LACK OF IDENTITY IN NEUTRALIZING AND HEMAGGLUTINATION-INHIBITING ANTIBODIES AGAINST INFLUENZA VIRUSES

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(Received for publication, September 9, 1949)

Following the development of the hemagglutination-inhibition technique for the measurement of antibodies directed against influenza virus, this *in vitro* procedure largely supplanted *in vivo* neutralization techniques. It has been generally considered that the hemagglutination-inhibition and neutralization techniques measure the same antibody. Justification for this assumption appears to rest mainly on the demonstration by Hirst (1) of a fairly good correlation between serum antibody titers measured by hemagglutination-inhibition and titers measured by neutralization in mice. Some support for the concept also was provided by absorption experiments of Wiener, Henle, and Henle (2) which showed that both hemagglutination-inhibiting and neutralizing antibodies were absorbed from immune serum by concentrated influenza virus.

Some evidence has been obtained, however, which suggests that the hemagglutination-inhibition and neutralization techniques may measure different antibodies. Burnet and Beveridge (3) and Stuart-Harris and Miller (4) have demonstrated marked discrepancies in the antibody titers of sera as measured by hemagglutination-inhibition and by neutralization in mice or *in ovo*. In addition, Friedewald (5) found that absorption of antiserum with influenza virus resulted in a proportionately greater reduction in neutralizing titer than in hemagglutination-inhibiting titer.

Because of the theoretical and the practical implications of this problem, it appeared to merit further study. The results obtained in the present investigation indicate that there is an exponential linear relationship between the quantity of virus and antibody in *in ovo* neutralization; that the concentration of neutralizing antibody can be measured with considerable precision *in ovo*. It will be shown that the amounts of hemagglutination-inhibiting and neutralizing antibodies which are absorbed by a given quantity of virus are predictable; that the reactivity of these antibodies is directly related to the extent of immunization. It will be demonstrated that there are marked discrepancies in correlation between antibody titers obtained by *in vitro* and *in vivo* techniques. Moreover, it will be shown that neutralizing antibody is preferentially absorbed by a given quantity of virus. On the basis of these results it

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appears probable that hemagglutination-inhibiting and neutralizing antibodies are not identical.

Materials and Methods

Virus.—The PR8 strain of influenza A virus was used. This strain previously had been passed many times in mice and in chick embryos. The virus was cultivated in the allantoic sac of White Leghorn chick embryos which had been incubated at 39°C. for 10 to 12 days. After inoculation with approximately $10^{4.5}$ E.I.D. of virus, the eggs were incubated at 35°C. for 48 hours and then chilled at 4°C. overnight or at −20°C. for 1½ hours before the allantoic fluids were harvested. Infected fluids used for *in ovo* neutralization experiments were diluted with 9 parts of sterile normal horse serum (previously heated at 56°C. for 30 minutes) and stored in nitrocellulose tubes at −70°C. Infected fluids used as a source of virus for serum absorption experiments were sterile pooled fluids from groups of 60 to 100 eggs and were sealed in glass ampoules and stored at −70°C. until used.

Virus Titrations.—Hemagglutination titrations were done in a manner similar to the technique described by Hirst (1). Serial twofold dilutions of allantoic fluid were made in saline buffered at pH 7.2. To 0.4 cc. of each dilution was added 0.4 cc. of a 1 per cent suspension of chicken RBC in buffered saline. Readings were made after the tubes had stood for 1 hour at room temperature. The end point was taken as the highest dilution which gave a definite 2+ pattern of RBC agglutination.

Virus infectivity titrations in chick embryos were done by the intra-allantoic technique in 10 to 12 day old embryos. Serial tenfold dilutions of allantoic fluids were made in sterile broth containing 10 per cent normal horse serum. A group of embryos was inoculated with each dilution and each embryo received 0.2 cc. Following incubation at 35°C. for 48 hours, the allantoic fluids were harvested individually and tested by the hemagglutination technique. The 50 per cent infectivity end point was calculated according to the method of Reed and Muench (6). Virus infectivity titrations in mice were carried out exactly as described previously (7).

Immune Serum.—Young adult rabbits were given an intravenous injection of 10 cc. of undiluted infected allantoic fluid. The rabbits were bled from the heart 2 weeks later. Prolonged immunization was carried out with a number of rabbits. Following the first bleeding they were given an intraperitoneal injection of 10 cc. of infected allantoic fluid. At 2 week intervals thereafter they were bled and reinjected with 10 cc. of allantoic fluid intraperitoneally until 3 or 4 injections had been given. The serum was stored frozen at −20°C. Human convalescent serum obtained from patients hospitalized with influenza A during the winter of 1943–44 was stored at 4°C. without preservatives. Before use in hemagglutination-inhibition or *in ovo* neutralization tests all serum was heated at 56°C. for 30 minutes.

Antibody Titrations.—Hemagglutination-inhibition titrations were carried out in a manner similar to that described by Hirst (1). Serial twofold dilutions of inactivated serum were made in saline. To 0.2 cc. of each dilution was added 0.2 cc. of infected allantoic fluid diluted so as to produce a final concentration of 8 hemagglutination units of virus in each tube. To each mixture was added 0.4 cc. of a 1 per cent suspension of chicken RBC. Readings were made after 1 hour at room temperature and the end point was taken as the highest dilution of serum which completely inhibited RBC agglutination. Titrations were always carried out in parallel with the same reagents when it was desirable to compare the antibody levels of two or more sera.

In ovo neutralization titrations were carried out by the intra-allantoic technique in 10 to 12 day old embryos. Serial twofold dilutions of inactivated serum were made in 10 per cent horse serum broth. To 0.5 cc. of each serum dilution was added 0.5 cc. of infected allantoic

fluid diluted in 10 per cent horse serum broth so as to contain the desired number of E.I.D. of virus. In routine titrations the mixtures were held at 4°C. for 30 minutes and each mixture was then inoculated into a group of 4 embryos; each embryo received 0.2 cc. Allantoic fluid was harvested from each embryo after 48 hours' incubation at 35°C. and tested by the hemagglutination technique. The serum dilution end points were calculated by the 50 per cent end point method of Reed and Muench (6). Each neutralization test was controlled by a titration of the virus done at the same time. The quantity of virus actually used in the neutralization test was determined by subtracting the final dilution of virus in the serum-virus mixtures from the virus dilution end point of the control virus titration. Neutralization tests in mice were carried out exactly as described previously (7).

Concentration of Virus.—Virus for use in absorption experiments with immune serum was concentrated from PR8-infected allantoic fluid by sedimentation in a high speed vacuum centrifuge (8). Large sterile pools of allantoic fluid with a hemagglutination titer of about 1:1024 were prepared for this purpose. When it was desirable to compare results obtained after absorption of aliquots of a serum or of aliquots of two or more sera, a single pool of allantoic fluid was used as a source of virus.

