GENETIC STUDIES OF INFLUENZA VIRUSES

I. Viral Morphology and Growth Capacity as Exchangeable Genetic Traits. Rapid in Ovo Adaptation of Early Passage Asian Strain Isolates by Combination with PR8*

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Plates 25 to 28

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Evidence for recombination or genetic interaction among strains of influenza viruses has been presented by several groups of investigators (1–3). Of special interest is the observation, originally made by Burnet and Lind (4), that virus rendered non-infective might participate in genetic interchange as a "parent" when mated *in vivo* with a differing and infective virus strain. Potential advantages of this type of recombination include: (*a*) the reduced chance of production of unstable, diploid, heterozygotes, (*b*) elimination of the vagaries of double infection, and (*c*) the ease of separating from the infective parent recombinant forms with differing characteristics through the use of a selective environment (e.g. antiserum) inimical to the infective progenitor.

The evidence that certain biologic and chemical characteristics of influenza viruses may be interchanged is not only of theoretical interest but augurs possible pragmatic applications of consequence. Thus, laboratory combination of a poorly growing strain of the desired antigenicity with a virus of greater growth potential should result in recombinant progeny more suitable for vaccine production. Indeed, the poor yields of Asian influenza virus in chick embryos during the 1957 influenza pandemic seriously curtailed vaccine production, and prompted efforts in several laboratories, including our, to produce a recombinant virus with better growth capacity. Such efforts proved futile.

During the conduct of recent studies in our laboratories of the interchange of filamentous and spherical viral morphology as genetic traits, the earlier goal of producing an (antigenically) Asian virus of increased growth capacity was

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realized inadvertently. The investigations here presented have defined the requisite conditions for strain-to-strain transfer of augmented growth potential in the chick embryo allantoic sac and its apparent concomitant of predominantly spherical viral morphology.

Materials and Methods

Viruses.—The classical PR8 strain of influenza A virus (now categorized antigenically as group A (5)), an Asian (group A2 (5)) strain A/217 isolated in this laboratory from the lung of a fatal case of influenza (6) and an inhibitor-sensitive clone of an Asian (A2) strain

TABLE I

Properties of the Parent Viruses (Experiments I to III)

	Viruses		
	A/217	PR8	RI-5+
Antigen	A2	A	A2
Morphology	Filamentous	Spherical	Filamentous
Hemagglutination titer*	128–256 (8–9 $\log_2$)	4096–8192 (12–13 $\log_2$)	64 (7 $\log_2$)
Hemagglutination sheep RBC	+	0	+
Mouse virulence	0	+	0
Inhibitor sensitivity	+0 (mixed)	0	+
Passage history			
isolated in:	1958	1934	1957
from:	Lung	Throat	Throat
egg passage:	3	100+	6

* Reciprocal of viral dilution at titration end point.

isolated in the Rockefeller Institute (RI/5+ (7))[1] were employed in the present study. Properties of these strains are summarized in Table I. The PR8 strain was passed at limiting dilution ($10^{-8.5}$) before preparation of seed virus for inactivation at 35°C. for 17 days. After similar limiting dilution passage, an aliquot of A/217 virus in the form of dialyzed allantoic fluid was exposed as a thin layer in a Petri dish with a magnetic stirrer to ultraviolet irradiation for 30 seconds from an 8 watt germicidal lamp 7 inches from the fluid surface. Two successive passages of both the PR8 and A/217 preparations as undiluted allantoic fluid virus in cortisone-injected eggs failed to induce the formation of detectable viral hemagglutinin, thus demonstrating the extirpation of residual infectivity. (Passage in cortisone-injected eggs,—also effected throughout the passage of recombinants to be described,—reduces the autointerference occasioned by injection of large doses of undiluted influenza virus (8).)

Eggs.—In the present experiment intact 10 to 12 day old white Leghorn chick Embryos were used for the propagation of virus.

"Cortisone".—The compound referred to herein as "cortisone" possesses high aqueous solubility (100 mg./ml.) and is actually hydrocortisone diethylaminoacetate hydrochloride. It was employed in a dosage of 0.01 mg./egg 1 hour prior to injection of virus.

[1] Kindly supplied by Dr. Purnell W. Choppin of The Rockefeller Institute.

Hemagglutination and infectivity titrations were carried out by methods described previously (6, 9).

Hemagglutination-inhibition tests for the antigenic identification of virus utilized antisera produced in rabbits by the intravenous inoculation of either PR8 (A) or Jap. 305 (A2) influenza viruses. Sera were inactivated prior to use at 56°C. for 30 minutes. When diluted initially 1:10, these sera were specific in inhibiting only homologous virus in a final concentration of 4 to 8 units.

Electron Microscopy.—Freshly harvested allantoic fluid from groups of 6 eggs was centrifuged at 2700 *g* for 15 minutes to remove any red blood cells, and the supernate ultracentrifuged at 26,360 *g* (average) for 1 hour in the S40 head of the Spinco preparative centrifuge. The sediment was resuspended in approximately 0.1 ml. of the supernate and kept at 4°C. overnight to allow complete disaggregation. Thin formvar (R) films were made by air-drying standard microscope slides which had been dipped in a solution of 0.3 per cent formvar (Shawinigan Resins Corp., New York). Subsequently, the film was floated off on a clean water surface; two standard electron microscope grids (150 mesh) were inverted on the floating film, and the film with grids was replaced on the slide in such a way that the grids were sandwiched between the film and the glass.

After these prepared slides were dry, a small drop of the virus sample was pipetted on to each coated grid and the slide was placed in a closed Petri dish containing 0.5 ml. of 2 per cent osmium tetraoxide for 10 to 15 minutes at room temperature. The slide was then removed and very gently flooded with 0.5 ml. of distilled water for 5 minutes. This was repeated three consecutive times, whereupon the slide was drained and air-dried. One grid of each pair was shadowed with chromium. This method produced highly consistent samples on repetition and gives substantially the same results as more complex methods while providing a high percentage of successful well fixed preparations. Some of the influenza strains in this laboratory (J. S. M.) give such a characteristic appearance that they can be consistently differentiated from each other in a blind experiment. For example, the Jap. 305 strain is largely very short filaments, and the Lee strain of influenza B virus is primarily spherical but has filaments which vary in diameter.

Estimation of "Predominance" of Spherical or Filamentous Particles by Electron Microscopy.—The viruses employed in the present experiments were selected for recombination on the basis of their disparate morphologic character. Thus, with the standardized preparatory procedure used, PR8 is never manifest as long filaments, and in every preparation is almost exclusively spherical, as in Fig. 1. The Asian strains, in contrast, were consistently observed to consist of a mixture of long filaments and spheres, with filaments being readily apparent in any microscopic field, as in Fig. 2. As the demands of the present experiment required the transfer of morphology as a "black and white" character, subtle changes in the sphere/filament ratio were not acceptable as evidence and therefore were not looked for. All readings of morphology have been "blind."

