

INDICATIONS OF HEREDITARY, SPATIAL REARRANGEMENT OF ANTIGEN COMPLEXES, IN THE INFLUENZA VIRUS*

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The purpose of this paper is to present additional data concerning the mechanism involved in the development, in a strain of influenza virus, of lessened susceptibility to the growth-inhibiting effects of an "immune" environment.

Data were presented in a previous report (1) concerning the passage of virus in mice vaccinated with the homologous and with closely related strains. The passage strain developed the capacity to multiply more freely in the "immunized" animals; the developed capacity was retained following frequent sub-passages—indicating that the change was of an hereditary nature. The "mutant" strain differed from the parent in dominant antibody-evoking antigen; however, analysis of the data from the dual aspects of antigenicity—antibody-evoking capacity and reactivity with homologous and heterologous antisera—suggested that the change involved quantitative redistribution and spatial rearrangement of existing antigen complexes. Those findings are enhanced and extended by the present data.

Materials and Methods

Strains of Virus.—Variant-1 (MV2-M12-M2-M7) (1) was used as starting material for the presently reported passage series. It was derived during passage of A/Berkeley-1/53 strain of influenza virus in mice which had been vaccinated with formalinized, partially purified, homologous, and closely related strains of virus: 10 passages were in mice vaccinated with the parent (A/Berkeley-1/53) strain, 4 were in mice vaccinated with both the parent and A/England-1/51 strains, and 9 were in mice vaccinated with the parent (A/Berkeley-1/53), A/England-1/51, and A/England-1/54 strains. The A/Berkeley-1/53 strain (isolated by Dr. E. H. Lennette) had been selected because at the start of those previous experiments, it was one of the most recently isolated strains available. It had not been passaged previously in this laboratory, and had been through only 3 amniotic and 6 allantoic passages in Dr. Lennette's laboratory. Such a strain suited the experimental design in that it seemed to offer the chance of obtaining dominant antigens which had not been dominant in known strains,—a condition which would free the experiments from the suspicion that resulting variants may have been due to laboratory contamination.

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The starting material (Variant-1) was derived from a single egg infectious unit of mouse passage virus. During early passage in the vaccinated mouse series, it had been necessary to resort to allantoic fluid in order to continue the series. The fluid selected was from the virus titration of the mouse lung suspension of the previous passage. In that titration virus was detected in allantoic fluid of only one of five eggs inoculated with the 10^{-5} dilution of the 10 per cent mouse lung suspension and in none of the fluids of eggs inoculated with the 10^{-6} dilution. The fluid from that single embryonate egg with apparent infection was used for passage.

Place and date of isolation of each of the thirty-two strains of virus used in the experiments are indicated by the strain designation in the text and tables. In those instances in which strain designations have not followed the procedure suggested by the World Health Organization, the designation which had been assigned by the respective worker is indicated along with place and date of isolation. All stock strains had been dried under vacuum, while frozen, and then stored at 4°C. Antibiotics or other antimicrobial agents were not used in passage of the stock material; bacterial contamination of any kind is viewed as evidence of inadequacy of virus technical procedures.

Vaccines.—Vaccines were partially purified virus suspensions which contained one part formalin in 5000. Allantoic fluids from embryonate eggs, 12 to 14 days old, which had been inoculated amniotically 2 or 3 days previously, were clarified in an angle centrifuge at 4000 RPM for 30 minutes; the virus then was sedimentated in a Spinco centrifuge at 40,000 RPM for 60 minutes, resuspended to one-fifth or one-tenth of the original volume in pH 7.2, M/15 phosphate buffer or in distilled water, and again clarified by centrifugation at 4000 RPM for 30 minutes. Formalin was then added. After 2 hours at 37°C and 24 hours or 48 hours in the refrigerator (4°C), the formalinized suspensions were tested for egg infectivity. None of the preparations contained detected egg infectious material.

Antiserums.—Antiserums were obtained from rabbits which had been given three inoculations, each of either 5 or 2.5 ml of the respective vaccine at weekly intervals. The animals were bled 1 week after the third inoculation, and 3 weeks after the first; in general, inoculations were intravenous, but in some series they were intraperitoneal. The antiserums were obtained aseptically, inactivated at 56°C for 30 minutes, and then stored at 4°C without a preservative.

The hemagglutination-inhibitory substances normally present in serums were removed, as indicated in the text, either by treatment with cholera filtrate (2) or by pepsin digestion followed by fractional separation of the globulins, by slight modification of the method reported by Glaubiger for the partial purification of diphtheria and tetanus antitoxins (3).

Vaccination and Passage.—Mice were vaccinated by intraperitoneal inoculation of 0.25 ml quantities of vaccine. Intranasal inoculations were made while the mice were under ether anesthesia; the inoculum, per mouse, was usually in excess of 0.1 ml. Because of the time required for vaccination, mice were from 15 to 20 gm in weight when given the passage material intranasally.