The allantoic fluid was distributed in nitrocellulose tubes (9 cc. per tube) and subjected to a mean gravitational field of 30,000 *g.* for 40 minutes or 37,900 *g.* for 30 minutes. After centrifugation the supernatant fluid, except for about 0.4 cc. above the sediment, was removed from each tube with a fine tipped pipette. The sediments were resuspended in the remaining supernate by repeated aspiration and ejection with a fine tipped pipette. Sufficient sterile saline, buffered at pH 7.2, was added to bring the volume to about $\frac{1}{10}$ the original and the fluid was again subjected to centrifugation as described above. After the second centrifugation all but about 0.3 to 0.5 cc. of the supernatant fluid was removed and sterile buffered saline was added to the sedimented material to bring the volume to the desired level, usually 1.0 cc. The sediment was again resuspended by repeated aspiration and ejection with a fine tipped pipette. The results of direct tests showed that each concentrated virus preparation contained at least 99 per cent of the virus originally present in the infected allantoic fluid pool.

Absorption of Antibody.—Immune serum was heated at 56°C. for 30 minutes. Serum which had been stored for a time sufficient to allow the development of a white amorphous precipitate was centrifuged and the clear supernatant serum was removed and used in the experiment. In general, 1.0 cc. of serum was added to an equal volume of concentrated virus and thoroughly mixed. At the same time 1.0 cc. of serum was mixed with 1.0 cc. of sterile saline as a control. The mixtures were held overnight at 4°C. and were then centrifuged at 4°C. in the high speed vacuum centrifuge in a gravitational field of 37,900 *g.* for 30 minutes. After centrifugation the supernatant serum, except for about 0.3 to 0.4 cc. above the sediment, was carefully withdrawn. The supernates were heated at 65°C. for 40 minutes to eliminate any unsedimented virus which might remain and their antibody content was measured by means of the hemagglutination-inhibition and the *in ovo* neutralization techniques. The absorbed serum and the unabsorbed saline control were always compared for antibody titer in the same test using the same reagents. The amount of antibody absorbed was calculated from the difference in titers shown by the absorbed serum and the unabsorbed control in the same test.

EXPERIMENTAL

Quantitative Relationship between Virus and Antibody in in Ovo Neutralization.—It has been shown (7) previously that a linear relationship exists between the quantity of influenza A virus neutralized and the quantity of serum employed in neutralization experiments in mice. Such a relationship also has been demon-

strated (9) *in ovo* with certain strains of influenza virus. This quantitative relationship between immune serum and virus *in ovo* was studied with the PR8 strain which was employed in the present investigation in order to obtain information as to the reproducibility of neutralization titers determined *in ovo* and the effect of varying amounts of virus upon such titers.

Dilutions of allantoic fluid infected with the PR8 strain ranging from 10^{-2} to 10^{-7} were each mixed with a number of twofold dilutions of anti-PR8 rabbit serum. Each mixture was inoculated intra-allantoically into a group of chick embryos. The details of the procedure employed and of the calculation of the quantity of virus used as well as the serum dilution end point are given above.

The results obtained in a series of neutralization experiments *in ovo* with two homologous antisera are presented graphically in Fig. 1. The logarithm of each serum dilution end point is plotted against the logarithm of the quantity of virus neutralized. Parallel straight lines which appeared best to fit the end points obtained with each serum were drawn. The slope of the lines shown in Fig. 1 is approximately 4.7, indicating that a change of 5.7 log units in the quantity of virus employed causes a change of only 1.0 log unit in the serum dilution end point. Similar surprisingly steep slopes in *in ovo* neutralization experiments with other strains of influenza A virus and either human or ferret immune serum have been reported previously by Burnet (9). It should be emphasized that in comparable experiments in mice the slope of the neutralization line was found (7) to be 1.44. Moreover, in hemagglutination-inhibition experiments *in vitro* the linear relationship between virus and antibody has been shown (1) to have a slope of 1.0.

Experiments similar to those illustrated in Fig. 1 also were carried out with the Lee strain of influenza B virus and homologous immune rabbit serum. Four different quantities of virus were employed. The slope of the neutralization line obtained was almost identical to that found with the PR8 strain.

It can be seen from the slope of the line drawn through the neutralization end points in Fig. 1 that antiserum rapidly loses efficiency in its ability to neutralize virus as it is diluted. It was found that when neutralization tests were carried out with dilutions of virus and a constant quantity of serum the results varied markedly within individual groups of embryos which resulted in the spreading of both positive and negative allantoic fluids over a number of virus dilution–serum mixtures. As a consequence, the reproducibility of neutralization end points obtained in this manner was relatively poor. With the 22 end points shown in Fig. 1 the mean deviation from the lines is $\pm$ 0.61 log unit along the y axis (vertically). On the other hand, when titrations were carried out with a constant virus concentration and dilutions of serum, the results were much sharper and far more reproducible end points were obtained. With the 22 end points shown the mean deviation from the lines is $\pm$ 0.14 log unit

along the x axis (horizontally). It is evident, because of the steep slope of the *in ovo* neutralization line, that in titrations carried out by this method the end points are but little affected by considerable variations in the amount of virus employed. A change of 1.0 log unit in the quantity of virus used results in a shift in the serum dilution end point of only 0.21 log unit.

On the basis of the results obtained in this study on the quantitative relationship between the quantities of serum and virus in neutralization experiments

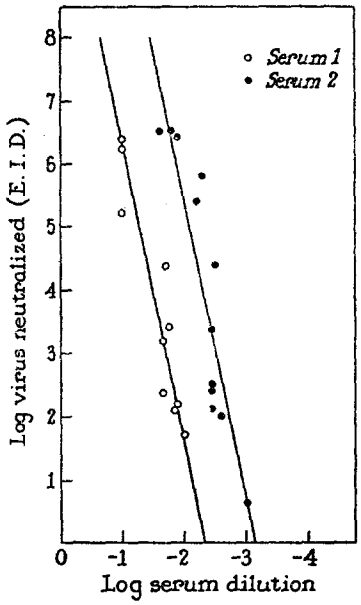

FIG. 1. Linear relationship between the quantity of virus and the quantity of immune serum in *in ovo* neutralization titrations with influenza virus (PR8). The slope of the lines shown is 4.7, indicating that a tenfold change in serum dilution end point (x axis) corresponds to a 500,000-fold change in the amount of virus neutralized (y axis).

in ovo, the constant virus–serum dilution technique was adopted for use in all further experiments.

