THE ANTIGENIC COMPOSITION OF INFLUENZA VIRUS MEASURED BY ANTIBODY-ABSORPTION*

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The first comprehensive investigations of the antigenic composition of various virus isolates from epidemics of influenza A demonstrated the complex nature of the immunologic problems to be considered with this disease (1–4). These data, obtained by mouse protection tests, portrayed antigenic variation and, in addition, were clearly interpreted as evidence of shared antigens among the strains of influenza virus then available (2, 3). It was pointed out that they represented a spectrum of interrelationships and that all strains did not fit readily even into loosely defined groups. Certain investigators (4), however, attempted to classify groups of strains in terms of types or "master" strains.

With the advent of hemagglutination-inhibition technics emphasis has again been toward relating all isolates to prototype strains (5–7). Recently Magill (8) and Hilleman (9) have classified strains by hemagglutination-inhibition with rabbit or chicken sera prepared against a limited number of strains employed as prototypes. Although the sera are commonly prepared in a manner which emphasizes the dominant antigen of a strain, they may still contain antibody to its lesser antigens. The results, therefore, do not indicate whether the reaction of a test strain with a given serum is the result of antibody to a single dominant antigen or to several components shared with the prototype strain. Hence, many differences and relationships among strains may not be evident.

A promising approach to a more precise definition of antigenic components is by absorption of antibodies from sera. That antibodies against influenza virus can be absorbed from serum has been repeatedly demonstrated (7, 10–15). Friedewald (12) found antibody absorption tests to be valuable in studying the antigenic composition of influenza virus and concluded that quantitative as well as qualitative differences in components provided antigenic distinction to strains. Hirst (15) absorbed crossing antibodies from rabbit hyperimmune sera and typed strains by their inhibition with sera absorbed so as to make them relatively specific for one of eight selected strains. This procedure tends to ignore the composite antigenic structure of influenza virus and demonstrates only that antigen shared with the prototype strain.

The foregoing review summarizes trends which emphasize either similarities or differences among strains and indicates the necessity of adequately mapping

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the antigenic constitution for a comprehensive understanding of strain relationships. It has been indicated that quantitative absorption procedures are an effective logical approach to the problem but have been limited in their application by the technical burdens involved in preparation of concentrated antigenic masses, in separation of added virus from absorbed serum, and by the difficulty of establishing quantitative standards of absorption by antigens.

The demonstration in this laboratory (16) that influenza virus adsorbed to erythrocytes can function efficiently to absorb antibody from serum stimulated the development of procedures for ensuring stable erythrocyte-virus complexes. The absorption of antibodies from sera is thus greatly facilitated. Information concerning different components which may be shared can then be derived by a systematic investigation of the antibodies absorbed by homologous and heterologous strains. Comparisons of the qualitative and quantitative capacity of the analyzed strain to absorb defined amounts of homologous and heterologous antibody from a standard serum mixture give accurate information concerning shared components. Standardization of the antibody-absorptive capacity of each lot of virus-coated cells against the homologous serum provides a standard for comparison so that antigenic composites or profiles of the strains of influenza virus can then be mapped. The information so obtained constitutes the basis of this report.

Materials and Methods

Procedures for Absorption of Antibody.—The union between erythrocytes and influenza virus offers a unique approach for the absorption of virus-specific antibody from serum. Virus held by other absorbents (norite, celite, calcium phosphate, etc.) may unite with the antibody, but the non-specific absorption of antibody by these chemicals makes them unsuitable. Unfortunately the equilibrating nature of the reaction between erythrocyte and active virus does not permit formation of a stable red cell–virus complex. Virus particles that have been inactivated by heat will adsorb firmly to erythrocytes (14, 16–18), but uniform inactivation of viral populations is difficult to obtain with heat. On the other hand, treatment of either the virus or the erythrocytes with periodate consistently yields irreversible adsorption of the virus to the cell receptor (19–21). Treatment of erythrocytes with periodate was studied in a series of experiments but was discontinued when it became apparent that such cells were not readily coated with a sufficient concentration of virus to effect complete absorption of antibody. Experiments with periodate-treated virus were more rewarding and the method outlined below was developed. The procedures involved are diagrammed as a flow sheet in Fig. 1.

(*a*) *Preparation of Formalinized Erythrocytes.*—Erythrocytes from human blood were washed three times in buffered saline (0.01 M phosphate in 0.85 per cent sodium chloride, pH 7.2), and 50 per cent cell suspensions were mixed with an equal volume of 30 per cent formalin. After storage at 4°C. for 48 hours the cells are very cohesive, dark brown masses, and the supernatant fluids are dark red, apparently due to methemoglobin. The cells were then washed 6 to 8 times in buffered saline over a period of several days until the odor of formalin was no longer detectable. The virus materials subsequently added are therefore not subjected to significant concentrations of formalin. As Flick (22) has shown, such cells are extremely stable. In our hands, they resist lysis for several weeks, adsorb influenza virus to a maximal degree, and are readily packed in the centrifuge.

(*b*) *Preparation and Treatment of Virus.*—Pools of 1 to 2 liters of allantoic fluid containing a given strain of virus were obtained by inoculating 11-day chick embryos with 0.1 ml. containing approximately 10^4 EID_{50} of the desired strain. After incubation at 37°C. for 48 hours the eggs were chilled at 4°C. for 2 hours, the fluids harvested with a suction apparatus, and then clarified by low speed centrifugation. The hemagglutinin titers of these pools vary from 1:160 to 1:1280 by a standard pattern method (23) with 0.5 per cent chicken erythrocytes. Pools were mixed with an equal volume of M/25 potassium periodate solution in buffered saline and held at room temperature for 1 hour. The excess periodate was neutralized by

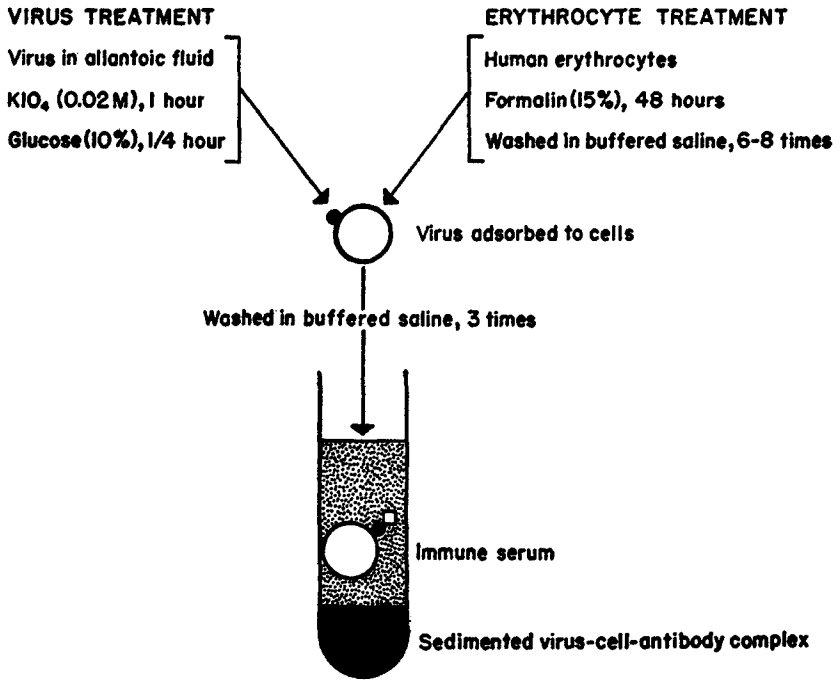


FIG. 1. Method for serum absorption.

one-half volume of 10 per cent glucose. Such treatment destroys the eluting capacity of the virus but not its hemagglutinating activity (20). Furthermore there is no evidence that the antigenicity of the virus is measurably altered.