EXPERIMENTAL RESULTS

Recombination of the PR8 and A/217 Strains of Influenza A Virus (Experiment I)

Earlier experiments in this laboratory (E.D.K.) have defined effective conditions for *in ovo* genetic combination of non-infective and infective influenza virus strains (10). These conditions include the prior inoculation of a large quantity of the inactivated virus, the subsequent injection into the allantoic sac of 10^6 to 10^8 EID_{50} of infective virus and the prompt harvesting of resulting viral yields at less than 24 hours. Under these conditions it can be shown by

infectivity titrations in the presence of antiserum suppressive to the infective parent that from 0.01 to 0.1 per cent of the yield will comprise virus of antigenic type different from the infective parent. Assuming the production of an equal number of recombinants of the opposite antigenic type, the total number of recombinant particles in the initial passage may be estimated as 0.02 to 0.2 per cent of the yield.

In the present experiment (Table II), 4 groups of 4 12-day-old chick embryos were first injected with either water or cortisone, then 1 hour later with either 1024 hemagglutinating units of heat-inactivated PR8 virus or phosphate buffered saline. One hour later, saline or $10^{6.5}$ EID_{50} of A/217 virus was injected. All injections were made into the allantoic sac. Twenty hours after the inoculation of infective virus, eggs were chilled for 1 hour at −30°C., then harvested individually for allantoic fluid virus. Subsequently, serial and separate passages

TABLE II

Recombination of PR8 (A) and A/217 (A2) Strains of Influenza Virus (Experiment I)

Group	No. of eggs	−2 hrs.	−1 hr.	0 hr.	+20 hrs.
1	4	Cortisone	Saline	A/217	Harvest
2	4	Cortisone	ΔPR8	A/217	Harvest
3	4	H_2O	ΔPR8	A/217	Harvest
4	4	Cortisone	ΔPR8	Saline	Harvest

Cortisone, 0.01 mg./egg of hydrocortisone diethylaminoacetate hydrochloride.
ΔPR8, non-infective virus, 1024 hemagglutinating units/egg.
A/217, $10^{6.5}$ egg infective $doses_{50}$/egg.

were made of the fluids from the individual eggs of the 4 different experimental groups. Thus, the experiment was done simultaneously in quadruplicate. In most passages allantoic fluid inocula were diluted only 1:2 ($10^{-0.3}$) to assure the transfer of recombinant progeny present in low concentrations. Passages were carried out in cortisone-injected eggs to reduce the potential viral autointerference of the concentrated inocula which otherwise might be expected to lead to the development of viral yields with low infectivity/hemagglutination ratios (incomplete virus). Experiments in our laboratories have demonstrated that cortisone does not significantly modify the ratio of spherical and filamentous particles observed by electron microscopy.

The results of the passages subsequent to recombination are detailed in Table III, in which the passage history of each individual egg line may be traced. In several instances, parallel or repeated passages were made. In this event aliquots of individual allantoic fluids were sometimes pooled for electron microscopy studies. Such additional passages are indicated by letter as 2 *a*, *b*, etc.

The Emergence of Virus of Changed Character on Serial Passage.—Following the initial *in ovo* combination of the filamentous Asian strain A/217 (Fig. 2), and the inactivated spherical PR8 strain (Fig. 1), allantoic fluids of individual

eggs were passed at low dilution (1:2 in antibiotic saline) in cortisone-injected eggs. The assumption was made that the spherical forms of virus characteristic of most egg-adapted strains were intrinsically better able to survive and flourish in the egg milieu than the filamentous virus of new isolates so that once intro-

TABLE III

Rapid Emergence Following Recombination of Asian (A2) Virus of Changed Morphology and Growth Potential (Experiment I)

Passage No.	Dil.	With antiserum *vs.*	Inocula of initial (recombination) passage (Table II)																	
			Group 1 (A/217 + cortisone)						Group 2 (ΔPR8 + A/217 + cortisone)						Group 3 (ΔPR8 + A/217)					
			Log₂ HA titer* Egg No.						Log₂ HA titer Egg No.						Log₂ HA titer Egg No.					
			1	2	3	4	Avg.	Antigen	Morphology	1	2	3	4	Avg.	Antigen	Morphology	1	2	3	4	Avg.	Antigen	Morphology
1	0.3	—	7	6	8	8	(7.2)	A2	—	8	9	7	6	(7.5)	A2	—	9	7	7	7	(7.4)	A2	—
2 *a*	0.3	—	4	9	6	7	(6.5)	A2	—	7	5	6	9	(6.7)	A2	—	5	7	6	6	(6.0)	A2	—
b	0.3	—	—	—	—	—	(8.0)	A2	Fil.	—	—	—	—	(8.0)	A2	Sph.	—	—	—	—	(8.0)	A2	Sph.
c	2.0	—	—	8	9	7	(8.0)	A2	—	12	9	11	9	(10.2)	A2	—	9	9	10	10	(9.5)	A2	—
3 *a*	0.3	—	—	—	—	—	(8.0)	A2	Fil.	—	—	—	—	(11.0)	A2	Sph.	—	—	—	—	(10.0)	A2	Sph.
b	0.3	A2	7	9	6	8	(7.5)	A2	Fil.	11	12	12	12	(11.8)	A	Sph.	11	13	12	11	(11.7)	A	Sph.
4	0.3	A	9	8	8	9	(8.5)	A2	Fil.	11	13	13	13		A2	Sph.	12	13	13	13		A2	Sph.
5	3.0	—	9	8	8	9	(8.5)	A2	Fil.	10	11	11	10	(10.5)	A2	Sph.	12	10	11	12	(11.2)	A2	—
6	3.0	—	—	—	—	—	(9.0)	A2	Fil.	—	—	—	—	(13.0)	A2	Sph.	—	—	—	—	—	—	—

* Log_2 of the reciprocal of the highest dilution of virus inducing hemagglutination.

Dil., log_{10} of reciprocal of dilution of allantoic fluid used in passage.

Avg., geometric mean of log_2 hemagglutinin titer of individual fluids; arithmetic mean titers when individual titers are not shown.

Fil., filamentous.

Sph., spherical.

Note: The passage history of group 4 (ΔPR8) (Table II) is not detailed here as in no instance did hemagglutinin appear on passage.

duced by selection, mutation, or recombination virus of predominantly spherical expression would outgrow the filamentous forms characteristic of new isolates. In assessing the significance of such replacement of predominantly filamentous with predominantly spherical virus, the great importance of adequate control passages of filamentous virus alone was appreciated. Choppin, Murphy, and Tamm (11) have observed the sudden change in morphology of Asian isolates followed by electron microscopy during serial passage at low (10^{-3}) dilution,

and Burnet and Lind (12) have reported the change of a predominantly filamentous virus to a spherical form after 12 to 15 passages at low dilution. For this reason the present experiments included serial passage of group 1 fluids (Tables II and III) initially derived from infection with the A/217 virus only. A further control, group 4, (Table II) retested the prior demonstration that the noninfective PR8 parent virus did not in fact possess residual infectivity. Results of passage of this group are not recorded in Table III as they were repeatedly and uniformly negative.

Examination of Table III demonstrates that as early as the second passage (2 *b*) after recombination, predominantly spherical virus was present in group 2 and 3 fluids while the virus of group 1, in which recombination was not effected, remained highly filamentous. Of further interest was the 2.7 to 4.6-fold greater yield of virus in recombinant groups 2 and 3 noted in a later repetition of the second postrecombination passage (2 *c*) when passage fluids were injected in 10^{-2} dilution. In the third passage (3 *a*) both the spherical morphology and the augmented yields of viral hemagglutinin were again apparent in the egg groups derived from PR8-A/217 interaction (groups 2 and 3), while virus derived only from the infective parent (A/217) retained its original characteristics.