The initial inoculum was allantoic fluid from eggs which had been infected with Variant-1 (MV2-M12-M2-M7) (1). In all but six instances subinoculations were made with suspensions of lungs from the 2 or 3 mice of the previous passage; the suspensions were approximately 10 per cent by weight and were prepared by grinding the lungs in sterile distilled water with alundum as an abrasive. An aliquot of each suspension was tested for virus content by egg infectivity tests. In six instances, indicated in Table I, passage was made with allantoic fluids from eggs which had been used for the egg infectivity tests of the preceding successful mouse passage material.

Egg Infectivity Tests.—In order to eliminate the hazards of contamination of the work areas, eggs were prepared before inoculation. The day preceding the test the shell and shell membrane were removed from the "butt" end of embryonate eggs, 10 or 11 days old; the open

TABLE I
Vaccination and Passage Data

Mouse serial passage	Vaccination	Passage					
	Strain of virus	Days after vaccination	Material inoculated	Egg infectivity	Day of death after inoculation	Lung lesions	Passage summary
1	Albany-9/51 N. Y.-1/53 Stockholm-1/54	6	Allantoic fluid Variant-1 (MV2- M12-M2-M7)	<i>log</i> 8.2	2	d, 3, 3	M-1
2	1st same 2nd Albany-9/51 N. Y.-1/53	8	M-1	5.6	3	1, 1, 2	M-2
		2					
	1st same 2nd "	12	M-2	4.8	2	1, 0, 0	
		6					
3	0	—	Allantoic fluid M-2	8.7	2	4, 3, 1	M2-NM1
4	0	—	M2-NM1	5.9	2	2, 2, 2	M2-NM2
5	England-1/51 Berkeley-1/53	4	M2-NM2	6.9	3	1, 2, 2	M2-NM2-M1
6	Same	7	M2-NM2-M1	5.1	3	0, 1, 2	M2-NM2-M2
7	"	10	M2-NM2-M2	6.8	3	0, 0, 0	M2-NM2-M3
8	"	13	M2-NM2-M3	5.1	2	0, 0, 0	M2-NM2-M4
9	"	15	M2-NM2-M4	6.9	2	1, 1, 2	M2-NM2-M5
10	Albany-9/51 N. Y.-1/53	8	M2-NM2-M5	6.1	3	2, 2	M2-NM2-M6
11	1st same 2nd "	11	M2-NM2-M6	4.0	2	0, 1, 1	M2-NM2-M7
		3					
12	1st " 2nd "	13	M2-NM2-M7	6.6	2	0, 0, 2	M2-NM2-M8
		5					
	1st " 2nd "	7	M2-NM2-M8	5.2	3	1, 2, 2	M2-NM2-M9
		2					
	1st "	10	M2-NM2-M9	4.9	2	2, 2, 3	M2-NM2- M10

TABLE I—Continued

Mouse serial passage	Vaccination	Passage					
	Strain of virus	Days after vaccination	Material inoculated	Egg infectivity	Day of death after inoculation	Lung lesions	Passage summary
	2nd "	5		<i>log</i>			
13	1st " 2nd "	14 9	Allantoic fluid M2-NM2-M8	7.9	3	d, 3, 3	M2-NM2- M8-M1
14	Albany-9/51 N. Y.-1/53 Berkeley-1/53 Variant-1a	7	M2-NM2-M8- M1	5.1	2	0, 0, 1	M2-NM2- M8-M2
15	1st same 2nd "	9 2	M2-NM2-M8- M2	5.7	2	0, 0, 0	M2-NM2- M8-M3
	1st " 2nd "	11 4	M2-NM2-M8- M3	6.0	3	2, 2	
16	England-1/51 Berkeley-1/53	5	Allantoic fluid M2-NM2-M8- M3	7.9	2	2, 3, 3 4	M2-NM2- M8-M3- M1
17	Same "	7 10	M2-NM2-M8- M3-M1 M2-NM2-M8- M3-M2	7.6 5.1	3 2	2, 2, 2 0, 0, 0	M2-NM2- M8-M3- M2 M2-NM2- M8-M3- M2-M1
18	1st same 2nd "	12 2	Allantoic fluid M2-NM2-M8- M3-M2	9.0	2	1, 2	M2-NM2- M8-M3- M2-M1
19	1st same 2nd "	14 4	M2-NM2-M8- M3-M2-M1	6.9	3	1, 1, 1	M2-NM2- M8-M3- M2-M2