In the experiments described above virus–serum mixtures were held at 4°C. for 30 minutes before inoculation of chick embryos. Other experiments in which virus–serum mixtures were held for 24 hours at 4°C. were also carried out. The same two rabbit antisera were used; 3 end points were determined with one and 4 with the other. It was found that holding mixtures for 24 hours did not affect the serum dilution end point when large amounts of virus were used; *i.e.*, in mixtures in which the concentrations of virus and serum were high. However, as the quantity of virus was reduced, the serum dilution end point tended to increase; this was especially evident in mixtures in which the

concentrations of virus and serum were low. The slope of the *in ovo* neutralization line obtained with mixtures held 24 hours at 4°C. was of the order of 3. A slight decrease in the slope of the neutralization line under similar conditions with ferret immune serum was reported by Burnet (9).

Correlation between Hemagglutination-Inhibition and in Ovo Neutralization Titers.—Previous workers (3, 4) have pointed out discrepancies between the results of the neutralization and hemagglutination-inhibition techniques as means of determining the concentration of antibodies against influenza viruses. A study of the correlation between antibody titers against the PR8 strain as measured by *in ovo* neutralization and hemagglutination-inhibition was undertaken.

Preliminary titrations of both neutralizing and hemagglutination-inhibiting antibodies were carried out with a number of anti-PR8 rabbit sera and convalescent sera from patients with influenza A. The techniques employed were identical to those described above; in hemagglutination-inhibition titrations a final concentration of 8 units of PR8 was used, in neutralization titrations 10^3 E.I.D. of virus was used. When two sera were found which showed approximately equal hemagglutination-inhibition titers but different *in ovo* neutralization titers, they were more closely studied in parallel titrations of both types.

Forty-one immune rabbit sera and 11 convalescent human sera were studied in this manner. Within this group there were 11 pairs of rabbit sera which did not show as much as a twofold difference in the hemagglutination-inhibition titers but did show, between themselves, differences in *in ovo* neutralization titers which ranged from four- to 13-fold. Similarly, three pairs of convalescent human sera were found which showed no difference in hemagglutination-inhibition titers, but had six- to ninefold differences in neutralizing titers.

One such pair of anti-PR8 rabbit sera was selected for intensive study. With these sera (sera 1 and 2) 8 hemagglutination-inhibition titrations were carried out in parallel. The titrations failed to reveal any consistent difference in the hemagglutination-inhibition titers of the two sera. The geometric mean of the 8 titrations was 1:1024 with serum 1 and 1:940 with serum 2. The results obtained when the two sera were compared by *in ovo* neutralization using a wide range of virus concentrations are presented in Fig. 1. It can be seen that, although the sera did not differ significantly in hemagglutination-inhibition titer, serum 2 had a mean neutralizing titer sixfold higher than serum 1 over the entire range studied.

It is evident that the correlation between hemagglutination-inhibition titers and *in ovo* neutralization titers in the group of sera studied was poor. The poor correlation raised the possibility that the hemagglutination-inhibition and neutralization techniques measure different antibodies directed against influenza virus. Further experiments were carried out to test this hypothesis.

Absorption of Specific Antibody with Influenza Virus.—In order to test more fully the possibility that hemagglutination-inhibition and *in ovo* neutralization

measure different antibodies, it was decided to utilize the classical method of antibody absorption.

Details of the procedure used are given above. For the purpose of controlling the procedure anti-PR8 sera were absorbed with a quantity of heterologous Lee virus equal to that used with PR8 virus in specific antibody absorptions. Several experiments demonstrated that absorption with Lee virus did not give results significantly different from those obtained when immune sera were mixed with saline and carried through all steps of the procedure. In subsequent experiments such saline controls were used for comparison with PR8-absorbed sera. Experiment showed that the amount of centrifugation employed as routine in the absorption procedure caused sedimentation of at least 99.9 per cent of the virus added to a 1:2 dilution of rabbit serum in saline and that heating the supernate of such a mixture at 65°C. for 40 minutes eliminated any demonstrable virus remaining in the supernate.

Preliminary experiments showed that both the hemagglutination-inhibiting and the neutralizing titers of anti-PR8 serum were reduced by absorption with PR8 virus; that the neutralizing titer was reduced to a considerably greater extent than the hemagglutination-inhibiting titer; that the neutralizing titer could be reduced to a very low level even though the serum still retained a high titer of hemagglutination-inhibiting antibodies. A typical experiment illustrating these points was carried out as follows:—

Aliquots of an anti-PR8 serum were absorbed with virus concentrated from 5, 10, and 20 cc., respectively, of PR8-infected allantoic fluid. After absorption the hemagglutination-inhibition and *in ovo* neutralization titers of each aliquot were determined in parallel with those of the unabsorbed saline control serum.

The results obtained are presented in Table I. It can be seen that, as increasing quantities of virus were used for absorption, there was a regular and a progressive reduction in the antibody titers of the serum as measured by both hemagglutination-inhibition and *in ovo* neutralization. It is evident, however, that there was a greater reduction in neutralizing titer than in hemagglutination-inhibition titer and that the discrepancy between the two titers increased as more and more antibody was removed until, with the use of virus from 20 cc. of allantoic fluid, the *in ovo* neutralization titer was reduced 76-fold while the hemagglutination-inhibition titer was decreased by only eight-fold. This represents a reduction in neutralizing titer 9.5 times greater than in hemagglutination-inhibiting titer.

Comparison of the Sensitivity of the Hemagglutination-Inhibition and the in Ovo Neutralization Techniques in Measuring Known Differences in Antibody Concentration.— Because hemagglutination-inhibition and *in ovo* neutralization are such markedly different procedures, one carried out *in vitro* and the other *in vivo*, it was considered possible that the apparently greater absorption of neutralizing antibody could be due to differences in the sensitivity of the two techniques in measuring changes in antibody concentration. Experiments

were carried out to compare the two procedures in the measurement of known changes in antibody concentration.

Anti-PR8 rabbit serum was diluted 1:4, 1:16, and 1:64 with normal rabbit serum. Aliquots of these dilutions, together with an aliquot of undiluted antiserum, were each further diluted 1:2 in saline, centrifuged at 37,900 *g.* for 30 minutes, and the supernatant serum heated at 65°C. for 40 minutes in order to reproduce the conditions of the absorption experiments. The antibody concentration of each of the specimens was then measured 3 separate times by the hemagglutination-inhibition technique and once by the *in ovo* neutralization technique. All titrations were carried out in parallel.

TABLE I

Absorption of Specific Antibodies with Influenza Virus (PR8)

Amount of virus	Antibody				Ratio of change in titers Neutralization / Hemagglutination-inhibition
	Hemagglutination-inhibition		Neutralization *in ovo*		
	Titer	Decrease in titer	Titer	Decrease in titer	
*cc. all. fl. per cc. serum**		*fold*		*fold*	
0	8192	—	835	—	
5	4096	2	354	2.4	1.2
10	2048	4	64	13	3.3
20	1024	8	11	76	9.5

* Anti-PR8 rabbit serum.

