THE PRODUCTION OF INCOMPLETE VIRUS PARTICLES AMONG INFLUENZA STRAINS: EXPERIMENTS IN EGGS

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CONCENTRATED allantoic inocula of the classical influenza strain PR8 give rise to the production of incomplete virus particles possessing the same antigenic and enzymic equipment as the parent virus but lacking infectivity (von Magnus, 1947). The first exception to this phenomenon was noticed incidentally in the course of some unrelated work. The observation, however, seemed interesting enough in its implications to warrant a systematic comparison of influenza strains in this regard. From the results we hoped to gain information on two specific points: first, the relationship between the von Magnus phenomenon and interference; and second, the nature of the difference between the initial and subsequent cycles of viral multiplication, a fact which has been explicitly recognized only recently (Cairns, 1952; Fazekas de St.Groth and Cairns, 1952).

A preliminary note (Fazekas de St.Groth and Graham, 1953), restricted to the behaviour of two contrasting strains, has appeared. Here the detailed findings with ten influenza virus strains are presented, and it will be shown that the von Magnus phenomenon, far from being a uniform feature of the strains tested, shows such quantitative variation as to give the impression of fundamental differences between the viruses at the opposite ends of the series.

MATERIALS AND METHODS.

Saline was made up as 0·90 per cent NaCl in distilled water. The diluent used in infectivity titrations was a mixture of 1 part Seitz-filtered horse serum and 9 parts sterile saline.

Red blood cells were obtained from venous blood of White Leghorn fowls, collected in a 2 per cent sodium citrate solution. They were washed in three changes of saline, packed and stored at 4°. Suspensions of 10 per cent strength (by volume) were made up daily. No cells older than three days were used.

Viruses used in this study were grown by the standard method laid down by Beveridge and Burnet (1946). Batches of six 11-day-old eggs were inoculated allantoically with 0·1 ml. sterile horse serum saline containing not more than 10^4 infective units of virus. After 42 hr. incubation at 35° the eggs were chilled for 2 hr. and the allantoic fluids harvested aseptically. The virus preparations were always used within 3 hr. of harvesting.

Type A strains:

- WSE sub-strain of WS, the prototype of influenza A virus (Smith, Andrewes and Laidlaw, 1933), adapted by extensive serial passage to grow and produce pocks on the chorio-allantois (Burnet, 1936);
- PR8 (Francis, 1934);
- MEL (Burnet, 1935);
- BEI (Burnet, Beveridge, Bull and Clark, 1942).

Type A-prime strains :
CAM (Anderson, 1947) ;
FM1 prototype of A-prime virus (Rasmussen, Stokes and Smadel, 1948).

Type B strains :
LEE prototype of influenza B virus (Francis, 1940) ;
BON (Beveridge, Burnet and Williams, 1944) ;
HUT (Burnet, Stone and Anderson, 1946).

Swine influenza :
SW the classical " Strain 15 " of Shope (1931).

Haemagglutinin titration.—Serial twofold dilutions of the test material were made in 0·25 ml. volumes of saline. To each tube a standard drop (0·025 ml.) of a 10 per cent fowl red cell suspension was added, the racks shaken and left standing at room temperature (18–24°). The pattern of the settled cells was read 20 min. later, a conventional degree of partial agglutination marking the end-point. The titre of a sample is the reciprocal of this end-point dilution. In Table I haemagglutinating units (HA) refer to 1·0 ml. volumes, *i.e.*, the actual titres have been multiplied by a factor of 4. As the error of haemagglutinin titrations shows a log-normal distribution, all titres have been conveniently expressed in $\log_{10}$ units.

Infectivity titration.—Serial 3·16-fold (log 0·5) dilutions of the test material were prepared in cold horse serum saline, and all tubes were kept in an ice-bath until used. Sufficient penicillin was added to the diluent to give a concentration of 100 units per egg. Of the dilutions 0·10 ml. volumes were inoculated allantoically into groups of five 11-day-old embryos. The eggs were incubated at 35° for 45 hr.

Then, without preliminary chilling of the eggs, a hole 3 mm. diam. was poked through the paraffin seal with a red-hot iron rod, and through it a sample of approximately 0·2 ml. of allantoic fluid was removed with a special " dipping pipette ". This pipette was made of 7 mm. external diameter glass tubing by pulling it out in a flame, sealing the tip, and blowing a small hole through the side of the drawn-out part approximately 4 mm. from the tip. This device is a small version of the " harvesting pipette " designed by Henle and Henle (1944) ; with its aid samples of allantoic fluid may be conveniently collected without any interference from extra-embryonic structures, which render sampling with ordinary Pasteur pipettes cumbersome. The dipping pipettes were sterilized in boiling water between each egg.

One standard drop of 10 per cent red cells was added to each sample of allantoic fluid, and the pattern of agglutination read after 20 min. As the reactions were invariably either complete agglutination or complete lack of agglutination, the results were recorded as positive or negative. The cumulative totals of positive (infected) and negative (non-infected) eggs were calculated, and the modal infectivity end-point determined by the method of Reed and Muench (1938). As with haemagglutinating units (HA), the infectivity titres were corrected to refer to 1·0 ml. volumes of allantoic fluid, and are expressed in $\log_{10}$ units. In Table I they appear under the symbol "ID".

