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**EXPERT COMMITTEE ON
INFLUENZA**

First Report

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EXPERT COMMITTEE ON INFLUENZA

First Session

Geneva, 8-12 September 1952

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EXPERT COMMITTEE ON INFLUENZA

First Report¹

The Third World Health Assembly emphasized the importance of influenza and approved a proposal that an Expert Committee on Influenza should be convened by the Director-General in 1951.² Since no expert committee could be established in 1951, the meeting was postponed until 1952.

The first session of the Expert Committee on Influenza was held in Geneva from 8 to 12 September 1952. The session was opened by the Director-General of the World Health Organization, Dr. Brock Chisholm. Professor P. Lépine was elected Chairman; Dr. T. P. Magill, Vice-Chairman; and Professor C. H. Stuart-Harris, Rapporteur.

The provisional agenda was adopted.

1. Introduction

Influenza is one of the most important infectious diseases still unconquered. Even in its present relatively mild form it can cause great economic loss and dislocation of essential services, especially as its ill effects are commonly concentrated within a few weeks. It can also be responsible for many deaths: for instance, in the Netherlands in 1949, within a short period, 2,200 people died of influenza and its complications. These facts alone would make it important to learn how to control the disease; but it is even more vital to discover how to prevent or repel the attack of a lethal influenza pandemic, such as that of 1918-9, which is believed to have killed at least fifteen million people throughout the world. We do not know what led to the appearance of this outbreak in 1918, nor whether a similar one will occur again.

¹ The Executive Board, at its eleventh session, adopted the following resolution:
The Executive Board

1. NOTES the first report of the Expert Committee on Influenza;
2. THANKS the members of the committee for their work, and
3. AUTHORIZES publication of the report.

(Resolution EB11.R13, *Off. Rec. World Hlth Org.* 46, 5)

² *Off. Rec. World Hlth Org.* 23, 47; 28, 43 (Resolution WHA3.71.3.5)

Considerations such as these led WHO to institute in 1947 a programme for the study and control of influenza. The present committee has been reviewing the work of the past few years and making plans for more-effective international collaboration in this field in the future. This is necessary because there is still very little knowledge about the origins of influenza outbreaks and the spread of the disease, and without such knowledge prevention and control are unattainable. Many, probably most, aspects of research into influenza are best carried out through the initiative of individuals and small groups; where the worldwide epidemiology of influenza is concerned, however, international collaboration is essential, or the needed information may never be forthcoming at all.

The present organization needs improvement in several respects. Information obtained from laboratories already well equipped can be better interpreted if workers therein are using the same, or at any rate comparable, techniques. Again, it is highly desirable to stimulate and assist the study of influenza in less developed countries; only if information comes from all over the world can the epidemiological picture be seen as a whole.

The influenza network of laboratories can already be of value to WHO Member States in several ways. WHO can be put in a position to advise them as to what control measures are useful, and which useless or harmful. Constant watch on the appearance and spread of influenza will help to make possible accurate forecasts of the times and places of epidemics. Antigenic studies of prevalent viruses should be able to afford guidance as to what strains should be incorporated in vaccines being made, or as to which vaccines in stock can profitably be used. The committee has discussed in detail the many technical difficulties to be overcome before the control of influenza can be said to be in sight.

2. Grouping of the Influenza Viruses

The committee considers that, although among the strains isolated so far there is a wide range of antigenic variation, it is desirable at present to limit subdivision into main groups based upon individual strains. As, and when, new strains appear in the future, this grouping can be extended.

Strain differences were first recognized in 1938, but the information concerning the earlier strains in their original state is incomplete; and the evidence is insufficient to indicate whether or not there has been a continuous variation of strains in the past. Although different methods of study have been used in different laboratories and there is still no agreed standardized procedure for the antigenic analysis of strains, a substantial measure of agreement exists as to the broad groups.

With these limitations in knowledge in mind, the committee recommends the subdivision of the influenza A virus into the following main groups, which are named after their prototype viruses and the date of their isolation :

WS (1933)
PR8 (1934)
FM1 (1947)

The recent A strains, of which FW/1/50 and A/England/1/51 are examples, though different from FM1, are not as divergent as the other main groups and are therefore considered to fall within the FM1 (1947) group. There is also a heterogeneous group of swine influenza viruses related antigenically to the human influenza A viruses.

The influenza B viruses should be subdivided into groups with the general characteristics of :

Lee (1940)
Bon (1943)

The most recent influenza B strains (1952) appear to diverge from Bon, and it may be found on the basis of future experience that further groups must be created.

In influenza C, only one antigenic group is so far known, of which 1233 (1947) is the prototype.

3. Importance of Continued Study of the Influenza Virus Antigens

Continued study of the antigens of influenza virus strains which may be isolated in the future is important chiefly because of the possible emergence of new strains which may have epidemiological significance. Nothing is known concerning the type of virus responsible for the 1918 pandemic of influenza, but the possibility of the recurrence of such an outbreak is always present. Such an event might be associated with a new antigenic variant which would need, therefore, to be detected at the earliest possible moment.

There appears to be no stability of strains in nature, and the mechanism by which new virus variants emerge is not known. One factor may be that during epidemic and interepidemic periods the virus is subjected to a process of biological selection by reason of its survival in hosts of varying degrees of immunity.

The method of spread of influenza may be elucidated by a continuation of recent studies on the geographical occurrence of influenza virus strains. These have shown two possible methods by which epidemics may be

originated. Geographical spread of virus from one country to another has been indicated in some studies, but others rather favoured the development of an epidemic from an endemic state. Future studies should include a collaborative investigation involving the comparison of strains isolated in the Americas with those recovered in Europe and elsewhere.

Another justification for the study of antigenic variants is the profound significance that these have in relation to artificial immunization (see section 8, page 10).

There is no evidence that swine influenza viruses are related epidemiologically to the human disease at the present time. Nevertheless, because of suggestive evidence in the past of epidemiological relationship between the human and swine diseases, the study of the viruses should be continued.

4. Methods of Comparing and Typing Strains

The committee agrees that the haemagglutination-inhibition test³ is the technique to be preferred for comparing and typing strains. A divergence of opinion exists, however, as to the antisera to be used in the test. There are two classes of antisera which can be employed, those that result from infection, e.g., in the ferret, hamster, or mouse inoculated intranasally, and those produced by artificial immunization, e.g., in the guinea-pig, rabbit, or fowl inoculated parenterally. It is agreed that the main disadvantages to the use of the ferret are that strict isolation of the infected animal must be preserved and that the yield of serum is relatively small. However, the use of ferret antisera has enabled differences to be discerned between virus strains which were not detected by the use of rabbit or fowl antisera. Nevertheless, some workers prefer to use fowl or rabbit antisera because of the ease of handling these animals and the large yield of serum. It is emphasized, however, that all types of antisera enable the main antigenic groups already set out to be readily differentiated, provided that the non-specific inhibitors present in these sera are eliminated.

Procedures which have proved satisfactory in the preparation of ferret, rabbit, and fowl antisera for these purposes and for the elimination of the non-specific inhibitors are given in Annex 1 (page 20).

