STUDIES ON THE ANTIGENIC COMPOSITION OF HUMAN INFLUENZA VIRUS A STRAINS

with the aid of the haemagglutination inhibition technique

J. VAN DER VEEN

STUDIES ON THE ANTIGENIC COMPOSITION OF HUMAN INFLUENZA VIRUS A STRAINS

with the aid of the haemagglutination inhibition technique

STUDIES ON THE ANTIGENIC COMPOSITION OF HUMAN INFLUENZA VIRUS A STRAINS

with the aid of the
haemagglutination inhibition technique

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE GENEESKUNDE AAN DE RIJKSUNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR-MAGNIFICUS DR B. A. VAN GRONINGEN, HOOGLERAAR IN DE FACULTEIT DER LETTEREN EN WIJSBEGEERTE, TEGEN DE BEDENKINGEN VAN DE FACULTEIT DER GENEESKUNDE TE VERDEDIGEN OP WOENSDAG 18 JANUARI 1950 TE 15 UUR

DOOR

JOHAN VAN DER VEEN

GEBOREN TE LEEUWARDEN

1950
H. E. STENFERT KROESE'S UITGEVERS-MIJ N.V.
LEIDEN

Promotor: Prof. Dr J. Mulder

ACKNOWLEDGEMENTS

This investigation was carried out in the Virus Laboratory of the Clinic for Internal Medicine, University of Leyden, a subdepartment of the Influenza Research Organization of the Institute for Preventive Medicine, Leyden.

The experiments were performed with the co-operation of Mr L. M. BRANS, Miss S. W. ENSERINK, Mr F. HEEMSKERK, Mr P. WEBBERS, Mr J. NIBBELINK and Miss I. DE NOOYER.

If it had not been for the continuous help of Prof. Dr W. J. DE HAAS, Prof. Dr C. J. GORTER and the scientific staff of the Kamerlingh Onnes Laboratory in providing us with technical advice on low temperature technique, our investigations would have been impossible.

The investigation was supported by General F. DAUBENTON, M.D., General J. TH. WILKENS, M.D. and Lieutenant-Colonel B. J. W. BEUNDERS, M.D. of the Dutch Army Medical Corps.

We are greatly indebted to Dr C. H. ANDREWES, London, Dr F. M. BURNET, Melbourne, Dr C. M. CHU, London, Dr H. COX, Lederle Laboratories, Pearl River, N.Y., Dr TH. FRANCIS, Ann Arbor (Mich.), Dr G. K. HIRST, New York, Dr G. LÖFSTRÖM, Upsala, Dr T. P. MAGILL, New York, Dr. P. VON MAGNUS, Copenhagen, Dr C. H. STUART HARRIS, Sheffield and Dr R. M. TAYLOR, New York, for sending us influenza strains with their laboratory data. We wish to express our gratitude to Prof. C. H. STUART HARRIS for reading the manuscript.

Financial support was provided by the Institute for Preventive Medicine, Leyden, N.V. Philips-Roxane, Weesp, the State Department of Science, the Jan Dekker Fund and the Curaçao Fund for Preventive Medicine.

CONTENTS

I.

FORMER STUDIES ON THE ANTIGENIC COMPOSITION OF HUMAN INFLUENZA VIRUS A STRAINS

Introduction. In the year 1933 a virus causative of human influenza was demonstrated for the first time by inoculating ferrets intranasally with garglings from patients suffering from the clinical disease (SMITH, ANDREWES and LAIDLAW(1933)). Later a similar virus was recovered in many different countries during influenza epidemics (survey by VAN BRUGGEN *et al.*(1947)).

Since 1940 two groups of influenza viruses — influenza virus A and B — have been recognized. As groups they are quite distinct from each other in their antigenic composition (FRANCIS(1940), MAGILL(1940)) but in both groups there exist strains with individual antigenic properties. The first communications on this subject, as regards the A group, were made by MAGILL and FRANCIS(1936, 1938) and WILSON SMITH and ANDREWES(1938).

In the present communication the A strains are indicated by the name originally given by the author, the year, and the country where the strain was isolated (table 1 shows the data on strains and sera).

Differences of antigenic composition in human influenza A strains, employing the cross-neutralization test in mice. By means of cross-neutralization tests in mice with ferret antisera 28 A strains (17 from England, 3 from the continent of Europe, and 8 from America), were classified by WILSON SMITH and ANDREWES(1938). These authors considered that there are 4 principal antigenic components present in the A group, and that these occur in most strains but in unequal proportions.

They therefore classified the strains into 3 groups. The first or "specific" group comprised 4 strains, WS(1933 E), TALMEY(1937 E), CHRISTIE (1937 E) and GATENBY(1937 E). In addition, they indicated a second group of "intermediate" strains, which showed a close relationship to more than one specific strain. Finally they described a third group, comprising strains which were related to all four specific strains and were relatively non-specific antigenically.

MAGILL and FRANCIS(1938) examined 23 human strains (17 from USA, 1 from Australia, 1 from Hungary, and 4 from England: WS(1933 E), TALMEY(1937 E), GATENBY(1937 E) and BH(1935 E)), with the aid of rabbit sera. They arrived at a classification in 5 groups, merging however more or less into each other. In their study WS(1933 E) also represented a distinct group, to which, however, none of the other strains belonged. TALMEY(1937 E) and GATENBY(1937 E) also fell into two different groups. One of the American strains, PR8(i.e. Puerto Rico 8,

TABLE 1

Data on the influenza virus strains used in this investigation

Strain designation	Location of donor	Year of isolation	Passage formula of the strain used for preparation of ferret antisera [1]
Lee(1940 USA)	United States of America	1940	$F_8 M_{137}$ and $F_8 M_{137} E_{146}$
Swine-15(1930 USA)	United States of America	1930	F M E_7
WS(1933 E)	England	1933	F M E_{19} and F M E_{26}
PR8(1934 USA)	United States of America	1934	$F_{198} M_{593} E_{59}$ and $F_{198} M_{593} E_{66}$
Phila(1935 USA)	United States of America	1935	$F_{246} M_{96} E_5$
Melbourne(1935 Au)	Australia	1935	F M E (E_1) [2]
Christie(1937 E)	England	1937	F M and F M E_6 and F M E_{33}
Talmey(1937 E)	England	1937	F M and F M E_1 and F M E_{21}
Gatenby(1937 E)	England	1937	F M E_7 and F M E_{30}
Burr(1937 E)	England	1937	F M E_3
Bos(1939 Ned)	Netherlands	1939	F_7 M
A(1941 Ned)	Netherlands	1941	$F_3 M_{38} E_{21}$
Sl(1941 Ned)	Netherlands	1941	$F_4 M_{36} E_{17}$ and $F_4 M_{36} E_{25}$
Schn(1941 Ned)	Netherlands	1941	$F_3 E_8$
Schn(1941 Ned), mouse-adapted	Netherlands	1941	$F_3 E_6 M_{12} E_5$
Weiss(1943 USA)	United States of America	1943	$F_3 M_{32} E_{64}$
965(1943 USA)	United States of America	1943	E_8
Hemsbury(1943 E)	England	1943	F M E_1
A 128(1943 Sw)	Sweden	1943	F E_9
Cam(1946 Au)	Australia	1946	E_{69}
Gg(1947 Sw)	Sweden	1947	$F_5 E_{15}$

[1]) The strain used as antigen in serum inhibition tests has the same passage formula, only having some more final egg-passages.

[2]) F M E (E_1): Number of previous egg-passages unknown and one egg-passage done in our laboratory.

TABLE 1 (continued)

Strain designation	Location of donor	Year of isolation	Passage formula of the strain used for preparation of ferret antisera
Barratt(1947 E)	England	1947	E (E_2)
Barratt(1947 E), mouse-adapted	England	1947	E M_{20}
Kunz(1947 E)	England	1947	E_7
Woiteki(1947 E)	England	1947	E_{13}
K(1947 Ned)	Netherlands	1947	F_3 E_{39}
K(1947 Ned), mouse-adapted	Netherlands	1947	F_3 E_{24} M_{13} E_9
FM1(1947 USA)	United States of America	1947	E M_8 E_{14}
Rhodes(1947 USA)	United States of America	1947	F_4 M_{14} E_{22}
1236(1947 USA)	United States of America	1947	E_{15}
1265(1948 USA)	United States of America	1948	E_{14}
FJS(1948 Au)	Australia	1948	E_{14}
AI(1949 Ned)	Netherlands	1949	E_8 and E_{16}
Hof(1949 Ned)	Netherlands	1949	E_8
Vr(1949 Ned)	Netherlands	1949	E_8 and E_{10}
Hes(1949 Ned)	Netherlands	1949	E_8 and E_{10}
Wagt(1949 Ned)	Netherlands	1949	E_8 and E_{14}
Heer(1949 Ned)	Netherlands	1949	E_8 and E_{15} and E_{20}
Heer(1949 Ned), mouse-adapted	Netherlands	1949	E_8 M_{20}

1934), was also by itself a representative of one group, but with the peculiarity of polyvalence, both in regard to the strain and to the antiserum against it [1]). There were considerable differences between the classifications of SMITH and ANDREWES and of MAGILL and FRANCIS, although with regard to some strains they resembled each other. SMITH and ANDREWES tried to account for the differences by means of the circumstance that different species of animals had been used for the production of the antisera, and also that the American investigators

[1]) Swine influenza (Swine-15 (SHOPE 1930)) was included in their experiment and though belonging to the A group stood out clearly from the above mentioned groups of human influenza A strains.

relied on differences which they were inclined to ignore. This investigation on a large scale with the mouse protection test has not been repeated since 1938. The British workers and the other European Centres provisionally held to the English classification, and compared newly isolated strains with the four "specific" strains.

BURNET and CLARK(1942) developed a third theory of the antigenic composition of human A-strains. They employed ferret sera, and defined the strains WS(1933 E), a strain isolated by them in Melbourne in 1935 (MELBOURNE(1935 Au)), and GATENBY(1937 E) as prototype of three groups, within which more specific but unstable differences may be observed. While ANDREWES had found (quoted from BURNET and CLARK(1942)) that Melbourne was an intermediate strain with TALMEY and CHRISTIE components, these investigators found no difference in antigenic composition in the three strains mentioned. To explain this, they assumed that after repeated mouse passages the strains gradually lost their more specific antigenic structure and passed into an intermediate form, represented by Melbourne. It remains an open question, however, whether this view has been adequately proved. Still, BURNET and CLARK's interpretation remains important, and it was put forward again by HIRST in 1947.

The PR8(1934 USA) strain. (FRANCIS(1934)). Smith and Andrewes' analysis placed this strain in the intermediate group, with a close relationship to the strains TALMEY(1937 E) and CHRISTIE(1937 E). They found more strains of this kind. From Magill and Francis' analysis there appears to be some relationship to TALMEY, and generally a closer relationship to most of the other strains that were examined. In consequence of this polyvalence and the fact that the strain is higher virulent for mice, it has been widely used in America to investigate the antigenic structure of newly isolated A strains.

New A strains isolated in Europe after 1937. After the great 1936/1937 epidemic in Europe numerous fresh influenza epidemics were recorded, and new strains were isolated. (PATOCKA(1939), MULDER(1940), STUART HARRIS *et al.*(1940), ANDREWES *et al.*(1941), DAVOLI and PARODI(1941), MULDER *et al.*(1941), TAYLOR and DREGUSS(1941), DREGUSS(1942), STUART HARRIS *et al.*(1943)). In so far as they were analysed, all showed the antigenic composition of the intermediate strains, which puts them in close proximity to the strains TALMEY(1937 E) and CHRISTIE(1937 E). In America new strains were found related to PR8. It can hardly be doubted that on both continents more or less identical A strains were recovered between 1937 and 1943, as PR8 is closely related to TALMEY and CHRISTIE.

The employment of polyvalent strains in the analyses of the antigenic structure. For a provisional analysis of the antigenic structure of newly isolated strains the employment of polyvalent strains had advantages. The English authors mentioned this already in 1938, but their most polyvalent strains (BURR(1937 E) and KOPP(1936 Germany)) showed a low virulence for mice. Afterwards the PR8-strain was also employed in the National Institute for Medical Research in London (STUART HARRIS *et al.*(1940)). It is also the strain now generally incorporated in influenza vaccines.

The epidemiological significance of the variations in the A group encountered in the year 1937. The epidemiological significance of the variations in the A group has never been clearly understood. During the big influenza epidemic of 1936/1937 in England the three specific strains, TALMEY, CHRISTIE and GATENBY were recovered in towns near each other (Chatham, Shorncliffe and Windsor). In addition a strain of the WS type (EO) was recovered in London, a polyvalent strain (BURR) in Uxbridge and also many intermediate strains. Nor has it been possible since 1937 to draw up a classification of epidemics caused by certain "specific" A strains.

Other methods of analysis of antigenic composition. The use of other procedures to study the antigenic properties of influenza strains within the A or the B group was not generally applied until the in vitro haemagglutination inhibition test became known. The complement fixation test with antigen from infected mouse lung did not prove sufficiently specific to differentiate the strains within the A group owing to "soluble antigen" (LENNETTE and HORSFALL(1941), EATON(1941), HOYLE(1945)). FULTON and DUMBELL(1949) showed however, that when using elementary bodies from allantoic fluid (by adsorption and elution from red cells or high-speed centrifugation) complement-fixation though elaborate does permit recognition of antigenic differences within the principal groups of A viruses.

The cross-immunity test in mice was performed on a large scale by SMITH and ANDREWES(1938) en MAGILL and FRANCIS(1938) and showed strain differences which in principle agreed with those obtained with the cross neutralization test in mice.

Analysis of the antigenic composition of strains of influenza virus with the haemagglutination inhibition test of Hirst. The newer methods of study of influenza virus firstly by means of propagation on the chick embryo (BURNET(1936)) and secondly by in vitro haemagglutination (HIRST(1941), MCCLELLAND and HARE(1941)) have provided totally different techniques for studying the antigenic composition of strains. This is due to the fact that the inhibition of haemagglutination by antisera proved to be very specific (HIRST(1943)).

Influence of the passage formula of strains and antisera on the antigenic structure. Owing to the propagation of old stock strains on embryonated eggs, and the fact that after 1941 numerous new strains were isolated directly in eggs, the adaptation to mice fell into disuse, and a series of strains has been developed with different "passage formulae". The "old" stock strains isolated before 1941 have all undergone ferret- and mouse-passages. By propagation on eggs their passage formula becomes F_p, M_q, E_r, with different values for p, q and r. Many strains isolated after 1941 have E_r as formula (including passages via amnion and allantois). Further there may exist F_p, E_r and E_rM_q strains, and possibly also M_q and M_qE_r strains. The antisera, too, have different formulae, according to their preparation from ferret-, ferret-mouse, ferret-mouse-egg, ferret-egg, or egg-passages of virus.

HIRST(1943) has pointed out that all A strains isolated by him in 1941 in the amnion of the chick embryo had the same antigenic com-

position (ferret sera being employed), and he supposed that the differences in antigenic structure of strains isolated in a single epidemic (such as in 1936/1937) may have been caused by repeated ferret- and mouse-passages. Moreover he has found (HIRST(1947 a)) that two egg strains had actually changed their antigenic properties after passage in mice. From this it would follow that the passage formula of the strain may influence the antigenic structure.

Analysis of the antigenic composition of human A strains investigated with the haemagglutination inhibition test. The second world war was responsible for the fact that the analysis of the antigenic structure of a large number of A strains by means of the agglutination inhibition test has as yet been little studied. The first investigation on this subject was made by HIRST(1943). He used for this purpose three American A strains of 1941 isolated in the chick embryo, the strain PR8(1934 USA) and the strains WS(1933 E), TALMEY(1937 E), GATENBY(1937 E), CHRISTIE (1937 E) and the swine influenza strain SHOPE(1930 USA). The old laboratory strains all had the formula FME, and the ferret antisera had been prepared with infected allantoic fluids. In his hands the 1941 strains differed sometimes considerably in antigenic structure from the other strains and had the closest relationship to PR8 and CHRISTIE.The antigenic relationship of the older laboratory strains that were examined agreed in the main with the results of SMITH and ANDREWES. The four "specific" strains of these workers were antigenically remote from each other. PR8 was most closely related to CHRISTIE and TALMEY. The swine virus of SHOPE differed greatly from the other strains. HIRST pointed out that, although clear differences between strains will always be found, the degree in which they appear to deviate from each other may differ considerably according to the particular antisera which are employed.

A new influenza A subgroup. In the years 1945, 1946 and 1947 influenza strains were recovered in Australia, Sweden, England, USA and Holland, belonging to the A group and showing considerable antigenic differences from the established laboratory strains as demonstrated by the cross-haemagglutination inhibition test (ANDERSON and BURNET (1947), ANDERSON(1947 a), FRANCIS *et al.*(1947), STUART HARRIS and MILLER(1947), DUDGEON *et al.*(1948), KALTER *et al.*(1948), LÖFSTRÖM (1949), MULDER and van der VEEN(1948), RASMUSSEN *et al.*(1948), SIGEL *et at.*(1948), TAYLOR(1949)) or by the cross-complement fixation test (FULTON and DUMBELL(1949)).

TAYLOR(1949) performed cross-agglutination inhibition tests with different strains from the American epidemic in the spring of 1947 and compared them with an A strain isolated in 1943, a swine strain and a strain of influenza B (LEE). Hamster and rabbit antisera were employed. With the exception of one strain, the 1947 strains comprised a separate coherent group, being not only quite different from the classic strains PR8 and LEE and the swine virus, but also from a 1943 strain of type A. The new type is denoted by American investigators as Influenza A-prime.

It was the aim of our study to make a more detailed investigation of the antigenic composition of human A strains by means of the new method of haemagglutination inhibition.

II.

