A STUDY OF INFLUENZA VIRUSES AND THE IMPLICATIONS IN REGARD TO THE EPIDEMIOLOGY AND PREVENTION OF INFLUENZA

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I. INTRODUCTION

The work on which this thesis is based was carried out partly in 1947-8 in the University Department of Medicine, Sheffield, under Professor C.H.Stuart-Harris, partly in 1948-50 in the Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia, under Sir Macfarlane Burnet, and mainly during the past 3 years in the World Influenza Centre, The National Institute for Medical Research, Mill Hill, under Dr. C.H. Andrewes. Many of the ideas in this thesis were not originally my own but came from the writings and conversations of my teachers; if I have borrowed too freely it is because of the compelling logic of their ideas rather than from a conscious plagiarism.

Until 1947, study of the influenza viruses as epidemic agents was carried out independently in a number of different countries. In view of the rapid way in which influenza apparently spreads across countries and even continents, it became obvious that only by study on an international basis could accurate information be obtained about the origin and spread of influenza epidemics. As a result the World Health Organisation set up a structure consisting of a chain of laboratories (Regional Influenza Centres) throughout the world, a World Influenza Centre in London under the direction of Dr. C.H. Andrewes, and an information service in Geneva.

During an influenza epidemic the Regional Laboratories try to isolate influenza virus strains which are then sent as quickly as possible to the World Influenza Centre where serological comparisons are carried out. The World Influenza Centre also maintains contact with an analogous Strain Study Centre for the Americas in New York under the direction of Dr. T.P. Magill. I have been fortunate enough to be responsible for the laboratory work of the World Influenza Centre and for the training of visiting workers during the past 3 years, and this has given me a good opportunity to study epidemic influenza from a most favourable vantage point. For this and for much more I am most grateful to all my teachers and particularly to Dr. Andrewes. I am also most grateful to Dr. T.P. Magill, with whom I had many helpful discussions during a visit to his laboratory in New York under the auspices of the World Health Organisation.

II. TECHNIQUES OF STUDY

Methods of Antigenic Analysis of Influenza Virus Strains

The agglutination-inhibition test

Many of the arguments which follow rest on antigenic similarities or differences found in strains of influenza virus. It seems worth while, therefore, to give a critique and a justification of the technique of antigenic analysis chosen.

Many techniques of antigenic analysis of influenza viruses are available. They can be broadly divided into:

(a) *in vivo* neutralisation tests and

(b) *in vitro* techniques.

In vivo neutralisation tests measure virus-neutralising antibodies, and for particular problems they cannot be supplanted by *in vitro* techniques. Virus-serum neutralisation tests in the mouse lung, the allantoic cavity of the chick embryo or in surviving fragments of chorio-allantoic membrane in tissue culture are useful for detailed antigenic analysis of small numbers of strains. They are impractical, however, for antigenic analysis of the large numbers of strains required in epidemiological studies. Also, neutralisation tests tend to be more specific than the *in vitro* tests and thus the broad similarities between strains may be obscured by fine differences.

In vitro tests are more practical for the present purpose. The choice lay between the complement-fixation test using virus elementary bodies and the haemagglutination-inhibition test.

The complement-fixation technique is not very specific and although it allows strain differentiation between the major antigenic types and subtypes of influenza viruses (Fulton & Dumbell, 1949) it does not clearly distinguish minor antigenic groupings (Chu, Andrewes & Gledhill, 1950; Isaacs, Gledhill & Andrewes, 1952). The agglutination-inhibition test does distinguish minor antigenic groupings and since it is technically very simple and can be carried out rapidly, it can be readily adapted to comparisons of large numbers of strains. These advantages made the agglutination-inhibition test the method of choice. However, the apparent simplicity of the test is offset by difficulties in interpreting the results, and these are in turn linked to the necessity for attending to many technical details. Two major technical arrangements and a number of minor details required to be settled before any uniformity could be expected of the haemagglutination-inhibition test as a tool for antigenic analysis.

Inhibitors of virus agglutination in normal animal sera: Since the earliest descriptions of agglutination-inhibition tests (Hirst, 1942) it was known that the sera of normal animals may inhibit agglutination by influenza viruses. Obviously difficulties in the interpretation of the results of agglutination inhibition tests would be increased enormously unless one could be certain of treating the sera in some way which would abolish the effects of normal inhibitors of agglutination without

affecting specific antibody titres.

This problem was complicated by conflicting reports and confusing nomenclature in the literature about normal serum inhibitors of agglutination. However, it now appears that the inhibitors in normal sera can be broadly divided into two classes.

(a) Some sera, notably those of the ferret and fowl, contain a heat-stable (100°C. for 15 min.) inhibitor which is particularly active against strains of influenza B heated at 56°C. for 30 minutes (Francis, 1947) and is inactivated on incubation with influenza viruses and with purified receptor-destroying enzyme (RDE) of V. cholerae (Anderson, 1948).

(b) Some sera, particularly those of the rabbit, guinea-pig and mouse contain a heat labile inhibitor which is particularly active against unadapted but not mouse-adapted lines of influenza A(Chu, 1951). It is not inactivated on incubation with influenza viruses and with purified RDE.

The former type of inhibitor is generally known as Francis inhibitor and the latter has been called Chu inhibitor, after the names of the workers who first pointed out the important characters of each. These names probably correspond to the α and β inhibitors in rabbit serum described by Smith, Westwood and Belyavin (1951). Sampaio (1952) has described the distribution of the two inhibitors in normal animal sera.

The first practical method of removing these inhibitors

from animal sera was the use of crude extracts of V. cholerae (Mulder, van der Veen, Brans & Enserink, 1949). At that time the two types of inhibitor had not been clearly distinguished and they were probably linked together under the title "non-specific inhibitors". Van der Veen and Mulder (1950), Appleby and Stuart-Harris (1950) and Chu, Andrewes and Gledhill (1950) confirmed the usefulness of crude V. cholerae extracts and concluded from their separate experiments that the active factor in V. cholerae filtrates was not the receptor-destroying enzyme (RDE).

Crude V. cholerae filtrates were obviously very imperfect tools of investigation however, since their mode of action was completely unexplained and there was no means of standardising the potency of different batches of filtrate. We therefore investigated the properties of the unknown factor in V. cholerae extract using ferret serum as a source of normal inhibitor; ferret serum was chosen by us and by earlier workers since anti-sera for routine typing of strains in this laboratory had always been prepared in ferrets. It was surprising to find that the properties of the unknown factor resembled very closely those of RDE, and in fact, when the hydrogen-ion concentration was controlled at pH 6.2, purified RDE was highly active in destroying the inhibitor in ferret serum (see Table 1). We concluded that the action of crude V. cholerae filtrates on ferret serum inhibitor was due to its RDE content (Isaacs &

TABLE I

Action of purified RDE on Francis inhibitor and non-specific inhibitor in normal ferret serum

RDE dilution	Inhibitory Titre	
	Francis Inhibitor	Non-specific Inhibitor
1:1	<50	<10
1:16	<50	<10
1:256	100	15
Nil	4800	80

Francis inhibitor was tested against heated and non-specific inhibitor against unheated influenza B virus.

Bozzo, 1951). This finding has been confirmed by Murphy (1952) who believes that the mistaken conclusion of the earlier workers was due to their neglecting the control of pH.

This work, and the conclusions reached, referred only to ferret serum which contains mainly Francis inhibitor (Sampaio, 1952). However, Professor Mulder told us that purified RDE had no action on rabbit serum inhibitor but that crude V. cholerae extracts were active (see also Chu, 1951). It seemed odd that V. cholerae extracts should contain two factors active against two different types of inhibitor and at first it was suspected that some factor such as pH or concentration of a particular ion was involved, although this did not prove to be the case. Professor Mulder sent us a sample of crude V. cholerae extract which was very active against rabbit serum inhibitor, and this was used for investigating the second active component of V. cholerae extracts. The active factor was found to be heat-labile, to be precipitated by half saturation with ammonium sulphate and to be adsorbed after filtration through collodion membranes. These preliminary observations suggested that the second component might be macro-molecular, possibly protein, and to test this possibility further the V. cholerae filtrate was incubated with trypsin. At first this experiment appeared to give a negative result but when a control test with trypsin

TABLE II

Effect of trypsin on crude V. cholerae extract

Material	Treatment	Inhibitory titre of rabbit serum
Saline plus rabbit serum	nil	384
" " " "	Heated 56°C. for 1 hour	192
V. cholerae plus rabbit serum	Heated 56°C. for 1 hour	<12
Trypsin-treated *V. cholerae* plus rabbit serum	Heated 56°C. for 1 hour	<12
Trypsin plus rabbit serum	Heated 56°C. for 1 hour	<12

Inhibitory activity was assayed against 8 agglutinating doses of A/Eng/1/51 virus.

alone was included it was found that the trypsin itself was highly active on rabbit serum inhibitor (see Table LI). It was known that V. cholerae filtrates contained a trypsin-like enzyme (Stone, 1949) and we found that the factor in crude V. cholerae extracts active against rabbit serum inhibitor was similar to trypsin in many of its properties. We concluded that the factor in crude V. cholerae extracts active against the Chu inhibitor in rabbit serum was probably a trypsin-like enzyme (Sampaio & Isaacs, 1953). We also found that crystalline trypsin was highly active in destroying both Francis and Chu inhibitors in ferret, fowl, rabbit, mouse and guinea-pig sera under conditions where specific antibody titres were unaffected. It seems possible that the Chu inhibitor is a protein and there is good evidence that the Francis inhibitor is a mucopolysaccharide. Trypsin is presumably active against the protein molecule of Chu inhibitor and the protein moiety of the mucoprotein molecule of Francis inhibitor whereas RDE probably acts on the carbohydrate prosthetic groups (Gottschalk, 1952) of the mucopolysaccharide molecule. Crude V. cholerae extract is probably active on inhibitors in different animal sera as a result of both its RDE and its trypsin-like enzyme. However, crude V. cholerae extracts are not easy to prepare and they are liable to contain variable amounts of the two enzymes. Also, the trypsin-like enzyme attacks the RDE enzymically

(Stone, 1949) and thus the enzymic composition of the V. cholerae extracts becomes altered on storage. Crystalline trypsin can be obtained commercially and its inhibitor destroying activity against each of the above animal sera is known on the basis of the weight of enzyme required/ml. of serum (Sampaio & Isaacs, 1953). It is also very simple to use - it is necessary to mix serum and trypsin and heat at 56°C. for 30 minutes only; the heat inactivates both the serum and the trypsin and the serum is then ready for immediate use. Trypsin is likely to replace V. cholerae extract in antihaemagglutinin tests and we have found it to be a most satisfactory agent for use in routine tests.