The Antigenic Nature of the Spherical Virus of Increased Growth Capacity Which Emerged during Serial Passage.—The hemagglutinating virus of each individual allantoic fluid of each group was subjected to antigenic analysis in hemagglutination-inhibition tests with antisera for PR8 (A) and Asian (A2) viruses. In every instance fluids of the passages described above were found to contain hemagglutinin inhibited with Asian (A2) antiserum but not with antiserum directed against the PR8 strain. Therefore, the spherical virus of increased growth potential did not represent mere reactivation of the heated PR8 virus which had been injected initially in groups 2 and 3, but was demonstrably derived from the infective Asian strain parent. However, the ability of the heated PR8 virus to contribute genetically is suggested not only by the evidence cited above that its morphology and growth potential might be acquired by virus of Asian antigenicity, but more directly by the evidence which follows that recombinants of PR8-like (A) antigenicity were produced.

Selection of Recombinants with Specific Antisera.—In a repetition of the third passage of fluids from eggs of passage 2 *a* (Table III), undiluted fluids were injected with initial 1:10 dilutions of Asian (A2) antiserum (passage 3 *b*). Predominantly spherical virus of high titer again emerged from recombinant groups 2 and 3. Antigenic analysis of the resulting virus yields showed the virus of groups 2 and 3 to be antigenically PR8 (A) and the virus of group 1 to be of Asian type, as expected. Thus, even a quantity of Asian antiserum inadequate to suppress significantly the multiplication of Asian virus, nevertheless permitted the emergence in one passage of PR8-like virus. A subsequent passage (4) with *PR8 (A) antiserum* demonstrated the continued latent presence in all

groups of virus antigenically Asian (A2). This virus in both groups 2 and 3 again contrasted with the parent Asian virus of group 1 in its markedly higher hemagglutination titer. Comparison of a parallel fourth passage for electron microscopy revealed once more the spherical nature of the recombinant virus and the persistence of a predominantly filamentous form of the A/217 parent. The continued persistence of enhanced growth potential and spherical morphology was still observable on two succeeding (5th and 6th) passages conducted in the absence of antiserum and using higher (10^{-3}) dilutions of virus. The stability of the A2 virus of increased growth potential has been verified in 4 subsequent (total 10) passages. The retention of spherical morphology was verified on the 7th postrecombination passage of group 3 fluids, as is illustrated in Fig. 3.

TABLE IV

Emergence of a Virus Antigenically PR8 (A) but of Predominantly Changed (Filamentous) Morphology Following Recombination with A/217 (A2) Virus

Postrecombination passage* No.	Passed with antiserum vs.	Virus antigenically	Geometric mean $\log_2$ hemagglutination titer	Predominant morphology
1	—	Asian (A2)	7.5	—
2	—	Asian (A2)	8.0	Spherical
3‡	Asian (A2)	PR8 (A)	11.0	Filamentous
4	—	PR8 (A)	9.0	Filamentous§
5	—	PR8 (A)	10.0	Filamentous
6	—	PR8 (A)	12.0	Filamentous‖

* Following recombination of A/217 (A2) filamentous Asian virus and PR8 (A) spherical influenza virus in the presence of cortisone (Experiment I, group 2, Table III).

‡ Different 3rd passage than passage 3 *b*, Table III.

§ No spheres seen (Fig. 4).

‖ Lower ratio of filaments to spheres than in previous passages.

The Emergence of a Virus Antigenically PR8 (A) but of Predominantly Filamentous Morphology and Decreased Growth Rate in Passage Following Recombination.—The possibility of a spontaneous evolution from filamentous to spherical morphology,—although well controlled in the present study,—is a bothersome one. Therefore, the observed emergence of virus with PR8 antigen and filamentous form provided more decisive evidence that viral morphology is a transferable trait, as spontaneous change from *spherical* to *filamentous* morphology has not been reported. The circumstances under which this filamentous PR8 strain emerged are summarized in Table IV. It will be noted that the use of Asian (A2) antiserum on the 3rd postrecombination passage 3 *b* (*not* shown in Table III) resulted in a yield of virus of augmented titer and filamentous form. So unequivocal was this change that on the next passage no spherical forms were observed on electron microscopy (Fig. 4). The continuing stability of this

form on low dilution passage is open to question, however, even though filaments still predominanted at the 6th passage as a lower ratio of filaments to spheres was observed on this passage. Also, a decreased growth rate noted on the 4th postrecombination passage was no longer evident with virus derived from the 6th passage.

A Comparison of Certain Biological Properties of the Parent Virus and Two Progeny (Recombinant) Strains. Virulence for Mice.—The isolation of a 'filamentous" PR8 and a "spherical" Asian strain prompted comparison of their properties with those of the parent viruses. It was of special interest to determine whether virulence for the mouse lung (possessed by the PR8 parent) was linked genetically with the antigen of the mouse virulent parent or to the morphology of its particle. The comparative virulence for the mouse of the parent and recombinant viruses was studied in an experiment in which varying dilutions of virus providing 10^5 to 10^8 EID_{50} were inoculated by the intranasal route into 18 gm. CFW male mice, 3/group under light ether anesthesia. A record of mortality was kept and surviving mice were killed on the 7th day after inoculation and their lungs scored for lesions. A percentile score of both mortality and lesion severity was calculated by a modification (13) of a method described by Horsfall (14). It was observed that recombinant 1 (Asian antigen-spherical morphology) was even less effective than A/217 in inducing pneumonia in mice. In contrast, recombinant 2 (PR8 antigen-filamentous morphology) was at least as virulent as the parent PR8 strain as measured by the minimum quantity needed to induce a lesion score of more than 50 per cent. These results are obviously inadequate to establish true linkage of antigenicity and virulence, but they do suggest a dissociation of viral morphology and viral virulence for the mouse lung. Furthermore, evidence is provided of an additional trait (virulence or avirulence) other than antigenicity—shared by each recombinant and its parent of similar antigenicity. A summary of comparative properties of parent viruses and two recombinant progeny is presented in Table V.

Agglutination of Sheep Erythrocytes.—A useful marker in previous genetic studies in this laboratory (E.D.K.) has been the capacity of certain influenza virus strains to agglutinate sheep red blood cells at 22° to 30°C. This property is possessed by all Asian strains so far isolated or examined here, and has appeared to be stable on passage. As the PR8 strain used in this laboratory does not agglutinate sheep red blood cells at 30°C., it was considered that agglutination of these cells would be useful in the present study. In passages of A/217 and possible recombinants prior to the use of selective antisera, the viruses of groups 1 (control) and 3 (Table III) retained their capacity to agglutinate sheep erythrocytes. In the case of group 2 in which recombination had been effected in the presence of cortisone, this property disappeared in 3 of 4 egg lines on the 3rd passage. Interpretation of this fact is confounded by the loss of sheep cell–agglutinating capacity of the A/217 virus itself after the 4th and subsequent

passages, perhaps related to the intervening use of rabbit antisera (see below). As shown in Table V, the capacity to agglutinate sheep erythrocytes was not acquired by virus manifesting PR8-like antigenicity and mouse virulence.

