and by individual bird sera collected at intervals after experimental inoculation, by Alexander et al (1976). These authors found that certain IB virus strains would haemagglutinate chick cells after a simple ultracentrifuge concentration followed by treatment with phospholipase C. Only Massachusetts-41, Connecticut, Holte and H120 have so far been found to haemagglutinate by this method. The titres of antisera against Massachusetts-41 correlated reasonably well with those given by the neutralisation test in eggs and the plaque reduction test in chicken kidney cells, but the peak antibody response to inoculation occurred earlier, at 2 weeks post-infection. This could indicate that the method is more sensitive for IgM, which was found by Gillette (1974) to give a peak at this time, than for IgG.

SUMMARY

The indirect haemagglutination test using tanned horse red blood cells was reproduced as described by Brown et al (1962). Specific titres were given by one batch of IB antiserum when certain batches of Beaudette allantoic fluid harvests were used as sensitising antigens. The specificity of the reactions was confirmed by inhibition tests, as described by Mohanty et al (1964). Difficulty was encountered in reproducing the reactions consistently with a variety of antigens and antisera. The end-points were not clear, and the haemagglutination patterns were very unstable and appeared to be formed by weak adhesion of the cells to the surface of the well. The method was not consistent enough for it to be applied to studies of antigenic relationships, or to diagnosis.

Chapter IV: COMPLEMENT FIXATION TEST

INTRODUCTION

The introduction to this chapter consists of an account of the preliminary studies and the reasoning which led to the selection of the method described in the body of the chapter.

The decision to concentrate on a direct test on chicken sera has been explained in Chapter I. There remained the choice of whether to work with untreated sera, along the lines described by Uppal (1970), or whether to heat-inactivate the sera and attempt to implement fixation by the addition of fresh chicken serum factors, following the methods described for other avian antibody systems by the workers at Minnesota (Brumfield and Pomeroy, 1959; Benson et al, 1961; Brumfield et al, 1961) and by Tsubahara et al (1958), Rice et al (1960) and Okazaki et al (1962). At the time of planning the work, the paper by Uppal (1970), and the earlier work by Brumfield and Pomeroy (1957) on untreated sera, were the only descriptions available to us of a direct test for IB on chicken sera. It was therefore logical to try this method first.

(1) Preliminary Studies

Before commencing studies on Uppal's method, a few preliminary studies were made on the effects of untreated chicken serum on sheep red blood cells and guinea-pig complement, omitting IB antigen or antibody altogether. These studies helped to familiarise us with some of the pecularities of chicken serum in the CF test, and confirmed the findings of Bushnell and Hudson (1927) and Rice (1947). Untreated chicken serum was found to be capable of exerting both pro- and anti-complementary effects. Which effect predominated in a particular test depended very largely on the timing of the addition of the reagents. The longer the time of contact with complement before addition of sensitised red cells, the greater the anti-complementary effect.

The pro-complementary effect was a lytic effect on sensitised red cells, independent of the presence of guinea-pig complement, and it was lessened by delays before adding the cells.

(2) The Method of Uppal (1970)

Uppal's method, like that of Brumfield and Pomeroy (1957), depended on overcoming the anti-complementary effect of untreated chicken serum by increasing the dose of guinea-pig complement. This dose was determined by a preliminary titration of guinea-pig complement in the presence of a $^{1}/_{8}$ dilution of the chicken serum. The fixation period was 3 hours at $4^{\circ}C$, followed by 30 minutes at $37^{\circ}C$, this having been found to be the best compromise for achieving specific fixation without the intrusion of serious anti-complementary effects.

The first studies were based on the use of a freshly collected batch of serum taken 15 days after infection of SPF chicks with M-41. A doubt arose immediately over the determination of the correct dose of complement, because its titre was found to vary with the dilution of the chicken serum. However, when we pressed on with the method as described, basing the dose of complement on the titre obtained with a $^{1}/_{8}$ dilution of the chicken serum, and titrating the serum against undiluted Beaudette allantoic fluid harvest, an encouraging result was obtained. Complete fixation was obtained up to a dilution of $^{1}/_{16}$ with the Beaudette harvest, while a CELO harvest and normal allantoic fluid gave no fixation.

When the studies were extended to a variety of sera, some very freshly collected and other stored at $4^{\circ}C$ or $-20^{\circ}C$ for varying periods, it was not possible to determine a dose of complement that would be correct for all. Also, after 2 months' storage at $-20^{\circ}C$, the batch used for the first studies had become more strongly anti-complementary, and required a large adjustment of the dose of complement.

Further difficulties in determining the dose of complement arose when

strong pro-complementary effects were encountered in some very freshly collected sera. This effect could be quite strong at the $^1/_8$ dilution used for the preliminary titration of the complement, preventing the reading of the complement titre.

It was concluded that Uppal's method was not suitable for our purposes, which required the ability to titrate accurately sera collected over a fairly long period of time.

(3) Early Studies On Inactivated Sera

Because of the difficulty of standardising the dose of complement involved in performing tests on unheated sera, it was decided to investigate the possibility of performing tests for IB on heat-inactivated sera, along the lines described by Brumfield and Pomeroy (1959) for the ornithosis system.

The effects of treatment at 56°C for 30 minutes on the properties of chicken serum concerned in the CF test were studied. These properties were analysed into: (1) the lytic, or pro-complementary effect; (2) The anti-complementary effect; (3) Specific antibody to IB; (4) The factor necessary for fixation of guinea-pig complement by chicken antibody-antigen reactions. The effects were as follows:

(1) The lytic effect was completely lost.

(2) The anti-complementary effect was completely lost.

(3) Specific antibody to IB was unaffected.

(4) The essential "implementing" factor was completely lost.

When IB antisera had been inactivated in this way, they lost their ability to react in the CF test, but fixation could then be achieved by adding small, known amounts of unheated normal chicken serum. (UNCS). Whatever pro-and anti-complementary effects were then observed were due entirely to the unheated serum added. Thus it was confirmed that Brumfield and Pomeroy's principle would apply to the IB system. This method clearly

presented a better means of standardising the dose of guinea-pig complement, because when the correct dose for a batch of UNCS had been determined, a large number and variety of serum samples could be tested without the need to adjust the dose. The selection of batches of UNCS having no lytic effect, and very low anti-complementary effect, was found to be feasible.

(4) Studies On The Lytic Effect

During these early studies, it was of interest to examine briefly the lytic effect of unheated chicken serum in relation to the implementing factor. The Minnesota workers regarded the implementing factor as avian C_1. If this were correct, it would perhaps be logical to expect that the lytic effect and the implementing factor would always be present together, and that loss of one would imply loss of both. However, in our batches of UNCS that were selected as suitable for providing implementing factor, the lytic effect had been lost. Was it therefore possible that the two properties were independent of each other?

In order to answer this question, tests were carried out on a batch of IB antiserum that had been freshly collected, and had a strong lytic effect. A test was performed in which the serum was titrated both with and without IB antigen, and also without the addition of guinea-pig complement. In the absence of guinea-pig complement, the serum caused complete lysis up to a dilution of $^1/_8$, and 50% lysis at $^1/_{16}$. When guinea-pig complement was present, but antigen absent, complete lysis occurred at all dilutions, but when both guinea-pig complement and antigen were present, complete fixation occurred up to $^1/_{32}$. It was concluded that the lytic effect of the chicken serum had been fixed simultaneously with the guinea-pig complement by the IB antibody-antigen reaction.

From these results, it seemed possible that the CF test might work without the addition of guinea-pig complement, by utilizing the complement present in

freshly collected chicken serum. Thus it might be possible, for example, to identify virus strains as IB by simply testing them against a freshly collected IB antiserum, used, perhaps, at a single, selected dilution such as $^1/_8$. To test this possibility, a variety of allantoic fluid harvests were tested undiluted against an IB antiserum at $^1/_8$, both with and without the addition of guinea-pig complement. The result was that fixation was obtained, to a fairly high degree, with all the harvests, including normal allantoic fluid and CELO virus harvests, when guinea-pig complement was omitted. In the presence of guinea-pig complement, fixation was obtained only with Beaudette harvest. It was concluded that the fixation of chicken complement was too non-specific to be relied on.

(5) The Method Of Marquardt And Newman (1971)

While these early studies on the use of inactivated sera were proceeding, Marquardt and Newman (1971) described a CF test for Mycoplasma, based on the principles of Brumfield and Pomeroy (1959). In their method, the reaction was implemented by the addition of UNCS, which was stored at 4°C. Attractive features of the method were that the UNCS was included in the complement aliquot, reducing the number of operations in the test, and the fixation period was only 1 hour at 37°C, which allowed two tests to be performed on the same day. The test described was performed in tubes.

When applied to the IB system, this method was found to work better than that of Uppal (1970), in that the standardisation of the complement dose was much easier, the test was more convenient to perform, and the serum titres obtained were significantly higher. It became the basis of the method finally adopted. Certain modifications were made. These included those described below under adaptation to the "Microtiter" system, and the methods of testing batches of UNCS and titrating complement described in Materials and Methods. Methods of preparing and testing antigens had of course to be developed for the IB system, and these are also described in Materials and Methods.

(6) Adaptation Of The Test To The "Microtiter" System

Parallel tests were carried out in tubes and on "Microtiter" plates, in which the dilutions prepared for the plates were in duplicate, one set being prepared on the plates by means of microdiluters, and the other set being aliquots of the dilutions prepared in tubes. Identical titres were obtained by the different methods. The only unsatisfactory feature of the "Microtiter" system was that the red blood cells tended to settle in spread-out patterns, which interfered somewhat with the readings. This effect, which was thought to be due to the haemagglutinin content of the rabbit anti-sheep haemolytic serum used for sensitising the cells, did not cause difficulty in the tube test, because the tubes were centrifuged immediately on completion of the reaction period, throwing the cells down into a button.

This slight difficulty was overcome by shaking the plates continuously throughout the reaction period, as described in Materials and Methods. This procedure was also found to improve the clarity of end points, by aiding the completion of haemolysis. Intermittent shaking was less satisfactory.

Haemagglutination effects were reduced by keeping the plates at a higher temperature during settling, such as 37°C. Settling was also more rapid at this temperature, enabling earlier reading. However, pro-zones and incomplete fixation effects were slightly increased at 37°C, and on balance room temperature gave more satisfactory results.

MATERIALS AND METHODS

(1) Basic Reagents

Diluent

The diluent used was barbitone-buffered saline, prepared as described in Appendix 3. Early studies had shown this to give identical results to the standard "veronal-buffered saline". (Appendix 2.)

Sheep Red Blood Cells

Sheep blood was collected into Alsever's Solution (Appendix 1), and stored for 1 week at 4°C, before the cells were removed by centrifugation with discarding of the supernatant liquid, and washed by 3 cycles of re-suspension in diluent alternating with centrifugation and discarding of supernatant liquid. The final centrifugation was carried out in graduated conical tubes at a standard speed and time. After reading the volume of packed cells, the supernatant fluid was discarded, and the whole quantity of packed cells in the tube was suspended in a volume of diluent calculated to result in a 3% suspension.

Haemolysin

Rabbit haemolytic antiserum to sheep red blood cells was obtained from Wellcome Reagents Ltd. Its titre was found to be stable over many months' storage at 4°C. On the day of the test, a small volume was withdrawn from the vial and diluted $^1/_{600}$ in diluent.

This dilution for use in the test was selected from the results of several block (2-dimensional) titrations of haemolysin with complement. For these block titrations, varying dilutions of haemolysin were used for sensitising the sheep red blood cells, and the complement aliquot consisted of varying dilutions of guinea-pig complement in diluent containing UNCS at a constant dilution of $^1/_{16}$. Apart from these 2 variables, the method used resembled the standard procedure described below in Section (7). Inspection of the results of these titrations gave a dilution of $^1/_{800}$ for the optimal sensitising concentration of the haemolysin. That is, the complement titre was seen to be maximal at all dilutions of haemolysin up to $^1/_{800}$, but to fall progressively at higher dilutions. It was also observed that $^1/_{800}$ haemolysin sometimes just failed to give complete haemolysis when the complement dilution was exactly that used as the standard dose in the test, namely 2 CH_{50}. (See Section (2) below.) A dilution of $^1/_{600}$ haemolysin, however,

never failed to give complete haemolysis with 2 CH_{50}, so, since it was considered near enough to the optimal sensitising concentration to make no difference, $^{1}/_{600}$ was selected as the standard dilution for use.

(2) Complement

Source Of Complement

Freeze-dried, preserved guinea-pig complement was obtained from Wellcome Reagents Ltd. Within each batch, the titre was found to be constant from vial to vial, and to remain constant up to at least 6 months' storage in the freeze-dried state at 4°C. By securing a large stock of vials of one particular batch, it was therefore possible to reduce considerably the number of preliminary titrations of complement that were needed.

However, not all batches were found satisfactory for use in the avian CF test. Some batches combined strongly with UNCS, to produce the effect of total suppression of complement throughout the test. This effect occurred with all batches of UNCS, and far exceeded in strength the variable anti-complementary effects shown by most batches of UNCS when freshly collected. (See Section (3) below.) This problem was overcome simply by avoiding the use of such unsuitable batches of complement, and laying down a large stock of a suitable batch.

Reconstitution And Dilution Of Complement

Vials of complement were reconstituted by dissolving the contents in sterile distilled water, as recommended by the supplier. In this form the complement was stored at 4°C, and could be used for the next 7 days without loss of activity. This material consisted, in effect, of guinea-pig serum plus Richardson's preservative. For this reason, it was slightly hypertonic. In order to prepare an isotonic solution for use in the test, the supplier's instructions were followed, and a $^{1}/_{8}$ dilution was made in distilled water, on the day of the test. This was referred to as "$^{1}/_{10}$ complement", and all further dilutions were made in diluent.

Titration of Complement

Complement was titrated both alone and in combination with UNCS, following the standard test procedure described in Section (7) below. Initially, various forms of dilution series were tried. The most convenient was found to be a 0.1 $\log_{10}$ series, prepared as follows:

First, the lowest dilution to be tested (often $^1/_{50}$) was prepared from the $^1/_{10}$ complement. Then, 3.85 ml. of this dilution was added to the first of a series of tubes containing 1 ml. of diluent. The contents of the first tube were mixed, and 3.85 ml. transferred to the next tube, and a series of such transfers was made. The resulting dilutions were regarded as: $^1/_{63}$, $^1/_{80}$, $^1/_{100}$, $^1/_{125}$, $^1/_{160}$, $^1/_{200}$, $^1/_{250}$, $^1/_{320}$, $^1/_{400}$, $^1/_{500}$. These figures were taken from antilog tables. This particular series had certain practical advantages, although it would not be correct to suggest that it is necessarily the best possible. It was found to be easily memorised. Being a geometric progression, each step produced an equivalent effect on the activity of the complement. Because there is a doubling on every third dilution, once the dilution giving 50% haemolysis had been recognised, it was only necessary to inspect the reading in the third tube below to see exactly what reading would be expected with the dose of 2 CH_{50} in the test. This check was necessary to ensure that the anticomplementary effect of the UNCS was not too strong.

Complement titrations were perfromed in the presence of a standard dose of antigen when new batches of antigen were checked for their freedom from anticomplementary effect.

The titre of complement was read as that dilution of the complement alone giving exactly 50% haemolysis. This corresponded to a reading of "2" on the notation used:

"4" = No haemolysis

"3" = 25% haemolysis

"2" = 50% haemolysis

"1" = 75% haemolysis

"tr" = Almost 100% haemolysis

"0" = 100% haemolysis.

Test Dose Of Complement

The standard test dose was selected to be twice the concentration giving 50% haemolysis (2 CH_{50}). The routine for preparing complement for the test proper was to first make up a suitable volume at 4 CH_{50}, and then, immediately before adding it to the plates, to mix this with an equal volume of $^1/_8$ UNCS.

Studies on the effect of varying the dose of complement showed that the margin within which the test would work satisfactorily was small, and that the width of the margin depended mainly on the quality of the UNCS in regard to freedom fron anticomplementary effect. A full range of readings was possible only with the highest quality of UNCS. Since most batches of UNCS were found to have some degree of anticomplementary effect, it was, in practice, often necessary to tolerate less than complete haemolysis in those areas of the test where complete haemolysis was desired, such as the antigen controls and the upper ranges of serum dilutions beyond the end-points. Readings of "tr" or "1" had to be allowed in these areas, provided that the effect was uniform throughout the test, and did not interfere with the recognition of end-points. The use of a higher dose of complement, such as 2.5 CH_{50}, could convert such readings into "0", but only at the cost of undesirable reductions in serum titres, or the appearance of incomplete fixation in those areas of the test where complete fixation was desired.

(3) Unheated Normal Chicken Serum (UNCS)

Preparation And Storage Of UNCS

To prepare a batch of UNCS, blood was collected at death from a group of 6 to 10 SPF chicks, of similar age within the range of 6 to 12 weeks old. The method of collection was as described for antiserum in Chapter II, Section (6). After natural coagulation and retraction of the clot, the serum was separated at the earliest opportunity, usually within 5 hours of collection of the blood. Serum that could not be separated quickly and cleanly, or contained any discolouration or marked opacity, was discarded. Birds of 6-12 weeks of age usually gave very clear serum, provided that food was withheld for 18 hours before the collection. The yield of serum varied between 40 and 120 ml. It was pooled and stored in a 4-ounce or 6-ounce medical flat bottle, at $4^{o}C$.

Within a few hours of being cooled to $4^{o}C$, the serum became opaque, but this change was ignored. Over the next 2 or 3 weeks of storage, the opacity slowly cleared, as a thick deposit appeared adhering to the bottom and sides of the bottle. The cleared serum was then decanted into a clean bottle and left at $4^{o}C$ for a few more days. If it remained clear, testing of the batch would begin. If it went opaque again, it was left for another week and then decanted again.

Sterile glassware was used for these operations. No problem of bacterial or fungal contamination of UNCS was experienced, so it was considered unnecessary to add a preservative at any stage, although merthiolate was used for this purpose by Marquardt and Newman (1971), apparently without any deleterious effects.

Batches of UNCS were prepared at intervals of 4-8 weeks. Because of the very small quantities needed for the kind of development work on which we were engaged, only the very best batches were selected for use in the test. One particularly good batch was in continuous use for 4 months. There was no

deterioration of this batch up to this time; in fact there was an improvement in its "quality" as shown by its anticomplementary effect declining until it disappeared altogether.

Testing of UNCS

Each batch of UNCS was tested for anticomplementary effect by comparing the titre of complement alone and combined with the UNCS at a final dilution of $^1/_{16}$ UNCS in the complement aliquot. To be satisfactory for use, the UNCS was required not to depress the titre of complement by more than 2 dilutions, and also not to cause readings greater than "1" when 2 CH_{50} of complement was present. Initially, other methods of testing UNCS for anti-complementary effect were tried. These involved titration of the UNCS with complement constant at 2 CH_{50}. It was found to be very difficult to obtain end-points, with which to measure anticomplementary effect, by these other methods.

If testing was begun soon after collection of a batch of UNCS, say within 3 weeks, it was usual to find a fairly high anticomplementary effect, shown by depression of complement titre by 4 or 5 dilutions. However, the effect usually declined during further storage at 4°C.

An unusual anticomplementary effect was observed after 6 months' storage at 4°C, in the particularly good batch of UNCS mentioned above as having steadily improved in quality during the first 4 months of storage. This effect appeared suddenly and was very strong. It appeared to differ in character from the effect seen during early storage, in that it could be inhibited totally by the presence of any small amount of heat-inactivated chicken serum. The dilutions of the test serum were effective in this inhibition, even at very high dilutions, and this led to some very intriguing patterns of readings. The batch was discarded at this stage, although it might have been possible to continue to use it, with the addition of a small amount of heat-inactivated normal chicken serum. It was

interesting to note the resemblance between this situation and the "C_1" preparation of Brumfield et al (1961).

The anticomplementary effect of freshly collected UNCS was found to increase during storage at -20°C, but to remain unchanged at -60°C. It is therefore logical to recommend -60°C for long term storage of UNCS, once it has passed its tests. These studies were not prolonged enough for the need for long term storage to have arisen.

The anticomplementary effect of UNCS was found to depend, to some extent, on the time of contact with complement. For example, the effect could be greatly increased by the use of a prolonged period of fixation, such as 18 hours at 4°C. Because of this, these two reagents were always kept apart until the last possible moment, in other words when the whole test was ready for addition of the complement aliquot.

(4) Antigens

Preparation of antigen from the Beaudette strain

After 9 or 10 days' incubation, 20 to 30 eggs were inoculated with 0.1 ml. of a $^1/_{100}$ dilution in antibiotic broth of Beaudette stock virus. 42 hours later, the allantoic fluid was collected from the dead eggs, pooled, and stored at 4°C. The live eggs were chilled at this time by placing them at 4°C, and their allantoic fluids were harvested 5 or 6 hours later. All the harvests were then pooled, and held at 4°C for 18 hours. The fluid was then clarified by centrifugation, aliquotted in 7 ml. volumes in universal container bottles, and stored at -60°C. Great care was taken during collection of allantoic fluid to avoid contamination with blood, and as a result these antigens usually appeared almost colourless and transparent.

Preparation of antigen from strains other than Beaudette

Ultracentrifugation followed by resuspension of the pellet in diluent at a reduced volume was rarely satisfactory, due to concentration of the anti-complementary properties present in the original harvest. However, one

satisfactory batch of Massachusetts-41 antigen was prepared in this way, the concentration factor required being X20.

A more satisfactory method was ultracentrifugation through a layer of 22% potassium tartrate, (prepared as in Appendix 4) with the object of removing from the harvest those particles of smaller size or lower density than the virus. The procedure was as follows:

Into each of three 25 ml. polypropylene centrifuge tubes was placed 6 ml. of 22% potassium tartrate. To each tube, 15 ml. of the harvest was added, running it in very slowly, without disturbing the tartrate below, so as to form a layer. The tubes were then balanced exactly for weight by adding harvest to the lighter tubes, using a syringe and needle. They were then centrifuged at 25,000 r.p.m. for 1 hour in the 3 x 25 ml. swing-out head of an M.S.E. Superspeed 40 centrifuge, with temperature set at 5°C. The supernates were then discarded, and the deposits were taken up into 1 ml. of diluent and resuspended, using a Pasteur pipette. This stock antigen was stored at -60°C.

Testing of batches of antigen

New batches of antigen were titrated with an excess of antiserum of the same virus strain, or the same type if this was known. When the titre of the antiserum was completely unknown, it was used arbitrarily at a dilution of $^1/_8$. Knowledge of the titres of such antisera had shown that $^1/_8$ would usually provide a comfortable excess, while being high enough to avoid low-dilution effects such as pro-zones and non-specific fixation. Simultaneously, the antigen was also titrated without the presence of the antiserum, in order to measure its anti-complementary properties.

These titrations usually commenced with undiluted stock antigen in the first well, and proceeded with doubling dilutions thereafter. The second component added to each well was the antiserum, at constant dilution. In the anti-complementary control titration, the place of the antiserum was taken by

diluent.

The titre of the antigen was read as the highest dilution to give a reading greater than "2" (i.e. "3" or "4"). The batch was useable at 2 dilutions below the titre, or, in other words, at 4 units of antigen, provided that the corresponding reading at this dilution in the anti-complementary control titration was not greater than "1", and preferably not greater than "tr".

(5) Antisera

The antisera prepared in SPF chickens by inoculation with the reference virus strains, described in Chapter II, were used for many of the studies on the development of the method and on the effects of antigenic differences. Samples from individual chickens, collected at intervals after infection with the Houghton 140 and T strains, were used for studies on the time of the antibody response. Individual and pooled samples from the field were used to confirm the applicability of the test for detection of field infection. Studies on the use of the test for assessing IB vaccination in the field employed individual samples collected during a vaccine trial. For demonstrating the specificity of the method, standard chicken antisera prepared in SPF birds against viruses other than IB were obtained from within the Poultry Department.

Collection and storage of antisera

The methods of collection and storage of the reference antisera have been described in Chapter II. In general, no particular precautions or unusual procedures were used for antisera. Unlike the procedure for the UNCS, in which speed was regarded as important, sufficient time was allowed for full separation from the clot, usually by leaving samples overnight on the laboratory bench. Storage was at -20°C, in the fresh (non-inactivated) state.

Inactivation of antisera

It was one of the essential features of the method that all antisera were treated at 56°C for 30 minutes before being tested. The effect of omitting this inactivation was to allow the appearance of a bewildering assortment of both pro- and anti-complementary effects, making interpretation of results impossible. Inefficient heat-treatment, such as occurred on one occasion due to leaving a group of samples in the water bath while still held tightly together by rubber bands, resulted in pro-zones, presumably caused by preservation of the lytic effect of fresh chicken serum at the lower dilutions.

When a series of tests was planned on the same serum samples, as for instance on the positive and negative control sera, it was found to be a better practice to store the bulk of the material in the fresh state, and immediately before each test to inactivate a small aliquot. This is because it was found that antisera stored at -20°C after inactivation underwent a slow deterioration, shown as a decline in specific titres and the appearance of non-specific (anti-complementary) titres. This effect was not observed after short periods of storage, such as 1 or 2 weeks, but was observed after a few months. Thus it was permissible to use the same inactivated aliquots of control sera throughout a series of tests if they extended for no more than about 2 weeks.

(6) Apparatus

Standard "Microtiter" equipment, manufactured by Cooke Engineering, U.S.A. and supplied by Flow Laboratories, Irvine, Scotland, was used. The use of this technique, which involves the addition of reagents by standard-sized drops and the making of simultaneous sets of dilutions by means of "microdiluter" loops, is now well recognised for the complement fixation test. The general methods are described by Lenette (1969). The use of the method for studies of antigenic relationships between virus strains was described by

Darbyshire et al (1972). Disposable plastic trays in sterile packs, containing 8 rows of 12 U-bottom wells, were used. Transparent, adhesive plastic film for sealing the trays, a shaker, and a mirror for reading the trays from below, were supplied as standard equipment.

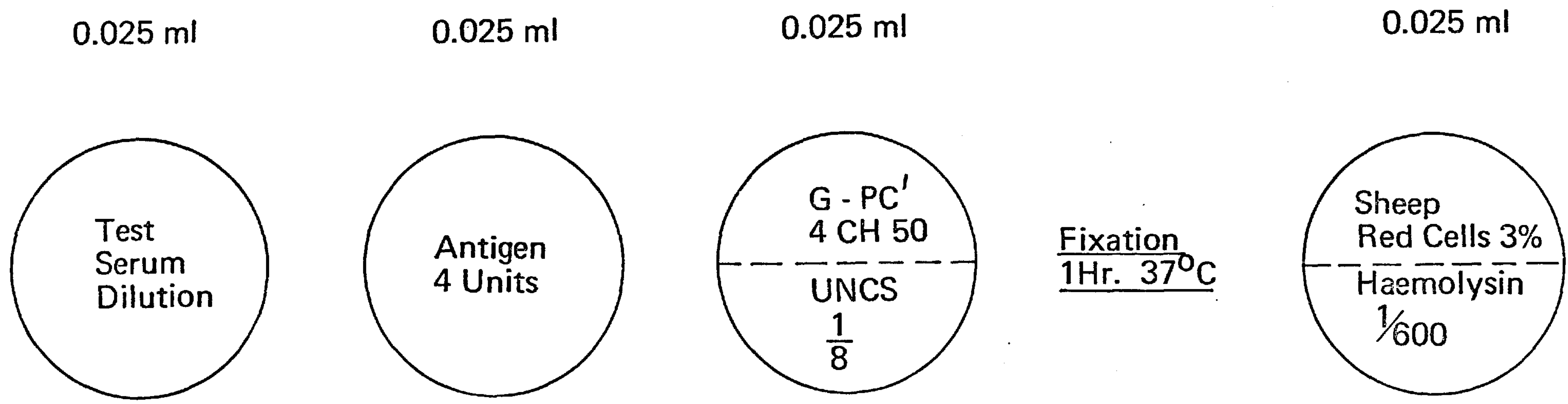
A hot-room running at 37°C was required for the stages of fixation and reaction. The shaker was employed inside the hot-room for agitating the trays during the reaction period. Water baths set at 37°C and 56°C were used for the sensitisation of the red blood cells and the heat-inactivation of sera, respectively. Graduated, conical, glass centrifuge tubes (size 5 ml.) were needed for measuring the packed red blood cells, and an M.S.E. "Minor" centrifuge with an appropriate swing-out head was used for this operation.

(7) Procedure

The final volume in each well was kept constant at 0.1 ml., using additional diluent to make up the volume in the control wells where particular reagents were omitted. The droppers and microdiluters used gave volumes of 0.025 ml. The addition of the components to a well in a serum titration is represented diagrammatically in Fig. 3. The procedure was as follows:-

1. 0.025 ml. of diluent was added to every well to be used in the test, including all control wells.
2. By means of the microdiluters, doubling dilution series were formed of the sera or antigens to be titrated.
3. To each well in a serum titration was added 0.025 ml. of antigen, containing 4 units. To each well in an antigen titration was added 0.025 ml. of the selected constant dilution of the antiserum. To each control well was added 0.025 ml. of either antigen, antiserum, or diluent only, depending on which reagents were being controlled.

