THE GENETIC CHARACTER OF O–D CHANGE IN INFLUENZA A.

F. M. BURNET, JOYCE D. STONE, A. ISAACS* AND MARGARET EDNEY.†

From the Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia.

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In 1943 Burnet and Bull showed that fluids obtained from initial isolations of influenza A virus by the method of amniotic inoculation gave unusual haemagglutination reactions. Fowl cells were not agglutinated or showed a very low titre of incomplete agglutination, but human or guinea-pig cells were agglutinated to a titre in conformity with the infectivity of the fluid. By transfer in the amniotic cavity at limiting dilutions it is possible to maintain the original, O phase, character. One of the 1942 strains, BEI, is still available in this laboratory in the O form. Without these precautions passage by any method in the chick embryo speedily results in the appearance of the D phase, agglutinating fowl cells to the same titre as human or guinea-pig cells.

In all the isolations of influenza A that have been made in Melbourne since 1942 virus was first obtained in the O phase. Workers in other laboratories have for the most part confirmed this finding (Dudgeon, Mellanby, Glover and Andrewes, 1948). A number of isolations have, however, been reported by the use of allantoic inoculation, with recognition of the virus by fowl cell agglutination (Rickard, Thigpen and Crowley, 1944). Nevertheless, all are agreed that allantoic inoculation is far less effective than inoculation of 13-day embryos in the amniotic cavity, and an interpretation of the significance of the results of Rickard *et al.* has already been published (Burnet and Stone, 1945*a*).

There has been no confirmation from other laboratories of maintenance of a strain indefinitely in the O phase by passage at limiting dilutions. This is necessarily a time-consuming and difficult task not likely to be undertaken by anyone not deeply interested in the specific problems. The D phase or an intermediate always appears at each passage and correct dilutions and appropriate timing of harvesting are needed to maintain the O character. In the light of our experience, a strain whose characters can be readily maintained by chick embryo passage is not in the O phase.

The stimulus to publish the work reported in this paper was the appearance of Magill and Sugg's (1948) paper in which it is claimed that the O–D change has no genetic significance, but represents merely a change which can be produced at will by altering the ionic environment of the virus. Briody (1948), in this laboratory, had previously shown that by simple heating, some strains with O type of agglutination reactions could be converted into material agglutinating fowl cells.

It seemed to us desirable that we should describe our own studies of this type on various O and intermediate phase strains of influenza A. It will be shown

* Rockefeller Travelling Research Fellow.
† Francis Haley Research Officer.

that influenza A virus can exist in a form which maintains its O character despite the application of any of these methods. It can be converted to the D only by the occurrence of an appropriate mutation in the course of multiplication in chick embryo tissues. It is only intermediate forms which can be modified *in vitro* to give O or D reactions.

We would point out that in our original paper O phase is ascribed to many strains which we should now regard as intermediates. In a more recent paper (Anderson and Burnet, 1947) a strain IAN–O was described which was said to be capable of growing in the O-phase in the allantoic cavity. This interpretation will be shown to be wrong. The strain is, in fact, a particularly good example of the intermediate type of virus which allows the various changes described by Magill and Sugg to be demonstrated.

MATERIALS AND METHODS.

Viruses.

BEL.—Influenza A strain isolated during Victorian epidemic, 1942.

O and intermediate (false O) phase virus was obtained by amniotic inoculation of material which had been maintained in the O phase during 26 amniotic passages at limiting dilutions (method of Burnet and Stone, 1945*b*).

D phase virus had received numerous allantoic passages.

IAN.—Influenza A strain isolated in 1946 from a sporadic case of influenza in Melbourne. The virus used was the substrain previously described as in the *O phase*. It was prepared by allantoic inoculation of material which had received 6 allantoic passages since its isolation in the amniotic cavity. Its properties are described by Anderson and Burnet (1947).

Oc.I.—Influenza A strain isolated from an epidemic in Ocean Island, 1948.

O and intermediate (false O and δ) phase virus was obtained during the first or second amniotic passage of the strain.

The following strains were propagated in the allantoic cavity :

WSE.—Egg-adapted substrain of the classical WS (influenza A) strain.

MEL.—Melbourne strain of influenza A.

LEE.—Classical strain of influenza B.

SW.—Shope's strain 15 of swine influenza virus.

NDV.—Newcastle disease virus isolated by Albiston and Gorrie, 1931.