It is important that the typing of strains should be carried out using early egg-passage virus before their characteristics have been altered by artificial cultivation. Freshly isolated strains should therefore be sent to the reference laboratories in London and New York.

³ An outline of the haemagglutination-inhibition test in use at the World Influenza Centre, London, for comparing or typing influenza virus strains will be found in: Isaacs, A., Gledhill, A. W. & Andrewes, C. H. (1952) *Bull. World Hlth Org.* **6**, 287.

5. Designation of Newly Isolated Influenza Virus Strains

The use of the patient's name for designating the infecting strain of virus is becoming increasingly inconvenient and a possible source of confusion. It is therefore fundamental that all laboratories should adopt in the future a standard system for the designation of newly isolated strains. The procedure which has been in operation for the last three years in some centres is recommended.

The strain isolated, whether or not immunologically identical with strains from elsewhere, should be named :

- (1) A, B, or C, according to type ;
- (2) by the name of the country, state, or large city from which it was recovered ;
- (3) by a serial number, to designate which of several strains isolated at the same place is referred to ;
- (4) by the year of isolation.

Thus a new strain might be called A/England/1/53.

With reference to (3), it is important that the serial number should not be duplicated, as for instance by two laboratories situated within the same city. It is therefore recommended that the serial number to be used should be obtained from a list of numbers allocated by the reference laboratories in London and New York upon application by the laboratory concerned. The serial number should be used once only, irrespective of the type (A, B, or C) of virus. The list of numbers should be exhausted before a new application for numbers is made, even if lasting for more than one year.

6. Methods of Collection and Distribution of Specimens

In general, the collection and distribution of three sorts of specimens need to be considered. These are, firstly, strains of virus already isolated in a laboratory ; secondly, throat washings or fresh sputum, etc., for testing for virus ; and thirdly, serum samples for diagnostic purposes.

It may often be difficult in countries without adequate laboratory facilities for the isolation of the virus to be attempted. This is regrettable because the information obtained from such virus strains in relation to the spread of the disease cannot be obtained by the examination of human sera. Nevertheless, serological methods utilizing human sera can give useful diagnostic and epidemiological information.

6.1 *Influenza strains*

Strains which may be sent to reference laboratories at a distance should be lyophilized if possible. If not, the material should be packed in a container in dry ice adequate for the duration of the journey.⁴ If dry ice is not available, ordinary ice may be used in a thermos flask, but only for short journeys (e.g., 36 hours' duration).

6.2 *Throat washings and other materials*

Materials for virus isolation should be sent to a laboratory only if it is possible to send them packed in ice or dry ice adequate for the duration of the journey. If no other method is available, specimens may be sent without refrigeration by ordinary post but should contain an equal volume of neutral glycerine. Throat washings should be obtained as early as possible in the disease (not later than the fourth day) by getting the patient to gargle with 15 ml of water, physiological saline, bacteriological broth, or skimmed milk. Five millilitres of bacteriological broth should immediately be added to throat washings carried out with water or saline, and the whole put as soon as possible in the cold.

6.3 *Sera*

A diagnosis of influenza A, B, or C can usually be made by the examination of two serum specimens from a patient, one taken as early as possible in the disease (and not later than the fourth day), another taken 10 to 14 days after the onset. The titre of influenzal antibodies in human sera is so variable that only by detecting a significant rise (at least fourfold) in titre in the course of illness can the diagnosis be established—hence the need for two specimens. At least 2 ml, preferably 5 ml, of each serum should be sent, the serum being removed from the clot before dispatch; whole blood may be sent if the journey to the laboratory occupies less than two days. Care should be taken to avoid contamination during the collection of the blood or the subsequent separation of the serum.

Serum samples can be examined either by the complement-fixation test or the haemagglutination-inhibition test. The complement-fixation

⁴ A variety of insulated containers for the transport of virus-containing specimens is available such as ones made from compressed fibre, aluminium foil, and vacuum flasks.

The simplest possible method, such as wrapping up with dry ice in layers of crumpled newspaper, has on occasion proved satisfactory, whereas vacuum flasks are expensive and are frequently broken. Actual specimens have also been broken when packed in flasks with an inadequate amount of ice or dry ice and insufficient resilient packing to allow for liquefaction or evaporation. It is felt that further investigation is needed before a standard container can be recommended for universal adoption.

test is to be preferred for diagnostic purposes in influenza A and B for reasons given below (see section 7, below).

7. Recommendations for Standard Diagnostic Procedures

7.1 *Standard serological procedures*

Standard procedures are now available for the diagnosis of influenza virus infection, and full details of the techniques recommended for two of these are given in Annex 2 (see page 24). These are described as a guide for workers unfamiliar with these techniques and are based upon methods which have proved satisfactory in the hands of experienced workers.

From the standpoint of clinical diagnosis, it is necessary to determine only which type of infecting virus is concerned. The so-called soluble antigens (A or B) employed in the complement-fixation test described will distinguish between infections caused by viruses A and B, avoiding difficulties introduced by the existence of variants within the A and B groups. Moreover, the antibody formed against this antigen persists for a shorter period than the haemagglutination-inhibiting antibodies. In the case of influenzal pneumonia in which the virus infection may precede bacterial complications by several days, the titre of antibodies may already have reached a high level at the time of admission to hospital. The results of the test are not affected, as with the haemagglutination-inhibition method, by the presence in the serum of non-specific substances which produce effects resembling those of specific antibody. Thus, in the complement-fixation test using the soluble antigen with serum from a case of influenza, the first or acute sample of serum has either small amounts or no detectable amount of antibody. The convalescent sample will give good complement-fixation.

The haemagglutination-inhibition test is relatively more specific than the complement-fixation test in that during convalescence antibodies may appear in the serum which are detected only by using an antigen prepared from related strains of virus. It is therefore essential to employ more than one type of antigen or else the actual infecting strain of virus, if that is known. Again, the haemagglutination-inhibition test is affected by non-specific inhibitors present in the serum. The acute sample of the serum will thus give a certain level of inhibition against the virus antigen, unless steps are taken to destroy the inhibitors, as already mentioned in the case of animal sera. Nevertheless, even if inhibitors are not destroyed, the convalescent serum will, by virtue of its enhanced content of antibody, produce a greater degree of inhibition against the appropriate virus.

7.2 *Standard reagents*

The committee considered the need for distribution of standard reagents for diagnostic tests. It is recommended that WHO should undertake an investigation as to the desirability of making such reagents available and the means whereby this might be effected.

8. **Influenza Virus Vaccines**

Experience in the past has shown that it is possible to reduce the incidence of influenza by means of immunization. Nevertheless, influenza virus vaccination is still, in the opinion of the committee, an experimental procedure, since success or failure is determined by a number of different factors which demand further experimentation.

8.1 *Choice of strain*

It has been demonstrated that the antigen must bear a definite relationship to the type of virus against which protection is desired. In practice this might be attained either by the use of all known antigens in a mixed vaccine or by the use of one or two strains responsible for the most recent outbreaks. In either case there is a risk that the forthcoming epidemic may in fact be caused by an antigenic variant not represented in the vaccine. Mixed vaccines, containing all known antigens, suffer from the disadvantages of bulk and of dilution of what may prove to be the one important constituent. In either case the antigenic potency of the strain as judged by antibody formation must be considered. Therefore, the committee is not at present able to recommend the selection of particular strains for incorporation in the vaccine, but international collaboration is advised in their selection. Some authorities recommend a monovalent vaccine in the present phase of experimentation, others advise the inclusion of both A and B viruses. Experiments on immunization with influenza C virus have not been undertaken because of the technical difficulties of cultivation and the present lack of knowledge of the importance of the infection.