Fig. 3 COMPLEMENT FIXATION TEST METHOD



Reaction 35 mins. 37 °C With Vibration

4. The complement was prepared, by mixing equal volumes of $^1/_8$ UNCS and the selected dilution of guinea-pig complement.

   The components required for the complement controls were prepared as follows:

   Complement Control 1. : guinea-pig complement only. A mixture of equal volumes of diluent and the selected dilution of guinea-pig complement.

   Complement Control 2. : UNCS only. A mixture of equal volumes of diluent and $^1/_8$ UNCS.

5. 0.025 ml. of complement was added to the wells involved in titrations of serum and antigen, to the 2 Antigen Control wells, and to the 2 wells composing Complement Control 3. Complement Controls 1 and 2, consisting of 2 wells each, received 0.025 ml. of the appropriate mixtures. 0.025 ml. of diluent was added in lieu of complement to the 2 wells destined to be the Cell Control.

6. The trays were stacked in a small pile in order to reduce evaporation, given a brief shake to mix the components, and left in the 37°C hot room for the Fixation Period of 1 hour.

7. The sensitisation of the cells was concurrent with the fixation period. A mixture of equal volumes of 3% sheep red blood cells and $^1/_{600}$ haemolysin was incubated in a water bath at 37°C for 1 hour.

8. To every well was added 0.025 ml. of the sensitised cells.

9. The trays were sealed, and attached to the shaker for continuous vibration throughout the Reaction Period of 35 minutes.

10. The trays were then left on a stationary, level surface at room temperature, for settling of cells to take place.

11. The trays were read from below by means of the mirror, 2½-3 hours after the end of the reaction period.

12. The readings of each well were recorded on standard, photo-printed work sheets. Titres, interpretations of controls, and conclusions, were written down after all the readings had been recorded.

(8) Interpretation Of Readings

Sera and antigens were titrated, for normal routine purposes, by means of a single row of wells per sample. Their titres were taken to be the highest dilution giving less than 50% haemolysis, i.e. a reading of "3" or "4".

2 Wells were used for each of the controls, as a check on technique. These pairs almost invariably gave identical readings. For the test to be valid, the following results were required:

Control	Acceptable Range Of Readings
Antigen	0, tr, 1
Complement Control (1)	0
Complement Control (2)	4
Complement Control (3)	0, tr, 1
Red Blood Cells	4

Control readings outside these ranges were interpreted as follows:

Red blood cells	< 4	: Cells autolysing. Batch of cells may be too old, or damaged. Diluent may be incorrectly prepared.
Complement Control (1)	> 0	: Inadequate dose of guinea-pig complement. Very rarely due to failure to sensitise cells.
Complement Control (2)	< 4	: UNCS possesses lytic property. (Assuming red blood cell control is satisfactory.)
Complement Control (3)	> 1	: Anti-complementary effect of UNCS is too high.
Antigen	> 1	: Antigen is too anti-complementary, or else is combining non-specifically with the UNCS in such a way as to fix complement.

It was expected that any wells above the end-points of serum titrations would have very similar readings to the antigen controls.

There was little need to use a serum control when titrating unknown antigens against a constant dilution of a known antiserum, because at the dilutions used ($^1/_8$ or over) anti-complementary effects of sera were always negligible, usually absent entirely. Practically the only things that could cause an anti-complementary effect in the serum would be gross negligence in either its storage or its heat treatment.

A standard antiserum was always included in any test involving titrations of unknown sera. Frequently a known negative serum was also included. Similarly, a known antigen was included along with titrations of unknown antigens. The titres of standard antisera were required to be not more than 2 dilutions different from the expected titre. Since the standard antigens were usually unconcentrated Beaudette harvests, with titres of only $^1/_4$ or $^1/_8$, a smaller permissible variation was necessary, and the titre was required to be not more than 1 dilution different from that expected.

The titres of both sera and antigens were expressed as $\log_2$ reciprocals.

(9) Experimental Treatments Applied To Certain Antigen Preparations

In order to obtain knowledge of the properties of the antigen, with the object of preparing antigens of higher potency and lower anti-cpmplementary effect, various methods of treatment were applied, as brief experiments. Several of these methods were simple, routine, physical treatments such as ultracentrifugation, heating and lyophilisation. Treatment with Arcton-113 (the fluorocarbon solvent manufactured by Imperial Chemical Industries) was carried out as described by Brown and Cartwright (1960) for foot-and-mouth disease virus.

Allantoic fluid harvests of the Beaudette and Massachusetts-41 strains were supplied to Dr. Reeve, who performed various physical and chemical treatments on them, and returned samples to us, to be titrated as antigens.

These treatments were the same used by Dr. Reeve and his co-workers at Hammersmith Hospital in their studies on Newcastle disease virus. They included: ultracentrifugation through sucrose density gradients; ultrasonics; concentration by precipitation with ammonium sulphate; treatments with 1% sodium desoxycholate, sodium dodecyl sulphate, trypsin and nonidat. The fractions from the sucrose density gradients were examined under the electron microscope; and their protein contents, and, for one gradient, their infectivity titres in embryonated eggs, were determined.

It was as a result of these collaborative studies that the methods of preparing the antigen were standardised as described in Section (4) above. The results also provided some knowledge of the nature of the antigen. They are described further in the Discussion.

RESULTS

(1) The Results Of Repeated Titrations Of A Standard Antiserum

The Beaudette antiserum batch As25 was included as a positive control serum in 15 separate tests over a period of 3 months. Its titre ranged from 5 to 8, with a mean of 6.6, standard deviation 0.828, and coefficient of variation 12.5%.

(2) The Effect Of Ultracentrifugation On The Antigen

A Beaudette allantoic fluid harvest with a very low anti-complementary effect (antigen control gave a reading of "0") was ultracentrifuged through 22% potassium tartrate, as described in Materials and Methods. The concentrated antigen and a sample of the original harvest were titrated in the same test. The titres were:

Original harvest = 2

Concentrate = 7

Thus, a concentration factor of X32 was obtained. This agreed very closely with the reduction in volume, indicating that no significant loss of antigen had occurred.

In order to control the anti-complementary effect, each dilution was also tested without the presence of the antiserum. The anti-complementary effect of the concentrate was very low, being just apparent when undiluted, as a reading of "tr".

Similar concentrates were prepared from 2 harvests of the T strain. One harvest was taken before any of the embryos had died, and the other was taken at the earliest opportunity after death. The titres were:

Material	Specific Titre	Anti-Complementary Titre
Concentrate of early harvest	2	< 1
Concentrate of late harvest	3	1

The concentrate of early harvest was suitable for use undiluted, since it provided 4 units with a negligible anti-complementary effect. The concentrate of late harvest was less satisfactory because of its higher anti-complementary effect. (At the $\frac{1}{2}$ dilution necessary to give 4 units, the anticomplementary effect was giving a reading of "2".)

A Massachusetts-41 allantoic fluid harvest with a very low anti-complementary effect was concentrated 20-fold by straightforward ultracentrifugation and resuspension of the pellet in diluent. This material gave a titre of 3, and was useable as antigen at a $\frac{1}{2}$ dilution. This harvest was however an exception. As a rule, harvests of strains other than Beaudette were too anti-complementary when concentrated by straightforward ultracentrifugation. In contrast, Beaudette harvests were easily concentrated by this method, and because of their high titre could be used at dilutions well beyond any anti-complementary effect. There was however, little motive for doing this, since Beaudette harvests were normally useable as unconcentrated harvests.

Allantoic fluid harvests of Gray and Iowa 609 were concentrated by

ultracentrifugation through 22% potassium tartrate. These concentrates were not suitable for use as antigens, because their titres were too low, and masked by anti-complementary effect. Several batches of concentrate prepared from the T strain by the same method were unsuitable for the same reasons.

(3) The Effect Of Heat On The Antigen

Samples of a Beaudette allantoic fluid harvest were treated at 56°C for 30 minutes, and brought to the boil for a few seconds. The titres were:

Material	Titre
Original harvest	2
Harvest treated 56°C	2
Boiled harvest	1

The anti-complementary effect was unchanged. The harvest was chosen for this study because it had an unusually high anti-complementary effect for a Beaudette harvest, so the effect of heat on this property could be observed.

(4) The Effect Of Arcton-113 On The Antigen

An unusually anti-complementary Beaudette allantoic fluid harvest was given 3 successive treatments with Arcton-113, as described by Brown and Cartwright (1960) for foot-and-mouth disease virus. The specific titre was unchanged after 1 or 2 treatments, and only slightly reduced after 3 treatments. The anti-complementary effect was unchanged.

(5) The Effects Of Conditions Of Storage On The Antigen

The standard method of storage was in a -60°C refrigerator. Under these conditions there was no loss of titre or increase of anti-complementary effect in either allantoic fluid harvests, or ultracentrifuge concentrates, throughout the period of these studies.

Beaudette allantoic fluid harvests were tested after 3 months at 4°C, and after 14 months in the lyophilised state. Both were found to be suitable for use as antigens. The harvest stored at 4°C had a somewhat higher anti-complementary effect.

(6) The Observation Of Variations In Strength Of Fixation

When the dose of complement and the other test variables were correctly balanced, positive control sera always gave complete fixation, as shown by readings of "4", at all dilutions below their end-point, when titrated with an antigen of the same strain or type of virus. Incomplete fixation, as shown by readings of "3" or less, was sometimes observed in wells in which it was logical to expect full fixation. Repeats of these tests showed that these effects were reproducible. Their mechanism and causes were not understood, but after some experience of them, it was possible to generalise to some extent about the conditions under which they were seen.

Firstly, the effects were found in serum samples from certain individual birds, and were more commonly found in birds that reacted to infection less strongly than the average. They tended to occur more often in the early or late stages of the response than in the middle of the response. In Table 7, the serum titrations of samples from 2 birds are compared. The samples were taken at intervals after experimental infection with Houghton 140. Incomplete fixation was clearly observed in the tests on bird no. 3, during both the early and late stages of its antibody response, but was far less obvious in those on bird no. 4. Similar comparisons could be made between other birds.

Secondly, the effects were found among the standard antisera, particularly those collected after a single inoculation, and especially when titrated against a virus strain of a different type. Antisera collected after a second inoculation generally gave complete fixation, although their homologous titres were not necessarily higher in general than those collected after one inoculation.

TABLE 7. Comparison of CF Test Readings to Show Variation in Strength of Fixation.
Serum Titrations on Samples from Two Birds After Experimental Infection.

BIRD No. 3.

Weeks Post-Infection	Serum Dilutions 1	2	3	4	5	6	7	8	9	10	11	12
2	3	2	1	1	2	2	2	2	1	1	1	1
$2\frac{1}{2}$	4	3	2	2	3	3	3	2	1	tr	tr	tr
3	4	4	3	3	3	3	3	2	1	tr	tr	tr
4	/	/	4	4	4	4	3	1	tr	tr	tr	tr
5	/	/	2	4	4	4	3	1	1	tr	tr	tr
6	4	4	4	4	4	4	4	4	4	3	1	1
7	4	4	4	4	4	4	4	2	tr	0	0	0
8	4	4	4	4	4	4	3	2	2	1	tr	tr
9	4	3	3	2	2	2	2	1	1	1	1	1
11	4	3	tr	tr	1	3	4	4	3	3	2	1
15	4	4	3	2	1	2	2	2	2	1	1	1

BIRD No. 4.

Weeks Post-Infection	Serum Dilutions 1	2	3	4	5	6	7	8	9	10	11	12
2	3	3	4	4	4	4	4	4	3	3	2	2
$2\frac{1}{2}$	4	4	4	4	4	4	4	4	4	3	2	1
3	4	4	4	4	4	4	4	4	4	4	3	2
4	/	/	3	4	4	4	4	3	1	tr	tr	tr
5	/	/	4	4	4	4	4	4	3	1	tr	tr
6	4	4	4	4	4	4	4	4	4	4	3	2
7	4	4	4	4	4	4	4	4	4	3	tr	1
8	4	4	4	4	4	4	4	4	4	4	4	4
9	4	4	4	4	4	4	4	4	4	2	2	1
11	4	4	4	4	4	4	4	4	4	4	4	4
15	4	4	4	4	4	4	4	4	3	3	3	2

(7) Studies On The Antibody Response To Experimental Infection

The antibody responses of individual birds were measured before, and at intervals after, their inoculation with two strains of IB virus thought to differ in clinical effects and antigenically: Houghton 140 and T. The objects were: to confirm the specificity of the test, and to acquire knowledge of the times of appearance, reaching peak titre, and persistence of antibody in individual birds. Some of the serum samples were also titrated by the plaque reduction test.

The Houghton 140 strain was selected as a British field strain capable of causing marked respiratory symptoms, and already known to stimulate high CF test tires against Beaudette antigen. 8 White Leghorn-type SPF chicks of age 6 weeks were inoculated intratracheally and intranasally with a total of 1 ml. each of undiluted allantoic fluid harvest. Serum samples were taken up to 15 weeks post-inoculation. Their titres are shown in Table 8.

The T strain was selected as an exotic strain thought to differ antigenically from the Massachusetts-like strains (Winterfield et al, 1964 a), and associated with a nephritis syndrome in the country of origin. 8 Brown Leghorn Lasswade chicks of age 6 weeks were inoculated in a similar manner to those that received Houghton 140. One bird died at 13 days post-inoculation, and was never sampled. Another died at 14 weeks, 2 more at 15 weeks, and another at 15½ weeks. Serum sampling of the surviving birds continued up to 19 weeks. Because of the difficulty of preparing adequate quantities of T strain antigen, only the 14- and 24- day samples were tested against this strain. Tests against Beaudette were carried out on the samples taken at 14, 24, 40 and 89 days post-inoculation. The titres are shown in Table 9. The effect of the antigenic difference between the Beaudette and T strains is shown in the lower titres obtained with Beaudette antigen. The deaths which occurred in this experiment are discussed in the Discussion section of Chapter V.

TABLE 8. Serum Titres, by Complement Fixation Test Against Beaudette Antigen, of 8 Individual Chicks, at Intervals After Infection with Houghton 140

Bird No.	Weeks Post-Infection														
	0	½	1	1½	2	2½	3	4	5	6	7	8	9	11	15
1	1	<1	1	1	6	8	8	7	6	8	6	8	8	2	2
2	1	1	1	<1	<1	2	7	2	1	1	2	3	3	1	2
3	1	<1	<1	<1	1	7	7	7	7	10	7	7	3	10	3
4	1	1	1	<1	8	9	10	8	9	11	10	>12	9	>12	11
5	1	1	1	<1	<1	6	8	6	2	2	3	6	7	<1	2
6	1	1	1	<1	11	11	11	10	11	12	11	>12	>12	>12	>12
7	1	<1	<1	<1	5	7	8	6	7	9	9	9	8	6	1
8	1	<1	1	<1	<1	7	8	6	5	4	1	3	2	<1	<1

TABLE 9. Serum Titres, by Complement Fixation Test Against T and Beaudette Antigens, of 8 Individual Chicks, at Intervals After Infection with T.

Bird No.	Test Antigen T		Test Antigen Beaudette			
	Days Post-Infection:		Days Post-Infection			
	14	24	14	24	40	89
1	3	4	<1	1	2	1
2	≥4	5	<1	1	3	1
3	≥4	7	<1	2	3	1
4	≥4	7	<1	2	2	1
5	≥4	5	<1	1	2	<1
6	≥4	<5	1	<1	2	<1
7*	-	-	-	-	-	-
8	4	5	<1	1	2	1

* This chick died at 13 days post-infection.

(8) The Effects Of Antigenic Difference

In Table 10, the standard antisera to Beaudette, Massachusetts-41 and Connecticut are shown titrated against each of these three strains. The classical antigenic difference between Connecticut and the other 2 strains was clearly shown. However, the Beaudette antiserum was much more effective than the Massachusetts-41 in reacting with Connecticut antigen.

In Table 11, antisera to the strains T and Allen are shown titrated against antigens of the T strain and the strains Beaudette and Massachusetts-41. The 2 latter strains had been shown to be of the same type as the Allen strain on the basis of neutralisation tests. By the complement fixation test, reductions in titre were obtained when the T antiserum was titrated against Beaudette, and when the Allen antiserum was titrated against T. However, there was little reduction when T antiserum was titrated against Massachusetts-41.

These inconsistencies could not be explained. The difficulties of interpretation were increased further by the phenomenon of incomplete fixation, which, as described in Section (6) above, was often observed in cross-type reactions. With some combinations of antiserum and antigen, the titre was very variable, seeming to depend on minute, uncontrolled variations in the test conditions which affected the delicate balance of the reaction. For example, 5 titrations of the Gray antiserum As21 against Beaudette gave titres of: 5, 3, 3, 2 and 4. The last 2 results were obtained in the same test, by a small variation in the dose of complement. In another example, the T antiserum As37 gave a titre of 2 in 2 tests against Beaudette, but while in one test complete fixation was obtained at the 2 lowest dilutions, in the other test no well gave complete fixation, and readings of "2" extended right up to the sixth well. The homologous titre of this serum was 5, and so this series of incomplete reactions obtained in the heterologous reaction could be regarded in that particular test as a weaker reflection of the homologous reaction.

TABLE 10. The Effect of Antigenic Differences on Complement Fixation Test Titres: (1) Cross-Fixation Tests between 3 Virus Strains.

Antisera		Test Antigens		
Batch No.	Virus Strain	Beaudette	Massachusetts-41	Connecticut
As 25	Beaudette	7	6	4
As 32	Massachusetts-41	7	7	1
As 16	Connecticut	1	3	6

TABLE 11. The Effect of Antigenic Differences on Complement Fixation Test Titres: (2) Titres of 2 Antisera, of Different Types, Against Antigens of the Same and Different Types.

Antisera		Test Antigens		
Batch No.	Virus Strain	Beaudette	Massachusetts-41	T
As 37	T	2	4	5
(10.9.70)	Allen	5	5	3

A similar series of readings of "2" was seen in a titration of the Iowa 97 antiserum As39 again st Beaudette antigen. In this test, the first 2 wells gave readings of "4" and "3" (indicating a serum titre of 2) but the next 5 wells gave readings of "2".

(9) Application To Diagnosis

The titration of field samples of chicken serum against Beaudette antigen was found to be easy and rapid, and therefore this appeared to be a promising method for testing large numbers of samples for diagnosis or monitoring of infection. Opportunities were taken to test samples from a variety of field situations where a provisional diagnosis of IB had been made on clinical grounds, and also from farms where IB was thought to be absent. The object of these tests were to obtain additional evidence of the specificity of the test, as well as to evaluate its usefulness for these purposes.

Table 12 shows the titres of samples taken before and after the appearance of IB in a commercial broiler house. The building was divided into 9 separately ventilated rooms, each housing 1000 birds. Each serum sample tested was a pool of 5 birds, caught at random from each room. In spite of the separate ventilation, the disease spread very rapidly through the building, affecting birds in all rooms at between 5 and 6 weeks of age. It will be seen that at 5 weeks of age all the samples were ≤ 1, and all were positive at 9 weeks, and showed a further increase at 11 weeks.

Table 13 shows the titres of samples collected from random individual birds on a broiler farm in a foreign country. IB was suspected to be the cause of a respiratory disease problem on this farm. It will be seen that at 15 days of age the titres were noticeably a little higher than at 32 days, at which time they were all ≤ 1. This was interpreted as probably due to persistence of maternal antibody at 15 days, and its disappearance at 32 days. At 55 days, 6 of the 9 samples tested were ≥ 5, and 3 samples were < 1. These results were considered to support the diagnosis of IB.

TABLE 12. CF Titres Against Beaudette Antigen of Pooled Sera from Chicks in a Commercial Broiler House

Age of Chicks When Sampled	Room Nos.								
	1	2	3	4	5	6	7	8	9
5 Weeks	< 1	1	1	1	1	< 1	1	1	< 1
9 Weeks	8	7	8	7	7	7	6	8	4
11 Weeks	10	10	10	10	8	9	11	10	10

TABLE 13. CF Titres against Beaudette Antigen of Individual Chicks from a Broiler Farm in a Foreign Country

Age of Chicks When Sampled	Titres of Random Individuals															
15 Days	1	3	2	2	2	2	2	3	3	1	1	2	<1	4	2	2
32 Days	<1	<1	<1	<1	<1	<1	<1	1	<1							
55 Days	<1	5	<1	<1	6	5	5	6	5							

Groups of samples, collected from individual birds on 13 separate broiler farms owned by the same company, were titrated. The farms were classified by the owners into: 6 farms in which there was a coli-septicaemia problem, and regular use was made of live IB vaccines; and 7 farms in which there was no coli-septicaemia problem, and IB vaccine was not used. From the former, supposedly IB-infected farms, 45 samples were titrated, taking at least 2 samples from each of the 6 farms. 40 of these samples gave titres $\geqslant 3$, regarded as "positive", and every farm gave at least one such "positive" sample. From the latter, supposedly IB-free farms, 38 samples were titrated, again taking at least 2 samples from each of the 7 farms. 2 of these samples gave "positive" titres, these being both from the same farm, and the remainder were "negative" (< 3). The distinction between "negative" and "positive" samples was very marked: the mean titre of the 42 "positive" samples was 7.9, while the majority ($^{34}/_{41}$) of the "negative" samples were actually flat negative (< 1).

A few more small groups of samples were obtained from other field situations in which IB was suspected, including one farm with a high incidence of nephritis. Nearly all samples gave strongly positive reactions, including those from the nephritis farm.

(10) Application To Vaccine Trials

The application of the CF test to the assessment of vaccination against IB under particular field conditions is under evaluation by the Respiratory Disease Unit of the Poultry Department. The author participated in one of the earlier experiments, in which live vaccines of two different strains of IB virus were compared for their effectiveness when given combined with a live Newcastle disease vaccine. The details of this work are described by Gdovinova et al, (1974).

DISCUSSION

(1) The Mechanism Of The Reaction

It was not one of our main objects to investigate the fundamental nature of the reaction forming the basis of the test. However, these studies would not be complete without some consideration of this aspect.

The studies of the Cambridge workers (Orlans et al, 1962) showed how complex were the interactions between mixtures of heated and unheated chicken and guinea-pig sera, even in the absence of any specific antibody-antigen reaction. These authors questioned the specificity of the results obtained by the Minnesota workers (Benson et al, 1961), because apparently similar "titres" could be produced by making certain mixtures of sera, in the absence of any known antibody-antigen reaction. We feel that although the Cambridge workers were correct in pointing out these complexities and possible pit-falls, their results did not invalidate those of Benson et al, (1961), because the evidence for the specificity of these authors' titres depended not only on the controls provided within their test, but also on their knowledge of the disease they were working with.

Like the Minnesota workers, and Marquardt and Newman (1971), our approach was also to use our knowledge of the disease and its agent to search for a method that would give results that were consistent with that knowledge. Although this approach may be open to criticism as arbitrary and based on trial and error, once reproducible results have been obtained, confirmation of their specificity is abundantly available. The explanation of their mechanism then becomes a matter for academic study. This kind of practical approach, avoiding the need for a previous understanding of the mechanism, has been taken in the past by most workers developing new methods.

The mechanism put forward by Benson et al (1961) is that fresh chicken serum contains a factor essential for the fixation of guinea-pig complement by chicken and turkey antibody-antigen reactions. They claimed to have

concentrated 3-fold, and partially purified, the factor, which they have identified by further studies as "avian C_1". That is, it is a heat-labile, water-soluble, early component of avian complement. They postulated that avian antibody reacting with antigen requires to react first with avian C_1 before the fixation of mammalian complement can occur.

Nothing in our own experience of using a similar method for IB would contradict these principles. However, a slight inconsistency has appeared regarding the heat-lability of the factor. Like Marquardt and Newman (1971), we found that the factor was very stable at $4^{\circ}C$. In this respect it is unlike mammalian C_1. In contrast, we found that storage at $4^{\circ}C$ caused complete disappearance of the lytic effect of chicken serum on sensitised sheep red blood cells. In this respect chicken serum is like mammalian serum. It seems to follow from this that although the factor is stable at $4^{\circ}C$ and labile at $56^{\circ}C$, there is another essential component in chicken complement which, like mammalian C_1, is labile at both $4^{\circ}C$ and $56^{\circ}C$. To refer to the factor as avian C_1, thereby equating it with mammalian C_1, seems to be an over-simplification.

Further studies on the mechanism of the reaction are required, but it would be advisable not to use the IB system for this purpose, because of the difficulty of preparing large amounts of antigen in a pure, concentrated form, and the lack of knowledge of the role of the corona in the reaction.

(2) IB Virus As Antigen

Although the CF test was found to work equally well with virus strains other than Beaudette, once satisfactory antigens had been prepared, the relative ease with which such antigens could be prepared from Beaudette was very striking, and requires explanation. The infectivity titres of Beaudette harvests are at least 10-fold higher than those of any of the other strains used in these studies, and 50-fold higher than most of them. It could be argued that the superiority of Beaudette was due simply to higher virus yields. Against this, the titre of antigen was shown, in the studies with Dr. Reed, to

be independent of the infectivity titre. Again, it could be argued that the virus particle content, as well as the infectivity, was much higher with Beaudette. This may be so, but it would be surprising, because Beaudette harvests are extremely early. With other viruses, for example foot-and-mouth disease virus, early harvests tend to have high infectivity titres, but low particle contents, and later harvests usually give higher CF antigen titres in spite of having lower infectivities. The explanation could lie in the relative purity of Beaudette harvests. Their comparative freedom from host cell debris and inflammatory exudates may mean less masking and aggregation of the virus particles.

The possibilities that the antigen may be an internal component of the virion, or a protein not incorporated in the virion, were investigated in the studies with Dr. Reed. When Beaudette harvests were centrifuged through sucrose density gradients, the virus particle peak, recognised by optical density and protein estimation, was found to coincide with the peak CF antigen titre, and also with the peak infectivity titre, although the absolute value of the latter had been severely reduced by the centrifugation. Under the electron microscope, the peak consisted of IB virus particles that were entire, but completely without a corona. Treatments that were calculated to destroy the virion, including sodium dodecyl sulphate and 1% sodium desoxycholate, caused the disappearance of the CF antigen. Treatments with trypsin and nonidat did not significantly alter the antigen titre. It was concluded that the Beaudette antigen was due entirely to whole virus particles, apparently without a corona. The possibility that the corona had been removed by the physical forces applied during centrifugation could not be ruled out.

Studies on the corona of 12 different IB virus strains by Harkness and Bracewell (1974) confirmed that the Beaudette strain normally appeared without a corona in negatively stained preparations of allantoic fluid harvests, while certain other strains, whose harvests were treated in an identical manner,

appeared with a prominent corona. It even seems possible that it is the very absence of a corona that accounts for the strikingly greater efficiency of Beaudette harvests as antigens in the CF test. This would, however, imply that it is possible for antibody molecules to become directly attached to the envelope of the Beaudette strain. Berry and Almeida (1968) showed antibody attached to the corona of typical IB virus particles and not to the envelope. It should be possible to resolve this apparent conflict by examining Beaudette virus in reaction with antibody under the electron microscope.

One explanation that would fit both these observations is that the viral antigen in a typical IB virus strain is carried on the corona, but that in the Beaudette strain it is carried on the envelope. To explain the atypical structure of the Beaudette virion, it may be regarded as a premature virion in which a corona never develops, but the viral antigen remains incorporated in the surface of the envelope. This concept was also advanced to explain the results of the plaque reduction test, and is dealt with more fully in the Discussion section of Chapter V.

(3) The Antibody Response To IB

The responses of individual birds following infection with IB, shown in Table 8, clearly indicated an early peak at 3 weeks in all birds. This finding agreed with the report of Uppal (1970), although the titres we obtained were considerably higher. Our higher titres were probably due mainly to use of a different test method, but perhaps a difference in virulence between the virus strains used for infection may have contributed. The titres shown in Table 8 declined after 3 weeks, in an irregular manner, in 6 of the 8 birds, but were maintained at a high level in 2 birds beyond the 15-week period of observation. This plateau effect was not observed by Uppal (1970). The reason for these wide differences between birds in the persistence of complement-fixing antibody is not known. However, the phenomenon strongly suggests that at least 2 different classes of immunoglobulins may be involved in the CF test; one which

is produced more transiently, declining from a peak at 3 weeks post-infection, and one which is much more persistent, but is less frequently triggered.

In the vaccine trial described by Gdovinova et al (1974), many birds failed to respond to vaccination on the CF test, although their responses on the neutralisation and plaque reduction tests showed that they had received an exposure to the virus. An association between CF test responses and clinical reactions was suggested. These findings support the view that a more intense or prolonged antigenic stimulus is required to trigger complement-fixing antibody than neutralising antibody. In the same vaccine trial, the challenge produced consistently high CF test titres in nearly all birds. Those that were already positive as a result of vaccination showed significant rises after challenge. The response to a virulent field infection, as shown in Table 12, was uniformly high. The finding of maternal antibody in young chicks, as in Table 13, suggested that persistent antibody may commonly result from field infection.