(*c*) *Preparation of RBC-Virus Complex.*—1000 ml. of virus suspension in allantoic fluid, periodate, and glucose are mixed with 50 ml. of 50 per cent formalinized cells and subjected to repeated gentle shaking and then placed at 4°C. overnight. Estimates of the amount of virus adsorbed were made by comparing the hemagglutinin titers of fluids before and after addition of the erythrocytes. When the hemagglutinin titer of the pool was low, adequate concentrations of virus on the cells were obtained by adsorption of the virus from several 1 liter volumes of fluid with the same lot of erythrocytes. The virus-cell complexes were washed 3 times in buffered saline and stored at 4°C. as 50 per cent cell suspensions.

(*d*) *Preparations of Antisera and Antibody Titrations.*—All ferret sera were obtained from convalescent animals. Ferrets were infected by intranasal instillation with 2.0 ml. of a 1:1000

dilution in broth of allantoic fluid containing the selected virus. Blood was obtained by cardiac puncture on the day of infection and 2 weeks later. 1 month after the original infection the animals were again inoculated intranasally with 2.0 ml. of infected allantoic fluid to enhance the antibody response to all antigenic components of the strain of virus. Blood was collected on the day of reinoculation and 2 weeks later. Convalescent sera were employed because the antigenic stimulus from each antigenic component is probably more readily realized from infection than from vaccination, and difficulties with non-viral antigens are not encountered.

The standard pattern test (23) was used to titrate hemagglutinin-inhibiting (H-I) antibody. Difficulties with non-specific inhibitors were circumvented by using inhibitor-resistant lines of virus and dilutions of serum beyond the range of inhibitor effect.

Virus-neutralizing antibodies were titrated *in ovo* by mixing approximately 500 EID_{50} and serial 4-fold dilutions of serum, incubating at 37°C. for 30 minutes, and inoculating 0.1 ml. of each mixture into four 10-day embryonate eggs. After incubation for 72 hours at 37°C. 0.5 ml. of the allantoic fluid of each egg was tested for virus hemagglutinin with 0.5 ml. of 0.5 per cent chicken erythrocytes.

The technic of complement fixation (CF) was similar to that described by Lennette *et al.* (24) except that incubation of the antigen-antibody mixture for 1 hour at 37°C. was substituted for overnight at 4°C.

Acute and convalescent phase sera were tested simultaneously with each strain of virus. Antibody titers are expressed as the reciprocal of the initial dilution in the CF test and of final dilutions in the H-I and neutralization tests.

(*e*) *Method of Antibody Absorption.*—Erythrocytes coated with the selected strain of virus were sedimented by centrifugation from stock 50 per cent cell suspensions and resuspended in dilutions of serum. The mixtures were held as routine overnight at 4°C., although comparable results have been obtained after 3 hours at room temperature. Cells were then packed by light centrifugation and the H-I antibody titer of the supernatant serum was compared with that of the unabsorbed serum. In the case of an homologous serum-virus system, essentially all the homologous H-I antibody is removed. No free hemagglutinin should be detected in either acute or convalescent phase sera absorbed in this manner. That the reduction of antibody titer is not merely the effect of virus released from the cells is shown by the fact that no virus is detected in normal serum so treated.

(*f*) *Quantitation and Standardization of Antibody-Absorptive Capacity.*—The results of preliminary absorption experiments indicated the need for quantitation of the antigen-antibody system. In the first experiments the concentration of hemagglutinin on the erythrocytes was estimated from titrations of the periodate-treated fluids before and after adsorption to formalinized RBC or by direct titration of the hemagglutinin on the virus-coated human cells with 0.5 per cent chicken cells in a pattern test. However, the capacity of the cell-virus complex to absorb antibody from sera is not measured by these methods. Results from several absorption experiments with many different antigen-antibody mixtures could be compared only when the amounts of absorbent antigens were standardized. The following procedure was developed to meet this need. The homologous antibody concentration of a standard serum for each strain of virus was adjusted by dilution so that the H-I titer would be 1:64. 2.0 ml. aliquots of the serum dilutions were mixed with several different volumes (0.12, 0.25, 0.5, and 1.0 ml.) of packed virus-coated cells. Homologous H-I antibody titers of the absorbed aliquots of serum were then determined. The minimal volume of cell-virus complex that would completely remove the 64 units of homologous H-I antibody in a single absorption was defined as one absorbing unit (A.U.). The A.U. volume of a preparation has been found to remain constant for several weeks when stored at 4°C. This relative stability therefore provides the obvious advantage of subsequent use in several absorption experiments with relative confidence in the comparability of results.

Strains of Virus.—The 42 strains of Type A influenza virus included in this study were selected as representative of isolates from epidemics of the disease occurring since 1933. The majority of these strains were from the files of this laboratory and have been described previously. The Henry, Hickcox, Cam, Coamo, and Netherland 1/49 were obtained from the Strain Study Center of Dr. T. P. Magill. Dr. G. K. Hirst of The Public Health Institute sent the Christie strain. Information concerning the date and place of isolation of each strain is given in Table I.

The Type B strains of influenza virus studied included Lee, isolated in Irvington, New York, in 1940, and Allen, from Ann Arbor, Michigan, in 1945.

All strains were well adapted to eggs by passage in the allantoic sac of 11-day chick embryos. Many strains also had ferret and mouse passages in their histories.

TABLE I

Strains of Type A Influenza Virus Studied

1. Swine	Iowa	1931*,‡	15. C112	Europe	1939*,‡	29. Berry	Mich.	1948
2. WS	Eng.	1933*,‡	16. Baum	Mich.	1940*,‡	30. CA7	Canada	1949*
3. PR8	P. Rico	1934*	17. Hick	N. Y.	1940*	31. Hood	Mich.	1949
4. Alaska	Alaska	1935*	18. Weiss	Mich.	1943*,‡	32. O. Is.	O. Is.	1949
5. BH	Eng.	1935*,‡	19. IA43	Iowa	1943*,‡	33. Roma	Rome	1949*,‡
6. Mel	Austr.	1935*,‡	20. Jacksi	Mich.	1943	34. Neth.	Neth.	1949
7. Phila.	Phila.	1935*	21. Ian	Austr.	1946	35. Chom	Austr.	1950
8. TF	N. Y.	1936*	22. Cam	Austr.	1946*	36. Sweden	Sweden	1950*
9. NY2	N. Y.	1936*,‡	23. Leiden	Neth.	1947	37. Cup	Wyo.	1950*
10. Henry	N. Y.	1936*,‡	24. FM1	N. J.	1947*,‡	38. Ten	Mich.	1950*,‡
11. Smithb	Eng.	1937	25. Rhodes	Mich.	1947*,‡	39. Lond	Eng.	1951
12. Gatenby	Eng.	1937*,‡	26. FjS	Austr.	1948	40. Boch	Mich	1951*
13. Talmey	Eng.	1937*,‡	27. Coamo	P. Rico	1948	41. Mor	Mich.	1951*,‡
14. Chris	Eng.	1937*	28. Dean	Mich.	1948	42. Jes	Mich.	1953*,‡

*, attempted preparation of strain-specific serum (29 strains).

‡, successful preparation of strain-specific serum (18 strains).