Inhibitor Sensitivity.—The parent viruses A/217 and PR8 differed with respect to their inhibitability by normal guinea pig and horse serum heat-inactivated at 56°C. for 30 minutes. Hemagglutination by strain A/217, like that of many Asian strains, is inhibited by dilutions of serum of 1:16 to 1:64, while 4 hemagglutinating units of PR8 are not inhibited by a dilution of 1:4. The usefulness of this possible marker was—like the sheep cell agglutinating trait—similarly invalidated by the loss of this trait on passage (with serum) by the A/217 virus. Since this study was initiated it has been shown that Asian strains

TABLE V

Properties of Parent Viruses and Two Recombinant Progeny (Experiment I)

	Parents		Progeny	
	A/217	PR8	1	2
Antigen	A2	A	A2	A
Morphology	F	S	S	F
Hemagglutination titer*	128–256 (8–9 $\log_2$)	4096–8192 (12–13 $\log_2$)	8192 (13 $\log_2$)	1024 (10 $\log_2$)
Hemagglutination of sheep RBC	+	0	0	0
Mouse virulence	0	+	0	+
Inhibitor sensitivity	+0	0	0	+
In ovo growth rate	+	+++	+++	++

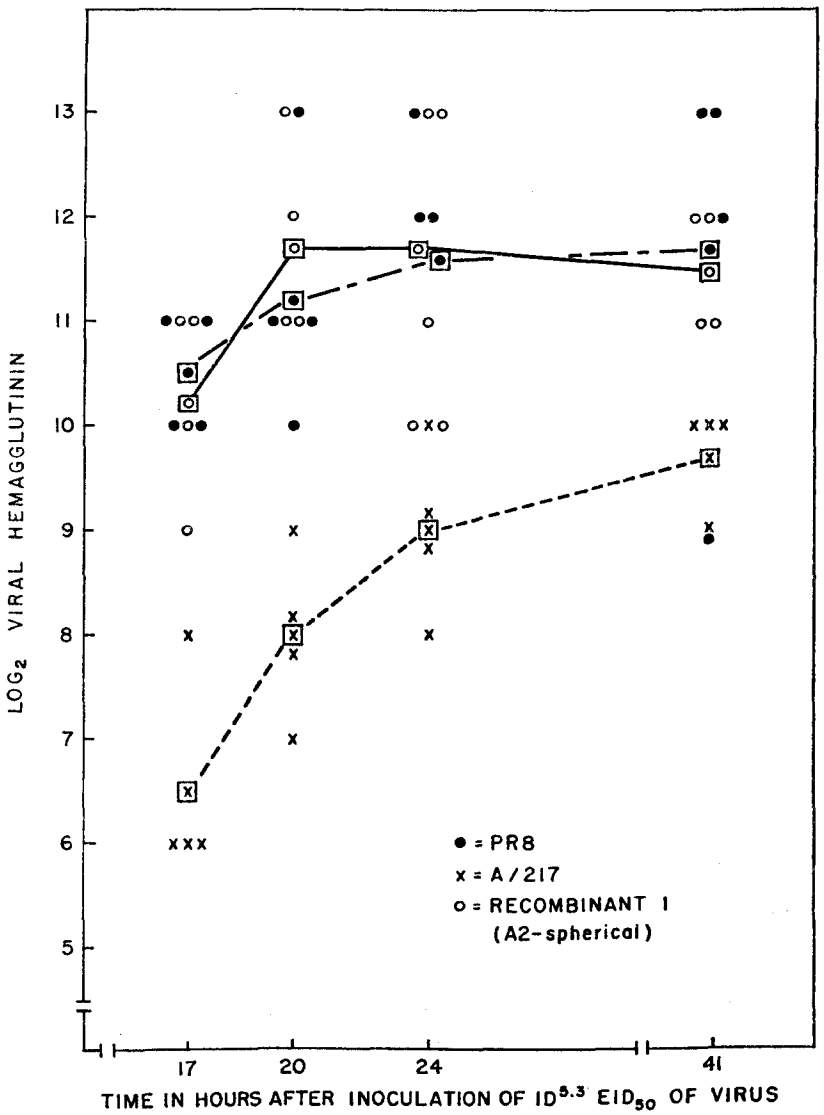
F, predominantly filamentous.

S, predominantly spherical.

* Reciprocal of viral dilution at titration end point.

of influenza A virus are mixtures of two discrete types of particles: inhibitor-sensitive and inhibitor-resistant (7). The probability exists that the parent strain in the present study comprised an unrecognized mixture of the two types of particles, and that the selective pressure of passage with serum removed the inhibitor-sensitive particles. It is notable, however, that recombinant 2 (PR8 antigen-filamentous morphology) possesses inhibitor sensitivity.

The Comparative Growth Rates of Parent and Recombinant Viruses.—To confirm the earlier assumption that A2 virus with acquired PR8-like growth potential might emerge following recombination, the *in ovo* rates of increase of recombinant 1 (Table V) and its parents were measured in the same experiment. Recombinant virus represented a pool from a repeated second post-recombination passage of the fluids of group 2 (Table III) at 10^{-2} dilution. On the basis of infectivity titration of this pool, an inoculum calculated to contain $10^{5.3}$ EID_{50} was injected into groups of 4 eggs. Identical doses of PR8 virus and



TEXT-FIG. 1. Comparative incremental rates of parent viruses PR8 and A/217 and A2 spherical recombinant 1 (Experiment I)(Table V). Rates measured by determination of hemagglutinin content of allantoic fluids. The values for individual eggs are shown; geometric mean values for each point in time are enclosed in squares. $10^{5.3}$ EID_{50} of each virus inoculated.

A/217 virus (control group 1, Table III) were inoculated simultaneously. Individual allantoic fluids were harvested and titrated for hemagglutinin at 17, 20,

24, and 41 hours thereafter. The individual and geometric mean values derived from these titrations are presented in Text-fig. 1. The virtual lack of overlap in the distribution of hemagglutinin concentrations of the parent Asian strain and the other 2 viruses is notable. So too is the close correspondence of the incremental curves of PR8 and recombinant 1. It is thus apparent that this spherical, antigenically Asian virus possesses not only the capacity to attain high concentration *in ovo*, but the capacity to increase more rapidly than its Asian parent. This attribute obviously affords a competitive advantage for the recombinant which might allow it to emerge to predominance even in the absence of strategems calculated to suppress its slower growing parent.

Recombination of ΔPR8 and Another Asian (A2) strain, RI/5⁺. The failure of Inactivated Filamentous Virus (A/217) to Effect Emergence of Spherical Virus (Experiment II)

Despite the evidence provided above that the emergence of a spherical Asian virus occurred in association with manifest recombination, the point could be raised that this result might be a non-genetic effect of the injection of inactivated virus. Therefore, in the present experiment (outlined in Table VI) the effect of ultraviolet inactivated A/217 (filamentous) virus as well as that of inactivated PR8 virus was studied. A further modification was the employment of another recently isolated filamentous Asian strain (inhibitor-sensitive RI/5⁺) as the infective parent. This strain had received 2 limiting dilution passages for a total of 6 allantoic passages. When subsequently passed with horse serum inhibitor at 10^{-3} dilution its *seeming freedom from inhibitor-resistant particles* had been demonstrated.

