STUDIES OF ANTIGENIC DIFFERENCES AMONG STRAINS OF INFLUENZA A BY MEANS OF RED CELL AGGLUTINATION

By GEORGE K. HIRST, M.D.

(From the Laboratories of the International Health Division of The Rockefeller Foundation, New York)

(Received for publication, August 3, 1943)

It has previously been demonstrated that the red cell agglutination reaction with influenza virus, when used for serum inhibition tests, is capable of demonstrating antigenic differences among influenza strains (1), a fact which has been repeatedly confirmed (2–6). This method is so much simpler than the usual techniques employed for demonstrating strain differences that it has become desirable to investigate more fully its advantages and limitations, and it was with this need in mind that the present work was undertaken. These studies were considerably facilitated by the fact that a large number of strains were available from the country-wide epidemic of influenza A which occurred in the winter of 1940–41. It is the comparison of these strains which is of the most interest.

Methods

Virus Strains.—Table I contains a summary of the pertinent data on the strains which were employed in the present study. The first group of six are old laboratory strains, all of which (except swine) were isolated by inoculation of throat washings into ferrets, and their virulence was enhanced by repeated passage in mice. All were obtained from epidemics prior to 1940.

All of the other strains shown in the table came from the 1940–41 epidemic of influenza in the United States and Hawaii. Every strain of this group (except S.F. and Haw.) was isolated by direct inoculation of filtered throat washings into chick embryos. Strains Haw. and S.F. were isolated by means of ferret and mouse passage and were supplied to us through the kindness of Dr. Monroe D. Eaton.

Preparation of Virus Strains.—All of the virus strains were prepared by inoculating infected allantoic fluid diluted to 10^{-3} into the allantoic sacs of 11 day old chick embryos. After 48 hours' incubation at 37°C. the eggs were placed at 4°C. overnight, and blood-free allantoic fluid was removed the following morning. The cellular debris was removed by a short centrifugation at 2000 R.P.M., and the viruses were stored at 4°C. in the fluid state.

Hemagglutinin and Antibody Titrations.—Titrations of the hemagglutinin content of virus suspensions and of the antibody level of sera were made by a standard technique which has been previously fully described (10). Antibody titrations were usually done in duplicate and the results ordinarily checked within ± 5 per cent. Special care was taken in antibody titrations to use a final concentration of 4 agglutinating units of virus by performing preliminary agglutination titrations in

quadruplicate with the same red cells to be employed in the final test. Any slight deviation from 4 units in the serum tests was compensated by correcting the final

TABLE I

Data on Influenza Virus Strains Employed

Strain designation	Location of donor	Institution No.	Date of isolation	Method of isolation
PR8*	Puerto Rico		1934	Ferret and mouse passage
W.S.‡	England		1933	Ferret and mouse passage
Christie (Chr)§	England		1937	Ferret and mouse passage
Talmey (Tal)§	England		1937	Ferret and mouse passage
Gatenby (Gat)§	England		1937	Ferret and mouse passage
Swine (No. 1976)‖	United States			Mouse passage
Dra-1	Alabama	1	Jan. 16, 1941	Chick embryo
Dra-2				
Dra-3				
Dra-4				
Dra-5				
Dra-6				
Dra-7				
Fla-I	Florida	2	Jan. 9, 1941	Chick embryo
Fla-II	Florida	3	Jan. 10, 1941	Chick embryo
Fla-III	Florida	4	Jan. 10, 1941	Chick embryo
Ala-I	Alabama	5	Jan. 17, 1941	Chick embryo
Ala-II	Alabama	6	Jan. 20, 1941	Chick embryo
Ala-III	Alabama	7	Jan. 14, 1941	Chick embryo
Ala-IV	Alabama	8	Jan. 15, 1941	Chick embryo
Ala-V	Alabama	9	Jan. 18, 1941	Chick embryo
N.Y.	New York		Feb. 1, 1941	Chick embryo
Haw.	Hawaiian Islands		Oct. 12, 1940	Ferret and mouse passage
S.F.	San Francisco, California		Nov. 20, 1940	Ferret and mouse passage

* See reference 7 of Bibliography.
‡ See reference 8 of Bibliography.
§ See reference 9 of Bibliography.
‖ Obtained from Dr. R. E. Shope.

antibody titers obtained. Thus if 3.8 units of hemagglutinin were used (5 per cent too low), the antibody titer obtained was reduced by 5 per cent to give the correct value for the use of 4 units. Such a correction was based on the finding that within fairly wide limits the antibody titer of a serum was inversely proportional to the amount of hemagglutinin used for the test (1, 3). When the variation from 4 units

was greater than 15 per cent, the results were discarded. Details regarding special techniques for certain tests will be included in the text.

Preparation of Immune Sera.—Sera from infected ferrets were used throughout as a source of antibody. Ferrets were inoculated intranasally with a 10^{-3} dilution of infected allantoic fluid. The animals were bled before inoculation and 2 weeks after infection. Usually 2 ferrets were used for each virus strain. In each case the preinfection bleeding was tested to insure the absence of antibodies.

All sera were heated at 56° for 30 minutes to inactivate heterologous hemolysins for chicken cells. When cross titrations of 7 or more virus strains were performed at one time, the serum dilutions were made up in large volume (about 10 cc.). These dilutions were then pipetted into small tubes for the test, thus insuring a much greater uniformity of serum dilutions than could be obtained by making them for each strain separately. This is an important point when small degrees of variation in titer are to be considered.