MICRO HIRST TECHNIQUE FOR THE HAEMAGGLUTINATION INHIBITION TESTS WITH INFLUENZA VIRUS STRAINS

In 1941 HIRST(1941) and MCCLELLAND and HARE(1941) found that chicken red cells were agglutinated by influenza virus, and this discovery was immediately applied to measure virus and serum antibodies quantitatively in vitro (HIRST (1942 a)). The technique of the virus and serum titration with Hirst's phenomenon varies slightly in the various laboratories (HIRST and PICKELS(1942), BURNET and CLARK(1942), SALK(1944)). In the virus laboratory of the Clinic for Internal Medicine, Leyden University, a modified micro method is practised (MULDER and GOSLINGS (1948)). The technique is as follows:

Preparation of dilutions. As diluting fluid for virus, serum and red cells we employ a 0.85 per cent NaCl solution, buffered with a 20 per cent sodium carbonate solution at pH 7.0. Comparative virus titrations in phosphate buffer (pH 7.0) and buffered saline yield the same results.

Preparation of red cell suspension. Blood of fullgrown hens is collected in a solution of 3.8 per cent sodium citrate, washed three times with saline, and next centrifuged for 10 minutes (at the rate of 2000 r.p.m.) in a graduated centrifuge tube. The red cell volume is made up with saline to make a 10 per cent suspension for storage. The required dilution is made immediately before the test. The red cells are not kept longer than five days at 2° C.

Micro technique for the titration of virus. The virus dilutions are made with bevelled Pasteur pipettes with nearly constant diameter in the concavities of a porcelain tile. The diameter of the holes is 11 mm and their depth is 9 mm. The final dilution of the red cells yields excellent sedimentation and agglutination patterns. The tiles are placed in a Petri dish with moistened filtering paper at the bottom to prevent desiccation. A series of twofold virus dilutions in saline is mixed with an equal volume (four drops) of one per cent red cell suspension (final dilution 0.5 per cent). The patterns, which are distinctly visible against the white background, are read after standing for half an hour at 2° C (plate 1, figure 1 and 2, opposite p. 8). Partial agglutination of the red cells, which can be clearly judged, is expressed as 1, 2 and 3 plus agglutination, depending on the size of the patterns. At 2° C the patterns remain unaltered for many hours. The titre of a virus suspension is recorded as the reciprocal value of the highest final dilution that gives complete agglutination,

and this is termed one agglutination unit (AU). As in the case of the HIRST-SALK method, the titres of the virus dilutions in the micro technique are inversely proportional to the concentration of the final red cell dilution (table 2).

TABLE 2

Titrations of the strain PR8 with a micro method using different final concentrations of red cells

Concentration of red cells (per cent)	Dilutions of virus							
	40	80	160	320	640	1280	2560	5120
1.5	++++	++++	++++	++++	+	0	0	0
1.0	++++	++++	++++	++++	+++	0	0	0
0.75	++++	++++	++++	++++	++++	++	0	0
0.50	++++	++++	++++	++++	++++	+++	0	0
0.375	++++	++++	++++	++++	++++	++++	++	0

Micro technique for the titration of serum. With a 1 ml pipette the serum is first diluted in saline, to make a 1 : 6 dilution. From this solution, sufficient for titrations with several strains, a series of twofold serum dilutions is made in saline in a porcelain tile, and next mixed with half a volume (two drops) of virus suspension. After interaction for half an hour at 2° C two drops of a 2 per cent red cell suspension are added. Readings are made after the tiles have remained undisturbed for half an hour at 2° C. The titre of the serum is expressed as the reciprocal value of the final serum dilution that gives partial (2 plus) agglutination. If this pattern does not occur, the dilution with 2 plus agglutination is calculated by means of interpolation from the dilutions showing larger or smaller agglutination patterns.

Concentration of the virus in the haemagglutination inhibition test. At least two AU of the virus are always employed. In order to determine the exact quantity of virus used in the serum tests, we perform, simultaneously with the latter, a virus titration with the same virus dilution and red cell dilution as that added to the serum. The number of AU units employed in the test is calculated as follows: if the virus dilution t gives 4 plus agglutination, and in the dilution $\frac{1}{2}$ t the red cells settle out completely (0), the dilution t contains 1 AU virus. If the dilution $\frac{1}{2}$ t shows partial agglutination (1, 2 or 3 plus), the virus dilution t contains respectively $1\frac{1}{4}$, $1\frac{1}{2}$ or $1\frac{3}{4}$ AU virus. From the number of AU virus present in the dilution t it may then be calculated how many AU have been employed in the serum dilutions as final concentration. The final concentration of the virus that is employed generally varies from $2\frac{1}{2}$ to $3\frac{1}{2}$ AU.

PLATE I

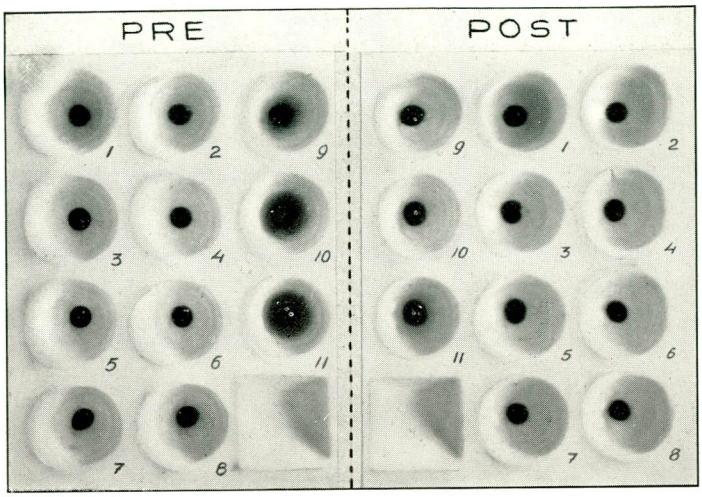

Fig. 1.

Titration of the pre- and post-infection ferret serum against the A-prime strain Barratt (1947 E) with the haemagglutination inhibition test in a tile of porcelain. The sera are not freed from non-specific inhibitor. The first serum dilution is 12.

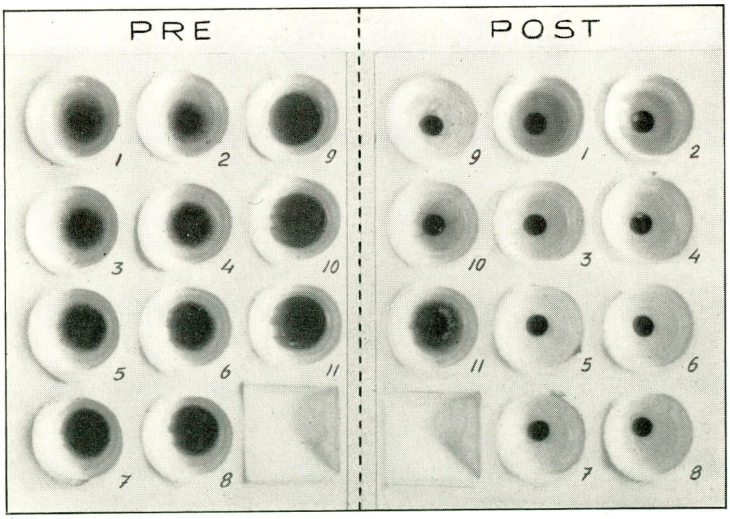

Fig. 2.

Titration of the same sera against the strain Barratt (1947 E), the sera being freed from non-specific inhibitor by interaction with crude filtrate of V. cholerae.

Ultimately the serum titre found by test is adjusted to a value equivalent to 3 AU virus, it being assumed that an inverse proportion exists between the serum titre and the number of AU of the virus that is added (table 3).

Accuracy of the micro titrations. Apart from the virus, the serum and the red cells, the following variables are of importance:

(1) *Pasteur pipette.* In order to prevent where possible errors arising from the employment of this pipette, each series of experiments is performed with the same pipette, which between the various actions is cleaned with saline, alcohol and ether, afterwards being dried in a flame. We also endeavour to keep constant other factors which may influence the size of the drops, such as the space of the opening, the position of the pipette, the rate of dropping, the volume that is present in the pipette, the absence of fluid on the outside of the pipette, and the temperature of the pipette.

TABLE 3

Ferret antiserum titration against the homologous strain PR8 with a micro method using increasing numbers of agglutinating units

Number of AU	Final dilutions of antiserum (ferret)							
	190	380	770	1540	3070	6140	12290	24580
3	0	0	0	0	0	++++	++++	++++
6	0	0	0	0	+++	++++	++++	++++
12	0	0	0	+++	++++	++++	++++	++++
24	0	0	+++	++++	++++	++++	++++	++++

(2) *The porcelain tiles.* After each experiment the tiles are cleaned with water, alcohol and ether, and then dried. All the tiles that are used, have been examined beforehand to see whether they yield good agglutination patterns of the red cells.

(3) *The size of the drops.* The drops are not always quite identical in volume but vary according to whether saline, serum or red cell suspension is used. The differences however proved to be slight [1]).

(4) *The temperature.* Unless stated otherwise, all tests took place at 2° C.

Determination of the standard deviation of the dilution errors resulting from the micro method. The standard deviation of the method was determined in a series of twofold dilutions of red cell suspension, mixed with

[1]) With the graduated pipette described by M. VAN RIEMSDIJK (1917) it is possible to drop more accurately than with a Pasteur pipette. As, however, the use of the van Riemsdijk pipette took longer than the Pasteur pipette, we have provisionally preferred the latter.

an equal volume (four drops) of saline. The number of red cells per cubic millimetre in certain red cell dilutions was counted by using a B-T-Haemocyter. This procedure was repeated ten times with 4 to 5 countings per dilution. The mean dilution of the red cells was then expressed as the ratio of this dilution to the original undiluted suspension (table 4). The experimental averages deviated from the dilutions that were theoretically expected. We have to consider the fact, however, that the red cell suspension has a different drop size and a different viscosity from the saline. In view of the purpose of these tests it is especially the standard deviation that is important, giving a measure of the accuracy of the drop technique. The test here described was performed in the same way as the antibody titration of serum. As it may be assumed that in both cases the errors of the drop technique are equal, we may conclude from the results that if a serum titration is performed 100 times, the deviation of the mean dilution will in 95 cases not exceed 30 per cent. As in titrations of different sera each dilution in itself always shows

TABLE 4

Standard deviation of a red cell dilution using a micro method

Theoretical dilution	Mean experimental dilution of red cells	Standard deviation	Standard deviation expressed in per cent
2	2.0	± 0.09	± 4.5
8	9.1	± 1.0	± 11
32	35	± 4.1	± 12
128	117	±14	± 15

practically the same deviations, and also because the absolute figures are of secondary importance for the serology of influenza, we have assumed that in practice the serum dilutions range in a purely geometrical progression.

In order to obtain an idea of the magnitude of the standard deviation in virus titrations and serum titrations, ten virus titrations were performed twice, side by side, and in exactly the same way, with one strain (PR8). The standard deviations of the ten titres were respectively ± 7 and ± 10 per cent. The standard deviations of two series of ten serum titrations performed side by side were respectively ± 14 and ± 14 per cent.

From these tests it is clear that the micro technique yields a reasonable degree of accuracy.

Comparison of the micro technique and Hirst method. In order to compare the two methods, virus suspensions of different strains were titrated simultaneously by the HIRST method and by the micro method.

Titration of virus by Hirst-Salk technique. We followed the technique of HIRST-SALK(SALK 1944), except that a 0.20 per cent final dilution of red cell suspension was employed. In the comparative agglutination tests the HIRST-SALK method yielded a titre which was two to six times

higher than that of the micro method. This difference could not be due only to the greater red cell concentration used in the micro tests, as the relative titres (HIRST-SALK/micro) were not constant. The ratio varied not only in the titration of different strains, but also with different egg-passages of the same strain. We did not investigate all the factors involved in this variation as this is of secondary importance for the purpose of this paper.

Titration of serum by Hirst-Salk technique. The exact number of AU virus was checked in the same way as in micro serum titration, viz. by performing a virus titration simultaneously with the antibody titration. These tests were also made at a temperature of 2° C. The titres were read one hour after the virus was added to the red cell-serum mixture, and were calculated in the same way as those in the micro method with regard to 3 AU virus. As in the micro haemagglutination inhibition tests higher antibody titres were found when a longer period of interaction took place between serum and virus (fig. 3). The red cells (contrary to the

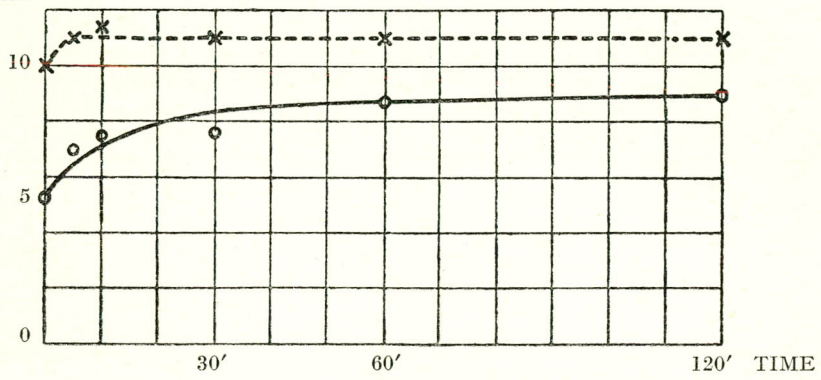

Fig. 3.
Comparison of the results of haemagglutination inhibition tests of normal and immune ferret sera after different periods of interaction of serum and virus. Titre expressed in terms of a geometrical series (using two-fold dilutions of serum 1 : 12). Time in minutes.
Solid line = non-specific titre of normal ferret serum against the strain K(1947 Ned), ferret-egg strain.
Broken line = antititre of PR8 serum against PR8 strain.

original HIRST-SALK method) were added to the serum half an hour after the addition of virus. The titres found with the HIRST-SALK technique were about twice as high as the titres of the micro titrations. The ratios of the heterologous titres to the homologous titres, however, were for the most part identical in the two methods, so that comparable results were obtained (table 5). SALK(1944) pointed out that the results of serum titrations are comparable, when the amount of antigen and the concentration of the red cells vary in the same ratio. HIRST(1942 a) found that the volume of the test does not influence the optimal ratio figure between antigen and antibody (see also WHITMAN(1947)). The tests described here confirm those views.

TABLE 5

Comparison of ferret antiserum titrations against homologous and heterologous strains with different methods of titration

Antiserum (ferret)	Technique of titration	Titres against virus			
		PR8 (1934 USA)	Christie (1937 E)	Talmey (1937 E)	Gatenby (1937 E)
PR8(1934 USA)	Hirst-Salk	1	1/1.8[1])	1/30	1/9.2
	Micro	1	1/2	1/30	1/11
Gatenby(1937 E)	Hirst-Salk	1/1.3	1/2.6	1/50	1
	Micro	1/1.3	1/2.9	1/60	1

[1]) Ratio of titres of the heterologous to the homologous strain.

Advantages of the micro technique. In this study the virus titrations and the antibody titrations were performed in micro for various reasons. The method saves glass ware, personnel, and biological materials. The technique is quick and easy to perform. The agglutination patterns including intermediate forms, are convenient to read and are very clearly readable after a short time.

III.

NEUTRALIZATION OF THE NON-SPECIFIC INHIBITING SERUM FACTOR IN FERRET SERA

As early as 1942 HIRST(1942 a) pointed out that normal ferret sera inhibit the haemagglutination of influenza virus strains to a greater or lesser degree. This non-specific inhibition is particularly strong against newly isolated egg-strains of influenza virus A (RICKARD *et al.* (1944), FRANCIS *et al.*(1946), FRANCIS(1947 a), HIRST (1947 b)). After being heated at 56° C, however, the LEE strain (influenza B) also shows a high non-specific titre in normal and antiserum (FRANCIS(1947 a)). The strong inhibitory action of normal sera against some strains is not due to a previous infection of the ferret (BURNET and MCCREA(1946)). It must probably be attributed to a muco-protein, which is present in the serum (BURNET *et al.*(1947), BURNET(1948 a)). BURNET and co-workers (1948 c) distinguish substances inhibiting the heated LEE strain, the "Francis inhibitor", and substances inhibiting non-inactivated strains, the "non-specific inhibitor". It is not only pre-infection ferret serum that inhibits the haemagglutinating property of influenza virus but the same phenomenon is shown by the sera of guinea-pigs, rabbits, and mice (HIRST(1942 a), MCCREA(1946). FRANCIS(1947 a), FRIEDEWALD *et al.*(1947), SVEC and FORSTER(1947), DE BURGH *et al.*(1948), WOOLLEY (1949)).

Non-specific inhibition. The degree to which influenza strains are inhibited by normal ferret sera varies greatly. Moreover the degree of inhibition is not always constant for a certain strain. FRANCIS(1947 a) reported that it is diminished considerably after repeated egg passages. So far our experience is as follows: some strains, such as PR8(1934 USA) show the inhibition only slightly when passed in the chick embryo. Others, such as A(1941 Ned) on the other hand, are much more strongly inhibited non-specifically. The strain K(1947 Ned) from the third ferret passage inoculated in the allantoic sac of chick embryos still showed the phenomenon at the 46th egg passage. The 24th passage was adapted to the mouse and was re-inoculated into the egg after 13 mouse passages. The strain was now hardly

susceptible to inhibition at all (except for the appearance of two plus patterns in the first dilutions (see table 6)). The strain Sl(1941 Ned), isolated in the ferret and propagated in mice, showed a gradual reduction of non-specific inhibition between the 13th and the 19th egg-passage. The egg strain BARRATT (1947 E), on the other hand, after adaptation to the mouse lung and after 20 mouse passages, showed hardly any change in non-specific inhibition, which in this strain was high (about 3000 in the ferret serum). A survey will be found in table 6. It was also found that the serum of the same ferret may differ in inhibitory action, depending on the time the blood is taken. In this way deceptive pseudo antititres may appear! ANDERSON *et al.* (ANDERSON *et al.*(1946), ANDERSON(1948)) have called attention to the fact that the non-specific serum titre greatly depends on the hens whose red blood cells are employed, because of the competition existing between the agglutinating capacity of the red cells and of the substance which inhibits agglutination non-specifically.