Some explanation should be attempted of the weak reactions (incomplete fixation effects) mentioned in Sections (6) and (8) of the Results. It could be that, like the individual bird variation seen during the decline of titres after 3 weeks in Table 8, these effects may be due to failure to trigger all the immunoglobulin classes that are potentially active in the reaction. For instance, it could well be that a particular sub-class of IgG is needed to give full fixation and high, persistent titres, and that the production of this immunoglobulin requires a degree of antigenic stimulation that is not always present in laboratory experiments.

(4) Application To IB Virus Typing

In these studies, we did not find the CF test to be helpful in tackling the difficult problem of antigenic variation between strains of IB virus. (Results, section (8)). Antigenic differences caused reductions in serum titres, but these reductions were either not large enough, or not consistent

enough, to serve as a basis for classifying virus strains. There were no signs that the CF test would indicate a totally different classification from that which had emerged over the years from the application of the neutralisation test. Rather it seemed that the same differences between reference strains were being shown, but with even poorer reproducibility.

Probably the major factor responsible for the failure of the method in this application was the weakness and variability of the complement-fixing antibody response to IB, discussed in section (3) above. In retrospect, it can be seen that the standard antisera produced for these studies, although suitable for the various forms of neutralisation test, were probably not ideal for the CF test, because the low virulence of many of the strains caused responses to be sub-maximal.

An important technical difficulty arose over the preparation of antigens from IB virus strains other than Beaudette. This was partly overcome by concentration and purification by centrifugation through potassium tartrate solution, but the time and labour required still severely limited the work.

Once it became clear that the plaque reduction test was giving reproducible and meaningful results in cross-type tests on the stock antisera, the decision was made not to pursue further the application of the CF test to this purpose.

(5) Applications To Other Purposes

Studies on the applications of the method to diagnosis and vaccine trials continued. Because of the ability to use unconcentrated Beaudette harvests as antigen, and the speed and economy inherent in the "Microtiter" system, the method appeared to be a promising new tool for these purposes. The reports by Bracewell (1973) and Gdovinova et al (1974) describe these applications. The evaluation of the CF test is continuing, to be carried out in the Poultry Department, under the supervision of Mr W H Allan.

It seems probable that the method will find a place among the other

serological tests for IB. Its speed and economy are perhaps somewhat offset by its greater technical complexity, and the inconsistency of the complement-fixing antibody response in IB. This latter feature, however, could perhaps be turned to its advantage when enough knowledge of the implications of reduced responses has been acquired.

One application to laboratory work with IB virus, for which the CF test could prove invaluable, is in the titration of IB virus in preparations in which the infectivity has been lost. Examples of this type of work are studies on the effects of physical and chemical treatments on virus structure, and analysis of virus components.

SUMMARY

Methods of performing direct CF tests for IB on chicken sera were compared. A test carried out on unheated sera was found unsuitable for comparative titrations on samples collected over a period of time, because the variations in pro- and anti-complementary properties prevented standardisation of the dose of complement, and also because the serum titres were limited by their content of a factor other than specific antibody. A test carried out on heat-inactivated sera was found to be more controllable and reproducible, and to give higher serum titres. In this method, the fixation of guinea-pig complement was found to require the addition of unheated normal chicken serum, and this was included in the complement aliquot. The "Microtiter" technique was used.

Studies were made of the reproducibility and specificity of this method, on the properties of IB virus as antigen, and on the antibody response to IB as shown by the CF test. Variations in strength of fixation were observed, especially in heterologous reactions.

Although serum titres were often markedly reduced by virus strain differences, the reductions were not consistent enough to be of use in classifying

strains into antigenic groupings. The weakness and variability of the complement-fixing antibody response to infection with IB virus strains of low virulence probably made the stock antisera unsatisfactory as material for the critical, comparative titrations that were needed. The technical difficulty of preparing adequate quantities of satisfactory antigens from strains other than Beaudette had a retarding effect on these studies.

The test using Beaudette antigen was found to be a practical, quick and economical method for titrating large numbers of chicken serum samples for diagnostic purposes and vaccine trials in the field.

Chapter V: PLAQUE REDUCTION TEST

INTRODUCTION

Surveys of literature on the use of cell cultures and the basic methods used for the neutralisation test, appear in Chapter I. The reasons for selecting the percentage plaque reduction test in CK cells as the method to receive the biggest effort, have also been given. This chapter describes the materials and methods used in preparing CK cell cultures, and applying them to the passaging and plaque assay of virus strains, and the method of performing serum titrations by percentage plaque reduction. The results of studies on the development of the method are described, including experiences in adapting virus strains to CK cells, the measurement of experimental error, the demonstration of specificity, and comparison with the results of the neutralisation test in eggs. This section is followed by the results of applying the method to the main objective (the defining of antigenic relationships) and the secondary objective (practical use in diagnosis, screening tests, and vaccine trials), and of studies on the antibody response to infection.

The discussion contains our conclusions and speculations on antigenic relationships, and a comparison of the practical value of the method with that of the neutralisation test in eggs.

MATERIALS AND METHODS

(1) Chickens

The chickens used to provide kidneys for CK cell cultures were obtained from several different sources. The birds hatched and reared from SPF eggs (Chapter II) would always have been preferred, because of their greater freedom from infectious agents, to those obtained from Coombelands and

Lasswade. However, the quality of the cultures from the SPF birds was less consistent. There were periods of several weeks together when SPF birds gave good results, but then periods of bad results followed, and recourse was made to the non-SPF birds.

Marek's disease virus plaques were seen from time to time, usually in small numbers, in the cultures derived from Coombelands birds. They were never seen in cultures from SPF or Lasswade birds. Interference with IB virus plaques, as reported by El Zein et al (1972), was observed on a few occasions when the number of Marek's plaques exceeded 10 per dish. This may have been one factor contributing to variation in virus titres between cell batches.

The virus titres obtained with cells from Lasswade birds were somewhat higher than with those from the other sources. The kidneys had a softer consistency, and broke up more rapidly during trypsinisation. The cell sheets that resulted were smoother and contained a smaller proportion of fibroblastic cells.

After some preliminary studies comparing the results obtained with birds aged 3 and 6 weeks, it was decided that the ideal balance between speed of growth and ease of maintenance lay somewhere between these ages, at 4 to 5 weeks of age. The yield of cells varied from about 100 ml. for a 3 week bird to 600 ml. or over for a 6 week bird. The yield of 300-400 ml. obtained from a 4 to 5 week bird was adequate for the usual week's work. This age of bird was similar to that used by Churchill (1965).

(2) Media

Initial studies in which some of the standard culture media in common use were compared led to the adoption of the formulae in Appendices 5, 6 and 7. Because of the intended use of open petri-dishes, it was decided from the outset to use Hanks-based media in preference to Earles-based media.

Eagles' medium and 199 were found to give CK cells with poorer virus susceptibility than the cruder lactalbumin-tryptose phosphate broth medium that was adopted. The gross appearance of the cell sheets was very similar, in fact better with 199, so it is quite possible that these other media would be more suitable for other viruses. The medium selected seemed to be particularly effective for preserving the epithelial-like cells which are most susceptible to IB virus. (Butler, 1965).

Two of the medium constituents, hepes and foetal calf serum, were given special attention, so they are described in more detail below.

Hepes

The use of hepes buffer (N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid) for control of medium pH in petri dishes was arrived at after an initial period of experimentation with other methods. These included gassing with 5% CO_2-air mixture (directly supplied from cylinder), and the use of Tris buffer. Gassing was able to control the pH effectively, but the sealed containers that were necessary with this method were cumbersome, and sources of contamination. Tris buffer (obtained from Sigma Chemical Company as "Trizma Base, Reagent Grade") was used as a stock 1M solution, in conjunction with an approximately equal proportion of 1M hydrochloric acid. It was found that the minimum concentration needed to obtain control was 15 mM, but this was very toxic for CK cells.

Hepes was obtained from B.D.H. Ltd. as a non-sterile powder, and used as a stock 1 M solution, stored at $4^{o}C$. This was added to the medium at 1.5 ml. per 100 ml. In order to achieve a final pH of 7.1, the same volume of $^{N}/_{4}$ sodium hydroxide solution was also added. The concentration of hepes in the medium was 15 mM. At this level it was found to give adequate pH control without any adverse effects on cell growth or virus susceptibility.

The pH of the medium when first made up was 6.7. Soon after being dispensed into petri-dishes, the pH rose to 7.1, under the influence of the bicarbonate which was present in the medium in the normal concentration used for Hank's medium. The pH then became stable at 7.1. When normal cell growth took place, the pH slowly fell, to about 6.9 on the third or fourth day. If cell growth was poor, the pH slowly rose, to reach values up to 7.3 or 7.4 by the fourth day. The resulting colour changes were useful as a rough guide to the quality of a batch of cells.

A comparison of hepes obtained from our usual supplier with that obtained from another supplier as a sterile 1 M solution, showed no advantage for the solution in terms of cell growth or virus titre.

Foetal Calf Serum

Sterile foetal calf serum was obtained in large batches from Biocult Ltd. or Flow Laboratories Ltd., and stored at -20°C. It was agreed with the suppliers that their batches would be tested in advance of purchase for their ability to promote the growth of CK cells. These tests were carried out by the Virology Department, who also carried out growth tests on other cells. CK cells were found to be the most sensitive to variations in the quality of the serum, and batches that were purchased as suitable for CK cells were always found to be good for any other cells. Growth tests were performed in Leighton tubes, and consisted of comparisons of the microscopic appearance of the test cultures with cultures of the same batch of cells in a serum known to be satisfactory.

Cheaper alternatives to foetal calf serum were examined. Heat-inactivated newborn calf serum was found to be suitable for use in the plaque overlay, diluent and maintenance medium. If used in the growth medium, it gave more

uniform results than the batches of foetal serum that were tested. Although better than the poorest batches of foetal serum, it was inferior to the best batches. Serum from SPF chicks aged 5 to 6 weeks gave very good cell growth, but virus susceptibility was greatly reduced.

(3) Petri Dishes

After a preliminary comparison with glass petri-dishes, which were found to have no advantage, disposable plastic petri-dishes were used throughout the studies. In the early stages of the work, these were "Falcon" 60 x 15 mm. vented dishes supplied by Becton, Dickinson Ltd., or "Nunclon" 50 x 13 mm. vented dishes supplied by Sterilin Ltd. A comparison of cell growth and titres in these dishes showed no significant differences. Another make of dish was found to be unsatisfactory because of poor adhesion of the cell sheet. In the later stages of the work, "Mini-Petri Plates" supplied by Flow Laboratories Ltd., were used. These dishes were 35 x 11 mm., and moulded in groups of 6, with a single, large, rectangular lid. This design was found to encourage speed and neatness. The cell growth and virus titres were the same as given by the Falcon and Nunclon dishes. The great majority of the plaque reduction tests were performed on Mini-Petri Plates.

In order to prevent evaporation of the medium, the petri dishes were enclosed in large, flat, rectangular, plastic food containers with self-sealing lids. Leaving the dishes exposed in an incubator or hot room was found to cause loss of medium by evaporation at a rate of about 10% per day. The use of these containers also reduced the amount of aerial contamination to negligible proportions. The containers were wiped out with methylated spirit before use. Incubation was carried out on the shelves of a hot room with a good air circulation. There was no problem of condensation inside the containers while in the hot room.

(4) Cell Culture Methods

Use of Cells other than Chicken Kidney

The following mammalian cell lines were tested briefly for susceptibility to the Beaudette strain:

BHK 21 clone 13 (Macpherson and Stoker, 1966).

VERO (African green monkey kidney) (Yasumura and Kawakita, 1963).

MDBK (Bovine kidney) (Madin and Darby, 1958).

Monolayers in 4 oz. medical flat bottles and Leighton tubes, in the appropriate media, were inoculated with 1 ml. and 0.2 ml. respectively, of both allantoic fluid and CK cell harvests. No cytopathic effects were seen on the first passage, or one further passage. Titrations of the culture supernates in eggs or CK plaques gave no evidence that virus replication had occurred.

Chick embryo kidney (CEK) cells were tried briefly for virus passage and plaque assay. 10 to 12 pairs of kidneys from Coombelands embryos of 18 days' incubation were used to prepare small batches of CEK cells, by a similar method to that described for CK cells. The cultures tended to overgrow, leading to detachment of the cell sheet. Plaques grew rapidly with irregular shapes and tended to become confluent too soon. Although the virus susceptibility was at least as high as the CK cells, it was apparent that further studies on medium constituents would be needed to prevent overgrowth of cells and confluency of plaques, and this was not worthwhile bearing in mind the much greater labour involved in preparing the cultures.

Trypsinisation

The trypsin solution was prepared immediately before use by reconstituting a vial of 5% trypsin (obtained from Wellcome Reagents Ltd.), and adding 5 or 10 ml. to 500 ml. of Dulbecco's phosphate buffered saline solution A (PBS 'A': Appendix 8). At a later stage of the work, the PBS 'A' was made

up from tablets supplied by Oxoid Ltd., which gave a solution that was similar in composition except that it contained no phenol red. The omission of phenol red made it easier to see how much blood was coming out of the tissue into the liquid.

The preparation of the kidneys before trypsinisation was performed in an identical manner each time, as far as possible, and all unnecessary delays were avoided. The time between killing the bird and starting trypsinisation was 25 minutes. During this time the bird was decapitated and allowed to bleed out, dipped in warm "Teepol" solution to wet the feathers, then pinned out on a board. Next, the kidneys were removed, using 2 sets of sterile instruments, and dropped into 200 ml. of PBS 'A' at 37°C. The ureters and blood vessels were then carefully removed, using another set of sterile instruments, and the remaining tissue was chopped up with a fine pair of scissors until it started to stick together and no prominent large lumps could be seen. It was then put into a 100 ml. conical flask and washed twice with about 50 ml. of PBS 'A' at 37°C. During this process much blood and floating debris was removed with the decanted fluid. Trypsinisation then began, with the addition of about 50 ml. of the trypsin solution at 37°C, and the magnet, (2.0 or 2.5 cm.).

This first wash in trypsin released much more blood from the tissue than a similar wash in PBS alone would have done at this stage. Further washes were carried out, adding 25 ml. of trypsin and stirring for exactly 3 minutes each time. The number of washes discarded before collection was begun was varied slightly, depending on the amount of blood to be removed, the consistency of the tissue, the fineness of the chopping it had received, and the observation of the beginnings of the disintegration of the tissue. Usually 3 or 4 washes in trypsin were discarded.

By the time that collection of harvests began, the temperature of the trypsin solution in the flask, and the degree of agitation, had been stabilised

by rigid adherence to the 3 minute periods, and constant watching of the magnet and correction of changes in the speed of stirring and the position of the flask. The trypsin was held in a 37°C water bath in pre-measured volumes of 25 ml. in universal bottles. The stirrer was positioned on the bench near to the water bath. Since the flask was exposed to the air in the room, the room temperature was maintained steadily at 26°C. In cold weather, the work was not started until the room had been made warm enough. The effect was that the temperature of the trypsin at the commencement of each 3 minute period was 37°C, and at the end it was 34° to 35°C. The flask was shielded from draughts, and insulated from contact with the metal base of the stirrer by standing it on several sheets of cardboard. The flask was placed slightly eccentrically, in order to produce a more irregular movement of the liquid. Occasionally the magnet had to be freed from a tangle of fibrous tissue that sometimes formed around it, by giving the flask a quick shake.

The harvests were taken off by decanting as much liquid as possible into a universal bottle containing 1 ml. of foetal calf serum. Initially, these bottles were held at 0°C in an ice bath, and the harvests were held there at 0°C until all had been collected, following the method of Churchill (1965). Later it was discovered that better growth was obtained by leaving the harvests on the bench, where they cooled slowly to room temperature.

The number of harvests collected varied between 8 and 10. The universal bottles were centrifuged, in sets of four or pairs, on an M.S.E. "Super-Minor" centrifuge which was set at positions determined to give 800 r.p.m., for 10 minutes. The supernatant liquids were poured off, and the deposits were suspended in growth medium by gentle mouth pipetting with a 10 ml. pipette. The deposits from 2 bottles were suspended in 5 ml. of medium.

When the last harvest had been dealt with in this way, the suspensions were pooled by pouring them through 2 layers of muslin supported in a glass funnel leading into a 300 ml. conical flask. The volume of the pool was

usually about 23 ml. This was divided equally between two 10 ml. graduated conical centrifuge tubes, which were centrifuged on an M.S.E. "Minor" at 1000 r.p.m. for $7\frac{1}{2}$ minutes. The volume of packed cells was measured by the graduations on the tubes, and 400 times this volume of growth medium was measured into a round bottle of capacity 1 litre or 500 ml. The usual volume of packed cells was about 1 ml., but there was considerable variation in the yield, the age of the bird being a big factor.

Cell Growth

Cultures were seeded on the basis of varying the volume of cell suspension according to experience of the particular containers, leaving the cell concentration constant at 1 part of packed cells per 400 ml. of medium. Cell counts on 9 consecutive batches of cells gave a mean count of 7.8×10^5/ml. for the cell suspension, with a range of 6.2 to 9.3×10^5/ml. The use of 0.1% trypan blue in the diluent for these counts showed that the proportion of dead or damaged cells was never more than 10%, and was usually too low to be counted easily. The proportion of erythrocytes present varied greatly, from almost complete absence to a maximum of 8×10^5/ml. None of these variations were correlated with the variation in quality of these cultures.

Mini-petri plates were seeded with 3.5 ml. per dish, and Falcon and Nunclon dishes with 5 ml. One-ounce medical flat bottles received 5 ml., and Leighton tubes 1.3 ml.

The cultures were usually ready for inoculation on either the third or the fourth day. The confluency of the cell sheets was checked microscopically before inoculation. Unsatisfactory batches were sometimes left until the fifth day, to be either used on that day or discarded. Variation in growth was frequently observed between cell batches, but there was very little variation between individual dishes, bottles or tubes seeded from the same cell batch. Lack of confluency caused marked reductions in virus susceptibility

(5) Passaging of Virus Strains

Adaptation of virus strains to CK cells was accomplished by means of between 5 and 14 straightforward passages in monolayers in 1 ounce medical flat bottles. Two bottles were used for each passage, as a safeguard against contamination or accidents, but usually their harvests were pooled. The inoculum for the first passage was usually 0.5 ml. of undiluted allantoic fluid harvest, which was added after the growth medium had been poured off. After 30 minutes at 37°C had been allowed for virus adsorption, the inoculum was tipped off, and 5 ml. of virus maintenance medium (Appendix 6) was added to each bottle. For later passages, the inoculum was a supernate of the previous harvest, applied either undiluted or at a low dilution such as $^1/5$ or $^1/10$, and usually not removed before addition of the medium.

The progress of the cytopathic effect was observed. In the early stages of adaptation, when the progress was very slow, the harvests were made on the 6th day. When definite cytopathic effects began to appear, the harvests were made at progressively earlier times, the object being to harvest soon after the effect had reached its maximum, regardless of the percentage reached. For example, on its 3rd passage a virus strain might reach 20% cytopathic effect on the 3rd day, and show no increase thereafter, so we would harvest late on the 3rd day, or on the 4th day, as soon as it was realised that no further increase was occurring.

Harvests were made by pouring off the medium into a universal bottle and centrifuging it to remove cell debris. The harvest was then stored at -60°C. A few variations on this basic method were tried, particularly when difficulty in adapting a strain was encountered. Sometimes the whole culture was frozen and thawed in the bottle a few times, before being centrifuged. At other times centrifugation was omitted; or cold storage was omitted, the harvest being used as inoculum while still warm. There was no evidence of any advantage in these variations.

(6) Plaque Assay Methods

Virus Dilution and Inoculation

A series of 10-fold dilutions in virus diluent (Appendix 6) were prepared in bijou bottles. The growth medium was removed by aspiration through a pasteur pipette, suction being provided by a small "Austin" air pump, via a 1-litre bottle fitted with a 2-way head to act as a trap for the aspirated medium. Two dishes were inoculated with each of the dilutions 10^{-3}, 10^{-4} and 10^{-5}. The volume of inoculum was 0.1 ml. per dish, measured out with a 0.2 ml. graduated pipette. The dishes were placed at 37°C for 30 minutes for virus adsorption, and then they were taken out and the overlay was applied. Removing the inoculum before applying the overlay was found to make no difference, so it was decided to omit that operation.

Overlaying

The overlay medium (See Appendix 7) contained Oxoid "Ionagar No. 2" at 0.84%. This was prepared for incorporation in the medium by dissolving 4.2 grammes in 100 ml. of distilled water by heating in a pressure cooker at 15 pounds per square inch for 15 minutes, followed by cooling of the bottle in air as quickly as was safely possible. When it was about 56°C (cool enough to handle) it was mixed with 400 ml. of medium containing all the other ingredients and warmed to 44°C in a water bath set at that temperature. The complete overlay was then held at 44°C until required. This was timed so as to occur towards the end of the virus adsorption period. A long period of waiting at 44°C was avoided, because we found that it impaired the setting of the agar. An automatic pipetting syringe, size 2 ml., was used to apply the overlay. 2 Shots of 1.8 ml. were put into each dish of the Flow Laboratories' "mini-petri" plate, to give 3.6 ml. in each dish. The plates were left on the bench for 15 minutes before being put back into the plastic food containers and then into the 37°C room.

Staining

The cell sheets of the virus controls were examined microscopically on the morning of the 2nd day after inoculation. Developing plaques could be seen clearly as groups of syncytia. If the plaques were sufficiently well developed, as happened with the better-adapted strains such as Beaudette and 927, the cells were stained at this time. The majority of strains were stained on the 3rd day. A few strains, for instance M-41, were sometimes best if stained late on the 2nd day. The best time for staining depended on the quality of the cells, the number of plaques actually developing, and previous experience of the behaviour of the virus strain.

The usual method of staining was to remove the agar, flood the dishes with crystal violet, wash off the stain with tap water, and leave them to dry before counting. A few small variations were tried. The agar could be removed either by a carefully controlled flow of water directed into the side of the dish, or by inverting the dish and levering the agar off with a scalpel. The latter method was found to be quicker and have less risk of detaching part of the cell sheet. It also had the advantage that the pieces of agar could be caught neatly in a polythene bag and disposed of by incineration. The crystal violet was applied from an aspirator held about 9 inches above bench level. The most effective formula for the stain was found to be 0.5% crystal violet in 25% ethanol.

Vital staining with neutral red was tried briefly, using two methods: incorporation in the initial overlay at $^{1}/10,000$; and incorporation at $^{1}/10,000$ in a second overlay applied on top of the initial, normal overlay, about 3 hours before reading the plaques. Incorporation in the initial overlay reduced the size and number of plaques. Incorporation in a second overlay gave the same results as crystal violet, but with slightly more labour, and the plaques were a little more difficult to see, and were not permanently stained as they were with crystal violet. The latter was an

important practical advantage for crystal violet, because the plaques could be counted whenever it was convenient, whereas with neutral red the counting had to follow closely on the staining.

The plaques were counted by examining the dishes from below against a white background, and marking each plaque as counted with a fine felt-tip pen.

Calculation of Virus Titres

Virus titres were calculated as the weighted mean plaque count over the 2 or 3 ten-fold dilutions at which there were countable numbers of plaques. Since the number of dishes inoculated with each dilution was always 2, the usual formula for calculating a weighted mean (Rhodes and Van Rooyen, 1968) (Appendix 9) was slightly modified as follows:

(1) The counts of the 2 dishes for each dilution were added and divided by 2 to give the mean count for each dilution.

(2) These mean counts were added, giving the total of the mean counts.

(3) This total was divided by 1.1, where 2 dilutions were used, or 1.11 where 3 dilutions were used.

This value gave the weighted mean count at the lowest dilution used.

(4) The virus titre per ml. was obtained from this by first multiplying by 10 for the dose factor and then expressing the result as $\log_{10}$ plaque-forming units per ml. (log PFU/ml.)

The preliminary calculation of the mean count for each dilution was considered to be useful in assessing the regularity of the plaque distribution. A very unequal distribution indicated a need for a repeat titration.

(7) Serum Titration Methods

Standardisation of Test Virus

When a virus strain had become sufficiently well adapted to CK cells, by passage in monolayers in one-ounce medical flat bottles, it was given a further passage in 2 to 4 twenty-ounce medical flat bottles, and this harvest

pool was stored at -60°C in 4 ml. aliquots in bijou bottles. Plaque assays were performed on aliquots withdrawn from this stock, to ensure that readable plaques could be produced and that the titre was accurately known. The dilution at which the virus was to be used in the plaque reduction test was calculated as follows:

(1) For Falcon or Nunclon plates, where 50 plaques per dish were aimed at, the figure of 3.0 was subtracted from the virus titre. This figure was composed from: log 50 (1.7) + log 10 for the dose factor (1.0) + log 2 for the dilution of the virus with an equal volume of serum dilution (0.3).

(2) For Mini-Petri dishes, where 40 plaques per dish were aimed at, the figure of 2.9 was subtracted. (1.6 + 1.0 + 0.3).

Test Procedure

The sera to be tested were treated at 56°C for 30 minutes, preferably on the morning of the test. If they were heat-treated on the day before, they were then stored at 4°C overnight before the test. Repeated heat treatments were avoided. The valuable standard antisera were stored at -20° in universal bottles in non-heated form, and small aliquots were usually heat treated as required. Serum dilutions were carried out in virus diluent (See Appendix 6), by pipetting in bijou bottles, usually by adding 0.5 ml. to 0.5 ml. to make a doubling dilution series. For very small samples 0.25 ml. volumes were sometimes used, or the series was started at 1/4 by adding 0.2 ml. to 0.6 ml. of diluent, and thereafter 0.4 ml. volumes were used. Serum dilutions were expressed as $\log_2$ reciprocals from the earliest stage, because it made easier the later recording of results and estimation of titres. For example, 1/4 was called -2, and 1/1024 was called -10. The dilutions always referred to the initial serum dilution before the addition of the test virus.

An average test, of the size performed twice a week when the routine was established, consisted of titrating 5 or 6 serum samples, each over 6 dilutions, using 2 dishes per dilution. For the virus control, 0.5 ml. of the test

virus was mixed with 0.5 ml. of virus diluent, left on the bench alongside the serum-virus mixtures for the 30 minute reaction period, and used to inoculate 4 or 6 dishes. The rest of the procedure was as described for plaque assay methods (Section (6)).

Estimation of Serum Titres

Percentage plaque reductions were calculated for each serum dilution, by subtracting the mean count at the dilution from the mean count of the virus controls, and converting the difference to a percentage of the virus control count.

The serum titres were taken as the $\log_2$ reciprocal of the serum dilution estimated to cause 75% reduction of the count obtained from the virus control. They were estimated graphically by plotting the results on graph paper that combined a probability scale with an equal division scale. (Chartwell Graph Data paper 5571: 80 Equal Divisions x Probability). The percentage reductions were shown on the probability scale, and $\log_2$ reciprocals of serum dilutions on the equal division scale. The points tended to lie approximately on a straight line, for the standard antisera, as shown in Fig. 4. The best straight line through the points was drawn by eye. The titre was read off from the intersection of this line with the vertical corresponding to 75%.

The value 75% was selected by inspection of the results of the 6 replicate titrations shown in Fig. 4. It was a value at which the titres showed minimal variation, and about which the observations were evenly distributed. With certain serum samples, for example the 2-weeks-post-infection samples shown in Fig. 5, the regression line was curved in the region of the lower percentage reductions, but even with these samples the line became approximately straight at 75%. The slope of the line varied significantly between serum samples, as also shown in Fig. 5.

Serum titres were expressed as $\log_2$ reciprocals, to the first decimal place.