The use of antisera prepared in different animals: The second main factor which required attention in the technique of the haemagglutination-inhibition technique was the type of animal to be used for preparing antisera. In this country and in many European laboratories it has been the practice to use ferrets for preparing antisera, following on the discovery that these animals were susceptible to infection with influenza viruses (Smith, Andrewes & Laidlaw, 1933). In other laboratories particularly in the United States of America, sera are frequently prepared by immunising rabbits or fowls, presumably because of the difficulty of keeping ferrets in isolation. *A priori* it might be expected that sera from animals convalescent from influenza would react differently from sera from immunised

animals and this has proved to be the case. There is some evidence that sera from animals immunised with influenza virus show cross reactions with extracts of the virus host cells, i.e. normal chick embryo tissues(Anthanaryan, 1953) and on theoretical grounds, therefore, post-infection sera are to be preferred. In practice, we found that two subtypes of influenza virus A, the Liverpool and Scandinavian (1951) subtypes, could be readily differentiated by ferret sera but not by fowl sera (Isaacs, Gledhill & Andrewes, 1952), a finding which agrees with that of Hilleman (1951). Various animal sera were then compared by Sampaio (1952) who concluded that post-infection sera from the ferret or hamster gave more specific results than immune sera from the fowl, rabbit, guinea-pig or mouse. It seems therefore that theoretical and experimental arguments support the use of the ferret for preparing antiserum. Dr. Magill finds, however (personal communication) that hyperimmune rabbit sera show reactions which are broadly similar to those of ferret sera.

Technique of the test: The agglutination-inhibition test carried out in our laboratory is a pattern test in plastic plates. Plastic plates are preferred to test tubes because of their uniformity and ease of handling. Serial dilutions of trypsin treated serum are prepared in saline in bulk and then distributed in 0.25 ml. amounts. An equal volume of red cell suspension is then added followed by a third volume of

virus. The cells are allowed to settle and the results read by the pattern of sedimented cells. Owing to unavoidable variations in the technical conditions of the test absolute agglutination or agglutination-inhibition titres may vary slightly from day to day. However relative titres of two sera or two viruses are largely independent of the factors causing day to day variation and in the results reported here only titres relative to those of controls tested at the same time are considered.

The red cells were used as a 0.5 per cent (v/v) suspension of washed fowl cells in saline calibrated in a photo-electric densitometer. The fowls chosen for bleeding were animals whose cells were known to be insensitive to agglutination by vaccinia virus (Stone, 1946) and of uniform sensitivity to inhibitors of influenza virus (Anderson, Burnet & Stone, 1946). The viruses were diluted in saline to a concentration of 8 agglutinating doses per 0.25 ml., usually titrated in advance of the main test. The source of virus for the tests was generally allantoic fluid from fertile eggs inoculated with influenza virus via the allantoic route by conventional techniques (Beveridge & Burnet, 1946).

Limit dilution technique

In some experiments it was desirable to test the homogeneity of preparations of influenza virus or to separate the components of mixtures of two viruses or to use virus preparations

which had a high probability of being pure in the bacteriological sense. These processes can be easily carried out with bacteria but not with viruses. The technique used was to inoculate eggs with virus at limit dilution, i.e. at a dilution at which the majority of eggs inoculated are not infected. Under these conditions there is a probability that in some of the infected eggs infection may have been initiated by a single infective dose so that a pure virus culture will result. The technique has in a number of instances been found to be successful in separating the components of a mixture (Isaacs & Edney, 1950 a, b; Isaacs, Gledhill & Andrewes, 1952). Nevertheless, caution is required in the use of this technique (Liu & Henle, 1953) particularly in view of the recent demonstration (Donald & Isaacs, 1954) that roughly 10 influenza virus particles correspond to one infective dose.

Growth of virus in eggs with antiserum

Experiments were carried out to test the effect of growing strains of influenza virus in the presence of homologous antiserum. A useful technique was evolved for adapting virus to grow in the presence of large amounts of antiserum (Isaacs, Gledhill & Andrewes, 1952). A large dose of virus was mixed with antiserum at a dilution which was just insufficient to prevent growth of virus (e.g. one half the minimal neutralising dose of serum). This was inoculated allantoically into eggs and after a few hours,

further antiserum was added and further injections, of up to 50 neutralising doses of serum, were continued for the next 24 hours. The virus recovered after 3 days was found to be highly resistant to the action of antiserum.

Antibody absorption

Friedewald (1944) and Hirst (1952) have studied sera which they had absorbed with influenza virus by a complicated technique involving high-speed centrifugations and ultra-filtration. Recently, Fiset and Donald (1953) in this laboratory have developed a much more simple technique in which virus firmly bound to chick red cells is used for absorbing the sera. Dr. Fiset and I have carried out a number of tests with sera absorbed by virus with this technique.

Other techniques are mentioned in the text.

III. ANTIGENIC STUDIES OF INFLUENZA VIRUSES

Antigenic Variation in Influenza Viruses

The antigenic variability of influenza viruses and particularly of the influenza A viruses is a great disadvantage in populations exposed to influenza and to those who are anxious to prepare influenza virus vaccines. It is of assistance in investigating the spread of influenza viruses however, since the distinctive antigenic characters of influenza viruses isolated in different epidemics serve as markers. Within a few years of the first isolation of influenza virus (Smith, Andrewes & Laidlaw, 1933) antigenic differences were detected in strains of influenza virus recovered from different epidemics (Magill & Francis, 1936; Andrewes, 1937; Smith & Andrewes, 1938), although the strains still showed a considerable antigenic overlap. The identification of strains of influenza B (Francis, 1940; Magill, 1940) and of the recently described influenza C (Taylor, 1949; Francis, Quilligan & Minuse, 1950) has drawn attention to viruses which appear to be pursuing independent courses from the influenza A viruses, both antigenically and epidemiologically. The influenza A viruses are antigenically much more variable than the others and they appear to be much more important epidemiologically. There is a great deal of evidence to support Hirst's (1943) contention that influenza A viruses recovered from a single epidemic and grown in eggs (in which they tend to remain antigenically stable) are antigenically

fairly homogeneous and differ from virus strains recovered in successive epidemics (van der Veen & Mulder, 1950; Hilleman, 1952; Magill & Jotz, 1952; Isaacs, Gledhill & Andrewes, 1952). Thus the picture which emerges is that of influenza A viruses showing progressive antigenic modification with the steady emergence of new serological patterns to replace the older ones which are gradually left behind.

Laboratory Contamination by Influenza Viruses

Before this picture can be accepted it is necessary to digress from the main theme in order to refer to the occasional reports of recoveries of strains of influenza virus which had been prevalent 10-20 years earlier. Should these reported isolations be genuine they would make it necessary to modify greatly the picture which has been described in the preceding paragraph. Accordingly, the reported isolations must be examined very critically before their authenticity is accepted. The striking characteristic which seems to be common to these reported isolations is that the viruses recovered show properties associated with well-adapted laboratory strains rather than those of recently isolated strains. In addition the strains "isolated" have always been found to be identical with a stock strain carried in that laboratory and recently under investigation and therefore the possibility of laboratory contamination requires consideration.

While laboratory contamination of bacteriological plates

is accepted as a commonplace there seems to be a widespread resistance among many virologists to the idea that laboratory contamination with viruses can occur. Yet a drop of infected allantoic fluid may contain as many as 10^9 infective doses of influenza virus and in many laboratories isolation of influenza viruses is attempted in the same room as serological tests with highly infective allantoic fluid. Further, there are a number of well-authenticated cases where contamination has been shown to occur (Andrewes, Glover, Himmelweit & Smith, 1944). Professor J. Mulder has told me that during attempts to isolate influenza viruses in his laboratory saline "blanks" are included as controls and that on two occasions influenza virus was recovered from the "blanks". We have recovered Lee strain of influenza B from a supposedly pure culture of influenza A, strain PR8, by growing the strain in the presence of PR8 antiserum. There was quite a small proportion of Lee virus present in the culture, insufficient to be detected by routine antigenic tests, and we were unable to determine whether the contamination had occurred in our laboratory or in the New York laboratory which had sent us the strain. It seems clear therefore, that unless the most rigid precautions are taken and perhaps even in spite of rigid precautions, laboratory contamination with influenza viruses can occur. It is necessary therefore, to consider the factors which distinguish a recently isolated from a laboratory-adapted strain and which might help to decide whether an isolation was

probably genuine or not.

O-D Variation: Burnet and Bull (1943) described unusual properties of freshly isolated influenza A virus (O, or original phase) which distinguished it from the same strain after further cultivation in eggs (D, or derived phase). O and D phases differ in a number of respects but are most readily differentiated by their behaviour in agglutination tests with fowl and mammalian (human or guinea-pig) cells. O phase virus shows a low titre or absence of agglutination with fowl cells but a high titre with guinea-pig cells (low F/G ratio) whereas D phase virus shows the same agglutinin titre with both types of cells (F/G ratio of 1). The change of O to D virus is believed to be a discontinuous mutation and Burnet and Stone (1945) have described intermediate stages between the O and D phases. Magill and Sugg (1948) disagreed and suggested that O phase virus could be changed to D phase virus *in vitro* by altering the pH and ionic constitution of the medium and hence that the O-D change was not a true mutation. However Burnet, Stone, Isaacs and Edney (1949) showed that the techniques used by Magill and Sugg were successful only with intermediate forms of virus which superficially resemble O phase virus, but had no effect on true O phase virus. It is generally accepted that if virus is recovered in the O phase it represents a genuine isolation.