The results of one experiment are presented in Table II. The titers found with each diluted serum specimen are recorded and it can be seen that both the hemagglutination-inhibition and *in ovo* neutralization titers all fall within less than 0.2 log unit of the theoretical titers computed on the basis of dilution. The reproducibility of the serum dilution end points obtained by either technique is evident when the titers are expressed in terms of the undiluted serum. As is seen, the deviation of such computed end points from the geometric mean was ± 0.07 log unit with hemagglutination-inhibition and ± 0.03 log unit with *in ovo* neutralization.

In Fig. 2 the results of two such experiments are presented graphically. The logarithm of the hemagglutination-inhibition titers of the diluted sera is plotted against the logarithm of the *in ovo* neutralization titers. Inasmuch as the experiments were carried out with two different immune rabbit sera which had different antibody levels, the two sets of experimental points do not fall along the same line. Lines with a slope of 45° which best fit the points obtained with each serum have been drawn. These lines represent the theoretical end points which should be obtained with known changes in antibody concentration. It can be seen that the experimental end points fall very close to the

TABLE II

Measurement of Known Changes in Antibody Concentration by Hemagglutination-Inhibition and in Ovo Neutralization

Dilution of immune serum* in normal serum	Antibody					
	Hemagglutination-inhibition			Neutralization *in ovo*		
	Titer found	Titer computed to undiluted serum	Deviation from geometric mean	Titer found	Titer computed to undiluted serum	Deviation from geometric mean
	log	*log*	*log*	*log*	*log*	*log*
0	−3.91	−3.91	+0.07	−3.10	−3.10	0.0
1:4	−3.31	−3.91	+0.07	−2.48	−3.08	−0.02
1:16	−2.51	−3.71	−0.13	−1.95	−3.15	+0.05
1:64	−2.01	−3.82	−0.02	−1.26	−3.07	−0.03
Geometric mean....		−3.84	±0.07		−3.10	±0.03

*Anti-PR8 rabbit serum.

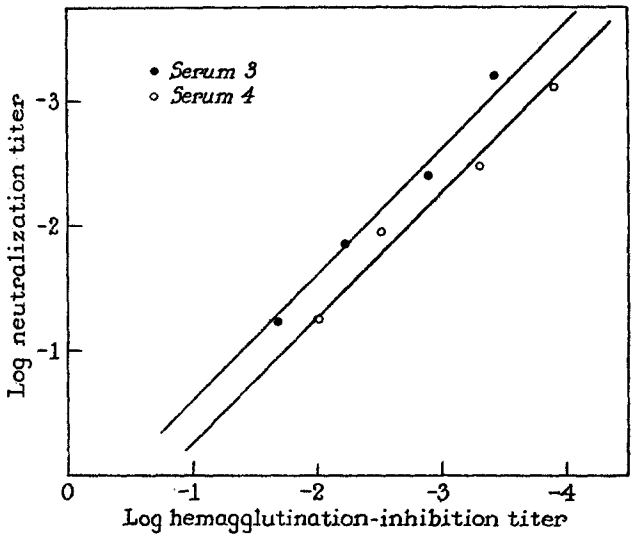

FIG. 2. Correlation between experimentally determined and theoretical end points measured *in vitro* and *in vivo* with known decrements in antibody concentration. The slope of the lines shown, which correspond to the thoretical end points, is 1.0.

appropriate 45° line, indicating that both methods of antibody measurement are sufficiently sensitive to detect the changes which were produced in antibody concentration by serial fourfold dilution of the sera. It is, therefore, evident that the disproportionate reduction in neutralizing antibody titer produced

by absorption of anti-PR8 serum with PR8 virus cannot be attributed to a difference in the sensitivity with which the *in vivo* and *in vitro* techniques measure changes in antibody concentration.

Absorption of Various Immune Sera with Influenza Virus.—Numerous experiments were carried out in which specific antibodies were absorbed from immune serum with concentrated PR8 virus. Both convalescent human and immune rabbit sera were used and various virus-antibody ratios were employed. The details of the procedure and a description of the controls used are given above.

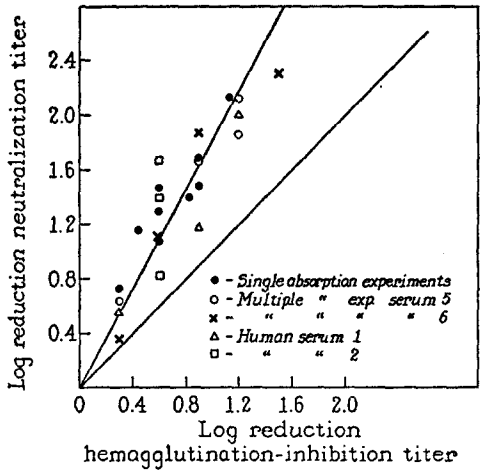

FIG. 3. Relation between quantity of neutralizing antibody and hemagglutination-inhibiting antibody absorbed from immune sera by influenza virus (PR8). Preferential absorption of neutralizing antibody is indicated by deviation of experimental line from theoretical line.

In Fig. 3 the results of 23 serum absorption experiments are presented graphically. Of the 23 experimentally determined points, 14 represent values derived from the geometric means of end points determined in two or more *in ovo* and *in vitro* titrations. The logarithm of the reduction in the hemagglutination-inhibition titer is plotted against the logarithm of the reduction in the neutralization titer produced by absorption of immune serum with PR8 virus. A straight line which appeared best to fit the experimental points was drawn. In addition, a line with a slope of 45° was drawn. This line indicates the values which, in theory, should have been obtained if the two techniques measured the same antibody.

It is evident that the experimental points deviate systematically from the theoretical values, indicating that consistently more neutralizing antibody was absorbed than hemagglutination-inhibiting antibody. It can be seen that as

progressively greater amounts of antibody were removed, the deviation from the 45° line becomes wider and wider. Preferential absorption of neutralizing antibody in like amount was demonstrated in neutralization titrations in mice with some of the absorbed sera.

Two experiments were carried out in which aliquots of an immune rabbit serum were absorbed with increasing quantities of virus. A different antiserum was used in each experiment. In the first, aliquots of serum 5 were absorbed with PR8 virus concentrated from 10, 20, 30, and 35 cc., respectively, of infected allantoic fluid. In the second, aliquots of serum 6 were absorbed with virus from 5, 10, 20, and 30 cc., respectively, of allantoic fluid.