Fig. 4 RESULTS OF SIX REPLICATE SERUM TITRATIONS

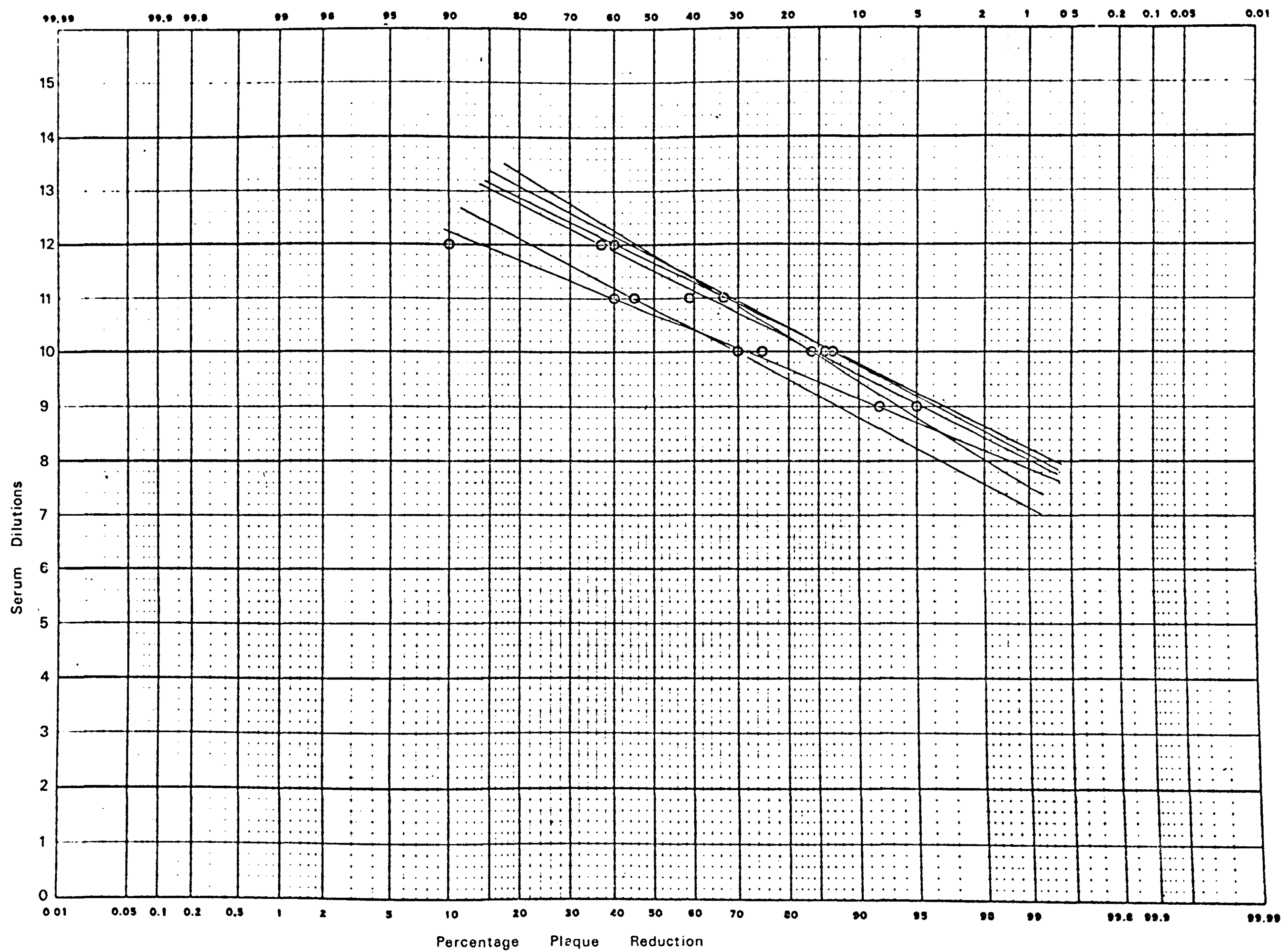
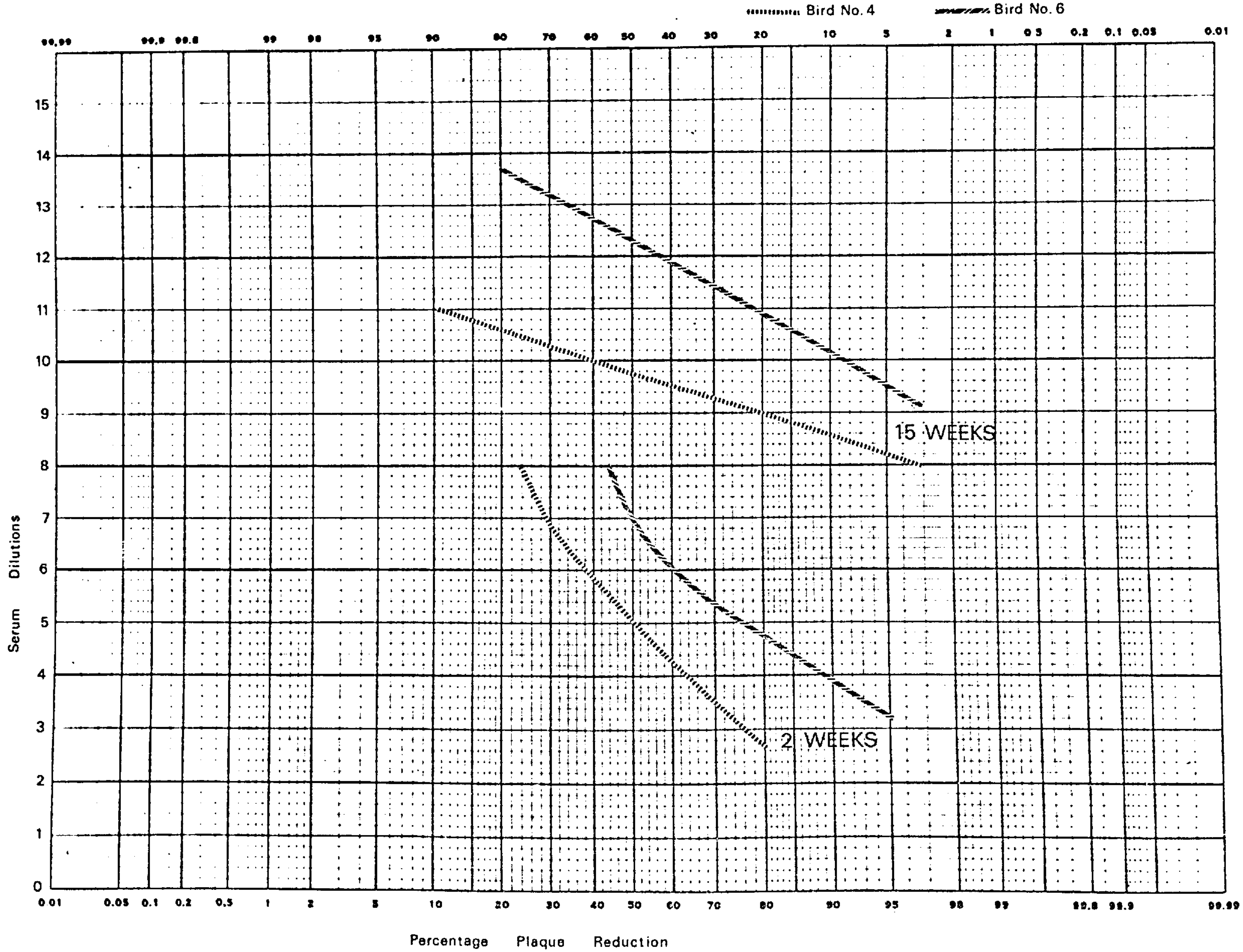


Fig.5 DIFFERENT SLOPES OF REGRESSION LINES OF EARLY AND LATE ANTIBODY



RESULTS

A. DEVELOPMENT OF METHOD

(1) Adaptation of Virus Strains to CK Cells

The following virus strains were passaged in CK cells until they produced large syncytia within 3 days, and were then laid down in 4 ml. aliquots at -60°C, and used for plaque assay:

Virus Strain	No. of Passages Given
Beaudette (Uncloned)	6
Beaudette (Cloned)	7
Massachusetts-41 (Cloned)	8
Connecticut (Cloned)	14
Iowa 609 (Cloned)	10
Gray (Cloned)	10
Holte (Cloned)	10
T (Cloned)	5
Houghton 140 (Uncloned)	6
227 (Cloned)	9
591 (Cloned)	7
604 (Cloned)	9
927 (Cloned)	5

The number of passages given was a rough indication of the difficulty experienced in adapting a strain. The majority of strains did not show syncytium formation until at least the 4th passage. The typical cytopathic effect in the early passages was the appearance of small groups of rounded cells, which slowly detached, leaving holes in the remaining cell sheet. As the degree of adaptation increased, the rounded cells became more numerous, more swollen, and detached earlier. Syncytium formation followed this stage, and it usually made its first appearance when the batch of cells was of particularly good quality, i.e. with a high proportion of smooth-looking epithelial-like cells.

Some strains produced syncytia immediately on the first passage. These

were: Beaudette (both cloned and uncloned), T, Houghton 140, and 927. All these strains were well adapted to egg embryos. Conversely, the slowest strains were the most poorly adapted to egg embryos. Adaptation failed with Iowa 97, which was particularly slow in eggs. In view of this clear association of CK adaptation with previous adaptation to eggs, no attempt was made to adapt those strains that were very slow in eggs. These included one of the reference strains (SE-17) and several of the British strains.

(2) Experimental Error

A batch of serum prepared by infecting chickens with the Allen strain (Allen batch B of 27/1/70) was titrated 6 times, in separate tests over a period of 9 months, against Beaudette virus. The titres were: 10.7 10.5 10.6 9.6 9.9 10.6.

The graphs obtained are shown in Fig. 4. The range of the 6 observations was 1.1, i.e. only a little over one dilution. Mean = 10.32; standard deviation = 0.452; standard error = 0.184.

The following antisera were each titrated on 2 separate occasions:

Antiserum	Test Virus	Titres (1)	Titres (2)
As 26 (Iowa 609)	Iowa 609	11.6	11.6
As 28 (Houghton 140)	Massachusetts-41	8.3	8.2
As 16 (Connecticut)	Connecticut	6.5	6.3
As 37 (T)	T	12.8	12.9

It will be seen that all 4 antisera gave virtually identical titres on the 2 tests.

(3) Specificity

Normal chicken sera, or antisera prepared against viruses other than IB under isolation conditions, were consistently negative on the plaque reduction test against IB virus strains.

The following sera all gave titres <1 against the test viruses shown:

Serum	Test virus
Normal SPF chick serum pool (1)	Massachusetts-41
" " " " " (2)	"
" " " " " (3)	927
" " " " " (4)	"
CELO (Ote) chicken serum	Beaudette
CELO (Phelps) " "	"
Infectious laryngotracheitis chicken serum	Massachusetts-41
Transmissible gastro-enteritis pig serum	"
Mouse hepatitis virus mouse ascitic fluid	Beaudette and 927
Human coronavirus OC 43 " " "	" " "

(4) Comparisons with the Neutralisation Test in Eggs

The Relative Sensitivities of the Methods

For assessing the relative sensitivities, 10 of the standard antisera were titrated against their homologous virus strains, using the beta (serum dilutions) method, as described in Chapter I, for the test in eggs. The results are shown in Table 14. The plaque reduction test gave a higher titre for every antiserum, and the mean difference in titre was 2.26, with a range of 0.6 to 4.7 and standard error of 0.385. This difference was highly significant. ($t = 5.87$, $P < 0.001$).

A Comparison of the Beaudette and Massachusetts-41 Strains as Test Viruses by the Two Methods

6 Of the standard antisera were titrated against both Beaudette and Massachusetts-41, using the beta method for the test in eggs. The results are shown in Table 15. The degree of dependence of the 2 variables (test virus strain and method) was expressed as the "overall difference". This figure was obtained by subtracting the titre in eggs from the titre in plaques, for each strain, and then subtracting the difference for Massachusetts-41 from the difference for Beaudette.

TABLE 14. Comparison of Homologous Titres of 10 Antisera by Plaque Reduction and Neutralisation Tests

Antiserum Batch No. and Virus Strain	Titres In: Eggs	Plaques	Difference (Titre in Plaques-Titre in Eggs)
As 43 Beaudette	7.3	10.6	3.3
As 32 Massachusetts-41	7.5	8.5	1.0
As 16 Connecticut	5.7	6.8	1.1
As 26 Iowa 609	8.7	11.6	2.9
As 21 Gray	9.3	9.9	0.6
As 18 Holte	8.1	10.3	2.2
As 37 T	10.9	12.8	1.9
As 40 227	8.9	11.3	2.4
As 44 591	8.7	13.4	4.7
As 41 604	8.5	11.0	2.5

TABLE 15. Comparisons of Titres of 6 Antisera against Beaudette and Massachusetts-41 by Plaque Reduction and Neutralisation Tests.

Antiserum Batch No. and Virus Strain	Test Viruses *	Titres In: Eggs (C)	Titres In: Plaques (D)	Overall Difference ø
As 43 Beaudette	(A)	4.7	4.8	3.2
	(B)	7.3	10.6	
As 32 Massachusetts-41	(A)	7.5	8.5	0.9
	(B)	4.7	6.6	
As 37 T	(A)	5.5	6.9	2.9
	(B)	4.3	8.6	
As 28 Houghton 140	(A)	6.9	8.3	1.0
	(B)	7.5	9.9	
As 40 227	(A)	6.5	7.0	1.4
	(B)	6.3	8.2	
As 41 604	(A)	5.7	6.7	1.6
	(B)	3.9	6.5	

* (A) = Massachusetts-41

(B) = Beaudette

ø Overall Difference = BD - BC - AD + AC

(Difference between the two strains of the excess titre in plaques over eggs).

The results were further analysed for significance by taking the mean differences in titre for the 6 antisera. These were as follows:

Virus Strain	Mean Difference in Titre (Plaques-Eggs)	Standard Error of Mean	Measures of Significance of Difference
Massachusetts-41	0.90	$\pm$ 0.210	$t = 4.29$, $P < 0.01$
Beaudette	2.73	$\pm$ 0.378	$t = 7.23$, $P < 0.001$

Also, the overall mean difference of 1.83 $\pm$ 0.401 was highly significant. ($t = 4.58$, $P < 0.01$).

B. APPLICATION OF METHOD

(1) Antigenic Relationships

The results of the main study on antigenic relationships are given in Table 16.

The 13 sera prepared against reference strains were fully tested against all 7 reference strains available as test viruses, but due to shortage of time it was necessary to omit about half of the possible combinations with the British strains.

TABLE 16. Plaque Reduction Titres obtained from Main Study on Antigenic Relationships

Antisera			Test Viruses											
			Reference Strains							British Strains				
Batch No.		Virus Strains Against Which Prepared	Beaudette	M-41	Connecticut	Iowa 609	Gray	Holte	T	Houghton 140	227	591	604	927
As 25	Reference Strains	Beaudette	7.0	7.0	5.5	3.5	3.5	3.7	3.5	6.5	6.9	-*	-	-
As 43		Beaudette	10.6	4.8	1.4	0	0.8	0.8	0.7	9.0	7.8	-	-	11.4
As 32		M-41	6.6	8.5	4.0	1.2	3.5	2.8	1.8	5.2	6.8	3.3	2.2	8.0
As 14		M-41	11.3	13.0	9.1	6.8.	8.6	9.4	5.7	-	-	-	-	-
As 15		M-41	7.8	10.6	5.8	4.5	5.0	4.5	4.0	-	-	-	-	-
As 16		Connecticut	0	0.5	6.8	0	1.1	0.3	0.5	0	-	-	-	0.7
As 39		Iowa 97	2.9	1.8	3.4	3.2	5.0	4.5	4.5	-	-	-	-	-
As 26		Iowa 609	4.7	5.3	5.3	11.6	6.8	6.6	4.8	4.5	4.3	-	-	-
As 21		Gray	5.5	4.0	4.9	3.6	9.9	9.4	4.0	-	-	-	-	5.8
As 18		Holte	5.0	5.4	3.4	3.5	10.3	10.7	4.0	-	-	-	-	-
As 36		SE-17	6.2	5.8	5.4	5.2	5.0	4.5	4.3	-	-	-	-	5.9
As 37		T	8.6	6.9	8.1	6.1	8.5	7.7	12.8	-	5.3	-	-	-
As 27		H 120	9.6	7.8	7.7	5.2	6.7	5.7	5.3	8.0	-	-	-	10.8
As 29	British Strains	Houghton 116	7.5	6.0	-	4.9	-	-	-	5.8	-	6.4	7.0	-
As 28		Houghton 140	9.9	8.3	6.5	5.5	6.0	6.1	5.1	9.4	-	-	6.3	11.0
(10.9.70)		Allen	9.6	8.4	6.6	2.7	5.0	-	5.1	-	-	4.4	5.4	-
As 51		183	-	-	-	-	5.4	-	-	-	-	4.8	5.1	-
As 45		225	8.5	6.6	8.1	6.0	9.5	-	7.2	-	-	8.4	7.8	-
As 40		227	8.2	7.0	5.8	3.3	5.0	4.8	4.3	8.3	11.3	4.3	5.0	-
As 42		317	3.3	2.9	4.7	2.8	4.3	4.1	3.3	-	-	-	-	-
As 49		317	-	6.1	-	-	6.3	-	-	-	-	6.4	8.0	-
As 46		551	-	2.8	-	-	4.0	-	-	-	-	4.6	5.3	-
As 44		591	8.3	7.2	8.6	7.0	7.6	8.7	8.0	-	5.0	13.4	9.7	-
As 41		604	6.5	6.7	4.6	7.7	6.4	6.0	4.8	-	-	5.8	11.0	-
As 48		690	-	-	-	-	5.9	-	-	-	-	4.4	5.3	-
As 47		860	-	7.4	-	-	7.1	-	-	-	-	7.2	8.0	-
As 22		927	4.5	3.3	2.5	1.0	1.4	1.0	0.4	-	-	-	-	6.0
As 50		927	-	10.1	-	-	6.0	-	-	-	11.0	6.4	6.6	14.6

* = Test not carried out.

(2) Diagnosis, Screening Tests and Vaccine Trials

Screening for IB of the Flocks at Lasswade and Compton

Before obtaining eggs from these flocks for work with IB virus, it was necessary to screen the flocks for IB antibody, among other tests. We employed a plaque reduction test in CK cells for this purpose, using Beaudette as test virus.

From Lasswade, 231 serum samples were tested, of which 209 were from the main flock, and 22 from a separate, subsidiary flock. Pools were prepared from groups of 10 birds by taking 0.1 ml. from each sample. The pools were tested at a dilution of -2 ($^1/4$) on a single petri dish each. The pools from the main flock caused no reductions, but the pools from the subsidiary flock both gave 100% reduction. 10 individual samples from this flock were then tested at dilutions of -2 and -3 ($^1/4$ and $^1/8$) on one dish each. 9 of these samples gave 100% reduction at both dilutions. 2 of these samples were then tested at dilutions of -5, -7 and -9, using 2 dishes per dilution. In both samples, plaques appeared only at -9. We concluded that the subsidiary flock had been exposed to IB.

The 2 latter samples were also titrated in eggs against Beaudette, using the beta method (See Chapter II) and starting at a dilution of -4. One sample gave a titre of -4, and the other gave a titre of less than -4. This comparison provided the first evidence of the greater sensitivity of the plaque reduction test.

A similar screening test was carried out on the Compton flock, with negative results. The same sera were also negative on the gel diffusion test.

A pool of sera from SPAFAS chicks was also negative, likewise a pool of sera from day-old Dalton chicks, which were tested with M-41 test virus.

Straightforward Diagnostic Tests

The plaque reduction test was used on a few occasions as an aid to diagnosis in the field.

A pooled serum sample received from abroad from a flock with respiratory disease and nephritis had a titre of 7.5 against Beaudette. (This sample also had a good positive titre on the CF test).

A pool of 8 samples from the Ministry's Experimental Husbandry Farm, Gleadthorpe, gave a titre >9.0 against Massachusetts-41.

Although perfectly effective for detecting the presence of IB-neutralising antibody, we felt that the plaque reduction test had no advantage over the routine neutralisation test in eggs for straightforward diagnostic tests of this kind.

Vaccine Trials

In collaboration with Dr. E. Gdovinova and Mr. W. H. Allan, three serological methods were compared for their usefulness in assessing the protection given by live IB vaccines as shown by a test challenge, and for their correlations with each other. The methods compared were the complement fixation test, the neutralisation test in egg-embryos, and the plaque reduction test in CK cells. This work is described in the paper attached.

It was concluded that the plaque reduction test was the most sensitive method, as shown by giving the highest rate of serological response to vaccination, and significantly higher titres than the neutralisation test in eggs. Its cost in labour and materials was similar to the test in eggs, but of course very much higher than the complement fixation test. As with the other serological tests, correlation with challenge results was very poor for individual birds, but acceptable on a group mean basis.

(3) The Antibody Response to Infection

The plaque reduction test was applied, to a limited extent, to studies on the antibody responses of individual birds to infection with two strains of IB virus, Houghton 140 and T. The main object of the experiment with Houghton 140 was to study the complement fixing antibody reaction, and these results were given in Chapter IV, Results, (7). Certain key samples were titrated by plaque reduction test against M-41 and T. These results are shown in Table 17.

TABLE 17. Plaque Reduction Serum Titres of 8 Individual Chicks after Experimental Infection with Houghton 140.

Bird No.	Weeks Post-Infection	Titre Against M-41 Test Virus	Titre Against T Strain Test Virus
1	2	4.7	- *
	2½	6.3	3.7
	3	8.1	-
	11	-	7.7
	15	11.1	-
2	6	6.4	-
	11	-	6.1
	15	10.6	-
3	2½	3.4	-
	15	10.9	-
4	2	3.1	-
	15	9.1	-
5	2½	3.0	-
	3	5.9	-
	11	7.2	-
6	2	5.1	-
	2½	6.6	-
	4	9.0	-
	7	-	3.1
	15	11.1	-
7	2	2.7	-
	3	-	3.7
	15	6.4	-
8	3	-	3.7
	11	7.7	4.6

* = Test not carried out.

These results were compared with the CF titres against Beaudette antigen in Table 8, and the following conclusions were made:

(1) At 2 and 2½ weeks post-infection, the plaque reduction titres were lower than the CF titres, but at 15 weeks they were higher, except for birds 4 and 6, which had persistent high CF titres.

(2) The plaque reduction titres rose steadily, while the CF titres gave an early peak at 3 weeks post-infection, then fluctuated widely.

(3) The titres against the T strain were significantly lower than against M-41, as expected from our studies on the standard antisera, that were prepared as pools. (In other words, type differences are shown by individual sera as well as by pooled sera).

An unexpected, additional observation was made on the slope of the percentage reduction curve. The sera collected at 2 weeks post-infection gave steeper slopes than the later sera. Below 50% reduction, the slope increased so much that the line became nearly vertical. (See Fig. 5). Following the theories of Burnet et al (1937) mentioned in Chapter I, Section (5), this effect was interpreted as indicating a slower reaction rate of the early antibody, with consequent failure to reach equilibrium. It gave further support to our choice of a 75% end-point, since in this region the line became more linear, and the slope less steep.

The main object of the experiment with the T strain was to study the relationship between the type titre and the group titre for individual birds, and in particular, possible changes in their values with time after infection. The type titre was measured by titrating the sera against T test virus, and the group titre by titrating them against M-41 test virus. It was also planned to titrate the sera against T and Beaudette antigens on the CF test. This work was not completed due to lack of time and the giving of priority to other work. The CF test results have been given in Chapter IV, Results, (7).

The plaque reduction titres are shown in Table 18.

TABLE 18. Plaque Reduction Serum Titres of 8 Individual Chicks after Experimental Infection with T Strain.

Bird No.	Titres, at Times after Infection									
	2 Weeks		$3\frac{1}{2}$ Weeks		6 Weeks		13 Weeks		19 Weeks	
	T	M-41	T	M-41	T	M-41	T	M-41	T	M-41
1	-*	1.1	-	-	-	-	-	-	(Died at	15 weeks)
2	-	0.9	-	-	-	-	-	-	(Died at	15 weeks)
3	7.8	0.3	9.3	0.4	8.6	-	10.8	4.4	(Died at	14 weeks)
4	8.0	2.0	10.3	1.5	8.0	2.4	9.4	3.6	-	8.2
5	5.6	1.0	8.4	0.0	10.2	3.4	12.3	5.0	(Died at	$15\frac{1}{2}$ weeks)
6	4.6	0.9	8.0	1.9	9.7	4.2	12.7	6.5	>18	9.1
7	(Found dead on 14th day after infection)									
8	8.0	1.5	9.1	2.7	12.1	5.1	11.4	6.3	16.3	7.4

* - = Test not carried out.

Birds 7 and 8 were male, the others female, of Lasswade stock.

Clinical symptoms and post-mortem findings were observed. During the week after infection, respiratory signs were observed in all birds except Nos. 5 and 8. These signs were mild and transient, except in No. 7, which was found dead on the 14th day. This bird had acute nephritis, with no calculus or urate formation, and no lesions in the respiratory tract, at the time of death. At $3\frac{1}{2}$ weeks after infection, definite respiratory signs were observed in No. 8, but in none of the other chicks. All the chicks then entered a period of good health and rapid growth. The deaths which occurred at 14 and 15 weeks were very sudden. The 4 birds which died at this time all had identical post-mortem findings. The kidneys were swollen and congested, but calculus and urates were absent. The livers were yellow. The three birds still alive at 19 weeks were still in very good health when killed shortly after. The post-mortem examinations showed no abnormality

except that birds 4 and 6 had yellow livers. There was nothing to suggest that an intercurrent infection had occurred. The sera collected at 19 weeks were negative to the Newcastle disease haemagglutination inhibition test. We think it likely that the cause of the deaths was the IB virus strain they were inoculated with. There was an extraordinary build up of the antibody titres between 13 and 19 weeks post-infection, suggesting continued presence of the virus, and there may have been allergic reactions of the antigen-antibody complex type, perhaps attached to the kidney tubule epithelium and leading to necrosis of the tubules. This explanation is of course only speculative. Although very interesting, we could not follow up these findings for fear of losing time on the main projects.

The steady increases of the type titres were accompanied by increases of the group titres. The relationship between the two remained about the same throughout the period of observation, and for each bird. The standard antiserum As 37 (T), prepared from pooled sera, had a similar relationship (T = 12.8, M-41 = 6.9) although it was prepared in White Leghorn type chicks, which had more severe respiratory symptoms and no nephritis. This consistency of the relationship between type and group titre suggested strongly that it was due to the inherent antigenic composition of the virus strain, and not to differences in host responses or immunoglobulin classes. It was obvious that the group reaction was not particularly associated with early antibody as it is for many other virus species where types are recognised; or for that matter with late antibody, to which the virus might have been more attracted by higher avidity.

DISCUSSION

(1) Antigenic Relationships

Four main conclusions were reached from the results in Table 16. These were:

(1) The existence of a group of strains closely related to Massachusetts-41 was confirmed. The corollary of this statement was that several other strains existed that were different from the Massachusetts group.

(2) These "different" strains usually differed from each other to the same degree as they differed from the Massachusetts group. Each of these strains therefore had equal standing as a "type".

(3) Each antiserum displayed two titres: its "type titre", a higher titre obtained when titrated against the homologous virus strain, or strains of the same type; and its "group titre", a lower titre obtained when titrated against strains of different type. These values varied greatly for different antisera, in both their absolute and relative values.

(4) Because of the "group titre" effect, it was not possible to quantify antigenic relationships. If the strains were "different", the degree of difference that was manifested depended on the relative values of the group and type titres of their antisera. Thus, strains which produced antisera with relatively high group titres would, on those results, appear to be fairly closely related to all other IB strains, whereas strains which produced antisera with relatively low group titres would appear to differ greatly from all "different" strains. In this situation, it was only possible to classify strains broadly as "similar" or "different". This is not to say that minor antigenic relationships could not be recognised (two examples are discussed below), merely that degrees of relationship could not be quantified.

Fortunately, the quality of antigenic relationships, that is, whether any two strains were similar or different, was very constant. These main conclusions are expanded below:

The group of strains closely related to Massachusetts-41 were clearly identifiable as the "Massachusetts Type" first recognised by Jungherr et al (1956) and since described by many later authors who used the neutralisation test in eggs. (Chapter I, Section 1). The following strains were found to fall within this group: Beaudette, H 120, Houghton 140, Allen, 227 and 927. The following reference strains were confirmed as being different from the group: Connecticut, Iowa 609, Gray, Holte and T. The British strains 591 and 604 were also different. Two-way tests were not carried out for the other British strains, but if the third conclusion, concerning "group" and "type" titres, was universally true, then there were strong indications that several of these other strains were also different from Massachusetts, as were the reference strains Iowa 97 and SE-17.

Outside the Massachusetts group, only two strains were found that had any marked relationship: Gray and Holte. A type classification of the usual kind would therefore be very unbalanced, since it would consist of on the one hand a fairly large group of similar strains, forming the Massachusetts type, and on the other hand a large number of less common types, many of which are at present represented by only one isolate.

The titres obtained with several antisera against a range of test viruses are shown as histograms in Figs. 6-9. The contrast between the type and group titres is demonstrated strongly by the Iowa 609, T, Houghton 140, and 591 antisera. With the Beaudette and Connecticut antisera, the group titre was very low, almost undetectable. With H 120 it was so high relative to the type titre that it would be difficult to recognise the type from these results alone. These extreme situations were unusual. With most antisera the group and type titres were readily recognisable.

Minor antigenic relationships probably contributed to the small variations in the value of the group titre of an antiserum obtained when it was titrated against various test viruses of different types. For example,

in Table 16, the group titre of As 32 (Massachusetts-41) is represented by the range of titres from 1.2 (given against Iowa 609) to 4.0 (given against Connecticut). A possible explanation is that the Connecticut strain is somewhat less different from Massachusetts-41 than is Iowa 609.