EXPERIMENTS.

Single-passage Tests for Production of Incomplete Virus.

The first set of experiments consisted essentially in the replication, with each strain to be tested, of the fundamental experiment described by von Magnus, namely the titration of infective and haemagglutinating units after allantoic inoculation of large or small quantities of influenza virus. To avoid any bias in favour of incomplete virus production, the test samples were always taken at the height of infectivity, as assessed in some preliminary trials. There is no need to give here the results of these sighting experiments in detail as they were in complete agreement with previously published findings of other workers. It will suffice to say that after inoculation of concentrated virus preparations (10^9–10^{10}

ID_{50}) the allantoic fluids were harvested after 16 hr. incubation; after dilute inocula (10^4–10^5 ID_{50}), where three or more cycles of multiplication are needed to reach maximum titres, the fluids were harvested after 42 hr.

All experiments were done to the following set pattern. Two days before the main tests the strain to be used was given a single allantoic passage at high dilution, usually 10^{-4}. By this means it could be ensured that the seed would contain no incomplete virus. The fluid obtained was titrated for haemagglutinin, and if found suitable, *i.e.*, of high titre, it was used within 3 hr. of harvesting. The experiment proper was done on two groups of six 11-day eggs. One group received 0·6 ml. of the undiluted seed virus allantoically, and was harvested after 16 hr. at 35°. The other group was given 0·05 ml. of a 10^{-4} dilution of the same seed in horse serum saline; the allantoic fluid of these eggs was removed after 42 hr. incubation. Immediately after harvesting approximately 1 ml. of each allantoic fluid was ampouled and stored in the frozen state at −20°. On the rest of the allantoic fluids haemagglutinin tests were performed on the day of harvesting.

TABLE I.—*Yield following Infection*

Strain.	Inoculum : ~ 10^9 ID_{50}.			Inoculum : ~ 10^4 ID_{50}.		
	HA/ml.	ID_{50}/ml.	ID/HA ratio.	HA/ml.	ID_{50}/ml.	ID/HA ratio.
Type A, WSE	3·40	6·75	3·35	3·97	9·25	5·28
	3·22	7·42	4·20	3·58	10·20	6·72
	3·43	7·46	4·03	3·67	10·40	6·73
	3·09	7·41	4·32	3·67	9·70	6·03
	3·10	7·75	4·65	3·58	10·20	6·62
	2·20	6·89	4·69			
	2·17	6·75	4·58	2·98	9·12	6·14
	Average ratio : 4·26 ± 0·18			Average ratio : 6·25 ± 0·23		
,, PR8	3·45	7·14	3·69	3·92	10·22	6·30
	3·30	7·72	4·42	3·44	10·34	6·90
	3·14	7·30	4·16	3·51	9·42	5·91
	3·42	7·55	4·13	3·22	10·30	7·08
	3·51	7·24	3·73	3·47	9·39	5·92
	3·22	7·70	4·48	3·30	10·19	6·89
	Average ratio : 4·10 ± 0·14			Average ratio : 6·50 ± 0·22		
,, MEL	3·19	7·80	4·61	3·40	10·10	6·70
	3·16	8·28	5·12	3·55	9·36	5·81
	3·31	7·50	4·19	2·53	8·75	6·22
	3·28	7·00	3·72	3·11	10·00	6·89
	3·25	7·75	4·50	3·41	10·00	6·59
	3·28	7·75	4·47	3·32	10·00	6·68
	3·28	7·25	3·97	3·53	9·40	5·87
	Average ratio : 4·37 ± 0·17			Average ratio : 6·39 ± 0·16		
,, BEL	4·03	8·90	4·87	4·05	9·88	5·83
	3·85	9·35	5·50	4·05	9·75	5·70
	3·52	8·33	4·81	3·23	9·80	6·57
	3·37	8·38	5·01	3·53	10·20	6·67
	3·52	7·43	3·91	3·57	9·65	6·08
	3·55	8·63	5·08	3·83	9·75	5·92
	Average ratio : 4·86 ± 0·21			Average ratio : 6·13 ± 0·16		

Infectivity was titrated later, on the frozen samples. Tests on the two experimental groups were always done in parallel. If any of the titrations proved technically unsatisfactory, there was enough of the frozen sample for several repetitions.

Table I gives the results in detail. All titres are expressed in $\log_{10}$ units and are directly comparable as they refer to 1·0 ml. volumes of allantoic fluid. The infectivity-to-haemagglutinin ratio of individual samples is shown, and the average ratio and its standard error has been calculated for all groups. The Table also contains a test of significance for the comparison of yields after the two inocula. Although only two averages are being compared at a time, the method applied is the analysis of variance instead of the more usual *t*-test. This was done mainly because the statistics so obtained could be used directly in later inter-strain comparisons. It should be clear, of course, that the two methods are bound to give identical results; indeed, Student's *t*-test is but a simpler special case of the more comprehensive analysis of variance.

with Concentrated or Dilute Seed.

Analysis of Variance.