The serum inhibition end points throughout are expressed as whole numbers representing the dilution factor of the serum necessary to reach the end point, and these figures are reciprocals of serum dilution. All concentrations, as heretofore, are expressed in terms of final concentration after adding all reagents.

Red Blood Cells.—Red cells from domestic chickens were used throughout. In some cases pooled cells from a slaughterhouse were used, but in most tests the cells from only one or two chickens were employed. In the latter case the blood was obtained from barred Plymouth Rock roosters by bleeding from a wing vein into sodium citrate solution. In any case the cells were washed three times before use. They were stored in concentrated form at 4°C. and were not employed when more than 4 days old. A final concentration of 0.75 per cent red cells was used for the tests.

EXPERIMENTAL

Before any extensive cross tests for strain differences by means of agglutination were attempted, the titration methods were reexamined in order to evaluate possible sources of error. There was no doubt from previous work (1) that large antigenic differences could be easily detected, but it was necessary to determine to what degree small differences could be recognized. It was found that two of the reagents used in the test introduced variations in the results of sufficient magnitude to be serious sources of error.

Variations in Titer Due to Red Cells.—Stuart-Harris has pointed out (11) that a considerable variation in serum titer results from the use of cells from different chickens, and he found the variations especially striking in tests for influenza B antibody. In order to obtain an idea of the variation in titers due to the red cells employed, blood was secured from five chickens of different domestic breeds. The different cells were all washed and handled in the same manner. Suspensions of each type of cell were carefully standardized with the photoelectric densitometer, and the hemagglutinin titer of a single preparation of PR8 virus was determined with each lot of cells. The results, shown in Table II, indicate a total variation in titer of about twofold. A similar variation was obtained with a preparation of the Lee strain.

Serum inhibition tests were then set up with PR8 serum and virus and the same lots of red cells (Table II). Four agglutination units of virus (as determined for each lot of cells) were used in each serum test. A similar test was performed with the Lee strain and its antiserum. Here again there was an approximately twofold variation in end point with different types of cells. Similar variations in titer were found to occur as the result of aging of cells at 4°C. Fresh cells were changed especially in respect to agglutinability after standing a few hours at room temperature.

TABLE II

Hemagglutinin and Serum Inhibition Titers with Different Lots of Chicken Red Cells

Lot No. of chicken cells	Agglutination titer with PR8 virus	Inhibition titer of PR8 ferret serum with 4 units of PR8 virus	Agglutination titer with Lee virus	Inhibition titer of Lee ferret serum with 4 units of Lee virus
1	84	4400	147	3010
2	104	5800	256	2700
3	158	6700	417	4720
4	120	7200	256	3820
5	104	7200	194	4096

TABLE III

Hemagglutinin Titrations and Serum Inhibition Tests on Five Virus Preparations of the Same Strain of PR8

Virus preparation No.	Agglutination titer	Inhibition titer with 4 units of virus		Ratio of inhibition titers PR8/W.S
		PR8 serum	W.S. serum	
1	208	9450	338	28
2	208	8800	315	28
3	275	7200	294	24
4	104	7650	447	17
5	417	3820	138	28

The variation demonstrated in Table II clearly indicates that in order to have accurate comparisons among different strains of influenza virus by means of inhibition titrations the tests for comparability must be done at the same time and with the same lot of red cells. It has also been found that the results were further improved when the cells from only one chicken were used for a particular test, and that is the procedure employed in the cross tests which will be subsequently described.

Variations in Serum Inhibition Titers with Different Preparations of the Same Virus Strains.—A much more serious source of error was discovered when different preparations of the same virus strain were tested against two antisera. For sources of virus the PR8 strain was inoculated into a number of eggs. The allantoic fluid from each of 5 eggs was removed and kept separately. These 5 lots of virus were used in serum inhibition titrations with an homologous (PR8) and a heterologous (W.S.) ferret antiserum. The results are shown in Table III.

The hemagglutinin titers of the 5 lots of virus varied from 104 to 417, which is not beyond the usual variation in individual eggs with an influenza strain. Four agglutinating units of each preparation were set up in inhibition tests with PR8 antiserum, and there was a variation of slightly more than twofold in the titers obtained. A similar and parallel variation was found with W.S. antiserum. This variation was well beyond the limits of error of an individual titration and indicates a definite difference in the amount of serum necessary to neutralize 4 agglutinating units of virus from different preparations of the same strain. It should be noted that the ratio PR8 titer/W.S. titer for a given virus preparation was, except in one instance, relatively constant, indicating that the variable factor influences results with two different sera in the same degree.

Since all preparations of virus used came from the same generation in egg passage and were treated in an identical manner, it seems unlikely that there were significant differences in antigenic pattern of the virus from one lot to another. A search was therefore made for some variable other than antigenic pattern which could cause the different preparations to require varying amounts of serum for inhibition of an agglutinating unit.

TABLE IV

Difference in Amount of Serum Required to Inhibit Agglutination by Fractions of a PR8 Suspension Separated by Centrifugation

Virus fraction tested*	Inhibition titer PR8 serum	Inhibition titer W.S. serum	Ratio of titers PR8/W.S.
Supernatant fluid after centrifugation	9450	891	11
Sediment virus resuspended	5050	417	12
Uncentrifuged virus suspension	6700	512	13

* Four agglutination units of each preparation used for testing.