The results of this experiment will be described but briefly as they comprise largely a repetition and confirmation of the previous experiment. As shown in Table VI, the simultaneous passage of inactivated PR8 (A) and infective A2 (RI/5⁺) virus resulted in the evolution of A2 virus of increased titer and predominantly spherical morphology. This virus (as recombinant 1 in the previous experiment) was found to possess a faster incremental rate than its infective parent. Simultaneous passage of RI/5⁺ alone or with inactivated A/217 resulted in no such changes. The non-infectivity of the inactivated viruses was confirmed.

An attempt was made to isolate a recombinant strain which was antigenically Asian (A2), spherical, and inhibitor-*resistant*, as indisputable proof of the genetic contribution of PR8 to the supposed pure clone inhibitor-sensitive (I⁺) RI/5⁺ virus. Such a strain was readily isolated by passage with heated normal horse serum (7). However, from one of the 4 egg lines of the control passage of RI/5⁺ alone similar virus could also be recovered. Re-examination of the starting seed (which had been doubly cloned) revealed that passage under the conditions of the experiment (*i.e.* at 1:2 dilution in cortisone-injected eggs) un-

veiled the presence of contaminating I (inhibitor-resistant) virus not remarked by conventional methods.

Definitive Evidence for the Genetic Contribution of ΔPR8 Virus to the Spherical, High Titer, Asian Virus Appearing after Recombination (Experiment III)

The important question remained as to whether the spherical, fast growing virus derived from the infective Asian strains possessed genetic characters other than these attributes which it might ultimately acquire spontaneously at low dilution passage. For this reason a third experiment was undertaken, this time

TABLE VI

Recombination of ΔPR8 (A) and RI/5$^+$ (A2) Strains of Influenza Viruses. Failure to Effect Emergence of Spherical Virus with an Inactivated Filamentous Virus (A/217 (A2)) Experiment II

Initial Passage					On 3rd passage*		
Group	No. eggs	−1 hr.	0 hr.	+20 hr.	Antigen	HA titer‡	Morphology
1	4	Saline	RI/5$^+$	Harvest	A2	8.5	Filament
2	4	u-v A/217	RI/5$^+$	Harvest	A2	8.5	Filament
3	4	ΔPR8	RI/5$^+$	Harvest →	A2	10.2	Sphere
4	4	u-v A/217	Saline	Harvest	No growth		
5	4	ΔPR8	Saline	Harvest	No growth		

u-v A/217, ultraviolet irradiated, non-infective A2 virus; 64 hemagglutinating units/egg.
ΔPR8, non-infective virus; 1024 hemagglutinating units/egg.
RI/5$^+$, inhibitor-sensitive A2 virus—$10^{6.3}$ EID_{50}/egg.
* 3rd passage subsequent to initial recombination passage.
‡ HA titer-$\log_2$ of reciprocal of highest dilution at titration end point (geometric mean of hemagglutination titers of 4 eggs).

employing once more the inhibitor-sensitive RI/5$^+$ virus, but this time a doubly cloned strain demonstrably free of I particles even when subjected to 2 successive passages at 1:2 dilution with horse serum in cortisone-injected eggs. The protocol for this experiment appears in Table VII. Once again, A2 virus of increased titer and spherical morphology emerged on the 2nd postrecombination passage. When individual allantoic fluids of groups 1 (control) and 2 (recombination) were passed in the presence of a 1:2 dilution of normal horse serum heated 56°C. for 30 minutes, no hemagglutinin was detectable after 40 hours of incubation in group 1, but virus identified antigenically as PR8 was present in all eggs of group 2. When these fluids were then passed with PR8 antiserum, A2 virus emerged to predominance. This virus was resistant *in vitro* to normal horse serum inhibitor. When studied by electron microscopy the virus proved to be spherical in shape. This A2I$^-$ virus could be isolated in a single step by passage

of group 2 (3rd passage) fluids with normal horse serum and PR8 antiserum. Thus, firm evidence of genetic contribution of ΔPR8 to the spherical Asian virus was obtained.

TABLE VII

Recombination of ΔPR8 (A) and RI/5$^+$ (A2) Strains of Influenza Virus (Experiment III)

Initial passage					On 2nd passage*		
Group	No. eggs	−1 hr.	0 hr.	+20 hr.	Antigenicity	HA titer‡	Morphology
1	4	Saline	RI/5$^+$	Harvest	A2	7.7	Filament
2	4	ΔPR8	RI/5$^+$	Harvest →	A2	9.5	Sphere
3	4	ΔPR8	Saline	Harvest	No growth		

ΔPR8, non-infective virus, 1024 hemagglutinating units/egg.

RI/5$^+$, inhibitor sensitive clone of A2 (Asian) virus; $10^{4.8}$ EID_{50}/egg.

* 2nd passage subsequent to initial recombination passage.

‡ HA titer = $\log_2$ reciprocal of highest dilution at titration end point (geometric mean of 4 individual hemagglutination titrations).

TABLE VIII

Evidence of the Genetic Contribution of ΔPR8 Virus to the Spherical Asian (A2) Virus. The Isolation of Recombinants by Selective Procedures (Experiment III)

	Parent viruses		Recombinant isolated by passage with:		
	PR8	RI/5$^+$	(1) anti-I$^+$ (NHS)	(2) anti-I$^+$ anti A	(3) anti-A2
Antigen	A	A2	A	A2	A A
Inhibitor sensitive (I$^+$)	0	+	0	0	+ 0
SCA	0	+	0	0	+ 0
Morphology	S	F	S	S	— —

Anti-I$^+$ (NHS), normal horse serum Δ56°C. for 30 minutes.

Anti-A, PR8 antiserum.

Anti-A2, Jap. 305 antiserum treated with trypsin to destroy inhibitor.

SCA, capacity to agglutinate sheep RBC at 30°C.

S, spherical.

F, filamentous.

That inhibitor sensitivity is a transferable genetic trait is further attested by the demonstration that when A2 (Japan 305) antiserum was heated with 2 per cent trypsin to destroy inhibitor, A (PR8) virus possessed of inhibitor sensitivity (AI$^+$) could be isolated from group 2 fluids (see Table VIII).

The Relation of Inhibitor Sensitivity to Sheep RBC Agglutination at 30°C.—The puzzling loss of ability of A2 virus to agglutinate sheep RBC at high tempera-

ture which was noted above following passage of virus with antisera, now appears explicable on the basis at: (*a*) such antisera contained inhibitor, and (*b*) the sheep RBC agglutination of early passage Asian viruses appears to be effected by I^+ (inhibitor sensitive) particles (which do not elute (7)). Evidence for this point may be inspected in Table VIII in which the coincidence of inhibitor sensitivity and (SCA) sheep cell agglutinating capacity are noted with both A and A2 recombinants.

TABLE IX

The Yield of Recombinant Virus on Initial (Recombination) Passage (Experiment III)

Virus	+ Anti-serum	Viral yield EID_{50}/0.1 ml.	Recombinant selected
Exp. III, group 1 ($RI/5^+$)	—	10,000,000	—
" "	A2	0	—
" "	NHS	0	—
Exp. III, group 2 ($\Delta PR8 + RI/5^+$)	—	5,000,000	—
Exp. III, group 2 ($\Delta PR8 + RI/5^+$)	A2 + NHS	500	AI^-
Exp. III, group 2 ($\Delta PR8 + RI/5^+$)	A + NHS	2.5	$A2I^-$

A2 antiserum, 1:4 dilution of rabbit antiserum *vs.* Jap. 305 virus.