Pathogenicity for mice: Strains of influenza virus which have been isolated and maintained in eggs are non-pathogenic in mice,

i.e. they multiply in mice but do not produce significant pulmonary consolidation, or death, until they have been adapted by serial growth in mice.(e.g. Hirst, 1947). Thus 26 strains of virus isolated in eggs in the 1950-51 European influenza epidemic were tested and all found to be non-pathogenic (Isaacs, Gledhill & Andrewes, 1952). Strains isolated before 1941 when eggs came into use have all been passaged in mice and generally kill mice after intranasal inoculation in high dilution. Hence a strain of virus highly pathogenic for mice has presumably been previously passaged in mice in the laboratory.

Chu inhibitor: Chu (1951) found that an inhibitor present in normal mouse, guinea-pig and rabbit sera was highly active against unadapted but not against mouse-adapted strains of influenza virus. This test can be used to confirm the results of the direct pathogenicity test in mice.

Presence of virus filaments: Chu, Dawson and Elford (1949) drew attention to the presence of numerous filaments in addition to the usual round elementary bodies in allantoic fluid of eggs infected with recently isolated strains of influenza virus A. These filaments were absent or represented by scanty short rods in old laboratory strains.

We have had an opportunity of examining most of the strains thought to represent "recent isolations" of strains such as WS and PR8, which were originally isolated nearly 20 years ago. In every case the strains were in the D phase, pathogenic for

mice in high titre, showed no virus filaments and were serologically identical with a strain carried in the laboratory at the time the virus was "isolated" (Isaacs & Andrewes, 1951). This last point was an interesting one; thus substrains of PR8 virus carried in different laboratories may show very slight antigenic differences from one another and in every case the supposedly newly-isolated strain of virus showed the peculiar characteristics of the stock strain carried in that laboratory. There is no known example of a strain of virus recently isolated and serologically similar to WS or PR8 for example, but which is in the O phase or non-pathogenic for mice or shows numerous filaments or was isolated and studied at a time when a stock virus with identical properties was not present in the laboratory. One case which appeared at first to be an exception to this statement was that of the strains isolated from an epidemic of influenza among Eskimos in Northern Alaska. Van Rooyen, McClelland and Campbell (1949) and Nagler, van Rooyen and Sturdy (1949) reported that from cases in a severe outbreak of influenza among Eskimos they had recovered from the spleen, heart blood and other unusual sites, a number of strains of virus closely related serologically to the PR8 virus. Furthermore the strains were isolated independently by two investigating laboratories which had no direct contact with one another. As this seemed to be an important case, a detailed investigation was commenced and

Dr. Nagler was invited to the World Influenza Centre to assist. The first point which was established was that although the isolations were carried out in different laboratories, the garglings were first taken to one laboratory and there divided into aliquots; the opportunity for contamination to have occurred was thus established. The second point was that all strains showed the characters of old laboratory strains, i.e. they were in the D phase, highly pathogenic for mice (LD_{50} about 10^{-6}) and showed no filaments on electron microscopy. Thirdly, the strain of PR8 "isolated" was slightly different from our standard PR8 strain in its serological reactions with the heterologous WS antiserum but was identical with the stock PR8 strain carried in the Canadian laboratories (see Table III). Fourthly, it was stated by Dr. Nagler, and we confirmed his observation, that one of the strains which he had isolated was an A-prime virus serologically similar to many of the strains recovered from other parts of Canada and elsewhere at this time. The Eskimos themselves showed an antibody rise to an A-prime virus and to PR8, but a greater rise to the former - a reaction characteristic of infection with A-prime viruses. Finally, one of the strains which we attempted to recover again from early passage material was found to be Lee (influenza B) virus in Dr. Nagler's second amniotic passage material and a mixture of PR8 and Lee viruses at the third amniotic passage. The latter was shown by growing

TABLE III

Haemagglutination-inhibition titres of the Canadian and London lines of PR8 virus and an Arctic strain tested with different sera

VIRUS	SERUM				
	PR8(London)	WS	FM1	Weiss	Arctic strain (CAP 2)
London strain of PR8	5120	160	60	960	1280
Canadian strain of PR8	1920	20	20	960	960
Arctic strain CAP 2	2560	30	20	960	1920

the culture in the presence of Lee or PR8 serum when the unneutralised virus grew out. Dr. Nagler later confirmed that he had also recovered Lee virus from the cultures and since the patient in question had no serological response to influenza B it seems difficult to escape the conclusion that both the PR8 and Lee viruses were laboratory contaminants.

A priori, there is no reason why strains prevalent 20 years ago should not reappear today. However, our view (Isaacs & Andrewes, 1951) is that no clear evidence of this occurrence has yet been produced. It is not easy to say whether any single isolation is genuine or whether laboratory contamination has occurred but as more and more strains are tested the direction of the serological changes seems to point continually forwards and there is no definite evidence yet to suggest that the virus occasionally looks back.

Antigenic Characters of Influenza A Viruses

The property which characterises the influenza A viruses is that they all produce the same "soluble antigen", a product of virus growth which can be separated from the virus by high-speed centrifugation (Hoyle & Fairbrother, 1937) and which is detected by complement-fixation tests. At first sight the influenza A viruses themselves present a bewildering antigenic complexity. This is partly due to the fact that many of the earlier strains were passaged repeatedly in animals, a procedure which tends to induce antigenic alterations (e.g. Hirst, 1947)

and partly to the techniques of antigenic analysis for the study of influenza viruses, which are less precise than the corresponding techniques for bacteria.

Friedewald (1944) studied a number of strains with virus-absorbed sera and he concluded that different influenza A viruses contained distinct antigenic components. In a more extensive study of the same type Hirst (1952) concluded that the influenza viruses had group-specific and type-specific antigens and that quantitative variations in the proportions of these antigens would explain the observed antigenic variability. It is not yet clear whether there is only a single group-specific antigen for all the influenza A viruses, or what relationship the group antigen bears to the "soluble antigen". So far Hirst has identified some six or seven type-specific antigens and recently Jensen and Francis (1953) have described eighteen influehza A antigens.

Our studies at the World Influenza Centre have been concerned with the antigenic characters of these viruses in so far as they reflect the epidemiology of influenza. We have therefore been concerned with studying the antigenic characters of influenza viruses primarily as a means of following the spread of influenza. Thus, much of our work consists in comparing strains isolated in an epidemic with one another and with previously isolated strains using un-absorbed sera.

The tendency in the past has been to describe three major subtypes of influenza A virus, excluding the swine influenza viruses (World Health Organisation Expert Committee on Influenza, 1953). These subtypes, the WS, PR8 and the so-called A-prime subtype are clearly distinguishable and Table IV shows the relationships found in our tests.

The antigenic change which occurred in the influenza A viruses in 1946 appeared to be very much greater than those of earlier years and the new viruses were called A-primes. However, since 1946 antigenic changes have continued and Dr. Magill, in a letter to Dr. Andrewes, has suggested that it is inappropriate to talk of major subtypes such as A-prime when there is considerable variability within the subtype. His view seems reasonable and there is much to be said for grouping similar strains from one epidemic and for considering one group as distinct from a second epidemic group whether there is a big or a small antigenic difference between the two groups. Some evidence will be presented to show that this type of classification of strains has epidemiological significance.

Investigations of Major Influenza A Epidemics

The first international investigation of a major influenza epidemic was carried out following the 1948-49 influenza outbreak (Chu, Andrewes & Gledhill, 1950). Altogether 20 influenza A virus strains were studied and found to be serologically homogeneous and to be related to, but distinguishable

TABLE IV

Cross haemagglutination-inhibition tests with WS, PR8 and FM1 viruses

Virus	Year isolated	SERUM		
		WS	PR8	FM1
WS	1933	960	60	20
PR8	1934	70	5120	35
FM1	1947	<10	<10	1280

from earlier (1947) strains. Chu, Andrewes and Gledhill concluded that the 1949 European epidemic had a single focus of origin, probably in Sardinia, and that the virus spread from there to the mainland of Italy to involve most of Western Europe. They suggested, however, that a more complex situation might be found in later epidemics. Nevertheless, this investigation seemed to answer one question; it appeared that true country-to-country spread of virus did occur during a major epidemic.

After this epidemic no influenza A was detected until June 1950 when Dr. Svedmyr reported a small outbreak in Stockholm from which he recovered four strains. These strains were examined in the World Influenza Centre and found to be serologically homogeneous but recognisably different from earlier strains. Since influenza A epidemics in Europe tend to occur every second year (e.g. 1947, 1949, 1951, 1953), it seemed possible that this outbreak might herald a major epidemic in Europe in the following winter caused by this serologically "new" strain of virus. We therefore wrote to Dr. Svedmyr suggesting this possibility and asked him to see if influenza could be detected by virus isolations or serological tests in the remainder of the summer and autumn. Dr. Svedmyr carried out numerous serological tests and attempts at virus isolation but all were negative. In November 1950, a sudden outbreak of ihfluenza

was reported from Denmark and shortly afterwards from Sweden. The virus strains recovered were closely related to those found 5-6 months earlier in Stockholm (Isaacs & Andrewes, 1951). In addition, Dr. von Magnus reported (personal communication) that the Danish outbreak appeared to start almost simultaneously in numerous foci in small islands not in direct communication with one another. Our conclusion was that this represented what Dr. Andrewes has referred to as "underground" spread of virus, in which the spread of virus is conceived of as preceding the outbreak. It is assumed that the epidemic then broke out at a number of points simultaneously when some factor, possibly climatic, had come into operation. This mechanism is similar to that postulated by Shope (1943) for epidemics of swine influenza. Multifocal origins of influenza epidemics are not invariable but they occur quite frequently. One might conceive, alternatively, that the virus could produce numerous sub-clinical infections during a pre-epidemic period and thus become widely seeded, but there is no direct evidence for this so far.