The reductions in antibody titers found in both experiments are indicated by distinctive symbols in Fig. 3. It should be pointed out that as increasing quantities of virus were used to absorb antibodies from a constant quantity of antiserum, *i.e.* as the virus-antibody ratio was increased, larger and larger amounts of both antibodies were removed, and that the selective absorption of neutralizing antibody became progressively more evident. In view of the results obtained with rabbit antisera, it was considered of importance to determine whether similar disproportionate reductions in neutralizing antibody would be produced by virus absorption of the serum of patients convalescent from influenza A.

Two convalescent human sera were used. The procedure employed was identical with that used for absorption of antibodies from rabbit serum. Absorption of each serum was carried out with three different quantities of virus.

The reductions in hemagglutination-inhibiting and neutralizing titers produced by absorption of convalescent human sera are shown in Fig. 3. It can be seen that the experimental points do not deviate markedly from the line shown and indicate that the results obtained with human sera were closely similar to those found with rabbit sera.

Relationship between the Quantity of Virus Employed and the Amount of Antibody Absorbed.—Antigen-antibody reactions have been extensively studied in recent years by means of the quantitative techniques devised and perfected by Heidelberger and Kendall (10, 11). They and other investigators have obtained much information concerning the course of reactions which occur in several protein-antiprotein and polysaccharide-antipolysaccharide systems. At the present time quantitative measurements, comparable to those employed in studies on precipitating systems, are not applicable to virus-antivirus systems unless marked purification and concentration of the virus are obtained and hyperimmune serum of very high potency is employed (12). Moreover, in the present study the objective was to determine the amounts of neutralizing and hemagglutination-inhibiting antibodies which react with influenza virus. To measure either antibody it was necessary to employ biological methods and to express results in relative rather than in absolute units. However, the

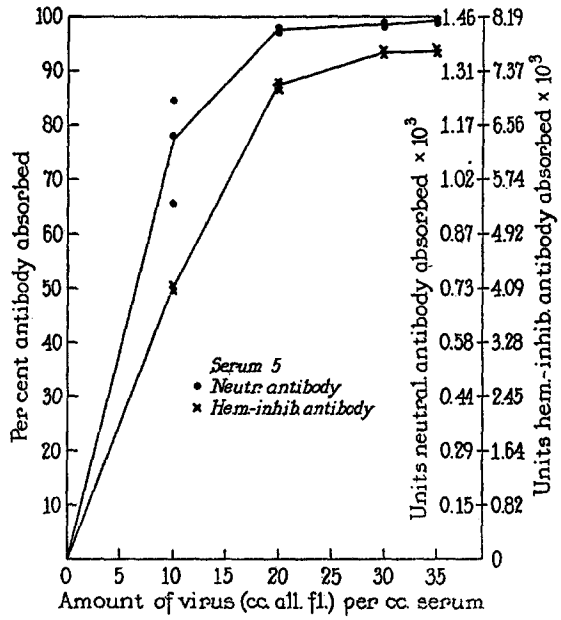

FIG. 4

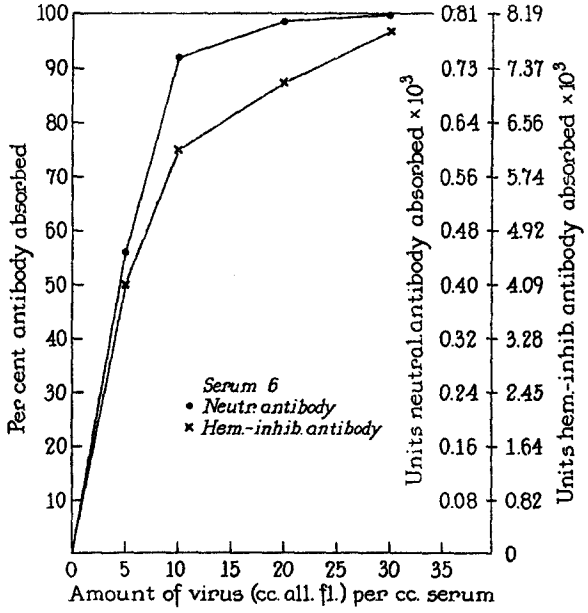

FIG. 5

FIGS. 4 and 5. Extent of absorption of neutralizing antibody and hemagglutination-inhibiting antibody from immune rabbit serum relative to the quantity of influenza virus (PR8) employed. In Fig. 4 the curves are drawn through the geometric means of the experimentally determined points.

reproducibility and apparent predictability of the results seemed to justify an analysis of the data in a manner similar to that used by Heidelberger and Kendall (10) with precipitating systems.

The relationship between the quantity of virus used and amount of both antibodies absorbed was studied in experiments with two different anti-PR8 rabbit sera (sera 5 and 6). The quantities of virus used with each serum are given in a preceding section and the reductions in antibody titers found after absorption of each serum are shown in Fig. 3. Titration end points were determined at least twice in experiments with serum 5.

In Figs. 4 and 5 the results obtained are presented graphically. The amount of virus used is plotted againt the per cent as well as the number of units of antibody absorbed. It can be seen that the curves obtained with both hemagglutination-inhibiting and neutralizing antibody resemble those obtained with precipitin systems by other workers (10). It should be noted that throughout the course of the reaction the amount of hemagglutination-inhibiting antibody absorbed differed from the amount of neutralizing antibody absorbed. The extent of the difference reached values of definite significance when the larger quantities of virus were used. It can be seen that virus from only 16 cc. of allantoic fluid was required to absorb 90 per cent of the neutralizing antibody from 1 cc. of serum 5, whereas virus from approximately 25 cc. was necessary for absorption of 90 per cent of the hemagglutination-inhibiting antibody. Comparable figures for serum 6 show an even greater difference; 10 and 25 cc. were required to absorb 90 per cent of neutralizing and hemagglutination-inhibiting antibodies, respectively.

With precipitin systems it has been clearly established that in the zone of antibody excess a straight line is obtained when the ratio of antibody to antigen in the precipitate is plotted against the antigen added (10). In the present study, when the ratio of absorbed antibody to virus was plotted against virus added, a straight line also was obtained in the region of antibody excess. This relationship with serum 5 and serum 6 is shown in Figs. 6 and 7. For the purposes of this analysis, the neutralizing titers of the sera were multiplied by the factor necessary to make the titers numerically equal to the hemagglutination-inhibiting titers in order to allow comparison of the slopes of the lines obtained.

It should be emphasized that a straight line is obtained only in the region of antibody excess. The points obtained after addition of large quantities of virus (*i.e.*, 30 cc. in Fig. 7) represent end points in the region of antigen excess and, as a consequence, deviate from the line in the manner noted with precipitin systems (10). It can be seen that with each serum the slope of the line relating neutralizing antibody and virus differs from that for hemagglutination-inhibiting antibody. The slopes of the lines and the intercepts on the y axis are also different for the two sera as might be anticipated from the findings with other antigen-antibody systems (10).