Effect of Particle Size on Serum Inhibition Titers.—The following experiment was performed to see if a preparation of influenza virus separated into two fractions on the basis of particle size would give different results in serum neutralization tests.

Forty cc. of allantoic fluid containing PR8 virus was centrifuged for 30 minutes at 12,000 R.P.M. in an angle centrifuge, which removed about 80 per cent of the agglutinin from the supernatant fluid. The top 5 cc. of fluid from each tube was carefully removed and saved, and the remainder of the supernatant was poured off and discarded. The sediment was resuspended to the original volume with buffered saline. Four agglutination units each of the original uncentrifuged material, the centrifuged supernatant, and the resuspended sediment were tested against a PR8 and a W.S. antiserum.

The results, shown in Table IV, indicate that the sedimented virus required about twice as much serum (either PR8 or W.S.) to neutralize four units as

did the virus from the supernatant. The untreated material gave an intermediate result. Again the titer ratios for each virus (PR8/W.S.) were uniform for each fraction tested.

The most obvious difference between the influenza particles in the supernatant and in the sediment was one of average size. From the above experiment it can be deduced that four agglutination units of influenza virus in large aggregates required more serum to inhibit the agglutinating effect than did four agglutinating units of small virus particles or aggregates. Chambers and Henle (12) have described the occurrence of virus aggregation in allantoic fluid, and they also found that the degree of aggregation varied among different preparations of the same strain. An uncontrolled variation in the degree of aggregation could explain, in part at least, the variation in serum required for neutralizing different virus preparations of the same strain when tested at the same agglutinating strength.

Method of Expressing the Results of Strain Difference Tests and of Compensating for Avidity Effects.—Whether or not the aggregation of virus particles is the explanation for the variable serum titers obtained above, it is obvious that such uncontrolled variation will have an important and equally uncontrolled effect on the results of cross tests by the agglutination method. Since the observed variation in serum required for inhibition of agglutination seems to be something apart from antigenic pattern, we shall refer to this property of a suspension as avidity. A virus suspension which requires a large amount of serum for inhibition per unit of agglutinin may be said to have a high avidity and *vice versa*. Inasmuch as cross tests are performed for the determination of antigenic pattern only, it becomes important to be able to rule out or compensate for the effect introduced by avidity so that the expressed results may be interpreted in terms of antigenic structure alone. As will be demonstrated below, the failure to compensate for this factor often leads to results which are not interpretable in terms of antigenic pattern.

To demonstrate the method we have employed for making graphic comparisons among strains and for compensating for the variable avidity of different virus preparations, a model example of cross inhibition tests has been prepared, showing the cross relationships among three virus preparations (Fig. 1).

Three virus strains, PR8-1, PR8–2, and W.S., were cross tested with two PR8 and one W.S. ferret antisera. Both PR8 suspensions were from individual eggs of the same passage. For purposes of illustration it has been assumed that the PR8–1 serum was homologous to the PR8–1 virus suspension and the same for PR8–2 virus and serum. Actually both sera were prepared by infecting ferrets with the same virus preparation.

In section *a* of Fig. 1 are shown the actual inhibition titers obtained when four units of each virus were used for the tests. On examination of the results with the PR8 sera it will be seen that the PR8-2 serum gave lower titers with all three

viruses than did the PR8-1 serum, indicating that the PR8-1 ferret gave a better antibody response, which was reflected in heterologous (W.S.) as well as homol-

