

STRAIN DIFFERENCES IN THE
NEWCASTLE DISEASE VIRUS

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(With 2 Figures in the Text)

Newcastle disease is wide-spread throughout the world and presents a major economic hazard to the poultry industry. Many species of birds suffer from the infection including domestic fowls, turkeys, guinea-fowls and pheasants; natural infection has also been reported in starlings (Gillespie, Kessel & Fabricant, 1950), gannets (Wilson, 1950), cormorants (Blaxland, 1951), peacocks (Jansen & Kunst 1952), ospreys and parakeets (Zuydam, 1952). Since Doyle (1927) first isolated the causative virus from an outbreak in Newcastle-upon-Tyne, epizootics have been reported from many countries in Europe, Asia, and Africa as well as from Australia.

The disease is highly infective, severe in nature, and carries a fatality rate of 90–95 per cent. It is characterized by hyperpyrexia, profuse diarrhoea, and a thick mucoid nasal discharge; the pathological lesions are those of haemorrhagic extravasations into nearly all the organs but mainly concentrated in the alimentary tract.

In the United States of America, however, the manifestations of the disease have been so different that it was not realized until 1944 that a disease known in that country as avian pneumo-encephalitis was immunologically identical with European Newcastle disease (Beach, 1944). The American form of the infection is milder than the European; the fatality rate varies from 5 to 50 per cent. The predominant symptoms are inco-ordination, tremor, and leg and wing paralyses with pathological lesions mainly situated in the central nervous and respiratory systems.

Further evidence that Newcastle disease occurs in different forms under natural conditions is provided by the work of Blanco (1949) who reported that vaccines of American origin failed to protect fowls against the indigenous virus of an epizootic in Spain, although a vaccine prepared from a local strain proved effective.

Despite these facts little evidence has been obtained to indicate that there are any marked differences between individual strains isolated in different parts of the world. Jungherr, Tyzzer, Brandly & Moses (1946) have concluded that strains of American origin are primarily neurotropic or viscerotropic and that those from Europe are viscerotropic. Brandly, Moses, Jungherr & Jones (1946), comparing an English strain with others obtained from diverse sources, were able to show only a minor degree of strain specificity with haemagglutination inhibition tests. Variations in the pathogenicity of 18 American strains for young mice inoculated by the intranasal route were reported by Hanson, Upton & Brandly (1951). Evans (1950) showed that human group O cells modified by the action of two different strains of the virus differed markedly in their agglutination properties in the presence of serum from cases of infectious mononucleosis and infective hepatitis.

So similar are the Newcastle and the influenza viruses that Burnet (1942) has suggested that they derive from a common origin. Together with the mumps virus they share the properties of great infectivity, wide geographical distribution and the power of haemagglutination. At this point, however, the resemblances cease, for it has been shown that the infectivity of the influenza virus is closely related to an antigenic instability and a tendency to frequent mutation, whereas the stability of the mumps virus is so marked that it is unlikely that such factors play a part in the ecology of mumps.

It is uncertain whether the Newcastle virus owes its wide distribution and great infectivity to the property of variation since little attention has been paid either to the growth characteristics or the antigenic structure of individual strains. For this reason it was decided to obtain strains representative of epizootics widely spaced both in time and geographical situation, and to attempt to define more closely their biological properties, pathogenicity, and antigenic structure.

MATERIALS AND METHODS

Virus strains

1. HERTS strain isolated by Doyle in 1933 from an outbreak in England.
2. Hebrides strain (HEB) isolated by Blaxland (1951) from a cormorant shot in the vicinity of the island of Harris.
3. Lasswade strain (LASS) originally the HERTS strain which, after losing some of its virulence for the adult fowl, was passaged through the cormorant. On recovery its virulence had been restored.
4. Victoria strain (VIC) isolated by Albiston & Gorrie (1942) from an outbreak in Australia.
5. TWISS strain, isolated by Mitchell & Walker (1951) from an outbreak in Canada.
6. California strain (CAL) isolated in California, U.S.A.
7. Massachusetts strain (MASS) isolated in Massachusetts, U.S.A.
8. Blacksbury strain (B.1) an American strain (Hitchner & Johnson, 1948) of obscure origin and low virulence. It has been used successfully as a live vaccine in the U.S.A. In addition, two strains freshly isolated and in their first egg passage were examined.