Source of variation.	Sum of squares.	Degrees of freedom.	Variance.
Between inocula	13·4067	1	13·4067
Error	2·9173	11	0·2652
Total	16·3240	12	

Variance ratio F = 50·55; P < 0·001.

Between inocula	17·2559	1	17·2559
Error	1·9295	10	0·1929
Total	19·1853	11	

Variance ratio F = 89·43; P < 0·001.

Between inocula	16·3623	1	16·3623
Error	2·3677	12	0·1973
Total	18·7300	13	

Variance ratio F = 82·93; P < 0·001.

Between inocula	4·8006	1	4·8006
Error	2·1923	10	0·2192
Total	6·9929	11	

Variance ratio F = 21·90; P < 0·001.

[*Table continued overleaf.*

TABLE I—

Strain.	Inoculum : ~ 10^9 ID_{50}. HA/ml.	ID_{50}/ml.	ID/HA ratio.	Inoculum : ~ 10^4 ID_{50}. HA/ml.	ID_{50}/ml.	ID/HA ratio.
Type A-Prime, CAM	2·95	8·10	5·15	2·83	8·90	6·07
	3·58	9·78	6·20	2·83	9·72	6·89
	3·10	9·25	6·15	3·37	9·06	5·69
	3·34	8·42	5·08	3·58	9·65	6·07
	3·16	9·30	6·14	3·43	9·25	5·82
	3·07	8·00	4·93	2·20	9·15	6·95
	3·28	8·60	5·32	3·37	9·60	6·23
	Average ratio : 5·57 ± 0·22			Average ratio : 6·25 ± 0·19		
„ „ FM1	2·68	7·50	4·82	3·01	10·35	7·34
	2·77	7·85	5·08	2·80	9·07	6·27
	2·95	8·35	5·40	3·40	8·20	4·80
	2·11	7·70	5·59	2·44	8·86	6·42
	3·07	8·88	5·81	2·95	7·90	4·95
	3·19	8·88	5·69	2·56	9·28	6·72
	Average ratio : 5·40 ± 0·16			Average ratio : 6·08 ± 0·41		
Type B, LEE	3·28	9·81	6·53	3·41	9·45	6·04
	3·23	9·20	5·97	3·28	9·80	6·52
	3·23	9·62	6·39	3·33	9·40	6·07
	2·92	9·82	6·90	3·23	9·02	5·79
	3·32	9·63	6·31	2·98	9·80	6·82
	3·23	9·30	6·07	3·10	9·30	6·20
	3·55	10·05	6·50			
	3·19	9·80	6·61	1·00	7·55	6·55
	3·49	10·05	6·56			
	3·37	8·92	5·55	3·48	9·17	5·69
	3·58	9·42	5·84	3·22	9·00	5·78
	3·04	8·63	5·59	1·72	7·86	6·14
	2·95	8·50	5·55	2·02	7·86	5·84
	3·37	9·38	6·01			
	3·07	8·76	5·69	3·04	9·66	6·62
	Average ratio : 6·14 ± 0·04			Average ratio : 6·17 ± 0·03		
„ BON	2·05	6·72	4·67	2·83	8·60	5·77
	2·05	6·35	4·30	2·92	8·06	5·14
	2·35	6·50	4·15	3·10	7·87	4·77
	2·05	6·10	4·05	2·68	8·00	5·32
	2·05	5·65	3·60	2·68	8·50	5·82
	2·92	6·28	3·36	2·74	9·00	6·26
	Average ratio : 4·02 ± 0·19			Average ratio : 5·51 ± 0·22		
„ HUT	2·68	7·42	4·74	2·68	8·93	6·25
	2·41	8·00	5·59	2·80	8·50	5·70
	2·38	7·22	4·84	2·20	7·85	5·65
	2·92	8·62	5·70	2·38	8·78	6·40
	2·27	7·42	5·15	2·65	8·36	5·71
	2·58	8·50	5·92	2·83	9·06	6·23
	Average ratio : 5·32 ± 0·20			Average ratio : 5·99 ± 0·14		
Swine Influenza, SW	3·10	7·43	4·33	3·37	9·20	5·83
	3·13	7·50	4·37	2·83	9·50	6·67
	2·83	7·58	4·75	3·13	8·81	5·68
	2·98	7·20	4·22	2·80	9·28	6·48
	3·28	8·32	5·04	3·41	10·00	6·59
	3·55	8·40	4·85	3·53	9·70	6·17
	Average ratio : 4·59 ± 0·14			Average ratio : 6·24 ± 0·17		

—*continued.*

Analysis of Variance.

Source of variation.	Sum of squares.	Degrees of freedom.	Variance.
Between inocula	1·6114	1	1·6114
Error	3·4102	12	0·2842
Total	5·0215	13	

Variance ratio F = 5·67 ; 0·05 > P > 0·01.

Between inocula	1·4076	1	1·4076
Error	5·7913	10	0·5791
Total	7·1989	11	

Variance ratio F = 2·43 ; 0·20 > P > 0·05.

Between inocula	0·0076	1	0·0076
Error	4·2463	25	0·1699
Total	4·2539	26	

Variance ratio F = 0·05 ; P > 0·5.