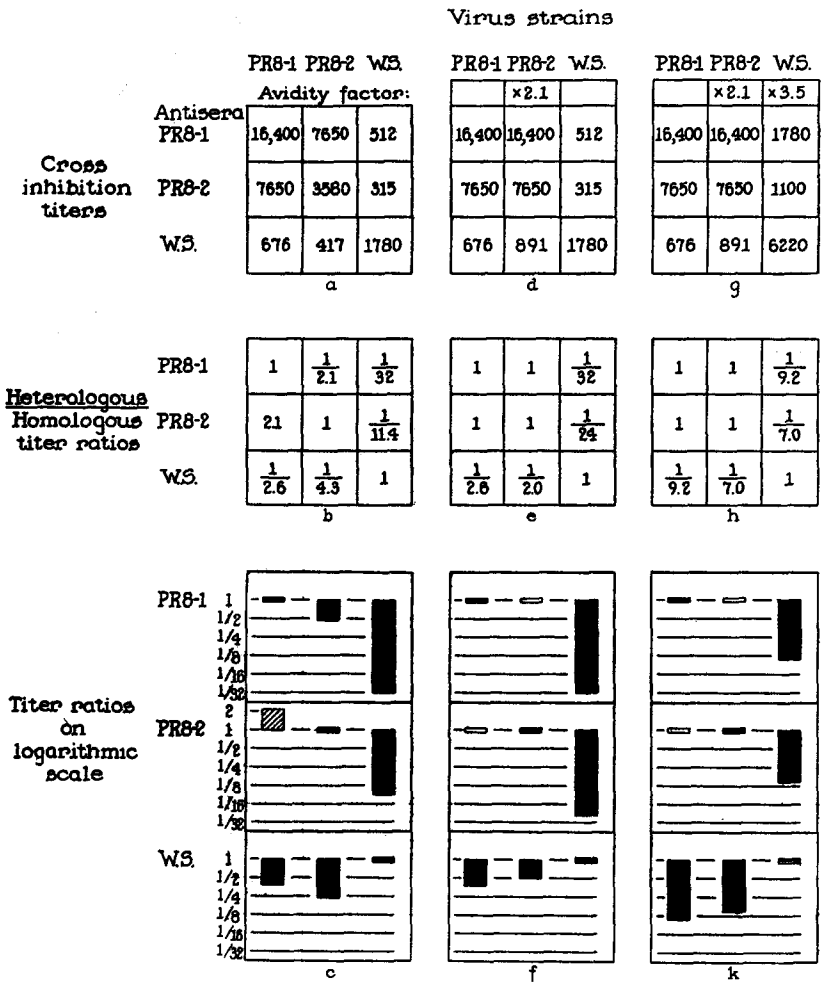

FIG. 1. A model of a cross inhibition test among two preparations of PR8 virus and one of W.S. virus and their respective ferret antisera. The length of the solid bar below the base line indicates the degree to which that serum titer fell below homologous titer. A hatched area above the base line indicates that the titer was higher than the homologous.

ogous antibody. Since the degree of antibody response of different ferrets was found to be highly varied, even after inoculation with the same virus, it seemed desirable to rule out this variable in expressing the results of strain differences, since it is not connected with antigenic pattern. Therefore the

convention was adopted of expressing the results obtained with a serum in terms of ratios of heterologous titer divided by homologous titer. This is a convention used by Burnet and Lush (13) who expressed the ratios in terms of per cent. This method presupposes that a constant fraction of the antibody present will be heterologous, regardless of the absolute level of antibody. The extent to which this is true will become more apparent later.

The ratios of the serum titers in section *a* are shown in section b. These heterologous/homologous ratios are expressed graphically on a logarithmic scale in section *c*. The length of the solid bar extending below the base line indicates the degree to which the heterologous titer fell below the homologous titer. A hatched area above the base line indicates that a given virus gave a higher titer than the homologous virus with that serum. The ratios for homologous titers are always unity.

From the ratios in section *b* and the graphs in section *c* it may be seen that the PR8-2 serum gave a higher titer with the "heterologous" PR8-1 virus than it did with the homologous PR8-2 strain. Such a result cannot be interpreted in this case to be a strain difference because of the nature of the starting material. From this same graph, however, (section *c*) it is apparent that with each serum the PR8-1 virus gave titers which were of the order of twice those obtained with the PR8-2 preparation. From this it may be deduced that the PR8-2 suspension had twice the avidity of the PR8-1 preparation. In order to compensate for this difference in avidity the titers obtained with PR8-2 virus were multiplied by a factor of 2.1 (Fig. 1, section *d*). This converted the titer ratios (section *e*) of the PR8 strains to unity and the graphic picture (section *f*) may now be interpreted as showing no antigenic differences between these two strains.

The relationship shown in section *f* between the W.S. and the PR8 strains was slightly different. In this case there was no paradoxical titer which was higher than the homologous result. From previous experience with the results of neutralization tests it has been found that a large and reciprocal degree of antigenic difference existed between these two strains. The same result was obtained by the agglutination method except that the lack of relationship was not completely reciprocal. Larger differences were found when W.S. virus was tested against PR8 sera than when PR8 virus was tested against W.S. serum. This lack of complete reciprocity may also have been due to differences in avidity, and the application of an avidity factor (3.5) to all titers obtained with W.S. virus established the relationship shown in section *k* where the titers are completely reciprocal. From the final result it may be seen that the amount of heterologous (W.S.) antibody in PR8-1 serum was less than in the PR8-2 serum. Larger variations were commonly found where the differences among strains were even greater. Nevertheless the individual response of ferrets in this respect was surprisingly uniform.

Since there is no direct method at the present time of determining the relative avidity of different virus suspensions, it has been necessary in calculating the corrective factor to use an indirect method. This consists in finding a factor for each virus preparation which, when multiplied by all the titers obtained with that suspension, converts the cross ratios under consideration into reciprocal

results. In using this method it is assumed therefore that under ideal conditions of testing, *i.e.*, with strains of equal avidity, strain differences are always reciprocal. The validity of this assumption will be discussed further after its use in other experiments has been described.

Cross Inhibition Tests among Strains of Influenza A Virus Isolated from the Same Institution on One Day.—Cross inhibition tests were performed with strains isolated from individuals in a single institution and from throat washings which were taken on the same day. It was felt that one could expect the antigenic patterns of such strains to show a maximum of homogeneity. The strains used were designated Dra-1, Dra-2, etc., up to Dra-7 (Table I) and were obtained from throat washings taken on January 16, 1941, from persons in an institution in Alabama. The strains were isolated from throat washing filtrates by chick embryo inoculation, and the fourth to the sixth egg passage was used for testing. Two ferret immune sera were prepared with each strain, and cross inhibition tests were set up between the 14 sera and the 7 strains at one time. The titers obtained with the 2 sera for each virus were averaged, and from these mean titers the ratios of heterologous titer/homologous titer were calculated for each pair of sera. These results are expressed graphically in the first section of Fig. 2.