Virus propagation

All the strains were cultivated in the allantoic cavity of 10-day-old chick embryos. It was important to standardize the temperature of incubation of inoculated eggs accurately at 37° C. A drop of 1° C. in the incubation temperature was found to prolong the time taken by the virus to kill the embryos by 9 hr., and the production of the infective, haemagglutinating, and haemolytic properties of the virus was delayed for periods varying between 9 and 15 hr.

Infectivity tests on embryonic fluids were made by inoculating 0·1 ml. of allantoic fluid, diluted in buffered saline to 1 in 1000, into four 9-day-old chick embryos.

Infectivity tests were also carried out with each strain in adult fowls. Groups of three young adult Rhode Island Red fowls received 1·0 ml. of infected allantoic

fluid by intramuscular inoculation. Only birds whose serum gave a negative haemagglutination inhibition test with the Newcastle virus were used.

Storage

All sera and infected egg fluids were stored at -35° C. in a refrigerated cabinet.

Immune sera

Antisera to five of the eight virus strains were prepared in the rabbit. Two intravenous inoculations of 1.0 ml. of infected allantoic fluid, spaced at 14-day intervals, were given.

Antisera to all eight strains were prepared in adult fowls. Pairs of adult Rhode Island Red fowls were isolated and inoculated intramuscularly with 1.0 ml. of infected allantoic fluid. With the exception of the birds inoculated with B.1 strain all developed Newcastle disease and four weeks later high titre immune sera were obtained from those which had survived inoculation with the HERTS, CAL and MASS strains. With the LASS, VIC, and TWISS strains all the birds used succumbed to infection and were replaced by birds which provided satisfactory sera after inoculation with infected allantoic fluid which had been exposed for 15 min. to irradiation with ultraviolet light. These latter fowls all developed symptoms of Newcastle disease but survived the infection. The B.1 strain did not produce an appreciable immune response when given intramuscularly but did so in full measure when given in a dose of 1.0 ml. by the intranasal route.

Human sera

Sera from seven cases of infectious mononucleosis were used; each gave a positive Paul Bunnell reaction after absorption, with titres of heterophile antibody of 1 in 80 or above. A single serum from a normal healthy adult was included for control purposes.

Haemagglutination tests

Progressive doubling dilutions of infected allantoic fluid were prepared in 0.2 ml. volumes in physiological saline. To each virus dilution was added 0.2 ml. saline and 0.2 ml. of 0.25% fowl erythrocytes. Readings were taken at 15, 30 and 60 min. and recorded according to the pattern of haemagglutination observed.

Haemolysin estimations were made colorimetrically by the following method. To 10.0 ml. infected allantoic fluid, clarified by low speed centrifugation, was added 0.25 ml. washed, packed, human, group O, erythrocytes, and the mixture was then incubated at 37° C. for $2\frac{1}{2}$ hr. Measurements of the absorption of the allantoic fluid before and after the addition of the erythrocytes were made in an E.E.L. colorimeter with a blue filter. The difference between the two readings gave a figure representing the degree of haemolysis that had occurred. It was essential to use freshly harvested allantoic fluid since storage at 4° C. or, more markedly, at -35° C. resulted in a great increase in haemolytic activity.

Haemagglutination inhibition tests

Two methods were used. In the first 0.2 ml. of a constant dilution of serum was tested against falling dilutions of the virus ranging from 1 in 1 to 1 in 124. The second method was a modification of that of Salk (1944) and was used in parallel with the first method in controlling the cross haemagglutination inhibition tests of the viruses and their specific antisera.