8.2. *Composition of vaccines*

All recent experimentation has involved the use of inactivated virus produced by egg cultivation. The vaccine has usually been suspended in physiological saline or buffer, but recently an emulsion in mineral oil (adjuvant vaccine) has been introduced. It is reported that the use of vaccine containing adjuvants results in greater antibody production, lasting

for a longer period of time and effective against a wider range of antigens, than does a vaccine in saline solution. While these observations hold promise, further studies are necessary before adjuvant vaccines can be recommended for general use. Moreover, the safety of the procedure is still in question, whereas the safety of saline or buffer vaccines has been established beyond doubt.

However, a major advantage of adjuvant vaccines might be their ability to produce immunity with very small quantities of virus antigen. This might be of critical importance in the rapid production of vaccines in the face of a virulent epidemic of influenza.

8.3 *Methods of use*

8.3.1 *Route of inoculation.* Saline or buffer vaccines may be injected either subcutaneously or intracutaneously. The advantages of the latter route are the smaller dose required for inoculation and the lessened risk of febrile reactions. However, further evidence is needed regarding the relative effectiveness of the appropriate doses given by the two routes in terms of antibody production.

8.3.2 *Time of immunization.* The evidence is that maximum protection obtained with saline vaccines appears to be limited to the period between the second and sixth week after inoculation. This should be taken into consideration by those authorities planning vaccination programmes.

8.3.3 *Risk of reactions.* Reactions both of a local and of a febrile character have occurred, particularly after the use of an unduly concentrated or imperfectly purified vaccine. With due care their incidence should be so low as to be of no importance. The dose of vaccine used for the immunization of children should, however, be reduced in relation to the age and body-weight. At all ages care should be taken not to inject the vaccine into egg-sensitive individuals.

There is no evidence that paralytic poliomyelitis has occurred as a consequence of influenza virus vaccination. Nevertheless, the risk has to be considered, and the committee recommends that wherever possible influenza vaccination should not be carried out during periods of prevalence of poliomyelitis.

8.4 *Potency tests*

A number of tests for the standardization of the potency of influenza virus vaccines have been utilized. These include, firstly, a mouse-protection test with pooled sera from groups of mice immunized with varying dilutions of the vaccine. This test, as used by the National Institutes of Health

(US Public Health Service), Bethesda, Md., USA, necessitates the employment of a mouse-adapted strain of virus in the neutralization test with the pooled sera. There are other disadvantages in the test, such as the biological variation in the immune response of individual mice.

Secondly, a neutralization test in eggs using sera from immunized mice. Non-mouse-adapted viruses can be used in determining the neutralizing power of the serum, which is a great advantage if vaccines are to be made from recently isolated strains.

The third method is immunization of groups of human volunteers and subsequent testing of their sera by various procedures. It is understood that the adaptation to virus vaccines of methods employed to standardize bacterial vaccines is under experiment. Other workers are engaged in trying to design a test on the lines of the assays used in the biological standardization of drugs. The committee considers that the lack of satisfactory means of standardizing influenza virus vaccines is a serious handicap to a comparison of different vaccines. Therefore, the committee recommends the comparison of the several procedures as they are evolved.

8.5. *Production methods*

These are largely the concern of manufacturers; and, in the present experimental state, no recommendation can be made. However, it is urged that the greatest possible purity of material should be maintained.

8.6 *Planning and assessing field trials*

The committee considered the question of planning and assessing field trials of influenza vaccines. It wishes to emphasize the importance of the proper selection of control groups, and the great difficulties met with in assessing the results. At present a decisive answer concerning the value of a vaccine can be obtained only in an outbreak with a relatively uniform infecting agent. The occurrence of other types of respiratory disease, not due to influenza virus, on any considerable scale vitiates the assessment of the vaccine on purely clinical grounds. Similarly, an alternative assessment by using serological methods of diagnosis of influenza has disadvantages such as the variation in individual antibody response and the fact that the test may fail to detect all cases of influenza, thereby weighting the assessment in favour of the vaccine.

It is understood that field trials on a large scale are in progress in the USA and in the United Kingdom, and the assessment of results is being made on the basis of clinical diagnosis with or without the aid of serological tests.

9. Collection and Distribution of Epidemiological Information Regarding Influenza⁵

It has already been pointed out that the virus of influenza shows no stability in nature, and that at present it is impossible to foresee when a serious epidemic may occur. If it should occur, the condition spreads with such rapidity that there is very little time in which to initiate control measures. The committee therefore stresses the need for national administrations to obtain early information of the occurrence of an outbreak of influenza within its territory, and within neighbouring territories and other countries with which it is in frequent contact, in order to give itself, and others, time for the institution of the practical measures which can be taken to meet the emergency created by an epidemic of the disease. Early knowledge of the strain of virus involved is also necessary if specific vaccines are to be used to protect selected groups in the community.

The committee agrees that the standard practice of notifying (reporting) individual cases, which is appropriate for diseases such as typhoid fever, is inadequate in the case of influenza because many cases are not notified, since they escape medical attention or because practising physicians are too overworked to report each case. It is also pointed out that in many countries influenza is not a notifiable disease; in others, only epidemics are reported; in still others, individual cases must be notified. Therefore, figures purporting to record actual numbers of cases mean very little, and there can be no comparison between the figures originating in different countries. What is needed is a way in which an abnormal prevalence of influenza (or influenza-like conditions) can be detected promptly and the course of the epidemic followed with the minimum time-lag. This might be done in a given area by the preparation in advance of a rough scale—to indicate the incidence of the disease—based upon local information which could readily be obtained, such as the daily records of absenteeism in one or more industrial plants, or among public transport

⁵ Attention is drawn to the following resolution adopted by the Executive Board at its third session:

“The Board heard with interest a statement . . . on the work carried out by the World Influenza Centre of WHO . . . during the recent influenza epidemic in certain European countries.

“The Board noted that it was desirable for national health administrations to obtain more rapid and detailed information—not only on the types of virus involved, but also on the epidemiology, the clinical description of the disease, the morbidity, mortality (with age groups affected), and any administrative action found to be of value in controlling the disease . . . It was decided that national health administrations should be asked to make such information available to WHO in Geneva for dissemination.”

(*Off. Rec. World Hlth Org.* 17, 15, Section 5.3.1)

workers ; absenteeism in schools ; the volume of new insurance claims where social insurance covers the bulk of the population ; requests for admission to hospital (especially for pneumonia), etc. A scheme by which a selected sample of practitioners voluntarily report to the local health authority the number of influenza-like diseases treated daily has recently been introduced experimentally in some countries and may prove very valuable. It is, of course, a matter for national health authorities to collect such information without delay from strategically located towns or areas. Such information should be notified promptly (telegraphically in time of epidemics) to the epidemiological services of WHO, which would in turn make the information available to other health administrations through its daily epidemiological broadcast bulletins and the several epidemiological weeklies issued and airmailed from Geneva, Washington, Alexandria, and Singapore. The information would also be passed on by WHO to the WHO network of influenza laboratories. This is necessary to initiate measures to isolate and identify the responsible virus. The results of these efforts would be made available as soon as possible to all national health administrations through the epidemiological services of WHO.