TABLE I—*Continued*

Mouse serial passage	Vaccination		Passage				
	Strain of virus	Days after vaccination	Material inoculated	Egg infectivity	Day of death after inoculation	Lung lesions	Passage summary
20	1st Berkeley-1/53	9		<i>log</i>			
	2nd England-1/51 Berkeley-1/53	2	M2-NM2-M8- M3-M2-M2	4.7	2	0, 0, 0	M2-NM2- M8-M3- M2-M3
21	1st same	11	M2-NM2-M8-	6.8	2	0, 0, 0	M2-NM2-
	2nd “	4	M3-M2-M3				M8-M3- M2-M4
22	1st same	13	M2-NM2-M8-	6.8	3	0, 0	M2-NM2-
	2nd “	6	M3-M2-M4				M8-M3- M2-M5
23	1st same	16	M2-NM2-M8-	4.8	2	1, 2	M2-NM2-
	2nd “	9	M3-M2-M5				M8-M3-
	3rd England-1/51 Berkeley-1/53 Variant-1a	2					M2-M6
24	1st same	18	M2-NM2-M8-	7.0	2	0, 1	M2-NM2-
	2nd “	11	M3-M2-M6				M8-M3-
	3rd “	4					M2-M7
25	1st same	20	M2-NM2-M8-	7.0	3	0, 0	M2-NM2-
	2nd “	13	M3-M2-M7				M8-M3-
	3rd “	6					M2-M8
	1st same	23	M2-NM2-M8-	4.8	2	0, 0	
	2nd “	16	M3-M2-M8				
	3rd “	9					
26	1st England-1/51 Berkeley-1/53 Variant-1a	7	Allantoic fluid M2-NM2-M8- M3-M2-M8	8.8	3	4, 4, 4	M2-NM2- M8-M3- M2-M8-
	2nd Variant-1	4					M1
27	1st same	10	M2-NM2-M8-	6.0	2	0, 0	M2-NM2-
	2nd “	7	M3-M2-M8- M1				M8-M3- M2-M8- M2 (Vari- ant-2)

end of the egg was covered with a glass staining dish, and the eggs incubated. The following morning, eggs containing embryos which had not survived the manipulation were discarded. Titrations were made in tenfold steps, each dilution being tested in six to eight eggs. All inoculations, each of 0.25 ml, were made with $1\frac{1}{2}$ inch, 23-gauge needles directly into the amniotic fluid. In order to reduce possible bacterial contamination from the mouse lungs, the culture broth used to make the dilutions contained sufficient penicillin and streptomycin to provide for 250 units and 0.025 mg respectively, per each 0.25 ml inoculum.

Hemagglutination-Inhibition Tests.—The test mixtures consisted of 0.2 ml volumes of antiserum, virus suspension, and $\frac{1}{2}$ per cent suspension of chicken erythrocytes. After 1 hour at room temperature, hemagglutination was read as complete (++), partial (+), or negative (0), on the basis of pattern.

Egg Protection Tests.—Eggs were prepared as described above (*egg infectivity tests*) and then inoculated amniotically with 0.25 ml of a mixture of equal volumes of serum and virus suspension. The mixtures contained from 10^8 to 10^4 egg infectious units and were kept at 37°C , in a water bath, for one-half hour before inoculation. Virus suspensions were prepared from stock passage material by dilution with trypticase-soy culture broth, containing per inoculum 250 units of penicillin and 0.025 mg of streptomycin. After 3 days' incubation, the respective allantoic fluids were tested for hemagglutinating activity. The antibody titers were determined by the 50 per cent end-point method of Reed and Muench (4).

EXPERIMENTAL

The plan of the experiment was to continue passage of the previously reported (1) variant strain in mice vaccinated with homologous and with closely related strains in an effort to produce strains which differed from known strains of influenza virus in a manner more marked than variant-1. The intent was to propagate the virus by mouse to mouse passage but difficulties were encountered when the strains included in the vaccines were changed, which necessitated resorting to allantoic fluid in six instances. Tests made during the course of the experiments failed to disclose the anticipated changes, and after 27 passages in vaccinated mice, the series was discontinued. Subsequently, following more extensive analysis, it was found that the passage strain had acquired the capacity to evoke antibodies reactive with older known strains, while retaining the capacity to evoke antibodies against recent strains. Data concerning vaccination and passage are summarized in Table I.

Lessened susceptibility to the deleterious effects of the "immune" environment may develop to a relatively high degree during passage in vaccinated mice. In the case of passages 23 (Table I) the mice had been vaccinated 16, 9, and 2 days previously with the single (Berkeley-1/53), the double (England-1/51 and Berkeley-1/53), and the triple (England-1/51, Berkeley-1/53, and Variant-1) vaccines, respectively. The passage inoculum contained $10^{4.8}$ units of virus per ml. When the mice were killed 48 hours later the lungs contained $10^{7.0}$ egg infectious units per ml.