Predictability of Specific Antibody Absorption with Influenza Virus.—Heidel-

berger and Kendall (10) derived a theoretical equation based on mass law considerations to express the relation between the amount of antigen added throughout the region of antibody excess. They found this equation to be applicable to several precipitin systems and by means of it were able to predict, with con-

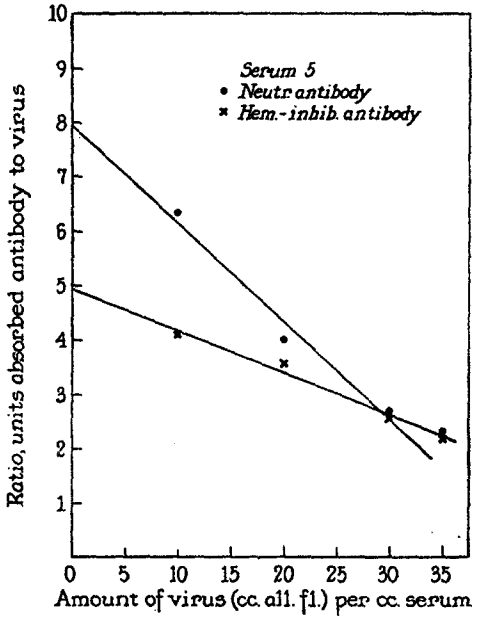

FIG. 6

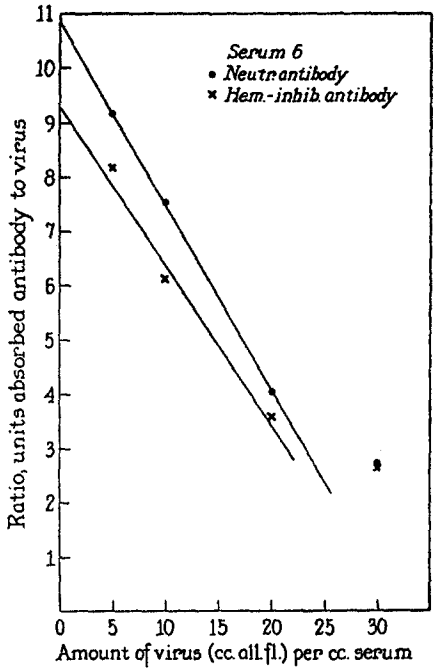

FIG. 7

FIGS. 6 and 7. Linear relationship between the quantity of influenza virus (PR8) employed and the ratio of the amount of antibody absorbed to the amount of virus used. Experimental points in the region of antibody excess are connected by straight lines. In Fig. 6 each point was calculated from the geometric mean of two or more titrations.

siderable precision, the amount of antibody precipitated with various quantities of antigen.

The equation, $N/S = 2R - \frac{R^2}{A} S$, which was employed by Heidelberger and Kendall (10) for calculation of the amount of antibody nitrogen precipitated, is readily altered merely by substitution of symbols so as to be applicable to the results obtained in the present study. In the form, $Ab/V = 2R - \frac{R^2}{A} V$, in which Ab = units of antibody absorbed, V = amount of virus used, and A = units of antibody present in the unabsorbed serum, their equation can be used to calculate the amount of antibody absorbed when $2R$, the ratio of absorbed antibody to virus (*i.e.*, the intercept on the y axis as shown in Figs. 6 and 7) is known. With this equation the necessary computations were carried out for the quantities of virus used with sera 5 and 6.

In Table III the calculated results for both antibodies are compared with those found experimentally. The deviations of the calculated from the experimental results are shown. It is evident that the results indicate that absorption of either antibody with PR8 virus is predictable over a considerable range in the region of antibody excess.

Increase in Reactivity of Antibodies with Prolonged Immunization.—The experiments on absorption of antibodies with PR8 virus described in preceding sections were carried out with antisera collected from rabbits after a single injection of virus. Exploratory absorption experiments revealed that anti-

TABLE III

Predictability of Absorption of Specific Antibodies with Influenza Virus

Serum No.	Absorbed with virus	Antibody					
		Neutralization units absorbed			Hemagglutination-inhibition units absorbed		
		Found	Calculated*	Deviation	Found	Calculated*	Deviation
	cc. all. fl. per cc. serum			*per cent*			*per cent*
4	10	1125	1182	+5	4096	4200	+3
4	20	1424	1455	+2	7168	6900	−4
4	30	1435	1150	−20	7680	8100	+5
5	5	451	448	−1	4096	4096	−2
5	10	741	705	−5	6144	6680	+9
5	20	794	715	−10	7168	8040	+12
Mean deviation				±7.2			±5.8

* Computed with the equation, $Ab/V = 2R - \frac{R^2}{A} V$.

PR8 antibodies in sera collected after multiple virus injections behaved in a somewhat different manner. Experiments were carried out to determine the extent of this difference.

An anti-PR8 serum (serum 7) was obtained from a rabbit after one intravenous and two intraperitoneal injections of PR8-infected allantoic fluid given at intervals of 2 weeks. Details of the immunization procedure are given above. Aliquots of the serum were absorbed with virus concentrated from 11, 16, 20, and 30 cc., respectively, of PR8-infected allantoic fluid. After absorption the hemagglutination-inhibition and *in ovo* neutralization titers of each aliquot were determined in parallel with those of the unabsorbed saline control serum. All titration end points were determined at least twice on different days.

The results obtained are presented in Table IV. It can be seen that the discrepancies between the decrease in hemagglutination-inhibition titer and the decrease in neutralization titer found in this experiment were much smaller

than those found in experiments with antisera obtained after a single injection of virus. It will be noted, too, that the extent to which neutralizing antibody was absorbed by a given amount of virus did not differ markedly from that found in experiments described above. However, absorption of hemagglutination-inhibiting antibody was much more complete and resulted in much lower titers than were attained previously with the same amount of virus.

TABLE IV

Absorption of Specific Antibodies after Prolonged Immunization with Influenza Virus(PR8)

Amount of virus	Antibody				
	Hemagglutination-inhibition		Neutralization *in ovo*		Ratio of change in titers: Neutralization / Hemagglutination-inhibition
	Titer	Decrease in titer	Titer	Decrease in titer	
*cc. all. fl. per cc. serum**		*fold*		*fold*	
0	5150	—	1580	—	—
11	1840	2.8	449	3.5	1.3
16	456	11.3	61	26	2.3
20	322	16	64	25	1.6
30	40	128	5	316	2.5

* Anti-PR8 rabbit serum 7.

In Fig. 8 the results obtained after absorption of serum 7 are shown graphically. It can be seen that the general shape of the curve representing absorption of neutralizing antibody appears quite similar to those shown in Figs. 4 and 5 (sera 5 and 6). However, the curve showing absorption of hemagglutination-inhibiting antibody closely approximates that for neutralizing antibody in both shape and height as was not the case with sera 5 and 6.