The comparisons in the first part of this figure show a rather extreme example of non-reciprocal crossing in which almost half of the heterologous titer ratios are greater than unity, or, in other words, about half of the heterologous titers were higher than the homologous titer. The virus strains were arranged in such order that from left to right each successive strain required more of a given serum for inhibition than did its predecessor, with a few minor exceptions. With serum Dra-1 every heterologous virus gave a lower titer than the homologous strain, while with serum Dra-7 all of the heterologous strains gave results which were higher than the homologous. The other strains were intermediate between these two extremes.

In order to present the picture more clearly the titer ratios were recalculated in terms of one virus strain rather than in terms of the homologous virus, and these ratios are shown graphically in the center section of Fig. 2. The ratios in this section were obtained by dividing a given titer by the titer obtained with Dra-1 virus. This graph shows even more clearly that in progressing from the results of testing with strain Dra-1 to those with Dra-7, each virus gave progressively lower serum titers and hence smaller ratios. Also when the ratios are calculated in this way, it is easily seen that the graphic picture presented by each serum is substantially the same. This strikingly similar behavior of the various sera is important and may be taken to mean that the antibody pattern resulting from infection of ferrets with these various strains was exceedingly uniform. There was a marked difference in the behavior of the virus strains used for testing, however, and this could easily have been due to differences in avidity of the various suspensions.

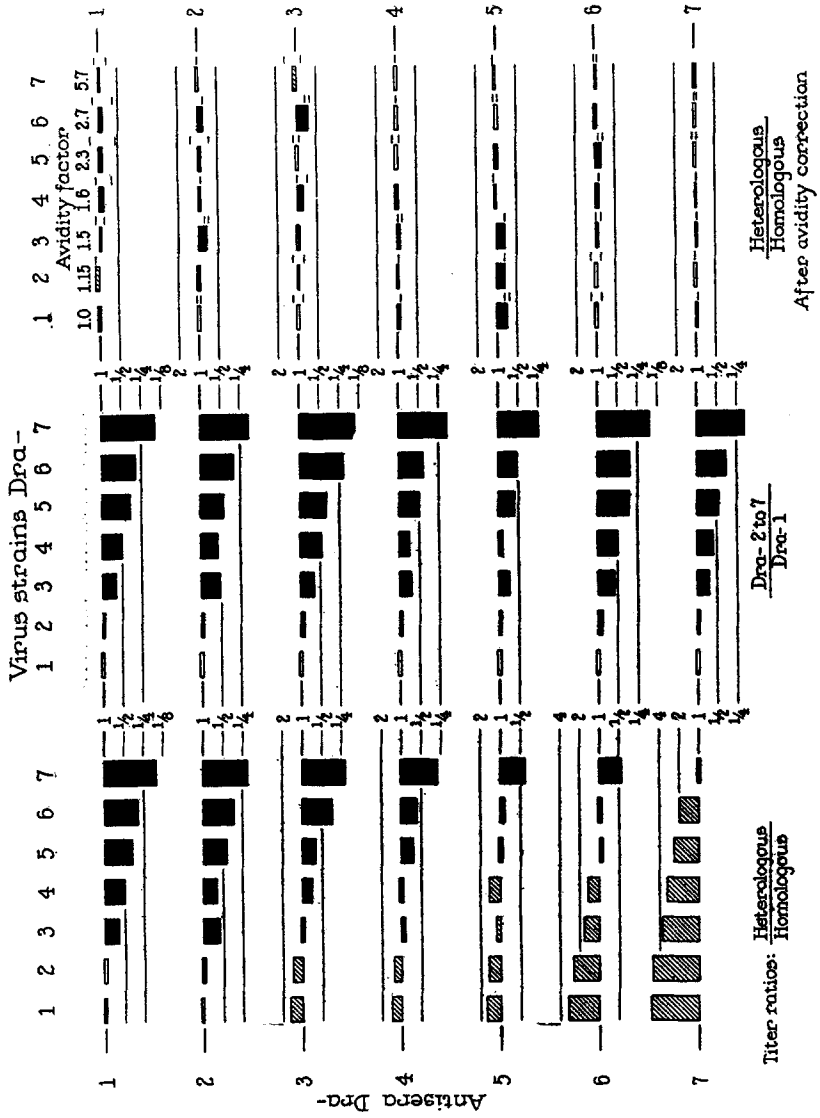

FIG. 2. A graphic presentation of a cross inhibition test among seven strains from the 1940-41 epidemic and their respective antisera. The strains were isolated from cases occurring in a single institution on one day. The solid and hatched areas in the first section of the figure indicate the degree to which the serum titers fell below or were higher than the homologous titers.

The results of the titer ratios in the second section of Fig. 2 make it clear what corrective factors are necessary in order to obtain reciprocal results and to eliminate the paradoxical ratios of greater than unity. The factors used are indicated at the top of the third section of Fig. 2, and after the application of these factors the heterologous/homologous ratios were recalculated. In this figure the discrepancies between the duplicate serum determinations for each titer are indicated by small horizontal lines to the right of each block in the graph. From a consideration of the results calculated in this way it is clear that such antigenic differences as obtained between these strains were negligible and were also well within the experimental error of the method. In most instances the discrepancy between results with duplicate sera was small except with the two Dra-1 sera where the differences approached twofold.