A minor antigenic difference between Massachusetts-41 and Beaudette was observed, confirming the findings of Chomiak et al (1963). This difference was seen most clearly in the titres obtained with As 43 (Beaudette) against the two virus strains. (Beaudette: 10.6; Massachusetts-41: 4.8)

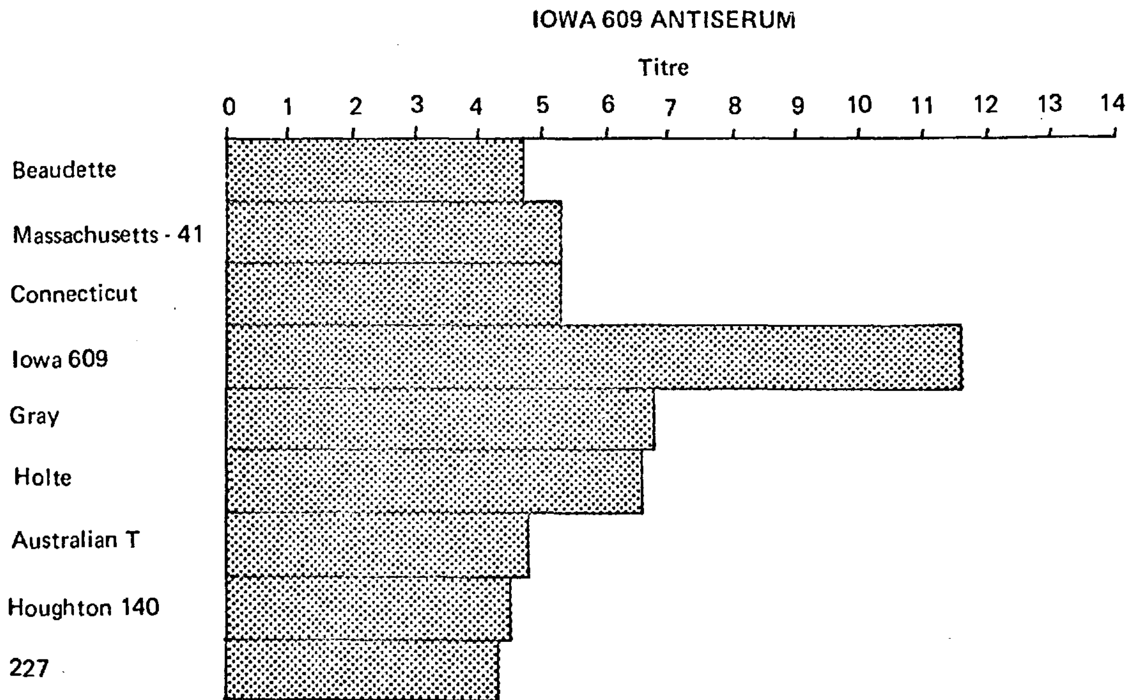
(2) The Significance Of Group And Type Titres

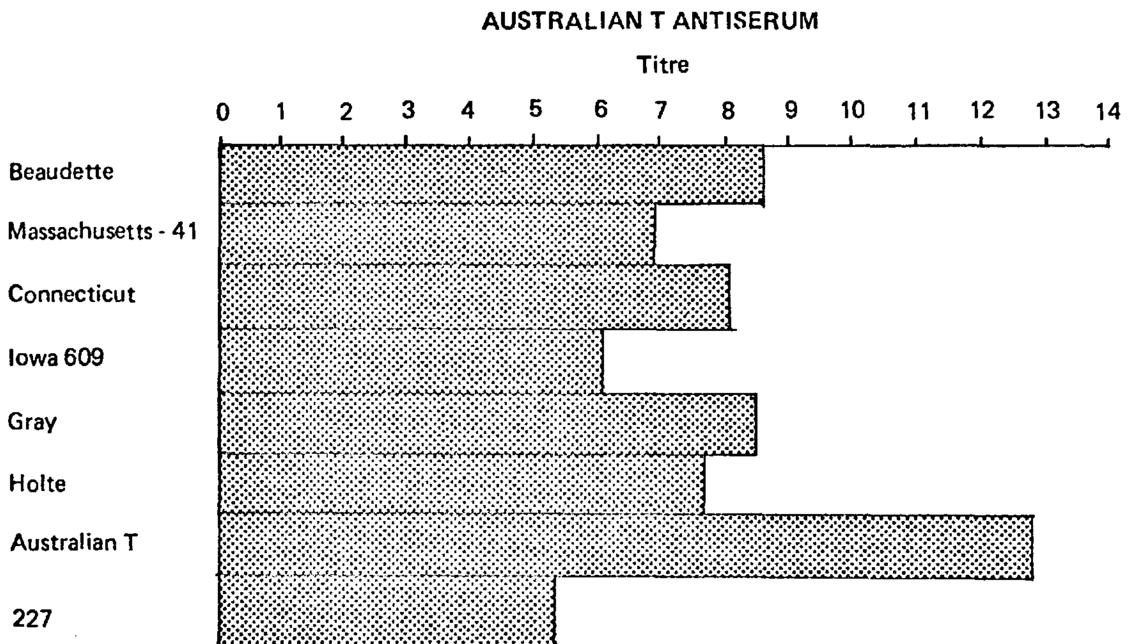
To the best of our knowledge, the group titre effect that we observed in IB antisera titrated by the plaque reduction test had not been described before. High degrees of cross-reaction have often been reported by previous authors, using the neutralisation test in eggs or cross-immunity tests (Hofstad, 1961; Winterfield and Hitchner, 1962; Winterfield, Cumming and Hitchner, 1964; Dawson and Gough, 1971), but a uniform pattern of the kind we have described has not been recognised. We considered it to be a new observation that had resulted from the application of a new method. (Bracewell, 1973b). Its implications are discussed in this section.

Was the group titre effect due to specific antibody against IB virus? There is a considerable weight of evidence among the results in Table 16 to support this. Non-specific neutralisation of IB virus was found to be particularly low in the plaque reduction test. (Results, section A (3)). In contrast, the group titres of several antisera were as high as 7 or 8 (corresponding to serum dilutions of $^1/_{128}$ or $^1/_{256}$). The remote possibility that the effect could be caused by inadequate heat treatment was ruled out by the fact that the titres shown for each antiserum were usually obtained from tests performed on separate occasions, which frequently involved the inactivation of fresh aliquots or the re-inactivation of the same aliquot. To explain the group titres as non-specific, one would need to imagine that

PLAQUE REDUCTION TITRES AS HISTOGRAMS

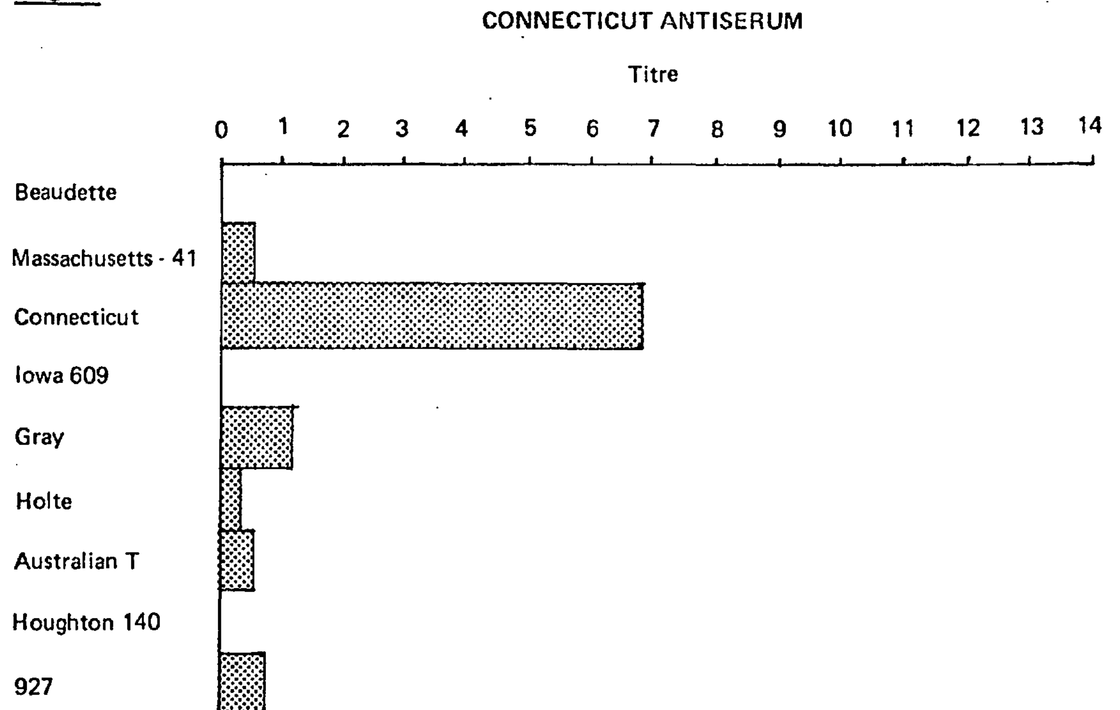
Fig. 6

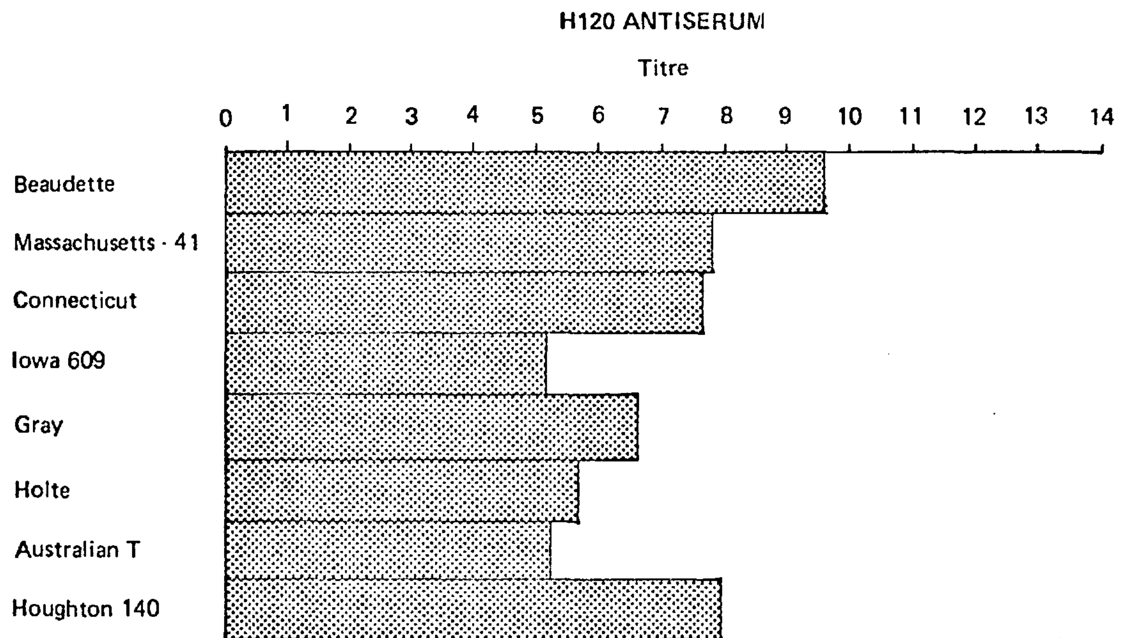




PLAQUE REDUCTION TITRES AS HISTOGRAMS

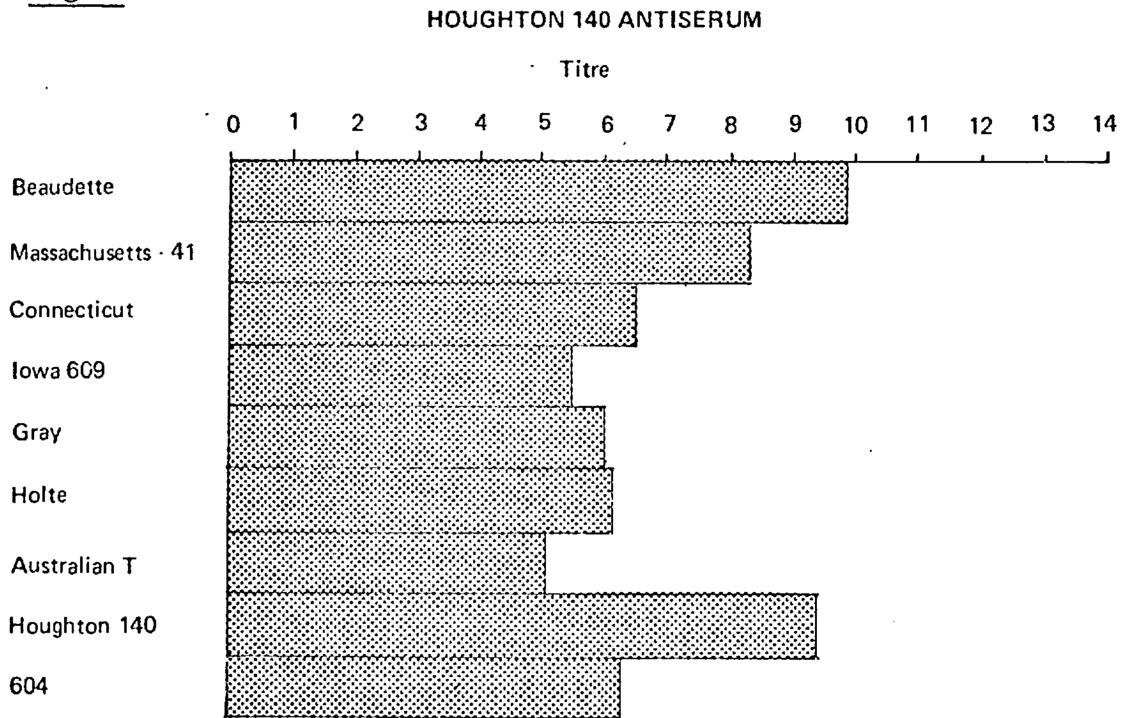
Fig. 7

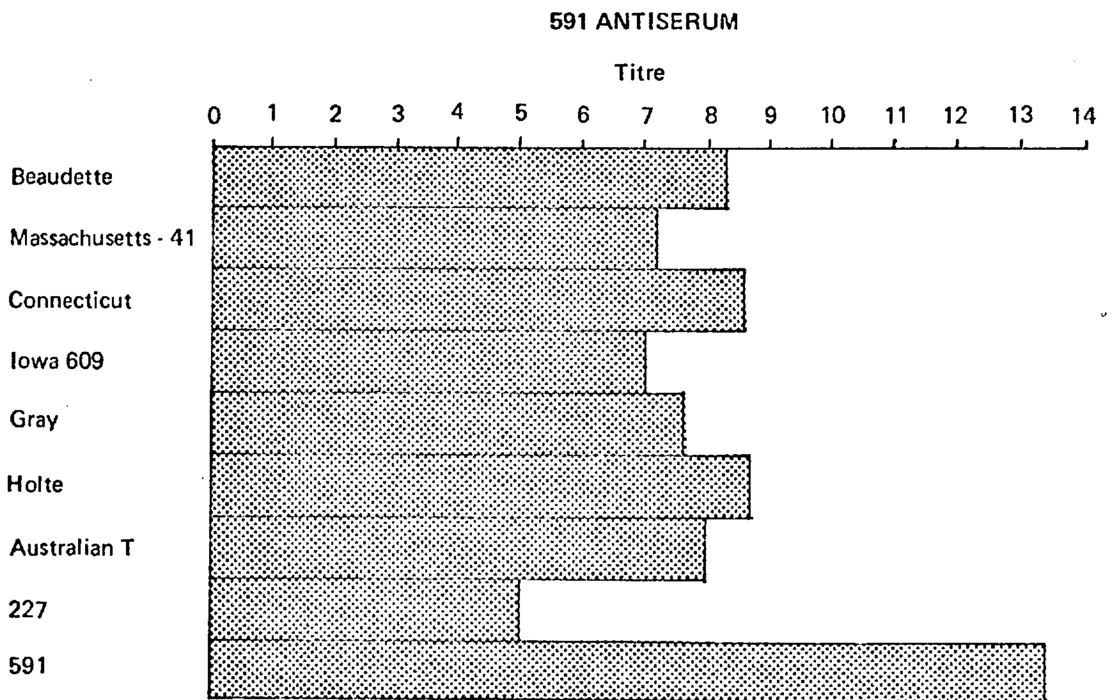




PLAQUE REDUCTION TITRES AS HISTOGRAMS

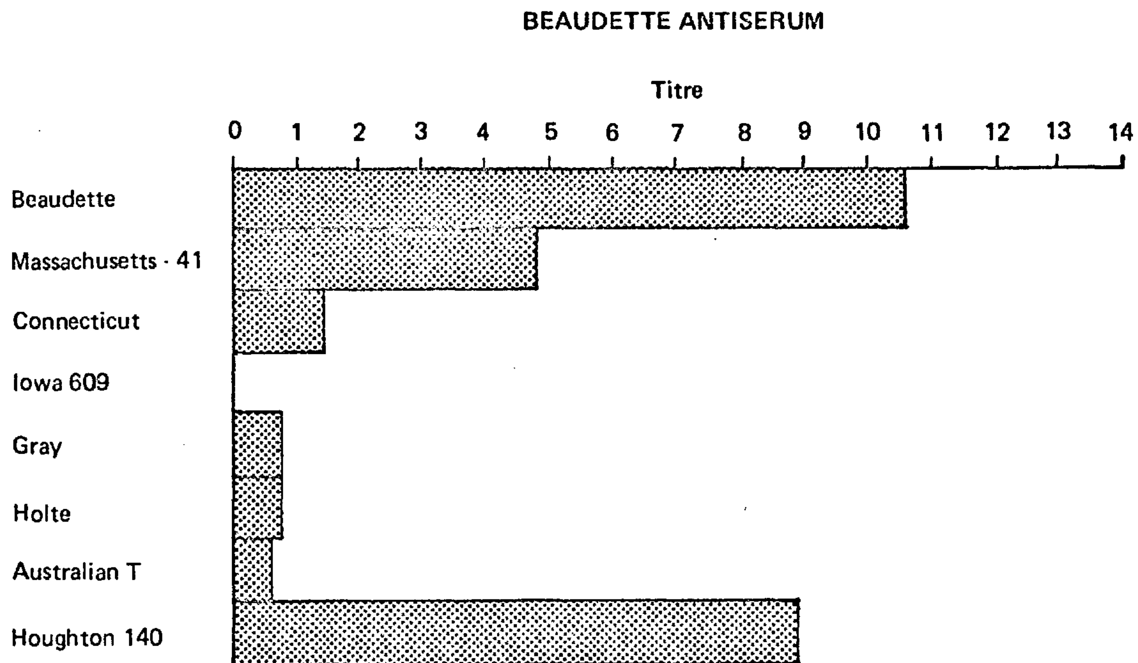
Fig. 8





PLAQUE REDUCTION TITRES AS HISTOGRAMS

Fig. 9



a non-specific neutralising factor, resistant to heating at 56°C for periods of 30 minutes, could occur in the serum of chickens that had been inoculated with IB virus, (but not in the serum of SPF chickens, or chickens that had been inoculated with Newcastle disease virus, infectious laryngotracheitis virus or avian adenoviruses) to titres that, for some antisera, exceeded the homologous titres of some other IB antisera. This is plainly much less likely than the simple view that the group titres were due to IB antibody.

Complete proof that this was so would have required the performance of satisfactory cross-absorption tests. The feasibility of absorbing out antibodies to IB virus was considered. Large quantities of purified, concentrated virus would have been required, from several different virus strains. Our experience of preparing such materials for use as antigens in the CF test led us to expect that even this first step would not be easy. The fragility of the virus, with its delicate-looking corona, was not encouraging. Although Berry and Almeida (1968) were able to obtain electron photomicrographs of IB virus-antibody complexes, the possibility of structural changes during ultracentrifugation was very real. The paucity of reports in the literature of work of this kind with IB virus tended to confirm our impression of special difficulties. Supposing that significant reductions in titre could have been achieved, we would have been very fortunate to obtain such a clean separation of group from type antibody that a firm conclusion could be reached from just one or two experiments. It seemed much more likely that we would need to repeat the experiments, and to use a variety of virus strains and antisera, because of the large differences in their characteristics that we have noted. Once studies of this kind had started to yield results, it would have been essential to continue until a definite conclusion had been reached. In view of the limited time available, and the weight of indirect evidence supporting the specificity of the group titres, we decided not to attempt cross-absorption tests.

It seemed more profitable to accept provisionally that the group titre effect was due to a specific antibody, or antibodies, reacting with an antigen, or antigens, held in common by all strains of IB virus, and to spend the little time available in considering the implications of this assumption.

A striking feature of the group titres was their great variation in value between antisera. For example, (from Table 16), the Houghton 140 antiserum As 28 had a homologous titre of 9.4 and a mean group titre of 5.9 (range 5.1 to 6.5), while the Beaudette antiserum As 43 had a homologous titre of 10.6, but a mean group titre of only 0.7 (range 0.0 to 1.4). If the common antigens theory was provisionally accepted, this variation suggested that there might be a corresponding variation in the ability of different virus strains to stimulate antibodies to the common antigens. This variable ability might be due to variations in their relationship with the host, such as their virulence, or their tissue tropisms. It might be due to variations in their morphology; either in the amount of common antigens present, or in the degree to which the common antigens were exposed. The contrast between the group titres of the Houghton 140 and Beaudette antisera was explained equally well by the differences in either virulence or morphology of these virus strains, since the Beaudette strain had long been known to be avirulent and of low immunogenicity, and had recently been observed by us to have no corona whatever (Chapter IV, Discussion, section (2)), while the Houghton 140 strain was a typical IB virus in both virulence and morphology. (McDougall, 1971).

In order to study the variations in morphology of virus strains, and to compare the correlations with group titres of morphology, virulence and number of egg passages, electron microscope studies were made of 12 strains, selected to cover a wide variation in these properties. (Harkness and Bracewell, 1974.) The preparations presented for elctron microscopy were allantoic fluid harvests that had been ultracentrifuged through 22% potassium

tartrate by the method used for preparation of antigens for the CF test. (Chapter IV, Materials and Methods, section (4)). This study confirmed that there were large variations between IB virus strains in morphology. The most striking variation was in the proportion of virions bearing a corona, and again the Beaudette strain appeared to be completely lacking in a corona. There appeared to be a better correlation between corona and group titre than between any of the other variables considered. It was appreciated that further experiments were required to establish the validity of a direct association between corona and group antigens.

Some aspects of the results of the plaque reduction test (Table 16) were still difficult to explain by a simple theory that the group antigens were carried on the corona. Strains lacking a corona, such as Beaudette and Connecticut, certainly seemed to give rise to antisera with very low group titres, but how could one explain their neutralisation by antisera of different types? Regarded in this direction, as test viruses, they behaved similarly to test viruses, such as Houghton 140 and T, that had a well-developed corona. One explanation could be that strains such as Beaudette regain their corona when passaged in cell culture. We have a small piece of evidence that this may be so. Electron microscopy of the harvest of the second Beaudette passage in CK cells showed some virions with a typical corona. However, a more complex explanation probably fits all the facts more neatly. This is that the viral antigens of all IB strains can potentially be borne either on a corona or on the surface of the envelope. Immediately after separation of the virus particle, by budding from the membrane of the endoplasmic reticulum, it is very rare to see a corona, and it seems a reasonable deduction that the corona is formed at a later stage in maturation. The corona may be formed by an outward, flowing movement of viral proteins. The rigidity of the envelope may be maintained by the host membrane, formed like a basket, in the holes of which lie the semi-fluid viral proteins.

To take this speculation to its logical conclusion, it can be imagined that the corona has a function in ensuring survival of the virus in its natural environment. For example, it may assist in attachment to the ciliated epithelial cells of the respiratory tract. Perhaps if the virus is passaged in the allantoic cavity the corona becomes unnecessary. Virus strains that have the genetic potential for earlier maturing would then change under selection in that direction. The Beaudette strain may be regarded as an example of the ultimate stage in this change, in which the virus is released into the allantoic fluid at an extremely early stage, at which it is fully infective for cells of the allantoic cavity, but has no tendency to develop a corona. The early death of the embryo then selects against the release of any slow maturing elements of the virus population. Even the Beaudette strain still retains the potential to develop a corona when it is made to replicate in a different host system, such as chicken kidney cell culture, as we have seen.

Another area of speculation concerns the position and significance of the type antigens. In strains lacking a corona, they are presumably carried at the surface of the envelope, like the group antigens. Their number and distribution may be such that when a chicken is inoculated with particles having this structure, it tends to form antibodies much more strongly against the type antigens than against the group antigens. But where are the type antigens in a particle with a corona? From the results (Table 16) it appears that strains with a corona, such as Houghton 140, T and H 120, stimulate in chickens antibodies against both type and group antigens. It may well be that both kinds of antigens are borne on the corona, but the altered spatial relationships increase the relative immunogenic effect of the group antigens. For instance, one may visualise the formation of a projection as occurring by a process of eversion, the group antigens being folded inwards on the original envelope surface, and outwards on the corona.

This brings us to a discussion of the significance of type differences in IB. Like other authors who have recently applied the plaque reduction test to IB typing (Hopkins, 1974; Cowen and Hitchner, 1973; Cowen, 1974), we consider it to be the most promising method available for elucidating this very confusing picture. Moreover, in spite of our finding of a constant pattern in the cross-reactions (the group titre effect), our own results in no way invalidate the numerous reports of genuine antigenic differences. (Chapter I, section 1.) On the contrary, all of the standard reference strains that we studied which were reported as different proved to be so in our hands, except for Gray and Holte, which appeared to be alike, although different from the others.

However, certain aspects of our findings lend support to the view that the importance of antigenic differences has been exaggerated by the application of laboratory techniques to IB, over the years since it was first cultivated. The highest degree of cross-neutralisation was observed in antisera against virus strains that had both a full corona and high virulence, that is, those nearest to the wild form. The most type-specific antiserum was produced with the most artificial strain, the Beaudette, having no corona and no virulence. Between these extremes, most strains fitted roughly into a spectrum of cross-reaction in which a general association could be discerned between group antigen, corona, virulence, and low number of egg passages. A notable exception to this rule was the vaccine strain H120. This strain appeared to have retained its corona, its group antigen, and, at least when experimentally inoculated, its virulence, in spite of its high number of egg passages. Could this be the reason for its success in the field?

The widespread use of live vaccines may have encouraged the emergence of new antigenic types. These vaccine strains have all been passaged extensively in eggs. It is possible that many of them have lost their corona, and so are deficient in stimulating group antibody, and, perhaps, by inference, group

immunity. They may then appear to protect well against a homologous type challenge, but not against heterologous types. This would lead to the selection of different antigenic types in the field. The increasing number of reports from the U.S.A. of vaccine breakdowns caused by new antigenic types (Winterfield et al, 1971; Cowen et al, 1971; Johnson et al, 1973) may be explained by the long use of vaccines in that country. In countries in which vaccines have not been used, natural infection will produce a broadly-based immunity, not conducive to antigenic variation. To go even further back, if the husbandry is primitive enough to allow mixing of young chicks with adults, then there will probably be frequent exposure of the chicks to infection at an early age, when they will carry maternal immunity, and they may develop active immunity without suffering from severe disease. The association of IB as a disease problem with intensive forms of husbandry is well known. However, since intensive husbandry must continue, because of its economic advantages, and to prevent infection from reaching birds in intensive units is still too difficult and expensive, protection by vaccination must continue to be sought. The right approach to the problem of antigenic variation is to seek vaccine strains that will confer a broad spectrum of immunity. Unfortunately this property in IB strains is often linked with virulence. However, there are signs that this linkage is not inevitable. As we have found with the plaque-reducing antibody, a broad spectrum of immunity may also be more closely associated with the presence of a full corona than with virulence.

(3) Comparisons Of Serological Methods

Unlike the complement fixation test, which detects an earlier, distinctly different, antibody, the plaque reduction test detects the same antibody as the neutralisation test in eggs. There is however a difference in sensitivity: plaque reduction titres are on average about two dilutions

higher than egg titres, as shown in Table 14. There is also a difference in the results obtained with particular virus strains, as shown in Table 15. This effect may be associated with the large differences in degree of adaptation to the egg assay system shown by IB virus strains. The amount by which the plaque reduction titre exceeds the egg titre is greater for the highly adapted Beaudette strain than the less well adapted Massachusetts-41 strain. Expressed another way, the Beaudette strain has a depressing effect on serum titres when used as a test virus in eggs, which it does not have in plaques. This fits in with the report of Box and Barnes (1967) that the use of a Beaudette strain for testing vaccinate sera had a lowering effect on their titres. It also agrees with the findings of Von Bülow (1967b) on the effect of degree of adaptation of test virus on the slope of the regression line. (See Chapter I, Section (5)).

When combined with its low experimental error, these features of the plaque reduction test give it a clear advantage over the neutralisation test in eggs for the study of antigenic relationships. It is doubtful whether the phenomenon of the group and type titres of antisera would have been recognised without the use of the plaque reduction test, although it is logical to assume that a similar effect is present underlying the results of the egg test, but obscured by other influences.