In general, whenever the infection was permitted to persist for 3 days the virus titer of the lungs tended to be lower than in those instances in which the infection was terminated in 48 hours. The fall in titer was especially marked

in those instances in which the mice were "hyperimmunized." For instance, the mice of passage 25 were of approximately the same vaccination status as mice of the preceding (24) passage, and the titers of the two inoculums were the same (10^7); however, the virus titer of the lungs in the former instance, in which the infection was terminated after 48 hours, was 10^7 ; whereas, in the latter instance, in which infection persisted for 72 hours, the titer decreased to $10^{4.8}$.

Another point which may be significant is that change of vaccine strains, even though closely related to the previous vaccine strains, rendered passage more hazardous. For example, the starting strain (Variant-1) had developed the capacity to multiply in mice which had been vaccinated with England-1/51, Berkeley-1/53, and England-1/54 strains (1). However, attempts were unsuccessful to passage Variant-1 in mice vaccinated with the related strains Albany-9/51, New York-1/53, and Stockholm-1/54 (passages 1 and 2, Table I).

Characterization of Strains on the Bases of Antibody-Evoking Capacity and of Surface Reactivity.—It was pointed out in an early study (5) that strains of influenza virus possess two kinds of antigenic characteristics—the capacity to evoke antibodies on the one hand, and the relative reactivity with homologous and heterologous antisera, on the other. The former characteristic was referred to as *antigenicity* and the latter—because protection tests were used, and for want of a better term—as *aggressiveness*. The one may be viewed as a measurement of the sum of the antigens included in and on the entire virus particle, capable of evoking detectable antibodies in a given animal, whereas the other may be viewed as a measurement of the reactivity of the antigens which are present on the actual exposed surface of the particle, or sufficiently close to the surface to be within the sphere of attraction. Those two antigenic characteristics were determined for the parent (Berkeley-1/53) strain and for the two derivatives of that strain.

Hemagglutination-Inhibition Tests with Serums Treated with Cholera-filtrate.—

The parent and two of the derived strains were compared with one another and with twenty-nine other strains. Antisera were treated with cholera filtrate to remove the normal inhibitory substances, and then tested in constant dilution for capacity to inhibit hemagglutination by serial threefold dilutions of the virus suspensions. The test mixtures contained equal volumes (0.2 ml) of the 1:25 dilution of cholera filtrate-treated antiserum, virus suspension, and $\frac{1}{2}$ per cent suspension of chicken erythrocytes.

The data from those tests indicated that during passage in vaccinated mice the passage strain lost the broad surface reactivity of the parent strain and developed a broader antibody-evoking capacity, and were interpreted to mean that a rearrangement of the structure of the virus particle had occurred. The findings seemed to have depended upon specific antigen-antibody reactions; treatment of the antisera with cholera filtrate seemed to have been effective

in removing the normal inhibitory substances because tests with strains of influenza B virus in no instance showed inhibition of more than 3 hemagglutinating units. Nevertheless, in view of the implication of the data, it seemed desirable to retest antisera from which the normal inhibitory substances had been removed by another procedure.

Hemagglutination-Inhibition Tests with Pepsin-Digested and Partially Purified Antisera.—

In addition to the procedure used to remove the non-specific inhibitory substances, the tests with the pepsin-digested antisera differed in two other aspects from the previous tests. Serial twofold dilutions of reconstituted antiserum globulin were tested against a constant quantity (not less than 4, nor more than 8 hemagglutinating units) of virus. Also, the pepsin-digested and partially purified material was from pools of two or three antisera which had been prepared at the same time and in the same manner. In the previous tests individual antisera had been selected, because of high homologous titer, from those same groups of two or three which subsequently were pooled for pepsin digestion.

RESULTS

Pertinent data concerning the tests with cholera filtrate-treated sera are summarized in Table II. The thirty-two strain designations listed in the left hand column (Table II) refer either to antisera evoked by those strains, or to the virus suspensions. When the designation refers to antiserum, the respective antiserum was used in tests with suspensions of the three strains of virus indicated by the headings of the three double columns to the right. Whenever the designations in the left hand column refer to suspensions of the respective virus, the suspensions were tested with antisera evoked by the strains listed in the three double columns to the right. Thus, for each of the three compared strains (Berkeley-1/53, Variant-1, and Variant-2) the left hand data in each double column (under the heading *Virus*) refer to surface reactivity with the inhibitory substances in the thirty-two antisera. The right hand data in each of the three double columns (under the heading *Antiserum*) refer to the capacity of the antiserum, evoked by that strain, to inhibit hemagglutination by the thirty-two strains of virus. Because the hemagglutination-inhibition tests were made with a constant dilution of serum and serial threefold dilutions of virus suspension, the results have been expressed exponentially to the base 3, in terms of hemagglutinating units of virus inhibited; that is, each numeral in the six columns of data indicates the number of tubes in the series of threefold dilutions of virus in which hemagglutination was completely inhibited. The various virus suspensions contained approximately the same number (3^5 to 3^6) of hemagglutinating units of virus.