A considerable non-specific inhibition is also found in the serum titrations performed by the HIRST-SALK technique. The titres of normal ferret sera against strains recently adapted to the egg varied from 192 to 3072.

It has also been found that sera from uninfected animals may neutralize certain strains in the mouse protection test (FRANCIS *et al.*(1947), BURNET and MCCREA(1946), WILSON SMITH and WESTWOOD(1949)).

Influence of non-specific inhibition on the formation of red cell patterns. When using normal ferret sera and strains which show non-specific inhibition, it may be observed that the transitional zone of the haemagglutination patterns from complete inhibition to complete agglutination is broader than in titrations of antisera with high titres against homologous and heterologous (related) strains. When using normal sera the transitional zone becomes broader when the test takes place at a higher temperature (20 to 37° C); the limits, on the other hand, become more distinct when using higher concentrations of red cells and when the temperature is + 2° C.

Influence of the period of interaction between serum and virus. Fig. 3 shows that the maximum antibody titre of an antiserum is reached after a shorter time of interaction than the maximum non-specific titre when the tests are performed at + 2° C.

TABLE 6

Pattern reading of titrations of normal ferret serum against influenza strains with different passage formulae

Experiment	Strain and passage formula	A.U. of virus	Final dilutions of serum									
			24	48	96	190	380	770	1540	3070	6140	12280
1	K(1947 Ned) F_3 E_{30}	$3\frac{1}{2}$	0	0	0	0	0	0	0	++	++++	++++
	K(1947 Ned) F_3 E_{24} M_{13} E_1	$3\frac{1}{2}$	++	++	++++	++++	++++	++++	++++	++++	++++	++++
2	K(1947 Ned) F_3 E_{22}	3	0	0	0	0	0	0	++	++++	++++	++++
	K(1947 Ned) F_3 E_{34}	3	0	0	0	0	0	++	++++	++++	++++	++++
	K(1947 Ned) F_3 E_{46}	3	0	0	0	0	0	0	++	++++	++++	++++
3	Sl(1941 Ned) F_4 M_{36} E_{13}	$2\frac{1}{2}$	0	0	0	0	0	0	0	++	++++	++++
	Sl(1941 Ned) F_4 M_{36} E_{16}	$2\frac{1}{2}$	0	0	0	0	0	±	+	++	++++	++++
	Sl(1941 Ned) F_4 M_{36} E_{19}	$2\frac{1}{2}$	0	0	0	0	±	++	++	+++	++++	++++
4	Barratt(1947 E) E_r [1]) (E_4)	$2\frac{1}{2}$	0	0	0	0	0	0	0	±	+++	++++
	Barratt(1947 E) E_r (E_1 M_{10} E_1)	3	0	0	0	0	0	0	+++	++++	++++	++++
	Barratt(1947 E) E_r (E_1 M_{20} E_1)	3	0	0	0	0	0	0	±	+++	++++	++++

[1]) E_r indicates, that the number of egg-passages is unknown. Between brackets has been placed the number of egg-passages, done in our laboratory.

HIRST found a similar phenomenon with respect to the "Francis inhibitor" (HIRST(1948 b)).

Elimination of the non-specific inhibitory action. Influence of the direct mixing of the virus in the red cell/serum mixture. By means of a direct mixing of serum, virus and red cells it is possible to reduce the intensity of the non-specific inhibition (fig. 3). Apart from the fact that this modification causes the antibody titre to be somewhat reduced, a more or less considerable non-specific inhibition also remains. GREEN and WOOLLEY(1947) found that rabbit serum may still exercise an inhibitory influence when it is added to the virus after the red cells.

Influence of the heating of the sera. The heating of normal ferret sera for one hour at 56° C proved to have little effect on the "Francis inhibitor", but it sometimes reduced the non-specific inhibition to a considerable degree (BURNET and MCCREA(1946), MCCREA(1946), HIRST(1948 b)), and sometimes slightly or not at all (FRIEDEWALD *et al.*(1947), SVEC and FORSTER(1947)). We found that the result of this treatment depended in great measure on the virus strain that was used and that the reduction in most cases was not complete. The serum factor is probably complex, and for various strains it has fractions which are in differing degrees sensitive to heat (BURNET and MCCREA(1946), HIRST(1948 b), BURNET (1948 c)).

Influence of the interaction between heated Lee virus and ferret sera. An excess of heated LEE virus was added to ferret antiserum, and after the mixture had been left standing for some hours at 2° C, the virus was removed by adsorption with chicken cells and centrifugation (18000 r.p.m.). Serum titrations were then performed with the supernatant liquid. The addition of chicken red cells did not in itself influence the non-specific inhibition (see also HIRST(1948 a)). It appeared that the serum titre of the supernatant against heated LEE was inversely proportional to the number of AU of heated LEE that was added. There apparently existed a relationship between the virus and the non-specific serum factor which was the same as that between the virus and antibodies (HIRST(1942 a), SALK(1944), WHITMAN(1947), FRANCIS(1947 a)). In this way it is possible partly to eliminate the non-specific inhibition effect while retaining the antibody titre (table 7). This method has some drawbacks, however. After such treatment non-specific inhibition may be unaltered in amount for some strains though reduced against others. Again, the addition of the virus, even if concentrated, causes the serum to be materially diluted, which is a particular drawback in the case of antisera with low titres against heterologous or homologous strains. The method is moreover rather complicated.

The influence of the interaction between unheated Lee virus and serum. On the analogy of an enzymatic interaction between influenza virus and red cells (HIRST(1942 b), STONE(1947 a and b), BURNET(1947), ANDERSON(1948), GREEN and WOOLLEY(1947)), a similar activity may

be presumed with regard to the non-specific serum factor (BURNET(1948 a)). In contradistinction to heated LEE virus, which probably combines with the inhibitor, unheated influenza virus (owing to its enzymatic action) is able to destroy the serum substance (DE BURGH *et al.*(1948), HIRST(1948 a and b)). Ferret sera were treated with unheated LEE virus in the same manner as with heated virus. The interaction between serum and virus took place, however, at a temperature of 37° C (see table 7). In this way it was possible to neutralize the non-specific inhibitor to a certain extent, but this method has the same objections as the treatment with heated LEE virus.

Destroying the non-specific inhibitor by periodate. HIRST and HOTCHKISS (cited by GREEN and WOOLLEY(1947)) observed that periodate destroys that component of the red cells which reacts with the virus. HIRST(1948 b) employed this discovery in order to destroy the non-specific inhibitor in the serum. He caused sodium periodate to act on serum, and after a sufficient period of interaction he added glucose, in order to discontinue the oxydative action of the periodate on the serum. We have compared this method with the destruction by cholera enzyme (see below).

TABLE 7

Reduction of non-specific inhibition of normal ferret serum against different strains after treatment with heated and unheated Lee virus

Treatment of normal ferret serum	Heated Lee	K [1] (1947 Ned)	Sl (1941 Ned)	Gg (1947 Sw)
Heated Lee 3 hours at 2° C.	16-fold	2-fold	4-fold	4-fold
Unheated Lee 3 hours at 37° C.	16-fold	2-fold	16-fold	4-fold

[1]) Ferret-egg strain.

One part of serum is mixed with two parts of a saturated solution of potassium periodate. After fifteen minutes' interaction at 56° C two parts of glucose 7 per cent are added. Control experiments with other strains which are strongly inhibited by normal sera, showed that this treatment is just adequate to destroy the non-specific inhibitor in a serum dilution of 1 : 12. When the time of interaction is longer, or when more potassium periodate is added, the serum becomes cloudy and in some cases the serum proteins are precipitated. But the above method, too, caused a certain turbidity in some sera. In less intensive treatment with potassium periodate, however, the non-specific inhibition against some strains remained, either completely or partly.

Table 8 gives a summary of the titrations of a number of antisera, respectively those untreated and those treated with cholera filtrate and potassium periodate against the homologous strain.

Neutralization of the non-specific inhibition by means of titration in the presence of anti-Calcium ions. BURNET and co-workers discovered that Calcium-ions are required for the activity of the enzymes in filtrate of Vibrio Cholerae (BURNET and STONE (1947), STONE(1947b), BURNET(1948c), EDNY(1949)) and for the enzymatic action of influenza virus on red cells (BRIODY(1948)). In the course of experiments aiming at the neutralization of the activity of V. cholerae filtrate on the non-specific inhibitor of normal ferret sera by the addition of a citrate solution, we were struck by the fact that even without the interaction of cholera

TABLE 8

Titres of antisera of ferrets, treated in different ways, against the homologous strains

Strain	Titres of homologous antiserum [1])		
	Untreated	Treated with crude cholera filtrate	Treated with KJO_4
Swine-15(1930 USA)	9560	3580	2990
Christie(1937 E)	14600	2050	2300
Talmey(1937 E)	10240	13650	6830
Lee(1940 USA)	9560	5380	2990
Sl(1941 Ned)	21500	21500	10750
Barratt(1947 E)	24000	10240	10240

[1]) The ferret antisera, untreated and treated with crude cholera filtrate and KJO_4 are titrated with the same egg fluid of the homologous strain on the same day.

filtrate the non-specific inhibition of a serum was less when titration was carried out in the presence of citrate solution. We then examined solutions containing other anti-Calcium ions, and it was found that all these solutions had a more or less similar action.

Technique. The following solutions were used in the experiments. Two per cent potassium oxalate (dissolved in phosphate buffer at pH = 7.0). Two per cent sodium metaphosphate, $NaPO_3$ (dissolved in phosphate buffer at pH = 7.0). One per cent sodium fluoride (dissolved in phosphate buffer at pH = 7.0).

3.8 per cent sodium citrate (dissolved in distilled water, pH=7.4). The sera with which the titrations were performed were diluted in one of these solutions, instead of in saline. Virus and red cells were suspended in saline, so that ultimately the solutions were diluted twice. Virus suspensions, when titrated in saline, contain the same number of AU as when titrated in one of the above mentioned solutions. Moreover the number of AU virus was checked in the same solution in which the serum was diluted.

Table 9 gives a survey of the titrations of a normal serum in different solutions with some A strains.

TABLE 9

Titres of non-specific inhibition of normal ferret sera diluted in different Calcium-removing salt solutions

Strain	Titres				
	Saline	Citrate	Oxalate	Fluoride	Phosphate
Heated Lee	8190	260	260	2040	130
Sl(1941 Ned)	7120	480	290	290	340
Barratt(1947 E)	5120	70	50	50	70
Rhodes(1947 USA)	290	—	<12	<12	<12
Gg(1947 Sw)	2300	<12	<12	<12	<12
K(1947 Ned) (Ferret-egg-strain)	2390	<12	<12	<12	<12
Christie(1937 E)	100	<12	<12	<12	<12
Talmey(1937 E)	140	<12	<12	<12	<12
Gatenby(1937 E)	1150	<12	<12	<12	<12

With many strains non-specific inhibition in a serum dilution of 1 : 12 was entirely eliminated, but with others some non-specific inhibition remained. Similar results were obtained with the HIRST-SALK technique and using rabbit sera. The best results were generally obtained in titrations in physiological oxalate solutions.

The phenomenon is probably attached to the problem of

tricomplex systems known in colloid chemistry and published by BUNGENBERG DE JONG *et al.* (1938, 1942).

Table 10 gives a survey of comparative titrations of pre- and post-infection ferret sera against the homologous strain in saline and in an oxalate solution. From these experiments it is clear that the antibody titre is unaffected by titration in an oxalate solution.

When Calcium-ions are withdrawn the non-specific titre decreases; on the other hand we do not observe any effect on the inhibitory action when titration takes place in the presence of excess of Calcium (0.1 per cent Calcium chloride in saline). Apparently the optimum concentration of Ca-ions for the non-specific inhibition is already present when the serum is diluted in saline.

TABLE 10

Titres of normal and antisera of ferrets, diluted in different salt solutions against the homologous strains

Homologous serum pairs (Ferret pre- and post-infection)	Titres	
	Saline	Oxalate
PR8(1934 USA)	100/14340	24/14340
WS(1933 E)	100/3070	30/2560
K(1947 Ned) Ferret-egg-mouse strain	50/1280	<12/1340

When this investigation had been completed, there appeared a publication by WOOLLEY(1949), in which it was stated that Calcium-ions are indispensable for the inhibitory action of apple pectin on the haemagglutination by the influenza virus. It is possible that the mechanism of the inhibitory action of normal serum is the same as that of apple pectin, and that both are influenced by Calcium-ions in the same way.

Neutralization of the non-specific serum factor by a filtrate of Vibrio cholerae. The best method of freeing the ferret sera from non-specific inhibition was found by applying the discovery of BURNET *et al.*(1946). We made use of the enzymatic action of a filtrate of a culture of V. cholerae which caused the inhibitor in the serum to be destroyed.

Preparation of the cholera filtrate (according to BURNET). The preparation was made as described by BURNET and STONE (1947). At first we used the cholera strain Ogawa, kindly sent to us by Dr W. Æ TIMMERMAN. After some months, however, the yield of enzyme of this strain decreased for unknown reasons. By means of the strain 4Z, sent to us by Professor BURNET, it was possible to obtain a much more constant yield of enzyme.

The strain is cultivated on 1 to 2 per cent nutrient agar, and passages are made every fortnight. Cultures yielding a potent enzyme are freeze-dried. One per cent nutrient agar plates (pH = 6.9) are inoculated with a culture of the cholera strain, previously cultivated for 6 to 8 hours in ordinary broth. After sixteen hours' incubation the agar is scraped off the plate and pressed through a Buchner funnel. The fluid is then centrifuged and filtered through a Seitz disc. The pH of the filtrate must not exceed 7.6.

The receptor destroying enzyme (R.D.E.) Activity of the crude filtrate may best be determined by heating 10 ml of it with 0.25 ml of a 20 per cent $CaCl_2$ solution for 40 minutes at 56° C and then centrifuging. In this way agglutinins for chicken red cells present in the filtrate are largely destroyed. To dilutions of this mixture, heated at 37° C, a suspension of chicken red cells (also heated at 37° C) is added in a final concentration of 0.5 per cent, and a virus suspension with a 5 to 10 AU final concentration. After this mixture has been left standing for exactly 30 minutes at 37° C, the red cell patterns are read. The titre of R.D.E. is recorded as the dilution at which haemagglutination by the virus is just partially (2 plus patterns) inhibited. When the incubation period at 37° C is continued, the titre rises. The titres of the R.D.E. preparations usually vary between 256 and 512, and with some filtrates between 1000 and 2000. As some filtrates show an active R.D.E. titre, but do not sufficiently neutralize the non-specific inhibition of the serum, the activity of the crude filtrate against ferret serum is always examined. For this purpose one part of a normal ferret serum is mixed with five parts of crude cholera filtrate, without calcium chloride, and incubated at 37° C for one night. Next the enzymatic activity of the filtrate is destroyed by heating the mixture for one hour at 56° C. Control experiments have shown that after this treatment R.D.E.-activity is no longer demonstrable. The serum is next titrated in the usual way against certain A strains

which are highly inhibited non-specifically. We found that a ratio of five parts crude cholera filtrate and one part serum (for all the sera that were examined) is amply sufficient to neutralize completely the non-specific inhibition in a final serum dilution of 1 : 12 against all the strains that were used (plate 1, fig. 1 and 2, opposite 8).

So far we have not yet been able to investigate the factors which play a part in the destruction of the non-specific inhibitors in ferret sera by crude cholera filtrate, but they are certainly complex.

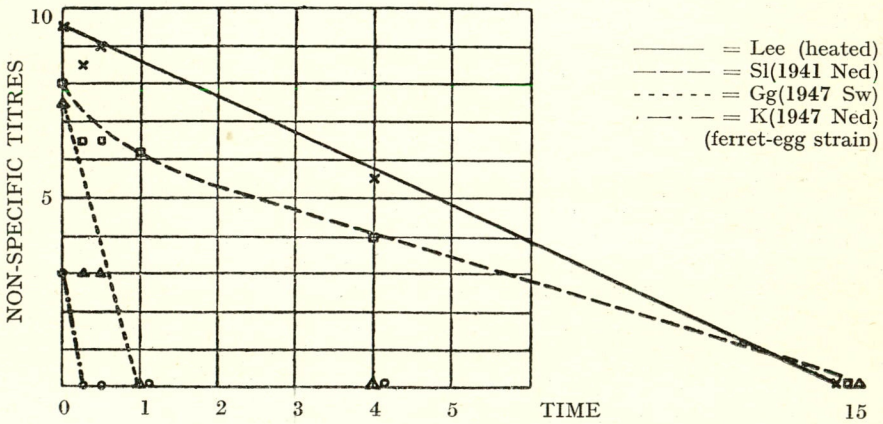

Figure 4.

Comparison of the results of haemagglutination inhibition tests of normal ferret serum against different strains after different periods of interaction of serum and crude cholera filtrate (1 : 5).
Titre expressed in terms of a geometrical series (using two-fold dilutions of serum 1 : 12). Time in hours.

Influence of the amount of enzyme and the period of interaction. It appeared that the reduction of non-specific inhibition against a virus depends on the quantity of filtrate that is added and the period of interaction, but the reduction is not necessarily the same for different virus strains (see fig. 4 and 5).

The activity of crude cholera filtrate on rabbit sera. It could be demonstrated that cholera filtrate has the same effect on normal rabbit as on ferret sera.