This particular virus, which was called Scandinavian subtype, spread to a number of European countries, Finland, Iceland, Holland, Belgium, Germany and Italy, and some strains were recovered in England and Ireland. Altogether, 46 Scandinavian strains were studied and everywhere this virus seemed to cause widespread but mild influenza with

few fatalities. Towards the end of 1950, however, a severe outbreak occurred in the Liverpool area with a large number of deaths and this was found to be due to a second apparently new group of influenza A viruses, which was called the Liverpool subtype. Liverpool viruses were then recovered from a number of countries around the Mediterranean - Spain, France, Italy, Greece, Turkey and Israel and they also seem to have spread across the Atlantic to the U.S.A. and Canada. The Liverpool strains were compared with previously recovered viruses and were then found to be antigenically identical with a number of strains of virus causing influenza in the southern hemisphere in their 1950 winter, i.e. 6 months before the European epidemic. One strain from Australia and 6 from South Africa, as well as 6 from India, were of Liverpool subtype and it seems likely that this represents the source of the virus causing the epidemic in the northern hemisphere. The Liverpool viruses were serologically homogeneous (see Table V) but the Scandinavian viruses showed minor antigenic variations within the group, some of which are considered in a later section under P-Q variation. It seems therefore, that the 1950-51 epidemic in Europe had two sources, one from activation of latent virus in Scandinavia and the other by spread from the southern hemisphere. The evidence from this epidemic again favoured true country-to-country spread of influenza virus A. Thus, 47 strains of Liverpool subtype were recovered

TABLE V

Cross haemagglutination-inhibition tests

with A/Ankara/1/51 and A/England/1/51 viruses

VIRUS	SERUM	
	A/Ankara/1/51	A/England/1/51
A/Ankara/1/51	560	640
A/England/1/51	960	960

from this epidemic and since this particular serological variety of virus was not known before 1950, i.e. 6 months before, it seems reasonable to conclude that the Liverpool subtype resulted from antigenic variation on a single occasion followed by rapid spread of the new variant.

After the 1950-51 epidemic of influenza A there was an interval of nearly two years before the disease again flared up, this time without any summer outbreak to herald its arrival. Towards the end of 1952 large epidemics were reported to have broken out at about the same time in Japan, the U.S.A., and in some countries of Western Europe - Sweden, Holland and Portugal - and in early 1953 the epidemic reached France, Germany and this country. Within the next few weeks one country after another was affected until practically all of the northern hemisphere (excluding the U.S.S.R. and its neighbours, about which there is no accurate information) became involved. In Britain the southern part of England was most affected and the midlands and Scotland tended to be spared, the reverse of the experience during the 1950-51 epidemic. The incidence of cases was quite high generally, but the epidemic was everywhere mild, deaths occurring predominantly in those over 65 years of age.

Preliminary serological studies implicated influenza A viruses which were similar to 1950-51 Scandinavian viruses, but on further study a more complex situation was revealed.

Two groups of strains were identified, one similar to but not identical with Scandinavian 1950-51 strains, the second identical with 1950-51 Liverpool strains. The antigenic differences between these two groups are considered in some detail later (see Table XIII) and the distribution of the two groups is shown in Table VI. Perhaps the most striking finding in this Table is the distribution of the two groups of viruses in particular countries such as Portugal and Italy. From three Portuguese laboratories 19 strains of Liverpool virus were recovered while from six Italian laboratories 17 strains of Scandinavian (1953) virus were obtained. From the United Kingdom, only 2 Liverpool out of 86 virus strains were found and of these one is suspect since it came from a colleague who had been spraying A/England/1/51 (Liverpool) virus in the laboratory 2 days before, as part of an experiment on counting influenza virus particles. Distributions of this type suggest strongly that a single serological group or subtype of influenza A virus is spreading within a country. Yet the results also suggest country-to-country spread since Scandinavian (1953) virus strains recovered from Japan, the U.S.A., this country and elsewhere were also very similar to one another. Table VII shows the similarity between representative 1953 American and English strains and the relationship with Scandinavian and Liverpool 1950-51 strains.

TABLE VI

Distribution of 1952-53 Influenza virus A strains

Country	Serological Variety	
	Scandinavian	Liverpool
England	84	2
Scotland	7	0
Eire	1	0
U.S.A.	16	0
Canada	1	0
Japan	2	0
Germany	7	0
Holland	7	0
Belgium	1	0
Denmark	3	0
Finland	19	4
Iceland	2	0
Yugoslavia	2	0
Portugal	0	19
Italy	17	0
France	4	5
Switzerland	0	2
Sweden	1	0

TABLE VII

Comparison of A/Eng/1/53 and A/Missouri/303/52 viruses in agglutination-inhibition tests

VIRUS	SERUM			
	A/Swe/3/50 (Scandinavian)	A/Eng/1/51 (Liverpool)	A/Eng/1/53	A/Missouri/303/52
A/Eng/1/53	200	40	320	320
A/Missouri/303/52	160	35	320	320

The 1953 Scandinavian strains were slightly different from the 1950-51 Scandinavian strains and may have evolved from them. The relationship between the two is illustrated in Table VIII. Table VIII shows that the two strains are closely related when tested with A/Sweden/3/50 serum but not with A/England/1/53 serum. The A/Sweden/3/50 serum actually gives a higher titre with the heterologous than the homologous virus. This non-reciprocal type of relationship, which is quite commonly met with in the serology of influenza A viruses, will be discussed further in the next section. 1953 Liverpool viruses were found to be identical with those recovered 2 years earlier; a comparison of a 1951 with a 1953 Liverpool strain is illustrated in Table IX.

Hirst (1943) had suggested that strains recovered from a single epidemic are homogeneous but differ from strains obtained in different epidemics. This statement requires slight modification in view of the above results, since it appears that more than one serological group of viruses may be responsible for an epidemic, and that one serological subtype may persist without antigenic alteration for a period of at least $2\frac{1}{2}$ years. Any explanations of how epidemics of influenza A arise must take into account these facts:

(a) On two occasions, i.e. in the 1950-51 and 1952-53

TABLE VIII

Cross haemagglutination-inhibition tests between A/Swe/3/50 and A/Eng/1/53 viruses

VIRUS	SERUM A/Swe/3/50	SERUM A/Eng/1/53
A/Swe/3/50	100	20
A/Eng/1/53	240	320

TABLE IX

Cross haemagglutination-inhibition tests between A/Eng/1/51 and A/Lisbon/9/53 viruses

VIRUS	SERUM A/Eng/1/51	SERUM A/Lisbon/9/53
A/Eng/1/51	1920	2560
A/Lisbon/9/53	1280	2560

winters, epidemics caused by two recognisably different viruses started at about the same time.

(b) It is possible for a strain of influenza A virus to survive a 2-year inter-epidemic interval while undergoing either a slight antigenic alternation or no obvious change.

The inference is that whether or not strains underwent a slight antigenic alteration between epidemics the same factors favoured their producing an epidemic at about the same time.

The Influenza B Viruses

It has been stated that influenza B outbreaks tend to occur every 4-5 years in contrast with A outbreaks which occur every 2-3 years. Our experience at the World Influenza Centre has been different, however. It was interesting to note that in the middle of the 1948-49, 1950-51 and 1952-53 influenza A epidemics a number of scattered outbreaks due to influenza B occurred and a number of strains of influenza B were sent to us from widely separated places. In the intermediate years when influenza A outbreaks did not occur, there were again small scattered outbreaks of influenza B sometimes starting at about the same time in places not in direct communication with one another. Occasionally the outbreaks were quite severe, e.g. the Glasgow epidemic of 1952 (Anderson, Grist, Landsman, Laidlaw & Weir, 1953) but most of the outbreaks were mild and involved small numbers

of people relative to an influenza A epidemic.

Preliminary tests with strains recovered between 1950-52 suggested that the viruses were rather homogeneous and closely related serologically to the Crawley (England, 1946) strain. This impression was confirmed in careful studies from this laboratory by Bozzo (1952) and Hennessen (1952) and independently by Brans (1952) in Holland. These workers have all found that strains of influenza B recovered and maintained in eggs during the period 1943-52 showed rather uniform antigenic characters. Strains recovered between 1940 and 1943 differed from one another and from later strains; but since the earlier strains were recovered in ferrets and usually maintained by passage in mice and eggs the observed differences may have been due to the techniques used. These conclusions differ slightly from those of earlier workers (Tamm, Kilbourne & Horsfall, 1950; Hilleman, Mason & Buescher, 1950) but again differences in technique may account for the discrepancies. In particular, Bozzo and Hennessen paid great attention to the use of ferret sera prepared in a constant manner, fowl cells of uniform sensitivity (Stuart-Harris, 1943) and the elimination of normal serum inhibitors of agglutination (Isaacs & Bozzo, 1951). It would appear therefore, that the influenza B viruses contrast with the A viruses both in the extent of antigenic variability and in the epidemiological pattern of the corresponding diseases;

in Chapter V an attempt will be made to relate these findings.

The Influenza C Viruses

Taylor (1949) described a strain of virus which bore some resemblances to the influenza viruses and was recovered from a man showing a mild febrile disorder, and Francis, Quilligan and Minuse (1950) later recovered a second, identical strain from an outbreak of influenza. These workers proposed the name influenza C for this virus. The virus has been studied in the laboratory (Hirst, 1950) and a few epidemiological observations have also been carried out (e.g. Taylor, 1951; Davenport, Hennessy & Francis, 1953) but it is at present difficult to assess its exact importance. Influenza C viruses do not appear to cause widespread overt infection and there have so far been no reports incriminating them as the cause of serious infections in individuals. We have not yet received any strains of influenza C except for those of Taylor and Francis and Minuse, and there has been no reported occurrence of influenza C infection in this country. Antibody studies of human sera suggest that the virus may cause widespread subclinical infection, however (Davenport, Hennessy & Francis, 1953).

IV. P-Q VARIATION IN INFLUENZA VIRUSES

Description and Natural Occurrence of P and Q Viruses

During study of the 1950-51 epidemic of influenza A it was observed that antisera prepared against some strains inhibited agglutination by heterologous viruses to considerably higher titre than homologous virus (Isaacs, 1951). It was thought at first that this was an original observation, but it was later found that van der Veen and Mulder (1950) had described this phenomenon in some detail. A similar phenomenon with Rickettsia burneti had been described earlier (Topping, Sheperd & Huebner, 1946; Stoker, 1953).