EXPERIMENTAL

Absorption of Antibody from Individual Ferret Sera by the Infecting Strain

The serum of a ferret convalescent from infection with a given strain of virus contains antibody to the infecting strain and ordinarily to related heterologous strains. Absorption of that serum with the infecting strain removes all heterologous as well as homologous antibody. Representative examples of this observation are presented in Table II. Serum from a ferret convalescent from an infection with the A-prime Rhodes (1947) strain of influenza virus contains antibodies that will not only inhibit the Rhodes strain but also reacts with the Type A PR8 (1934) strain. After absorption with the Rhodes strain the supernatant serum no longer inhibits either of these strains of virus. This relationship is also found between the Type B strains of influenza virus. Thus the anti-Allen (1945) serum also inhibited the Lee (1940) strain, but the serum did not

react with either strain after absorption with the infecting strain. The specificity of this reaction is demonstrated by the failure of absorption with strains completely different antigenically to have any reducing effect on antibody titers,

TABLE II

Antibody Absorption with Homologous Strains of Influenza Virus

Serum		Test viruses			
Infecting strain	Absorption strain	PR8 (1934)	Rhodes (1947)	Lee (1940)	Allen (1945)
Rhodes	Nil	640*	2560	<32	<32
	Rhodes	<32	<32	<32	<32
	Lee	640	2560	<32	<32
Allen	Nil	<32	<32	320	2560
	Allen	<32	<32	<32	<32
	Rhodes	<32	<32	320	2560

* Reciprocal of final dilution of serum that inhibits 4 units of hemagglutinating virus.

TABLE III

Complete Removal of Antibody by Absorption with a Heterologous Strain of Influenza Virus

Test strains	Henry (1946) convalescent serum	
	Unabsorbed	Absorbed with TF (1936)
Swine (1931)	80	<40
WS (1933)	80	<40
PR8 (1934)	80	<40
Alaska (1935)	160	<40
BH (1935)	320	<40
Mel (1935)	160	<40
TF (1936)	160	<40
NY-2 (1936)	80	<40
Henry (1936)	640	<40
C12 (1939)	160	<40
Baum (1940)	80	<40

as shown by the absorption of the Rhodes (Type A) convalescent serum with the Lee (Type B) strain.

These results are to be expected since the crossing antibodies are presumably reacting with antigens shared by the two strains and the entire group of antigenic components is present when the homologous virus is used for absorption.

Absorption with Related Heterologous Strains

The extent to which antigenic components are shared among strains is further emphasized by the results of serum absorption with related heterolo-

gous strains of influenza virus. The antigenic prominence of a component varies among strains so that the results of antibody-absorption from a given serum by different strains will depend upon this relationship. Absorption with one strain representing a particular antigenic mosaic which has multiple

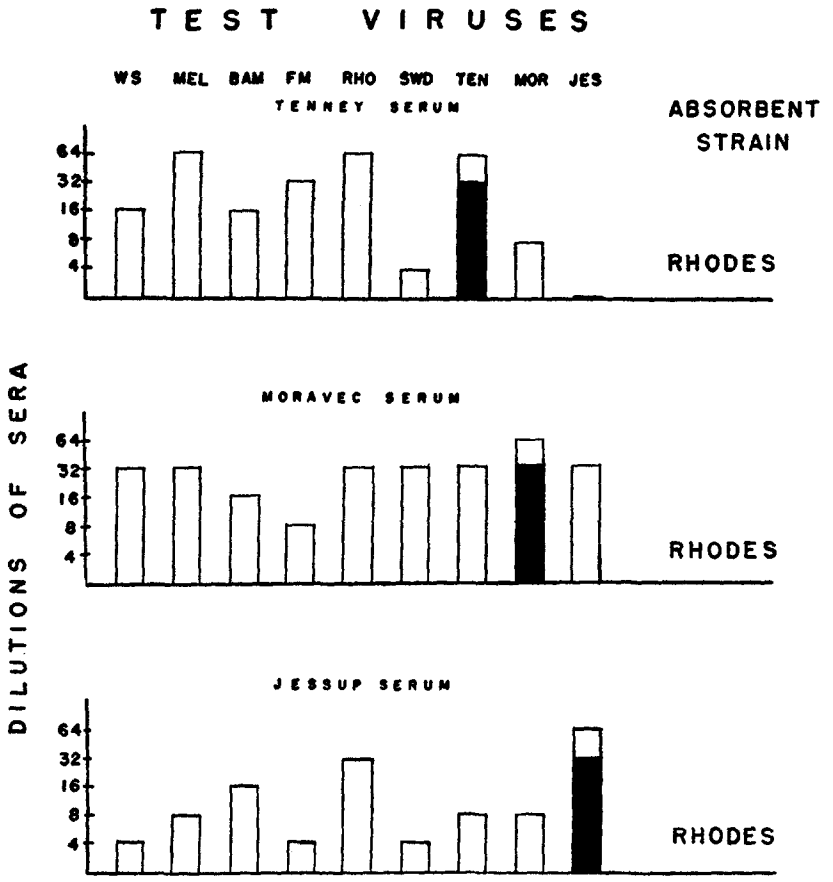


FIG. 2. Absorption of Tenney, Moravec, and Jessup ferret sera. Sera were initially diluted 1:20. Outlined areas indicate H-I titer against the various strains with unabsorbed sera and solid areas represent antibody after absorption.

components in common with the infecting strain may produce one of several results.

(*a*) All detectable antibody against homologous and heterologous strains may be removed (Table III). When the serum of a ferret which had been infected with the Type A Henry (NY-1936) strain is absorbed with the TF strain (NY-1936) the serum no longer reacts with any of the other 42 strains of influenza virus tested. The TF strain apparently contains all the components

of the Henry strain in sufficient concentrations to unite with all the anti-Henry strain antibodies and to leave no antibody that will react with the other strains of influenza virus.

(*b*) Absorption of a serum with a related heterologous strain may result in the complete reduction of all antibody which inhibits the absorbent or other heterologous strains, but leaves antibody that will inhibit the infecting strain

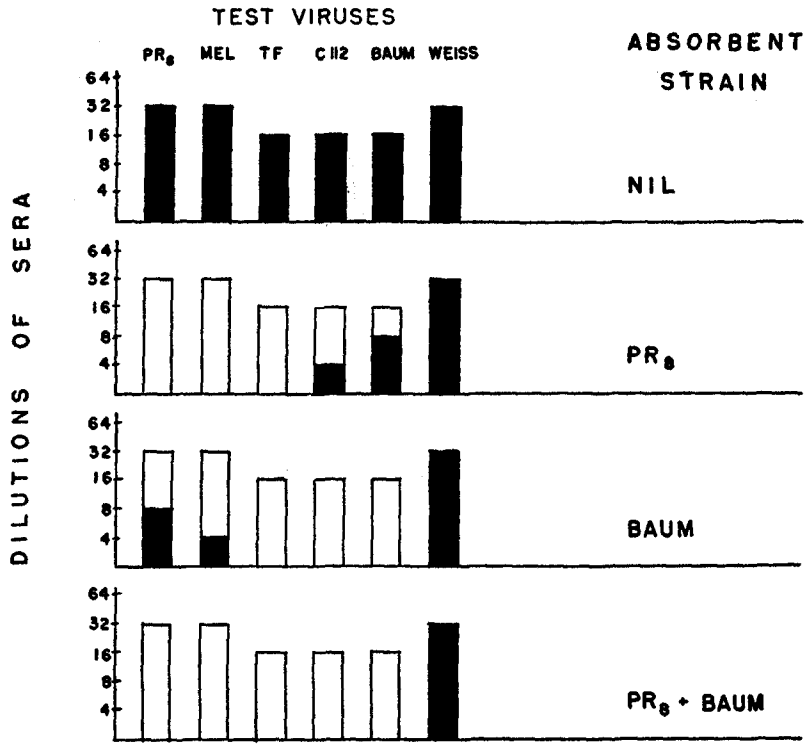


FIG. 3. Absorption results with Weiss ferret serum.

of virus. Fig. 2 summarizes the results of absorbing sera from each of several ferrets convalescent from infections with certain strains of virus that are related in this manner. In each example absorption with the Rhodes strain removes all antibody that reacts with heterologous strains, but the serum still reacts to high titer with the homologous strain. Here the antigenic complex of the absorbent strain shares many of the components of the infecting strains but is deficient in at least one component of each of the other strains. This allows production of strain-specific sera.