Influence of crude cholera filtrate on the titre of antibodies. It was very important to examine the influence of crude cholera filtrate on the titre of the serum antibodies. A number of immune ferret sera, treated with cholera filtrate and untreated, were titrated in parallel against homologous virus strains which were

scarcely or not at all inhibited by normal sera (table 11). In all the comparative tests the same suspensions of virus and red cells were used. Virus suspensions, titrated in saline and in an inactivated solution of cholera filtrate (one part filtrate and five parts saline), contained the same number of AU.

From table 11 it is clear that, in general, the antibody titre in ferret sera is not reduced by treatment of the serum with crude cholera filtrate. In one of the 11 experiments (SWINE-15 serum) a reduction was noted, but when the experiment was repeated afterwards with the same serum but a different filtrate,

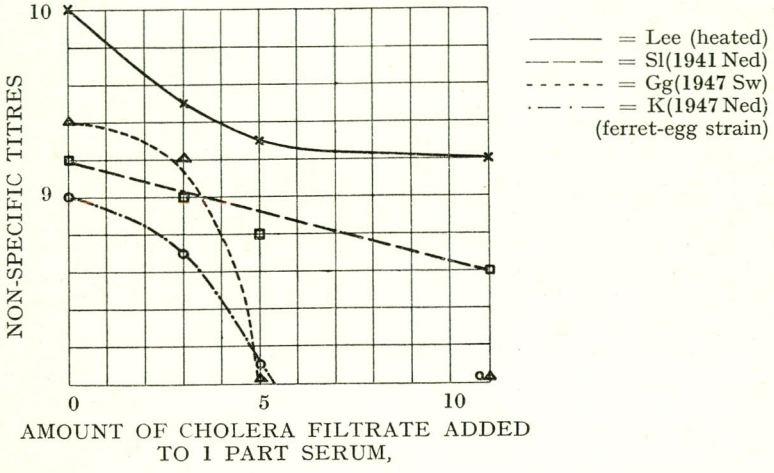

Figure 5.
Comparison of the results of haemagglutination nhibition tests of normal ferret serum against different strains after interaction with different amounts of crude cholera filtrate. Interaction of crude cholera filtrate and serum for $3^1/_2$ hours at 37° C. Titres expressed in terms of a geometrical series (using two-fold dilutions of serum 1: 12).

much less reduction was obtained. The last experiment of this table shows that crude cholera filtrate has no influence on the titre of an anti-typhoid rabbit serum.

Table 12 shows the results of three comparative haemagglutination inhibition tests with serum pairs (treated and untreated) against homologous and heterologous influenza A strains (A strains and A-prime strains). In these tests the homologous titres remained the same, but the heterologous titres of the PR8 antiserum entirely disappeared after treatment with cholera filtrate. This was not the case with the heterologous titre of the serum A(1941 Ned) against the strain Rhodes(1947 USA),

TABLE 11

Antibody titrations of pairs of sera from ferrets against homologous virus strains with and without treatment with crude cholera filtrate

Homologous serum pair of ferrets (Pre- and post-infection)	Strain	Titre [1]) untreated	Titre [2]) treated
Swine-15(1930 USA)	Swine(1930 USA)	96/8190 D	<12/3070 D
Swine-15(1930 USA)	Swine(1930 USA)	380/5120 S	<12/3070 S
WS(1933 E)	WS(1933 E)	<12/1540 D	<12/1540 D
WS(1933 E)	WS(1933 E)	170/5380 S	<12/5380 S
PR8(1934 USA)	PR8(1934 USA)	12/10240 S	<12/10240 S
Christie(1937 E)	Christie(1937 E)	96/2760 D	<12/2760 D
Talmey(1937 E)	Talmey(1937 E)	140/10240 D	<12/10240 D
Lee(1940 USA)	Lee(1940 USA)	24/10240 D	<12/6140 D
Lee(1940 USA)	Lee(1940 USA)	84/10750 S	<12/10750 S
Lee(1940 USA)	Lee(1940 USA)	75/10750 S	<12/10750 S
K(1947 Ned), mouse-adapted	K(1947 Ned), mouse-adapted	<12/3070 D	<12/3070 D
Antityphoid serum (rabbit)	typhoid laboratory strain	6400 (+)	6400 (±)

[1]) Inactivated 30 minutes at 56° C.
[2]) Inactivated 60 minutes at 56° C. after treating with crude cholera filtrate. D = tests done in duplicate. All these sera were treated with the same cholera filtrate. S = single test. These sera are treated with the same cholera filtrate, with the exception of the second test against Lee and the test against the typhoid strain.

which before and after treatment was practically the same, and had a low value. From this it follows that low heterologous antibody titres, too, are not diminished by treatment with crude cholera filtrate. The last test of table 12 (serum A I (1949 Ned) against the strain PR8 (1934 USA)) is important owing to the fact that the ferret used for immunization showed antibodies against the strain PR8 in the pre-infection serum and a further strong antibody response against this strain after inoculation with the heterologous A-prime strain. After treatment with cholera filtrate both titres remained practically unchanged.

Pseudo-reduction of the amount of antibodies when there is a

TABLE 12

Antibody titration of serum pairs (ferret) against homologous and heterologous strains untreated and treated with crude cholera filtrate

Experiment	Serum pair of ferrets (Pre- and post-infection)	Strain	Titre [1] untreated	Titre [2] treated
1	PR8(1934 USA)	PR8 (1934 USA)	<12/3070	<12/3070
		Gg (1947 Sw)	770/1540	<12/<12
		K (1947 Ned)	770/1540	<12/<12
2	A(1941 Ned)	A (1941 Ned)	3070/12290	<12/12290
		Rhodes (1947 USA)	48/320	<12/290
3	AI(1949 Ned) [3]	PR8 (1934 USA)	140/12290	**190**/9200

[1]) Inactivated 30 minutes at 56° C.
[2]) Inactivated 60 minutes at 56° C. after treating with crude cholera filtrate.
[3]) Ferret with low-titre pre-infection antibodies against PR8, showing a strong serological response against this strain after inoculation with an A-prime strain.

TABLE 13

Antibody titrations of serum pairs (ferret) against homologous strains with and without treatment with crude cholera filtrate

Homologous serum pair of ferrets (Pre- and post-infection)	Strain	Titre [1] untreated	Titre [2] treated
Sl(1941 Ned)	Sl (1941 Ned)	6140/12290	24/3070 [3]
Heer(1949 Ned)	Heer (1949 Ned)	1540/6140	<12/1540
Lee(1940 USA)	Lee (1940 USA) Heated	5970/>57340	<12/10750

[1]) Inactivated 30 minutes at 56° C.
[2]) Inactivated 60 minutes at 56° C. after treating with crude cholera filtrate.
[3]) Small remnant of non-specific inhibition owing to a weak enzyme preparation.

high non-specific inhibition in the serum and a low antibody titre. In some tests we noted the curious phenomenon that the antibody titre of the treated sera was greatly reduced, as compared with that of the untreated serum. Table 13 gives an example of this for three ferret sera. In all the treated post-infection sera the titre is lower than (or about as high as) that of the non-specific inhibition of the corresponding untreated pre-infection sera.

In table 14 the same is found in human sera from the 1949 epidemic in Holland. The two sets of sera in these tests were similarly treated, except for the addition of filtrate to the treated samples. (The untreated sera were diluted with saline in a ratio of 1 : 5, incubated for one night at 37° C and then heated for one hour at 56° C). Here, too, a distinct reduction is seen in the

TABLE 14

Haemagglutination inhibition tests with human serum pairs against different strains of influenza A virus (untreated and treated with a crude filtrate of V. cholerae). All tests with each serum pair were done simultaneously

Experiment	Human serum pairs	Titres					
		PR8(1934 USA)		Rhodes(1947 USA)		Heer(1949 Ned)	
		Untreated	Treated	Untreated	Treated	Untreated	Treated
1	Le Gr(1949)	130/200	84/130	20/140	<12/96	84/370	<12/48
2	Hoof(1949)	48/2390	42/2690	15/1380	<12/1380	112/1340	<12/370
3	Hoof(1949)	40/2050	36/2300	16/1380	<12/1150	96/1380	<12/320
4	Hoof(1949)					250/2560	<12/290
5	Herms(1949)	96/320	64/160	16/510	<12/260	160/2050	<12/320
6	Herms(1949)					580/6140	<12/160
7	Mas(1949)			36/580	<12/80	290/2300	<12/320
8	Goldb(1949)					70/2560	<12/1020
9	Boh(1949)					770/12290	20/1540
10	Muusse(1949)					190/1540	<12/160
11	v. d. Ende(1949)					1540/36860	36/32770

titres of the convalescent sera against the strain HEER(1949 Ned), while the titres against the strains PR8 and RHODES (with the exception of RHODES in experiment number 7) in the same sera remain practically the same after treatment with cholera filtrate. This fact indicates that the reduction of the titre against the strain HEER(1949 Ned) may be due to some special circumstance, and cannot be accounted for by assuming that the cholera enzyme

has partly destroyed the antibodies against the strain HEER.

When one surveys the figures in the tables 13 and 14, it is clear that the post-infection titres of the treated sera are much lower than those of the corresponding untreated sera in those cases only when the titre of the non-specific inhibition in the pre-infection samples is much the same as, or greater than, the titre of the antibodies of the treated sera. Moreover the reduction in the titre does not depend on the strain that is used (see, for instance, the reduction in the titre of the serum MAS(1949) against the strain RHODES (experiment number 7), and the absence of the reduction in the treated serum of V. D. ENDE(1949), the absolute value of which is very high, which was checked with a reference serum (experiment number 11)).

Table 15 shows a series of agglutination inhibition tests of the sera from a patient which were taken daily in the course of the influenza. All the tests were performed in duplicate with untreated and treated sera. They were repeated against the same strain, but with a different egg fluid, which showed a much lower non-specific inhibition compared with that which was used in the first experiment. From both tests it appears clearly that the reduction of the titre of the treated sera decreases as the absolute value of the antibody titre increases.

Explanation of the fact of the reduced antibody titre in treated antisera. In order to exclude the chance possibility that the post-infection sera in question might have higher non-specific titres than the pre-infection sera, both untreated sera were titrated against heated LEE virus; no essential difference was found in non-specific inhibition between the pre-infection and the post-infection sera. Though this concerns only the content of „Francis inhibitor" in the sera, it is improbable that the higher titres of the untreated post-infection sera are to be attributed to the fact that the post-infection sera have a higher content of non-specific inhibitor than the pre-infection specimens.

Table 16 shows the readings of the haemagglutination patterns of a patient's serum pair which was titrated (treated and untreated) against the strain HEER(1949 Ned) (one experiment). It is certain that the combination of antibody with antigen takes place more quickly than the combination of the antigen with the non-specific inhibitor (fig. 3). Now it is very probable that in the first dilutions of the treated antiserum, when haem-agglutination begins to make its appearance, the remaining

TABLE 15

Haemagglutination inhibition tests of the sera from one patient which were taken daily in the course of influenza, against the strain Heer(1949 Ned). In experiment 2 an egg fluid of the strain Heer(1949 Ned) has been used, showing a non-specific inhibition much weaker than that which was used in experiment 1. All the tests were performed in duplicate

Patient's sera	Strain	Experiment 1			Experiment 2		
		Titres		Ratio untreated: treated	Titres		Ratio untreated: treated
		Untreated	Treated		Untreated	Treated	
Hok(1949 Ned) I	Heer (1949 Ned)	2390	<12	>199	340	<12	>28
		2390	<12		340	<12	
Hok(1949 Ned) V		3550	170	27	600	80	6.2
		5380	170		450	80	
Hok(1949 Ned) VI		5380	750	6.9	1340	670	2
		4780	750		1340	670	
Hok(1949 Ned) VII		10750	2690	4.4	2990	2690	1.2
		14340	2990		3550	2690	
Hok(1949 Ned) VIII		21500	9560	2.6	7170	5970	1.4
		28670	9560		9560	5970	
Hok(1949 Ned) IX—XIII (pool)		21500	10750	2.1	11950	10750	1.1
		23890	10750		11950	10750	

portion of virus is entirely combined with the non-specific inhibitor, which may be inferred from the partial first positive haemagglutination patterns of the untreated serum HERMS I. The consequence is that the titre of the untreated serum II becomes considerably higher than that of the treated serum. In preliminary virus titration experiments with serum dilutions

TABLE 16

Haemagglutination patterns of a patient's serum pair treated and untreated with a crude filtrate of V. cholerae against the strain Heer(1949 Ned) (One experiment)

Serum pair	Serum dilutions									
	24	48	96	190	380	770	1540	3070	6140	12290
Herms I untreated	0	0	0	±	+	+++	+++	++++	++++	++++
Herms II untreated	0	0	0	0	0	0	0	++	+++	++++
Herms I treated	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
Herms II treated	0	0	0	0	+++	++++	++++	++++	++++	++++

in which haemagglutination begins to make its appearance we could show that the quantity of remaining virus in these dilutions really increases gradually. Minute further investigation by means of an exact method of quantitative virus determination has not yet been concluded.

WILSON SMITH and WESTWOOD(1949) state that when using an accurate reading of the haemagglutination inhibition with a haemodensitometer a drop in the antibody titres occurred after treatment of the sera with crude cholera filtrate, owing to the presence of proteinase. In a personal communication WILSON SMITH states that this drop is slight, however.

From the above it is probable that with filtrates of the cholera strain 4Z a real drop in antibody titre cannot be measured and that it lies within the error limit of the titration method used.

Criteria for the complete absence of the non-specific inhibition in antisera after treatment with cholera filtrate. We generally used the pre-infection serum of the same ferret which yielded the antiserum, and which was treated with the same cholera filtrate as the antiserum. As it sometimes happens that the serum of the same ferret shows different titres of non-specific inhibitor at different times, this check is not absolutely reliable, but as each filtrate is also always checked against two strains in our collection with a very high non-specific inhibition (*viz.* BARRATT (1947 E) and A(1941 Ned)), this method may be looked upon as sufficiently reliable. One can also use a treated antiserum against an A strain that is antigenically unrelated and which is more strongly inhibited by normal sera. The use of heated LEE virus is less reliable, as in general the non-specific inhibition against this virus is more easily eliminated by crude cholera filtrate than that against some A strains.

Other applications of cholera enzyme in influenza virus research.

1. *Neutralization of the non-specific inhibition in human sera.* Several investigators have pointed out that the capacity of human sera to inhibit haemagglutination by influenza virus may be caused in part by a non-specific factor (FRANCIS(1947a), FRIEDEWALD *et al.*(1947), SVEC and FORSTER(1947), DE BURGH *et al.* (1948)). On the analogy of the experiments with ferret sera it is probable that this non-specific substance in human serum could also be destroyed by cholera enzyme.

The influence of cholera filtrate on the human serum may be seen in table 14. The sera are from persons suffering from influenza

during the influenza epidemic in Holland (January 1949).

From these experiments it may be deduced that different human sera may give rather high titres without treatment and that after treatment with cholera filtrate a rise or fall of antibodies can be demonstrated in a much more sensitive manner.

Another important result of the application of cholera enzyme to human sera is that it is possible to demonstrate in a simple way the probability of the presence or absence of pre-infection

TABLE 17

Haemagglutination inhibition tests with stock sera against certain young egg strains of influenza A virus (1949 Ned) (A-prime)

Pairs of serum (Ferret) Pre- and post-infection	Strains					
	Hok (1949 N) AMN. 1 [1]	Ende (1949 N) AMN. 1 [1]	Sips (1949 N) AMN. 1 [1]	Mark (1949 N) AMN. 2 ALL. 1 [2]	Mas (1949 N) AMN. 2 ALL. 1 [2]	Homologous strains [3]
WS(1933 E)	<12/<12	<12/<12	<12/20	<12/<12	<12/<12	<12/8300
PR8(1934 USA)	...	<12/<12	<12/15	<12/<12	<12/<12	<12/13650
A(1941 Ned)	<12/<12	<12/<12	<12/<12	<12/<12	<12/<12	<12/8450
Barratt(1947 E)	...	<12/640	<12/340	<12/340	<12/340	<12/5490
Rhodes(1947 USA)	<12/670	<12/380	<12/110	<12/170	<12/170	<12/8360
Lee(1940 USA) [4]	<12/<12	<12/<12	<12/<12	<12/<12	<12/<12	<12/7590
Swine-15('30 USA) [4]	<12/<12	<12/<12	<12/<12	<12/<12	<12/<12	<12/2300

[1]) AMN. 1 = Amniotic fluid from first inoculated egg.

[2]) AMN. 2. ALL. 1 = Allantoic fluid from first allantoic passage after two amniotic passages.

[3]) Mean of 3 experiments during the period of these investigations.

[4]) The experiments with Lee and Swine-15 serum were done separately with a higher egg-passage number.

antibodies in man. Thus it appeared that during the influenza epidemic in Holland in the winter of 1949 only few adults had a basic antibody titre against the then prevailing influenza virus strain (A-prime).

2. *The demonstration of basic antibodies in ferrets bought from the trade* (MULDER *et al.*(1949a)). A low titre owing to basic antibodies in ferrets is easy to demonstrate when the ferret sera have been freed from non-specific inhibition (table 12, serum A I (1949 Ned)).

3. *Serological experiments with recently isolated influenza virus strains* (MULDER *et al.* (1949b)). Influenza virus strains newly isolated in eggs are often strongly non-specifically inhibited by ferret sera so that serological analysis of such young strains is rendered difficult unless the non-specific inhibition is neutralized. It was shown possible to use the first positive amniotic fluid as antigen in serological experiments, if it had an sufficient titre (table 17).