The committee considers it desirable to record here a brief outline of some of the existing arrangements under the WHO influenza programme.

Additional epidemiological information is obtained through the WHO network of laboratories. This information, which may refer both to the incidence of the disease and to the results of laboratory tests on sera and virus isolations, is sent (by telegram if necessary) in parallel both to the epidemiological services of WHO and to the reference laboratory in London. WHO distributes this information in the same way as that obtained from national health administrations. In the Region of the Americas special arrangements are considered necessary. From the USA and Canada the information is first collected by the appropriate influenza information centre and transmitted in parallel to the WHO Regional Office (Pan-American Sanitary Bureau), the reference laboratory in New York, and WHO Headquarters in Geneva. Elsewhere in the Region of the Americas information is sent in parallel to the WHO Regional Office, WHO Headquarters and the reference laboratory in New York. The reference laboratory in London is kept informed by the reference laboratory in New York as well as by the influenza information centres and by WHO Headquarters. In addition, newly isolated strains of virus are exchanged promptly between the two reference laboratories in order that an overall picture of the world epidemiology may be obtained.

10. Control Measures against Severe Epidemics of Influenza

10.1 *General measures*

The control measures which have been used in the past for combating particularly severe epidemics of influenza are based first of all on standard public-health procedures. These include quarantine (see below), restriction of movement of individuals, avoidance of crowds in cinemas, public meetings, etc., and, of most importance, the provision of extended hospital services. Other measures, such as the wearing of masks, and adequate ventilation and disinfection of the air (aerosols and ultra-violet light) in selected buildings are of more doubtful value. Public anxiety in the country affected and in other countries must be allayed as far as is practicable. In regard to quarantine, the committee felt that the isolation of groups within a community has not been shown to limit the spread of infection. Quarantine at a national level would be likely to be effective only in the case of islands and geographically isolated communities. The role of quarantine in regard to large countries or continents cannot be assessed while knowledge concerning the spread of influenza is still imperfect. There is no evidence, however, that in the case of such large areas quarantine could be applied effectively. The probable value of restriction of travel between countries can be judged in the light of the above remarks; and, particularly in the case of mild epidemics of influenza, it is totally unjustified.

10.2 *Role of vaccines*

The committee is hopeful of the use of vaccines in relation to epidemics, but the limiting factor in the utilization of this measure is the speed of production. Pharmaceutical houses are reluctant to prepare for storage supplies on a mass scale which in any case might not be of the proper antigenic type. It is clear, however, that if there was a dangerous world-wide epidemic it would be impractical to manufacture enough vaccine from eggs for universal use. Key persons could, however, be immunized; and economy in the quantity of vaccine might be attained by intracutaneous inoculation or by the use of adjuvants. The possible use of a live non-attenuated virus vaccine, either alone or with simultaneous injection of antibody, could hardly be recommended in epidemics such as those currently experienced but might be considered practicable in the face of a really lethal outbreak. In any case, virulent epidemics such as the 1918 pandemic particularly affected certain age-groups; and these might, therefore, be selected for attempted protection in such an event in the future. The committee recommends that WHO should carry out a survey of the potential production capacity of influenza vaccines at present available.

10.3 *Role of antibiotics*

Very large quantities of antibiotics might be needed in a severe epidemic of influenza for the treatment of the inevitable bacterial complications. Present stocks of antibiotics would probably prove inadequate, and there would be a danger of the imposition of export restrictions by individual governments. The possible need for rapid expansion of commercial plants for the production of antibiotics must be visualized. On the basis of present experience, the antibiotics which would be likely to be most useful would be, first, penicillin and, secondly, those derived from species of *Streptomyces*, including aureomycin, oxytetracycline,⁶ and chloramphenicol. The possible value of sulfonamides is more difficult to assess but should not be ignored.

It should be emphasized, finally, that the responsibility for mortality in influenza epidemics probably depends in part upon the character of the virus. The exact measure of success to be expected with antibacterial agents in a virulent pandemic is therefore to some extent unpredictable.

11. Therapeutic Measures in Influenza

There is at present no specific treatment available for the treatment of influenza virus infections. In recent epidemics the mortality has been related to bacterial complications. But there is a gap between the academic knowledge of the nature and treatment of complications and the practical treatment of individuals in their homes. An example of this exists in the widespread use, in certain areas, of sulfonamides for the treatment of severe influenzal pneumonia at home. This may cause undue delay before the institution of the effective antibiotic treatment needed in such cases. It is clear from recent experience that the bacterial infections chiefly to be feared are those caused by the staphylococcus. In contrast to the usually favourable response in pneumococcal infections to the exhibition of penicillin, influenza virus staphylococcal-pneumonia may be relatively unresponsive to this and other antibiotics. Therefore, high dosage of penicillin at frequent intervals is essential, and treatment must be commenced at a very early stage of illness. Experience with other antibiotics, such as aureomycin, oxytetracycline and chloramphenicol, is insufficient for a statement to be made on their effectiveness as compared to penicillin; but, in the case of infection with penicillin-resistant organisms, one or another should, of course, be used. The committee considers that further data as

⁶ Oxytetracycline is the international non-proprietary name for "Terramycin" (see *Chron. World Hlth Org.* 1953, 7, 41).

to the nature of the species of bacteria concerned in influenzal pneumonia should be collected from as many countries as possible.

The committee feels that publication by WHO of educational papers concerned with the diagnosis and treatment of the complications of influenza would be most valuable.

12. Training of Laboratory Workers

The present facilities for training laboratory workers at the reference centres of the WHO network of influenza laboratories are limited to one person per year. This is clearly inadequate, particularly because there are countries without any laboratory workers properly trained for influenza virus work. It is therefore recommended that WHO should explore the possibility of increasing the facilities, having regard to the fact that the minimum period of training which is of any value is at least one year.

13. Exchange of Publications

The committee considers that publications from panel members and laboratories within the WHO influenza network should be freely distributed within the network in order that the maximum interchange of information should be attained as speedily as possible. It is pointed out, however, that approximately 80-100 copies of each publication would be needed and that it might not be possible for the cost of these copies to be borne by the laboratory from which they originate.

The committee also suggests that a list of recent publications should be sent by WHO at regular intervals to all panel members and laboratories within the WHO influenza network.

14. General Conclusions

The bearing of antigenic variation of influenza viruses upon the occurrence of clinical influenza in the partially immune population and upon the effectiveness of the vaccine has been clearly demonstrated. Research during the past few years has led to the development of satisfactory methods for the rapid antigenic analysis of these agents, and it is felt that principal emphasis may now be given to the application of this basic knowledge in the study of the epidemiology of this disease. Consideration should also be given to an elucidation of the factors, besides antigenic composition

of the virus, which may be of importance in the generation of disease in the human host. Foremost among these are the characters responsible for virulence of the particular virus strain and the non-specific factors which may condition the resistance of the host. The possible role of non-specific inhibitor in limiting the spread of virus in the respiratory tract and of bacterial flora in the enhancement of parasite virulence or reduction of host resistance are worthy of consideration in this regard. Such factors may have particular bearing upon the generation of the pandemic form of this disease.