Haemagglutination titrations.—Equal volumes (0·25 ml.) of virus dilutions and 1 per cent red cells were mixed and allowed to settle. Readings were made by observing the pattern of sedimented cells as described by Burnet (1942). Partial (+) agglutination was taken as the end-point. Tests were carried out at room temperature or at 4° C. as specified in the text. Comparative titrations were made with fowl and guinea-pig cells and the titres are expressed as ratio of the two end-points, F/G.

Amniotic inoculation.—The techniques have been described in previous communications from this laboratory. After inoculation the eggs were incubated for 40–42 hours at 35° C. Samples of amniotic fluid were then withdrawn at regular intervals until fluids were obtained which agglutinated human cells ; the amniotic fluids were then harvested. The lungs were removed, ground with alundum, suspended in 1 ml. normal (0·9 per cent) saline and lightly centrifuged. The supernatants were used as lung emulsions.

Receptor-destroying enzyme of V. cholerae (RDE) was prepared by the method of Burnet and Stone (1947).

EXPERIMENTAL.

Character of Variants of a Recently Isolated Strain.

The characteristics of an epidemic of influenza in Ocean Island in October, 1948, will be described and the isolation of the virus reported elsewhere.

A detailed study was made of one strain, isolated in the first passage in the form of amniotic fluid with an F/G titre of $<10/480$ when tested at room temperature. When this fluid was titrated in eggs by amniotic and allantoic methods in parallel the results obtained were as shown in Table I.

TABLE I.—*Results of Passage of Ocean Island O Virus by Amniotic and Allantoic Routes at Different Dilutions.*

Dilution of inoculum.	Amniotic route.		Allantoic route.	
	Number infected.*	Phase.†	Number infected.*	Phase.†
10^{-7}	1/5	0	—	—
10^{-6}	4/4	000	0/6	—
10^{-5}	8/8	000	0/6	—
10^{-4}	5/6	000	1/6	D
10^{-3}	—	—	4/6	δ
10^{-2}	8/8	000 D	5/5	δδδDD
10^{-1}	—	—	6/6	δδ DDD
10^{-0}	—	—	5/6	δδDD

* Number giving haemagglutination of guinea-pig cells and number surviving.
† Phase character of representative fluids (Burnet and Stone, 1945*b*).

It will be seen that the situation is similar to that reported for earlier strains in the O phase.

Eighteen embryo lung emulsions which gave no agglutination of fowl cells at a dilution of 1 : 10 at room temperature were tested at 4° C. Seven of these gave no agglutination at 1 : 10, with agglutinin titres for guinea-pig cells ranging from 100 to 320. Five others gave fowl cell titres at 4° C. which were one-tenth or less of the guinea-pig cell titres. These twelve can be regarded as true O phase virus. Six lung emulsions gave the following values for fowl cell agglutination at 4° C, >80, >80, 80, 40, 40, 20. These last which gave no apparent agglutination at room temperature (18 to 20°) are referred to as " false O " phase virus. As was pointed out in the paper by Burnet and Stone (1945*b*) it is impossible to draw sharp dividing lines in classifying the actual materials, amniotic fluid or embryo lung emulsion, from infected embryos. Preparations can be obtained showing every graduation from " true O " which shows no fowl cell agglutination at any temperature to the full D phase.

In vitro *Characteristics of " True O " Phase Virus in Contrast to " False O " Phase Virus.*

Parallel experiments were carried out on (1) O and other variants of our standard strain BEL, which has been maintained in the O phase for seven years.

(2) The strain IAN–O which might be called the most blatantly false O in our possession and (3) the new strain Ocean Island 1948 (Oc.I.).

Influence of temperature at which haemagglutinin titration is made.

Our primary criterion of true O behaviour has been the absence of agglutination of fowl cells at 4° C. by a 1 : 10 dilution of virus giving a titre of at least 80 with guinea-pig or human cells. When false O strains, e.g. IAN–O, are studied at room temperature, close observation will show that in the early stages of settling a definite pattern is formed on the bottom of the tube. By the time settling is complete there is at most only a slight roughness of the edge of the central " button " of cells. If the cells are reshaken and again allowed to settle there is no trace of agglutination at any stage. This finding, which is common to all the false O viruses tested, provides the clue to the understanding of the behaviour of these strains, viz. that they very rapidly destroy by enzymic action the small proportion of fowl cell receptors to which they can be adsorbed. Anything which prevents or slows down this elution of virus by its own enzymic action will increase the apparent haemagglutinating action on fowl cells.