TABLE 18

Titres of antisera of ferrets against reference strains. The ferrets were inoculated with garglings of patients or mucosa of trachea and bled twice after inoculation. Pre-infection titre of all sera against all strains < 12

Expt.	Ferret antisera	Days after inoculation	Titres against virus				
			WS (1933 E)	PR8 (1934 USA)	A (1941 Ned)	Barratt (1947 E)	Rhodes (1947 USA)
1	Hof(1949 Ned)	8	<12	<12	<12	80	50
		13	<12	14	90	2990	3550
	Burl(1949 Ned)	8	<12	<12	50	370	220
		13	<12	<12	50	5970	4780
	Kra(1949 Ned)	8	<12	<12	40	90	60
		13	<12	60	140	750	670
2	Oord(1949 Ned)	8	<12	<12	90	80	60
		13	<12	<12	130	320	300
	Behr(1949 Ned)	8	<12	<12	<12	<12	<12
		13	<12	<12	14	580	670
3	Hee(1949 Ned) † Mucosa of trachea	13	<12	<12	<12	3070	4100

4. *Serological experiments with sera from ferrets inoculated with garglings, sputa, or autopsy material.* Table 18 gives an example of this. The animals were bled 8 and 13 days after inoculation. Also in this way it is possible to establish quickly the A subgroup to which the influenza virus in question belongs. The sera taken after 13 days yielded clearer results than those taken after 8 days. It is possible that antisera obtained in this way are the most suitable specimens in cross-haemagglutination tests, because they are derived from the first animal passage infected with the original human virus material.

IV.

CROSS-HAEMAGGLUTINATION INHIBITION TESTS OF INFLUENZA A STRAINS AFTER NEUTRALIZATION OF THE NON-SPECIFIC INHIBITOR IN THE ANTISERA

The following preliminary investigation into the cross-haemagglutination inhibition tests was limited in scope to practical issues. Some variable factors, which may influence the results of these tests, are analysed first.

Preparation of antisera. Ferrets were employed. The animals were bled before inoculation by heart puncture. The pre-infection serum was tested to ensure the absence of specific and non-specific inhibition after treatment with the same cholera filtrate as that used against the post-infection antiserum. It was found to be very important to perform this serum control regularly, for in the course of the investigation two uninoculated ferrets of our stock proved to possess antibodies against respectively WS(1933 E) + PR8(1934 USA) and against PR8 + A(1941 Ned). The ferrets came from a Dutch zoological garden and were more than one year old. These spontaneous titres were low (WS : 160 and PR8 : 40; PR8 : 190 and A(1941 Ned): 80) and could only have been found after elimination of the non-specific inhibition (MULDER *et al.* 1949a). The occurrence of spontaneous infection in ferrets with influenza virus was also observed by FRANCIS and MAGILL(1935) and by BELL and DUDGEON(1948).

The ferrets, anaesthetized with ether, were inoculated with fresh undiluted allantoic fluid, or (in the case of a highly virulent strain) with allantoic fluid diluted 1 : 10, of which a quantity of about 0.5 ml was aspirated into each nostril. The haemagglutination titre of the egg fluids varied from 480 to 960 (using $\frac{1}{2}$ per cent red cells in the final dilution). The number of egg-passages of ferret-mouse strains varied (table 1). In case the strain had been recently isolated in chick embryo from 8 to 16 egg-passages were performed before inoculating ferrets. After a

period of twelve days the convalescent ferret serum was recovered by means of heart puncture. For the purpose of checking the absence of non-specific inhibition in antisera a heterologous strain was occasionally used, which was known not to give cross-haemagglutination inhibition with the strain to be examined.

The sera were stored at + 2° C without preservative but we found sodium-azide (0.08 per cent final dilution) suitable in this respect as it does not interfere with the action of crude cholera-filtrate on the non-specific inhibitor and the antibody-titre.

Influence of red cells of different fowls. STUART HARRIS and MILLER (STUART HARRIS(1943), STUART HARRIS and MILLER (1947)) have pointed out that the titre of the serum may vary when using red cells of different chicks. This phenomenon may perhaps partly be explained by the presence of non-specific inhibiting substances in these sera (see Chapter III). When using red cells of different chicks we found that the differences in titre of sera treated with cholera filtrate were very slight.

Influence of different allantoic fluids. The variations in titre of an antiserum against virus suspensions of different eggs, inoculated with the same strain, were as far as possible eliminated by mixing the fluids of a number of eggs of the same egg generation, and performing the serum titrations with these pools (HIRST(1943)).

Influence of the individual response of the ferret. The individual serological response of the ferret to the inoculation with virus of the same egg passage may vary considerably (table 19). Three ferrets were inoculated with the allantoic fluids from the same egg-passage of TALMEY(1937 E) and CHRISTIE(1937 E). The allantoic fluid from another egg-passage of the two strains was used in the cross-haemagglutination inhibition tests with the antisera of these ferrets. Two other TALMEY and CHRISTIE sera which had been produced with mouse-lung virus of the ferret-mouse strains and from an early egg-passage of the same strains were titrated in the same experiment. From table 19 it is not only clear that the level of the antibodies against the homologous strains varies, but the ratio between the heterologous and the homologous titres is also seen to differ greatly. In this way, therefore, the results of comparative serum titrations may be disturbed (for the differences in titre of the TALMEY sera against the CHRISTIE sera see below). It would be desirable always to pool a number of antisera against the same strain, and to perform

the tests with these. Owing to the limited number of ferrets at our disposal we made serum pools only from certain representative strains (three ferrets per pool).

Influence of the egg-passage number on the titre of the antisera. It is probable that the serological response of the ferret also depends on the number of egg-passages of the strain which is

TABLE 19

Titres of antisera, prepared with different passages of the same strain, against the homologous and a heterologous strain (one experiment)

Ferret antiserum prepared with	Titres against virus		Ratio Heterologous titre / Homologous titre
	Talmey (1937 E)	Christie (1937 E)	
Christie(1937 E) F M	220	3070	1/14
Christie(1937 E) F M E_6	60	2050	1/34
Christie(1937 E) F M E_{33} A *)	60	10240	1/150
Christie(1937 E) F M E_{33} B	110	18430	1/170
Christie(1937 E) F M E_{33} C	60	5120	1/85
Talmey(1937 E) F M	2990	2300	1.3/1
Talmey(1937 E) F M E_1	5970	6140	1/1
Talmey(1937 E) F M E_{21} A	9490	1150	1/8
Talmey(1937 E) F M E_{21} B	7170	380	1/19
Talmey(1937 E) F M E_{21} C	9560	580	1/16

*) The ferret antisera A, B and C are prepared with the same virus suspension.

used as antigen. An example of this influence on the antigenic potency of a strain is the strain GATENBY(1937 E). It appeared that after seven egg-passages of the ferret-mouse strain it was poorly antigenic. After another twenty-three egg-passages, however, GATENBY elicited an antiserum with much higher titres against the homologous and the heterologous strains (Table 30 and graph. 2[1])). The haemagglutination titres of the virus

[1]) Graph 2 is found at the end of the publication.

suspensions with which the ferrets were infected, were the same in both cases.

The same phenomenon was observed in the case of a young egg strain from the epidemic of 1949 (HEER(1949 Ned)). Although after six egg-passages this strain elicited a poor antiserum in the ferret, it was a much better antigen after fifteen egg-passages. It follows moreover from table 19 that by continued egg-passages of ferret-mouse strains antisera may be produced with a lower titre against heterologous strains of the same subgroup.

Influence of mouse adaptation on the titre of the antisera. Mouse adaptation of ferret-egg and egg strains also proved important

TABLE 20

Titres of antisera prepared with different strains before and after mouse-adaptation against the homologous and heterologous strains

Ferret antisera [1])	Titres against strains						
	WS (1933 E)	PR8 (1934 USA)	A (1941 Ned)	Barratt (1947 E)	Rhodes (1947) USA)	Homologous strains	
						(ferret)-egg-strain	mouse-adapted
Sch(1941 Ned) Ferret-egg-strain	120	1920	4100	80	100	2690	16380
Sch(1941 Ned), mouse-adapted	<12	960	4100	<12	<12	2390	10240
K(1947 Ned) Ferret-egg strain	<12	<12	<12	640	260	320	1790
K(1947 Ned), mouse-adapted	30	40	240	640	770	240	3550
Barratt(1947 E) Egg strain	<12	<12	380	8190	13000	8190	12000
Barratt(1947 E), mouse-adapted	<12	<12	190	770	2050	770	5970
Heer(1949 Ned) Egg strain	<12	80	30	1340	2690	2560	3840
Heer(1949 Ned), mouse-adapted	160	600	370	370	7170	640	5120

[1]) Antisera against the ferret-egg line and the mouse-adapted line of the same strain are always titrated against WS, PR8, A(1941 Ned) and the homologous strains on one day and against Barratt and Rhodes on one day.

to the antigenic potency of some strains. Antisera were prepared against the ferret-egg strain Sch (1941 Ned), the ferret-egg strain K(1947 Ned), the egg strain BARRATT(1947 E) and HEER (1949 Ned) and against the same strains after complete mouse-adaptation. Table 20 shows that with the exception of BARRATT (1947 E) all the mouse-adapted strains produced a more potent antiserum than the homologous egg strains.

Influence of mouse-passage on the behaviour of influenza strains in cross-haemagglutination inhibition tests. In the following text the word *strain behaviour* is used provisionally in the place of antigenic composition where there could be doubt as to the existence of real differences in antigenic structure.

Mouse-passage caused a distinct change in behaviour of the influenza virus strains in the haemagglutination inhibition tests. It was found in the case of tests with four strains that after mouse-adaptation titres were much higher than with the original

TABLE 21

Comparison of titres of ferret antisera, prepared with 5 reference strains against different strains before and after mouse adaptation

Ferret antisera [1]	Titres against virus							
	Sch(1941 Ned)		K(1947 Ned)		Barratt(1947E)		Heer(1949 Ned)	
	Ferret-egg strain	Mouse-adapted	Ferret-egg strain	Mouse-adapted	Egg-strain	Mouse-adapted	Egg-strain	Mouse-adapted
WS(1933 E) (Pool)	<12	30	<12	16	<12	<12	<12	20
PR8(1934 USA) (Pool)	<12	90	<12	<12	<12	20	<12	<12
A(1941 Ned) (Pool)	510	2390	<12	50	60	190	<12	200
Barratt(1947 E) (Pool)	<12	<12	120	1610	8190	12000	1790	2990
Rhodes(1947 E) (Pool)	<12	40	60	640	770	2690	1190	5970
Homologous antiserum	2690	10240	320	3550	8190	5970	2560	5120

[1] The ferret antisera are always titrated against the ferret-egg line and the mouse-adapted line of the same strain on one day.

ferret-egg or egg strains (table 21 and 22). Especially interesting in this respect was the behaviour of the ferret-egg strain Sch(1941 Ned) which after mouse-adaptation showed a great change in titre pattern as compared with two ferret-mouse-egg strains from the same epidemic (table 22) (all strains were isolated in the same town of Groningen).

TABLE 22

Cross-haemagglutination inhibition tests with three different strains isolated from one epidemic (1941 Ned) (One experiment)

Ferret antisera	Titres against virus			
	A (1941 Ned) Ferret-mouse-egg	Sl (1941 Ned) Ferret-mouse-egg	Sch (1941 Ned) Ferret-egg	Sch (1941 Ned) Ferret-egg-mouse-egg
A(1941 Ned) Ferret-mouse-egg	**6140**	4780	120	4780
Sl(1941 Ned) Ferret-mouse-egg	12290	**19110**	80	11950
Sch(1941 Ned) Ferret-egg	3410	5970	**3410**	28670
Sch(1941 Ned) Ferret-egg-mouse-egg	4100	2690	1380	**14340**

HIRST(1947a), performing the same sort of experiments, found that egg strains after mouse adaptation showed lower titres against the antisera of the original egg strains. As our experiments show the reverse phenomenon, the whole problem has to be studied in further detail, but complete neutralization of non-specific inhibitor in the antisera is absolutely necessary in experiments of this kind.

Cross-haemagglutination inhibition tests with different "lines" of the same strain of influenza virus. From table 23 it may be seen how complicated the behaviour of the system of cross-haemagglutination inhibition may be, even with the same strain.

Two LEE (influenza B) ferret antisera (one obtained from Dr P. von MAGNUS, Copenhagen, and one from our laboratory and both prepared with virus suspensions of the ferret-mouse-egg strain) were used against two different LEE fluids, one

centrifugate from Dr von MAGNUS and one allantoic fluid from our laboratory. It was shown in several experiments that the Copenhagen anti-Lee serum gave a much lower titre with the Leyden line of LEE virus than with the Copenhagen strain. The Leyden anti-Lee serum, however, gave the same titre with both lines of virus, so that any influence of the condition of the virus (centrifugate or plain allantoic fluid) would seem to be improbable. Moreover an allantoic fluid purified by a precipitation

TABLE 23

Cross-haemagglutination inhibition tests with two anti-Lee ferret sera [1]) *against two lines of Lee-virus*

Experiment	Ferret antiserum (not treated with cholera-filtrate except in exp.4)	Strain		
		Lee (Copenhagen) (centrifugate)	Lee (Leyden) (Allantoic fluid)	Lee (Leyden) (purified)
1	Lee(Copenhagen)	10240	2560	...
	Lee(Leyden)	9220	10240	...
2	Lee(Copenhagen)	10240	2560	...
	Lee(Leyden)	10240	8530	...
3	Lee(Copenhagen)	13650	3550	4600
4	Lee(Copenhagen)	10240	3410	...
	Lee(Copenhagen) (treated)	5120	1150	...

[1]) Both antisera are prepared with virus suspensions of the ferret-mouse-egg strain, which has undergone many egg passages.

method (ZWART VOORSPUY(1949)) from the Leyden Lee strain gave the same difference in titre with the Copenhagen serum. As our laboratory possesses only one B strain (LEE), a confusion with a different B strain can be safely excluded. An explanation for the differences in titre of the two lines of virus could possibly be that the Copenhagen strain was recently derived from a mouse-passage strain, the Leyden-line being passed only in eggs. This supposition proved to be wrong however.

A good instance of variable titres of various antisera of a certain strain against the same heterologous strain (of the same subgroup) is found in table 19 (one experiment). From this it is clear that the TALMEY sera, made from infected mouse lung

and the first allantoic passage of it, show high titres with the strain CHRISTIE, but that three sera made from a subsequent egg passage give much lower titres with this strain. Conversely all CHRISTIE antisera, including those derived from infected mouse lung and the sixth allantoic passage of it, influence the TALMEY strain weakly.

Individuality of the system: passage formula of influenza virus strains and the corresponding ferret antiserum. We cannot account for the discrepancies in table 23 and 19, but it is clear that such observations lead one to be cautious concerning the significance of the results. It does not follow that the latter indicate essential differences in antigenic structure between influenza virus strains, and it is also probable that the combination of a single passage of a virus strain with the corresponding ferret antiserum possesses a certain individuality.

Moreover it follows from the above experiments from table 19, 21, 22 and 23 that the phenomenon of a strain showing low titres with antisera against heterologous strains from the same subgroup or with an antiserum of a different line of the same strain *may be reversible and that it is very important in this respect to know exactly the passage formula of each strain.*

Influenza virus strains which show low titres against the (corresponding) homologous and heterologous antisera. One of the greatest difficulties we encountered in the cross-haemagglutination inhibition tests was the fact that some influenza virus strains showed very low titres with the homologous and heterologous antisera of the same subgroup. FRANCIS and MAGILL(1938) noted the same phenomenon in their cross-neutralization tests in mice. Table 24 shows diagrammatically how a group of strains belonging to the same subgroup behave differently in the haemagglutination inhibition tests. The number of plus-signs (++...) is a measure of the titre. It appeared in our cross tests that there are strains ("Q") which do not show only low titres with heterologous antisera, but also with the homologous antiserum. Another type of strain ("P") shows a high titre with the homologous but a much lower with heterologous antisera. "R" strains show high titres both with homologous and heterologous antisera (with only a few exceptions). Generally speaking, Q-strains produce poorer antisera in the ferret than the P- and R-strains. P-, Q- and R-variations occur within the A-(PR8) and the A-prime subgroup.

Examples of a "P"-strain are TALMEY(1937 E) and GATENBY (1937 E), while K(1947 Ned) and WOITEKI(1947 E) are instances of "Q" strains (table 29 and 30 and graph. 1 and 2[1])). There are transitional patterns and there is a tendency of some strains to show the highest titres with the homologous antiserum, particularly in the A-prime group.

The fact that a strain showed a low titre with the corresponding antiserum proved to be independent of the circumstance whether the strain was primarily isolated in the ferret or in chick embryo. We gained the impression that, generally speaking, new and still young egg strains show low titres with sera against the same subgroup, but that after continued egg passages the titres became higher.

TABLE 24

Diagram of serological patterns of influenza strains within the same subgroup

Ferret antisera	Titres against virus		
	P	Q	R
P	++++	+	++++
Q	+	+	++++
R	+	+	++++

Influenza virus strains which combine to high titre with heterologous antisera of the same subgroup. Several strains were found which gave high titres with most antisera against strains of the same subgroup. Hence these strains ("R"-strains from table 24) were most useful as reference strains for antisera of strains to be classified. Of the pre-1946 strains we found that PR8(1934 USA) and BURR(1937 E) were very suitable for this purpose, and particularly also A(1941 Ned). Of the A-prime strains isolated after 1946, those useful as reference strains were BARRATT(1947 E) (both the egg strain and the mouse-adapted strain), and RHODES (1947 USA), a ferret-mouse-egg strain.

In our laboratory the strain FM1 (1947USA) (SMADEL(1947)) was only recently examined in two directions. The strain is used

[1]) Graph 1 and 2 are found at the end of the publication.

by American investigators as a representative of the A-prime group and falls as clearly in this group as the RHODES and BARRATT strains. In a cross-test it behaved practically as well as the RHODES strain, which has been used throughout all our experiments (table 25).