Between inocula	6·6751	1	6·6751
Error	2·5775	10	0·2578
Total	9·2526	11	

Variance ratio F = 25·90 ; P < 0·001.

Between inocula	1·1532	1	1·1532
Error	1·9244	10	0·1924
Total	3·0776	11	

Variance ratio F = 5·99 ; 0·05 > P > 0·01.

Between inocula	8·1013	1	8·1013
Error	1·4004	10	0·1400
Total	9·5017	11	

Variance ratio F = 57·85 ; P < 0·001.

Plentiful production of incomplete virus is evident with the strains WSE(A), PR8(A), MEL(A) and SW. The von Magnus phenomenon is as conspicuous in these as it was with the two PR8 sub-strains on which the work of the Scandinavian authors had been performed. The ID/HA ratios dropped from the normal average to values lower by about two orders of magnitude. The BEL(A) and BON(B) strains formed relatively smaller proportions of non-infective particles after inoculation of undiluted seed, although the reduction of the ID/HA ratios is still highly significant. The two A-prime viruses, CAM and FM1, and the HUT(B) strain are borderline cases, the drop in the average ID/HA ratios being, at the most, suggestive but statistically not significant. The LEE(B) strain, on the other hand, did not even give a hint of incomplete virus production. Since this behaviour was in such striking contrast to the strains exhibiting the von Magnus phenomenon, four separate series of tests were made with the LEE strain. The results proved to be reproducible, showing consistently no difference between the yields of virus after concentrated or dilute infective inocula.

Statistical.

The data in Table I thus give the impression of considerable diversity among influenza strains in incomplete virus production. As it seemed desirable to assess the contribution of different variables entering into the experiments, the results were submitted to an analysis of variance. This method is applicable provided the variances of all sub-groups to be compared are homogeneous. However, there was some reason to doubt whether this postulate would be automatically fulfilled, as further sources of variation might have emerged with the production of incomplete virus. Consequently, the variances of all sub-groups were first compared by Bartlett's test. According to the formula

$$\chi^2_{(k-1)} = \frac{k(n-1)\ln \bar{V} - (n-1)\Sigma \ln V}{1 + [(k+1)/3(n-1)k]},$$

where V is the variance of any of k sub-groups containing n replicates, the value of $\chi^2_{(19)} = 17{\cdot}045$. This corresponds to a probability of $P = 0{\cdot}62$, *i.e.*, there is no significant deviation from homogeneity, and an analysis of variance may be set up in its standard form. This result is equivalent to saying that whatever further sources of error have been introduced by the presence of incomplete virus in some of the specimens, their total effect is negligible compared with the inherent inaccuracy of infectivity titrations.

TABLE II.—*Statistical Evaluation of the Results in Table I*

Total Analysis of Variance.

Source of variation.	Sum of squares.	Degrees of freedom.	Variance.	Variance ratio.	Probability.
Inocula	45·5303	1	45·5303	191·19	<0·001
Strains	22·0573	9	2·4508	10·29	<0·001
Interaction	23·4313	9	2·6035	10·93	<0·001
Error	28·5769	120	0·2381		
Total	119·5958	139			

Analysis of Interaction-variance in Respect to Strains.

Strains at 10^9 ID_{50}	41·5475	9	4·6164	22·45	<0·001
Error	12·7477	62	0·2056		
Sub-total	54·2952	71			
Strains at 10^4 ID_{50}	3·9411	9	0·4379	1·60	0·16
Error	15·8292	58	0·2729		
Sub-total	19·7703	67			

The analysis of variance on all data obtained from the single-passage experiments is given in Table II. The important finding is the absence of difference among the strains after dilute infective inocula, as opposed to the highly significant difference in the yields after infection with large amounts of virus. The former confirms the conclusion we reached in an earlier study (Fazekas de St.Groth and Cairns, 1952), namely that the ID/HA ratio is identical for all well-adapted influenza strains when grown in the allantois from dilute seed, and that under these conditions no incomplete virus is being formed. The latter provides statistical evidence that the von Magnus phenomenon cannot be elicited uniformly with all influenza viruses. This point is stressed also by the highly significant interaction between strains and inocula, that is, while in some strains the size of the inoculum makes a difference, in others it does not.

We have then within the group of influenza viruses strains which produce less than 1 per cent viable offspring if inoculated into the allantois in amounts of 10^9 ID_{50}. At the other extreme there are strains in which incomplete virus is not detectable by the technique employed. As these differences comply with the strictest criteria of significance, the production of incomplete virus has to be regarded as a property with wide quantitative variation among influenza viruses. On the basis of the above results a *gradient of incomplete virus production* may be established, giving the series PR8 > WSE, MEL > SW > BON, BEL > HUT, CAM > FM1 > LEE, in the order of decreasing proneness to form non-infective particles.

Serial Passage Tests for Production of Incomplete Virus.