A straight line relationship also was obtained for both antibodies with serum 7 when the ratio of absorbed antibody to virus was plotted against the amount of virus employed. These relationships are shown in Fig. 9. It is apparent that the points representing the ratios obtained with the two antibodies fall very close together in contrast to those obtained with sera 5 and 6 as illustrated in Figs. 6 and 7.

Heidelberger and Kendall have shown previously (13) progressive changes in the combining characteristics of antibody from individual rabbits after successive courses of immunization. They found with precipitin systems that in the region of antibody excess a given amount of antibody combined with less antigen after each course of immunization. This resulted in precipitates with a higher ratio of antibody to antigen after each successive immunization. They interpreted their findings as indicating that on continued immunization antibody was formed which was progressively more reactive with antigen.

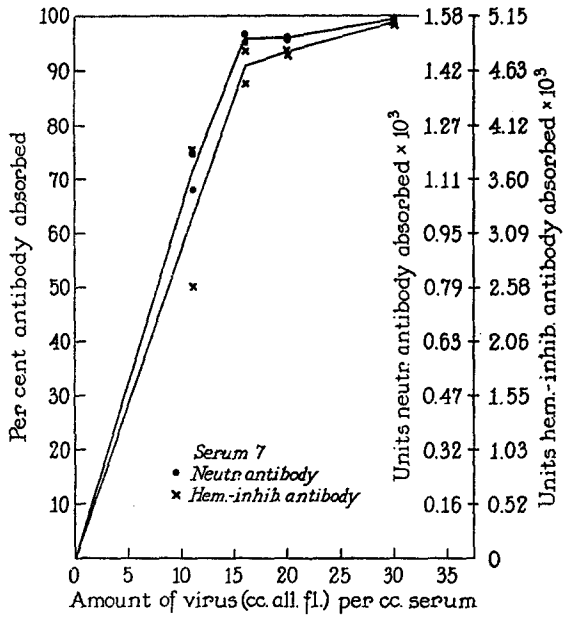

FIG. 8. Effect of prolonged immunization upon the extent of absorption of neutralizing antibody and hemagglutination-inhibiting antibody relative to the quantity of influenza virus (PR8) employed. Curves are drawn through the geometric means of the experimentally determined points.

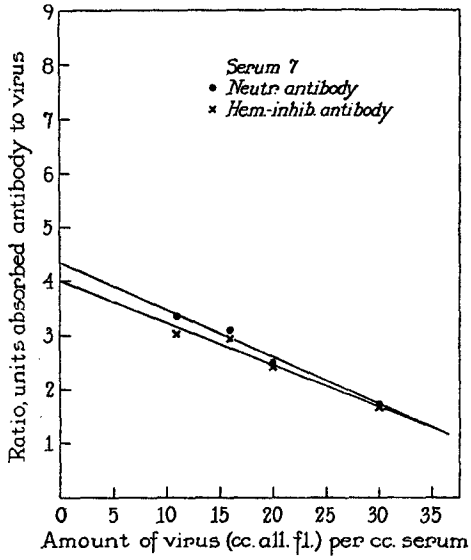

FIG. 9. Effect of prolonged immunization upon the ratio of absorbed antibodies to virus relative to quantity of influenza virus (PR8) employed. Each point was calculated from the geometric mean of two titrations.

It seemed possible that the results obtained in the experiments with serum 7 might be due to similar changes in the reactivity of one or both antibodies following continued immunization with PR8 virus. An experiment was carried out to test this possibility. A comparison was made of results found after absorption of sera obtained early and late in the course of immunization of the same rabbit.

Serum was obtained after one and after four injections of PR8 virus during the course of immunization of a rabbit. Aliquots of each serum were absorbed with virus concentrated from 10 and 20 cc., respectively, of PR8-infected allantoic fluid. After absorption the hemagglutination-inhibition and *in ovo* neutralization titers of each aliquot were determined in parallel with those of the unabsorbed saline control serum. All titration end points were determined at least twice on different days.

TABLE V

Comparison of the Absorption of Specific Antibodies from Early and Late Immune Serum of the Same Rabbit

Immune serum obtained after	Amount of virus	Antibody				
		Hemagglutination-inhibition		Neutralization *in ovo*		Ratio of change in titers Neutralization / Hemagglutination-inhibition
		Titer	Decrease in titer	Titer	Decrease in titer	
	cc. all. fl. per cc. serum		*fold*		*fold*	
1 injection of virus	0	4096	—	376	—	—
	10	2048	2	70	5.4	2.7
	20	1450	2.8	26	14.5	5.2
4 injections of virus	0	5790	—	1305	—	—
	10	1024	5.7	155	8.4	1.5
	20	64	90.4	6 or less	21.7 or more	2.4 or more

The results are presented in Table V and graphically in Fig. 10. In Table V it can be seen that even though the hemagglutination-inhibition titers of the 2 unabsorbed sera were similar, those found after absorption with equal amounts of virus were very different. As example, the titer of the early serum decreased only 2.8-fold on absorption with 20 cc. of virus, whereas the titer of the late serum was reduced 90.4-fold on absorption with an equal amount of virus. Similarly, the neutralizing titer of the late serum was reduced considerably more than that of the early serum even though the titer of the unabsorbed late serum was more than threefold higher than that of the early serum.

It should be pointed out that, as is shown in Table V, continued immunization frequently caused an increase in the neutralization titer without causing an increase in the hemagglutination titer. Among 8 rabbits given more than

one injection of virus, 5 showed fourfold rises in neutralizing titer associated with either no change or a two- to fourfold decrease in hemagglutination-inhibition titer.

When the ratio of units of absorbed antibody to virus added is plotted against virus added, after appropriate adjustment of values to correct for differences in antibody levels, the changes which have occurred on continued immunization are apparent. In Fig. 10 it can be seen that the ratio of ab-

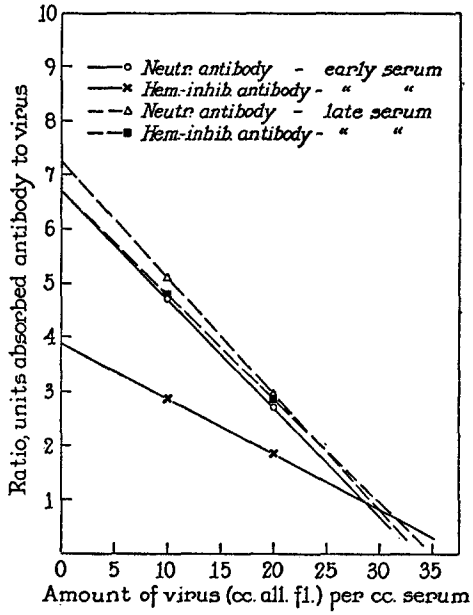

FIG. 10. Relative reactivity of neutralizing and hemagglutination-inhibiting antibodies in early and late immune serum from the same rabbit. Each point was calculated from the geometric mean of two titrations.

sorbed hemagglutination-inhibiting antibody to virus increased markedly on continued immunization. The ratio for neutralizing antibody likewise increased, but to a lesser extent. It appears that on continued immunization with PR8 virus there was an increase in the reactivity of both antibodies directed against the agent. The increase in reactivity of the hemagglutination-inhibiting antibody exceeded that of the neutralizing antibody. The marked increase in reactivity of hemagglutination-inhibiting antibody brought the ratios for the two antibodies much closer to equality. Sera were not available to determine whether, with more extensive immunization, the two would become equal or whether the ratio for hemagglutination-inhibition would exceed that for neutralizing antibody.