Apparent conversion of false O to D by treatment of the virus to reduce its enzymic activity.

By appropriate treatment any influenza virus can be converted into an indicator strain, still producing haemagglutination to nearly full titre, but lacking enzymic activity and being inhibited by those mucoid materials which serve as a substrate for the untreated virus (Stone, 1949*a*). Simple heating will accomplish this with some strains, others require to be diluted in a mildly alkaline medium containing citrate or oxalate. When these methods are applied to O and false O materials, the former show no development of a capacity to agglutinate fowl cells. The false O viruses characteristically develop a power to agglutinate fowl cells at room temperature to a titre approaching that to which guinea-pig, human or pigeon cells are agglutinated. The titre is never greater than that given by the untreated virus with fowl cells at 4° C. Results with Oc.I. and IAN are shown in Table II.

TABLE II.—*Effect of Heat on the Agglutination Titres with Fowl and Guinea-pig Cells of True O and False O Virus.*

Strain.	Phase.	Treatment.	F/G titres at room temperature.	
			Original.	After heating.
Oc.I.	O	56° C. 45 min.	<10/210	<10/80
Oc.I.	False O	,,	<10/430	40/160
IAN	,,	52° C. 15 min.	<10/120	140/120

With BEL false O strains, heating to 52° for 15 minutes in the presence of 1 per cent potassium oxalate allowed agglutination of fowl cells at room temperature, but the agglutination was of weaker type than that shown with guinea-pig cells. Similar treatment of the true O phase virus produced no capacity to agglutinate fowl cells.

The method used by Magill and Sugg (1948), suspension of virus in McIlwaine's phosphate-citric acid buffer at pH 5·6 was applied to the Oc.I.–O and false O

material used above (Table II); F/G titres at room temperature produced by this treatment were <10/210 and 80/430 respectively.

Very mild treatment of IAN false O, including incubation for an hour at 37° C. in the presence of borate buffer pH 8·5 plus 0·5 per cent sodium citrate, allowed a full titre D type of agglutination of fowl cells at room temperature.

Agglutination of fowl cells treated with periodate.

Fazekas de St. Groth (1949) has shown that at a certain level of periodate treatment red cells adsorb influenza viruses irreversibly, the normal enzymic action failing to occur. By treating a 25 per cent suspension of fowl cells with an equal volume of M/4000 KIO_4, cells were obtained which at room temperature were agglutinated by false O specimens of IAN or Oc.I to the full titre, and by BEL false O to about half the titre obtained with normal fowl cells at 4° C. True O phase virus of either Oc.I. or BEL was without effect on such cells.

The position in the receptor gradient of O and false O variants.

Previous experience in testing O and D phases of the same strain for their position in the receptor gradient of cells, human or guinea-pig, susceptible to agglutination by both has generally shown that the two phases lie fairly close to one another, the O being earlier than the D (Burnet, McCrea and Stone, 1946). In view of the results described above it was of considerable interest to compare the position in the gradient for fowl cells of false O and D phases of the same strain. Since true O never shows any agglutination of fowl cells it cannot be tested. The experiment with BEL, false O and D, which is shown in Table III followed the usual plan of such tests. Fowl cells were treated with graded amounts of the vibrio filtrate, RDE, and the action stopped by the addition of citrate. After washing with saline the cells were made up to 1 per cent suspensions. Cells were also treated with BEL false O and NDV until stablized. A modified series of viruses was then used to test the agglutinability of each type of cell at 4° C., five agglutinating doses (A.D.'s) being used. The false O material (BEL) was in the form of an embryo lung emulsion with a titre of 1600 with guinea-pig cells. The fowl cell titre at room temperature was <10, at 4° C., 200. It can be seen from Table III that BEL false O is at the " extreme left " of the gradient, quite separated from the D form of the same strain.

TABLE III.—*Relative Positions of BEL false O and BEL–D in Fowl Red Cell Receptor Gradient.*

Red cells treated with.	Haemagglutination by 5 A.D.'s at 4° C.						
	BEL false O.	NDV.	MMF.	WSE.	BEL–D.	SW.	LEE.
RDE							
1 : 10	−	−	−	−	−	−	−
1 : 50	−	−	−	−	−	−	+
1 : 250	−	−	−	−	++	++	++
1 : 1250	−	+	++	++	++	++	++
BEL false O	−	++	++	++	++	++	++
NDV	−	−	+	++	++	++	++
Control	++	++	++	++	++	++	++

DISCUSSION.

