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A Method Applicable to the Standardization of Influenza Virus Vaccines.*

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One of the problems that came to the fore as a result of the successful trials of influenza vaccination was that of establishing standards for the control of vaccine potency. In the investigations of the Commission on Influenza[1] the vaccine employed in studies in man had been tested for capacity to immunize mice against intranasal infection. The results of such tests[2] were then used to define a standard of potency.

Because of certain features of the mouse immunization test,[3] it seemed desirable to investigate other methods that might be used for estimating potency in order to find a more sensitive and simpler procedure that required less time for completion. For this purpose the hemagglutinating property[4] of the virus provided an obvious lead. However, when an attempt was made to correlate hemagglutinating and immunizing capacities, certain difficulties were encountered. One of the chief difficulties was the matter of the relatively greater sensitivity of the methods for measuring the hemagglutinating factor as compared with the methods used for measuring immunizing effect. At best, it has been possible thus far merely to ascertain the existence of a rough correlation between these two properties.[2,5-7]

In the course of investigating this question further, a technique was employed for measuring, quantitatively, the antigenic content of preparations of influenza virus. For convenience the test is referred to as the antigenic extinction titration. It is desired to report the details of the method at this time because of its possible application to the standardization of influenza virus vaccines. The test to be described is not suggested as a direct test of the immunizing capacity of a vaccine, but rather as a quantitative test for the amount of antigen that is capable of inducing antibody formation in the mouse. Although the results may reflect immunizing effect, the present report does not deal with this question. The problem of the relationship between hemagglutinating activity, the capacity to induce neutralizing antibody, and immunity will be the subject of a later communication.

Procedure. The purpose of the test is to determine how far an antigen, or vaccine, may be diluted and still induce a measurable antibody response in groups of mice given single intraperitoneal inoculations. The procedure may best be described by illustrating its application to the determination of the antigenic extinction titer of three different preparations

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1 a. Members of the Commission in Influenza, Army Epidemiological Board, *J. A. M. A.*, 1944, **124**, 982; b. Francis, T., Jr., *Am. J. Hyg.*, 1945, **42**, 1; c. Rickard, E. R., Thigpen, M., and Crowley, J. H., *Am. J. Hyg.*, 1945, **42**, 12; d. Hale, W. H., and McKee, A. P., *Am. J. Hyg.*, 1945, **42**, 21; e. Eaton, M. D., and Meiklejohn, G., *Am. J. Hyg.*, 1945, **42**, 28; f. Hirst, G. K., Plummer, N., and Friedewald, W. F., *Am. J. Hyg.*, 1945, **42**, 45; g. Salk, J. E., Menke, W. J., Jr., and Francis, T., Jr., *Am. J. Hyg.*, 1945, **42**, 57; h. Magill, T. P., Plummer, N., Smillie, W. G., and Sugg, J. Y., *Am. J. Hyg.*, 1945, **42**, 94.

2 Francis, T., Jr., *Am. J. Hyg.*, 1945, **42**, 1.

3 Francis, T., Jr., *J. Exp. Med.*, 1939, **69**, 283.

4 Hirst, G. K., *Science*, 1941, **94**, 22; *J. Exp. Med.*, 1942, **75**, 49.

5 Pearson, H. E., *J. Bact.*, 1944, **48**, 369; Stanley, W. M., *J. Exp. Med.*, 1945, **81**, 193.

6 Henle, W., and Henle, G., *J. Exp. Med.*, 1947, **85**, 347.

7 Studies to be published.

of formalinized allantoic fluid containing the PR8 strain of influenza virus Type A. The 3 preparations had been heated for different periods in studies of the rate of thermal inactivation of the antigenic factor.[7]

The test materials were diluted serially, in 2-fold steps, using beef heart infusion broth. The range of dilutions tested is shown in Table I. A single inoculation of 0.5 cc of the respective dilutions was given, intraperitoneally, to each of 10 mice, the weights of which were approximately 18 to 22 g.