Absorption tests

To 10.0 ml. of immune serum diluted to contain a standard amount of antibody, 1.0 ml. of the absorbing antigen was added in the form of virus-laden erythrocytes. After 10 min. at 4° C. the erythrocytes were removed by centrifugation in the cold and the serum was then ready for examination by the haemagglutination inhibition test.

Absorbing antigens were prepared for each of the eight virus strains as follows. All reagents and glassware were chilled to 4° C. before use and the centrifugation was carried out at the same temperature. To 10.0 ml. of infected allantoic fluid was added 1.0 ml. of a saturated solution of potassium meta-periodate and 1.0 ml. of washed, packed fowl red blood cells. After thorough shaking, the cells were deposited by slow speed centrifugation and washed three times with saline.

Sera were titrated with virus modified human group O cells by the haemagglutination method of Evans (1950).

Serum neutralization tests

Cross neutralization tests *in ovo* were carried out by the method of Cunningham (1951). Neutralization indices were calculated by the method of Reed & Muench (1938).

RESULTS

Pathogenicity experiments

The pathogenicity of eight strains of the Newcastle virus was studied in two hosts, the adult fowl and the chick embryo. From 3–7 days after intramuscular inoculation in groups of three fowls, five of the strains constantly induced typical and fatal Newcastle disease with pathological lesions of the European type. In the groups which received MASS and CAL fatal diseases occurred in two instances; death was deferred to the 10th and 31st days respectively. When the sera of surviving birds was examined it was found that high levels of specific antibody had developed. The B.1 strain failed to elicit more than a trivial antibody response and all the birds receiving it appeared to be quite unaffected.

A similar pattern of behaviour was found when the eight strains were inoculated under strictly identical experimental conditions into the allantoic cavity of chick embryos. Five strains killed 100% of the embryos in 42–45 hr. MASS required 54 hr. to produce the same effect, CAL 57 hr., and B.1 72 hr. These findings were remarkably constant for each strain and were obtained repeatedly throughout the investigation. Infective virus was present from the 9th hr. onwards in harvests

from the five strains, whereas it could not be detected until the 12th hr. in the case of the three American strains, MASS, CAL and B.1 (Table 1).

Growth curves

The rates of multiplication of the eight strains in the chick embryo were compared using as criteria of growth the development of infectivity, haemagglutinin and haemolysin. The first property of the virus which could be detected is that of infectivity some 9 hr. after inoculation; at 21 hr. haemagglutination was present and 3–6 hr. later haemolysis. Five of the strains, HERTS, VIC, LASS, HEB and TWISS showed closely similar patterns (Fig. 1).

The three American strains, MASS, CAL and B.1 grew more slowly and a more gradual rise to maximum values was associated with the prolonged killing time

Table 1. *The pathogenicity of strains of the Newcastle virus for the adult fowl and the chick embryo*

| Virus strain ... | HERTS | VIC | HEB | LASS | TWISS | MASS | CAL | B. 1 |
|---|-------|-----|-----|------|-------|------|-----|----------|
| Fowls inoculated/ fowls died | 3/3 | 3/3 | 3/3 | 3/3 | 3/3 | 1/3 | 1/3 | 0/3 |
| Average time in days between inoculation and death of fowls | 7 | 3 | 3 | 5 | 3 | 10 | 31 | survived |
| Time in hours at which 100 % of chick embryos died | 42 | 45 | 42 | 45 | 45 | 54 | 57 | 72 |

Table 2. *The reciprocals of haemagglutination titres of eight strains of the Newcastle virus for the erythrocytes of four species*

| Virus strain ... | HERTS | VIC | HEB | LASS | TWISS | MASS | CAL | B. 1 |
|--------------------|-------|-----|-----|------|-------|------|-----|------|
| Fowl erythrocytes | 320 | 160 | 320 | 160 | 160 | 640 | 640 | 320 |
| Ox erythrocytes | 160 | 320 | 80 | 320 | 80 | 10 | 5 | 5 |
| Pig erythrocytes | 160 | 80 | 80 | 160 | 0 | 5 | 5 | 20 |
| Sheep erythrocytes | 40 | 80 | 40 | 40 | 10 | 0 | 0 | 0 |

already described for these viruses. The development of infectivity by these strains was delayed until the 12th hr., haemagglutinins did not appear until the 24th hr., and haemolysins at the 24–30th hr.