It is the committee's opinion that the best approach to the prevention of influenza, at the present time, lies in prophylactic immunization, and that little hope may be expected from quarantine procedures, except perhaps in remote and isolated areas. The methods for preparing vaccine are still in the developmental stage, and further research will be needed to select the proper strains for inclusion in the vaccine, to develop appropriate production and quantitative potency-assay procedures, and to determine the best method for administering the material to the human host. Considerable hope is expressed in the usefulness of adjuvants in broadening the antigenic response to the strains in the vaccine and in extending the duration of immunity. While it is realized that the available supply of vaccine will greatly limit the number of persons who can be immunized, there is hope for increase in this respect through reduction of the dose of virus by use of adjuvants.

Methods which may assist materially in the diagnosis of influenza are at present available in the complement-fixation and haemagglutination-inhibition tests. While the haemagglutination-inhibition technique has certain advantages over the complement-fixation test as a research tool, it is to be noted that the complement-fixation procedure is simpler and superior for routine purposes.

Significant scientific advance has been made in the past in the study of influenza and may reasonably be expected in the future through the efforts of private or institutional initiative. There are certain areas of activity, however, in which the accomplishment may be greatly augmented through active participation of WHO:

- (1) First is the continued and expanded programme for collection, correlation, and distribution of information regarding occurrence, epidemiology, and laboratory findings from places not presently covered. Such information is needed to provide the bases upon which methods for prevention and control of influenza may be developed. There is also need for greater and more rapid dissemination and exchange of scientific information among the various workers throughout the world. Provision for purchase and distribution of reprints of published papers may assist materially in this regard.

(2) Second is the provision for training of qualified persons who will collect essential information on strain occurrence and epidemiological data from areas of the world in which there is no representation at the present time. Means should also be found to provide support and laboratory space for the trained person in his area of observation.

(3) Third, provision should be made to supply diagnostic materials to designated laboratories which are unable to supply their own. It is felt that such material should be prepared in a single laboratory (preferably commercial) and that it be distributed free of charge to the user. Consideration should also be given to the supplying of antisera to the basic prototype strains for rapid preliminary analysis of newly isolated strains in these laboratories.

Another item of supply urgently needed is a suitably insulated shipping container of light weight which may be used for transport of frozen virus or clinical specimens by air.

Annex 1

**METHODS OF PREPARATION OF ANTISERA
FOR THE COMPARISON AND TYPING OF STRAINS
OF INFLUENZA VIRUS****1. Preparation of ferret antiserum**

After a preliminary removal, under anaesthesia, of blood from the heart, two ferrets are inoculated intranasally with 1 ml of a 10^{-2} dilution of freshly harvested, infected, allantoic fluid. The animals are kept under strict isolation and are bled out 12 days after the original inoculation. To each serum after separation 1/10,000 merthiolate is added, and the sera are then stored at 2°C. Alternatively the sera may be stored at -70°C or lyophilized.

After treatment with cholera filtrate, the pre-infection serum specimen is tested for influenzal antibody in order to exclude natural infection of the ferret prior to inoculation. The post-infection serum can be used for strain analysis only if the pre-infection serum is devoid of specific inhibitory activity in the haemagglutination-inhibition test.

Treatment with cholera filtrate as prepared below (see page 22) is considered necessary by some workers. One volume of serum plus four volumes of crude cholera filtrate are incubated overnight at 37°C; the mixture is then heated for 1 hour at 56°C in order to destroy cholera enzyme activity. Other workers (Burnet & Stone¹) recommend treatment of ferret antiserum with the receptor-destroying enzyme of *Vibrio cholerae*, which is prepared from cholera filtrate by absorption with 5% fowl cells for 1 minute at 2°C followed by elution into normal saline for 30 minutes at 37°C. The eluate is diluted in acetate buffer, pH 6.0, and titrated for its ability to destroy non-specific inhibition in normal ferret serum using a technique similar to that described below. The eluate is then used with immune ferret serum at the highest effective dilution. This dilution is mixed with serum and the whole incubated overnight at 37°C, followed by heating at 56°C for 1 hour.

2. Preparation of rabbit antiserum

The antigen consists of infected allantoic fluid from embryonated eggs. Ten-day-old eggs are inoculated into the amniotic sac with 0.25 ml of a 10^{-4} dilution of high-titre, egg-adapted seed virus. The inoculated eggs

¹ Burnet, F. M. & Stone, J. D. (1947) *Aust. J. exp. Biol. med. Sci.* **25**, 227.

are incubated for three days, after which they are cooled and the allantoic fluids are removed and tested for haemagglutinating activity. Fluids of high haemagglutinating titre are pooled and tested for bacterial contamination (no antibiotic or other antibacterial material is employed).

Healthy young adult rabbits of either sex are inoculated intraperitoneally three times at weekly intervals with 5-ml quantities of whole, infected allantoic fluid, and are bled one week after the third inoculation.

The crude serum is treated for non-specific inhibitors in the manner described below.

3. Preparation of fowl antiserum

The antigen is derived from embryonated eggs, previously incubated for 9 days at 39°C, which are inoculated with 0.4-ml amounts of the appropriate dilution of seed virus (usually 10^{-4} infected allantoic fluid) into the allantoic cavity. After inoculation the eggs are incubated at 35°C for 40-44 hours, and the allantoic fluids are harvested following chilling in the refrigerator. The haemagglutination titre of the fluid for immunization should be at least 1/320 (initial dilution); titres of 1/640 or 1/1280 are desirable.

Following preliminary bleeding, 5-8 pound (2-3½ kg) roosters (cockerels) are injected with 5 ml of the infected allantoic fluid intravenously and 10 ml intraperitoneally. For newly isolated strains in which the haemagglutinin titres may be low (1/320) it is best to repeat the injections on the following day.

Ten days after the initial injection, the chickens are bled from the heart. (With large roosters, 80 ml can be removed on 3 successive days.) The blood is allowed to clot, is cut into small pieces, and is stood overnight in the ice-box, followed by 5 or 6 hours at room temperature (to aid contraction of clot). The serum is removed, inactivated at 56°C for 35 minutes, and lyophilized in 0.5-ml or 1-ml amounts and stored at +4°C. Storage of the undried serum at -20°C may also be acceptable.

The pre- and post-immunization serum specimens are titrated against the homologous antigen in order to determine the agglutination-inhibition titre. The immune serum should have a titre of at least 1/800 (initial dilution) with homologous antigen; 1/400 may prove acceptable. The pre-vaccination serum should not inhibit in a dilution of 1/50. Most viruses are not inhibited by normal rooster serum diluted 1/50, but exceptionally a non-specific inhibition titre of 1/200 may be encountered with newly isolated strains. For strain analysis, sera must be treated to remove non-specific inhibitor by incubating for 6 hours at 37°C, with 4 volumes of crude cholera filtrate prepared as indicated below. The

cholera enzyme activity is finally destroyed by heating at 56°C for 50 minutes, and there is no demonstrable loss of antibody.