Two weeks after inoculation all mice were bled. Bleeding was done by amputating the right fore-leg as close to the attachment to the trunk as possible. This method for collecting blood is far simpler than bleeding from the heart. The yield of serum from 10 mice of the size used here is 2 to 3 cc. For the bleeding, the mouse is lightly anesthetized with ether and is then held in the left hand so that the right fore-leg of the animal is the most dependent part. The mouse is kept in anesthesia by holding its nose against the lower end of a slanted tube that is open at both ends and contains a plug of cotton saturated with ether. When the foreleg is amputated close to the trunk, the skin retracts and the spurting blood is collected in a test tube. If the animal survives it is then sacrificed with chloroform. It has not been found necessary to use an antiseptic on the skin in order to obtain a sterile sample since the serum is heated at 56° C. for one-half hour after prompt separation from the clot and the red cells.

Finally, for the detection of neutralizing antibody in the pooled serum samples, the procedure followed was essentially the same as the one described by Hirst,[8] employing the chick embryo as the indicator for virus neutralization. Serum and virus were mixed in equal volumes and incubated for 30 minutes at 35° to 37° C. before inoculation into 10 to 11 day old embryonated eggs. Four eggs were used for each serum-virus mixture and 0.1 cc was introduced intra-allantoically. The serum was used undiluted, and the virus, in the form of infectious allantoic fluid containing the same passage of PR8 virus present in the vaccine, was diluted in beef heart infusion broth so as to contain approximately 1000 50% egg-infective doses. For control, a sample of normal serum was mixed with the virus dilution used in the test. A virus titration in eggs was also done. The diluent used for the virus titration was beef heart infusion broth. These controls were incubated in the same way as the test samples.

Following inoculation the eggs were incubated for 2 days at 35° to 37° C. At the end of this period 0.5 cc of allantoic fluid was removed from each egg to test for the growth of virus. This was done by adding to the fluid removed from each egg an equal volume of 0.5% suspension of washed chicken red blood cells. The multiplication of virus in the egg is indicated by the presence of sufficient virus hemagglutinin to cause the cells to settle in a characteristic pattern.[9]

The results of this experiment are shown in Table I. It will be noted that the pool of serum from the mice inoculated with the lower dilutions of vaccine contained sufficient antibody to neutralize the virus; whereas, no detectable neutralizing antibody was present in the serum from the mice given the highest dilutions of the respective materials that were tested. It is evident from these representative data that a point can be defined, even in a 2-fold dilution series, at which the serially diluted test material no longer contains sufficient antigenic material to induce the formation of a measurable amount of antibody.

The question of the interpretation of the end-point deserves some comment. Since the antigenic extinction titer expresses the extent to which the antigen can be diluted and still induce antibody formation in the mouse, one of 2 criteria may be adopted for estimating the end-point of the titration: The end-point may be considered to be the highest dilution of antigen that induces sufficient antibody in the pool of serum from 10 mice to neutralize completely the amount of virus used in the neutralization test (approximately 100 to 1000 50% egg-infective doses). Alternatively,

[8] Hirst, G. K., *J. Immunol.*, 1942, **45**, 285.

[9] Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

the end-point might be considered as the highest dilution of antigen that induces the formation of sufficient antibody to neutralize enough of the virus used in the test so that infection occurs in not more than half of the eggs inoculated. The latter criterion is preferable, and for this reason it would be better to use 6 eggs rather than 4 eggs for each serum-virus mixture.

The results obtained using this method for the determination of the antigenic extinction titer of a vaccine containing 3 strains of virus will be presented later in this report.

A Consideration of the Variables Involved in the Test. 1. *The influence of the quantity of virus used in the neutralization test upon the titer of neutralizing antibody in the serum.* Since the virus neutralization test is employed here to detect the presence of neutralizing antibody in a fixed dilution of serum, it appeared desirable to determine the influence of varying the dose of virus upon the sensitivity of the reaction. This was done in the following way. Serial dilutions were made of the 2 samples of mouse serum, one having a low antibody titer and the other a high titer. The high titer serum was obtained from mice given one intraperitoneal inoculation of 0.5 cc of a 1:4 dilution of unformalized allantoic fluid containing the PR8 strain (F198 M593 E60); the low titer serum was obtained from mice given a 1:1024 dilution of the same material. Each serum dilution series was set up in quadruplicate and a different concentration of virus was added to each. The respective serum-virus mixtures were inoculated into eggs and after 2 days' incubation the allantoic fluids were tested for the presence of virus hemagglutinin. The results are shown in Table II.