DISCUSSION

The extraordinarily steep slope of the line relating the two variables in *in ovo* neutralization of influenza virus by antibody provides confirmation of the results obtained previously by Burnet (9). It is of interest that evidence for a similarly steep slope was obtained also in *in ovo* neutralization of Newcastle disease virus (9). The implications of this relationship are important: any change in the *in ovo* virus-neutralizing titer of a serum is reflected by an enormous change, of the order of 50,000 times greater, in the *in ovo* virus-neutralizing *capacity* (7) of the serum. Under these circumstances, it is evident that serious difficulties in interpretation may arise when attempts are made to relate results obtained *in ovo* by the constant serum-varying virus technique to those obtained by the constant virus-varying serum procedure either *in vivo* or *in vitro*. In the present study all measurements of antibody concentration were carried out by the latter procedure and, as is shown, direct comparisons can be made between results obtained *in vivo* and *in vitro* under these conditions.

The finding that the correlation between hemagglutination-inhibition titers and *in ovo* neutralization titers was poor with the immune sera studied in the present investigation raised the possibility that the two procedures might not measure the same component of serum. This possibility has been considered by others (4, 5). The further finding that both the hemagglutination-inhibiting and the neutralizing titers of anti-PR8 rabbit serum, as well as convalescent human serum, were reduced in different degree by absorption with PR8 virus; that on absorption the neutralizing titer was reduced to a significantly greater extent than the hemagglutination-inhibiting titer; that the neutralizing titer could be reduced by absorption to a low level or even eliminated and yet the serum retained a high hemagglutination-inhibiting titer, provided strong evidence that the two antibodies were not identical.

Although it is obvious that hemagglutination-inhibition *in vitro* and neutralization *in ovo* are wholly different procedures, the data presented indicate that neither the poor correlation in the two titers nor the disproportionate absorption of neutralizing antibody can be attributed to differences in the precision with which the two techniques measure changes in antibody concentration. Furthermore, since discrepancies of the same order were demonstrable by neutralization in mice, it is evident that the findings are not due to peculiarities of neutralization *in ovo*.

On prolonged immunization the reactivity of both types of antibody changed. In this case, too, there was a difference between the hemagglutination-inhibiting and neutralizing antibodies for the change in reactivity of the former was much more marked than that of the latter. The changes observed in antibody reactivity on continued immunization are a further indication that the consistent, though aberrant, behavior of the two antibodies in sera obtained early in

immunization is not to be explained on the basis of differences in the techniques employed for their measurement.

As a consequence, it appears highly improbable that the antibodies measured by the hemagglutination-inhibition technique on the one hand, and by the neutralization technique on the other, are identical. If this is correct, the present findings have both practical and theoretical importance: Both the laboratory diagnosis of influenza and the evaluation of vaccines rest largely on the demonstration of an increase in the concentration of antibodies directed against influenza virus. Because hemagglutination-inhibiting and neutralizing antibodies appear in general to rise in parallel (1), use of the rapid and simple hemagglutination-inhibition technique alone may yield useful information. However, a failure to find a rise in antibody titer by the hemagglutination-inhibition technique does not necessarily indicate that there has been no increase in neutralizing antibody. Stuart-Harris and Miller (4) have pointed out some of the difficulties which may be encountered in this regard in studies of influenza epidemics.

In recent years many investigations have been carried out on the efficacy of influenza virus vaccines and particular attention has been directed to their capacity to stimulate the production of antibodies. The hemagglutination-inhibition technique has been used in the great majority of such investigations and latterly the rise and fall of antibody levels in vaccinated animals and human beings have been followed almost exclusively with this procedure. If the orientation of the antibody measurable *in vitro* is different from that measurable *in vivo*, it follows that the assessment of vaccine efficacy, on the basis of antibody levels produced, will have different significance depending upon the technique employed.

Of theoretical importance is the fact that, if two distinct antibodies directed against the virus actually develop on immunization, it can be predicted that the virus contains at least two corresponding and distinct antigens. Because it appears that influenza virus may also elaborate at least two complement-fixing antigens (14, 15), it is evident that this medium sized virus may possess an antigenic structure which approaches in complexity that of the large viruses, as for example vaccinia (16).

With the hope of separating the hypothetical antigens responsible for the production of hemagglutination-inhibiting and neutralizing antibodies, respectively, several attempts were made to bring about differential degradation of influenza virus (PR8). Although heating at 56°C. destroys the capacity of the virus to elute from RBC (17), such treatment had no significant effect on the antigenic properties of the virus. Heating at 65°C. destroys the hemagglutinating capacity of the virus (17) and also reduced its effectiveness as an absorbing antigen by about 75 per cent, but failed to cause any quantitative difference in absorptive capacity. Similarly, treatment of the virus with acid

(pH 5.0) or alkali (pH 10.0 to 11.5) was ineffective in bringing about selective inactivation of an antigenic constituent.

SUMMARY

There is an exponential linear relationship between the quantity of influenza virus neutralized and the quantity of immune serum employed in *in ovo* neutralization. The slope of the neutralization line is extremely steep. The concentration of neutralizing antibody can be measured with considerable precision *in ovo* if the constant virus-varying serum technique is utilized.

The amounts of hemagglutination-inhibiting and neutralizing antibodies which are absorbed by a given quantity of influenza virus (PR8) were found to be predictable and the degree of reactivity of these two antibodies was shown to be directly related to the extent of immunization. It was demonstrated that there are marked discrepancies in correlation between antibody titers obtained by *in vitro* hemagglutination-inhibition and *in vivo* neutralization techniques and that neutralizing antibody is preferentially absorbed by a given quantity of virus. Inasmuch as the results were found not to be attributable to peculiarities of the techniques employed, it appears that the antibodies measured by hemagglutination-inhibition *in vitro* and by neutralization *in vivo* are not identical.

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