A antiserum, 1:10 dilution of rabbit antiserum *vs.* PR8 virus. (This dilution calculated not to inhibit 1.0 EID_{50} of $RI/5^+$ virus.)

NHS, normal horse serum in 1:2 dilution (inhibitor of I^+ virus).

An Analysis of the Initial Yield of Recombinants.—Serial decimal dilutions of pooled virus yielded during the initial recombination passage of this experiment (group 2) were injected together with low dilutions of inhibitor and antibody into groups of 3 eggs as indicated in Table IX. As all progeny of the A2 infective parent are I^+ (inhibitor-sensitive), any virus emerging in the presence of NHS inhibitor is recombinant. The suppression of A2 non-recombinant virus (group 1) was demonstrated. It may be observed that the yield of $A2I^-$ recombinants was extremely low, and was only revealed by a further passage of individual egg fluids. On the other hand, the yield of AI recombinants was 200-fold greater, comprising 0.001 per cent of the total yield. This finding is baffling if one assumes (*a*) that "reactivation" of the inactivated parent PR8 may not occur unless effected by recombination involving equal genetic contribution from both parents and (*b*) that the *in ovo* environment is equally non-selective for all recombinants. Neither assumption is necessarily valid, but on the basis of present evidence for both bacteriophage (15) and influenza virus (16), virus induced viral reactivation as a non-genetic viral effect seems unlikely.

The relative paucity of A2I$^-$ virus gleaned from the first passage may be related to a competitive disadvantage. When grown with A2I$^+$ as in recent experiments directed at the isolation of these recombinants in later passages of Experiment III, A2I$^-$ proved difficult to isolate even in the presence of horse serum. It is probable that the sustained dominance of A2 virus in the present experiments is the result of multiple matings involving the infective A2 parent and its recombinant progeny (see Text-fig. 2). The total gene pool of the resulting population should therefore include more A2, which coupled with genes carrying PR8 growth potential will result in the eventual predominance of such A2 virus over the original infective parent and recombinants derived from the lesser pool of A antigen. This hypothesis is in accord with the observed lag of 2 to 3 passages before A2 virus of spherical morphology and increased multiplication potential emerges. If these viral attributes are determined by more than a single gene, then repeated recombination involving several loci may be required for the production of virus with optimal survival advantage. This possibility is supported by the recent report of Fraser (17) that a graded increase in virulence may be noted in successive cycles of double infection with the N-M recombinant strain and the neurotropic NWS strain of influenza virus.

x = PR8 gene pool, in whole or in part (including A antigen, rapid growth rate)
y = RI/5$^+$ gene pool, in whole or in part (including A2 antigen, slow growth rate)
if reactivation of ΔPR8 implies its recombination with RI/5$^+$ (16) then:

$$\Delta x + y \rightarrow x^*y + y^*x + y$$

then, on subsequent matings involving recombinants, as well as infective virus y:

$$xy + yx + y \rightarrow xy + \underline{yx} + y$$
$$(xy - yx) + (\underline{yx - xy}) + (xy - y)$$
$$(\underline{yx - y}) + (\underline{y - yx}) + (\underline{y - xy})$$

thus, the eventual predominance of y (A2) antigen combined with x (PR8) survival advantage (growth rate) is predictable in the absence of a selective environment. (Such potential strains on the basis of the first mating involving recombinants are underlined above.)

* First letter represents antigenic type of the recombinant.

TEXT-FIG. 2. An hypothetical scheme to explain the emergence and predominance of recombinant virus antigenically (A2) Asian but with A-like growth potential (Experiment III).

A Review of Earlier Failures to Transfer Increased Growth Potential from a Laboratory Strain to an Asian Strain.—Earlier experiments in this laboratory had failed to transfer the property of increased *in ovo* growth potential to the Asian (A2) prototype Jap. 305 strain of influenza virus, or to the A/217 strain, although recombination of these strains with the PR8 (A) strain was demonstrably accomplished. A review of these experiments in the light of the present

successful ones discloses that: (*a*) all such experiments employed inactivated Asian viruses and infective PR8 (so that later selective pressure with PR8 antiserum would induce the evolution of virus of the requisite *Asian* antigenicity), and (*b*) antiserum was used in the *first* postrecombination passage, as suggested by other investigators (3). In the present experiments two additional passages were allowed for the multiplication of possible recombinants before the use of antiserum (the use of the latter, indeed, proving to be unnecessary). It seems likely, in retrospect, that even had 2 additional passages been used in these earlier experiments the chance of outgrowth of recombinants in the presence of an infectious parent of high growth capacity (*i.e.* PR8) would be less than in the present instance in which the poorly multiplying Asian viruses A/217 and RI/5$^+$ were the infective viruses used.

DISCUSSION

The present studies have demonstrated that "adaptation" of a newly isolated influenza virus to a laboratory host may be effected rapidly by appropriate genetic interchange with an "adapted" virus. More precisely, conditions have been defined for the production and isolation of recombinant influenza A virus possessing predetermined antigenicity and growth characteristics. The conditions which apparently are required for this process include: (*a*) the use of noninfective "adapted" virus and (*b*) two to three passages after recombination for the manifest emergence of the new virus. The success of this experimental design in contrast to the failure of earlier methods in which an *infective* adapted virus was used evidently depends on circumventing a situation in which a competitive survival advantage over recombinant progeny, intrinsically possessed by the fast growing, adapted parent, interferes with both the recognition and isolation of such recombinants. A further detriment to the emergence of desired recombinants when an infective parent of the non-requisite antigenicity is used, is the necessity of using an environment selective against this parent (*i.e.* antiserum) which may be simultaneously and inadvertently suppressive to progeny possessing the susceptibility to serum inhibitors and antibody characteristic of many new isolates.

In contrast, the method employed here of recombining *inactivated* adapted virus with an infective recent isolate has evidently substantiated its theoretical basis of producing recombinant virus with a survival advantage (faster incremental rate) over the infective parent, which initially predominates. The remaining alternative of devising recombination experiments in which both strains are infective is predictably less satisfactory as: (*a*) the use of selective antiserum is precluded in separating the desired recombinant from its antigenically similar parent and (*b*) other selective environments (*i.e.* mouse lung, chorioallantoic membrane) will usually favor the multiplication of the better adapted "laboratory" parent strain.

It now seems clear that the production of filaments by an influenza virus as

its consistent morphologic expression is a genetic attribute of the strain which is almost always exhibited by recent isolates grown in the chick embryo allantoic sac (12, 18, 6). Even with established laboratory strains, the relative number of filaments produced by a strain seems to be characteristic for the strain (19). Other evidence on this point has been presented by Burnet and Lind (12) who were able to maintain the filamentous character of 2 strains on high (limiting) dilution passage, but who noted a stepwise change to a predominantly spherical morphology on passage at low dilution. Thus, the adaptive change to the spherical morphology common to virtually all laboratory stock strains appeared to occur under conditions favoring mutation or selection of a minority constituent and was not related merely to the number of host passages or viral generations. The same investigators obtained some evidence of recombination of homologous filamentous and spherical strains but were themselves concerned with the possibility that the observed loss in filamentary character resulted from spontaneous mutation before recombination (12, 20).