The results reported in this paper will, we hope, clarify the confusion in regard to the genetic significance of the O–D series of changes in influenza A viruses. In a subsequent paper a detailed study of the enzymic activity of O, D and intermediate phases of BEL will be reported. This has given results in line with the interpretation which can be placed on the findings given in this paper.

Our view is simply that when first isolated from human material influenza A virus particles are incapable of adsorption to and therefore of enzymic action on the receptor substance of fowl or mouse erythrocytes. In any large population of such virus particles a proportion of mutants appear which are capable of adsorption to the most " accessible " of the fowl cell receptor groupings. This proportion presumably is selectively favoured in the environment of the chick embryo and, in accordance with the now generally accepted views on microbial variation, further variants appear within the favoured clones by which the changed character is accentuated (Demerec, 1945). As the change goes on the virus becomes capable of adsorption to progressively less accessible receptor groupings until it finally reaches its appropriate position in the fowl cell gradient of fully " egg-adapted" strains. In the early stages only a few receptor groupings are accessible and a brief period of contact between virus and cells results in their enzymic destruction with disappearance of the initial cell aggregation. Anything which hinders this enzymic destruction of the most accessible receptors will at this stage of virus adaptation produce an apparent conversion of virus from O to D. The simplest way to inhibit enzymic action is to lower the temperature, but the same result can be achieved by heating virus in the region of 52–56° C. in an appropriate ionic environment, or by modifying the cell receptors with small amounts of periodate.

Even if it were shown that " true O " virus as obtained in primary isolations by the amniotic method, could be induced to give fowl cell agglutination by some physical or chemical treatment, the data provided in this and earlier papers as to the conditions under which fowl cell agglutinating virus appears would still demand a genetic interpretation. The fact that any true O strain will, on passage, give intermediate and D descendants with complete regularity, yet cannot be induced by any manipulation *in vitro* to agglutinate fowl cells, wholly invalidates the view that the O–D phase variations represent merely transformations in agglutinating activity induced by the ionic environment.

SUMMARY.

Influenza A virus strains in true O phase as isolated from the human subject, cannot be manipulated by physicochemical means to produce fowl cell agglutination.

The behaviour of intermediate forms which show such an apparent conversion is explicable in terms of their enzymic activity on cell receptors.

The change from O to D phase on chick embryo passage is due to processes of mutation and selective survival.

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AYFIVIN: AN ANTIBIOTIC FROM *B. LICHENIFORMIS*: PRODUCTION IN POTATO-DEXTROSE MEDIUM.

A. ARRIAGADA, M. C. SAVAGE, E. P. ABRAHAM, N. G. HEATLEY AND A. E. SHARP.

From the Sir William Dunn School of Pathology, Oxford.

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MAGARÃO, Arriagada and Thales (1944) reported that cultures of *Myco. tuberculosis* contaminated by a strain of *B. subtilis* underwent lysis, and that products of the contaminating organism were able to modify the course of tuberculosis in guinea-pigs. Cultures of the *B. subtilis* which had been brought to Oxford were found to contain two species of aerobic spore-forming bacilli. One was a strain of *B. subtilis* and the other was a strain of *B. licheniformis*. When tested by the cross-streak procedure on nutrient agar both organisms inhibited the growth of *Staph. aureus*, *C. xerosis* and *Myco. phlei*, but showed little activity against *Bact. coli* or *Ps. pyocyanea*. Antibacterial substances produced by the strain of *B. subtilis* have already been described (Newton, 1949). The present paper is concerned with an antibiotic formed in certain liquid media by the strain of *B. licheniformis*. This organism was originally called A-5 and the antibiotic has been named ayfivin (Florey, Chain, Heatley, Jennings, Sanders, Abraham and Florey, 1949).

EXPERIMENTAL.

The organism.

The strain A-5 showed the usual characteristics of *B. licheniformis* (Gibson, 1944). It was maintained for routine use on slopes of heart-extract agar, which were stored in the refrigerator until required. Fresh subcultures were made every two weeks.