The screening of influenza A strains with haemagglutination inhibition tests with the homologous antiserum. When the variable differences of behaviour in series of A strains and their respective antisera in the cross-haemagglutination inhibition tests are

TABLE 25

Cross-haemagglutination inhibition tests with four different strains from two epidemics (1947 and 1949) (One experiment)

Ferret antisera	Titres against virus			
	Barratt (1947 E) Egg	Rhodes (1947 USA) Ferret-mouse-egg	FM1 (1947 USA) Egg-mouse-egg	Heer (1949 Ned) Egg
Barratt(1947 E) (Pool) Egg strain	**2560**	1540	1380	380
Rhodes(1947 USA) (Pool) Ferret-mouse-egg	380	**6140**	640	210
FM1(1947 USA) (Pool) Egg-mouse-egg	1540	2050	**6140**	430
Heer(1949 Ned) (Pool) Egg strain	1380	1540	2050	**850**

considered, a rigid serological classification by this technique of such a series becomes a wellnigh insoluble problem. Hardly any two strains prove to be quite identical.

From literature, and also from our own experience, however, it had already become probable that two important subgroups of human influenza A strains may be distinguished, viz. strains isolated prior to 1946, and after that date. Our preliminary investigation was therefore based on this distinction and we have examined a number of strains, isolated between the years 1933 and 1949, with a view to finding their serological relationship to each of these subgroups. As the strain WS(1933 E) seems most probably to occupy a separate place among the human

influenza A strains, we also included this strain in our investigation. Along with this strain we used as reference the ferret-mouse-egg strains PR8(1934 USA) and A(1941 Ned) for the pre-1946 group, and the egg strain BARRATT(1947 E) and the ferret-mouse-egg strain RHODES(1947 USA) for the post-1946 group. The screening tests were performed in one direction, so that antisera against the strains to be tested were put up against the reference strains. Since reference strains were used which showed high titres against most heterologous strains, it was also possible to test antisera with a low homologous titre ("Q"-strains of table 24).

It appears that such a screening test gives a clear picture of the broad antigenic relationships of the influenza A strains which were examined, and shows the different subgroups very distinctly. Graph 1 [1]) illustrates the results of these tests (see also table 29). The strains that were isolated during the various epidemics in the period 1934—1943 (inclusive), though originating from different countries, are all closely related to PR8(1934 USA) and A(1941 Ned) and they are widely divergent from the A-prime strains. The strains isolated after 1945 are all related to the A-prime strains BARRATT(1947 E) and RHODES(1947 USA). Several strains of the PR8- and the 1947-types show a minor relationship to WS(1933 E). None of the various A strains from the three subgroups that were tested against the influenza B strain LEE(1940 USA) showed any relationship to it.

Hence it appears to be possible by this method to compare the antigenic composition of new strains quickly and reliably with the structure of certain selected representatives of known subgroups.

Cross-haemagglutination inhibition tests. We have cross-tested a number of strains completely in both directions. For this purpose we used the influenza B strain LEE(1940 USA), the swine influenza strain SHOPE-15(1930 USA), the stock laboratory strains WS(1933 E) and PR8(1934 USA), the 1937 strains isolated in England: CHRISTIE, TALMEY, GATENBY and BURR, two ferret-mouse-egg strains from the 1941 epidemic in Holland, viz. A(1941 Ned) and Sl(1941 Ned) and four 1947 strains, viz. K(1947 Ned) (ferret-egg and ferret-egg-mouse strain), Gg(1947 Sw) (ferret-egg strain), BARRATT(1947 E) (egg-and egg-mouse strain), and

[1]) Graph 1 is found at the end of the publication.

RHODES(1947 USA) (ferret-mouse-egg strain). Graph 2[1]) illustrates the results of these tests (see also table 30). Although the titrations were not performed on one day, they are yet comparable, since the same sera, the same egg fluids and red cells from the same chick were used and the tests were done in as short a time as possible. A titration against the homologous strain was always included. Only the analysis of the strains BURR, GATENBY (E_{30}) and LEE was done separately with the same sera but with different egg fluids.

It follows from the whole experiment that not a single one of the strains showed any relationship to the influenza B strain LEE(1940 USA). It is quite clear that the pre-1947 and the post-1947 strains belong to two different groups (A and A-prime), but a minor relationship is shown by the fact that strains from one group cross to a limited extent with strains from the other group by some heterologous sera. The tests also show that the strain WS(1933 E) displays some relationship to the pre-1947 strains and more so than to the A-prime strains.

Diagram showing the results of the cross-haemagglutination inhibition tests. Table 26 presents the results of the tests in the form of a diagram. The number of plus-signs (++...) is a measure of the titre of antibodies against the particular virus. While the influenza-B strain LEE is very clearly separated from the A strains, the A group shows four subgroups. They comprise three subgroups of human strains: A-(WS), A-(PR8) and A-prime strains, and a fourth subgroup of A-swine virus. The mutual serological relationships of these four subgroups are recorded in the table.

The subgroups of influenza A strains.

The A-(swine-15) influenza virus. The swine-15 strain exhibited a wide disparity from the other strains, which fact is in agreement with former studies (FRANCIS and MAGILL(1938), EATON and PEARSON(1940), BURNET and CLARK(1942), HIRST(1943), FRIEDEWALD(1944)). It shows a minor relationship to a few A-prime strains.

A-(WS) (1933 E). Owing to its relatively slight antigenic relationship to the other subgroups, WS occupies a separate place. This agrees with the findings of other workers (MAGILL

[1]) Graph 2 is found at the end of the publication.

and FRANCIS(1938), WILSON SMITH and ANDREWES(1938), BURNET and CLARK(1942) and FRIEDEWALD(1944)). The strain shows more relationship with the A-(PR8) than with the A-prime group.

It is possible that in the years from 1934 till 1937 some more strains were isolated which are closely related to WS (SMORODINTSEV *et al.*(1936), OAKLEY (See SMITH and ANDREWES(1938)). Clearly defined epidemics however caused by such strains have not been described.

TABLE 26

Diagram of serological patterns of influenza strains with different antigenic composition

Ferret antisera	Titres against virus				
	A strains				B strain
	A Swine 15 (1930)	A WS (1933)	A (1934—1946)	A-prime (1946—1949)	B Lee (1940)
A-Swine-15(1930)	++++	±	±	+	0
A-WS(1933)	±	++++	+	±	0
A(1934—1946)	0	+	++++	+	0
A-prime(1946—1949)	±	±	+	++++	0
Lee(1940)	0	0	0	0	++++

The strain shows a particularly high degree of pneumotropy in the ferret and extremely high mouse virulence and is rapidly adapted to the chorio-allantoic membrane of the chick embryo producing focal lesions (BURNET and LUSH(1938), STUART-HARRIS(1939)). When fully adapted to the chick-embryo it causes haemorrhagic manifestations and encephalitis and STUART-HARRIS(1939) could produce a neurotropic variety of the strain in mice (confirmed by FRANCIS and MOORE(1940)). For the present,

therefore, it would seem justified to look upon the strains WS as a representative of a separate subgroup.

Strains of the A-(PR8) subgroup. After FRANCIS had isolated the strain PR8 in 1934, various epidemics occurred and were described in the years from 1934 till 1943. They were very probably all caused by strains showing close relationship to this strain. The mouse virulence of most strains is high. Neurotropic tendency seems to be slight or lacking (FRANCIS and MOORE(1940)).

Strains of the A-prime group. The group of strains, isolated between 1946 and 1949 shows definite antigenic differences from the swine and PR8 group. Recent publications (DUDGEON *et al.* (1948), KALTER *et al.*(1948), RASMUSSEN *et al.*(1948), TAYLOR 1949)) describe the relationship of the strains isolated in 1947 to stock laboratory strains. While our experiments are qualitatively in agreement, the deviations found by us are quantitatively greater, perhaps because of the neutralization of the non-specific inhibitor in the antisera. The first studied human representative of this subgroup is the strain CAM, which was isolated in Australia during a small epidemic in a school in Melbourne in May 1946 (ANDERSON and BURNET(1947), ANDERSON(1947)). Although CAM is perhaps more related to the A-(PR8) subgroup than any of the other A-prime strains which were examined, yet it clearly belongs to the A-prime group, which is in agreement with the findings of TAYLOR(1949).

The 1947 strains which we examined came from epidemics in different countries. Other differences with the PR8-group are that the strains show a feebler antigenic power and perhaps that their mouse virulence when fully adapted, is generally also lower.

Varieties of strains of influenza A virus within the same subgroup. The best way to approach this problem is perhaps to start from the remarkable fact that in 1947 there appeared for the first time an antigenically deviating A group in many countries of the world in the same winter. In Sweden the epidemic was fairly extensive (LÖFSTRÖM(1949)). Strains were isolated in that year in Sweden, England, Holland and the USA, although the morbidity outside Sweden was probably considerably lower than in that country itself. This was certainly so in Holland, where influenza occurred only sporadically in January and February.

When the behaviour in the cross-haemagglutination tests of

the strains recovered in the different countries in 1947 are compared with each other, rather considerable differences are found, and complete reciprocal identity does not occur.

As autochthonous origin of a new virus in all those countries is unthinkable, we must assume that the new group spread over the world in the winter of 1947 (or perhaps earlier) and that there was some common original strain having the composition of the new A-prime group. In this light the mutual (minor) variations of the new strains would be relatively unimportant. It is almost certain that the differences arise partly owing to the fact that some strains "combine badly" with the homologous (and the heterologous) antisera (for example K(1947 Ned) and WOITEKI(1947 E)) and may be therefore perhaps attributed to some unknown intervening biophysical property of those strains. Other differences may perhaps be explained by slight modifications in the antigenic pattern during passage in community, but in our opinion we are not as yet entitled to attribute epidemiological significance to such differences.

The 1949 influenza strains, isolated in Holland. The epidemic of influenza A-prime prevailing in Holland during the winter of 1949 was certainly imported from Belgium, and its course could be approximately traced throughout Holland [1]).

Six strains from different places, isolated in eggs, were cross-tested on two occasions (table 27 and 28).

Table 27 shows (preliminary) crossings of six 1949 strains in 6 experiments (one serum used at the same time against all strains) and table 28 in one experiment (performed on the same day). The experiment of table 28 was done with ferret antisera prepared from the eighth egg passage of each strain, two ferrets being inoculated. The ferret antiserum with the highest homologous titre was used in the crossings. The allantoic fluids of the 1949 (Ned) strains (from table 28) were prepared from the same (eighth) egg-passage. The titres within the 1949 A-prime group in table 28 are higher than those in table 27. There is a distinct difference from the FM1 strain, but it has to be considered that this strain has undergone 8 mouse passages. Strain AI(1949 Ned) E_8 (table 28) is more sensitive to inhibition as AI(1949 Ned) E_{17}. Strain Vr(1949 Ned) shows itself in both experiments not very sensitive to inhibition.

[1]) In total 38 strains were isolated in Holland and it was distinctly shown that they all fell into the A-prime group.

TABLE 27

Cross-haemagglutination inhibition tests with reference strains and six different strains isolated from one epidemic (1949 Ned)

Experiment	Sub-group	Ferret antisera	Titres against virus										
			WS 1933 E	PR8 1934 USA	A 1941 Ned	Barratt 1947 E	Rhodes 1947 USA	AI 1949 Ned E_{17}	Hof 1949 Ned E_{7-11}	Wagt 1949 Ned E_{16-19}	Hes 1949 Ned E_{8-14}	Vr 1949 Ned E 12-13-15	Heer 1949 Ned E_{15-17}
1	A-(WS)	WS(1933 E) (Pool)	**5120**					<12	<12	<12	<12	<12	<12
	A-(PR8)	PR8(1934 USA) (Pool)		**10750**				<12	<12	<12	<12	<12	<12
2		A(1941 Ned) (Pool)			**7680**			<12	<12	<12	20	<12	<12
3	A-prime (1947)	Barratt(1947 E) (Pool)				**4270**		320	320	960	1920	770	750
		Rhodes(1947 USA) (Pool)					**7680**	12	480	530	960	260	170
4	A-prime (1949)	AI(1949 Ned) E_{16} F_1	<12	<12	<12	320	770	**90**	170	200	1190	80	370
		Hof(1949 Ned) E_8 F_1	<12	<12	<12	430	2300	90	**670**	450	670	90	450
		Wagt(1949 Ned) E_{14} F_1	<12	<12	<12	200	580	80	220	**300**	340	90	220
5		Hes(1949 Ned) E_{10} F_1	<12	<12	<12	750	1380	110	220	210	**2990**	200	640
		Vr(1949 Ned) E_{10} F_1	<12	<12	60	600	1380	60	90	130	450	**220**	580
6		Heer(1949 Ned) E_{15} F_1	<12	<12	40	4100	2690	960	1020	900	3070	2730	**3550**

TABLE 28

Cross-haemagglutination inhibition test with 6 A-prime(1949 Ned) strains and the strain FM 1 (1947 USA) performed on the same day. The titres of the antisera are expressed in the actual serumdilution and also in relation to the homologous serum titre

Sub-group	Ferret antisera	Titres against virus						
		F M 1 1947 USA EM_8E_{22}	AI 1949 Ned E_8	Hof 1949 Ned E_8	Wagt 1949 Ned E_8	Hes 1949 Ned E_8	Vr 1949 Ned E_8	Heer 1949 Ned E_8
A-prime (1947)	F M1(1947 USA) $EM_8E_{14}F_1$	**12290** **1,000**	220 0,018	580 0,047	750 0,061	100 0,008	510 0,042	1540 0,125
A-prime (1949)	A I(1949 Ned) E_8F_1	1380 1,156	**1190** **1,000**	380 0,322	450 0,375	510 0,429	380 0,322	640 0,537
	Hof(1949 Ned) E_8F_1	1540 1,500	900 0,875	**1020** **1,000**	1610 1,572	510 0,500	380 0,375	1020 1,000
	Wagt(1949 Ned) E_8F_1	1540 1,143	670 0,500	770 0,571	**1340** **1,000**	380 0,286	380 0,286	1150 0,856
	Hes(1949 Ned) E_8F_1	4610 2,250	2690 1,317	2050 1,000	2390 1,167	**2050** **1,000**	1380 0,674	2560 1,260
	Vr(1949 Ned) E_8F_1	4100 3,556	1610 1,398	1020 0,888	1190 1,036	1020 0,888	**1150** **1,000**	2560 2,222
	Heer(1949 Ned) E_8F_1	5120 1,250	3550 0,866	1380 0,435	1790 0,438	1540 0,375	1540 0,375	**4100** **1,000**

It appears moreover from table 28 that amongst the 6 strains examined the best representative of the A-prime (1949 Ned) strains would probably be the strain Heer.

The results of the above mentioned experiments are sufficiently clear-cut to prove that the 1949 strains in the haemagglutination inhibition test show distinct differences to the same extent and of the same nature as the 1947 A prime-strains. In spite of these differences, however, it is difficult to question the unity of the strain-type as an epidemiological agent. This means again that probably a part of the demonstrable differences in behaviour of the strains must be explained in another way.

So far, the differences between A strains from the PR8 subgroup have been investigated much more thoroughly than those from the A-prime subgroup, with the aid of the very

comprehensive cross-mouse protection tests of MAGILL and FRANCIS(1938) and WILSON SMITH and ANDREWES(1938). It is, however, difficult to decide how far the serological differences between strains from the same subgroup, as found with the somewhat crude technique of the mouse inoculations, indicate real and fixed differences in antigenic structure.

The strain diagram of Wilson Smith and Andrewes (see also chapter I). Out of the four specific strains distinguished by them, *viz.* WS, TALMEY, CHRISTIE and GATENBY, the strain WS can be identified as a separate group with the technique of the cross-haemagglutination inhibition test.

The Talmey and Christie strains. In our experiments too, there is a certain amount of difference in the way they behave. BURNET was not able to note any differences. It is therefore possible that his antisera resembled those of the first two TALMEY sera of table 19. The strain CHRISTIE resembles very much the strain PR8, but the strain TALMEY only shows a high titre with the homologous antiserum.

The Gatenby strain. It was looked upon by WILSON SMITH and ANDREWES as a separate strain. Related strains are seldom mentioned in medical literature, as opposed to the TALMEY and CHRISTIE strains. In our experiments the conduct of this strain resembled that of the TALMEY strain, except for the fact that one of the two Gatenby antisera also gave a low homologous titre. For the rest the strain undoubtedly belongs to the PR8 subgroup (graph. 2) [1]).

The intermediate group. WILSON SMITH and ANDREWES found 15 intermediate strains, 13 of which were related to TALMEY or CHRISTIE or to both, one to TALMEY, CHRISTIE and GATENBY and one to GATENBY. The strain PR8 belonged to the first-mentioned strains (related to TALMEY and CHRISTIE). Moreover they found four strains which were closely related to all four specific strains (WS, TALMEY, CHRISTIE and GATENBY). From this it follows already that the TALMEY and CHRISTIE strains represent a group to which the greater part of the strains from the 1934—1937 period belongs. This fact has been confirmed by new strains from the winter of 1939 (STUART HARRIS *et al.* (1940), TAYLOR and DREGUSS(1941), MULDER and BIJLMER(1941)).

In view of what we know from our cross-haemagglutination

[1]) Graph 2 is to be found at the end of the publication.

inhibition tests it is easy to understand that the CHRISTIE strain was often found related to strains isolated between 1934 and 1943.

With regard to the TALMEY strain the antiserum against this strain shows high titres with heterologous strains from the PR8 subgroup but the strain itself generally shows low titres against heterologous antisera from this group. We cannot account for the fact that the GATENBY strain has always proved to be a separate strain in the cross-mouse protection tests, as in our experiments its behaviour is practically identical with the TALMEY strain.

Polyvalent strains with respect to heterologous subgroups. Polyvalent strains were first described by WILSON SMITH and ANDREWES as non-specific (master) strains. As examples they mentioned the strains BURR(1937 E), KOP(1936 Berlin), HAM (1937 E) and CRI(1937 E). The strain BURR was examined by us. The antiserum of the strain classified it as being one of the PR8 subgroup. In our experiments, too, the strain showed the highest heterologous titre with the WS antiserum that was seen by us, but it did not show inhibition by A-prime antisera (graph. 2). We observed the same phenomenon with one antiserum of the A-prime strain CAM when tested against the strain A(1941 Ned). So far, however, there are not yet any detailed studies of such strains available.