In the present experiments, the possibility of spontaneous change in the filamentous strains has been carefully controlled in simultaneous passages conducted in parallel with those involving recombinants. In three successive experiments employing two A2 strains in early passage, a striking change in predominant viral morphology has been attended by the increase in viral yield and viral growth rate which is characteristic of strains adapted to cultivation in the allantoic sac. The present data are inadequate to establish a genetic linkage of these three attributes, but this possibility is further suggested by the *reduced* titer and growth rate noted in a filamentous A (PR8) recombinant in Experiment I. If the production of filaments represents an inefficient process of viral multiplication as suggested by Ada and Perry (21) then it is not surprising that low yields and low multiplication rates should go in concert with filamentous morphology.

If the findings of the present studies prove to be generally applicable to other strains of influenza virus, then an unintended but highly practical result has been realized. For it should now be possible to effect prompt adaptation to the desired cultural milieu of future strains of new antigenic constitution and thus facilitate their production for immunization or diagnostic purposes.

SUMMARY

The passage of newly isolated, filamentous Asian (A2) influenza viruses in the presence of non-infective PR8 (A) virus results in the rapid emergence of virus of Asian (A2) antigenicity but PR8-like growth capacity and spherical morphology.

Evidence is presented that this effect results from genetic interaction of the infective Asian and non-infective PR8 viruses rather than from spontaneous change of the Asian strain.

It is concluded that influenza viral morphology, growth rate and growth

capacity are associated genetic traits which distinguish unadapted from adapted strains, and which are transferable by recombination.

A pragmatic consequence of these experiments is the fact that conditions have been defined for the rapid adaptation of early passage influenza virus isolates to the chick embryo allantoic sac. Such adaptation is attended by an increase in viral yield which has obvious implications for vaccine production during future epidemics with new antigenic types.

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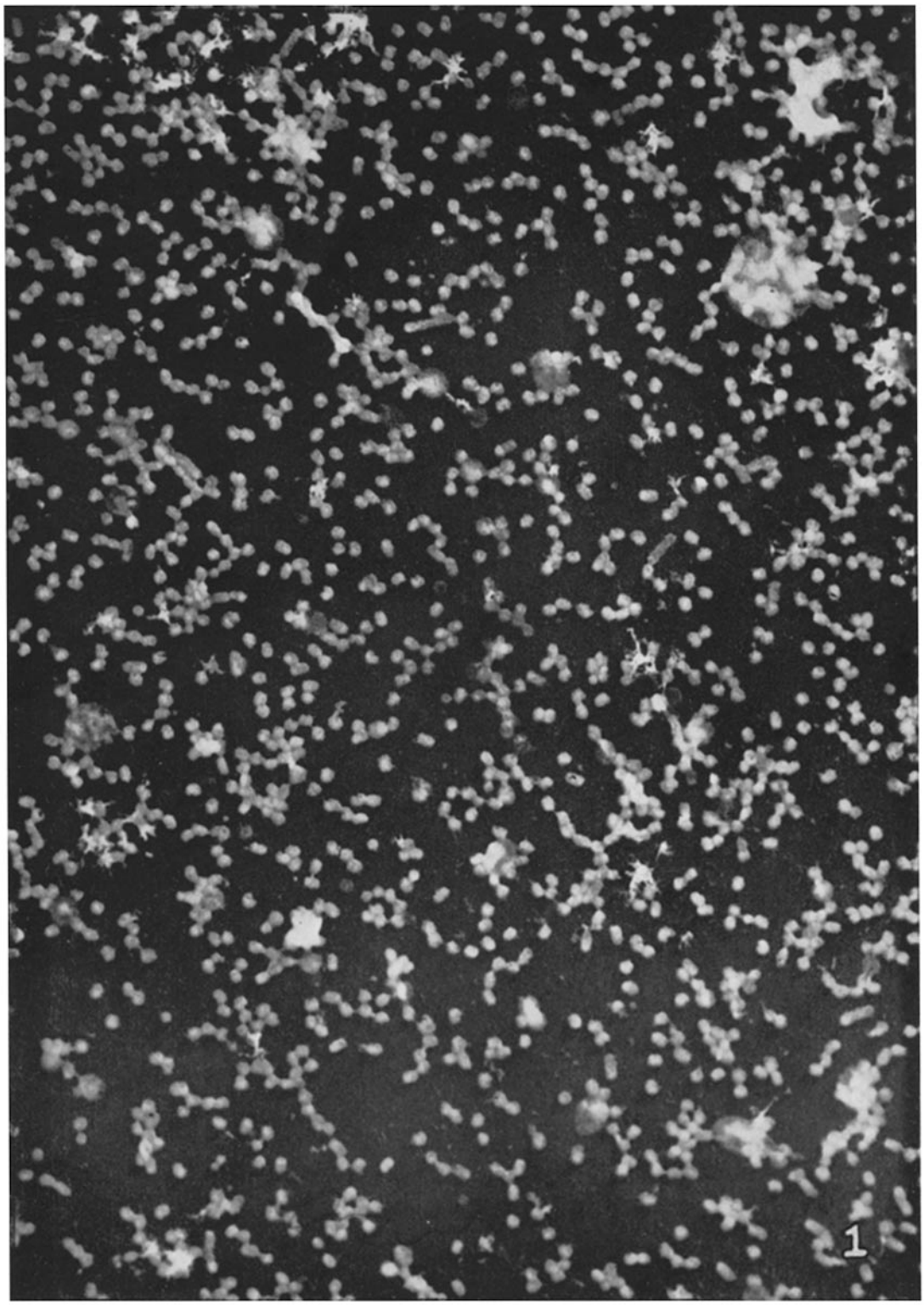
EXPLANATION OF PLATES