Strain analyses are carried out by the standard haemagglutination-inhibition method, using human red cells. In strain analysis, it is essential that the tests be read after a standard incubation period such as 55 minutes.

4. Preparation of crude cholera filtrate for the destruction of inhibitors

Preparation of culture

The strain, *V. cholerae* 4Z (Burnet & Stone) is cultivated in nutrient agar containing 2% agar and 2% peptone, pH 7.6, subcultures being made every 3 weeks. Subcultures yielding a potent enzyme should be lyophilized as the potency in terms of enzyme production is inconstant and may be decreased by serial subculture.

The seed culture for the preparation of a filtrate is a 6-8-hour growth in nutrient broth containing 2% peptone at pH 6.9. From this culture agar plates containing 0.8% agar and 2% peptone (pH 6.9) are inoculated and incubated for 16 hours. A heavy growth should be obtained. The agar is scraped off the plates, pressed through sterile gauze (8 layers), the liquid thereby obtained being subsequently filtered through an asbestos bacterial filter. The filtrate, whose final pH should not exceed 7.6, is stable for long periods at 2°-4°C, but should be tested at frequent intervals for potency as indicated below. The quality of the peptone used in the media is a most important factor in preparing a potent filtrate. Different brands of available peptone should be compared, and that found to be most effective should be desiccated and sealed in vacuo. It may then be stored at 4°C.

Potency test

The test for potency of the filtrate is carried out by mixing normal serum with the filtrate in a proportion of 1 of serum to 4 of filtrate, incubating overnight at 37°C, after which the mixtures are heated for 1 hour at 56°C. Sera from ferrets, rabbits, or fowls are all suitable but require separate titrations.

With the treated material, haemagglutination-inhibition tests are set up against two strains of virus. The viruses² are selected so that one is mouse-adapted and yet retains its sensitivity to inhibitor, and the other a recently

² Strains of virus which have proved satisfactory in the above test are the mouse-adapted strain A/Nederland/1941 and the egg-adapted strain A-Barrett/(England)/1947.

isolated influenza A strain of the FM1 group, cultivated only in eggs, which is also highly susceptible to inhibitor.

The actual test is carried out by mixing one volume of test virus containing 4 haemagglutinating units and two volumes of the undiluted serum-filtrate mixture. The whole is placed at 2°-4°C for half an hour, and one volume of 2% fowl red-cells in saline is then added. The final dilution of serum in this mixture is 1/10. At this strength of serum no inhibition of haemagglutination should be obtained with either virus. Dilutions of the serum filtrate mixture are usually prepared and tested at the same time as an indication of the relative potency of weak filtrates. The procedure of mixing virus and serum together half an hour before adding the red cells greatly increases the sensitivity of the test for inhibitor.

Because some batches of cholera filtrate may contain excessive amounts of calcium, which will stabilize to heat the red-cell receptor-destroying enzyme, it is necessary to include appropriate controls to ensure that the period of heating at 56°C has, in fact, produced inactivation. If not, the batch should be discarded.

Annex 2

TECHNIQUES RECOMMENDED FOR STANDARD DIAGNOSTIC PROCEDURES

1. Complement-Fixation Test

The following technique is based upon the procedure recommended by Hoyle,¹ but the antigens are prepared from infected eggs instead of mouse lungs as in the original method.

1.1 Reagents

1.1.1 *Antigens*

The antigens are prepared in one of two ways, both of which have been found satisfactory in different laboratories.

The PR8 or WS strain of influenza A and the Lee strain of influenza B are used in both methods.

Method A

Chick embryos are inoculated allantoically after 10 or 11 days' incubation with 0.1-ml amounts of a 10^{-3} or 10^{-4} dilution of infected allantoic fluid. After 42-48 hours' further incubation at 35°C, the membranes are removed and suspended in physiological saline (1 ml per membrane), frozen rapidly in a mixture of alcohol and dry-ice, and allowed to thaw out slowly at room temperature. The freezing and thawing are carried out three times, and the suspension then centrifuged at 3,000 r.p.m. for 15 minutes. The supernatant fluid is removed, and 2% chloroform is added to it. This is shaken up thoroughly and allowed to stand overnight at 4°C. The suspension is again centrifuged and the supernatant removed and used as the source of soluble antigen. It can be stored at 4°C, but it is recommended that the material should be dried from the frozen state for storage for periods longer than one month.

Method B

Seed. Nine-day embryonated eggs are inoculated with 0.4-ml amounts of a 10^{-4} dilution of virus-infected allantoic fluid. Following further incubation at 35°C for 40-44 hours, the eggs are chilled and the allantoic

¹ Hoyle, L. (1948) *Mon. Bull. Minist. Lab. Serv.* 5, 114

fluids removed. The fluids, which should titre 1/640 to 1/1280 by the haemagglutination method, are used immediately or stored for an indefinite period in glass-sealed ampoules in the dry-ice box at -70°C .

Preparation of antigen. Eleven-day embryonated eggs are inoculated into the allantoic cavity with 0.5-ml amounts of the undiluted allantoic fluid described above. After 6 hours' incubation at 35°C , the eggs are opened and the chorio-allantoic membranes are removed. These are thoroughly washed in 3 separate vessels of physiological saline solution, drained on blotting paper, and weighed. They are then ground in a Waring blender for 3 minutes with an equal weight of physiological saline solution to give a 1/2 dilution. The material is then centrifuged at 3,000 r.p.m. for 10 minutes to sediment the tissue, and the supernatant is removed. Infectivity is destroyed by minimal ultra-violet irradiation, the exact conditions for which will need to be worked out for the individual lamp. Tests for infectivity are made in eggs, using antigen undiluted and diluted 10^{-1} and 10^{-3} to obviate interference effects.

Normal control-antigens are prepared from uninoculated 11-day embryonated eggs in an identical manner.

These preparations are stable for an indefinite period when stored at -20°C or at 4°C .

1.1.2 Sera

These are inactivated at 56°C for 30 minutes. If not used at once, either they should be stored at -20°C , or 0.08% sodium azide should be added and the sera kept in the refrigerator (4°C).

1.1.3 Complement

Pooled guinea-pig serum is stored at -70°C . At this temperature the complement maintains its titre over a long period.

In the absence of storage facilities, complement may be preserved by the boric-acid sorbitol azide method of Richardson.²

Solution A

Boric acid (H_3BO_3)	1.55 g
Saturated NaCl	to 100 ml

Solution B

Sorbitol ($\text{C}_6\text{H}_{14}\text{O}_6, \frac{1}{2} \text{H}_2\text{O}$)	9.55 g
Sodium azide (NaN_3)	0.81 g
Saturated NaCl	to 100 ml

² Richardson, G. M. (1941) *Lancet*, 2, 696

Solution C

Sodium azide	0.81 g
Saturated NaCl	to 100 ml

To each 8 ml of serum add 1 ml of solution A and 0.5 ml each of B and C. Store in the refrigerator, and for use dilute 1/8 with distilled water; this gives a 1/10 dilution of complement.