Van der Veen and Mulder (1950) adopted the letters P, Q and R to describe three types of virus as shown by their reactions with immune sera and Table X, taken from van der Veen and Mulder's monograph, shows the characteristic reactions of these viruses. R strains have been uncommon in our experience; P strains are frequently encountered and show the type of serological behaviour thought to be most characteristic of influenza viruses. But Q strains have been found quite frequently, and the fact that the four inter-epidemic strains recovered from Sweden in June 1950 from the outbreak which heralded the epidemic in the following winter were all Q strains (Isaacs & Andrewes, 1951) focused attention on the possible significance of this phenomenon. It should be stressed that the properties on which these

TABLE X

Diagrammatic representation of results of haemagglutination-inhibition tests with P, Q and R viruses and sera

VIRUS	SERUM		
	P	Q	R
P	+ + +	+	+
Q	+	+	+
R	+ + +	+ + +	+ + +

characters depend appear to be inheritable and the strains maintain their characters on continued passage in eggs.

Laboratory Induction of P-Q Variation

On theoretical grounds it might be suggested that a Q strain, with a low avidity for antibody, would be better adapted to survive in a partially immune population than a P strain. The possibility was considered that when a P strain came in contact with specific antibody in a partially immune individual any Q variants naturally present in the strain would have their survival favoured. Since 1948, the possibility of producing antigenic variants of influenza virus by growing them in homologous immune serum had been tested with limited success (Isaacs, 1948; Isaacs & Edney, 1950b) and it seemed worth while to extend these experiments to test whether P strains could be "changed" into Q strains in this way.

In preliminary experiments virus was grown in eggs for a number of passages in the presence of gradually increasing amounts of immune serum but later the method was modified (see Techniques, p. 12) so that the concentration of anti-serum was gradually increased during the course of a single passage. In experiments of this type it was possible to induce the production of Q variants from P strains (Isaacs & Andrewes, 1951; Isaacs, Gledhill & Andrewes, 1952) and recent experiments by Drs. Fiset and Depoux with 1953

influenza strains confirm these findings.

During observations on 1950-51 Scandinavian viruses it was noticed that some strains recovered early in the epidemic and the inter-epidemic Scandinavian strains were Q, whereas many of those isolated later appeared to be serologically similar but showed a higher avidity for antibody. The possibility was considered that on passage through susceptible individuals change of Q to P virus might be favoured. An attempt was made to mimic this change in the laboratory by passaging Q strains of virus in mice, and it was found on a number of occasions that after one or two passages in mice followed by re-inoculation into eggs change to a P type of strain took place (Isaacs & Andrewes, 1951). Table XI shows a comparison of the behaviour of a laboratory-induced P variant of a Scandinavian Q strain with the naturally-occurring Scandinavian P strain, A/London/1/51. The resemblance between the laboratory-induced and natural P strains is striking.

Evidence for Natural Occurrence of P-Q Variation

Following these results, the provisional hypothesis was adopted that the P to Q change represented a biphasic variation which could be readily induced in the laboratory. It is necessary to examine next the evidence which suggests that P-Q variation is more than a laboratory manipulation but is occurring also under natural conditions. Three pieces of

TABLE XI

Haemagglutination-inhibition tests with P and Q Scandinavian 1950-51 strains

VIRUS	DESCRIPTION	SERUM A/Swe/3/50	SERUM A/Eire/1/51	SERUM A/Lon/1/51	SERUM A/Madrid/3/51
A/Swe/3/50	Scandinavian Q strain	40	10	50	10
A/Swe/3/50 M.1	Scandinavian Q strain after 1 mouse passage	120	60	360	25
A/Lon/1/51	Scandinavian P strain	60	60	320	60

evidence favour this idea. The first, referred to above, is the fact that in 1950 the appearance of Q viruses was soon followed by that of P viruses of the same serological type (cf. Table XI). The second is the fact that influenza virus strains may show evidence of heterogeneity in respect of their P and Q characters. It has been found (Isaacs & Edney, 1950a) that on growth of influenza virus at limiting infective dilution, i.e. under conditions where only the minority of eggs are infected, it is sometimes possible to separate the components of a mixture. A number of our strains have been tested by growth at limiting dilution and in five cases it was found that both P and Q strains were present side by side (Isaacs, Gledhill & Andrewes, 1952; Isaacs, 1953). Since P and Q viruses breed true on growth in eggs the assumption is that both components were originally present in the garglings. The third piece of evidence comes from the study of Q variants of a Liverpool strain. These variants were induced in the laboratory in 1951 by growth of a Liverpool strain in the presence of homologous immune serum (Isaacs, Gledhill & Andrewes, 1952). One year later two strains were isolated from garglings sent from the Persian Gulf to the Central Public Health Laboratory, Colindale. The strains were antigenically closely similar to the laboratory induced variants, and furthermore, both the Persian strains showed P and Q components when grown at limiting infective dilution

TABLE XII

Cross haemagglutination-inhibition tests with A/Johannesburg/7/50 and

(a) its laboratory-induced variant

(b) the naturally-occurring A/Persian Gulf/2/52

VIRUS	SERUM	
(a)	A/Johannesburg/7/50	A/Johannesburg/7/50 variant
A/Johannesburg/7/50	1920	960
A/Johannesburg/7/50 variant	80	800
(b)	A/Johannesburg/7/50	A/Persian Gulf/2/52
A/Johannesburg/7/50	1920	960
A/Persian Gulf/2/52	80	320

(Isaacs, 1953). A comparison of the behaviour of the natural and laboratory induced variants is shown in Table XII. These findings suggest that changes similar to those which had been induced in the laboratory had occurred under natural conditions, the garglings having been taken at a time when both variants were present side by side.

Dr. Magill has recently found evidence (personal communication) that the serum used in laboratory experiments acts by selecting pre-existing variants. Variants similar to those which we have found could be selected from a strain after precipitating it with antiserum <u>in vitro</u> and growing virus from the supernatant fluid. By this technique Dr. Magill hopes to be able to estimate the proportion of variants present in any strain. Presumably antibody in nasal secretions, or when added in the laboratory, selectively favours the growth of these variants which are poorly neutralised by antibody.

The Mechanism of P-Q Variation

We have earlier suggested that P and Q strains may have a similar antigenic composition but differ in the arrangement of their component antigens (Isaacs, Gledhill & Andrewes, 1952). Thus, as a convenient working hypothesis, one might suppose that the dominant antigen is present as a surface antigen in P viruses but is deeply situated within the virus particle in Q variants. Presumably the virus particles are broken up by antibody-forming cells; hence P and Q strains would be

equally efficient in inducing antibody production. However the P virus would combine with antibody more efficiently since the dominant antigen is present near the virus surface. This hypothesis has received striking support from some antibody absorption experiments carried out along with Dr. Fiset and using the technique of Fiset and Donald (1953). Table XIII shows the cross-serological relationships between a Liverpool 1950-51 strain (A/England/1/51), a Scandinavian 1950-51 strain (A/Sweden/3/50) and a Scandinavian 1952-53 strain (A/Missouri/303/52) when each serum is absorbed with each virus and tested with each virus. Fig. 1 also illustrates the results obtained after the third absorption.

The results show that A/England/1/51 and A/Missouri/303/52 have distinct major antigens and share minor antigens. A/Sweden/3/50 shares minor antigens with each strain but also shares a major antigen with A/Missouri/303/52. This latter fact can only be shown in tests with the A/Missouri/303/52 virus, however, and the reason for this can be seen when the central square of Table XIII is examined. It is clear from that that A/Sweden/3/50 virus is able to induce antibody to A/Missouri/303/52 virus but is unable to absorb the antibody, although homologous antibody is completely absorbed, a finding which may be unique in immunological investigations. This is not an isolated experimental result since Drs. Fiset and Depoux have noted similar findings with other influenza A

TABLE XIII

Cross absorption tests with A/Swe/3/50, A/Eng/1/51 and A/Missouri/303/52 viruses and sera

Absorption No.	A/Eng/1/51 serum tested with			A/Swe/3/50 serum tested with			A/Missouri/303/52 serum tested with		
	A/Eng/1/51	A/Swe/3/50	A/Mis/303/52	A/Eng/1/51	A/Swe/3/50	A/Mis/303/52	A/Eng/1/51	A/Swe/3/50	A/Mis/303/52
				I. Absorbed with A/Missouri/303/52					
1	1280	40	< 40	80	80	80	< 40	< 40	480
2	1280	< 40	< 40	40	60	< 40	< 40	< 40	80
3	1280	< 20	< 20	30	20	< 20	< 20	< 20	< 20
Control	1280	120	160	240	240	480	100	120	1920
				II. Absorbed with A/Swe/3/50					
1	1280	< 40	< 40	80	60	320	< 40	< 40	640
2	1280	< 40	< 40	40	< 40	320	< 40	< 40	640
3	960	< 20	< 20	< 20	< 20	240	< 20	< 20	640
Control	1280	80	80	240	160	480	120	120	1280
				III. Absorbed with A/Eng/1/51					
1	640	40	< 40	40	120	320	< 40	40	480
2	180	< 40	< 40	< 40	80	320	< 40	< 40	480
3	< 20	< 20	< 20	< 20	30	320	< 20	< 20	480
Control	1280	60	60	160	160	480	60	60	640