The plaque reduction test has, however, two disadvantages that limit its usefulness. One is the technical difficulty of maintaining the supply of suitable cell cultures, which should not be underestimated. The other is the difficulty of adapting some virus strains to the cell system. This tends to limit the number of strains that can be studied. The number chosen for these studies turned out to be more than could be fully adapted, so that some strains could only be examined by one-way tests on their antisera.

For straightforward diagnostic tests, eggs are probably preferable

because of their technical simplicity. The complement fixation test has a considerable advantage for this purpose because of its greater speed and economy. (See Chapter IV).

The higher sensitivity of the plaque reduction test may make it more useful than the egg test for flock screening or monitoring for IB. A positive sample should be more easily detected among a large pool of sera. Also, the test can be abbreviated to one petri-dish per serum pool initially, which makes it very economical if large numbers are to be tested.

Because of its high reproducibility, the plaque reduction test could play a useful part in vaccine trials, for making critical comparisons of antibody responses. However, the lack of correlation between the serum titre of an individual bird and its degree of protection, as shown by the degree of suppression of symptoms after challenge, must be borne in mind when interpreting serological responses in vaccine trials.

For studies on the immunology of IB, the plaque reduction test should be particularly useful, by virtue of its high sensitivity and reproducibility.

SUMMARY

After a brief examination of alternative cell systems, primary kidney cell cultures from chickens 4-5 weeks old were selected for the plaque reduction test. The growth medium selected was Hanks-yeast-lactalbumin with 5% tryptose phosphate broth, 10% foetal calf serum, and hepes buffer at 15 mM. Passaging of virus strains was varried out in 1 oz. medical flat bottles, and plaque assays in small, disposable plastic petri-dishes. Serum titrations were performed by allowing a series of 2-fold dilutions of serum to react for 30 minutes with a constant virus dilution that was calculated to give 40 plaques per dish. The percentage reductions in count at each dilution, when compared with the virus controls, were plotted on probability paper, against

$\log_2$ reciprocals of the serum dilutions, and the best straight line drawn by eye. The serum titre was read off at 75% reduction, and expressed as the $\log_2$ reciprocal, to the first decimal place.

7 Reference strains and 5 British strains of IB virus, that had been previously adapted to growth in eggs, were passaged in the cell cultures until they gave readable plaques, then used to titrate the antisera to 10 reference strains and 13 British strains, in order to study their antigenic relationships. The lack of similarity between the reference strains was confirmed, and also the existence of a number of strains similar to Massachusetts-41, as described by previous authors. At least 2 British strains were found that were as distinctly different as the reference strains.

Each antiserum was found to be capable of reacting at one of two distinct titres, depending on its antigenic relationship to the test virus. With a similar test virus it gave a higher titre, called the "type titre", and with all different test viruses it gave a lower titre, called the "group titre". The relative values of these 2 titres varied for different antisera, and seemed to be characteristic of the virus strain used to prepare the antiserum. There was possibly an association between the proportion of virus particles bearing a corona and the size of the group titre of the antiserum prepared.

Compared with the neutralisation test in eggs, the plaque reduction test was more sensitive, giving serum titres about 2 dilutions higher on average, and probably less affected by variation in degree of adaptation of test viruses. Applications in which these features were advantageous included flock screening tests, vaccine trials, and studies on the antibody response to infection.

GENERAL SUMMARY

A survey of the literature on antigenic relationships between strains of IB virus indicated a confused situation, in which the existence of antigenic differences was recognised, but, in spite of the importance of this finding to methods of diagnosis and control, no system of classification had emerged, and the significance of the differences was poorly understood. The serological method that had been most extensively applied to the problem was the neutralisation test in eggs. The reasons for this were the necessity for eggs to be used for the initial isolation of IB virus and its adaptation for easy handling in the laboratory, and the convenience and historical importance of the egg assay system. In view of the known limitations of the method, and the unsatisfactory state of knowledge that had resulted from its application to the problem, it was decided to examine alternative serological methods. The primary object was to be an examination of their suitability for defining antigenic relationships, and the secondary object was to be a study on their general applications, in diagnosis, vaccine trials and research on the disease and the virus.

Three methods were selected for study: the haemagglutination test, the complement fixation test, and the plaque reduction test. Preliminary studies of the alternatives within these basic methods narrowed the selection further to: the indirect (tannic acid) haemagglutination test, the direct complement fixation test on heat-inactivated chicken sera, and the percentage plaque reduction test in chicken kidney cell cultures.

Reference strains and British isolates were obtained from previous authors, and cloned by means of three successive passages at limiting dilution in SPF eggs. Antisera were prepared in SPF chickens, preferably by means of a single inoculation of the respiratory tract, only using a second inoculation when the serum titre resulting from the first was inadequate for study.

The tannic acid haemagglutination test was found to be unsatisfactory as a technique for IB virus, and the studies on this method were discontinued at a fairly early stage of the work, in order to devote more time to the other methods.

The complement fixation test was found to give reproducible results when applied to heat-inactivated chicken sera, provided that fixation of the guinea-pig complement was implemented by mixing it with unheated normal chicken serum. When adapted to the "Microtiter" system, this test was found to be a practical method for rapid titration of large numbers of serum samples, and it should prove useful in diagnosis, vaccine trials, and research. It was also a practical method for titrating viral antigen after its exposure to physico-chemical methods that destroyed the infectivity, and should prove useful in investigations of virus structure and properties. It was less satisfactory when applied to antigenic relationships. There were technical difficulties in preparing satisfactory antigens from many of the virus strains which were to be studied, and the cross-fixation titres between strains of different antigenic type were not sufficiently reproducible to be of much help in defining relationships. Improved methods of preparing and purifying antigens would be required, and the preparation of antisera defined more exactly for the purpose of the complement fixation test, before the method could be suitable for this purpose.

The plaque reduction test was found to give highly reproducible results from both homologous and heterologous reactions. Its suitability for defining antigenic relationships was better than that of the neutralisation test in eggs. This was shown by its ability to confirm the main conclusions already reached by use of the egg test, (namely the existence of a large group of strains similar to Massachusetts-41, and several distinctly different reference strains,) and furthermore to demonstrate for the first time a constant pattern in the cross-reactions, the effect of which was to increase confidence in, and

illuminate the nature of, the antigenic differences. The titre given by any particular antiserum when titrated against virus strains of different type was found to be sufficiently constant to be recognised, and was named the "group titre". This titre was usually considerably lower than, and therefore easily distinguished from, its titre when titrated against virus strains of the same type, or the homologous strain, which was called the "type titre".

The relative values of the group and type titres varied greatly for different antisera, and seemed to depend more on a characteristic of the virus strain used to prepare the antiserum than on any other factors considered, such as time of collection after inoculation, or whether a second inoculation had been given. The possibility that this variation may be associated with variation in the morphology of the virus strains was investigated, by electron microscopy of twelve strains for which titres of antisera were available. There was some association shown between the proportion of virus particles bearing a corona and the value of the group titre. More direct studies on antigenic components of the virus would be necessary to prove this association.

Compared with the neutralisation test in eggs, the plaque reduction test was found to be more sensitive, giving titres that were on average about two dilutions higher, and less affected by differences in degrees of adaptation of test viruses. It is however less easily applied to new virus strains, because adaptation of a strain to chicken kidney cells can apparently only be achieved after preliminary adaptation to eggs, and not all strains are suitable even then. This may prove to be a limitation on the use of the test for typing new isolates, since the degree of reliance that can be placed on one-way tests, where convalescent sera are tested against a limited range of standard test viruses, is probably not high. Perhaps a critical re-appraisal of the egg test for this purpose, backed up by information from the plaque reduction test about group and type titres of sera, may be more likely to lead to a practical typing test for the field. However it is suggested that the plaque reduction test offers a sounder basis for a type classification system for IB virus.

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BW

Appendix 1. ALSEVER'S SOLUTION

Sodium Chloride	0.42 gramme
Sodium Citrate	0.8 gramme
Glucose	2.05 grammes

Made up to 100 ml. with distilled water, and pH adjusted to 6.1 by addition of 10% citric acid solution.

Sterilised by autoclaving at 10 pounds per sq. inch for 15 minutes.

Appendix 2. VERONAL-BUFFERED SALINE

X5 Stock Solution

Sodium Chloride	850	grammes
Di-ethyl Barbituric Acid	57.5	"
Sodium Diethyl Barbiturate	37.5	"
Magnesium Sulphate	20.36	"
Calcium Chloride	2.94	"
Distilled Water	20	litres

This stock solution was kept at $4^{o}C$, and used at a $^{1}/_{5}$ dilution in distilled water, in these studies. (In Diseases of Breeding Department the stock solution was mixed with 4 times its volume of 0.04% Difco bactogelatin to make the working strength.)

The barbituric acid and calcium chloride dissolve very slowly in cold water, so it is usual to dissolve them separately in hot water (boiling for the barbituric acid) before adding the other components.

Appendix 3. DILUENT FOR CF TEST

CF Test diluent tablets, Code BR16, manufactured by Oxoid Ltd., London, S.E.1., were used. One tablet was dissolved in 100 ml. of warm distilled water. This solution was kept at 4°C, for use within the next few days. The formula, according to the manufacturers, was:

Barbitone	0.575	grammes per litre
Sodium Chloride	8.5	" " "
Magnesium Chloride ($6H_2O$)	0.168	" " "
Calcium Chloride	0.028	" " "
Barbitone Soluble	0.185	" " "

(Expected pH = 7.2)

Appendix 4. DENSITY OF POTASSIUM TARTRATE SOLUTIONS

Reeve and Alexander (1970)*, in studies on the isopycnic separation of Newcastle disease virus from allantoic fluid harvests, found that many of the particles smaller than entire virus particles that were present in such harvests had a density of about 1.14. Arguing that similar particles were probably present in IB virus harvests, it was decided to select a concentration of potassium tartrate that would be expected to just exclude them. The figures below are taken from International Critical Tables of Numerical Data, Physics, Chemistry and Technology (1928) 3, 91. (McGraw-Hill Book Co., U.S.A.)

%	d^{20}_{4}
18	1.1236
20	1.1387
22	1.1540
24	1.1696
26	1.1855
28	1.2017

22% was selected from this table. These percentages are weight/weight, so the procedure for making up the solution was to dissolve 22 grammes in distilled water and then make up to 100 grammes of final solution.

Since the solution supports well the growth of contaminants, it should be made freshly, or stored at 4°C for not more than a few days.

* Reeve, P. and Alexander, D. J. (1970).
Isopycnic separation of Newcastle disease virus on sucrose gradients using zonal ultracentrifuge rotors.
Microbios, 2, 53-58.

Appendix 5. CELL GROWTH MEDIUM

The medium for growth of CK cells consisted of "HYL" (Hanks' balanced salt solution with 0.01% "yeastolate" and 0.5% lactalbumin hydrolysate) to every 100 ml. of which was added 10 ml. of foetal calf serum, 1.5 ml. of 1M Hepes and 1.5 ml. of $^{N}/4$ sodium hydroxide. The medium was prepared on the day before use, stored overnight at $4^{o}C$, and warmed up to room temperature ($25^{o}C$) before adding to the cells.

"HYL" was prepared from stock solutions that had been prepared in large batches in the Virology Department and stored at $4^{o}C$, as follows:

Distilled water	800 ml.
Hanks' solution A	50 ml.
Hanks' solution B	50 ml.
Hanks' solution C	100 ml.
7.5% Sodium bicarbonate	4 ml.
Antibiotics	5 ml.

Preparation of Stock Solutions

Hanks' A

Sodium chloride	800 grammes
Potassium chloride	40 "
Magnesium sulphate ($7H_2O$)	20 "
Calcium chloride (anhydrous)	14 "
0.2% Phenol red solution	500 ml.
Distilled water	4500 ml.

Hanks' B

Disodium monohydrogen phosphate ($2H_2O$)	6 grammes
Potassium dihydrogen phosphate	6 "
Dextrose	100 "
Difco "Yeastolate"	10 "
Distilled water	50,000 "

Hanks' C

Lactalbumin hydrolysate	500 grammes
Distilled water	10,000 "

Solutions A and B were dispensed into 50 ml. amounts in 2 ounce medical flat bottles, and solution C in 100 ml. amounts in 4 ounce round bottles. All solutions were autoclaved at 5 p.s.i. for 10 minutes, then solutions A and B were stored at 4°C and solution C at room temperature.

Antibiotics

This mixture contained 20,000 units/ml. of penicillin, 20,000 microgrammes/ml. of streptomycin, and 5,000 units/ml. of nystatin. These concentrations were such that the final concentrations in the medium, after adding 5 ml. of the mixture per litre, would be:

Penicillin	100 units/ml.
Streptomycin	100 microgrammes/ml.
Nystatin	25 units/ml.

This mixture was prepared by dissolving the required number of vials of Glaxo "Crystamycin" and Squibb "Mycostatin" in sterile distilled water, using aseptic technique. The nystatin did not dissolve completely, so it was necessary to shake the mixture immediately before withdrawing some for use. A large volume such as 1 litre or 1.5 litres was usually made up, and aliquotted into 50 ml. bottles for storage at -20°C.

The vials of "crystamycin" contained either 500,000 units of benzylpenicillin sodium + 500,000 microgrammes of streptomycin sulphate BP, or 2,500,000 units/microgrammes of each.. The vials of "mycostatin" contained 500,000 units of nystatin.

Sodium Bicarbonate

The stock solution of sodium bicarbonate consisted of 7.5% sodium bicarbonate with 0.01% phenol red. When added to the medium at 4 ml./litre it gave a final concentration in the medium of 0.03%, which was slightly lower than the normal concentration for a Hanks'-based medium (0.035%). It was prepared as follows:

Sodium bicarbonate	75 grammes
0.2% Phenol red solution	50 ml.
Distilled water	950 ml.

This was dissolved without heating, and dispensed into 1 ounce McCartney bottles, which were filled completely and the caps screwed on tightly. These were autoclaved at 5 p.s.i. for 10 minutes, then stored at 4°C.

Phenol Red

The stock 0.2% solution of phenol red was prepared by grinding 2 grammes of phenol red in a mortar, then dissolving it in 30 ml. of $^{N}/10$ sodium hydroxide and making the volume up to 1 litre with distilled water. It was stored at 4°C.

Appendix 6. CELL MAINTENANCE MEDIUM AND DILUENT

The same medium was used for maintaining CK cells during virus growth and for making serial dilutions, of virus harvests for inoculation on to CK cell cultures, and of sera for the plaque reduction test.

The formulae for Hanks' solutions A, B and C, and antibiotics, are as described in Appendix 5.

To prepare "HYL", mix:

Distilled water	800 ml.
Hanks' A	50 ml.
Hanks' B	50 ml.
Hanks' C	100 ml.
7.5% Sodium bicarbonate	4 ml.
Antibiotics	5 ml.

Store at 4°C for up to 5 days.

To every 100 ml. of "HYL" add immediately before use:

Heat-inactivated calf serum (stored at -20°C) (Wellcome Reagents Ltd.)	2 ml.
1M Hepes	1.5 ml.
N/4 Sodium hydroxide	1.5 ml.

Appendix 7. PLAQUE OVERLAY MEDIUM

The formulae for Hanks' solutions A, B and C, and antibiotics, are described in Appendix 5.

To prepare "HYL (Overlay)", mix:

Distilled water	600 ml.
Hanks' A	50 ml.
Hanks' B	50 ml.
Hanks' C	100 ml.
7.5% Sodium bicarbonate	4 ml.
Antibiotics	5 ml.

Store at 4°C for up to 5 days.

To every 360 ml. of "HYL (Overlay)" add a few hours before use:

Tryptose phosphate broth (dilute form for addition to Eagle's medium. Wellcome Reagents Ltd.)	15 ml.
Heat-inactivated calf serum (stored at -20°C) (Wellcome Reagents Ltd.)	25 ml.
1M Hepes	7.5 ml.
N/4 Sodium hydroxide	8.5 ml.

This 400 ml. of medium was held at 44°C to await the addition of 100 ml. of 4.2% Oxoid "Ionagar No. 2" as described in Chapter IV, Materials and Methods, Section (6).

Appendix 8. PHOSPHATE-BUFFERED SALINE, SOLUTION "A" (PBS"A")

PBS"A" was initially made up from the stock solution prepared in the Virology Department, which contained phenol red. The formula of this is given below. Later it was made up from Oxoid tablets, in order to avoid the inclusion of phenol red.

PBS"A" Stock Solution

Component	Amount
Sodium chloride	400 grammes
Potassium chloride	10 "
Disodium monohydrogen phosphate (anhydrous) (or, with 2 H_2O - 72.0 grammes)	58 "
Potassium dihydrogen phosphate (anhydrous)	10 "
0.2% Phenol red solution (Appendix 5)	250 ml.
Distilled water	8 litres

This was dispensed into 160 ml. volumes and autoclaved at 5 p.s.i. for 10 minutes, then stored at 4°C.

Immediately before use, 160 ml. of stock solution was added to 800 ml. of sterile distilled water. The fact that the resulting solution would be very slightly hypertonic without the addition of another 40 ml. of water was ignored.

The Oxoid tablets were dissolved in water directly at the final concentration, making use of the autoclaving (at 5 p.s.i. for 10 minutes) to dissolve the tablets. The required volume (1 or 2 litres) was made up at approximately 1 week before use, and stored at 4°C after autoclaving.

Appendix 9. CALCULATION OF WEIGHTED MEAN FOR PLAQUE ASSAY

The general formula for calculation of the weighted mean (Rhodes and Van Rooyen, 1968) is:

$$m = \frac{N}{\sum dr}$$

where:

N = Total number of plaques counted.

d = The dilution.

r = The number of plates counted at the dilution.

The dilutions are conveniently expressed as decimal fractions of the lowest dilution used. For example, if plaques are counted at 10^{-3}, 10^{-4}, and 10^{-5} then "d" would be: 1, 0.1 and 0.01 respectively, and the weighted mean obtained would be X 10^{3}.

This formula assumes that the counts can be regarded as samples from a Poisson distribution. The standard error of the weighted mean can be calculated from a single titration by the formula:

$$s_m = \sqrt{\frac{m}{\sum dr}}$$

FOR OFFICIAL USE ONLY

ANTIGENIC RELATIONSHIPS BETWEEN STRAINS OF INFECTIOUS BRONCHITIS VIRUS AS SHOWN BY THE PLAQUE REDUCTION TEST IN CHICKEN KIDNEY CELL CULTURE

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Weybridge,
Surrey,
England.

SUMMARY

The antigenic relationships between 9 reference strains, 13 British field strains and 1 vaccine strain of infectious oronchitis (IB) have been studied, using antisera prepared in chickens and assayed by a plaque reduction test in chicken kidney cell culture. By this method, each antiserum was found to possess 2 titres : its "type titre", given against its homologous virus strain or strains of the same type; and its "group titre", given against virus strains of different type. The value of the group titre varied very widely between antisera. The ability of a virus strain to stimulate a high group titre was associated with the presence of a corona on a high proportion of the virions.

INTRODUCTION

Dawson and Gough (1.) of this laboratory examined the antigenic relationships between 15 British field strains and 7 reference strains of IB virus, using the neutralisation test in egg embryos. They found that it was not feasible to define a serotype classification for IB of the kind described for other virus groups, because of the complexity of the pattern of cross-reactions obtained.

In this extension of their work, many of the same strains have been examined using a plaque reduction test in chicken kidney cell culture.

MATERIALS AND METHODS

Antisera were prepared in specific-pathogen-free chickens by either a single intratracheal-intranasal inoculation, or two such inoculations at about 6 weeks' interval. For the Beaudette strain and two other strains of low immunogenicity, intra-abdominal inoculation was also used.

Chicken kidney cells were prepared by a modification of the method of Churchill (2.), in which pH control was obtained by including Hepes buffer at a final concentration of 15 mM in a medium based on Hanks-yeast-lactalbumin with the addition of 5% tryptose phosphate broth and 10% foetal calf serum. Disposable plastic petri dishes were used for plaque assays.

7 of the reference strains and 5 of the British strains were adapted to cell culture, by 5 to 14 passages, to give readable plaques, and stocks of these test viruses were stored in aliquots at -60°C.

Before test, the antisera were treated at 56°C for 30 minutes. Doubling dilutions of serum were mixed with equal volumes of virus at the dilution expected on the result of a preliminary titration to give 40 plaques from 0.1 ml. of the mixture. After 30 minutes at room temperature, 2 dishes were inoculated with 0.1 ml. each of each serum dilution. 6 Virus control dishes were inoculated. After 30 minutes at 37°C, the overlay was added. This was similar to the growth medium, except that foetal calf serum was replaced by 5% of heat-inactivated calf serum, and agar was included. After 2 or 3 days at 37°C, the overlay was removed and the cells stained with crystal violet. By comparisons with the mean control count, percentage reductions were calculated for each serum dilution. These were plotted against $\log_2$ serum dilution on probability graph paper, and the best straight line was drawn through the points. The dilution estimated to give 75% reduction was read from this line, and the serum titre was finally expressed as the $\log_2$ reciprocal.

RESULTS

The titres of 7 selected antisera against a range of test viruses are shown diagrammatically in Figures 1-7. They illustrate our observation that for each antiserum there was a common level of cross-reaction with test viruses of different type, which we have called the "group titre". This was usually significantly lower than the maximum titre obtainable with the antiserum, which was expressed against the homologous virus or virus of the same type, and called the "type titre".

A typing classification was possible on this basis. For example, from the results shown it was concluded that Iowa 609, Australian T, Connecticut and 591 were all different from each other, and from the Massachusetts-like strains Houghton 140 and Beaudette. The classification of H120 was less certain, because of its exceptionally high group titre in relation to its type titre, but it was nearer to the Massachusetts-like strains than it was to the other reference strains.

DISCUSSION

From the results shown, it will be seen that the value of the group titre varied greatly between antisera. Electron microscopic studies by Harkness and Bracewell (3.) have shown that strains of IB virus vary greatly in the proportion of virus particles seen to be carrying a corona, and that there

appears to be an association between presence of corona and ability to stimulate a high group titre in chickens. For example, H120 showed a corona on almost all particles; while Connecticut and Beaudette showed an almost complete absence of corona, which correlated with the absence of a group titre in their antisera.

ACKNOWLEDGMENTS

The technical assistance of Mr. C.A. Perkins, Mr. R.K. Eddy and Mr. J. Lukey was much appreciated.

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(To be published).