The single-passage experiments have revealed differences among influenza strains in regard to incomplete virus production. Yet, due to the inherent limitations of the technique, it was not possible to settle the question whether these differences were absolute, *i.e.*, whether some of the strains produced no incomplete particles at all. We sought to solve this problem by carrying concentrated inocula through several consecutive passages ; thus the effect can be cumulated while the error of the titrations remains constant. Were no incomplete particles produced, the series should be expected to behave like a serial passage of dilute inocula, *viz.*, maximal infectivity and haemagglutinin titres would be obtained throughout. If, on the other hand, even small amounts of incomplete particles were produced, these would progressively interfere with the yields of virus in subsequent passages, and finally a reduction of both infectivity and haemagglutinin titres should be expected. In experiments of this type special care has to be taken (*a*) to expose the great majority of cells to the initial inoculum, *i.e.*, to work at high multiplicities, and (*b*) to avoid contamination of the first-cycle yield with virus from later cycles which were initiated under different conditions. The latter precaution is particularly important in view of the observation that infection of a larger number of cells acts as a mitogenetic stimulus on the allantoic wall, resulting in the appearance of new infectible cells in numbers sufficient to obscure the results (Fazekas de St.Groth and Graham, 1954).

The technique finally adopted and applied to all strains was as follows : The seed virus was given one allantoic passage at 10^{-4} dilution immediately before the experiment proper. Then three 11-day eggs were inoculated allantoically with 2·0 ml. each of undiluted seed, and incubated for 12 hr. The fluids were then harvested through the hole used for inoculation, without previous chilling of the eggs. Three ml. volumes of each fluid were used to prepare pools within each group, and of these pools again 2·0 ml. volumes were inoculated into each of a group of three eggs, and the procedure repeated through four passages. The original seed, each of the first, second and third passage pools, and all the three

fluids of the final passage were titrated individually for infectivity and haemagglutinin content either immediately or after storage at −20°.

The results with six representative strains are shown in the Figure. The strains were so chosen that the group should contain a virus of each immunological type exhibiting the von Magnus phenomenon (PR8(A), BON(B) and SW), and also three strains which did not produce significant amounts of incomplete virus in the single-passage experiments above : CAM(A′), FM1(A′) and LEE(B). The four strains not shown in the figure behaved in every respect like the viruses PR8, BON and SW.

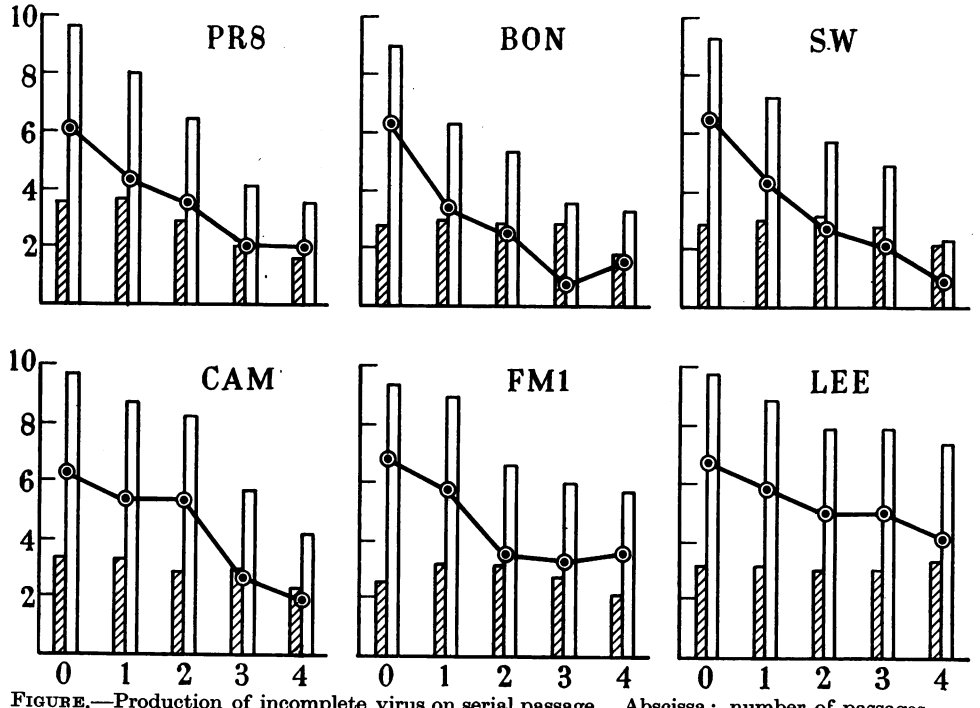

FIGURE.—Production of incomplete virus on serial passage. Abscissa: number of passages. Ordinate : titre in $\log_{10}$ units. Open columns : infectivity titre. Hatched columns : haemagglutinin titre. Continuous line : ratio of infectivity/haemagglutinin. (For experimental details, see text.)