(*c*) Absorption of serum with a particular strain may give reduction of antibody for only some of the related heterologous strains and again have

little effect on the titer of inhibiting antibody for the infecting strain. Successive absorption of that serum with two or more selected strains, however, can effect the creation of a serum that will inhibit only the infecting strain. This type of result is illustrated in Fig. 3 with the subsequent production of a serum which is specific for the Weiss (1943) strain following absorption of the ferret serum with the PR8 (1934) and Baum (1940) strains. The effect is obtained by either successive or simultaneous absorptions with these related strains and indicates that the critical combination of antigenic components is represented in the total antigenic complex of the two absorbent strains.

(*d*) It is also possible that absorption may result in a serum that will not inhibit the homologous strain but H-I antibody may be found for certain

TABLE IV

Absorption of Homologous Antibody with a Heterologous Strain of Influenza Virus

Test strains	Cam (1946) convalescent serum	
	Unabsorbed	Absorbed with Hickcox (1940)
Alaska (1935)	320	<40
TF (1936)	160	<40
Baum (1940)	320	<40
Hickcox (1940)	640	<40
Cam (1946)	640	<40
Rhodes (1947)	320	80
Tenney (1950)	320	<40
Moravec (1951)	80	80

heterologous strains. An example of this situation is found when the Hickcox (1940) is used to absorb antibody from a ferret convalescent from infection with the Cam (1946) strain (Table IV). In this case the absorbed serum does not react with the Cam or Hickcox strains but will inhibit the hemagglutinins of the Rhodes (1947) and Moravec (1951) strains of influenza virus. It is concluded that the antigenic composite of the Hickcox strain contains the principal antigens of the Cam strain mosaic but some antibodies stimulated by Cam are relatively less involved, qualitatively or quantitatively, in the inhibition of the homologous strain than of the Rhodes and Moravec strains.

Removal of Non-Specific Inhibitors by Absorption

Observations from several experiments indicating that non-specific inhibitors (25, 26) might be removed from serum by absorption with a virus-cell complex prompted additional experiments to investigate this possibility. Normal sera and sera from animals immune to Type A strains of influenza virus were absorbed with the antigenically unrelated Type B Lee strain so as

not to disturb the antibody. The data presented in Table V demonstrate that absorption of rabbit or ferret sera with the Lee strain invariably removes the materials that non-specifically inhibit the hemagglutinin of the heat-inactivated (inhibitor-indicator virus) Lee strain.

The irregular results of H-I tests obtained with the various lines of the Wright (1953) strain may be interpreted as evidence of non-specific hemagglutinin-inhibitory substances which do not unite as readily with the Lee strain of influenza virus and have a variable effect on the hemagglutinin of different lines of this isolate.

TABLE V

Removal of Non-Specific Inhibitors from Serum by Absorption with a Virus-Erythrocyte Complex

Animal sera	Phase of bleeding	Absorption with Lee strain	Test viruses			
			Lee ($F_8M_{12}E_{160}$)		Wright (AE_1E_{10})	Wright ($M_1AE_1E_2$) ($M_{26}E_4$)
			Active	Heated*		
Rabbit	Normal	Nil	<32‡	1024	256	512
Immunized with Wright (AE_1E_2)		4 A.U.	<32	<32	<32	<32
	Immune	Nil	<32	1024	1024	1024
		4 A.U.	<32	<32	256	256
Ferret	Acute	Nil	<32	512	128	256
Infected with Wright (AE_1E_2)		4 A.U.	<32	<32	<32	128
	Convalescent	Nil	<32	512	512	1024
		4 A.U.	<32	<32	256	1024

* 56°C. for 30 minutes.

‡ Reciprocal of final dilution of serum that inhibits 4 hemagglutinin units.

Evidently more than one kind of heat-stable non-specific inhibitory material may be found in sera as suggested by Chu (27) and Sampaio (26), but the effect on hemagglutinin is not necessarily overcome by adaptation to mice as was believed by these investigators. Additional investigations of the relationship between the inhibitors acting upon heat-inactivated virus and those inhibiting newly isolated strains are indicated.

Absorption of Neutralizing and Complement-Fixing Antibodies

Friedewald (12) and Walker and Horsfall (28) observed a reduction of antibody titer as determined in neutralization and complement fixation tests when antisera are treated with virus, and it was of interest to determine whether similar results would obtain with the virus-cell complex. The results of such an experiment are summarized in Table VI. Antibody absorption was

tested with CF and *in ovo* neutralization tests as well as by H-I tests with the sera of ferrets convalescent from infection with Type A Rhodes strain of influenza virus. Absorption of the sera with the infecting strain completely removes homologous and heterologous antibody as measured by all three serologic tests. Absorption with a related heterologous strain of virus (Moravec) results in a decreased titer of antibody for the absorbent and other heterologous strains, while the effect on homologous antibody is less marked. Again the specificity of absorption in relation to shared antigens is noted, and it is interesting to find the CF test also reflecting a strain-specific decrease in antibody titer following absorption with the heterologous strain. This is surprising since that degree of strain specificity is usually not observed with the CF test.

TABLE VI

Absorption Results with Convalescent Ferret Serum (Rhodes, 1947)

Serum Absorbent (1 absorbed unit)	Antigens tested after absorption					
	Rhodes (1947)			Moravec (1951)		
	H-I	CF	E.N.	H-I	CF	E.N.
Nil	4096*	320	>5120	512	160	640
Rhodes	<32	<40	<80	<32	<40	<80
Moravec	512	80	2560	<32	40	<80

E. N., egg neutralization.

* Reciprocals of final dilutions of serum.

Preparation of Strain-Specific Sera

With an increased understanding of the quantitative aspects of the absorption technics devised, a comprehensive study of the antigenic constitution of strains of influenza virus was begun. Exploratory absorbing experiments promised the preparation of a series of sera that would inhibit only the respective strains of virus used to infect the animals. Convalescent ferret serum was therefore prepared against each of 29 selected Type A strains and cross-tested by H-I with all these strains. The serologic relationships between strains as defined by these results governed the choice of absorbent strains for the preparation of strain-specific sera. In 10 cases absorptions with combinations of two heterologous strains resulted in sera that would react only with the infecting strain. On the other hand, 8 specific sera were produced by absorption of heterologous antibody with a single closely related strain. A summary of this aspect of the study is shown in Table I. A total of 18 strain-specific sera were prepared from 29 different convalescent ferret sera and tested with 42 related strains of virus. The strains with which attempts have been made to prepare specific serum are marked with an asterisk, and the double dagger

signs indicate those strains for which the highly specific sera have been prepared. Since 18 different strain-specific sera were demonstrated, it suggests that there are at least 18 different antigenic components in the Type A influenza virus, and evidence of other components is also available from the unsuccessful attempts to prepare strain-specific sera. Several sera were prepared that would react only with 1 or 2 strains other than the homologous strain which indicates a subgroup of strains sharing a specific antigen. When the problems of separation of these strains have been solved, an additional number of antigenic components may be as readily demonstrated as the 18 described above. The results forecast the extreme complexity of antigenic constitution that may eventually be defined for this group of viruses.