Virus haemagglutination

The haemagglutinating properties of the eight virus strains for the erythrocytes of fifteen different species were investigated. All the strains gave constant and closely similar results with the cells of the fowl, duck, turkey, cormorant, rook, seagull, man, dog, guinea-pig and mouse. The erythrocytes of the horse and the cat were not agglutinated by any of the strains. Ox, sheep and pig erythrocytes showed a lesser susceptibility to virus agglutination, a feature which was most marked with the three American strains (Table 2).

In order to determine whether any variation in the haemagglutination properties of the Newcastle virus occurred in a manner resembling the O→D variation of the influenza viruses, two strains freshly isolated from Newcastle disease were obtained.

During their first ten passages in the allantoic cavity of the chick embryo no significant alteration could be detected in the haemagglutination titres for fowl, guinea-pig and human group O erythrocytes.

Haemagglutination inhibition

Although the haemagglutination inhibition test has been used extensively in the antigenic analysis of the influenza viruses its application to those of Newcastle disease has been neglected.

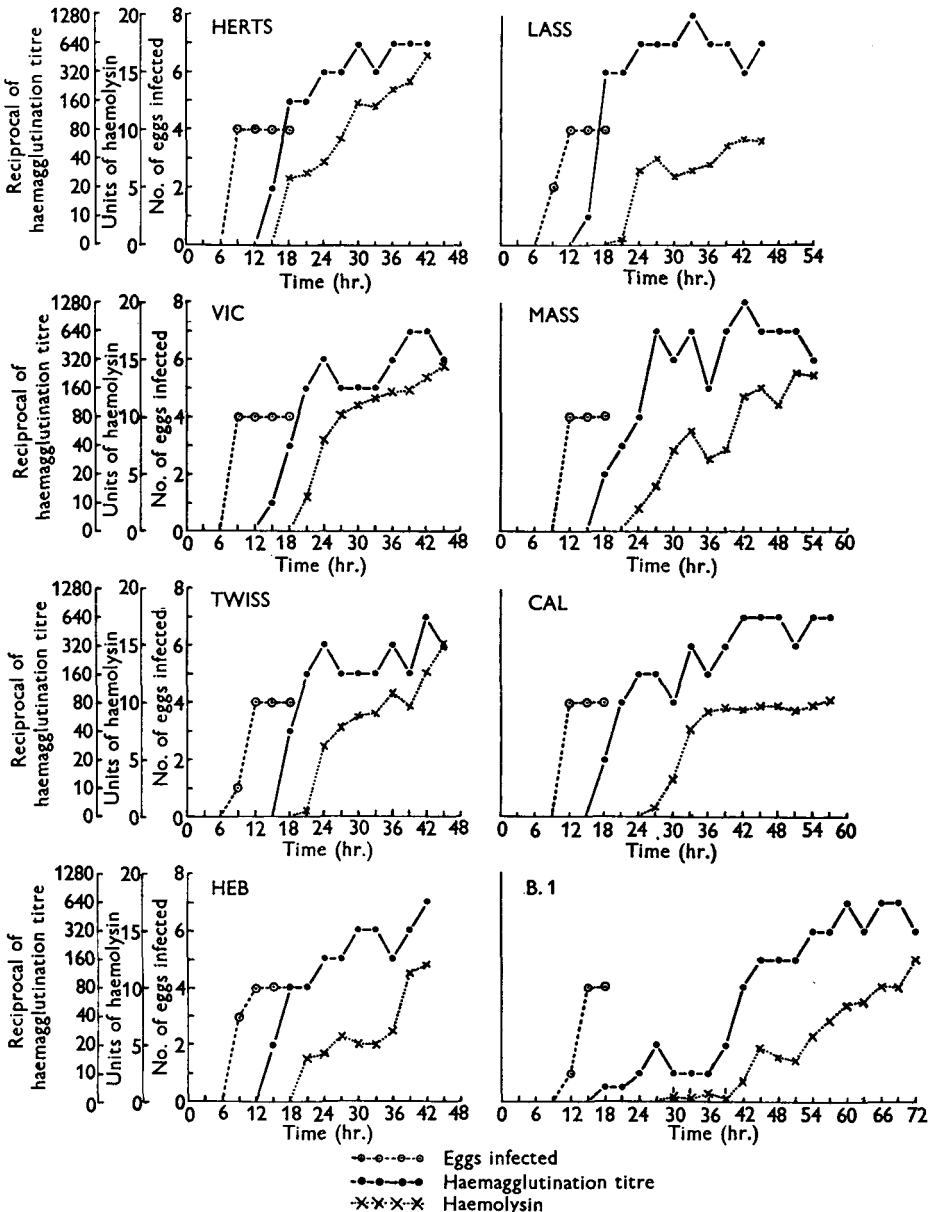


Fig. 1. Growth curves of eight strains of Newcastle virus.

Using antisera to HERTS, LASS, CAL, MASS and B.1 prepared in the rabbit, no significant difference in the inhibitory titres could be detected. With immune fowl sera, however, slight variations of antigenic structure were detected and the results of the experiments could be reproduced by either of the two techniques used. The sera always exerted their maximum inhibitory effect on the homologous viruses but considerable degrees of inhibition could be demonstrated against all

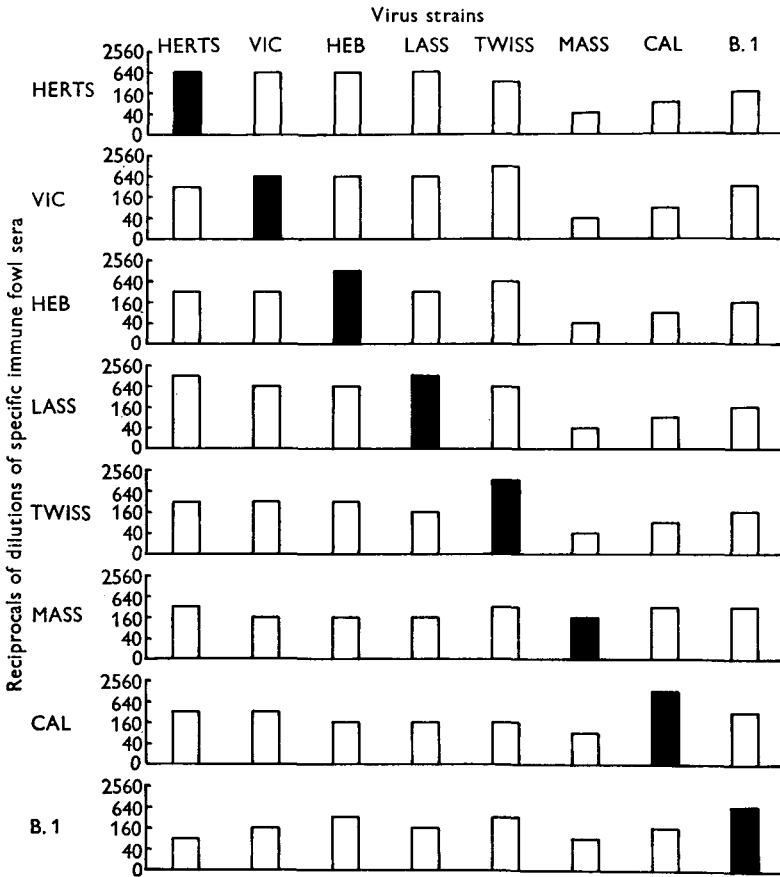


Fig. 2. Haemagglutination inhibition of eight strains of Newcastle virus by homologous and heterologous immune sera.

the heterologous strains (Fig. 2). The HERTS immune serum showed equal inhibitory powers for the homologous virus, LASS and VIC; while the VIC immune serum affected the same three viruses to an equal extent.