The findings with the strains PR8, BON and SW are a replication of von Magnus' findings : consecutive passage of large inocula progressively reduced the proportion of infective virus in the yields, and the ID/HA ratios are approximately a thousand times lower than the normal ID/HA ratio ($10^{6.26}$) already in the second passage and onwards. It is worth noting that from the stage where the absolute amount of infective virus in the inoculum dropped to a level below 1 per cent of infectible cells, that is to less than 10^6, the haemagglutinin yield of the eggs was also reduced. This phenomenon is well accounted for by the fact that under these conditions a second cycle of multiplication became possible, and in this cycle the interfering effect of the originally inoculated excess of incomplete virus became noticeable. It is known from previous work (Fazekas de St.Groth, Isaacs and Edney, 1952) that the establishment of heterologous inter-

ference is not instantaneous, and thus the first-cycle yields of active virus are the same whether the initial seed contained infective particles only or a mixture of infective and inactivated particles. A numerical estimate of this reduction may be supplied by comparing the mean haemagglutinin titres of the " O " and " I " passages with the average titre of the three fluids harvested after the fourth passage. The reduction for PR8 is 3·62 — 1·59 = 2·03 $\log_{10}$ units, *i.e.*, 100-fold ; for BON 2·83 — 1·78 = 1·05, *i.e.*, 11-fold ; for SW 2·89 — 2·16 = 0·73, *i.e.*, 5-fold. These differences are all significant, the error of a single haemagglutinin titration being $\pm$ 1·3-fold.

Of the two borderline cases, CAM(A′) and FM1(A′), the former obviously belongs to the group of viruses which exhibit the von Magnus phenomenon. In the single-passage experiments the CAM-strain showed a production of incomplete virus which was significant at the 5 per cent but not at the 1 per cent level. This behaviour was maintained through two consecutive passages, but from the third onwards the cumulative effect showed up quite clearly and the ID/HA ratios dropped well below the normal, the difference being over 1000-fold. Also, there is a distinct reduction in haemagglutinin yields with a difference of 3·33 — 2·21 = 1·12, *i.e.*, 13-fold.

The other A-prime strain, FM1, cannot be classed with the previous group of viruses inasmuch as the total reduction of infective offspring never reaches the low levels attained by the strains showing the typical behaviour described by von Magnus. Whereas the first four viruses represented in the figure produce an excess of incomplete particles of the order of 30,000 to 1 by the end of the fourth passage, the FM1-strain reaches a ratio of $<1000:1$ after the second passage and stays at this level for the subsequent passages. There can be no doubt, however, that significant amounts of incomplete virus have been produced and this became detectable by the more rigorous test afforded by the repeated-passage experiments ; there was also a definite drop in haemagglutinin yields, namely 2·88 — 2·14 = 0·74, *i.e.*, 5-fold.

The LEE strain in which no difference could be detected by the single-passage test, could also be shown to produce some incomplete virus under these conditions. The reduction of the ID/HA ratios is, however, evidently less than in any of the other strains tested, being of the order of 100 even after four consecutive passages. It is also significant that, though the multiplicity of infection was kept at the level of 50 particles for each infectible cell throughout the four passages, there is no reduction of haemagglutinin yields within the series. The average haemagglutinin titres compared as in the other strains show that the final yield was 3·06 — 3·23 = —0·17, *i.e.*, unchanged, the minimal rise (1·2-fold) being insignificant.

The general conclusion to be drawn from this set of experiments is that production of incomplete virus can be elicited from every one of the influenza virus strains tested. The differences found are thus, although extreme, of a quantitative nature rather than absolute. From the practical point of view the behaviour of haemagglutinin titres may be of importance. Their trend indicates that by the technique generally used, *viz.*, repeated passaging at high concentrations, no useful further reduction of the ID/HA ratio can be hoped, as below a limiting concentration of infective particles in the seed the resultant haemagglutinin yields will drop. Thus they not only fail to give preparations with a larger proportion of incomplete particles in them, but actually lead to a lower total output of particles per egg.

DISCUSSION.

Tests for incomplete virus are necessarily bound up with the observation of a lowered yield of infective particles, suggesting some interference along the course of their normal reproduction. It is not surprising then that the two earliest theoretical accounts of the von Magnus phenomenon postulate a mechanism resembling that of heterologous interference. The simultaneous infection of a cell by two or more particles, as a prerequisite of incomplete virus production, is the common feature of the hypotheses of von Magnus (1947 ; 1951*a* ; 1951*b* ; 1952) and of Gard (1952). In the extensive studies of von Magnus and his associates this requirement of multiple infection was satisfied, and for this reason his hypothesis appears adequate. Yet, one must not lose sight of some technical peculiarities of the experiments on which this explanation was based.

If a great majority of available cells is infected by the original inoculum, it is immaterial at what time the fluids are harvested as one will always deal with the yield of the first cycle, slightly contaminated perhaps by whatever has been produced in the minority of cells, *viz.*, those that have escaped infection at first. If incomplete particles are found in such experiments, it is safe to infer that they descended directly from the initial seed. The situation is quite different if the number of particles is only equal to or less than the number of infectible cells. Then a fraction—perhaps a small minority only—of cells will be infected at first. Their output will come into contact with large numbers of non-infected cells, and will have an opportunity to go through a second, perhaps a third cycle of multiplication. Obviously, the *final yield* will say very little about the behaviour of the original inoculum, and no valid inferences can be drawn. The method to follow at low multiplicities is to examine the *first-cycle yield* which, by definition, shows the response to the initial inoculum. This technique has such advantages, even when the host cells are outnumbered by infective particles, that no other should be considered when setting up crucial tests.