Assay of Antigenic Composition of Strains

The search for specific antigens is of obvious interest and must be continued, but a primary objective was to compare the composite antigenic structures of strains of influenza virus. The procedures which had been so fruitful in furnishing the foregoing were therefore adapted to this problem with gratifying results. If a serum were available which contained equivalent titers of antibody to each of the antigenic components, absorption with a standard amount of any strain of virus would remove antibody to the components present in that strain, and the extent of the absorption of the various antibodies would describe the antigenic structure of the test strain.

For this purpose sera from ferrets convalescent from infection with one of 17 strains chosen to represent different components were pooled in such concentration that the pool contained a comparable titer of H-I antibody for each of the 17 strains. Therefore the final titer of antibody for each strain represents homologous antibody and the amounts of cross-reacting antibody furnished by the sera against heterologous strains which contained the same antigens. Aliquots of the pool were absorbed with a standard number of absorbing units of each virus-cell complex. Each aliquot was then tested for antibody to all of the strains represented. The results of absorption with the Rhodes (1947) strain are given in Fig. 4. Three successive absorptions with 2 A.U. were carried out to investigate the antigenic constitution of the strain and to establish a practical limit for the amount of absorbent antigen to be employed for adequate analysis. The most complete reduction in antibody titer following this absorption was for those strains isolated in the years from 1947 to 1950. Antibody content was also decreased, but to a lesser extent, for several other strains. It is concluded that absorption with 6 A.U. is a practical level since only small changes in antibody titers were noted between the second and third successive absorptions with 2 A.U. in this experiment and in several tests with other absorbing strains. Antiserum for the Type B, Lee, strain was included in the pool to demonstrate the specificity of the reaction and to control the dilution factors.

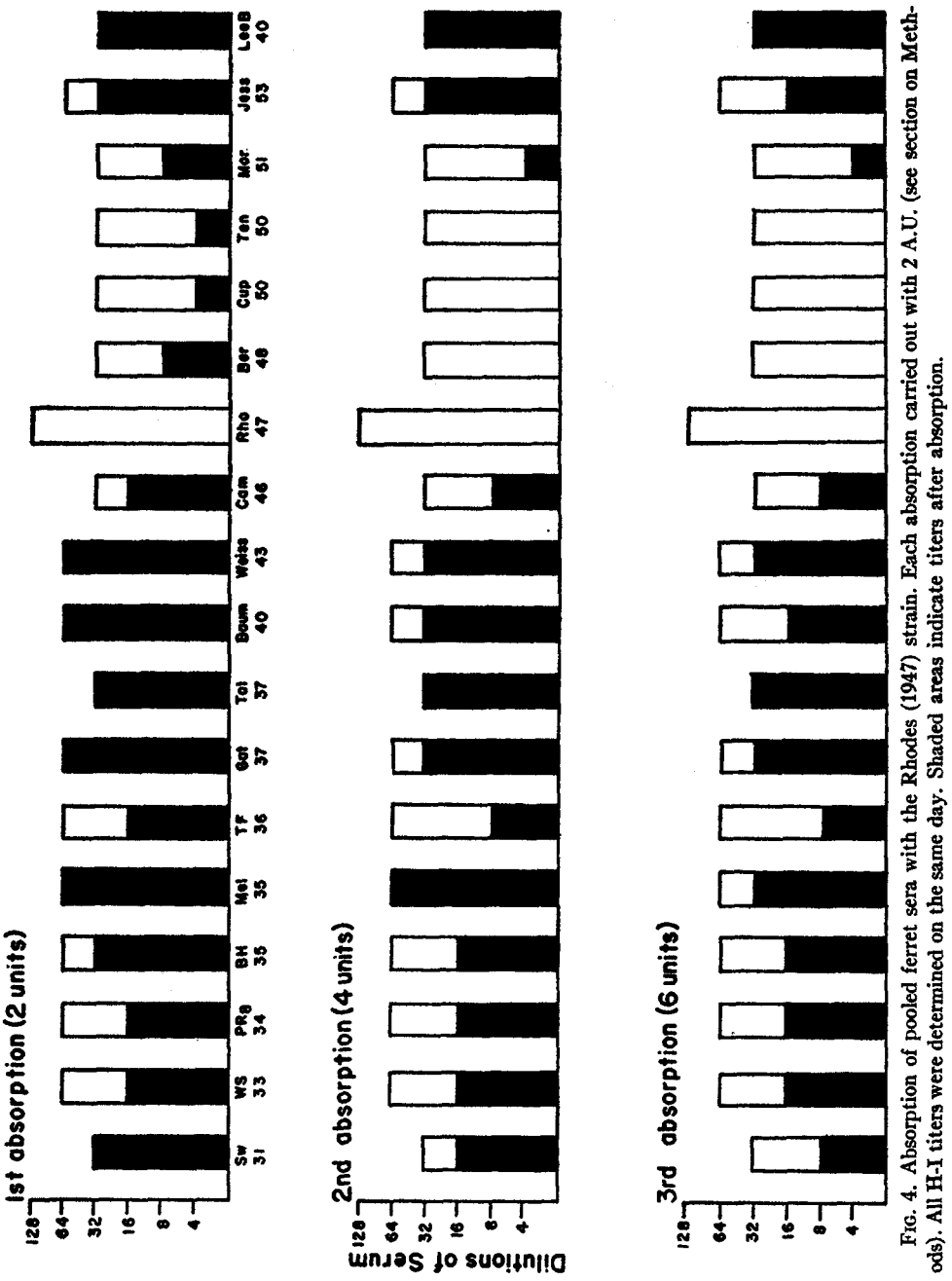
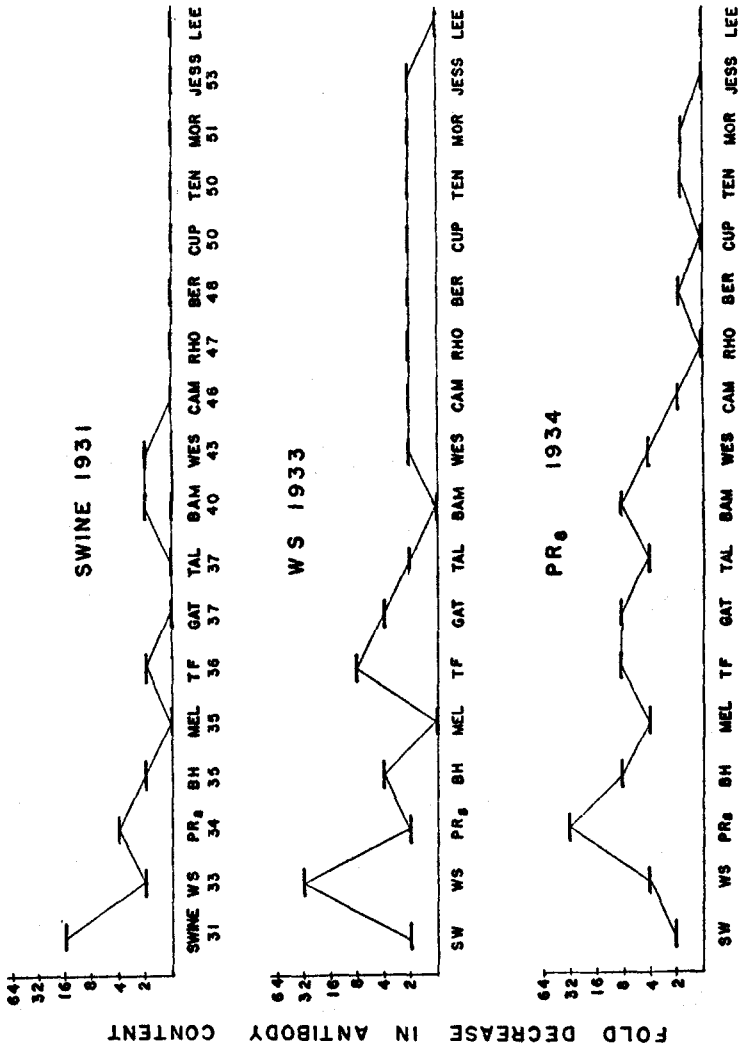