Absorption experiments

An attempt to define the antigenic composition of the Newcastle viruses was made using the method which Jensen & Francis (1953) applied successfully to a study of the influenza viruses. Newcastle virus, however, is absorbed only to a minimal extent by formalized human erythrocytes, and it was necessary to devise another method of providing a stable erythrocyte virus union as an absorbing

agent. Periodate-treated virus held on the surface of fresh fowl erythrocytes at 4° C. proved satisfactory for the purpose.

Table 3 shows the results of the absorption of the eight specific immune fowl sera with their homologous and heterologous viruses.

Although antibody absorption was always maximum by the homologous virus, a lesser and varying degree of absorption could be demonstrated with each of the heterologous strains. Two absorption patterns were seen: HERTS, VIC, HEB, and LASS all appear to have similar antigenic structures while MASS, CAL, and B.1 are closely related to each other although they clearly differ significantly from the first group. The virulent Canadian TWISS strain possesses an antigenic composition which is intermediate between these two patterns.

Table 3. Amount of antibody absorbed from immune sera diluted to standard antibody content. Expressed as the fold reduction in titre in terms of amounts of virus inhibited

| Virus strains | HERTS | VIC | HEB | LASS | TWISS | MASS | CAL | B. 1 |
|---------------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| HERTS | 32 | 8 | 8 | 16 | 8 | 8 | 8 | 8 |
| VIC | 32 | 32 | 16 | 16 | 16 | 8 | 8 | 8 |
| HEB | 32 | 32 | 32 | 16 | 8 | 8 | 8 | 8 |
| LASS | 32 | 32 | 16 | 32 | 16 | 16 | 8 | 8 |
| TWISS | 8 | 8 | 4 | 4 | 32 | 4 | 8 | 8 |
| MASS | 16 | 16 | 8 | 8 | 16 | 32 | 32 | 8 |
| CAL | 8 | 8 | 4 | 4 | 16 | 8 | 32 | 8 |
| B. 1 | 2 | 4 | 8 | 2 | 8 | 8 | 8 | 32 |

Table 4. Neutralization indices of immune fowl antisera to homologous and two heterologous strains of the Newcastle disease virus

| Antiserum to | Virus strain | | |
|--------------|---------------|-----------|--------------|
| | HERTS | CAL | MASS |
| HERTS | 10,000 | 6 | 10 |
| CAL | 10 | 63 | 10 |
| MASS | 100 | 6 | 1,000 |

Virus neutralization

The HERTS, MASS and CAL virus strains and their appropriate fowl antisera were selected for comparison in *in ovo* neutralization tests. Again the immune sera showed their maximum effect against the homologous strain of virus in every case; their neutralizing powers against heterologous strains were considerably weaker.

Haemagglutination of virus treated human erythrocytes by sera from cases of infectious mononucleosis

Human group O erythrocytes, after modification by the action of Newcastle disease virus, become agglutinable by the sera from cases of infectious mononucleosis and by the sera of animals immunized against the Newcastle virus (Burnet & Anderson, 1946). No strain specificity with immune fowl sera could be demonstrated but human glandular fever sera showed marked differences in their power to agglutinate erythrocytes modified by the action of the eight virus strains

studied (Table 5). It will be seen that the haemagglutination titres of seven Paul Bunnell positive sera for erythrocytes treated with HERTS, VIC, LASS, HEB and TWISS are almost identical and that very low titres occurred with cells which had been modified by MASS, CAL and B.1.