PLATE 25

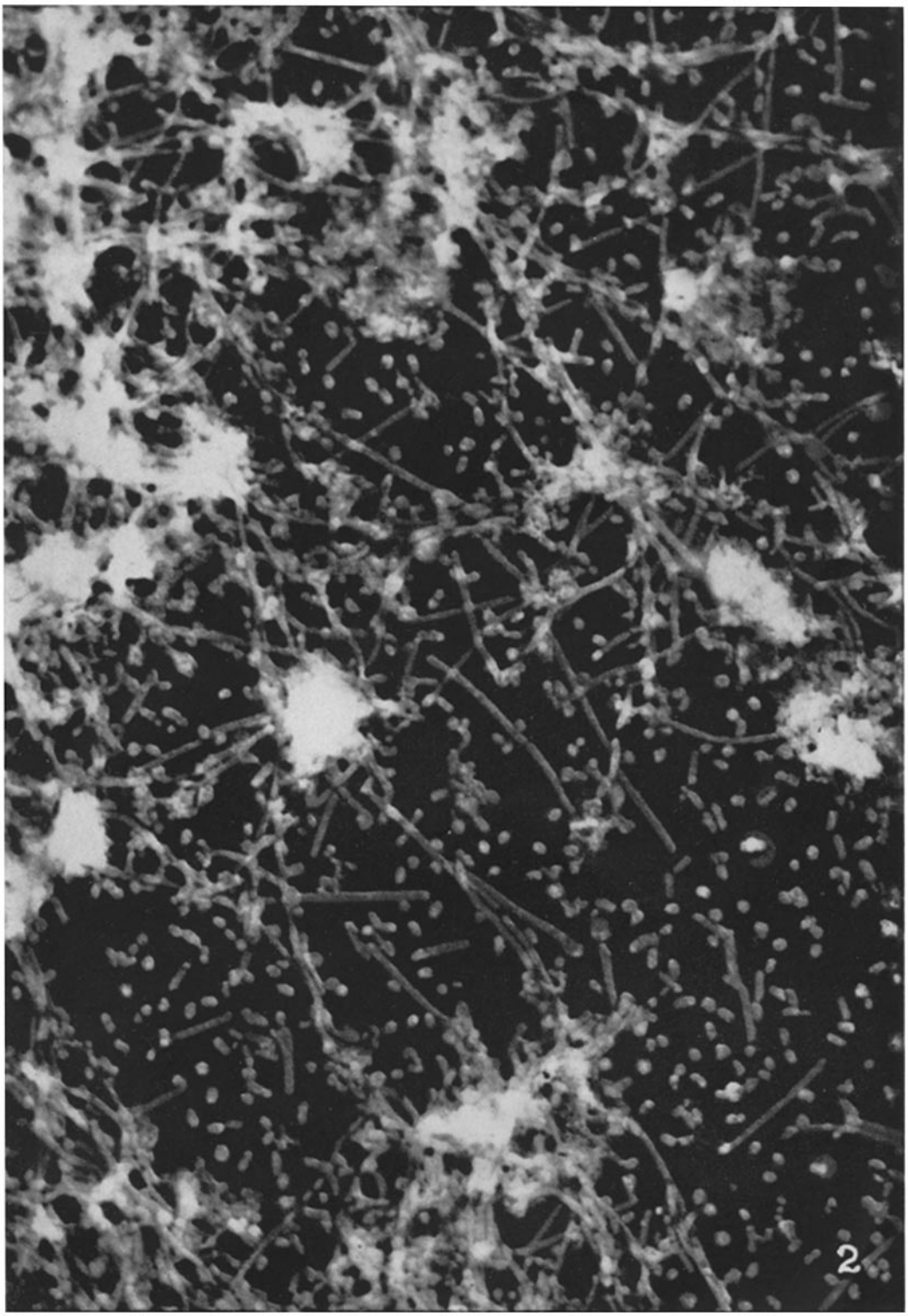
FIG. 1. The typical spherical appearance on electron microscopy of PR8 virus. Chromium shadow, OsO_4 fixation. Note the virtual absence of filaments. × 17,500.



(Kilbourne and Murphy: Genetic studies of influenza viruses)

PLATE 26

FIG. 2. Representative appearance of A/217 virus (Experiment I, group 1) at 7th control passage after initiation of experiment. The large proportion of filaments as seen here is indistinguishable from comparable pictures of the virus in earlier passage. The picture is also representative of the appearance of RI/5+ virus in all passages (Experiments II and III). × 17,500.



(Kilbourne and Murphy: Genetic studies of influenza viruses)

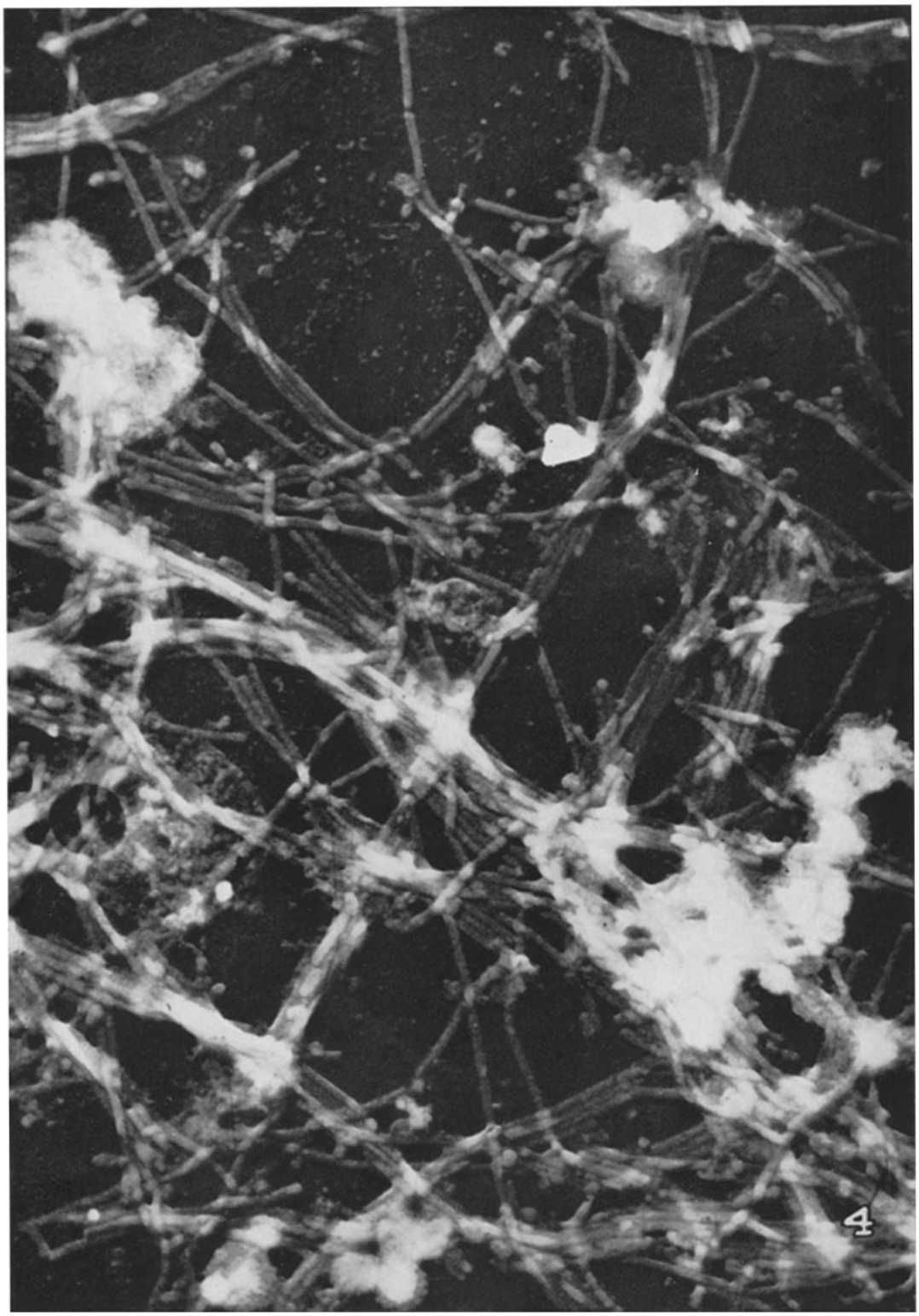
PLATE 27

FIG. 3. Predominantly spherical morphology of recombinant 1 virus (Experiment I, group 3) at 7th postrecombination passage. (Same passage as that of control, A/217 virus in Fig. 2). This picture is representative of the appearance of all passages of this virus from the second postrecombination passage on. × 17,500.

(Kilbourne and Murphy: Genetic studies of influenza viruses)

PLATE 28

FIG. 4. Predominantly filamentous morphology of recombinant 2 (Experiment I group 2) which is antigenically PR8 (A), at 4th postrecombination passage. × 17,500



(Kilbourne and Murphy: Genetic studies of influenza viruses)