Summary of the technique used routinely in the classification of influenza A strains of unknown antigenic constitution by the haemagglutination inhibition test. Certain points should be kept in mind in determining the subgroup of an influenza virus strain of unknown antigenic constitution by the technique of the cross-haemagglutination inhibition test. We feel that it is probably necessary to pass the standard representative laboratory stock strains to be used in the cross-test and for the manufacturing of standard antisera at regular intervals in mice in order to keep their antigenic activity and sensitivity to inhibition as great as possible; this will probably ensure that the standard sera will give a high titre with heterologous strains from the same subgroup. In the case of ferrets both the pre-and post-infection serum are collected in order to detect pre-infection antibodies in uninoculated animals. All sera are completely freed from non-specific inhibitor by crude filtrate of V. cholerae and tests

are made to determine the absence of inhibitor after such treatment, particularly in the corresponding pre-infection serum. The antiserum of at least three animals is pooled to constitute the standard stock serum against each strain. Each pool is crossed with strains representative of all known subgroups of influenza-virus and if found correct a portion is freeze-dried together with the virus of the same passage as that is used in the manufacture. This procedure helps to disentangle possible aberrant results due to laboratory contamination or mistakes in registration.

Strains newly isolated in chick embryo are put up as soon as possible (to avoid laboratory-contamination) against the pools of pre- and post-infection serum of at least the B (LEE) strain, the A-Swine-15 strain, A-(WS) and preferably two selected representatives of the A-(PR8) and the A-prime group showing high titres with most heterologous strains from the corresponding subgroup. The strains PR8 and FM 1 are included to assist international comparison. We do not rely on the PR8 strain alone because there are examples of A strains with a somewhat broader pattern of high titres against sera of the corresponding subgroup. As to the A-Swine and influenza-B group the future alone will decide in how far the use of a single antiserum representing these groups is a reliable procedure. The titres of the antisera should be verified at frequent intervals or in the actual test of the new virus.

As the classification of a new strain may give difficulties when the antibody titres using standard sera are low, it is generally more reliable to prepare a pool of antiserum against the new virus from two of three ferrets and to test this against the standard laboratory strains as well as homologous virus.

If a new strain is to be classified with certainty in any particular group, there must be considerable differences between the titres of inhibition of the antiserum against the virus strains representative of this group and those against other groups. Instances of height of titres which can be expected in routine-work are given in table 29, 30 and 31. The titre against the homologous strain may be low but against one or both of the representatives of the same subgroup inhibition may simultaneously be high. When the homologous titre against the unknown strain is high the titre against one or both the representatives of one of the subgroups must also be high or else the classification remains uncertain.

When classifying long established laboratory strains we feel that a cross-test with the A-(WS) strain should always be included, as WS has been so widely used in different laboratories that a chance of contamination with this strain may exist.

Continued research into possible antigenic structural differences of influenza strains within a subgroup. One of the most important problems waiting to be solved would for the present seem to be the low titres (or avidity) of some influenza antisera against the homologous strains. As long as this has not been clarified, it is scarcely possible to speculate about antigenic differences within the subgroups. The same remark holds good for the difference in behaviour between some egg strains and the mouse-adapted strains derived from them. The investigation of DINGER and WOLFF(1948) concerning a method to eliminate an avidity factor by means of fractionated adding of the virus suspension in haemagglutination inhibition tests is perhaps attached to this problem.

Cause of appearance of deviating human A strains. We are completely in the dark about the cause of the sudden appearance of antigenically deviating human A strains. Whether in future new strains will be found with deviating and as yet unknown antigenic structure, when this can be expected, what circumstances will influence it, all these are questions about which little can be said just now. In view of prophylactic measures, however, it is imperative to keep them in mind. In this connection we would refer to an article by TAYLOR(1949) who informs us that he has isolated a strain which does not show any relationship whatever to PR8, a 1943 strain (965 USA) and to A-prime strains. The World influenza-strain typing centres, set up by Dr C. H. ANDREWES, London, Dr P. MAGILL, New York and Dr F. M. BURNET, Melbourne, will be of great help in the future solution of this difficult problem.

Antigenic structure and vaccination. The antigenic difference between the strains of the A-(PR8) and the A-prime subgroups is seen clearly in the lack of immunity against A-prime strains after vaccination with a vaccine containing the strain PR8 (1934 USA) and another A strain, WEISS(1934 USA) (FRANCIS *et al.*(1947)). The failure of vaccination against influenza in the 1947 epidemic was generally attributed to the fact that the causal agent of the epidemic was not related to the strains present in the vaccine (FRANCIS *et al.*(1947), LOOSLEY *et al.*(1948), VAN

TABLE 29

Instances of titres of antisera, elicited by influenza strains, against the reference strains WS, PR8, A(1941 Ned), Barratt, Rhodes and against the strains Lee and Swine-15

Antisera (ferret)	Virus strains							
	Lee 1940 USA	Swine 15 1930 USA	WS 1933 E	PR8 1934 USA	A 1941 Ned	Homologous	Bar 1947 E	Rhodes 1947 USA
Lee (1940 USA)	**770**	<12	<12	<12	<12		<12	<12
Swine-15 (1930 USA)	<12	**4100**	40	12	<12		380	<12
WS (1933 E)	<12	60	**2730**	380	270		<12	80
PR8 (1934 USA)	<12	<12	30	**13650**	5520		<12	15
Phila (1935 USA)			15	14330	1950	**5380**	<12	<12
Melbourne (1935 Au)			<12	14340	1150	**3550**	<12	<12
Christie (1937 E)	<12	<12	50	1380	3660	**3410**	<12	<12
Talmey (1937 E)	<12	<12	20	6830	5970	**6830**	<12	<12

Antisera (ferret)	Virus strains							
	Lee 1940 USA	Swine 15 1931 USA	WS 1933 E	PR8 1934 USA	A 1941 Ned	Bar 1947 E	Rhodes 1947 E	Homologous
Cam (1946 Au)			80	200	1610	1610	5970	**3550**
Gg (1947 Sw)	<12	20	<12	<12	20	510	450	**1790**
Barratt (1947 E) Egg	<12	50	<12	<12	380	**8190**	13000	
Barratt (1947 E) Egg-mouse	<12	<12	<12	<12	190	770	2050	**5970**
Kunz (1947 E)			<12	30	70	1150	1920	**4610**
Woiteki (1947 E)			<12	<12	40	1380	1610	**290**
K (1947 Ned) Ferret-egg	<12	<12	<12	<12	<12	640	260	**320**
K (1947 Ned) Ferret-egg-mouse	<12	<12	30	40	240	640	770	**3550**

Gatenby E_7 (1937 E)	<12	<12	40	580	2560	**770**	<12	<12	FM1 (1947 USA)			<12	20	50	750	1380	**3070**
Gatenby E_{30} (1937 E)	<12	<12	430	10750	14340	**6140**	20	580	Rhodes (1947 USA)	<12	<12	80	20	80	770	**8190**	
Burr (1937 E)	<12	12	130	2390	2620	**4100**	40	510	1236 (1947 USA)			<12	<12	90	2990	3550	**2130**
Bos (1939 Ned)	<12	<12	<12	1020	290	Lost	<12	<12	1265 (1948 USA)			<12	20	200	1380	1610	**10240**
A (1941 Ned)	<12	<12	100	5380	10240		60	320	FJS (1948 Au)			<12	<12	<12	290	480	**290**
Sl (1941 Ned)	<12	<12	16	5120	10060	**16380**	40	50	AI (1949 Ned)	<12	<12	<12	<12	<12	320	770	**90**
Sch (1941 Ned) Ferret-egg			120	1920	4100	**2690**	80	100	Heer (1949 Ned) Egg	<12	<12	<12	<12	40	1340	2690	**2560**
Sch (1941 Ned) Ferret-egg-mouse			<12	960	4100	**10240**	<12	<12	Heer (1949 Ned) Egg-mouse			160	600	370	370	7170	**5120**
Weiss (1943 USA)			15	5120	3840	**16380**	<12	160	Hes (1949 Ned)	<12	<12	<12	<12	<12	750	1380	**2990**
965 (1943 USA)			<12	270	2990	**3070**	<12	<12	Hof (1949 Ned)	<12	<12	<12	<12	<12	430	2300	**670**
Hemsbury (1943 E)			<12	3550	11950	**7170**	<12	<12	Vr (1949 Ned)	<12	<12	<12	<12	60	600	1380	**220**
A 128 (1943 Sw)			<12	600	1790	**3410**	50	50	Wagt (1949 Ned)	<12	<12	<12	<12	<12	200	580	**300**

TABELE 30

Titres of cross-haemagglutination inhibition tests with influenza A strains [1])

Antisera (ferret)	Virus strains															
	Lee 1940 USA	Swine 15 1930 USA	WS 1933 E	PR8 1934 USA	Tal 1937 E	Christ 1937 E	Gat 1937 E	Burr 1937 E	A 1941 Ned	Sl 1941 Ned	Gg 1947 Sw	Bar Egg 1947 E	Bar Mouse 1947 E	K Egg 1947 Ned	K Mouse 1947 Ned	Rho-des 1947 USA
Lee(1940 USA)	**770**	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12
Swine-15(1930 USA)	<12	**4100**	40	12	<12	12	<12	30	<12	<12	110	380	12	12	<12	<12
WS(1933 E)	<12	60	**2730**	380	<12	510	40	1790	270	20	<12	<12	<12	<12	16	80
PR8(1934 USA)	<12	<12	30	**13650**	430	6830	1070	3110	5520	450	40	<12	20	<12	<12	15
Talmey(1937 E)	<12	<12	20	6830	**6830**	13650	580	6520	5970	1340	<12	<12	<12	<12	<12	<12
Christie(1937 E)	<12	<12	50	1380	190	**3410**	340	2880	3630	350	<12	<12	40	<12	<12	<12
Gatenby(1937 E) E_7	<12	<12	40	580	12	270	**770**	1280	2560	2980	<12	<12	<12	<12	<12	<12
Gatenby(1937 E) E_{30}	<12	<12	430	10750	170	9560	**6140**	8190	14340	2070	<12	20	<12	<12	220	580

Burr (1937 E)	<12	12	130	2390	160	1920	600	**4100**	2620	340	<12	20	60	<12	<12	510
A (1941 Ned)	<12	<12	100	5380	640	3410	170	1150	**10240**	2390	<12	60	190	<12	50	320
Sl (1941 Ned)	<12	<12	16	5120	600	9560	1090	3980	10060	**16380**	150	30	70	<12	<12	50
Gg (1947 Sw)	<12	20	<12	<12	50	<12	<12	<12	20	<12	**1790**	510	1340	60	110	450
Barratt (1947 E) Egg-strain	<12	50	<12	<12	20	<12	<12	12	380	12	340	**8190**	12380	120	1610	8190
Barratt (1947 E) Egg-mouse strain	<12	<12	<12	<12	110	<12	<12	20	190	<12	<12	770	**5970**	<12	80	2050
K (1947 Ned) Ferret-egg strain	<12	<12	<12	<12	<12	<12	<12	<12	<12	<12	200	640	2690	**320**	1790	260
K (1947 Ned) Ferret-egg-mouse strain	<12	<12	30	40	<12	100	50	<12	240	12	20	640	1610	240	**3550**	770
Rhodes (1947 USA)	<12	<12	80	20	<12	100	30	40	80	<12	110	770	2690	60	640	**8190**

[1]) All titrations have been performed with the same sera, chicken red cells and the same egg fluid of the virus strains, except the titration of the antiserum against Gatenby (1937 E) FME_{30} and the cross-titrations with Burr (1937 E) and Lee (1940 USA). These strains have been tested separately.

RAVENSWAAY(1948), RASMUSSEN *et al.*(1948), SARTWELL and LONG(1948), SIGEL *et al.*(1948)). It was therefore considered necessary to increase the number of strains in the vaccine by a representative of the A-prime group [1]).

From our experiments it cannot be inferred which strain would be most suitable for this purpose, as the ferrets are inoculated

TABLE 31

Instances of titres of cross-haemagglutination inhibition tests with the strains A128, Cam and FM1 and the reference strains WS, PR8, A(1941 Ned), Barratt and Rhodes

Antisera (ferret)	Virus Strains							
	WS 1933 E	PR8 1934 USA	A 1941 Ned	A128 1943 Sw	Bar Egg 1947 E	Rhodes 1947 USA	Cam 1946 Au	FM1 1947 USA
WS(1933 E)	**9560**			160			510	100
PR8(1934 USA)		**17070**		190			60	<12
A(1941 Ned)			**10750**	1380			80	20
A128(1943 Sw)	<12	600	1790	**3410**	50	50	40	30
Barratt(1947 E) Egg strain				<12	**9560**		240	3840
Rhodes(1947 USA)				80		**10240**	530	3410
Cam(1946 Au)	50	270	1380		1150	9560	**4780**	2210
FM1(1947 USA)	<12	20	50	<12	750	1380	20	**3070**

with active virus. The experiments show that the strains BARRATT (1947 E), RHODES(1947 USA) and FM1(1947 USA) have a broad antigenic composition, and from this point of view they are suitable for incorporation in a vaccine. Vaccination experiments on animals and human beings, however, must decide which strain should be employed by preference. Regular mouse-passage of the strains seems to enhance their antigenic potency and should

[1]) According to a personal communication from Dr TH. FRANCIS and Dr H. COX the A-prime strain FM1 (1947 USA) has been incorporated in the American vaccine.

be recommended. As to the PR8 group our experiments showed that the strain A(1941 Ned) is somewhat more polyvalent than PR8(1934 USA). Further investigation must show which of the two strains is more suitable to be incorporated in a vaccine as a representative of this A subgroup. Since 1937 no strains have been found showing a clear relationship to WS(1933). For the present, therefore, it would not seem necessary to incorporate the strain WS in a vaccine. It will be wise, however, always to keep the existence of strains of this type in mind, because WS probably is the most pneumotropic of all known strains and may return one day as the cause of a new epidemic.

SUMMARY

The antigenic structure of human influenza A strains was examined by means of the cross haemagglutination inhibition test (HIRST) with ferret sera. The investigation was limited in scope to practical issues. For this purpose a modified form of the HIRST technique was employed with a micro method, using tiles of porcelain with concavities. The findings of the serum titrations performed with this micro method and with the HIRST-SALK technique can be readily compared. The micro HIRST technique saves glass ware and biological material. It is very quick, and the red cell patterns are easy to read. The method gives a reasonable degree of accuracy, and is very suitable for routine laboratory work.

For the proper study of the antigenic structure of influenza strains it is essential to eliminate the non-specific inhibiting substances from the ferret sera, particularly in testing newly isolated egg strains which are often strongly inhibited by normal ferret sera. By diluting sera with a physiological solution of sodium citrate or some other physiological solution containing anti-Calcium ions, the non-specific inhibition against some strains is completely eliminated, but is still demonstrable with other strains. The antibody titre does not change in this process. By adding potassium periodate to the ferret sera the non-specific inhibiting substances are also destroyed. The quantity of potassium periodate to be added must be separately calculated for each serum.

A good method of freeing sera from non-specific inhibiting substances is to treat them with crude filtrate of *V. Cholerae*. The filtrate is prepared according to the method described by BURNET. After the interaction between the cholera filtrate and the serum the non-specific inhibition against all the strains used by us is completely eliminated in a serum dilution 1 : 12. The antibody titre is hardly or not at all modified. The reduction of the titre of some antisera is due to the superimposition

of the titre of the non-specific inhibition upon that of the antibodies; it is only observed when there is little difference between the quantity of each or when the titre of the non-specific inhibition is higher.

The treatment of sera with crude cholera filtrate has been successfully used to classify freshly isolated egg strains (first positive amniotic fluid). In some sera from bought ferrets it was moreover possible to demonstrate low antibody titres which must be due to former infections. As human sera also contain non-specific inhibiting substances, treatment with cholera filtrate could be employed to reveal their actual antibody levels more accurately. Post-infection increases of the titre were also demonstrated more minutely and it was possible to obtain a clue as to the presence of pre-infection antibody for influenza virus in human sera.

Cross-haemagglutination inhibition tests are characterized by many difficulties. Inconstant results may result from (1) varying antibody contents of different ferret sera of the same strain against heterologous strains; (2) varying behaviour of different egg-passages or "lines" of the same strain; (3) the fact that some antisera give much lower titres with the homologous strain than with the heterologous strains from the same subgroup; (4) in our tests mouse-passages of egg strains showed a broader pattern of high titres with heterologous sera from the same subgroup than the original egg strains. Hence it is very important to know exactly the passage formula of each strain.

Cross-haemagglutination inhibition tests with human influenza-A strains isolated between 1933 and 1946 identified the A and the B groups as absolutely distinct.

The A-(PR8) group (strains from 1934 to 1943) could be clearly distinguished from the A-prime group (strains from 1946 to 1949) with only a few exceptions.

The strain WS(1933 E) is clearly separated from the A-prime group, and reasonably clearly from the A-(PR8) group. The Swine-influenza strain (SHOPE-15) was also distinct from human strains with some affinity to the A and A-prime group.

There was hardly ever a reciprocal identity of antigenic structure between the strains of the same subgroup. It was only possible to establish their relation to one of the subgroups A-(PR8) or A-prime with certainty by testing the antiserum of the strain to be classified with one or more selected representative viruses

of these subgroups which gave high titres with most of the heterologous antisera of the same subgroup. Examples of such strains from the A-(PR8) group are the strain PR8 itself, the strain CHRISTIE(1937 E) and a Dutch strain A(1941 Ned). In the A-prime group such strains are FM1(1947 USA), RHODES (1947 USA) and BARRATT(1947 E).