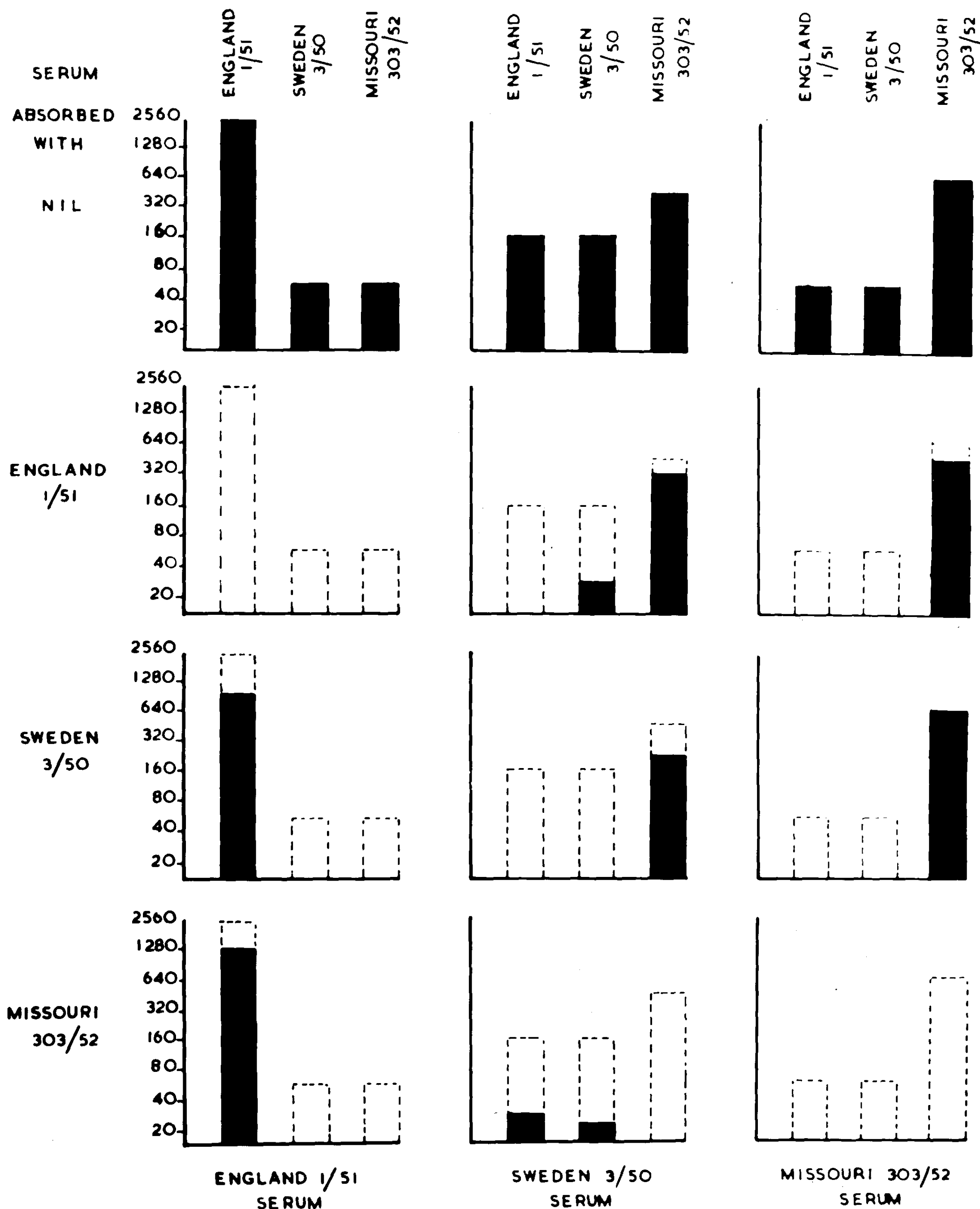

FIG. I. Anti-haemagglutinin titres with A/England/1/51, A/Sweden/3/50 and A/Missouri/303/52 viruses; results of cross-absorption tests.

viruses. The findings are compatible with the above hypothesis and they suggest that the P-Q variation can be considered as essentially a modification in the arrangement of the dominant antigen within the virus particle. The fact that the Scandinavian 1953 antigen was present within the 1950-51 strain and later emerged as a surface antigen may be of importance in the evolution of apparently new antigenic patterns of influenza viruses.

V. SOME EPIDEMIOLOGICAL CONCLUSIONS FROM THE ANTIGENIC STUDIES

We would like to suggest, based on the foregoing studies, that influenza viruses undergo two main types of variation. The first is antigenic variation in which a variant acquires a new antigen which cannot be demonstrated in the parent strain. The second is P-Q variation which we would propose occurs by re-arrangement of the antigenic components present in the parent strain. Antigenic variation occurs less frequently than P-Q variation under natural conditions and there is no convincing evidence that antigenic variation has ever been induced in the laboratory. Thus the changes induced in influenza viruses as a result of adaptation to mice (Hirst, 1947) or passages along with heterologous antibody (Archetti & Horsfall, 1950) are not easy to dissociate from P-Q changes or to the use of heterogeneous virus populations. Antigenic changes can be detected in influenza A viruses every 1-2 years and it is assumed that the presence of a new antigen confers survival advantages on the virus.

It is tempting to try to relate the antigenic patterns of the influenza viruses to their epidemiological behaviour. The influenza A viruses are highly variable and vary progressively, apparently by acquiring new antigenic components and discarding older ones. The controversy whether these progressive changes

are finite or infinite is one which has engendered more heat than light and which is immaterial to the present argument. The influenza B viruses show considerably less antigenic variability and strains recovered nearly 10 years apart have shown no significant antigenic differences, a situation unparalleled with the A viruses. Very few influenza C viruses have been recovered so far but all have been antigenically similar. Correspondingly, influenza A viruses are highly efficient epidemic agents, B viruses much less so and influenza C seems to be an endemic disease, no serious outbreaks having been recorded so far. It seems not unreasonable to believe that a virus which can produce frequent antigenic variants will be able to produce major epidemics by continually keeping ahead of the immunity of the host population.

The importance of previous exposure to antigenically related viruses on the reactions of a population to an influenza epidemic were very clearly shown by an epidemic which occurred in Ocean Island (Isaacs, Edney, Donnelley & Ingram, 1950). This island, which is almost entirely given over to phosphate mining, has three different racial populations. Gilbert and Ellice islanders are brought to Ocean Island for 1-2 year periods, a European group is responsible for supervising, and periodically Chinese are brought from near Hong Kong to supplement the Gilbert and Ellice islanders. It is a well-known epidemiological observation that something akin to

'influenza frequently breaks out among the islanders within a few days of the arrival of the Chinese and this state of affairs again occurred in 1948. On this occasion, however, we had the opportunity of confirming by bacteriological investigations the impression that this was an outbreak of influenza. The incidence of influenza in the three populations, which incidentally mixed freely with one another, was most striking. Most of the 290 Ellice islanders became ill, many severely so and there were 5 deaths. About 10 per cent of the Europeans were involved, with mild infections, an incidence which is not much higher than we would expect in an epidemic in this country. None of the Chinese became sick and there was no reported sickness among the Chinese on the boat to Ocean Island. Influenza A viruses were isolated from 10 out of 12 garglings tested and many patients showed sharp antibody rises to the infecting strain during the course of their illnesses. In addition, many of the Chinese showed high levels of antibody to the influenza A soluble complement-fixing antigen, a result strongly suggestive of recent infection with the virus. We would interpret these observations as indicating that influenza was endemic in the Chinese population, maintaining itself by producing occasional subclinical infections. The Gilbert and Ellice islanders indicate the effects which an otherwise rather harmless virus can produce in a highly susceptible population.

How does the virus apparently acquire "virulence" in its spread through a susceptible population? The virus strains recovered did not differ from other strains we have studied in any characteristic tested and at present we have no laboratory test which can be related to virulence for human beings. It seems worth while to attempt to relate virulence to known properties of the virus. The toxicity of influenza viruses is a well-known property in the laboratory (e.g. Henle & Henle, 1946) and Sugg (1949) has suggested that the toxicity of influenza viruses given intranasally to mice is a function of the virus dosage rather than a result of actual virus multiplication. One might postulate therefore that influenza viruses are toxic for man when they reach a certain critical concentration. When influenza viruses infect man they may first meet some opposition from small amounts of antibody or normal inhibitors present in the respiratory tract. The latter, while not usually considered to affect the infectivity of influenza viruses, may nevertheless slow down the rate of multiplication of certain virus strains (Andrewes, Isaacs & Marmion, 1954). Thereafter the outcome of infection may be conceived of as a race between the rate at which virus multiplies and antibody is produced and mobilised. Antibody production is presumably more rapid in people with past experience of antigenically related viruses. If antibody production is more rapid, the critical virus level is never reached and mild

or subclinical infection will result. If virus production is more rapid the critical level may soon be reached and toxic symptoms result. In addition, a person with a high concentration of virus in his respiratory tract is liable to spread large amounts of virus. Hence susceptible individuals may be infected with large doses of virus, an effect which would further assist the virus in its race against antibody production. In this way the virus would apparently acquire "virulence" in its passage through a susceptible population. Some indirect evidence for these speculations comes from the Ocean Island epidemic. The garglings were shipped to Australia in the cold room of a ship without any special precautions to preserve virus infectivity. The voyage to Australia took 5 weeks and yet in spite of these very far from ideal conditions, virus was isolated with the greatest of ease from 10 out of 12 garglings. This unusually high proportion of success suggests that the virus was initially present in the garglings in high concentration. Unfortunately we have no further direct evidence on this point.

The results described in Chapter III leave little doubt that true spread of influenza virus can occur across continents. The fact that within a few months of the appearance of a new antigenic variant the virus can be recovered from every continent of the world is most simply interpreted by assuming that antigenic variation is a relatively uncommon event

and that the new variant has sufficient antigenic novelty to allow it to find susceptible individuals everywhere. Trans-equatorial spread of virus is also indicated by the results of the 1950-51 epidemic but the puzzle of how the virus survives between epidemics remains unsolved. There are two main theories. The first assumes that influenza virus is endemic in certain populations and spreads by causing infrequent subclinical infections until a sufficiently large susceptible population is built up for an outbreak to occur. Presumably Q variants of virus would be able to survive well under these conditions and might therefore play an important role in the inter-epidemic survival of virus. Alternatively virus may survive in certain individuals in a latent form. This is the case with herpes simplex virus which persists in herpetic individuals from the time of first infection in infancy throughout the individual's life. Andrewes (1942) has suggested that influenza viruses may similarly survive in certain individuals in a "basic" form and recent work with symbiotic bacteriophages offers another analogy. With regard to influenza, both theories seem extremely difficult either to prove or disprove. Perhaps the period shortly before an epidemic is expected would repay further study but unfortunately prediction of influenza epidemics is still hazardous.

VI. PREVENTION OF INFLUENZA

The prevention of outbreaks of influenza usually conjures up a picture of vaccination in the minds of those concerned with this subject. It is generally accepted that quarantine measures are in most cases of little avail in halting an influenzal outbreak.

Vaccination against influenza has had its successes and its failures. The American trial carried out in 1943-44 showed clearly that the right vaccine given at the right time can materially and significantly reduce the incidence of clinical influenza (Commission on Influenza, 1944). On the other hand, trials in 1947 in the U.S.A. (Francis, Salk & Quilligan, 1947) and in this country (Mellanby, Andrewes, Dudgeon & Mackay, 1948) showed little effective action by influenzal vaccine, and a large and carefully organised trial in Britain in the 1952-53 winter showed that vaccine caused only a very slight, although statistically significant, reduction in influenzal illnesses (Committee on Influenza, 1953).