*Surface Reactivity of the Berkeley-1/53 Strain.—*The virus suspensions of the parent strain, Berkeley-1/53, reacted with a broad spectrum of antisera (Table II). Among the strains isolated in 1940 or earlier, significant reactivity

TABLE II
 Comparison of the Two Derived Strains with the Parent Strain (Berkeley-1/53) with Respect to
 Antibody-Evoking Capacity and to Surface Reactivity

No.	Antibody† vs. Virus vs.	Inhibition* of hemagglutination					
		Berkel.-1/53		Variant-1		Variant-2	
		Virus	Anti-serum	Virus	Anti-serum	Virus	Anti-serum
1	Swine-15/30	0	1	0	1	0	1
2	Engl.-W.S./33	0	0	0	0	0	1
3	PR-8/34	2	0	0	0	0	1
4	Phila./35	2	0	1	0	1	1
5	Alaska/35	0	0	0	0	0	0
6	N. Y.-Henry/36	2	0	1	0	0	1
7	N. Y.-Patnode/36	1	0	0	0	0	0
8	Aust.-Reid/39	2	0	1	0	0	0
9	N. Y.-Coyle/40	1	0	0	0	0	0
10	N. Y.-Niel./40	2	0	1	0	0	2
11	Aust.-Bel./42	1	0	0	0	0	0
12	Mass.-Beard/43	1	0	1	0	0	3
13	Ft. C.-Weiss/43	2	1	1	2	1	4
14	Hirst 982/43	1	0	0	1	0	2
15	FM1/46	1	2	1	4	1	4
16	PR-Coamo/48	2	1	3	4	5	3
17	St. John 2/49	4	0	3	0	3	1
18	Sweden 3/50	5	3	5	2	3	2
19	Brooklyn-M/50	3	0	1	0	1	1
20	F. Warren/50	3	1	1	1	0	2
21	England-1/51	5	2	4	3	2	2
22	Albany-9/51	4	3	3	3	4	2
23	India-Nich./52	1	3	0	1	0	1
24	Chile-1/53	5	3	5	2	3	2
25	N. Y.-1/53	5	3	5	4	5	3
26	Berkeley-1/53	5	5	4	5	4	5
27	Variant-1	5	4	5	5	5	5
28	Variant-2	5	4	5	5	5	5
29	England-1/54	4	4	3	4	2	4
30	PR-301/54	4	4	5	4	4	4
31	England-19/55	4	2	3	1	4	2
32	Albany-1/55	4	3	5	3	3	2

* The results are expressed exponentially to the base 3 in terms of threefold units of hemagglutinin inhibited by the 1/25 dilution of respective serum.

† The listings in the first column refer to *antiserum* whenever reference is made to data included in columns to the right which are headed *Virus*. They refer to *virus* whenever reference is made to data included in the columns headed *Antiserum*.

(inhibition of 9 hemagglutinating units of virus) was evident in tests with antisera evoked by PR-8/34 (No. 3), Philadelphia/35 (No. 4), New York-Henry/36 (No. 6), Australia-Reid/39 (No. 8), and New York-Nielson/40 (No. 10) strains. Also, surface reactivity was marked with antisera evoked by most strains isolated after 1946.

Antibody-Evoking Capacity of the Berkeley-1/53 Strain.—The antibody-evoking capacity was more limited. Antibodies evoked by the Berkeley-1/53 vaccines did not include those specific for older strains in quantities detectable by the methods employed, except for the suggestive inhibition of 3 hemagglutinating units in the case of swine. That is, whereas the respective antigens seem to have covered an area of the surface of the Berkeley-1/53 virus particle sufficiently extensive to be detected by antibodies evoked by those five pre-1941 strains (PR-8/34, Philadelphia/35, New York-Henry/36, Australia-Reid/39, and New York-Nielson/40), those antigens seem to have been quantitatively insufficient to evoke detected antibodies reactive with virus suspensions of those same pre-1941 strains.

Surface Reactivity of the Derived Strains.—Virus suspensions of both of the derived strains reacted with fewer of the thirty-two antisera than did the parent strain (Berkeley-1/53). Accordingly, if the assumption is correct that the inhibition of hemagglutination in those tests was a measure of specific surface antigen, the antigen complexes for which test antibodies were available were more limited on the surface of the derived strains than on the surface of Berkeley-1/53. None of the antigen complexes specific for antibodies evoked by the fifteen pre-1948 strains were present in significant quantities (exponents greater than 1, Table II) on the surface of either variant; moreover, there is a suggestion that the loss may have been progressive in that there were fewer instances of suggested inhibition (exponent 1) in the case of Variant-2 than in the case of Variant-1.