FIG. 4. Absorption of pooled ferret sera with the Rhodes (1947) strain. Each absorption carried out with 2 A.U. (see section on Methods). All H-I titers were determined on the same day. Shaded areas indicate titers after absorption.



FIGS. 5 and 6. Antigenic profiles of virus strains. Pool was absorbed with 6 A.U. of indicated strains.

FIG. 5

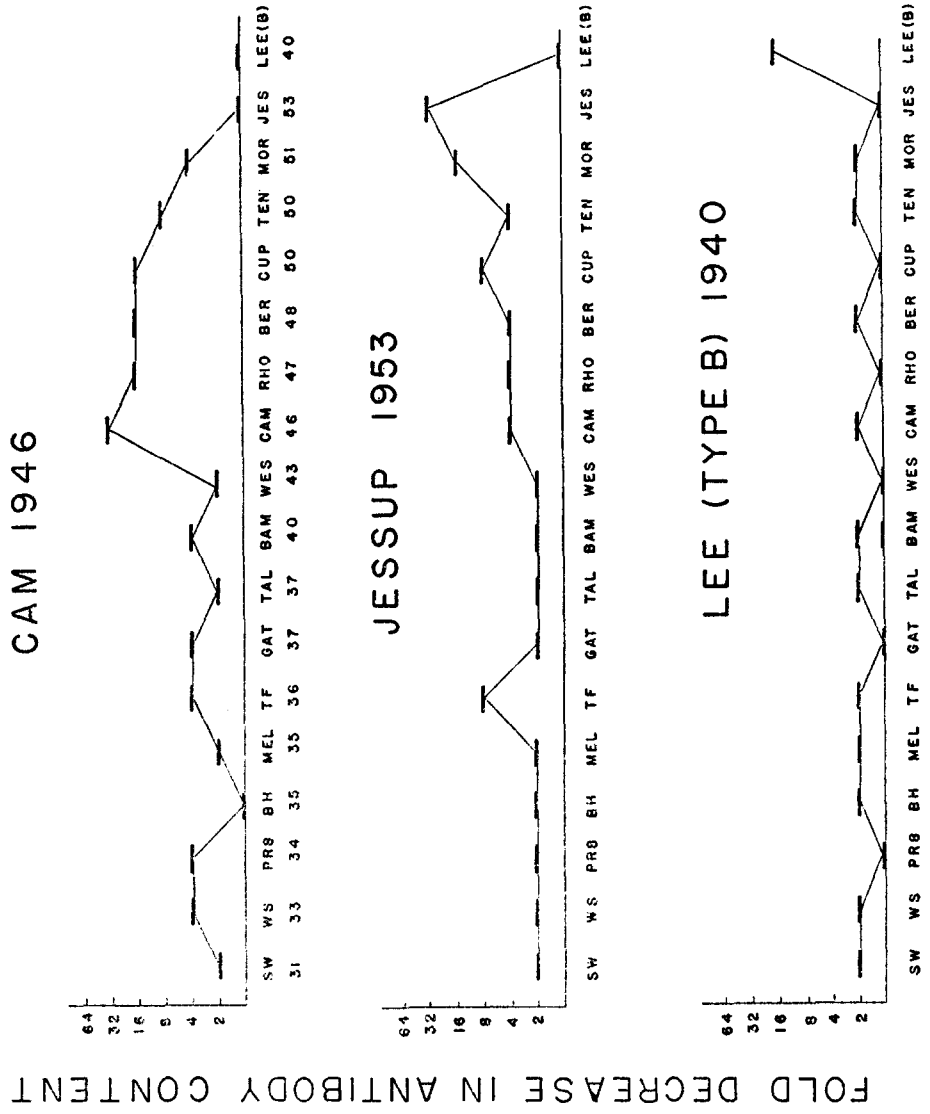

CAM 1946
JESSUP 1953
LEE (TYPE B) 1940
FOLD DECREASE IN ANTIBODY CONTENT
64
32
16
8
4
2
SW WS PR8 BH MEL TF GAT TAL BAM WES CAM RHO BER CUP TEN MOR JES LEE(B)
31 33 34 35 35 36 37 37 40 43 46 47 48 50 50 51 53 40

FIG. 6

A diagram of the decrease in antibody titers following absorption with 6 A.U. gives a measure of the antigenic pattern of the absorbent strain. The data diagrammed in this manner provide a positive picture of the antigenic profile of the virus studied. Profiles observed with the swine, WS, PR8, Cam, and Jessup strains are shown in Figs. 5 and 6. High points are seen at the positions where the antigenic reflection is greatest, and lesser elevations identify

TABLE VII

Prominent Antigenic Features

Analyzed strains	Antigen representative strains																
	SW '31 A1	WS '33 A2	PR8 '34 A3	BH '35 A4	Mel '35 A5	TF '36 A6	Gat '37 A7	Tal '37 A8	Bam '40 A9	Wes '43 A10	Cam '46 A11	Rho '47 A12	Ber '48 A13	Cup '50 A14	Ten '50 A15	Mor '51 A16	Jes '53 A17
Swine (1931)	1M		3m														
WS (1933)		2M		4m	5m	6m	7m										
PR8 (1934)		2m	3M	4m	5m	6m	7m	8m	9m	10m							
Phila (1934)		2m	3M	4m	5M	6M	7m	8m	9M	10M							
Alaska (1935)		2m	3M		5M	6M	7m		9M	10m	11m	12m		14m	15m	16m	
BH (1935)		2M	3m	4M		6m	7m				11m	12m	13m	14M	15m	16m	
Mel (1935)		2m	3M	4m	5M	6M	7m		9m	10m						16m	
Henry (1936)		2m	3m	4m	5m	6m	7m	8m	9m	10m					15m		
TF (1936)		2m	3M	4m		6M			9m	10m		12m					
Gat. (1937)	1m	2m	3m	4m	5m	6M	7M		9m	10m							
Hickcox (1940)	1m	2m	3m	4m	5m	6m	7m	8m	9m	10m	11M	12m	13m	14m			
IA43 (1943)		2m	3m	4m	5m	6m		8m	9m	10m							
Weiss (1943)		2m	3m	4m	5m	6m	7m	8m	9m	10M							
Cam (1946)		2m	3m			6m	7m		9m		11M	12M	13M	14M	15m	16m	
FMI (1947)		2m	3m	4m	5m	6m	7m		9m	10m	11m	12M	13M	14M	15m	16m	
Rhodes (1947)	1m	2m	3m	4m		6m			9m		11m	12M	13M	14M	15M		17m
Berry (1948)		2m				6m					11m	12m	13M	14M	15m	16m	
Roma/2 (1949)		2m	3m	4m	5m	6m	7m	8m	9m	10m	11m	12M	13m	14M	15M	16m	
Cuppett (1950)		2m		4m	5m						11m	12m	13M	14M	15m		
Tenney (1950)		2m	3m		5m	6m	7m		9m		11m	12M	13M	14m	15M	16m	17m
Sweden/3 (1950)		2m	3m	4m	5m	6m	7m	8m	9m	10m	11m	12m	13m	14m	15m	16M	
Moravec (1951)			3m	4m		6m	7m	8m			11m	12m				16M	17m
Jessup (1953)						6m					11m	12m	13m	14m	15m	16M	17M
Wright (1953)		2m	3m	4m		6m	7m	8m		10m	11m				15m	16M	17M