The foregoing experiment was repeated several times with the same sera but using different virus preparations of the same strains, and after corrections for avidity variations the end result was substantially the same as that reported, *i.e.*, no significant strain differences were encountered. As might be anticipated, however, it was found that the avidity factors applied in the experiment illustrated in Fig. 2 were not necessarily characteristic of the individual strains. Different preparations of the same strain often required the application of different avidity factors. This again points to the conclusion that avidity is a variable factor associated with a given preparation and is not clearly allied with strain differences.

It may be emphasized at this point that the method of correction we have proposed and used serves to cancel out an uncontrolled variable in the observations. In altering the results in such a way that strain differences are made reciprocal or abolished, experiments like the previous one are converted from a result which is not interpretable in terms of strain differences to one which gives a simple and reasonable answer. It may also be pointed out that the application of corrections does not change the fundamental differences found by experimentation but only alters their distribution in the final expression (Fig. 1).

Comparison of Strains Isolated from Widely Separated Localities in the 1940–41 Epidemic of Influenza A.—The epidemic of influenza A which occurred in the United States during the winter of 1940–41 began, it is believed, in the Hawaiian Islands during the early part of October, 1940. By November 15 the disease appeared on the mainland in California, and a wave of the disease spread eastward, reaching a peak incidence in the New York area about February 1, 1941.

The strains compared in the next experiment were obtained from cases occurring at different stages of this epidemic (Table I). Strain Haw. was isolated from a resident of the Hawaiian Islands in October, 1940, shortly after the beginning of the epidemic there. Strain S.F. came from a patient in San Francisco shortly after the appearance of the disease on the mainland. Strains Fla-I, Fla-II, and Ala-I were isolated from inmates of different institutions in Florida and Alabama during January of 1941.

Strain NY was isolated from a case occurring in Yorktown Heights, New York, on February 1. All of these strains were isolated by the inoculation of throat washing

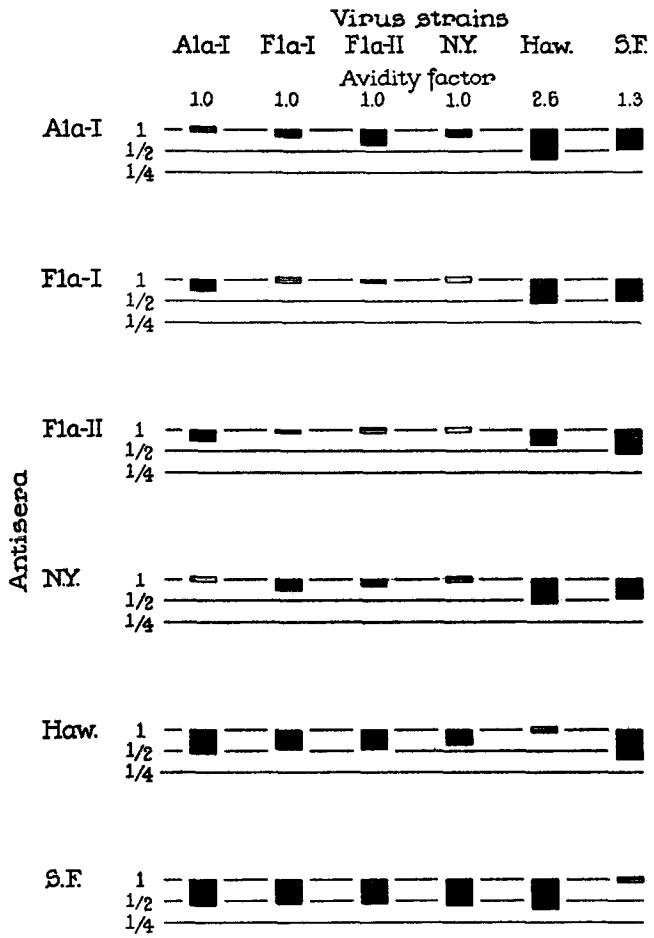

FIG. 3. A graphic presentation of heterologous/homologous titer ratios found when six strains from the 1940-41 epidemic were cross tested. The avidity corrections appear at the top of each column. The strains were obtained from geographically widely separated cases of influenza.

filtrates into chick embryos, except strains Haw. and S.F., which were isolated by ferret inoculation and repeated mouse passage.

Cross inhibition tests among these 6 strains and their respective ferret antisera were performed. The heterologous/homologous titer ratios are shown graphically in Fig. 3. Correction for avidity was necessary only for the mouse-adapted strains. The results show no significant antigenic difference among the first four strains, but

a small, definite, and reciprocal difference was obtained between the first four and the last two strains (Haw. and S.F.). Also strains Haw. and S.F. differed from each other in about the same degree as each differed from the first four strains.

In addition to the above results, similar comparisons have been made among five additional strains isolated by egg passage. These strains also came from cases in Florida and Alabama (Ala-II, Ala-III, Ala-IV, Ala-V, and Fla-III), and each one came from a different institution. No graphic analysis has been presented of these results since they were so similar to the comparisons made among other strains from the same locality and from New York. No significant strain differences were found.

In the experiments just described eighteen strains of influenza A virus were compared in three groups by means of cross inhibition tests. These strains all came from cases occurring in a single country-wide epidemic. Some of the cases were widely separated geographically and their occurrence was far apart in point of time. The striking thing about these strains was their marked homogeneity and, with two exceptions, the lack of significant strain differences. It may be worth noting that the two strains which were different by a slight degree (twofold) were isolated by means of ferrets and mice, while the remainder were obtained by the direct inoculation of chick embryos.