It is on this ground that von Magnus' demonstration of the necessity of multiple infection is unacceptable, his technique being unwittingly biased in favour of his own hypothesis. In the experiment designed for the assessment of the smallest inoculum after which the phenomenon still occurred (Experiment 3, von Magnus, 1952), incomplete virus simply *could not* be detected at multiplicities of less than 1, where over 30 per cent of the cells escaped immediate infection. He found that " mainly incomplete virus was produced in embryos following inoculation with undiluted or tenfold diluted seed, while the 10^{-2} and 10^{-3} diluted inocula resulted in the formation of fully active virus ". This is a perfectly correct statement if the yields at 24 hours, at the end of the third cycle, are considered. It is significant, however, that at 10 hours the eggs contained $10^{8.6}$, $10^{8.0}$, $10^{6.9}$ and $10^{5.7}$ ID_{50} after inocula of $10^{8.9}$, $10^{7.9}$, $10^{6.9}$ and $10^{5.9}$ infective doses respectively. This lack of change is in contrast with the behaviour of haemagglutinin titres : they rise conspicuously from the 4th hour onwards, as seen with the two larger inocula after which performance of haemagglutinin tests was feasible. This means, obviously, that only minute fractions of the first-cycle yields were infective, the bulk consisting of incomplete particles. From the growth curves it is also clear that by the end of the second cycle (at 15 hours) the eggs contained $10^{8.1}$, $10^{8.9}$, $10^{9.7}$ and $10^{9.5}$ infective doses respectively. Thus after smaller infective inocula a distinct second cycle has occurred,

and there can be no doubt that the virus produced in this cycle was fully infective. The quantitative data also show that the first-cycle yields of the two smaller inocula were completely masked by the produce of later cycles, the infectivity titres rising by a factor of 100 and 7000 between the 10th and 24th hour for the 10^{-2} and 10^{-3} inocula. For the undiluted and 10^{-1} seeds the titres show a 2-fold drop and a 3-fold rise respectively, neither of which can be regarded as significant change.

Recognition of such a technical flaw does not, of course, invalidate the hypothesis ; it merely deprives it of what was regarded as its crucial proof. The demonstration by Cairns and Edney (1952), on the other hand, is in itself enough to discount any theory based on interference. In an elegant experiment, exemplary both in design and accuracy, they were able to show that incomplete virus was being produced at multiplicities as low as 0·02, *i.e.*, where only 2 per cent of the cells had been infected. It adds to the value of these tests that they were done with the PR8 strain, that used by von Magnus.

That multiplicity of infection is not a sufficient cause for the production of incomplete virus should be evident also from the fact that no non-infective particles are formed after infections with dilute seed. Yet, however small the initial inoculum, from the time when more than one-fiftieth of the cells have already yielded, the conditions are those of multiple infection. As each cell produces roughly one hundred new particles, at least half of the cells will be infected by two or more particles in the last cycle. This point is emphasized by our finding of strains where multiple infection even by the initial inoculum does not give rise to non-infective particles.

It is implicit in the auto-interference theory of von Magnus and explicitly stated in the theoretical treatment of Gard that more concentrated inocula, by virtue of giving higher multiplicities of infection, should give issue to a smaller number of complete virus particles than more dilute inocula. The observations of von Magnus, if examined from this point of view, do not support his own hypothesis but appear to be identical with those of Cairns and Edney, who showed that a thousandfold change in the initial inoculum of PR8 virus was not followed by *any* significant variation in the amount of infective particles produced. Our own experiments show that this behaviour applies to influenza strains generally.

The main finding of the present study, namely the gradation in incomplete virus production among influenza strains could be accounted for by the auto-interference theory only with the greatest difficulty. At least two subsidiary assumptions would have to be made : first, that the mechanism was based not on simultaneous infection by two active particles, but rather by an active and an inactive one ; and second, that the various strains contained different amounts of inactive particles, even when grown from dilute seed. However, both of these assumptions would be at variance with observed fact. The first is untenable on quantitative grounds, and would imply increasing effect on increasing the initial inoculum, which is not the case. Or it could be upheld by the further postulate of such a close and regular association between active and inactive particles that defied randomness after inoculation. There is no evidence that anything like this happened ; indeed, the fact that dilute inocula do not produce incomplete particles all but rules it out of consideration. The second assumption appears unjustifiable in the light of the finding that the initial ID/HA ratio of all strains is, for practical purposes, identical while there is more than a hundredfold difference in the degree of incomplete virus production. Also, there is no evidence that

fresh virus preparations grown from dilute seed would contain non-infective particles. Up till recently this has been the expressed opinion of von Magnus (1951*a*), and we have published several points of independent evidence to support this view (Fazekas de St.Groth and Cairns, 1952).