1.1.4 *Diluting fluid*

Physiological saline is used in connexion with the above antigens, though other types of antigens prepared from eggs may require the use of buffers such as that recommended by Mayer et al.³

1.1.5 *Haemolytic system*

A 2% suspension of packed sheep cells is used, and to this is added an equal volume of haemolysin dilution containing 10 Minimal Haemolytic Doses (MHD).

1.2 Titration of complement

A series of tubes is set up containing 0.2 ml of complement dilution (1/10, 1/15, 1/20, 1/25, 1/30, 1/35, 1/40, 1/50, 1/60, 1/70, 1/80, 1/90, 1/100). To each tube are added 0.4 ml saline and 0.4 ml sensitized red blood-cells. The tubes are incubated in the water-bath at 37°C for 30 minutes. A reading is made of the highest dilution giving 100% haemolysis, and also of the highest dilution giving 50% haemolysis (made by throwing down the cells by centrifugation and matching the supernatant fluid against a 1/2 dilution of a tube giving complete haemolysis). For use in complement-fixation tests the complement is diluted so as to contain in unit volume 2½ MHD by the 100% haemolysis reading or 4½ MHD by the 50% haemolysis reading. These two readings usually correspond exactly; when any discrepancy occurs, the 50% haemolysis titre is the more accurate.

Preserved complement retains its titre unchanged for some weeks. After it has once been accurately titrated, it is better to use the same dilution each day rather than to titrate it afresh every time a test is done. In other words, it is better always to use the same amount of complement and to ignore minor variations in sensitivity of the haemolytic system.

³ Mayer, M. M., Osler, A. G., Bier, O. G. & Heidelberger, M. (1946) *J. exp. Med.* **84**, 535

1.3 Titration of antigen

Each batch of liquid antigen prepared is titrated against an excess of a known positive serum. Tubes are set up containing 0.2 ml of antigen dilution (1/1, 1/2, 1/4, 1/8, 1/16, 1/32), 0.2 ml of complement, and 0.2 ml of serum dilution (e.g., serum titre 1/64 would be used diluted 1/8). Tubes are incubated for 1 hour in the water-bath at 37°C; 0.4 ml of sensitized cells are added; the tubes are incubated again for 30 minutes, the cells allowed to settle, and readings made. Any tube showing less than 50% haemolysis is regarded as positive; 50% haemolysis or more is read as negative.

This test gives a reading of the maximal antigen titre. The optimal amount of antigen for use in serum titrations is three times the maximal titre, but in practice four times the maximal titre is used, as it is better to have a slight excess of antigen rather than too little.

1.4 Standard test for titration of antibody in serum

Each tube in the test contains 0.2 ml antigen, 0.2 ml complement, and 0.2 ml serum dilution.

For normal sera, serum dilutions of 1/2, 1/4, 1/8, 1/16 are used, for convalescent sera dilutions of 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, 1/128, 1/256.

In addition, a serum control is set up in which a serum dilution of 1/2 is used and the antigen is replaced by saline.

A fixation period of 1 hour at 37°C is used, the tubes being incubated in the water-bath and shaken after 30 minutes. After 1 hour, 0.4 ml of sensitized cells are added, the tubes are incubated for 30 minutes, the cells allowed to settle, and readings made.

Tubes showing less than 50% haemolysis are read as positive, and 50% haemolysis or more as negative, except in the case of the 1/2 dilution of serum where a reading of exactly 50% haemolysis is read as doubtful.

Sera may therefore be regarded as negative, doubtful (1/2), or positive at titres ranging from 1/2 to 1/256.

1.5 Small-volume and micro-tests

The standard test requires the use of large volumes of antigen. When testing numerous sera, it may be desirable to use smaller volumes of reagents.

<i>Standard test</i>		<i>Small-volume test</i>		<i>Micro-test</i>	
Antigen	0.2 ml	Antigen	0.1 ml	Antigen	0.05 ml
Complement	0.2 ml	Complement	0.1 ml	Complement	0.05 ml
Serum dilution . .	0.2 ml	Serum dilution . .	0.1 ml	Serum dilution . .	0.05 ml
Sensitized cells . .	0.4 ml	Sensitized cells . .	0.2 ml	Sensitized cells . .	0.1 ml
Total volume . . .	1.0 ml	Total volume . . .	0.5 ml	Total	0.25 ml

For the micro-test some form of constant-volume pipette is necessary to deliver 0.05 ml. With care the micro-method can be made almost as accurate as the standard test.

The test using 0.1-ml volumes of antigen, complement, and serum and 0.2 ml of sensitized cells has been found both satisfactory and economical in reagents and can be recommended.

2. Haemagglutination-Inhibition Test

2.1 Materials Required

1. Saline solution (0.85% NaCl in distilled water)
2. 2% sodium citrate (in saline solution)
3. 1-ml serological pipettes, graduated in 0.01 ml
4. Kahn tubes (12×75 mm) with evenly rounded bottoms; and suitable racks
5. Standard erythrocyte suspension (see section 2.2)
6. Standard antigens (see section 2.3)
7. Standard antisera (see section 2.4)

2.2 Standard erythrocyte suspension

The standard suspension should consist of 0.5% chicken erythrocytes in saline solution (human "group O" erythrocytes may be substituted if desired).

Obtain blood from wing vein or from heart and add immediately to 0.2 volume of 2% sodium citrate. The citrated blood may be preserved for not longer than 1 week at 4°C.

The standard erythrocyte suspension is prepared fresh for each day's work in the following manner. Wash approximately 10 ml of the citrated blood three times, each time with approximately 50 ml of saline solution. After the third washing, suspend the cells in approximately 10 ml of saline solution and transfer to a 15-ml conical, graduated, centrifuge tube. The tube should be held in a 50-ml centrifuge cup (No. 320, International) employing a reducing cap (No. 312, International) to adapt the cup to the 15-ml tube.

Centrifuge in a horizontal centrifuge at a gravity force of 3,200 for 10 minutes. Determine the volume of cells by direct reading of the tube graduations; discard the supernatant fluid; and then suspend the cells in enough saline to make a 0.5% suspension (199 volumes).

2.3 Standard antigens

For the present, it is recommended that antigens should be prepared from type A PR8 and FM1, and Lee or Bon strains of B virus. Each should be from a single pool of allantoic fluid. The pooled allantoic fluid should be from eggs inoculated with seed material selected for the purpose, and harvested from eggs containing embryos not older than 12 days.

In order to lessen the formation of precipitate on standing, the pooled allantoic fluids should be diluted 1/4 in saline solution and should contain 1/10,000 merthiolate as a preservative. This 1/4 dilution containing 1/10,000 merthiolate is the "stock antigen" and should titre not less than 1/64. It must be stored in a refrigerator at approximately 4°C.

2.3.1 *Haemagglutinating unit*

The haemagglutinating unit is contained in 0.25 ml of the highest dilution of stock antigen which completely agglutinates the standard chicken erythrocyte suspension. The unit should be determined as follows:

1. Remove 1.0 ml of stock antigen from the container, and dilute with 3.0 ml of saline solution. This is the "working dilution".