This homogeneity of strain pattern has not in general been found by other workers in the past who have examined a large number of strains. Magill and Francis (14), found that strains isolated from the same epidemic were more similar than strains isolated in different years, but in many cases they found what was considered to be definite differences among the former. Both Smith and Andrewes (9) and Burnet and Lush (13) found great and striking differences among strains isolated even in the same region in one epidemic. Tal and Gat were two such strains (Fig. 4) isolated from a small area in 1937, and yet these differed as much from each other as any two strains of influenza A yet described. The possible significance of this difference in findings will be discussed later.

Antigenic Comparison between a 1940–41 Strain and Six Stock Laboratory Strains.—A cross inhibition test was performed between one of the 1940–41 strains (Dra-3) and six stock laboratory strains of influenza virus. All the strains of this latter group were isolated from patients (except strain swine) by ferret and mouse passage. All are highly virulent for mice. Four of these strains (W.S., Tal, Gat, and Chr) have been designated by Smith and Andrewes as "type" strains, and the results of cross neutralization tests in mice with these strains show large antigenic differences. PR8 was designated by these authors as an "intermediate" type strain which showed minor relationships with all of the type strains. The intermediate group in their hands usually showed the closest relationship with strains Chr and Tal. Swine virus was included as an example of a strain distantly related to all of the human A strains. The graphic results of these tests are presented in Fig. 4. A correction for avidity was ap-

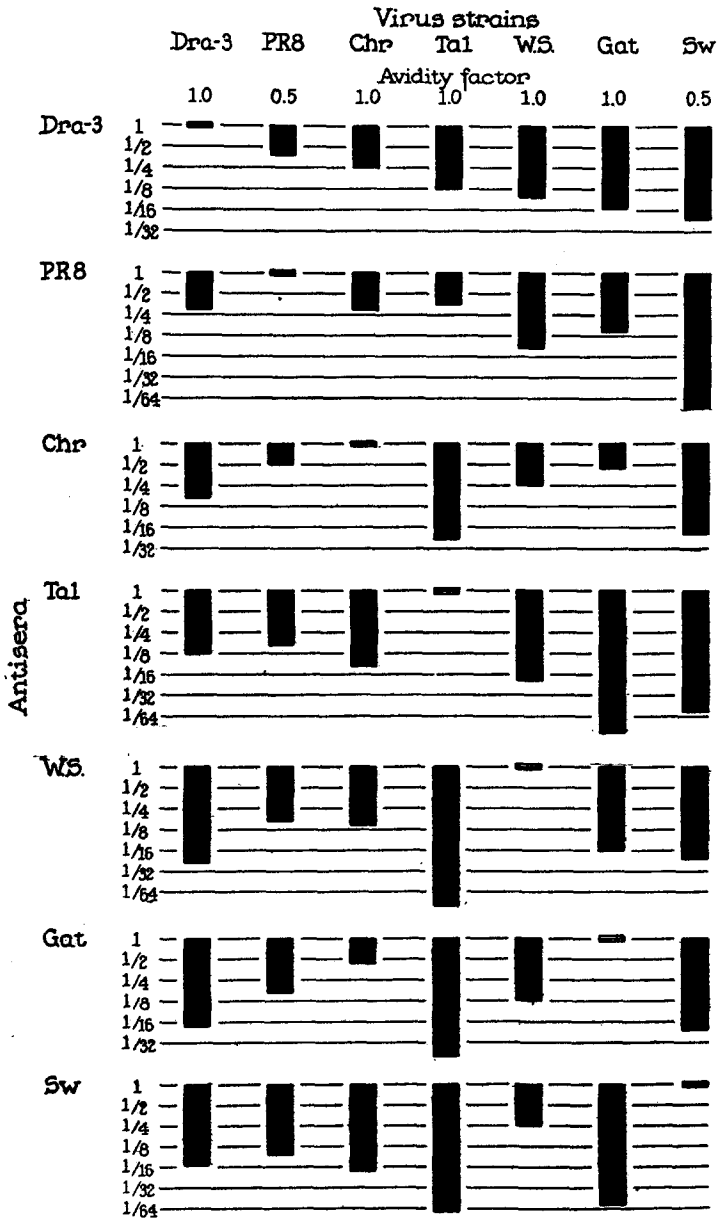

FIG. 4. Cross inhibition tests between a representative of the 1940-41 strains and six stock laboratory strains.

plied to the titers obtained with the PR8 and swine strains. The viruses were arranged on the chart in the order of their relationship to strain Dra-3. It is

quite clear that Dra-3 shows differences from all the other strains, in some cases marked differences, but it appears most closely allied to PR8 and Chr. On these grounds Smith and Andrewes would probably classify it as an intermediate strain.

The relationships among the other strains tested are in substantial agreement with previous work on these cross relationships. The "type" strains all show a wide disparity from one another. PR8 was most closely allied to Chr and Tal. In many instances in which the differences were great there was far from complete reciprocity of the differences, and this lack of reciprocity could not be completely corrected by the use of avidity factors. It was also found when dealing with large differences that duplicate sera did not always give similar results. Most duplicate sera gave results which checked within a twofold range, but some pairs of sera checked only within a fourfold margin of variation. That the ratio of homologous to heterologous antibody content should vary so much from ferret to ferret when the antigens concerned are quite different is not surprising. Because of this variation, however, the strain comparisons shown in Fig. 4 are not necessarily valid in finer detail. Any set of sera made with these strains would probably show these same marked strain differences, but the degree of difference between certain ones might vary considerably.