M, major antigen; reduced antibody titer 16- 64-fold.
m, minor antigen; reduced antibody titer 4- 8-fold.

those components less well expressed. These profiles clearly demonstrate strain differences and similarities. The quantitative variation presented in the patterns furnishes an estimate of the relative concentration of each antigen in the composite virus. Some difficulty is encountered in assessing the significance of the lowest elevation in the diagrams, however, because a 2-fold decrease of antibody titer is within the limit of error in the method as demonstrated by the results of absorbing the pool with the Type B Lee strain (Fig. 6). Here 2-fold decreases in antibody titers against several of the representative strains were seen. This may be explained on the basis of absorption of nonspecific inhibitor as discussed in a previous section. On the other hand a reduc-

tion of antibody titer by 16- to 64-fold reflects a relatively prominent or major antigen in the mosaic while less prominent antigens, spatially or quantitatively, are mirrored by the 4- to 8-fold reductions of antibody titers. The information gained concerning the relative concentrations of these components expressed as major or minor status is given in Table VII for several Type A strains of influenza virus. It is apparent that many antigens are often shared by these strains in either minor or major status. Noteworthy too is the trend toward a continuing change in antigens expressed as a major feature in the antigenic profiles. But the fact that the components now considered representative of older strains are readily demonstrable in recent strains points out the probability that variation can proceed in any direction.

The antigenic mosaics of some of these strains derived from the same data are pictorially presented in a different manner in Fig. 7. The radial lines spatially arranged on circles represent antigens demonstrated in the analyzed virus. The relative concentration of each antigen is pictured by the length of these radii to indicate major or minor status. From these profiles the shared antigenic relationship between the PR8 (1934) and Weiss (1943) strains is readily grasped, and the major antigenic differences in their composites become equally apparent. And some of the older strains (Alaska, BH, Hickcox) possess significant amounts of antigens most prominent in strains of recent years. In the FM1 profile several different components are prominently featured and yet many of the antigens demonstrated in strains isolated at an earlier date are evident. The number of the old antigens reflected is seen to vary in the more recently isolated strains. The antigen represented by the 1951 strain is again readily observed in the 1953 isolations, but an antigen not often expressed previously is also featured in a major role in the latter.

Selection of Strains for a Complete Vaccine

The selection of strains of virus for inclusion in a vaccine against influenza has been a continuing problem. Information gained from the above studies again suggests that broad coverage can be obtained by combining strains whose profiles fill the range of known antigens through their dominant and secondary components. From the data summarized in Table VII it appeared that a mixture of swine, WS, PR8, Gat, Weiss, FM1, and Jessup strains would satisfy the need. Accordingly, the pool of 18 sera which contained high levels of antibody to the entire complement of antigens was subjected to simultaneous absorption with 4 A.U. of each of these strains. The titer against the Type B, Lee, strain was unaltered, but all other antibody was absorbed except for small residues (1:4) against Talmey, BH, and Cam. It is apparent then that such a combination should be efficient in stimulating antibody to a wide range of antigens which have been identified and probably to others which, currently less prominent, may be dominant later. The results also recommend the pro-

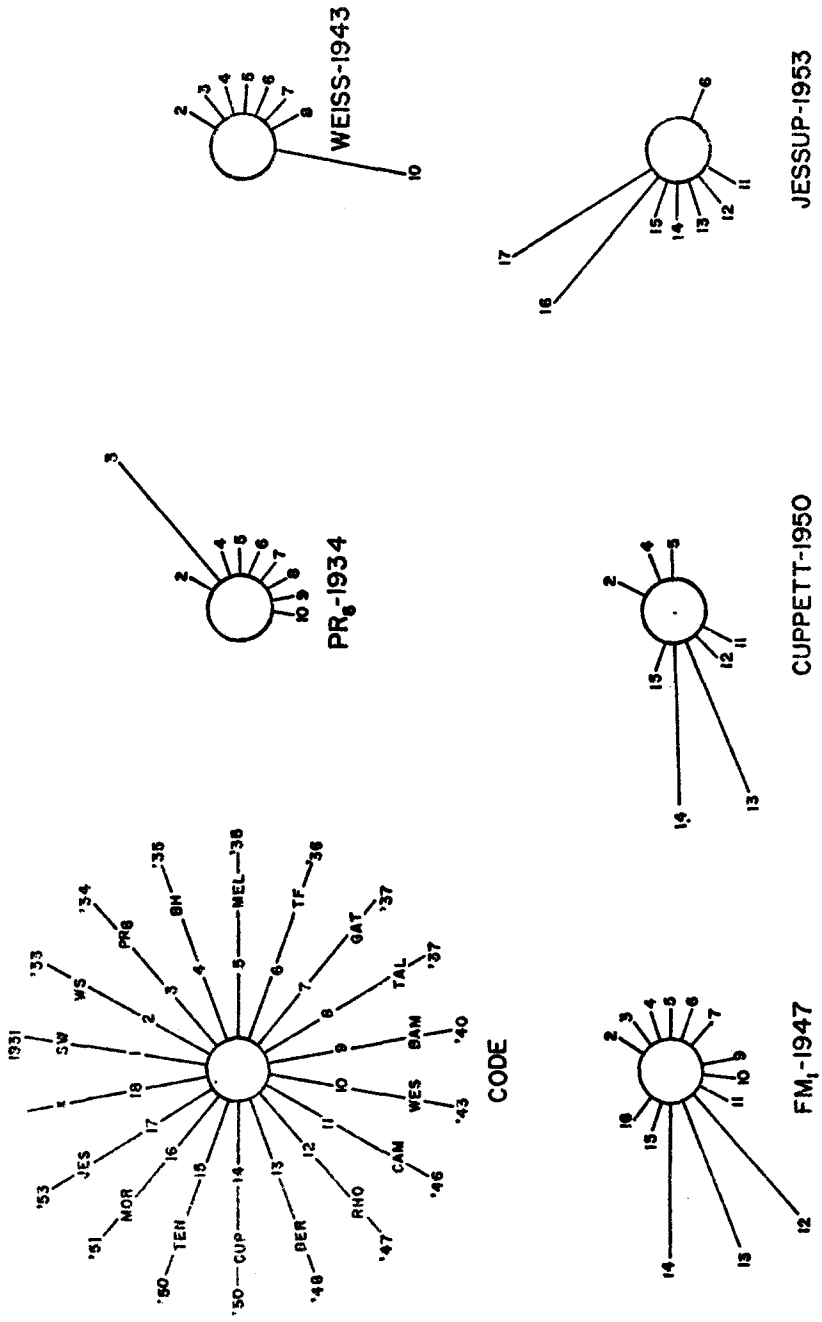


FIG. 7. Antigenic mosaics.

cedure for screening strains for their antigenic potency and for prompt detection of variants which might present quite different characteristics.