There has been a tendency on the part of enthusiastic protagonists of influenzal vaccination to assume a priori that vaccines should work and that where they do not work there is always a good reason for it. It may be worth while to examine these reasons and discuss what measures can be taken to counteract them, before considering further the rationale of parenteral vaccination against influenza.

Assessment of Vaccine Trials

One of the factors which interferes most with the assessment of influenzal vaccine trials is the presence of non-influenzal respiratory infections beside influenzal cases. The commonest infection is "febrile catarrh" (Stuart-Harris, Andrewes & Smith, 1938) or "acute respiratory disease" (ARD) (Commission on Acute Respiratory Diseases, 1946). This infection frequently causes large outbreaks among recent recruits to the army and it appears to be commoner in the U.S.A. than in this country. Since there is no laboratory test for this infection its laboratory diagnosis usually rests on the negative results of tests for influenzal infection. However, if the antihaemagglutinin response is used as a serological test for influenza, vaccination may produce such a high antibody titre that no further rise can be detected following an influenzal infection; it is therefore difficult to distinguish influenza in a vaccinated individual from febrile catarrh in this way. Many of the heroic efforts to present the results of vaccination trials in the most favourable possible light (e.g. Salk & Suriano, 1949) founder on this difficulty. On the other hand, the two infections are readily distinguished if virus isolation or complement-fixation with influenza soluble antigen are used. But best of all the assessment of influenzal vaccine trials can be made in civilian populations not heavily exposed to outbreaks

of febrile catarrh, and on the basis of clinical (and not laboratory) attack rates. This is essentially the approach adopted by the Medical Research Council's Committee on Influenza.

Importance of the Strains used

The success of the vaccine used in the 1943 trial contrasted with the failure of vaccine in the 1947 trials is generally attributed to the fact that the epidemic strain was closely related antigenically to one of the strains in the 1943 vaccine but not in the 1947 vaccine. This would appear to be a most important factor although it is difficult to say whether it is the only factor. Obviously, with the continued antigenic changes which influenza viruses are undergoing a vaccine should contain strains antigenically as close as possible to the current epidemic strain. It may occasionally be possible, as in 1943, to incorporate in the vaccine a strain of virus antigenically identical with the infecting strain. More commonly, a closely related strain can be made available. Occasionally, the influenza virus can be expected to show an abrupt antigenic change and in such cases the vaccine is liable to lag behind. One of the functions of the World Health Organisation programme on influenza is to obtain and make available the most up-to-date information on the antigenic characters of strains of virus in circulation. These strains can be distributed to anyone interested in preparing vaccine.

The Use of Adjuvants

One of the major disadvantages of influenzal vaccines is that the antibody response following vaccination is of short duration. The use of adjuvants based on the principles elaborated by Ramon and Freund has materially increased the duration of the antibody response. At first, however, adjuvants themselves were not without risk since sterile abscesses resulted from their use (Henle & Henle, 1945) but recently Salk has claimed that the use of a mineral oil (Bayol F) and an emulsifying agent (arlacel) injected intra-muscularly gives a safe vaccine (Salk, Bailey & Laurent, 1952). Careful enquiry reveals, however, that nodules may appear at the injected site about 1 year after vaccination in 1 per cent of vaccinated individuals. The risk of carcinogenesis from the injection of these oils is a possibility and it may be questioned whether the use of adjuvants is wholly desirable.

There are two main advantages claimed for adjuvant vaccines (Salk, 1953). They are said to increase the breadth of antibody response and to decrease greatly the amount of virus required to be used. The last factor is important since toxic effects due to the virus itself can be virtually eliminated and also because more doses of vaccine can be manufactured from a limited supply of eggs. Salk originally claimed that the use of ~~vaccine~~ adjuvant increased by a factor of about

100 the efficiency of a vaccine. Other workers find a figure closer to 10 and recently Salk has told me that the figure of 100 applied only to one particular line of the PR8 strain and that with other strains and even with another line of the PR8 strain the figure of 10 is more accurate.

While it seems clear that adjuvants increase greatly the efficiency of influenza virus as an antibody-producing agent, there has been no evidence yet that the protective effect of vaccines is increased. Most of the trials carried out in the U.S.A. have shown no protective effect since the emulsions used were unstable, but one trial which appears to be technically satisfactory has demonstrated that vaccine diluted 1 in 5 plus adjuvant gives about the same order of protection as undiluted saline vaccine against clinical influenza(Dorland Davis, personal communication). So far, no information about the duration of protection with adjuvant vaccines is available.

<u>Adaptation of Virus Strains to Mice</u>

It is well known that recently isolated strains are much poorer antigens than well established laboratory strains (Salk, Laurent & McGinnis, 1949; Appleby, Himmelweit & Stuart-Harris, 1951; Meiklejohn, Weiss, Shragg & Lennette, 1952). The possibility was considered that since all the earlier strains had been adapted to mice while recently isolated strains were generally isolated and maintained in eggs, adaptation to mice might play a significant role in the

antigenicity of a strain.

A new technique was evolved for assaying the antigenicity of influenza virus vaccines, based on the methods used for biological assays. Groups of mice are inoculated intraperitoneally with serial ($\sqrt{10}$-fold) dilutions of the vaccine to be tested. Fourteen days later the mice are bled and their sera tested individually for their antihaemagglutinin content. The results of a test of this type are shown in Fig. 2.

Figure 2 shows that there is a large variation in the response of individual mice, a fact which makes the use of pooled sera undesirable. However, there is a linear relation between the log dose of vaccine and the log antibody response within the limits shown.

This technique was used to compare the antigenicity of an unadapted strain with that of the same strain after mouse adaptation. The results are shown in Fig. 3.

It will be seen that the mouse adapted line is a much better antigen than the unadapted strain, the differences between the two being highly significant statistically (Isaacs & Sampaio, 1953). In addition, the difference between the two lines increases with increasing virus dosage. The findings were readily confirmed with the A/Sweden/3/50 virus.

Tests in man have already shown that a mouse-adapted strain was a better antigen than the corresponding unadapted strain (Isaacs & Sampaio, 1953) and recent experiments by the

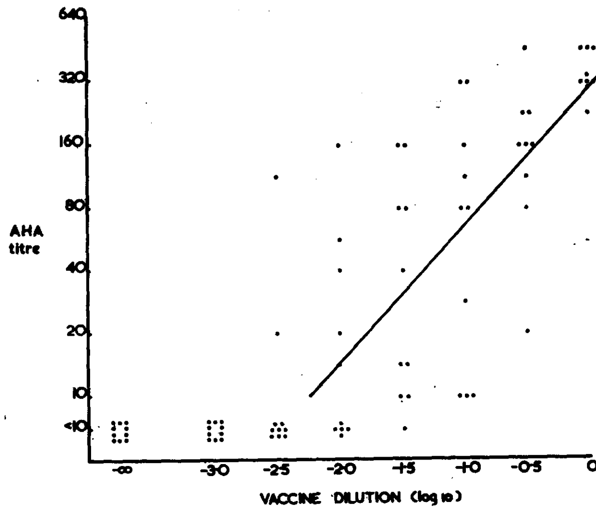

FIG. II. Anti-haemagglutinin titres (ordinate) of mice injected with different dilutions (abscissa) of influenza vaccine intraperitoneally. The spots represent the titresof individual mice.

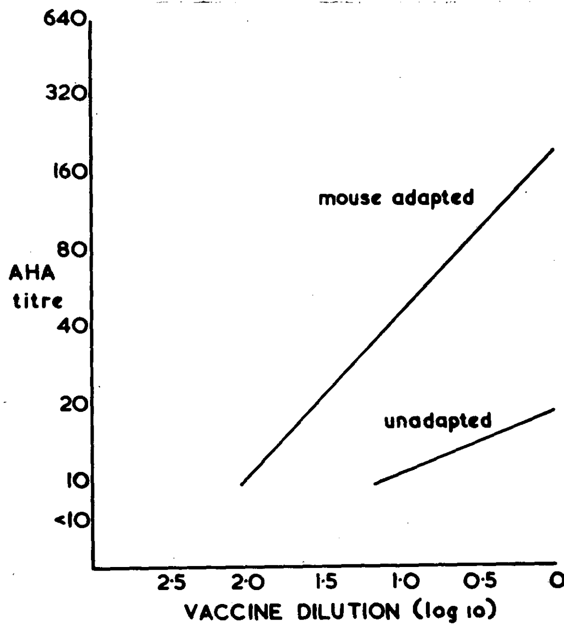

FIG. III. Geometric mean anti-haemagglutinin titres of mice injected with different dilutions of unadapted and mouse-adapted A/England/1/51 viruses.

Medical Research Council's Committee on Influenza appear to confirm this finding. No information about the relative protective values of the two lines is yet available.

Pathotopic Potentiation of Immunity

Francis (1941) pointed out that vaccination against influenza produced a large amount of antibody in the patient's serum but that probably only a small fraction of the antibody produced reached the respiratory tract. Francis, Pearson, Sullivan and Brown (1943) demonstrated a small rise in antibody in the respiratory tract following vaccination and this finding was later confirmed by Mulder, Brans and Hers (1952).

Fazekas de St. Groth and co-workers tried to increase the efficiency of vaccination in mice by combining intraperitoneal vaccination with the inoculation of an adjuvant by the intra-nasal route. The adjuvant was thought to increase the permeability to antibody of the barrier between the circulatory and respiratory systems (Fazekas de St. Groth, 1950). Attractive as this theory appears, extremely careful experiments in our laboratory have failed to confirm the experimental findings (Depoux & Mussett, 1954). The only difference from Dr. Fazekas' experiments was in the strain of mice used, and it must be concluded therefore that the phenomenon described under the term "pathotopic potentiation of immunity" is not of universal application.