DISCUSSION

The foregoing experiments indicate clearly that the agglutination reaction may be a useful tool in the study of strain differences among influenza strains. While the method has certain drawbacks such as the need for introducing corrective factors, it is felt that these are to a large measure offset by the speed and relative simplicity with which results can be obtained and especially by the fact that it is possible to test non-mouse-adapted strains.

The most interesting fact to emerge from the studies just reported is the extraordinary homogeneity of the strains from the 1940–41 epidemic. The discrepancy between this finding and that of other investigators has already been mentioned. The number of possible reasons for this difference are many, and at the present time it is difficult to rule out any of them as explanations. The following seem to be the most probable reasons.

1. Variations in Epidemics.—The outbreak in England in 1937 and in Australia in 1939 may have been fundamentally different from the American epidemic of 1940–41, in that the former were caused by multiple types of strains and the latter was not. The occasional appearance of influenza B simultaneously with epidemics predominantly caused by influenza A shows definitely that multiple etiologic agents may occur in a single outbreak. More experience with future epidemics will be necessary to assay accurately this possibility.

2. Different Methods of Testing for Strain Differences.—Although it has been shown that the agglutination method is sensitive enough to measure small strain

differences and that the findings with this method correlate well with the results of the neutralization test, nevertheless it is impossible to say at present that both methods give similar results under all circumstances.

3. Strain Differences May be Created by Animal Passage.—It seems possible that the change in virulence of a virus strain which occurs when it is passed through a series of animals may be accompanied by a change in antigenic pattern. Magill and Francis (14) compared the 27th and the 285th mouse passages of the PR8 strain and found no important differences in antigenic structure. However, it is possible that important changes may have taken place in the first 27 passages. This is a possibility which can now be examined experimentally by means of the agglutination technique for making comparisons, and the effect of animal passage on strain pattern is being tested in this laboratory at the present time. The results of such tests will be reported later.

The work we have done with stock laboratory strains confirms previous work with these same viruses and emphasizes again the large antigenic differences present. Among this group of strains the agglutination test does not give any additional information beyond or in conflict with that obtained by neutralization tests in mice. The data are too complex to offer any interpretation beyond saying that there are antigens which seem to be common to all the strains. It seems likely that cross adsorption studies will be required to clarify these relationships further. For this purpose the agglutination test may be of value as a means of following the adsorption.

SUMMARY

A study of cross inhibition tests among strains of influenza A virus and their antisera showed that the results obtained were subject to a certain amount of variation due to the red cells, the virus suspensions, and the ferret antisera employed. Methods have been demonstrated for handling the data obtained from such tests, so that these variables were corrected or avoided, making it possible to use the agglutination technique for antigenic comparisons.

The antigenic pattern of eighteen strains of influenza A virus, obtained from the 1940–41 epidemic in the United States, has been compared by means of agglutination inhibition tests with ferret antisera. No significant antigenic differences were found among sixteen of these strains (all isolated from throat washings by the inoculation of chick embryos) although they were obtained from individuals in widely separated regions of the country. Two strains, from cases occurring early in the epidemic and isolated from throat washings by ferret and mouse passage, showed a slight but significant strain difference from the other strains and from each other. One of the 1940–41 strains on cross test resembled the PR8 strain more closely than any other stock strain tested.

BIBLIOGRAPHY

1. Hirst, G. K., *J. Exp. Med.*, 1942, **75,** 49.
2. Gordon, I., *J. Immunol.*, 1942, **44,** 231.
3. Burnet, F. M., and Clark, E., Influenza, Monographs from the Walter and Eliza Hall Institute of Research in Pathology and Medicine, Melbourne, No. 4, 1942.
4. Burnet, F. M., Beveridge, W. I. B., Bull, D. R., and Clark, E., *Med. J. Australia,* 1942, **2,** 371.
5. Hudson, N. P., Sigel, M. M., and Markham, F. S., *J. Exp. Med.*, 1943, **77,** 467.
6. Personnel of Naval Laboratory Research Unit No. 1, U.S.N.R., *U. S. Naval Med. Bulletin*, 1943, **41,** 114.
7. Francis, T., Jr., *Science,* 1934, **80,** 457.
8. Smith, W., Andrewes, C. H., and Laidlaw, P., *Lancet,* 1933, **2,** 66.
9. Smith, W., and Andrewes, C. H., *Brit. J. Exp. Path.*, 1938, **19,** 293.
10. Hirst, G. K., and Pickels, E. G., *J. Immunol.*, 1942, **45,** 273.
11. Stuart-Harris, C. H., *Brit. J. Exp. Path.*, 1943, **24,** 33.
12. Chambers, L. A., and Henle, W., *J. Exp. Med.*, 1943, **77,** 251.
13. Burnet, F. M., and Lush, D., *Australian J. Exp. Biol. and Med. Sc.*, 1940, **18,** 49.
14. Magill, T. P., and Francis, T., Jr., *Brit. J. Exp. Path.*, 1938, **19,** 273.