Antibody-Evoking Antigens of the Derived Strains.—Antigens which were not evident in either Variant-1 or Variant-2 virus in tests with antisera to the fifteen pre-1948 strains were nevertheless present in sufficient quantities in the virus suspensions to evoke antibodies which reacted with suspensions of pre-1948 strains of virus. As measured by reactivity with the FM-1 antiserum (No. 15), significant quantities of FM-1 antigen were not detected in either variant. However, vaccination with the variant suspensions evoked relatively high titers of FM-1 antibodies; the 1:25 dilution of antiserum in each instance inhibited 81 (3⁴) hemagglutinating units of FM-1 virus. In tests with Weiss/43 (No. 13), a similar relationship occurred but was more marked in the case of Variant-2. In the tests with F. Warren/50 (No. 20), Hirst 982/43 (No. 14), Massachusetts-Beard/43 (No. 12) and New York-Nielson/40 (No. 10), significant antibody-evoking antigens in the absence of significant surface-reacting antigens were evident in the case of Variant-2 but not Variant-1.

Results of Tests with Pepsin-Digested Antiserums.—Data concerning the tests with pepsin-digested and partially purified antiserums are summarized in Table III, the arrangement of which is the same as that of Table II. However, because serial twofold dilutions of the reconstituted serums were tested with a constant quantity of virus the results are presented exponentially in terms of the greatest twofold initial dilution of antiserum which completely inhibited the hemagglutinating activity of the test aliquot of virus.

An obvious discrepancy between the results with the cholera filtrate-treated serums (Table II) and those with the pepsin-digested serums (Table III) concerns the surface reactivity of Berkeley-1/53. The former (Table II) suggest a broad range of antigens, reactive with antibodies evoked by some of the oldest known strains; the latter (Table III) indicate that the surface reactivity with serums evoked by pre-1941 strains was no broader in the case of Berkeley-1/53 than in the case of the two derived strains. Although the possibility exists that the discrepancy depends upon the more effective removal of inhibitory substances by pepsin digestion, it probably depends in part upon loss of antibody because of the drastic treatment.

A discrepancy of a somewhat different kind occurred in tests with Massachusetts-Beard/43. In tests with the cholera filtrate-treated serums (No. 12, Table II) no inhibition was evident with Berkeley-1/53 antiserum, whereas the Variant-2 antiserum inhibited 27 units (3^3) of Beard hemagglutinin. However, in the tests of pepsin-digested antiserums (No. 7, Table III), both Berkeley-1/53 and Variant-2 antiserums effected significant inhibition of hemagglutination. It is noteworthy that in neither test did the Variant-1 antiserum effect significant inhibition.

From the viewpoint of broadening of the antibody-evoking capacity, the data (Table III) agree well with those of the tests summarized in Table II. The broadening of the antibody-evoking capacity to include antibodies reactive with the early strains is evident in the case of New York-Nielson/40 (No. 6, Table III; No. 10, Table II) and Fort. C.-Weiss/43 (No. 8, Table III, No. 13, Table II). In the tests with pepsin-digested antiserums, the broadening seems also to include England-W.S./33 (No. 1, Table III). At the same time, the capacity to evoke antibodies for the more recently isolated strains seems not to have been impaired.

It is of interest that on the basis of the data in Table III, the antibody-evoking capacity of Variant-1 is more limited than that of its parent strain (Berkeley-1/53). The seeming decrease in apparent antigenic relationship is in accord with the original, more restricted analysis of Variant-1 (1); that is, there seems to have been a continuation of progression in replacement of dominant antigens. The differences between antibody-evoking capacity of Variant-2 as compared with that of its antecedent Variant-1 are striking (Table III). Variant-2 exhibited significantly greater antibody-evoking capacity

against England-W.S./33, New York-Nielson/40, Massachusetts-Beard/43, and Fort C.-Weiss/43 (Nos. 1, 6, 7, and 8, Table III).

Demonstration, by Egg Protection Tests, of Decreased Susceptibility of the Derived Strain to Action of Antibodies.—The lessened susceptibility of Variant-2 as compared with the parent (Berkeley-1/53) strain to antibody activity, which was evident in the tests with cholera filtrate-treated antisera (Table II), was

TABLE III
Hemagglutination Inhibition Tests with the Globulin Fraction of Pepsin-Digested Antisera*

No.	Antiserum † vs..... Virus vs.....	Berkel.-1/53		Variant-1		Variant-2	
		Virus	Anti-serum	Virus	Anti-serum	Virus	Anti-serum
1	Engl.-W.S./33	0	0	0	0	0	2
2	PR-8/34	0	0	0	0	0	0
3	Phila./35	0	0	0	0	0	0
4	N. Y.-Henry/36	0	0	0	0	0	0
5	Aust.-Reid/39	0	0	0	0	0	0
6	N. Y.-Niel./40	0	0	0	0	0	3
7	Mass.-Beard/43	0	3	0	1	0	4
8	Ft. C.-Weiss/43	0	2	2	1	0	5
9	FM-1/46	3	5	4	4	0	5
10	F. Warren/50	2	2	3	0	2	1
11	Engl.-1/51	4	5	6	4	4	5
12	Berkeley-1/53	8	8	9	6	7	5
13	Variant-1	5	9	9	9	7	8
14	Variant-2	5	8	8	7	7	7
15	Engl.-1/54	9	7	10	6	9	5

* Inhibition of hemagglutination is expressed exponentially to the base 2, in terms of *twofold* dilutions of respective antiserum which inhibited the hemagglutinating effect of the fixed amount of virus (4 to 8 units).