Moreover, data, to be published later, show that the significance of cross-relationships between strains can be further illuminated by application of the method to the study of antibody in human sera. It is of interest that single strains may actually remove all detectable antibody from the sera of persons who have had extensive experience with influenza. The influence of strains in inducing or heightening homologous and heterologous antibody by vaccination of man is also being subjected to analysis.

DISCUSSION

Application of the methods described here for antigenic analysis by absorption of antibody has resulted in a more precise definition of the shared components and a more complete analysis of the composite antigenic structure of strains of influenza virus than has been previously obtained. The demonstration of many shared antigenic components in strains widely separated in time of isolation leads to the conclusion that all Type A strains probably possess the potential expression of each of these shared antigens, any of which might become strain-dominant. It becomes obvious that myriads of antigenically distinguishable strains could be found by rearrangement of components in space or quantity even if the number of components were limited to 18. This is a very conservative estimate of the number of antigenic components because only 29 strains have been studied with these methods, and strains may be later encountered in which other components may be prominent.

The probability of the appearance of completely unrelated antigens and complete disappearance of old antigens appears small. Even with the limited number of strains studied there are no examples of the expression of an antigen not demonstrated in strains isolated several years before, nor are antigens completely lost, never to reappear. Moreover, the interrelationships are such as to retain characteristics of Type A virus in all the strains.

Several mechanisms for the apparent rearrangement of antigens can be suggested. Perhaps all the possible configurations exist in a pool of influenza virus molecules which are distributed throughout the world and from which highly infectious strains arise to be spread in epidemics of the disease. Higher antibody levels of the human population may tend to inhibit strains with major antigens which were dominant in recently prevalent strains and to select strains with different profiles for distribution. It is also possible that antibody may act directly on the nucleoprotein to inhibit the formation of certain prosthetic groups or indirectly accelerate the formation of other particular chemical groupings.

The work of Burnet and Lind (29) and Hirst and Gotlieb (30) suggests an-

other series of reactions by which antigenically different composites may be selected. When critical concentrations of two strains of influenza virus are simultaneously inoculated into a susceptible host, another strain which contains components shared by the test strain may be recovered. Although these investigators believed the result to be a recombination of the heredity-directive forces from the "parent" strains in the new strain, it appears equally probable that dynamic selective processes could be involved. The virus particles of any strain are heterogeneous, and antigenic analysis of these suspensions reflect the composition of the majority. If an inoculum contains particles from two populations which are antigenically different but share many components, there may be mutual interference in the processes of replication of the representative particles from each strain since they are dissimilar, while minority particles from either strain are multiplied. This could result in the recovery of strains that have antigenic structures capable of reacting with specific sera prepared for both of the "parent" strains and feature antigens that were not well expressed in other strains.

The results and concepts discussed here, in general, support the thesis (31) that the vagaries of strain variation probably are not insurmountable in relation to immunization procedures, and effective prophylaxis against influenza should be available by incorporation of several strains with known antigenic profiles in a vaccine to yield an antigenic mass in which all recognized components are adequately represented and which, in turn, should stimulate the production of antibody to protect against any possible combination of antigenic components in a strain of influenza virus of a given type.

SUMMARY

The application of methods herein described for the absorption of antibody from sera by influenza virus adsorbed to erythrocytes has greatly facilitated absorption studies with this virus and has resulted in a more comprehensive demonstration of strain differences and relationships. It is now evident that there are at least 18 different antigenic components which can be measured in Type A strains of influenza virus. Utilization of quantitative absorption technics allows the simultaneous detection of variable quantities of several different antigens so that the antigenic composite of the strain is more clearly defined than was previously possible. The wide sharing of antigenic components leads to the conclusion that no completely new antigens have formed of late nor have the antigenic components of strains isolated several years ago disappeared. Some mechanisms for antigenic variation among strains are suggested, and the significance of the variation in antigenic components is discussed in relation to specifications for an effective vaccine.

BIBLIOGRAPHY

1. Magill, T. P., and Francis, T., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1936, **35**, 463.
2. Magill, T. P., and Francis, T., Jr., *Brit. J. Exp. Path.*, 1938, **19**, 273.
3. Francis, T., Jr., and Magill, T. P., *Brit. J. Exp. Path.*, 1938, **19**, 284.
4. Smith, W., and Andrewes, C. H., *Brit. J. Exp. Path.*, 1938, **19**, 293.
5. Hirst, G. K., *J. Exp. Med.*, 1943, **78**, 407.
6. van der Veen, J., and Mulder, J., Studies on the Antigenic Composition of Human Influenza Virus A Strains, Leiden, Stenfert Kroese, 1950.
7. Hilleman, M. R., Mason, R. P., and Buescher, E. L., *Proc. Soc. Exp. Biol. and Med.*, 1950, **75**, 829.
8. Magill, T. P., and Jotz, A. C., *J. Bact.*, 1952, **64**, 619.
9. Hilleman, M. R., *Proc. Soc. Exp. Biol. and Med.*, 1951, **78**, 208.
10. Smorodintsev, A. A., Drobyshevskaya, A. I., and Shishkina, O. I., *Lancet*, 1936, **2**, 1383.
11. Burnet, F. M., *Australian J. Exp. Biol. and Med. Sc.*, 1937, **15**, 369.
12. Friedewald, W. F., *J. Exp. Med.*, 1944, **79**, 633.
13. Sigel, M. M., *J. Immunol.*, 1949, **62**, 81.
14. Kozinski, A. W., *Med. Dosw. i Mikrob.*, Warsaw, 1951, **3**, 83, (abstr., in *Bull. Hyg.*, 1952, **27**, 207).
15. Hirst, G. K., *J. Exp. Med.*, 1952, **96**, 589.
16. Francis, T., Jr., and Minuse, E., unpublished data.
17. Hirst, G. K., *J. Exp. Med.*, 1948, **87**, 315.
18. Burnet, F. M., *Australian J. Exp. Biol. and Med. Sc.*, 1952, **30**, 119.
19. Anderson, S. G., *Australian J. Exp. Biol. and Med. Sc.*, 1947, **25**, 163.
20. Hirst, G. K., *J. Exp. Med.*, 1949, **89**, 233.
21. Fazekas de St. Groth, S., *Australian J. Exp. Biol. and Med. Sc.*, 1949, **27**, 65.
22. Flick, J. A., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 448.
23. Committee on Standard Serological Procedures in Influenza Studies, *J. Immunol.*, 1950, **65**, 347.
24. Lennette, E. H., Clark, W. H., and Dean, B. H., *Am. J. Trop. Med.*, 1949, **29**, 527.
25. Francis, T., Jr., *J. Exp. Med.*, 1947, **85**, 1.
26. Sampaio, A. A. C., *Bull. World Health Org.*, 1952, **6**, 467.
27. Chu, C. M., *J. Gen. Microbiol.*, 1951, **5**, 739.
28. Walker, D. L., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1950, **91**, 65.
29. Burnet, F. M., and Lind, P. E., *J. Gen. Microbiol.*, 1951, **5**, 67.
30. Hirst, G. K., and Gotlieb, T., *J. Exp. Med.*, 1953, **98**, 41.
31. Francis, T., Jr., *Fed. Proc.*, 1952, **11**, 808.