DISCUSSION

Eight strains of the Newcastle virus have been found to fall into two distinct groups. The first includes all the highly virulent strains from Europe and Canada, the second comprises American strains of medium or low virulence. Common antigens and a similar pattern of reproduction are shared by both groups. After a silent phase of 6–9 hr. the developing virus is first demonstrable by infectivity tests. Following an interval of 3–6 hr. the property of haemagglutination is

Table 5. *Haemagglutination titres of infectious mononucleosis sera from human group O erythrocytes modified by the action of eight strains of the Newcastle virus*

| Serum no. | Reciprocal of serum haemagglutination titre with virus-treated erythrocytes | | | | | | | | |
|---|--|------|------|------|------|----|------|-----|--|
| | Normal control | 34 | 39 | 38 | 18 | B | 28 | 19 | |
| Virus strain used to treat erythrocytes | | | | | | | | | |
| HERTS | 20 | 2560 | 2560 | 2560 | 640 | 80 | 2560 | 10 | |
| VIC | 20 | 2560 | 2560 | 2560 | 1280 | 80 | 2560 | 10 | |
| HEB | 20 | 2560 | 2560 | 2560 | 640 | 80 | 2560 | 10 | |
| LASS | 0 | 2560 | 2560 | 2560 | 640 | 40 | 2560 | 10 | |
| TWISS | 10 | 2560 | 2560 | 2560 | 640 | 40 | 2560 | 20 | |
| MASS | 0 | 10 | 80 | 80 | 320 | 20 | 10 | 40 | |
| CAL | 0 | 0 | 40 | 20 | 80 | 0 | 0 | 20 | |
| B. 1 | 10 | 20 | 40 | 20 | 40 | 10 | 0 | 5 | |
| Reciprocal of titre of heterophile antibody | > 10 | 2560 | 2560 | 160 | 160 | 80 | 80 | 640 | |

developed and after a further 3–6 hr. that of haemolysis appears. With standardized inocula diluted to 1 in 100 this general pattern of virus growth was repeatedly observed with all the eight strains (Fig. 1).

Although these observations on the manner of growth of the Newcastle virus are confirmed by the work of Gordon, Birkeland & Dodd (1952), they differ from the results of experiments by Nadel & Eisenstark (1955). The latter authors, using the CAL strain as a model, were able to obtain embryonic fluids which contained moderate amounts of haemagglutinin before any trace of the power to infect could be detected. This apparent discrepancy in the results of two similar investigations may be due to differences in the sizes of inocula and to varying experimental conditions. Anderson (1947) has shown that Newcastle virus in infected allantoic fluid is largely present in the form of aggregates composed of some 40 virus particles and that it is thus not in homogeneous suspension. When the virus pools are stored at -35°C . the aggregates are dispersed and there is a rise of some fourfold in the haemagglutination titre together with an even greater increase in haemolytic

activity (Table 6). This dispersal of the clumped elementary bodies results in the liberation of the virus in a form which Anderson considers to be less active since, although it can attach itself to erythrocytes, it cannot subsequently be eluted. Conditions of storage thus markedly influence the nature of the virus in infected embryonic fluids and it is likely, after storage for more than 24 hr. at -35°C ., that a considerable proportion of virus is present in an inactive form.

It may be that Nadel and Eisenstark's experimental results were influenced by the presence of modified virus in their inocula and that this less active virus has affected the growth processes. Further work on this aspect of the nature of the Newcastle virus will be necessary before it is possible to accept or disprove the hypothesis made by these authors that here is a reproductive process characterized by an 'incomplete' phase in a maturation cycle similar to that of the influenza viruses.

Although the two groups of viruses are most strikingly distinguished from each other by their differing virulence, each has a number of other characteristics which are shared by all the members within the group. The highly pathogenic strains (HERTS, VIC, HEB, LASS and TWISS) all have the same rapid rate of growth

Table 6. *The rise in haemagglutination and haemolytic activity of allantoic fluids on storage at -35°C .*

| Virus strain | Reciprocal of haemagglutination titre after | | | | Haemolytic activity in units after | | | |
|--------------|---|--------|--------|--------|------------------------------------|--------|--------|--------|
| | 0 hr. | 24 hr. | 48 hr. | 7 days | 0 hr. | 24 hr. | 48 hr. | 7 days |
| HERTS | 320 | 640 | 1280 | 1280 | 12.4 | 75 | 100+ | 100+ |
| B. 1 | 20 | 40 | 80 | 80 | 0.3 | 11 | 25 | 100+ |

and a similar pattern of development; they agglutinate the same range of erythrocytes of 15 different animal species, and they have the same power to modify human group O cells and thus to render them agglutinable by the serum from cases of infectious mononucleosis. Furthermore, the antigenic structures of four of these five strains as seen when studied by haemagglutination inhibition and absorption methods are almost identical.