† The listings in the first column refer to *antiserum* whenever reference is made to data included in columns to the right which are headed *Virus*. They refer to *virus* whenever reference is made to data included in the columns headed *Antiserum*.

evident also in egg protection tests, results of which are summarized in Table IV.

In order to be reasonably assured of their purity, the test virus suspensions were derived from three serial passages with single egg infectious units. The test antisera were untreated and included five which had been used in the previous tests, and two pairs (W.S. and PR8) which had been recently prepared. Because the antisera were tested in twofold dilutions, the results (Table IV) are presented exponentially to the base 2, in terms of 50 per cent infectivity.

The antisera evoked by the pre-1944 strains of virus were even more effective in preventing infection of embryonate eggs (Table IV) by the Berkeley-

1/53 than in inhibiting hemagglutination (Table II) by that strain. Antiserums evoked by all seven of the test strains effected significant protection against infection by the Berkeley-1/53 strain, whereas the same antiserums exerted relatively little egg protective capacity *versus* the derived Variant-2.

Specificity of the Berkeley-1/53 Surface Antigens for Antibody Evoked by the Swine Strain of Virus.—In order to obtain additional assurance of the specificity of the reactions in the foregoing tests, aliquots of the pooled swine virus antiserums (R55-22 and R55-23, Table IV), were adsorbed with partially purified Berkeley-1/53 strain of virus and then tested for capacity to prevent infection of embryonate eggs by that strain. The virus previously had been through 3

TABLE IV
Effectiveness of Antibodies Evoked by Pre-1944 Strains in Egg Protection Tests with Berkeley-1/53 and Variant-2 Strains

Antiserum	Virus	
	Berkeley-1/53	Variant-2
Swine R55-22 and R55-23	2.9*	1.2
W.S. R60-26	2.8	<1.
W.S. R60-27	2.8	0
PR8 R60-59	2.9	0
PR8 R60-63	>3.0	0
Phila./35 R55-16	1.8	0
Aust.-Reid/39 R55-47	1.9	0
N. Y.-Nielsen/40 R55-21	1.8	1.6
Mass.-Beard/43 R55-49	3.0	<1.

* Expressed exponentially to the base 2, in terms of the 50 per cent end-point of infectivity.

serial passages with single egg infectious units—a reasonable assurance that the passage material contained but a single strain.

Allantoic fluid was clarified in an angle centrifuge at 4000 RPM for 30 minutes; the virus then was sedimentated in a Spinco centrifuge at 40,000 RPM for an hour, suspended in distilled water to $\frac{1}{10}$ th its original volume, and again centrifuged at 4000 RPM for one-half an hour. Two 5.0 ml aliquots of the concentrated virus suspension were centrifuged at 40,000 RPM for an hour, and sediment from one was suspended in a 2.5 ml aliquot of the swine virus antiserum. After overnight refrigeration (4°C), the serum-virus mixture and, also, an untreated 2.5 ml aliquot of the antiserum were mixed with a sufficient quantity of packed chicken erythrocytes to provide a 5 per cent suspension. After 20 minutes at 0°C, the erythrocytes were removed by centrifugation. The adsorbed aliquot of antiserum then was absorbed a second time in the same manner. Both specimens of antiserum, then, were adsorbed twice with chicken erythrocytes. In order to inactivate any virus which had not been removed by adsorption with chicken erythrocytes, the aliquots of antiserum were heated at 56°C for 60 minutes. Equal volumes of serial twofold dilutions of the two aliquots of antiserum were mixed with a 1/500 dilution of Berkeley-1/53 allantoic fluid, which provided $10^{3.7}$ egg infectious units of virus per inoculum.

The 50 per cent egg protection titers of the two aliquots of antiserum were as follows:

Not adsorbed	1:12
Adsorbed	0

Control tests of the adsorbed aliquot of antiserum were made by inoculating eggs with the 1:2 dilution to which no additional virus had been added. None of the inoculated eggs showed any evidence of infection, indicating that the virus used for adsorption had been completely removed, or inactivated.