Nasal Vaccination

On theoretical grounds it might be expected that the most efficient immunising agent against influenza would be a live attenuated virus vaccine given intranasally. The best vaccines available for other virus diseases all contain live virus, e.g. smallpox, rabies, yellow fever. The natural route of infection would appear to be the most logical to use for vaccination and there is experimental evidence to suggest that it produces the most favourable distribution of antibody between the respiratory tract and blood (Fazekas de St. Groth, 1950). In the event of a pandemic the amount of vaccine which could be produced for parenteral injection would be limited by the number of fertile eggs available. At present one egg supplies roughly one dose of vaccine and even if sufficient adjuvants were immediately available one egg could probably supply no more than 10 doses of vaccine. With live virus one egg might supply, theoretically at least, 10,000 to 100,000 doses, and thus bring rapid overall vaccination within the realms of theoretical possibility.

Unfortunately, past experience with nasal vaccination has not been encouraging (e.g. Francis, 1940; Burnet, 1943; Henle, Henle & Stokes, 1943).

A careful examination of these trials shows, however, that this technique may have been too readily condemned. Thus, extremely large doses of virus have been generally

used and these are known to be toxic per se (Henle & Henle, 1946; Sugg, 1949). It is conceivable that the reactions following nasal instillation of large doses of virus were due to toxic effects. Smaller doses of virus were found generally to produce no detectable antibody response and their protective effect against natural influenzal infection has not yet been investigated. It is very commonly assumed in studies with influenza vaccination that serum antibody is equated directly with immunity to infection, an unwarrantable assumption in view of the results to be described.

Our experiments, carried out in mice, have involved the comparison of mouse-adapted and unadapted lines of the A/England/1/51 virus given in the active form by the intranasal route. The technique used was to inoculate groups of mice intranasally with serial dilutions of the virus to be tested. Fourteen days later, half the mice of each group were challenged with 100 LD_{50} of mouse-adapted A/England/1/51 virus; the remainder were bled out and their sera tested individually. The mouse-adapted A/England/1/51 virus kills mice up to a dilution of 10^{-4}. The results of an experiment of this type using dilutions of 10^{-4} to 10^{-8} are shown in Table XIV.

Table XIV shows that at 10^{-5} and 10^{-6} dilutions some mice show protection to challenge and some show the development of serum antihaemagglutinin, the two activities running roughly

TABLE XIV

(a) Antihaemagglutinin titres and (b) Lung lesions developing on challenge in mice given a preliminary intranasal inoculation of A/Eng/1/51 mouse-adapted virus

(a) Antihaemagglutinin titres (to A/Eng/1/51 mouse-adapted virus)

No.	Dilution of A/Eng/1/51 (mouse-adapted) 10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	Broth saline control
1	160	20	<10	<10	<10	<10
2	15	20	15	<10	<10	<10
3	480	30	<10	<10	<10	<10
4	80	60	30	30	<10	<10
5		320	30	<10	<10	<10
6		240	15	<10	-	-

(b) % consolidation of lungs after challenge (with 100 LD_{50} of A/Eng/1/51 mouse-adapted virus)

No.	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	Broth saline control
1	Remaining mice died after 1st ihoculation	20	100	100	100	100
2		5	40	100	100	100
3		5	0	100	100	100
4		5	5	100	100	100
5		0	0	100	100	80
6		0	0	0	-	-

parallel. At 10^{-7} and 10^{-8} dilutions there is neither significant protection nor antihaemagglutinin.

By contrast the results of a similar experiment with the unadapted line of A/England/1/51 virus are shown in Table XV. The virus does not cause macroscopic lung lesions per se and at low dilutions it induces resistance to challenge at a time when no antihaemagglutinin could be detected. In other experiments protection up to a 10^{-3} dilution was observed. The observation that unadapted virus will immunise against mouse-adapted virus has been made on a number of occasions in the past (e.g. Shope, 1935); the present experiments are interesting because they tend to throw some light on the possible mechanism of this protective effect.

The possibility was considered that the unadapted virus protects by sensitising the antibody-producing mechanism so that the mouse responds to the challenge virus by rapid production of antibody. Some evidence for this theory was obtained. Mice were given a dose of A/England/1/51 virus intranasally and a second dose 14 days later and their antihaemagglutin responses measured. The results are shown in Table XVI. Controls showed that neither the first nor the second dose alone produced measurable antihaemagglutinin, but the first dose sensitised the animals so that they responded to the second injection by producing antihaemagglutinins to give quite high titres. It may be supposed therefore that the adapted line had

TABLE XV

(a) Antihaemagglutinin titres and (b) Lung lesions developing on challenge in mice given a preliminary intranasal inoculation of A/Eng/1/51 virus

(a) Antihaemagglutinin titres (to A/Eng/1/51 virus)

No.	Dilution of A/Eng/1/51 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Broth saline control
1	<10	<10	<10	<10	<10	<10	<10
2	<10	<10	<10	<10	<10	<10	<10
3	<10	<10	<10	<10	<10	<10	<10
4	<10	<10	<10	<10	<10	<10	<10
5	<10	<10	<10	<10	<10	<10	<10
6	<10	<10	<10	<10	<10	<10	<10

(b) % consolidation of lungs after challenge (with A/Eng/1/51 mouse-adapted virus)

No.	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Broth saline control
1	0	0	100	100	100	100	100
2	0	0	100	100	100	100	100
3	0	0	100	0	100	100	100
4	0	0	100	40	10	100	100
5	0	100	0	40	20	100	100
6	-	-	8	50	80	0	0

TABLE XVI

Antihaemagglutinin titres of mice given a preliminary intranasal inoculation of A/Eng/1/51 virus 10^{-2}, followed by a second inoculation of different dilutions of the same virus

Antihaemagglutinin titres:-

No.	Dilution of 2nd inoculum 10^{0}	10^{-1}	10^{-2}	10^{-3}	Broth saline control
1	80	<5	<5	<5	<5
2	<5	120	<10	<5	<5
3	20	80	20	<10	<5
4	10	15	<5	20	<5
5	-	320	20	<5	<5
6	-	-	-	<5	<10

TABLE XVII

Antihaemagglutinin and complement-fixing antibody titres of mice infected with A/Eng/1/51 and A/Eng/1/51 mouse-adapted viruses

No.	Antihaemagglutinin titres A/Eng/1/51	Antihaemagglutinin titres A/Eng/1/51 mouse-adapted	Complement-fixing titres A/Eng/1/51	Complement-fixing titres A/Eng/1/51 mouse-adapted
1	<2	64	2	2
2	<2	32	2	2
3	<2	32	3	2
4	<2	48	<2	2
5	<2	64	2	4
6	<2	-	2	-

virus. Oakley and Warrack (1940) had shown that intranasal infection produced a broader antibody response than parenteral injection. Groups of 10 mice were inoculated intranasally with A/England/1/51 virus and challenged 14 days later with a dose of approximately 100 LD_{50} of different challenge viruses. Table XVIII shows that infection with A/England/1/51 virus produced quite a broad protection, which was active against 100 LD_{50} of related mouse-adapted "A-prime" viruses although not significantly so with the distantly related Ws, PR8 and MEL virus strains. This result points clearly to the fact that the protection rests on an immunological basis and is not concerned with the interference phenomena or with some non-specific mechanism of immunity.

These results suggest that the possibility of intranasal vaccination of man with live influenza virus may be worth re-investigation. We have not found any reports in the literature of tests of immunity to natural infection or the development of complement-fixing antibody following the intranasal instillation of small doses of egg-grown influenza virus. Dr. Andrewes and I hope to start experiments of this type in the near future.

Essentially the experiments will be designed to answer three questions, in the following order:-

1. Can small doses of egg-grown influenza virus be given to man under conditions in which one can be sure that

TABLE XVIII

Protection by A/Eng/1/51 virus against challenge with different Influenza virus A strains

Mice given	Average percentage consolidation of mice challenged with virus						
	WS	PR8	MEL	FM1	A/Swe/3/50	A/Eng/1/51	A/Missouri/303/52
A/Eng/1/51	88	93	65	0	27	0	0
Broth-saline control	100	100	100	78	100	95	72

no illness will result?

2. Can a dose of virus be found sufficiently small to be non-toxic, but sufficiently large to produce antibody to the complement-fixing antigen?

3. If questions 1 and 2 can be answered satisfactorily, it would suggest that the virus could be given in doses in which it would multiply (since antibody to the soluble antigen is an index of virus multiplication) without producing illness. It would then be possible to pose the third question, whether protection to natural infection would result.

The experiments may take a few years to carry out but we are approaching them in a spirit of cautious optimism. Whether influenza vaccination should be advocated as a general measure is quite another question; our present objective is merely to try to gain the knowledge of whether it can be made to work.

VII. SUMMARY

The subject is introduced in Chapter I. Chapter II contains an account of the techniques of study used in the laboratory investigations. Chapter III considers the antigenic characters of the influenza viruses. All the evidence points to the belief that antigenically new varieties of influenza A viruses are continually emerging to replace older varieties. This statement prompts a digression into the recently reported isolations of strains of virus identical to strains recovered 10-20 years earlier; it is shown that the evidence that these strains represent genuine isolations is far from complete and that the possibility of laboratory contaminations in the cases investigated cannot reasonably be excluded. An account is given of the antigenic characters of older and more recent influenza A viruses, the latter received as part of the World Health Organisation's programme on influenza. The influenza B and C viruses are discussed briefly. Chapter IV describes the so-called P-Q variation in influenza viruses. The natural occurrence and laboratory induction of these variants are discussed and an attempt is made to estimate the epidemiological significance of the phenomenon. Evidence is presented for the theory that P-Q variation represents an alteration in the arrangement of the antigenic components within the virus particle. In Chapter V some conclusions are drawn about the epidemiology of influenza

from these results. An attempt is made to relate the characteristic epidemiological behaviour of influenza A, B and C viruses to their different degrees of antigenic variability. The importance of the antigenic variability of the virus and the past experience of a population to infection with antigenically related viruses is illustrated by an account of an influenza epidemic in Ocean Island. The evidence for transcontinental spread of virus is presented and the origin of influenza epidemics discussed. Chapter VI is concerned with the prevention of influenza. The successes and failures of parenteral vaccination are considered and methods for improving its efficiency are described. Finally, laboratory evidence is adduced that nasal vaccination with live attenuated virus has a sound theoretical basis and deserves further experiments in man.

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