Serological classification of strains within one of the subgroups was not possible by the cross-haemagglutination inhibition test, owing to the lack of reciprocal identity of the strains. It is possible that there are differences in antigenic structure of the strains within the same subgroup, but it must be pointed out that part of the differences found can possibly be explained by a difference (partly reversible) of behaviour of the strains owing to some unknown intervening biophysical property which may be separated from the actual antigenic structure.

REFERENCES

ANDERSON, S. G., F. M. BURNET and J. D. STONE: 1946, Austr. J. exp. Biol. med. Sci. **24**, 269.

ANDERSON, S. G.: 1947, Austr. J. exp. Biol. med. Sci. **25**, 243

ANDERSON, S. G. and F. M. BURNET: 1947, Austr. J. exp. Biol. med. Sci. **25**, 235.

ANDERSON, S. G.: 1948, Austr. J. exp. Biol. med. Sci. **26**, 347.

ANDREWES, C. H., R. E. GLOVER, N. P. HUDSON, D. LUSH and C. H. STUART HARRIS: 1941, Lancet II, 387.

BELL, F. R. and J. A. DUDGEON: 1948, J. comp. Path. Therap. **58**, 167.

BRIODY, B. A.: 1948, J. Immunol. **59**, 115.

VAN BRUGGEN, J. A. R., L. BIJLMER, W. A. HOEK, J. MULDER and L. J. ZIELSTRA: 1947, "Studies on the influenza-A-epidemic of January-March 1941 at Groningen (Holland)", Verhandelingen van het Instituut voor Praeventieve Geneeskunde, Leyden VII.

BUNGENBERG DE JONG, H. G.: 1938, Proc. Kon. Akad. v. Wetensch. Amsterdam, **41**, 788.

BUNGENBERG DE JONG, H. G. and C. H. RERING: 1942, Proc. Kon. Akad. v. Wetensch. Amsterdam, **45**, 697, 707, 713.

DE BURGH PATRIC, M., P. C. YU, C. HOUR and M. BOVARNICK: 1948, J. exp. Med. **87**, 1.

BURNET, F. M.: 1936, Brit. J. exp. Path. **17**, 282.

BURNET, F. M. and D. LUSH: 1938, Brit. J. exp. Path. **19**, 17.

BURNET, F. M. and E. CLARK: 1942, "Influenza"; Monograph from the Walter and Eliza Hall Institute of research in pathology and medicine, Melbourne, Number 4, 8.

BURNET, F. M. and J. F. MCCREA: 1946, Austr. J. exp. Biol. med. Sci. **24**, 277.

BURNET, F. M., J. F. MCCREA and J. D. STONE: 1946, Brit. J. exp. Pathol. **27**, 228.

BURNET, F. M., J. F. MCCREA and S. G. ANDERSON: 1947, Nature **160**, 404.

BURNET, F. M.: 1947, Austr. J. Sci. **10**, 21.

BURNET, F. M. and J. D. STONE: 1947, Austr. J. exp. Biol. med. Sci. **25**, 227.

BURNET, F. M.: 1948*a*, Lancet, Jan. 3, 7.

BURNET, F. M.: 1948*b*, Austr. J. exp. Biol. med. Sci. **26**, 71.

BURNET, F. M.: 1948*c*, Austr. J. exp. Biol. med. Sci. **26**, 389.

DAVOLI, R. and E. PARDI: 1941, Sperimentale **95**, 493, 609.

DINGER, J. E. and H. L. WOLFF: 1948, Antonie van Leeuwenhoek, **14**, 145.

DREGUSS, M.: 1942, Arch. Virusforsch. **2**, 3, 306.

DUDGEON, J. A., H. MELLANBY, R. E. GLOVER and C. H. ANDREWES: 1948, Brit. J. exp. Pathol. **29**, 132.
EATON MONROE, D. and H. E. PEARSON: 1940, J. exp. Med. **72**, 635.
EATON MONROE, D.: 1941, J. Immunol. **41**, 382.
EDNEY, M.: 1949, Austr. J. exp. Biol. med. Sci. **27**, 253.
FRANCIS, TH.: 1934, Science **80**, 457.
FRANCIS, TH. and T. P. MAGILL: 1935, J. exp. Med. **62**, 505.
FRANCIS, TH. and T. P. MAGILL: 1938, Brit. J. exp. Path. **29**, 284.
FRANCIS, TH.: 1940, Science **92**, 405.
FRANCIS, TH. and A. E. MOORE: 1940, J. exp. Med. **72**, 717.
FRANCIS, TH., J. E. SALK and W. M. BRACE: 1946, J.A.M.A. **131**, 277.
FRANCIS, TH.: 1947*a*, J. exp. Med. **85**, 1.
FRANCIS, TH.: 1947*b*, Proc. Soc. Exp. Biol. Med. **65**, 143.
FRANCIS, TH., J. E. SALK and J. J. QUILLIGAN: 1947, Am. J. Pub. Health **37**, 1013.
FRIEDEWALD, W. F.: 1944, J. exp. Med. **79**, 633.
FRIEDEWALD, W. F., E. S. MILLER and L. ROSS WHATLEY: 1947, J. exp. Med. **86**, 65.
FULTON, F. and K. R. DUMBELL: 1949, J. gener. Microbiol. **3**, 97.
GREEN, R. H. and D. W. WOOLLEY: 1947, J. exp. Med. **86**, 55.
HIRST, G. K.: 1941, Science **94**, 22.
HIRST, G. K.: 1942*a*, J. exp. Med. **75**, 49.
HIRST, G. K.: 1942*b*, J. exp. Med. **76**, 195.
HIRST, G. K. and E. G. PICKELS: 1942, J. Immunol. **45**, 273.
HIRST, G. K.: 1943, J. exp. Med. **78**, 407.
HIRST, G. K.: 1947*a*, J. exp. Med. **86**, 357.
HIRST, G. K.: 1947*b*, J. exp. Med. **86**, 367.
HIRST, G. K.: 1948*a*, J. exp. Med. **87**, 301.
HIRST, G. K.: 1948*b*, J. exp. Med. **87**, 315.
HOYLE, L.: 1945, J. Hygiene **44**, 170.
KALTER, S. S., O. D. CHAPMAN, D. A. FEELEY and S. L. MACDOWELL: 1948, J. Immunol. **59**, 147.
LENNETTE, E. H. and F. L. HORSFALL: 1941, J. exp. Med. **73**, 581.
LÖFSTRÖM, G.: 1949, Acta Med. Scand. **133**, 162 and 253.
LOOSLEY, C. G., J. SCHOENBERGER and G. BARNETT: 1948, J. Lab. clin. Med. **33**, 860.
MAGILL, T. P. and TH. FRANCIS: 1936, Proc. Soc. Exp. Biol. Med. **35**, 463.
MAGILL, T. P. and TH. FRANCIS: 1938, Brit. J. exp. Path. **19**, 273.
MAGILL, T. P.: 1940, Proc. Soc. Exp. Biol. Med. **45**, 162.
MCCLELLAND, L. and R. HARE: 1941, Canad. Pub. Health J. **32**, 530.
MCCREA, J. F.: 1946, Austr. J. exp. Biol. med. Sci. **24**, 283.
MULDER, J.: 1940, Acta Med. Scand. **104**, 81.
MULDER, J. and L. BIJLMER: 1941, Arch. Virusforsch. **2**, 2, 213.
MULDER, J. and W. GOSLINGS: 1948, Acta Med. Scand. **131**, 431.
MULDER, J. and J. van der VEEN: 1948, Antonie van Leeuwenhoek **14**, 184.
MULDER, J., J. van der VEEN, L. M. BRANS and S. W. ENSERINK: 1949*a*, Antonie van Leeuwenhoek, in press.

MULDER, J., J. van der VEEN, L. M. BRANS and S. W. ENSERINK: 1949b, Antonie van Leeuwenhoek **15**, 125.
PATOCKA, F.: 1939, Tsjech. Aertzebl. 875.
RASMUSSEN, A. F., J. C. STOKES and J. E. SMADEL: 1948, Am. J. Hygiene **47**, 142.
VAN RAVENSWAAY, A. C.: 1948, J.A.M.A. **136**, 435.
RICKARD, E. R., M. THIGPEN and J. H. CROWLEY: 1944, J. Immunol. **49**, 263.
VAN RIEMSDIJK, M.: 1917, Ned. T. v. Gen., I, 517.
SALK, J. E.: 1944, J. Immunol. **49**, 87.
SARTWELL, PH. E. and A. P. LONG: 1948, Am. J. Hygiene **47**, 135.
SIGEL, M. M., F. W. SHAFFER, M. WIENER KIRBER, A. B. LIGHT and W. HENLE: 1948, J.A.M.A. **136**, 437.
SMADEL, J. E.: 1947, Bull. U.S. Army Med. Dept. **7**, 795.
SMITH, W., C. H. ANDREWES and P. P. LAIDLAW: 1933, Lancet II, 66.
SMITH, W. and C. H. ANDREWES: 1938, Brit. J. exp. Path. **19**, 293.
SMITH, W. and M. A. WESTWOOD: 1949, Brit. J. exp. Path. **30**, 48.
SMORODINTSEV, A. A., A. I. DROBYSHEVESKAYA and O. I. SHISHKINA: 1936, Lancet II, 1383.
STONE, J. D.: 1947*a*, Nature **159**, 780.
STONE, J. D.: 1947*b*, Austr. J. exp. Biol. med. Sci. **25**, 137.
STUART HARRIS, C. H.: 1939, Lancet I, 497.
STUART HARRIS, C. H., W. SMITH and C. H. ANDREWES: 1940, Lancet I, 205.
STUART HARRIS, C. H.: 1943, Brit. J. exp. Path. **24**, 33.
STUART HARRIS, C. H., R. E. GLOVER and K. C. MILLS: 1943, Lancet II, 790.
STUART HARRIS, C. H. and M. H. MILLER: 1947, Brit. J. exp. Path. **28**, 394.
SVEC, F. A. and G. F. FORSTER: 1947, Proc. Soc. Exp. Biol. Med. **66**, 20.
TAYLOR, R. M. and M. DREGUSS: 1941, J. infect. Dis. **68**, 79.
TAYLOR, R. M.: 1949, Am. J. Pub. Health **39**, 171.
WHITMAN, L.: 1947, J. Immunol. **56**, 167.
WOOLLEY, D. W.: 1949, J. exp. Med. **89**, 11.
ZWART VOORSPUY, A.: 1949, Experientia, in press

GRAPH 1

Titres of sera of ferrets, inoculated with influenza virus strains (A, A-prime and B), against the reference strains WS, PR8, A(1941 Ned), Barratt and Rhodes

Ferret antisera	Virus Strains [1])						
	Homologous (Lee) (Swine)	WS 1933 E	PR8 1934 USA	A 1941 Ned	Homologous	Bar 1947 E	Rhodes 1947 USA
Lee(1940 USA)	7						
Swine-15(1930 USA)	9	3	1			6	
WS(1933 E)		9	6	5			4
PR8(1934 USA)		2	11	10			1
Phila(1935 USA)		1	11	8	10		
Melbourne(1935 Au)			11	8	9		
Christie(1937 E) FME_6		3	8	9	9		1
Talmey(1937 E) FME_1		2	10	10	10		
Gatenby(1937 E) FME_7		3	6	9	7		
Gatenby(1937 E) [2]) FME_{30}		6	11	11	10	2	6
Burr(1937 E)		4	9	9	9	3	6
Bos(1939 Ned) [3])			7	6	Lost		
A(1941 Ned)		4	10	11		3	6
Sl(1941 Ned)		1	10	11	11	3	3
Sch(1941 Ned) *ferret-egg*		4	8	9	9	4	4
Sch(1941 Ned) *ferret-egg-mouse-egg*			7	9	11		
Weiss(1943 USA)		1	10	9	11		5
965(1943 USA)			5	9	9		
Hemsbury(1943 E)			9	11	10		
A128(1943 Sw)			7	8	9	3	3

Ferret antisera	Virus Strains [1])					
	WS 1933 E	PR8 1934 USA	A 1941 Ned	Bar 1947 E	Rhodes 1947 USA	Homologous
Cam(1946 Au)	4	5	8	8	10	9
Gg(1941 Sw)			2	6	6	8
Barratt(1947 E) *egg*			6	10	10	
Barratt(1947 E) *egg-mouse*			5	7	8	10
Kunz(1947 E)		2	3	8	8	10
Woiteki(1947 E)			3	8	8	5
K(1947 Ned) *ferret-egg*				7	5	6
K(1947 Ned) *ferret-egg-mouse-egg*	2	3	5	7	7	9
FM1(1947 USA)		2	3	7	8	9
Rhodes(1947 USA)	4	2	4	7	10	
1236(1947 USA)			4	9	9	8
1265(1948 USA)		2	5	8	8	11
FJS(1948 Au)				6	6	6
A(I/1949 Ned)				6	7	4
Heer(1949 Ned) *egg*			3	9	9	9'
Heer(1949 Ned) *egg-mouse-egg*	5	7	6	6	10	10
Hess(1949 Ned)				7	8	9
Hof(1949 Ned)				6	8	7
Vr(1949 Ned)			3	7	8	5
Wagt(1949 Ned)				5	7	6

[1]) The serum dilutions are expressed by figures. The figures are the expression of the titres and correspond with the following table:

0 = < 12	6 = 288— 576
1 = 12— 18	7 = 576— 1152
2 = 18— 96	8 = 1152— 2304
3 = 36— 72	9 = 2304— 4608
4 = 72—144	10 = 4608— 9216
5 = 144—288	11 = 9216—18432

[2]) The anti-GATENBY serum, made from a higher egg-passage number, shows a higher homologous titre and some overlapping with A-prime reference strains.

[3]) Strain lost during the war.

GRAPH 2

Cross-haemagglutination inhibiiion test with influenza strains [1])

Ferret antisera	Virus Strains [2])															
	Lee 1940 USA	Swine-15 1930 USA	WS 1933 E	PR8 1934 USA	Tal 1937 E	Christ 1937 E	Gat 1937 E	Burr 1937 E	A 1941 Ned	Sl 1941 Ned	Gg 1947 Sw	Bar 1947 E *egg*	Bar 1947 E *mouse*	K 1947 Ned *egg*	K 1947 Ned *mouse*	Rhodes 1947 USA
Lee(1940 USA)	7															
Swine-15(1930 USA)		9	3	1		1		2			4	6	1	1		
WS(1933 E)		3	9	6		6	3	8	5	2					1	4
PR8(1934 USA)			2	11	6	10	7	9	10	6	3		2			1
Talmey(1937 E) FME_1			2	10	10	11	6	10	10	8						
Christie(1937 E) FME_6			3	8	5	9	6	9	9	6			3			
Gatenby(1937 E) FME_7			3	7	1	5	7	8	9	9						
Gatenby(1937 E) FME_{30}			6	11	5	10	10	10	11	8		2			5	5
Burr(1937 E)		1	4	9	5	8	7	9	9	6		2	3			6
A(1941 Ned)			4	10	7	9	5	8	11	9		3	5		3	6
Sl(1941 Ned)			1	10	7	11	7	9	11	11	5	2	3			3
Gg(1947 Sw)		2			3				2		8	6	8	3	4	6
Barratt(1947 E) *egg*		3			2			1	6	1	6	10	11	4	8	10
Barratt(1947 E *egg-mouse-egg*					4			2	5			7	10		4	8
K(1947 Ned) *ferret-egg*											5	7	9	6	8	5
K(1947 Ned) *ferret-egg-mouse-egg*			2	3		4	3		5	1	2	7	8	5	9	7
Rhodes(1947 USA)			4	2		4	2	3	4		4	7	9	3	7	10

[1]) All titrations have been performed with the same sera, chicken red cells and the same egg fluid of the virus strains, except the titration of the second antiserum against GATENBY(1937 E) FME_{30} and the cross-titrations with BURR(1937 E) and LEE(1940 USA).

[2]) The serum dilutions are expressed by figures. The figures are the expression of the titres and correspond with the following table:

0 = < 12	6 = 288— 576
1 = 12— 18	7 = 576— 1152
2 = 18— 36	8 = 1152— 2304
3 = 36— 72	9 = 2304— 4608
4 = 72—144	10 = 4608— 9216
5 = 144—288	11 = 9216—18432

STELLINGEN

I

Voor een onderzoek van de antigene structuur van influenza-virus-stammen met behulp van de haemagglutinatie-remmings-proef is het noodzakelijk, dat de niet-specifiek remmende stoffen in de sera verwijderd worden.

II

De thans geïsoleerde menselijke influenza-A-stammen behoren op grond van hun antigene structuur tot 3 verschillende sub-groepen.

III

Interne Geneeskunde als specialisme bestaat niet meer, maar zij is opgebouwd uit verschillende orgaan-specialismen en heeft als zodanig een overkoepelende taak.

IV

Voor een onderzoek van faeces op Salmonellae en Shigellae is het noodzakelijk, dat gekweekt wordt op meer dan 2 verschillende voedingsbodems.

V

Het verdient aanbeveling de plattelandsbevolking in Nederland te vaccineren tegen tetanus.

VI

Er bestaat reden te twijfelen aan de geldigheid van de waarnemingen, dat het oplosbare antigeen van pneumococci type I (het oplosbare antigeen van Dubos) antilichamen opwekt, die in staat zijn muizen te beschermen tegen heterologe typen pneumococci.

VII

De door Pfeifer beschreven arteriën in de hersenen van zoogdieren zijn in werkelijkheid venen en de door hem beschreven venen zijn arteriën.

VIII

De verandering van de huid-weerstand bij de psychogalvanische reflex is voornamelijk een gevolg van een wijziging in de toestand der huidvaten.

IX

Indien verdenking bestaat op simulatie van nachtblindheid, is het wenselijk aan de hand van het electroretinogram tot een definitieve uitspraak te komen.