DISCUSSION

Both the previously reported experiments (1), and those reported in the present paper were part of the same plan to test the idea that the influenza virus may have undergone changes under the impetus of biologic pressures exerted by the environment in which the virus is naturally propagated—an environment provided by the host population which is being progressively “immunized” during the very process of virus propagation. It had been anticipated, in keeping with the concept that the influenza virus had undergone a series of changes characterized by a progression of dominant antigen complexes (6-8), that the derived strains would be characterized by dominant antigen complexes which had not been dominant in previously known strains.

It was noted in early studies that the components which were dominant in more recently isolated strains had not been dominant in earlier strains (9), but the basis for the differences was not clear at that time. It had been suspected that the virus was unstable and might be subject to change under the impetus of environmental pressures (10), but it remained for Hirst to first present experimental evidence of the unstable nature; he showed that passage in mice of a strain that previously had been passaged only in embryonate eggs might result in significant antigenic change (11). Archetti and Horsfall showed that changes develop when mixtures of virus and heterologous antiserum are passaged in embryonate eggs (12). Isaacs and Andrewes obtained antigenic changes during passage in embryonate eggs of virus and homologous antiserum (13); they considered the changes, however, to be an expression of the P-Q-R variation of Van der Veen and Mulder (14). Subsequently, Gerber, Loosli, and Hamre obtained antigenic changes during passage of the virus in mice which had been vaccinated with the homologous strain (15), and they showed that the “new” dominant antigen complex had been present during early passages as a minor component. Similar findings, that the dominant antigen of the derived strain had been present in the parent strain, were reported independently from this laboratory (1).

The evidence that antigen complexes which were dominant in the older known strains persist as less obvious components of more recent strains, is in keeping with the findings of Jensen and Francis; those workers tested representative strains by adsorption techniques, and concluded “that no completely new antigens have been found

of late nor have the antigenic components of strains isolated several years ago disappeared" (16).

With respect to Variant-1, the data presented in this report are in keeping with the previously reported results which indicated a progression in replacement of dominant antigen complexes (1); the data indicate a contraction of range as measured by antigens which were dominant in the older known strains, both with respect to surface reactivity and to antibody-evoking capacity. Continued passage of Variant-1 in vaccinated mice, however, did not effect the anticipated continued progression of dominant antigens. Instead, the virus developed an increased capacity to evoke antibodies which reacted with the older known strains of virus; that is, antigen complexes which had been dominant in earlier strains regained prominence in the derived strain.

It might be reasoned on the basis of present biologic concepts that the recurrence of the previously dominant antigen complexes was a back-mutation, in a *qualitative* sense; that is, antigens which had been lost through successive qualitative changes had been regained through a series of reverse qualitative changes. The present data, however, suggest that the antigens had not been lost, but had been present in the parent strain in minor amounts.

The data indicate that during passage in the vaccinated mice an alteration occurred which involved an exchange of surface reactivity for antibody-evoking capacity; that is, there seems to have been a quantitative redistribution and a spatial rearrangement of the antigen complexes. The parent, Berkeley-1/53, strain possesses a mosaic of readily accessible complexes which react with antisera evoked, not only by recent strains, but by the older known strains. The broad surface reactivity seems not to be associated with a mixture of strains because it persists after serial passage of single infectious units of the virus. Also, the data (especially those derived from egg protection tests) suggest that the surface reactivity depended upon specific antigen-antibody reactivity.

The concept of a structure in which the virus surface is antigenically distinct from inner virus bulk was proposed in one of the earliest studies of the problem (5); Isaacs, Depoux, and Fiset (17) suggested a similar explanation for the P-Q-R variation of Van der Veen and Mulder (14); and it seemed to offer a plausible explanation of the data included in the previous report (1).

The altered arrangement of the virus particle has persisted through numerous subpassages over a period of 6 years. Accordingly, the change must be considered to have been hereditary. And, in view of the concept that phenotypic expressions are controlled by individual hereditary units, the hereditary, quantitative change in phenotype suggests a quantitative change in genotype. The data, thus, add weight to the suggestion (1) that influenza virus variation (*i.e.*, "mutation") may result from a rearrangement of existing hereditary elements, rather than from a qualitative change in one or more of the elements.

SUMMARY

During passage in mice which had been vaccinated with the homologous, and with closely related strains of influenza virus, the passage strain developed a lessened susceptibility to the deleterious effects of the "immune" environment, concomitant with which was a developed capacity to evoke antibodies which reacted with earlier strains of virus—a capacity which was inapparent in the parent strain. However, the parent strain exhibited a relatively broad range of surface reactivity which was not apparent in the derived strain.

The data are interpreted to mean that the hereditary change resulted from spatial rearrangement and quantitative redistribution of antigens in the virus particle (in which the surface is viewed as being distinct from the inner bulk), and are viewed as enhancing the idea that influenza virus variation (*i.e.*, "mutation") may result from a rearrangement of existing hereditary elements.

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