The third hypothesis (Cairns and Edney, 1952) came from an entirely different approach. Indeed, it cannot be called merely a hypothesis but rather an interpretation of experimental facts which rendered earlier hypotheses untenable and pointed in a direction along which a valid theory might be built from material provided by future research. In brief, Cairns and Edney observed that incomplete virus was being produced by inocula which caused negligible (less than 1 in 10,000) multiple infection. Realizing that this happened *only* in the first cycle of multiplication, they postulated that an uninfected egg contained a limited amount of some unknown substance and this set an upper limit to the number of infective virus particles that could be produced immediately. The process of viral multiplication was supposed to stimulate the infected tissue to provide more of this essential substance, and thus in the second and later cycles all of the virus produced might be infective. No attempt was made to define either this substance or the process by which it became available in later cycles. This explanation can account for all available data on incomplete virus production, such as its occurrence without multiple infection of cells, its quantitative independence of the primary inoculum at increasing multiplicities, and also the fact that the von Magnus phenomenon occurs only in the first cycle of multiplication. It should be added that it is consistent also with our findings, and should serve therefore as the basis of their interpretation.

The factual results of our study may be summed up in two points :

1. All influenza strains that produce non-infective particles in the allantois do so above a characteristic level of first-cycle infection. This level is independent of the multiplicity of infection, and is an inherent property of the virus, as are its antigenic make-up, enzyme activity, etc. The proportion of infective particles in the yield cannot be reduced by increasing the initial inoculum. Strains which do not produce incomplete virus fail to do so even at average multiplicities of infection of 10 or higher.

2. The different influenza virus strains form a gradient according to their capacity to produce non-infective particles. This gradient cuts across the line dividing the types, and is independent of the history of the strains as well as of the extent of their adaptation to the allantois.

The first of these points is the confirmation and extension of Cairns and Edney's thesis, which can now be regarded as a general statement holding for all influenza virus strains. The second point, on the other hand, requires the amplification of their hypothesis and its statement in more specific terms.

The variation in the extent of incomplete virus production among the strains could be satisfied by the assumption that their requirements for the " missing factor " are unequal. Yet, operationally this further assumption would be of little use as it gives no lead whereby the mechanism underlying these differences could be better understood ; neither does it suggest an experimental approach simpler than the random testing of all conceivable substances for their effect on incomplete virus production. It might be more profitable then to think in terms of a " missing process " rather than a " missing substance ". In this respect it is noteworthy that the gradient of incomplete virus production corresponds

closely to the gradient of enzyme action of these viruses on the allantoic membrane or, which is saying the same, the order in which infection can be prevented by the use of the " receptor destroying enzyme " of *V. cholerae* (Stone, 1947, 1948).

Whether this view is correct or not, it has the merits of a working hypothesis inasmuch as it immediately prompts several types of experiment by which its validity can be tested. It implies both that the " incomplete virus gradient " should be different in tissues which have different receptor gradients (*e.g.*, allantoic membrane and mouse lung), and that agents acting on receptors might alter the behaviour of influenza strains even in the allantois. An investigation on these lines will be reported later.

One more point calls for some discussion since our observations have a close bearing on it : it is the prolonged simultaneous passage of two influenza strains in the allantois and in mouse lungs. First described by Sugg and Magill (1948) and further investigated by Sugg (1951) and Liu and Henle (1951), the phenomenon appeared highly paradoxical, as from consideration of both cycle times and cyclic increments of influenza A and B strains, one would have postulated a rapid suppression of the B virus by the more quickly growing A strains. This indeed happens when dilute inocula of the two viruses (LEE and PR8) are used, even if the B started at a majority of 5 to 1 (Liu and Henle, 1951) ; yet by keeping the inoculum at the level of 10^7–10^9 particles the mixture could be carried through 52 consecutive passages (Sugg, 1951). The interpretation offered, namely that the cycle time of the LEE(B) strain was considerably shortened after large inocula (Liu and Henle, 1951), is not entirely satisfactory as another A strain " CC " is capable of suppressing LEE even when large initial doses are applied (Sugg and Magill, 1948). Moreover, although the apparent cycle time of LEE is shortened, its cyclic increment is not increased under the same conditions, as evident from Liu and Henle's published data (Fig. 4, 5, 6),

A simpler explanation can be offered now on the grounds of our findings. The slower growth rate of the LEE strain is compensated for by the large fraction of non-viable offspring the PR8 strain produces after concentrated inocula. The CC strain, producing a smaller amount of incomplete virus, will readily outgrow the LEE strain whether starting at high or low dilutions. It is significant that this CC versus LEE behaviour applies *only* to the allantois and not to mouse lungs, where the two viruses could be carried through five passages without any diminution of their initial ratio, and through further passages with only slight drop of the LEE component (Sugg and Magill, 1948). This latter difference is in agreement with the postulate of our working hypothesis that the amount of incomplete virus produced should vary from tissue to tissue.

SUMMARY.

Ten representative influenza virus strains have been tested for production of non-infective elementary bodies after allantoic inoculation of concentrated seed. The strains were found to differ widely in their capacity to yield incomplete offspring, and to fall into the gradient PR8(A) > WSE(A), MEL(A) > SW > BON(B), BEL(A) > HUT(B), CAM(A′) > FM1(A′) > LEE(B), in the order of decreasing incomplete virus production.

By serial passaging of undiluted inocula it could be shown that even the strains at the lower end of the gradient produced some non-infective particles under special conditions.

The hypotheses covering incomplete virus production are discussed in detail, and a new interpretation is offered to account for the serial simultaneous passage of two influenza strains in various hosts.

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