The three strains of the second group (CAL, MASS and B.1) in addition to being less virulent, grow at a slower rate and the development of their virus activities is generally retarded. The modification of group O erythrocytes by these strains is minor in degree and their capacity to agglutinate the cells of the ox, pig, and sheep is minimum. These strains all are closely similar in antigenic structure.

It is thus evident that there are at least two distinct types of the Newcastle disease virus. The two types resemble each other in that they share antigens and a common pattern of multiplication; they differ in rate of growth, haemagglutinating activity and pathogenicity. One or more antigenic components present in the first type are lacking in the second.

The hypothesis that the high virulence of strains recovered from the European form of the disease is related to the presence of an additional antigen might be made were it not for the fact that such a component is poorly represented in the

Canadian TWISS strain, which is perhaps the most virulent of all the strains examined. The pathogenicity of the two types is more closely related to their rates of multiplication and their capacity to modify or agglutinate erythrocytes than to any antigenic instability.

Although the eight strains examined were selected from sources widely separated both in time and geographical situation, the degree of antigenic variation among the virulent strains is not sufficiently great to warrant the assumption that here, as in influenza, lies the secret of the great infectivity of Newcastle disease. Indeed there is no detectable difference between the antigenic structures of the HERTS strain and the HEB strain which was recovered from an epizootic 18 years later. The antigenic similarity of virulent strains originating in epizootics of the European form of Newcastle disease in many parts of the world is striking. Nevertheless it is evident that variation does occur, for different antigenic patterns are found both in the less virulent American strains and the highly pathogenic TWISS strain from Canada.

The Newcastle virus thus has a tendency to antigenic variation which is intermediate in degree between that of the influenza and mumps viruses. In this respect it occupies a position similar to that in which it is placed by virtue of its haemagglutinating activity on the 'receptor gradient' of Burnet, McCrea & Stone (1946). It is of interest that no difference could be detected in the power of the two types of this virus to alter the virus receptors of human erythrocytes.

SUMMARY

The biological characters of eight strains of the Newcastle disease virus, isolated in different parts of the world between 1933 and 1951, have been studied and compared in detail.

Two types of the virus have been distinguished, one from the Newcastle disease prevalent in Europe and Australia, the other from that occurring in the United States of America.

Viruses of the first type are highly virulent, multiply rapidly and successively develop the properties of infectivity, haemagglutination and haemolysis. Their haemagglutination pattern is constant and wide; they profoundly modify human group O erythrocytes, and they are antigenically homogeneous.

Viruses of the second type are of weaker virulence. They grow at a slower rate in the allantoic cavity of the chick embryo, and the development of their characters is retarded; they have a reduced haemagglutination pattern and only a minimum capacity to modify human group O cells. Antigenically strains of the second type are homogeneous.

From haemagglutination inhibition and absorption studies it is concluded that major antigens are shared by both types, and that the first type possesses one or more antigens lacking in the second.

The highly virulent Canadian TWISS strain possesses the characters of the first type and an antigenic constitution with features of both types.

The significance of the results is discussed in relation to the ecology of Newcastle disease.

We acknowledge most gratefully all the kindly encouragement and stimulating criticism given to us throughout the whole of this work by the late Prof. T. J. Mackie. We are greatly indebted to Dr J. E. Wilson of the Ministry of Agriculture and Fisheries Veterinary Laboratory at Eskgrove, Lasswade, for placing the resources of this laboratory at our disposal. Our thanks are also due to Drs J. D. Blaxland, C. A. Mitchell, A. W. Stableforth, and to Sir Macfarlane Burnet for supplying us with the virus strains we examined.

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(MS. received for publication 16. XI. 55)