2. Set up two rows of Kahn tubes, 10 tubes in each row. Place 1.0-ml amounts of saline solution in each tube of the front row. Then prepare and dispense serial twofold dilutions as follows:

With a 1-ml pipette, add 1.0 ml of the working dilution to the first tube of the first row; discard the pipette. Mix well with a fresh pipette, and transfer 1.0 ml of the mixture to the second tube of the first row, and 0.25 ml to the first tube of the back row; discard the pipette. With a fresh pipette, mix the contents of the second tube of the first row, and then transfer 1.0 ml of the mixture to the third tube of the first and 0.25 ml to the second tube of the back row. Continue through the series, until each tube of the back row contains 0.25 ml of serial twofold dilutions. Discard front row of tubes.

3. To each tube, add 0.25 ml of saline solution (to bring the volume up to 0.5 ml).

4. Add 0.5 ml of the 0.5% chicken erythrocyte suspension to all tubes.

5. Include an erythrocyte control of 0.5 ml of saline solution and 0.5 ml of the erythrocyte suspension.

6. Mix well by shaking.

7. Incubate at 22°-24°C; after 60 minutes, read the results on the basis of the pattern. (In hot countries where room temperature is over 24°C the alternative of an ice-box technique (0°-4°C) should be permitted, results to be read after 2 hours, immediately after removal from the cold.)

The 0.25-ml quantity of the highest dilution of the working dilution which completely agglutinates the standard erythrocyte suspension is one unit (i.e., contains one unit of activity).

2.3.2 Test antigen. The test antigen should contain four haemagglutinating units of virus. This is provided by diluting the working dilution so that it will be four times more concentrated than the highest dilution of the working dilution which showed complete haemagglutination in the above test.

Example : If the greatest dilution of the working dilution which completely agglutinated the erythrocyte suspension was 1/64 (6th twofold dilution), the desired dilution of the working dilution is 64/4, that is, 1 part working dilution, plus 15 parts of saline solution.

The antigen so prepared (test antigen) should be tested in the manner described above (*haemagglutinating unit*, paragraphs 2, 3, and 4) in order to ensure that it contains 4 haemagglutinating units. Complete agglutination of erythrocytes must be present in the first two tubes of the series, and the third tube must show less than complete haemagglutination.

2.4 Standard antisera

Sera with known inhibitory properties against each of the test antigens should be included in the test.

2.5 Test for haemagglutination-inhibiting antibody content of sera

Pairs of sera are absolutely necessary for diagnostic tests. The antibody titres of unknown sera are determined by comparing in the same test the pairs of sera, and known antisera, against the standard antigens, in the following manner :

(1) All test sera should be inactivated at 56°C for 30 minutes.

(2) For each serum to be tested, set up four rows of Kahn tubes, 10 tubes in each row. Add 1.0 ml of saline solution to each tube of the first row. To the first tube of the first row, add 1.0 ml of a 1/8 dilution of the serum (0.2 ml of serum plus 1.4 ml of saline solution). Mix well, and transfer 1.0 ml of the mixture to the second tube of the first row, using a 1-ml pipette. Mix well, and transfer 1.0 ml of the mixture to the third tube of the first row. Continue through all ten twofold serial dilutions of this row. With the same pipette, now distribute 0.25 ml of the 10th twofold dilution to each of the last tubes of the other three rows. Then transfer 0.25 ml of the 9th dilution to each of the corresponding tubes of the other three rows. Continue with the 8th, 7th, and other dilutions

until all have been distributed. The tubes in the front row are discarded and not used in the final test. If it is desired to use two strains of influenza virus B, the volumes indicated will need to be modified accordingly.

(3) Prepare suitable dilutions of the known antisera.

(4) After the serum dilutions have been prepared, add 0.25-ml amounts of each of the test antigens to each tube of the appropriate row.

(5) To the serum-virus mixtures, add 0.5 ml of the 0.5% erythrocyte suspension.

(6) Shake well and examine to be sure that all cells are well suspended.

(7) Incubate at 22°-24°C for 60 minutes, and then read on the basis of the pattern of the settled cells.

The titre is expressed as the highest initial dilution of serum which effects complete inhibition of agglutination.

The test antigens must be retitrated at the same time that the haemagglutination-inhibition tests are performed, in the manner described for the retitration of the test antigen under the *Haemagglutinating unit*, above; again, complete agglutination should occur in the first two tubes of the series of twofold dilutions, and the third tube should show less than complete agglutination.

Titration of virus and antisera may be modified to some extent according to individual preferences. The use of individual pipettes in preparing serial dilutions of antigens or antisera is not necessary, provided adequate care is taken in the use of a single pipette.

3. Haemagglutination-Inhibition Test in Influenza C Infections

The diagnostic haemagglutination-inhibition test for influenza C has not been entirely satisfactory in all hands, primarily because of the difficulty in preparing antigen in the amniotic cavity. The test has, however, met with success in some laboratories; and a procedure found satisfactory is given below:

Influenza C virus grows poorly in the allantoic cavity of embryonated eggs, but the virus can be propagated in high titre in the amniotic sac. The 1233 strain of virus is preferred since it has been found to give higher titres than some of the other strains. Embryonated eggs, incubated for 10 days at 39°C, are inoculated into the amniotic cavity with 0.1 ml of a 10^{-2} dilution of suitable seed virus preparation. After further incubation at 35°C for 26-30 hours, the eggs are chilled and the amniotic fluids are harvested; these materials are used immediately or stored in 0.5-ml amounts in lyophilized form.

Antiserum to influenza C virus can be prepared in roosters using the basic technique outlined above in Annex 1. However, a single intravenous injection of 2.5 ml and 2.5 ml given intraperitoneally will usually suffice. Haemagglutination-inhibition titres as high as 1/25,600 have been obtained. Since all sera tested to date, both human and animal, have appeared to be free of non-specific inhibitors, cholera filtrate should not be used.

Haemagglutination and haemagglutination-inhibition titrations are performed by a modification of the standard haemagglutination-inhibition technique described in this annex. Because influenza C virus rapidly elutes from red cells at room temperature, titrations of serum and antigen must be made in the cold (4°C). The virus titrations are read after 75-90 minutes, incubation, and the haemagglutination-inhibition tests after 60-70 minutes. Human red cells are employed in the tests.

4. Modification of the Haemagglutination-Inhibition Test for Use with Plastic Plates

Titrations of virus and of sera can be carried out in plastic plates instead of tubes.

Serial twofold dilutions (0.25 ml⁴) of virus are prepared in physiological saline and equal volumes of 0.5% fowl cells are added. Readings are made by the pattern method and the end-point taken as partial (50%) agglutination. The 50% end-point is determined by observing the pattern made by a 1/2 dilution of virus showing just complete agglutination. Where partial (50%) agglutination does not show in any dilution, the end-point is determined by interpolation.

For titration of serum antibodies, serial dilutions of serum (0.25 ml) are made in saline and an equal volume of red cells added, followed immediately by a third volume of antigen. The antigen is made up to contain 8 partial agglutinating doses of virus per 0.25 ml, and a control test of the concentration of virus actually present is carried out along with the anti-haemagglutinin test. Partial agglutination is taken as the end-point, and interpolations for the end-point are again made where necessary.

⁴ The volumes given are those used in conjunction with the plates as distributed by the World Influenza Centre. Plates with different-sized cups will require different volumes.