Fig. 1

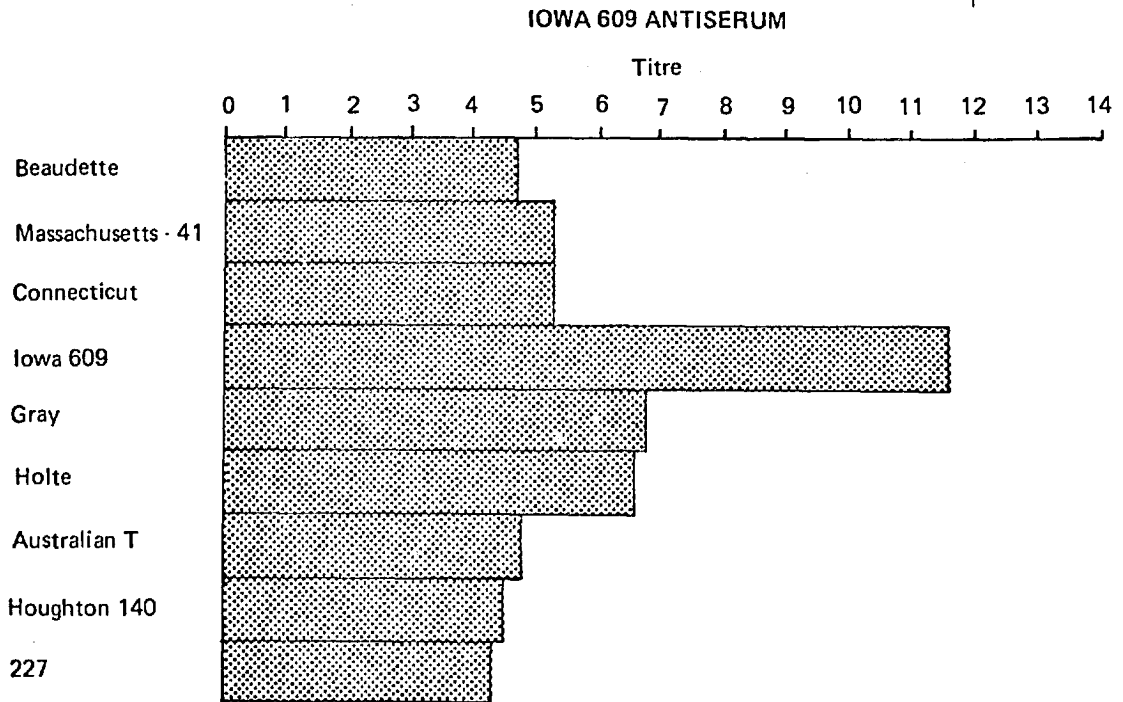


Fig. 2

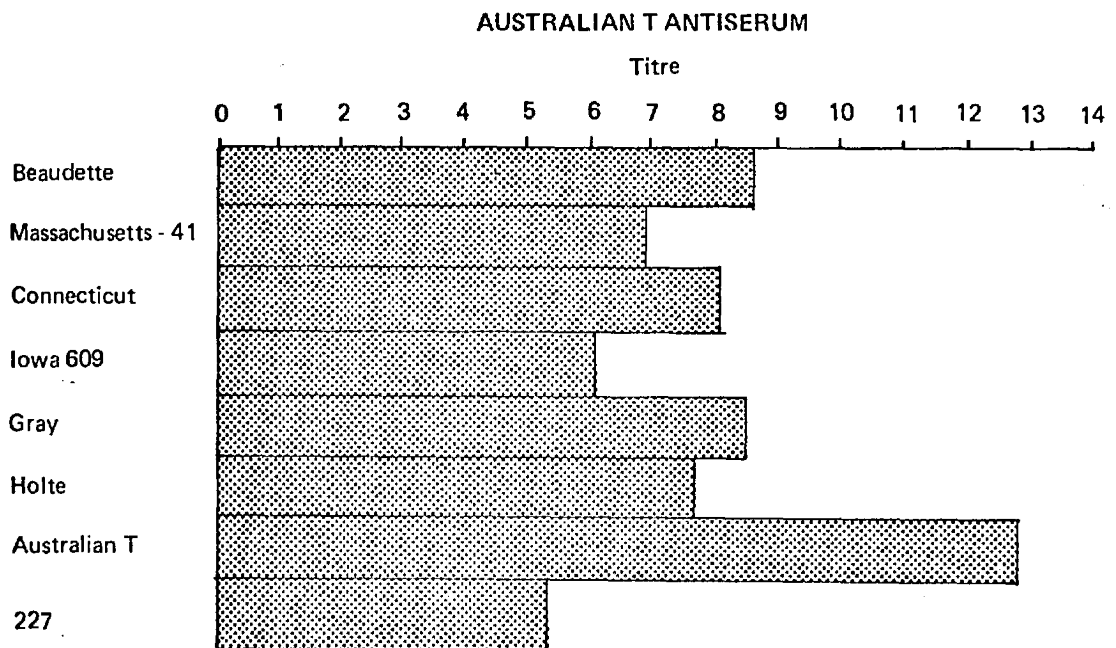


Fig. 3

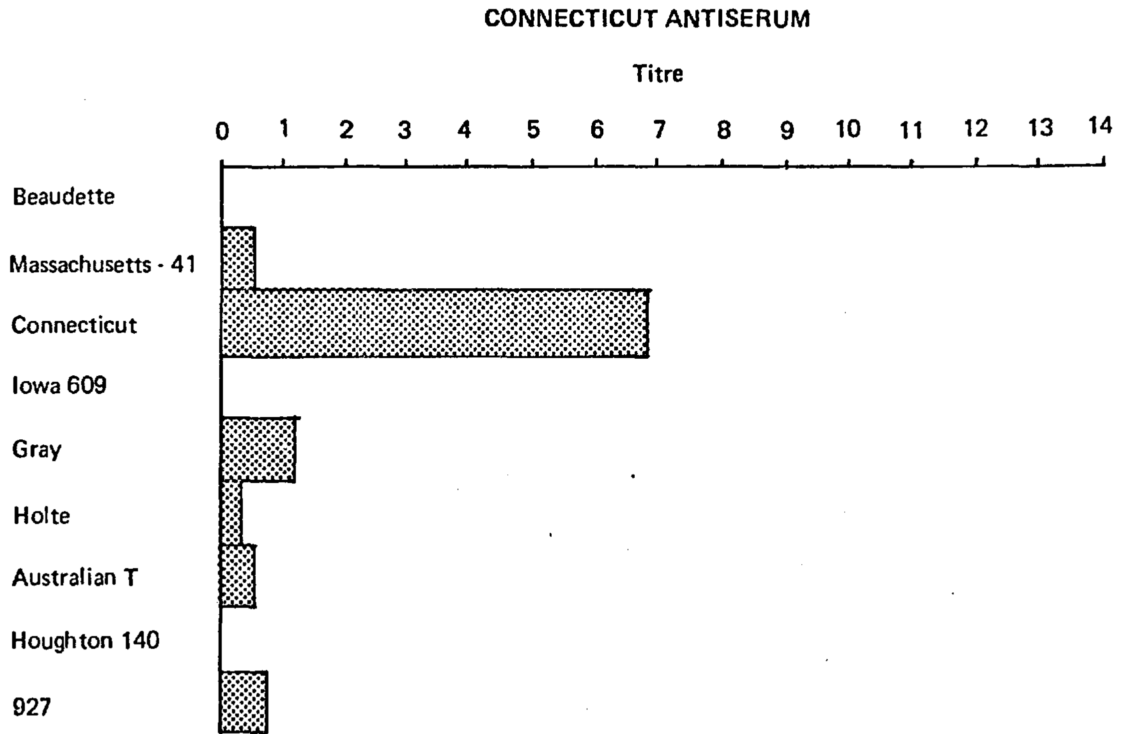


Fig. 4

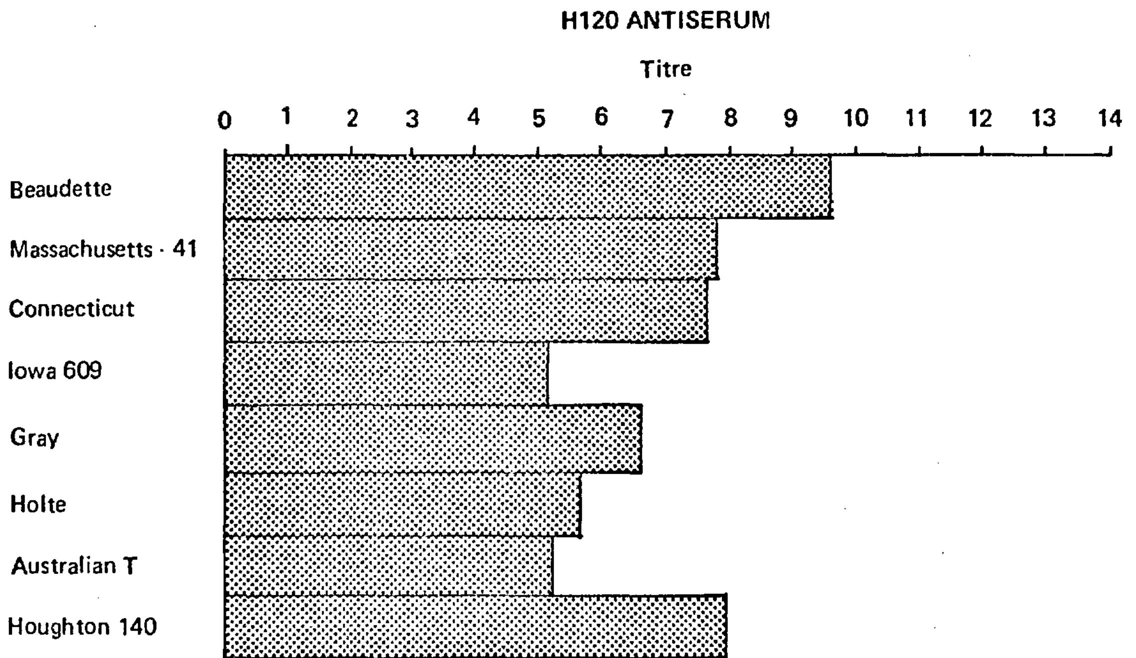


Fig. 5

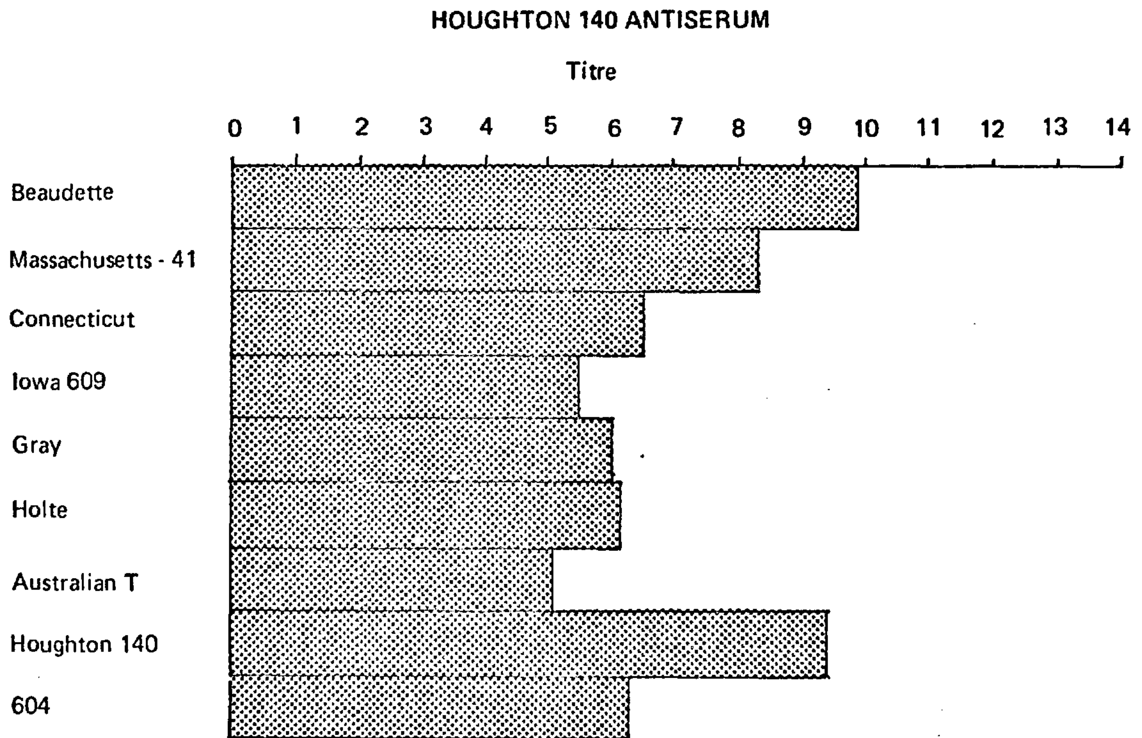


Fig. 6

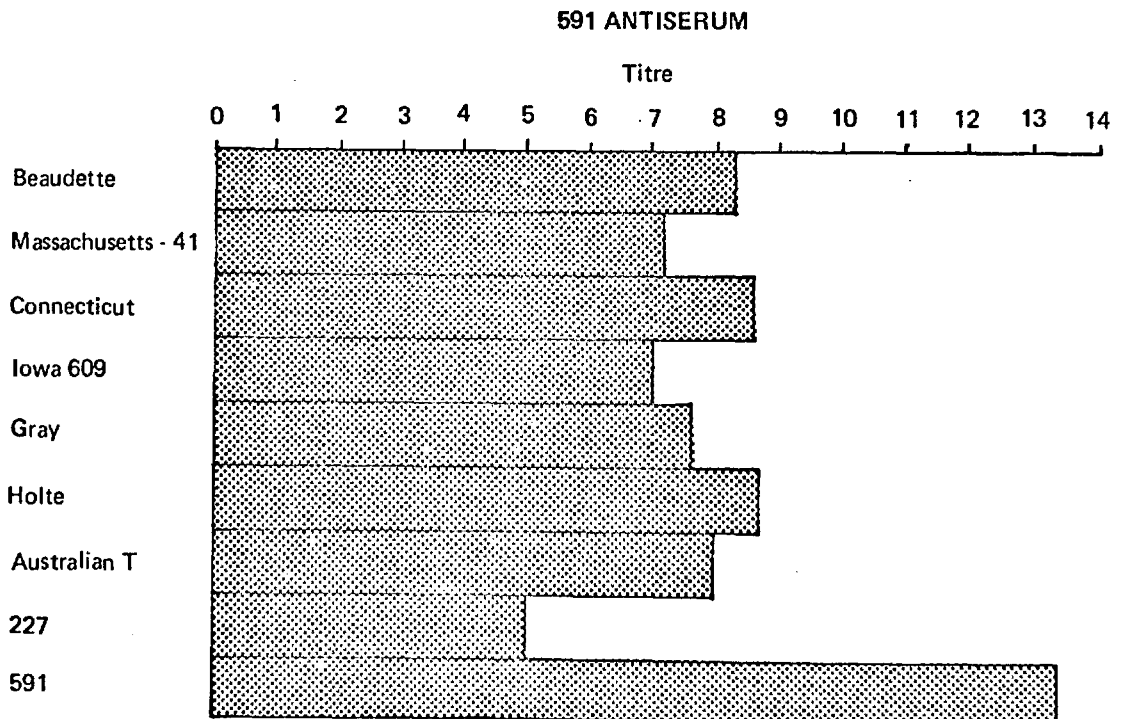
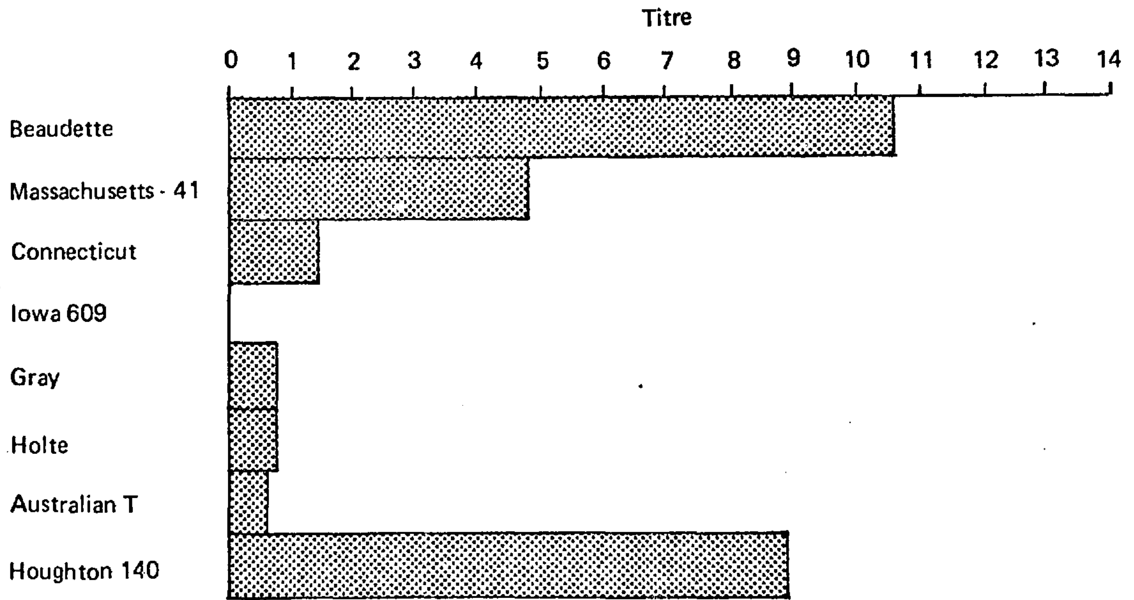


Fig. 7



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Scientific Letters

A Direct Complement Fixation Test for Infectious Bronchitis Virus Using Heat-inactivated Chicken Sera

Sir,—Because of the difficulty of estimating the serological response to infectious bronchitis (I.B.) in individual birds without recourse to the expensive and time-consuming serum neutralisation tests in embryos or cell cultures, it was felt that development of the complement fixation test for I.B. might be of value. The method adopted has been based on the approach reported for various non-viral systems by Brumfield and Pomeroy (1959), Rice, Magwood and Annau (1960) and Marquardt and Newman (1971), and consists in principle of the addition of unheated normal chicken serum (U.N.C.S.) to a system in which the test serum has been inactivated at 56° C. The purpose of this communication is to report the success of this approach for the individual assay of I.B. responses.

The method described for I.B. by Uppal (1970) was based on the use of unheated test sera. This method was found to have several serious disadvantages compared with the one adopted. The dose of complement had to be continually adjusted to match the variety of anti- and pro-complementary properties of the test sera, whereas heat-inactivation of the test sera abolished all these properties and so allowed the use of a constant dose of complement. Uppal's method also relied on the presence in the test sera of adequate amounts of the essential heat-labile factor needed for fixation of complement, but this was found to be variable and unstable on storage at −20° C. Also, this factor was inevitably diluted progressively in the act of titrating the serum, leading to limitation of serum titres to about 1/32 or below, whereas by the adopted method a full range of titres was possible, the highest being over 1/2,000.

Method

Diluent was prepared from "Oxoid"* barbitone buffer tablets. Complement and rabbit haemolytic serum were obtained from Wellcome Reagents.† The haemolytic system was formed by mixing equal volumes of 1/600 haemolytic serum and 3 per cent. sheep red blood cells. "Microtiter" equipment, supplied by Flow Laboratories,‡ was used including disposable, rigid styrene plates with 8 × 12 U-bottom wells, 0.025 ml. droppers and microdiluters, plate-sealers, shaker and mirror.

The final test volume was 0.1 ml. per well, composed of one 0.025 ml. drop of test serum dilution, antigen, complement, and haemolytic system. The complement dose used was prepared by mixing equal volumes of the required dilution of guinea-pig complement (4 CH_{50}—see over) and a $\frac{1}{8}$ dilution of U.N.C.S. The fixation period of one hour at 37° C. was arranged to be concurrent with the incubation of the haemolytic system. After adding the haemolytic system, the plates were sealed and given continuous shaking for 35 minutes at 37° C., and then left at room temperature to settle. Serum titres were read as the highest dilution giving less than 50 per cent. haemolysis, and expressed as $\log_2$ reciprocals. Use was made of the usual scoring system (4, 3, 2, 1, Trace, 0) where detailed recording of wells was desired.

The test controls included the titration of known positive and negative sera; the control for anti-complementary effect of the antigen (which should not exceed a score of 1) and three complement

* Oxoid Ltd., London, S.E.1.
† Wellcome Research Laboratories, Beckenham, Kent.
‡ Flow Laboratories Ltd., Victoria Park, Heatherhouse Road, Irvine, Scotland.

(Continued on page 453)

controls: (1) the final mixture of U.N.C.S. and complement (required to score 0); (2) the complement only (also required to score 0); (3) the U.N.C.S. only (required to score 4).

U.N.C.S. was stored at 4° C. in batches of about 100 ml. New batches of complement received from the manufacturer were given two preliminary titrations: alone and in the presence of both U.N.C.S. and antigen. The titre of a batch was read as that dilution giving exactly 50 per cent. haemolysis (a score of two) when titrated alone. It was then used at four times this concentration (4 CH_{50}) for preparing the complement dose for the test. It was necessary that the presence of U.N.C.S. and antigen should not depress the complement titre by more than two dilutions in a 0.1 $\log_{10}$ series. New batches of U.N.C.S. were also tested in this way for absence of pronounced anticomplementary effect, and unsuitable batches were discarded. Occasionally, certain batches of complement were found to give very strong, non-specific fixation with all batches of U.N.C.S., and these batches of complement were discarded.

New batches of antigen were titrated with excess of homologous type antiserum. The titre was read as the highest dilution giving less than 50 per cent. haemolysis, and four times this concentration (four units) was used in serum assays. Allantoic fluid harvests of the Beaudette strain (a highly embryo-adapted strain with a very long laboratory history) usually provided four units of antigen and could be used without concentration, but most other strains required a concentration of about 25-fold, which could be achieved by ultracentrifugation followed by resuspension in test diluent. Harvests collected before death of the embryo had less of an anticomplementary effect. Further work on improving the antigens from strains other than Beaudette is in progress.

Results

Table I shows the individual serum titres of eight chicks at intervals after experimental infection with the Houghton 140 strain (kindly supplied by Mr. J. S. McDougall of Houghton Poultry Research Station, Huntingdon) at the 7th embryo passage. These results illustrate the kind of responses resulting from infection with a moderately pathogenic virus strain. Natural infection in the field can give rise to higher titres, as shown in Table II. Infection by live vaccine in the drinking water gives lower titres and a variable proportion of non-reactors, as shown in Table III. This table also shows the titres of the same birds three weeks after aerosol challenge with the fairly pathogenic Massachusetts-41 strain. A significant increase in titre occurred in most of the birds.

TABLE I
INDIVIDUAL SERUM TITRES OF EIGHT CHICKENS AT INTERVALS AFTER INTRATRACHEAL INOCULATION WITH HOUGHTON 140

Bird No.	1	2	3	4	5	6	7	8
10 days	1	<1	<1	<1	<1	<1	<1	<1
14 days	6	<1	1	8	<1	11	5	1
17 days	8	2	7	9	6	11	7	7
21 days	8	7	7	10	8	11	8	8
6 Weeks	8	1	10	11	2	12	9	4
15 Weeks	2	2	3	11	2	12	1	1

Antigen: Beaudette. Titres expressed as $\log_2$ reciprocals.

TABLE II
SERUM TITRES (POOLS OF FIVE BIRDS) FROM A COMMERCIAL BROILER HOUSE DIVIDED INTO NINE PENS. RESPIRATORY DISEASE APPEARED AT FIVE-SIX WEEKS OLD

Pen No.	1	2	3	4	5	6	7	8	9
Titre at 5 weeks old	<1	<1	<1	<1	<1	<1	<1	<1	<1
Titre at 9 weeks old	7	6	7	6	4	5	5	7	4
Titre at 11 weeks old*	10	10	10	8	9	11	10	10	10

*Some birds maintained to this age for serum sampling.
Antigen: Beaudette. Titres expressed as $\log_2$ reciprocals.

TABLE III
INDIVIDUAL SERUM TITRES OF 12 CHICKENS VACCINATED AT TWO WEEKS OLD WITH NOBILIS H120 IN DRINKING WATER AND CHALLENGED AT FIVE WEEKS BY AEROSOL OF MASSACHUSETTS-41

Bird No.	1	2	3	4	5	6	7	8	9	10	11	12
Titre at three weeks Post-Vacc.	6	5	8	2	1	4	2	2	<1	6	<1	<1
Titre at three weeks Post-Chall.	3	8	9	8	12	8	8	7	8	8	7	1

Antigen: Beaudette. Titres expressed as $\log_2$ reciprocals.

These results show that the complement fixation test can be used to give a rapid assessment of individual bird responses to infectious bronchitis, which may be of use in analysing immunity and the effect of challenge in the field.

Studies are continuing on the relationships between the serum titres shown by this test and the plaque reduction test in chicken kidney cell cultures and the neutralisation test in embryos. Although obviously also resulting from I.B. infection, complement fixation titres do not run parallel with neutralisation titres and the test should be regarded as an alternative system rather than a substitute for the neutralisation test.

The effect of antigenic differences between virus strains is also being studied, and will be reported at a later date.

Acknowledgments.—The technical assistance of Mr. C. A. Perkins and Mr. R. K. Eddy is gratefully acknowledged. The serum samples in Table II were kindly supplied by Ross Poultry Ltd., who also

carried out the vaccination of the birds in Table III.

January 9th, 1973. Yours faithfully,

C. D. BRACEWELL.

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Assessing infectious bronchitis vaccines

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ONE approach to the assessment of infectious bronchitis (IB) vaccines is to carry out strictly controlled trials under laboratory conditions. Some newer methods used in such trials have been described by Winterfield and Fadly (1971), Winterfield and others (1972), and Coria and Booth (1972). This approach is especially needed in developing new vaccines. However, we have found that poultry farmers are asking for more direct evidence that IB vaccines are effective under their own particular field conditions. This laboratory is engaged in a series of vaccine trials in collaboration with poultry farmers, in which vaccination is carried out on the farm on large numbers of commercial chicks, and serological responses are monitored. In some trials, representative samples of birds are transferred to the laboratory for challenge. This communication reports the results of one such trial, in which live vaccines of two different strains of IB virus were compared for their effectiveness when given combined with a live Newcastle disease (ND) vaccine.

At 14 days of age, three groups of 3000 broiler chicks were vaccinated via the drinking water, as recommended by the manufacturers, with the following vaccines:

Group 1 : ND (La Sota) + IB (H120)*
Group 2 : ND (La Sota) alone
Group 3 : ND (La Sota) + IB (MM)†.

At 29 days of age, 24 chicks from each group were transferred to the laboratory. At 35 days of age, these chicks were challenged by an aerosol of IB virus of strain Massachusetts-41. The virulence of this strain had been demonstrated during two recent bird-to-bird passages.

Individual respiratory symptoms were observed pre-challenge and at three, nine and 14 days post-challenge. On each occasion each bird was scored from 0 to 3 according to severity of symptoms. Serum samples were collected pre-challenge and at 21 days post-challenge. Individual sera were titrated by three different methods: the complement fixation test (CFT) (Bracewell 1973a), against Beaudette antigen; the plaque reduction test (PRT) (Bracewell 1973b), against Massachusetts-41; and the serum neutralisation test in eggs (SNT) (Dawson and Gough 1971), against H120. Titres were expressed as $\log_2$ reciprocals.

A high proportion of negative titres was obtained from the pre-challenge sera, not only in Group 2 as expected, but also in Groups 1 and 3, especially with the SNT in Group 3, and the CFT in both groups. This was considered to indicate a low level of response in this trial as a whole. Because of this the pre-challenge titres were analysed as the number of positives, defined as titres greater than 2·0 (Initial serum dilution $\frac{1}{4}$). The low responses may have been caused by maternal antibody, which was shown, by means of the CFT, to have been present during at least the first week of life in this batch of chicks.

The results are summarised in Table 1.

TABLE 1: Results of assessment of 3 vaccine combinations against IB

Group	Pre-challenge: No of birds positive to: CFT	PRT	SNT	Post-challenge: Group mean titres (Log_2 reciprocals) CFT	PRT	SNT	Group means (± SEM) of total symptom scores per bird for three occasions
1	5	23	18	6·9	8·1	4·9	2·96±0·37
2	0	1	2	7·1	7·9	3·7	5·71±0·30
3	2	16	2	8·6	7·8	3·9	4·04±0·27

The serological responses to vaccination were on the whole significantly more numerous in Group 1 than in the other groups, although the number of positives with the CFT was too low in this trial to permit analysis of this test. This finding correlates with the post-challenge symptom scores, in which Group 1 was significantly lower than Group 2 ($P<0·001$) and Group 3 ($P<0·05$). The post-challenge serological responses were equally strong in all groups. At this time all birds were positive to the PRT and SNT, and all but four to the CFT (three of these were in Group 1).

None of these results correlated well for individual birds, emphasising the need for large groups in such trials. Twelve birds showed respiratory symptoms pre-challenge. Five of these were in Group 1, six were in Group 2, and one was in Group 3. In Groups 1 and 3, which had received IB vaccine, four out of the six affected birds were positive to the CFT, indicating perhaps some correlation between symptoms and positive CFT reactions. Although the CFT appears to be somewhat less sensitive than the other methods, it is easily the most rapid, and is convenient for testing the much larger numbers of sera which are involved in the other trials now in progress.

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PRELIMINARY EVALUATION OF THE HAEMAGGLUTINATION AND HAEMAGGLUTINATION INHIBITION TESTS FOR AVIAN INFECTIOUS BRONCHITIS VIRUS

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SUMMARY

Four of the 9 strains of infectious bronchitis virus which were concentrated and treated with phospholipase C type 1 showed haemagglutination activity. These strains, Holte, Massachusetts 41 (M41), H120 and Connecticut, were distinguishable by the haemagglutination inhibition (HI) test but showed much closer relationships than could be detected by the plaque reduction (PR) test. The four haemagglutinating strains were used to compare the HI and PR titres of 17 anti sera prepared against reference and field virus strains. In most cases titres were similar although there was a tendency for the HI titres to be higher than the PR titres especially with M41 antigen.

HI titrations of the pooled sera from 20 birds infected with M41 showed a peak of activity at 14 days after infection which was not detected by serum neutralisation or complement fixation tests. HI titres of individual sera from birds infected 21 days previously with M41 virus compared favourably with serum neutralisation titres but showed little relationship to the complement fixation titres.

INTRODUCTION

The long-established serum neutralisation (SN) test in eggs (Cunningham, 1973) is time-consuming, expensive and difficulty is often experienced in determining endpoints due to the problem of identifying infected embryos. These disadvantages of the SN test led to the development of a complement fixation (CF) test for the detection of antibodies to infectious bronchitis virus (IBV) (Bracewell, 1973a). Routine use of the CF test in this laboratory has shown that it can be employed for monitoring for IBV infection in flocks. However, the antibodies detected by the CF test do not always appear in every bird exposed to infection and their presence may be transient. There is, therefore, a need for a rapid and inexpensive method capable of detecting antibodies to IBV with, at least, the consistency of the SN test.

The typing of strains of IBV has also been difficult because the SN test has failed to

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give a clear pattern of antigenic relationships between strains (Dawson and Gough, 1971). More recently, in spite of the problem of adapting IBV to tissue culture which may take many passages, workers have used the plaque reduction (PR) test to study the antigenic relationships of IBV strains (Bracewell, 1973b; Hopkins, 1974; Cowen, 1973). The PR test has produced much better results than the SN test for typing IBV strains. Because of its greater accuracy, sensitivity and tendency to be less disturbed by variations in the virulence of test viruses, it may become accepted as the definitive method for such studies. However, there is still a need for a further serological technique, preferably not based on neutralisation of infectivity, to clarify the more complex and confusing aspects of the antigenic relationships between IBV strains.

Bingham *et al.* (1975) have shown that IBV treated with phospholipase C (type 1) will agglutinate red blood cells and that this haemagglutination (HA) may be specifically inhibited by antisera. In the present paper we report a preliminary study of HA and haemagglutination inhibition (HI) tests for IBV. We have shown that the HI test can be used for the detection of antibodies to IBV and for this purpose it compares favourably with the SN and CF tests.

MATERIALS AND METHODS

Viruses

The strains used, their origins, methods of growth and adaptation to chick kidney cells have been described (Dawson and Gough, 1971; Harkness and Bracewell, 1974; Bracewell, 1973b). H52 virus was obtained as a commercial vaccine (Intervet Ltd.).

Antisera

Reference antisera were prepared as described by Bracewell (1973b).

In experiments involving the immune response to infection by Massachusetts 41 (M41) 3-week-old specific pathogen free white leghorn chickens, from the Central Veterinary Laboratory flock, were infected by aerosol so that each bird received an estimated 100 infectious doses of M41 virus. The birds were bled at 3, 5, 7, 10, 12, 14, 17, 19, 21 and 42 days after infection.

All antisera were incubated for 30 minutes at 56°C.

Serum neutralisation, plaque reduction and complement fixation

The procedures for the titration of antisera by SN, PR and CF were those described by Dawson and Gough (1971), Bracewell (1973b) and Bracewell (1973a) respectively. Some of the sera were tested for antibodies by the PR test and stored at -20°C for up to two years prior to the HI titrations. Several of these sera were retested by PR test against homologous virus at the same time as the HI titrations were done, the PR titres were almost identical to those originally obtained. It was, therefore, considered unnecessary to re-titrate all antisera by the PR test.

Haemagglutination and haemagglutination inhibition

The method used to prepare haemagglutinating virus was a simplification of the technique used by Bingham *et al.* (1975). The allantoic fluid was harvested from infected eggs at the onset of embryo death or 72 hours after infection. The virus in the allantoic fluid concentrated 100-fold by pelleting at 30,000g for 45 min and resuspending in 0.01M Tris/HCL buffer at pH 6.4. An equal volume of phospholipase C type 1 (Sigma Ltd.) containing one unit of enzyme per ml in phosphate buffer at pH 6.4 (Witter *et al.*, 1973) was added to the virus suspension and the mixture incubated

for 30 min at 37°C. HA and HI titrations were done at 4°C using the microtiter system as described by Bingham *et al.* (1975). Unless stated otherwise 1% chicken red blood cells were used in all HA and HI tests.

RESULTS

Haemagglutination by different strains

Nine strains of IBV including one at 2 passage levels (H52 and H120) were concentrated, treated with phospholipase C (type 1) and tested for HA activity (Table 1). Only 4 strains, H120 (but not H52), M41, Connecticut (Conn.) and Holte, agglutinated chicken red blood cells. M41 frequently produced the highest titres although the titre varied between 64 and 1024 after treatment on different occasions of the same batch of virus with the same batch of enzyme. Use of guinea-pig red blood cells produced considerably higher titres for all the strains that agglutinated chick red blood cells but none of the strains that failed to agglutinate chicken red blood cells agglutinated guinea-pig cells.

Table 1. Haemagglutination (HA) by strains of infectious bronchitis virus

Virus x 100 conc.[a]	Enzyme treatment[a]	HA titre[b]
M41	–	4
M41	+	1024
Conn	–	<2
Conn	+	256
Holte	–	<2
Holte	+	32
H120	–	<2
H120	+	256
Iowa 97, Iowa 609, Grey, Beaudette, H52, T, T x 1000	–	<2
Iowa 97, Iowa 609, Grey, Beaudette, H52, T, T x 1000	+	<2

a Virus was concentrated and tested for haemagglutination activity both before and after treatment with phospholipase C (type 1). T x 1000 was subjected to a further tenfold concentration.

b HA titres are expressed as the reciprocal of the dilution giving 75% agglutination.

It was found necessary to store enzyme-treated virus in a frozen state. Overnight storage at 4°C caused a 16-fold drop in HA titre for M41 and H120 but storage overnight at -20°C or for several weeks at -60°C did not affect the titre. Similarly virus diluted to 4 or 8 HA units (HAU) could be stored at -60°C without loss in titre.

The end point of haemagglutination was not always easy to determine, agglutination tended to 'tail-off' rather than produce a distinct end point. It was found that reproducible results were most easily obtained if a settling pattern covering 75% of the area of full agglutination, estimated by eye, was used for end-point determination. This criterion enabled fully reproducible results to be obtained in repeated tests on any enzyme-treated batch of virus.

Dilution of virus did not necessarily give a linear relationship with HA (Table 2) and to achieve 4 HAU for HI tests it was usually necessary to allow a factor of 2 or 4 for the dilution.

Table 2. Effect of dilution on haemagglutination (HA) by infectious bronchitis virus

Dilution factor[a]	HA titre[b]	HA titre x dilution factor
1	256	256
2	128	256
4	16	64
8	8	64
16	4	64
32	2	64
64	<1	<64

a The dilution factor is the reciprocal of the dilution made.

b The HA titre is expressed as the reciprocal of the dilution showing 75% agglutination. Conn virus was used.

Haemagglutination inhibition

HI tests using sera from normal uninfected chickens usually gave titres of less than 4 against any of the 4 viruses that haemagglutinated although occasionally titres as high as 16 were recorded. The most important consideration in obtaining low non-specific inhibition was the age of the red blood cells. Titres obtained with normal chicken sera were highest when red blood cells were relatively old. Non-specific inhibition was lowest if the red blood cells were used within 48 hours of bleeding.

The reproducibility of the HI test was good. Duplicate titrations of sera or repeated titrations separated by a period of time usually gave similar results even with different batches of the same virus. However, to maintain a high level of reproducibility it was necessary to use fresh red blood cells.

Specific HI was obtained with antisera against all 4 haemagglutinating viruses and the results of cross HI tests were compared with those of cross PR tests (Table 3). The most notable difference was the much closer relationship between the viruses obtained using the HI compared with the PR test. A relationship between M41 and the other 3 viruses was shown by analysis of the cross HI results using the method of Archetti and Horsfall (1950). Conn was apparently the most closely related to M41 ($r = 0.35$) while the other 2 strains had r values of 0.25. Similar relationships were not detected by cross PR tests.

The 4 haemagglutinating viruses were also tested against 17 antisera prepared against reference and British field strains (Table 4). Although direct comparison of antibody

Table 3. Comparison of cross haemagglutination inhibition (HI) and plaque reduction (PR) tests

Antisera	Antigens			
	Conn	Holte	M41	H120
	HI			
Conn	32[a](1)[b]	<2(0.06)	16(0.35)	2(0.125)
Holte	64	512(1)	64(0.25)	8(0.06)
M41	32	64	128(1)	16(0.25)
H120	64	64	128	256(1)
	PR			
Conn	110(1)	<2(0.008)	<2(0.02)	ND[c]
Holte	11	1600(1)	42(0.02)	ND
M41	16	7	360(1)	ND
H120	200	50	240	ND

a Results are expressed as the reciprocal of the dilution giving the required inhibition

b The bracketed figures are the *r* values calculated by the method of Archetti and Horsfall (1950).

c Not done because tissue-culture-adapted H120 was not available

titres obtained by different methods may not be valid, high or low titres should coincide for each test. To this extent similar results were obtained by the PR and HI tests for most of the antisera. Where differences did exist they were usually within the range of the difference between PR and SN tests for homologous virus. The largest discrepancies were seen with Holte antigen and Gray antisera: HI 4.0; PR 9.4; M41 and Iowa 97: HI 6, PR 1.8; and M41 and Beaudette: HI 9, PR 4.8. The last mentioned result is of interest as these viruses are regarded as antigenically similar and therefore would be expected to give a titre close to the homologous PR titre of 10.6.

Immune response in birds

The immune response of 20 birds to infection with M41 was examined by applying the HI, SN and CF tests to pooled sera collected at intervals after infection (Text fig. 1). In the HI and SN tests the sera were titrated against the homologous virus but for the CF test the Beaudette strain was used (the Beaudette strain is routinely used in this laboratory for the detection of CF antibodies). The antibody responses as measured by the 3 methods were parallel up to 12 days post inoculation (p.i.). However a marked peak in HI titre was apparent at 14 days p.i. (Text-fig.1), after which time it decreased.

Sera from individual birds 21 days after infection had HI titres of 64-512, SN titres of 55-420, and CF titres of 2-128 (Table 5). There was a good correlation between the HI and SN titres which was not found between these titres and the CF titres.

DISCUSSION

Following concentration and treatment with phospholipase C (type 1) only 4 of the 9 IBV strains tested were able to agglutinate red blood cells. Comparison of our results

Table 4. Haemagglutination inhibition (HI) and plaque reduction (PR) tests with antisera to field and reference strains

Antisera	Antigens									
	Conn		Holte		H120		M41		Homologous	
	HI	PR	HI	PR	HI	PR	HI	PR	PR	SN
Conn	5[a]	6.8[a]	<1	0.3	1	–[d]	4	0.5	6.8	5.7[a]
Holte	6	3.4	9	10.7	3	–	6	5.4	10.7	8.1
Gray	7	4.9	4	9.4	6	–	9	4.0	9.9	9.3
Iowa 609	6	5.3	6	6.6	4	–	6	5.3	11.6	8.7
H120	6	7.7	6	5.7	8	–	7	7.8	ND[cd]	6.7
M41	5	4.0	6	2.8	4	–	7	8.5	8.5	7.5
T	7	8.1	7	7.7	7	–	7	6.9	12.8	10.9
Iowa 97	5	3.4	8	4.5	2	–	6	1.8	9.7	9.7
Beaudette	4	1.4	<1	0.8	6	–	9	4.8	10.6	7.3
SE 17	5	5.4	5	4.5	ND	–	7	5.8	6.2	9.5
227[b]	6	5.8	8	4.8	6	–	8	7.0	11.3	8.9
604[b]	8	4.6	9	6.0	7	–	8	6.7	11.0	8.5
317a[b]	3	4.7	ND		4	–	5	2.9	ND	
317b[b]	5	ND	7	7.0	5	–	9	6.1	ND	
591[b]	6	8.6	7	8.7	7	–	8	7.2	13.4	8.7
225[b]	9	8.1	10	ND	8	–	8	6.6	ND	
551[b]	ND		ND		ND		5	2.8	ND	

a Log$_2$ of the reciprocal of the dilution giving the required endpoint
b Sera against British field strains
c ND = Not done
d No tissue-culture – adapted H120 was available

Table 5. Immune response in individual birds 21 days after infection with Massachusetts 41 (M41) strain of infectious bronchitis virus (IBV)

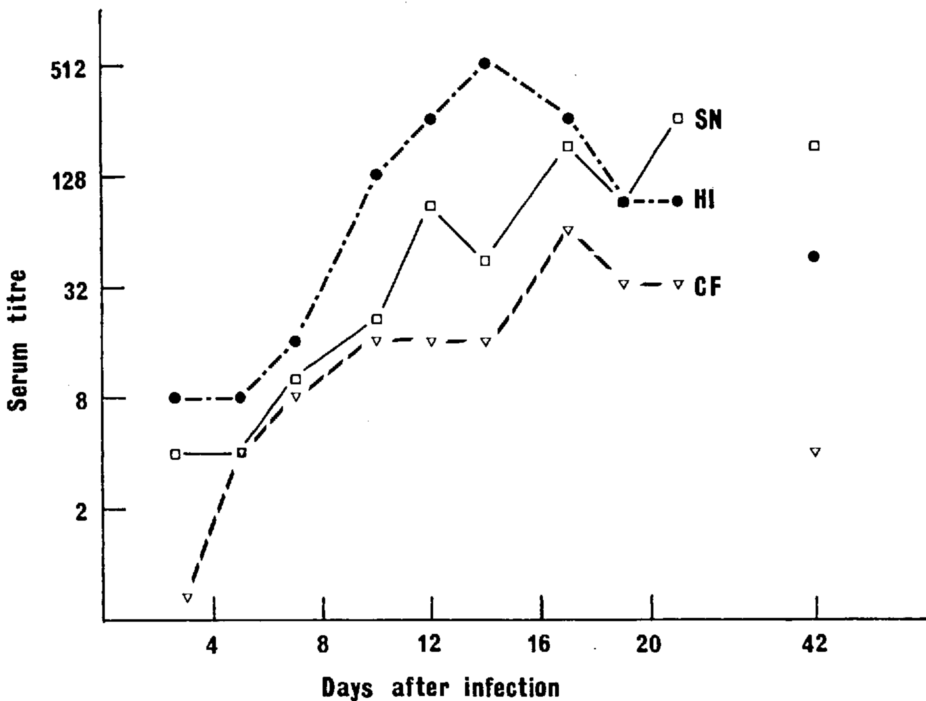
Bird Number	HI[a] titre[d]	SN[b] titre[d]	CF[c] titre[d]
1	64	55	16
9	64	75	16
16	64	76	32
15	64	106	32
2	64	110	4
13	64	125	16
10	64	150	<2
14	128	32	ND[e]
12	128	125	64
18	128	128	4
7	128	166	4
19	256	64	2
17	256	64	64
11	256	89	128
3	256	105	32
20	256	128	8
5	256	150	16
6	256	157	32
4	512	128	8
8	512	420	32

The sera were tested against M41 virus in the SN and HI tests but strain Beaudette was used in the CF test. Results are expressed as the reciprocal of the dilution giving the required end-point.

a = Haemagglutination titre using M41 strain of IBV
b = Serum neutralisation titre using M41 strain of IBV
c = Complement fixation titre using Beaudette strain of IBV
d = Titre expressed as the reciprocal of the dilution giving the required end-point
e = ND – Not done

with those of Harkness and Bracewell (1974) indicated that no apparent serological or morphological characteristic governed the ability of a strain to agglutinate red blood cells. Possibly HA activity was related to differences in the number of virus particles, although the failure of the 1000x concentrated T strain to haemagglutinate and lack of increase in the number of strains with HA activity when the more sensitive guinea-pig red blood cells were used, suggest this is not the case. The lack of a linear relationship between HA and virus dilution may have been due to mechanical carry over which would be enhanced by a high concentration of virus.

The levels of HA activity obtained following enzyme treatment varied amongst the 4 haemagglutinating strains and this was apparently a property of each strain. M41 consistently gave the highest HA titres and Holte the lowest. Because the viruses were only concentrated 100-fold from infected allantoic fluid this difference may be due to the number of virus particles present, however, if the HA to particle ratio differs from strain to strain comparative HI titres could be affected due to the larger number



Text-fig. 1. Immune response in birds infected with Massachusetts 41 (M41) strain of infectious bronchitis. The pooled sera from 20 chickens infected with M41 virus were titrated, at the specified times after infection, by haemagglutination inhibition (HI) (●), serum neutralisation (SN) (□) and complement fixation (CF) (▽) tests. The 42 day titres are the geometric mean of 20 individual tests.

of antibodies needed to inhibit strains with lower ratios. Although results with the Holte strain (Table 4) gave no indication of this, further work is required to determine the exact physical and chemical nature of the HA process.

A much higher degree of cross reaction was seen amongst the haemagglutinating strains when the HI rather than the PR test was used. M41 showed the closest relationship to the other strains, and the highest heterologous HI titres against 17 sera representing reference and field strains were obtained with M41. This indicates that M41 may be a good candidate for use in the routine surveillance for antibody to IBV by HI testing.

Detection of antibodies by HI in the pooled sera of birds infected with M41 indicated a response roughly parallel to that detected by CF and SN. However there was a marked peak of HI activity at 14 days after infection which, in this experiment, was not detected by SN or CF although a similar peak for the CF response was reported by Uppal (1970). Gillette (1974) has shown a similar pattern for the presence of IgM in the sera of birds following infection, whereas IgG neutralising antibodies showed

no such peak. This may suggest that IgM is inefficient at neutralising virus rather than more efficient than IgG at inhibiting HA.

Comparison of HI, SN and CF titres of the sera of individual birds 21 days after infection with M41 showed a much closer relationship between the HI and SN titres than between these titres and the CF titres. This may be due to the use of the Beaudette strain rather than M41 in the CF test, but is more likely to be due to the variable nature of the CF response.

We conclude that the HI test as described by Bingham *et al.* (1975) may be used to detect antibody levels to IBV in infected birds and it compares favourably with existing tests which are more expensive and laborious. The HI test may provide information which will help to understand the complicated antigenic relationships between strains of IBV. However further work is required to determine why only some of the strains will agglutinate red blood cells after enzyme treatment and, if possible, to develop a technique which will produce HA by all strains of IBV.

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RESUME

Evaluation préliminaire des épreuves d'hémagglutination et d'inhibition de l'hémagglutination pour le virus de la bronchite infectieuse aviaire

Quatre parmi 9 souches de virus de bronchite infectieuse, après concentration et traitement par la phospholipase C type 1 ont montré une activité hémagglutinante. Ces souches, Holte, Massachusetts (M41), H120 et Connecticut, peuvent être distinguées par l'épreuve d'inhibition de l'hémagglutination (HI), mais elles ont des relations bien plus étroites pour pouvoir être détectées par la réduction des plaques (PR). Les quatre souches hémagglutinantes ont été utilisées pour comparer les titres de HI et de PR de 17 sérums anti préparés contre des souches de références et sauvages. Dans la plupart des cas, les titres sont semblables bien que les titres de HI aient tendance à être plus élevés que les titres PR, spécialement avec l'antigène M41.

Les titrages HI d'un mélange des sérums de 20 oiseaux infectés avec M41 ont montré, 14 jours après l'infection, un pic d'activité qui n'est détecté ni par la séro-neutralisation, ni par la fixation du complément. Les titres HI de sérums individuels d'oiseaux infectés 21 jours avant avec le virus M41 peuvent être comparés favorablement avec les titres de séro-neutralisation, mais n'ont que peu de rapport avec ceux de la fixation du complément.

ZUSAMMENFASSUNG

Vorläufige Bewertung des Hämagglutinations- und des Hämagglutinations-Hemmungstestes für den Nachweis des "Infektiöse Bronchitis-Virus" in Vögeln

Vier von den 9 Stämmen des "Infektiöse Bronchitis Virus", die konzentriert und mit Phospholipase C Typ 1 behandelt wurden, zeigten haemagglutinierende Aktivität. Diese Stämme: Holte, Massachusetts 41, H120 und Connecticut waren im Haemagglutinationshemmungstest unterscheidbar, zeigten dabei aber engere Beziehungen zueinander als im Plaque Reduktions-Test (PRT). Diese 4 haemagglutinierenden Stämme wurden benutzt, um die Haemagglutinationshemmungstiter und die Plaque Reduktions-Titer von 17 Seren gegen Feldvirusstämme und Referenzstämme miteinander zu vergleichen. In den meisten Fällen war die Titerzunahme ähnlich. Es bestand aber eine Tendenz zu höheren Haemagglutinations-Hemmungstitern als PRT-Titern besonders beim Mass.41 Antigen.

Haemagglutinationshemmungs-Titrationen der Sammelseren von 20 Vögeln, die mit Mass. 41 infiziert worden waren, ergaben die höchsten Werte 14 Tage nach der Infektion. Diese Aktivität konnte jedoch weder im Serumsneutralisations- noch im Komplementbindungstest festgestellt werden. Die Haemagglutinationshemmungstiter von Einzelseren von Vögeln 21 Tage nach der Infektion mit Mass. 41 Virus entsprechen in etwa den Serumneutralisationstitern. Sie zeigten aber wenig Übereinstimmung mit den Komplement-Bindungs-Titern auf.

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Morphological Variation among Avian Infectious Bronchitis Virus Strains

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SUMMARY. Twelve strains of infectious bronchitis virus examined in the electron microscope showed differences in the morphology of the projections forming the corona. These differences were not related to the virulence or egg passage history of the virus, but showed some correlation with the ability of the virus to elicit a group antibody response.

THE MOST DISTINCTIVE morphological feature of infectious bronchitis virus (IBV) seen in negatively stained preparations is the possession of a corona of petal-shaped projections attached to the virus envelope (Berry *et al.*, 1964). These structures measured 20 nm in length and 9–11 nm in width at the outer edge (McIntosh *et al.*, 1967; Berry & Almeida, 1968). Not all authors, however, agree over the exact shape of these projections (Estola & Weckström, 1967; Nazerian & Cunningham, 1967). A collection of IBV strains was available at this laboratory, and it was of interest to examine morphological differences between strains and to attempt to correlate them with egg passage history, virulence and antigenicity.

MATERIALS AND METHODS

Virus Strains

Twelve known strains of IBV were used for electron microscopic study and antiserum preparation. Four of these were American reference strains: Beaudette, Massachusetts-41, Connecticut and Gray. The Australian 'T' strain and the H120 live vaccine strain of Dutch origin were included. The 6 remaining strains were British isolates. The Houghton 140 strain was supplied by Mr J. S. McDougall of Houghton Poultry Research Station, and the rest were isolated in the Poultry Department at Weybridge. The strains Beaudette, Massachusetts-41, H120, Houghton 140, and 927 were of the same antigenic type, but the other strains were all antigenically dissimilar, on the basis of a plaque reduction test (Bracewell, 1973). The test viruses used for the plaque reduction test included the American reference strains Iowa 609 and Holte and the British Field strain 227.

Serological Tests

Antisera were prepared in groups of 6 to 10 SPF chickens aged 6 to 9 weeks. During the course of this work, the virulence of the strains was assessed by the presence or absence of respiratory symptoms in the birds after inoculation. The antisera were titrated by a plaque reduction test in chicken kidney cell cultures, as described by Bracewell (1973). Serum titres were expressed as the $\log_2$ reciprocal of that dilution estimated to give a 75% reduction in plaque numbers.

Electron Microscopy

Allantoic fluid was harvested after 2 days incubation, usually before death of the embryos, pooled, and clarified in a bench centrifuge at approximately 400 *g* for 10 min. Forty-five ml was divided into 3 × 15 ml volumes which were layered on to 3 × 8 ml of 22% potassium tartrate and centrifuged in the 3 × 25 ml swing-out head of the MSE Superspeed 40 ultracentrifuge at 70,000 *g* (average) for 1 h. The pellet was resuspended in 1 ml of veronal-buffered saline, pH 7·2.

Virus preparations were examined within 2 h of centrifugation, using the negative staining technique of Brenner & Horne (1959). Carbon coated Formvar grids were stained with phosphotungstic acid (PTA) pH 6·6, and the preparations were examined in the Philips EM300 electron microscope.

RESULTS

Serology

In Table I, for most of the antisera shown, the titres can be seen to lie at two levels: a higher level which includes the homologous titre and also titres against virus strains of similar type, and a lower level which includes titres against virus strains of different type. This phenomenon was observed by Bracewell (1973) to be a general rule for all IB antisera when tested by this method. The higher level was called the 'type titre', and the lower level the 'group titre', of an antiserum. Where no homologous result was available, as for H120, 225 and 317, it was not possible to establish the type precisely. However, since H120 is known to be of similar type to Beaudette, Massachusetts-41 and Houghton 140, its type titre was estimated approximately by taking the mean of the titres against these 3 strains.

The results in Table I are summarized in Table II under 'Antiserum characteristics'. The values given for the group titre were arrived at by taking the mean of titres against virus strains of different type, and they are therefore approximations, depending on the use of the results available. The values given for the type titre are in fact the homologous titres, except for

TABLE I

SERUM TITRES (LOG_2 RECIPROCALS) BY PLAQUE REDUCTION TEST

Antisera	Test viruses: Beaudette	Massachusetts-41	Connecticut	Iowa 609	Gray	Holte	T	Houghton 140	227	591	604	927
Beaudette	10·6	4·8	1·4	0	0·8	0·8	0·7	9·0	ND*	ND	ND	ND
Massachusetts-41	6·6	8·5	4·0	1·2	3·5	2·8	1·8	5·2	6·8	3·3	2·2	8·0
Connecticut	0	0·5	6·8	0	1·1	0·3	0·5	0	ND	ND	ND	0·7
Gray	5·5	4·0	4·9	3·6	9·9	9·4	4·0	ND	ND	ND	ND	5·8
T	8·6	6·9	8·1	6·1	8·5	7·7	12·8	ND	5·3	ND	ND	ND
H 120	9·6	7·8	7·7	5·2	6·7	5·7	5·3	8·0	ND	ND	ND	ND
Houghton 140	9·9	8·3	6·5	5·5	6·0	6·1	5·1	9·4	ND	ND	6·3	ND
225	8·5	6·6	8·1	6·0	9·5	ND	7·2	ND	ND	8·4	7·8	ND
317	3·3	2·9	4·7	2·8	4·3	4·1	3·3	ND	ND	ND	ND	ND
591	8·3	7·2	8·6	7·0	7·6	8·7	8·0	ND	5·0	13·4	ND	ND
604	6·5	6·7	4·6	ND	6·4	6·0	4·8	ND	ND	5·8	11·0	ND
927	4·5	3·3	2·5	1·0	1·4	1·0	0·4	ND	ND	ND	ND	≥5·0

* ND = Not done

TABLE II

COMPARIONS OF 12 IB VIRUS STRAINS BY THEIR HISTORY, MORPHOLOGY, VIRULENCE AND ANTISERUM CHARACTERISTICS

Virus strain	History: number of egg passages	Virulence	Antiserum characteristics: Type titre	Group titre‡	Corona
Beaudette	Approx. 250	—	10·6	0·7	—
Massachusetts-41	11	+	8·5	2·7	+
Connecticut	25	—	6·8	0·4	—
Gray	20	—	9·9	4·6	++
T	8	+	12·8	7·3	+++
H 120	122	+	8·5†	6·1	+++
Houghton 140	9	+	9·4	5·9	++
225	14	—	Not known	7·8	++
317	24	+	Not known	3·6	—
591	11	+	13·4	7·6	+++
604	20	+	11·0	5·8	+
927	13	—	> 5·0	1·3	—*

* 927 showed unusual projections. See text
† Estimated
‡ Mean of titres against strains of different types

H120, as explained above, and are therefore precise values.

Electron Microscopy

The technique adopted for virus concentration resulted in preparations containing a high concentration of virus particles but little background material. Virions having no corona of projections were identified by their size, shape and staining characteristics. For each strain of virus, a large number of virus particles were examined and photographed and each particle was scored according to presence or absence of projections. Particles with a complete or partly complete corona were scored as positive, and those without as negative. The preparation as a whole was then evaluated according to the proportion of

particles displaying a corona. Thus strains carrying no projections were scored –, while those for which all virions showed projections scored ++++. (In fact, in no case did all virions display a corona.) The results are summarized in Table II, together with the results for virulence, egg passage history, and the titres of type and group antibody elicited by inoculation of virus. Quite marked pleomorphism was noted for some strains in respect of virion shape and apart from the presence or absence of a corona some variation was seen in the shape of projections. Strain 927 showed no corona but a few particles carried the type of projection referred to by Estola & Weckström (1967). These were thin radiating spikes, about 18 nm in length, some of which possessed a small knob-like dilatation at the outer extremity. It is not clear what relationship, if any, these spikes bear to the 'normal' petal-shaped projections of other strains (Fig. 1).

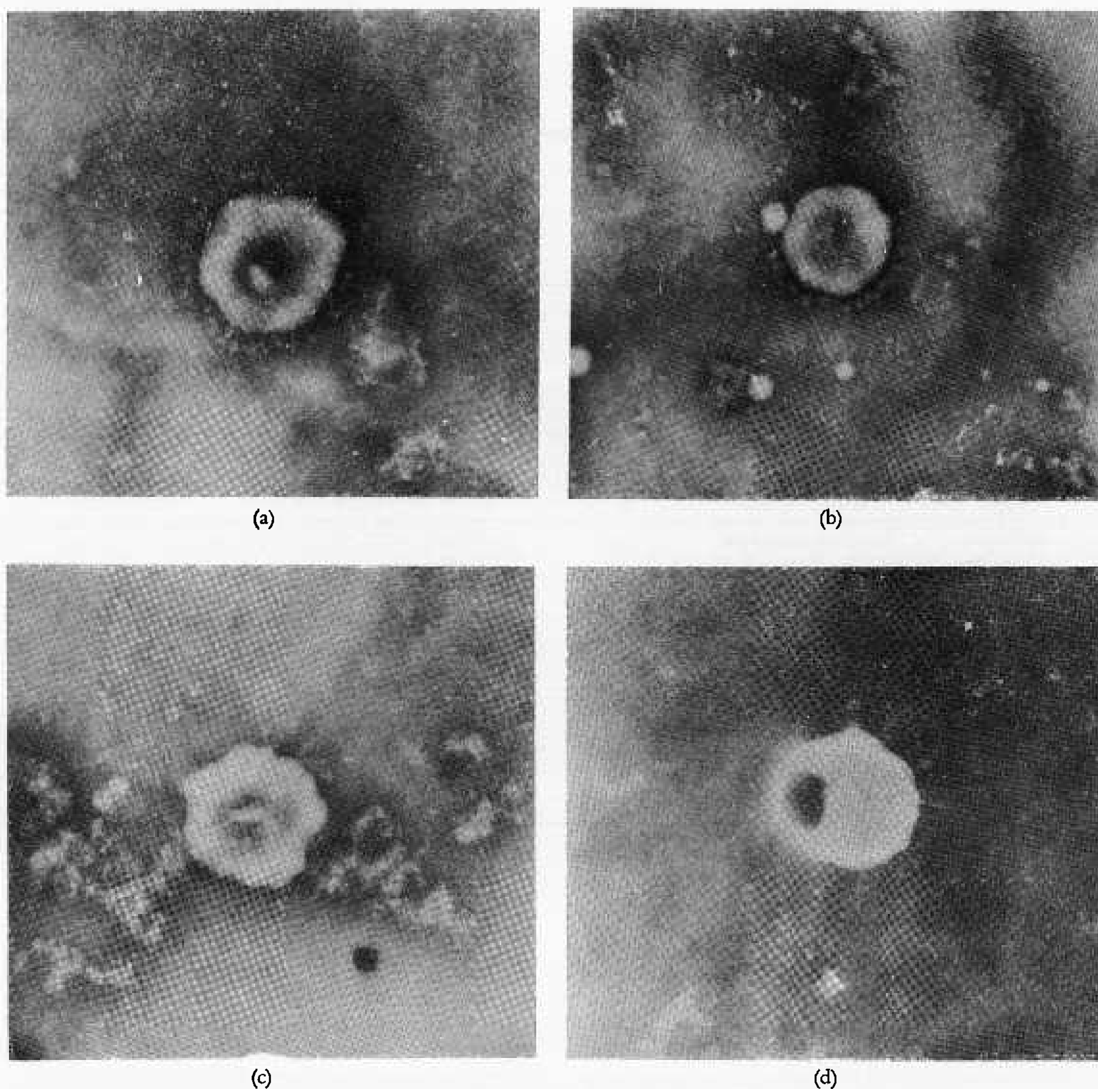

Fig. 1. (a) H120 strain: virion showing complete corona, (b) H120 strain: virion showing incomplete corona, (c) H120 strain: virion showing no corona, (d) 927 strain: virion showing unusual projections. All × 120,000.

DISCUSSION

The results of the study indicate that differences in morphology do exist between strains of IB virus prepared in the manner described, and it is unlikely that these differences could have arisen through variation in manipulative procedures alone. Such differences might, however, reflect variation in the fragility of the projections to centrifugal forces. The physical forces involved were of the order of 90,000 *g* and were probably responsible for the appearance in most preparations of particles with an incomplete corona. An attempt to discover whether virus in freshly harvested allantoic fluid from eggs infected with 'naked' strains carries a corona was made difficult by the relatively infrequent encounters with virions and the relatively high background in uncentrifuged allantoic fluid. For the Beaudette strain results indicated that the virus in such material did not have projections. If this is so, it would imply that the morphological variations are the result of virus-coded differences in projection size and shape.

The presence of a corona may confer upon the virus some biological advantage, such as better adsorption to ciliated epithelium, or better stability, which is not expressed when replicating in the allantoic cavity, and the projections may therefore tend to be lost upon continued passage in eggs. As Table II shows, however, there is little correlation between the egg passage history of the virus and the presence or absence of a corona. For example, Strain H120 had a full corona in spite of a large number of egg passages. Similarly, there is no direct relationship discernible between virulence and virus morphology. On the other hand, there appears to be some correlation between the morphology of the virion and its ability to elicit a group antibody response; those strains possessing a prominent corona producing the highest levels of group antibody in inoculated birds. This suggests that the corona may contain or be associated with a group antigen. The presence or absence of a corona might then be considered important in the selection of strains for vaccine production since only strains carrying a corona would induce a heterologous neutralizing antibody response. Clearly the association between corona and group antigen requires further experimentation to establish its validity.

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