It is apparent from these data that variation in the number of infective doses from approximately 10 to 1000 results in no more than a 2-fold difference in antibody titer. Therefore, unless the antibody content of a particular sample of serum, when used undiluted, is near the limit of detection, then the quantity of virus used in the neutralization test can be varied within rather broad limits without affecting the results of the antigenic extinction titration. As the test is set up, an all-or-none effect should occur except under circumstances where the actual antibody titer of the serum sample is in the region of 1:2. Serum samples of borderline titer are found only in the region of antigenic extinction.

2. *Stability of the antigens used in the virus neutralization test.* Throughout these studies the preparations of antigen employed in the neutralization test were allantoic fluids containing different strains of active virus. These materials were stored, without preservative, at 4°C. A systematic study has not been made of the stability of the infective properties of the various strains used, but the data shown in Table III summarize the experience gathered thus far. It is evident that the preparations are sufficiently stable in the liquid state in the ordinary refrigerator, so as to be useful without re-titration immediately before use, within limited periods of time. The significance of the differences in stability of the infective titer of the different preparations shown in Table III has not been determined.

3. *The influence of the interval between inoculation and bleeding of mice upon serum antibody titer.* In the course of these experiments it was found that the interval between inoculation and bleeding has a marked effect upon the titer of serum antibody. This finding, which is illustrated in Table IV, has been studied more extensively in a series of experiments yet to be reported. The fact that the titer of neutralizing antibodies increased progressively, in the pooled serum collected at intervals between the 7th and 21st days after inoculation, was of considerable interest. It was of further interest that the titer of antibody, as measured by the agglutination-inhibition reaction, approached its maximum level in 7 days and did not continue to increase thereafter as did the level of the antibody measured by the *in vivo* technique. That the results of the agglutination-inhibition test measured antibody and not some nonspecific factor is indicated by the inhibition of agglutination by the homologous Type A virus and the absence of any inhibition of the

agglutinating effect of the Type B virus. The implications of these observations will be discussed in a more detailed report to be published. For the present purpose it is suggested that mice be bled 14 days after inoculation.

4. *The importance of strain and line specificity.* The test for antigenic extinction titer was done on the vaccine now being used as the standard of reference by the National Institute of Health. It is referred to as the Provisional Control Vaccine (Lot F2) and represents a lot of material of average potency, prepared by adsorption-elution using the red cells of the embryo.[10-11] This control vaccine, which was made by a manufacturer of biologicals, contains the prescribed strains, *i.e.*, PR8 and Weiss, both Type A, and Lee, Type B. The experiment to be described is presented because the findings emphasize the importance, particularly as it applies to the preparation and potency testing of vaccines, of antigenic variations that occur under diverse laboratory passage of the same strain.

The experiment was carried out in essentially the same manner as that described in the experiment shown in Table I. It differed, however, in that 26 g mice were used rather than 18 to 22 g mice. The results of the present test are summarized in Table V. It will be noted that the sera were tested for antibody to the PR8 and Weiss strains of Type A influenza virus and to the Lee strain of the Type B virus. Moreover, 3 different lines of the PR8 strain were compared.

The reason for making the comparison between the 3 different lines of the PR8 strain was as follows. In the first test, using the F198 M593 E64 line of PR8 virus, the results obtained were spotty. That these results were not due to technical factors was revealed by repeat test with similar findings. The spottiness of the results was surprising, in view of the clear-cut data that had been obtained in other tests (See Table I). In all previous tests the virus used in the neutralization test was the same, with respect to passage-line, as the antigen in the material used for the immunization of mice. The possibility was considered, therefore, that the PR8 virus present in the NIH control vaccine was different, in antigenic structure, from the PR8 virus used as antigen in the virus-neutralization test. On the supposition that the PR8 virus in the F2 vaccine was prepared from an egg-passage line not far removed from the mouse line, the regular mouse passage virus maintained in this laboratory was transferred to the egg for the preparation of antigen to be used in a repeat test for antibody. The results obtained with the F198 M720 E2 antigen were quite sharp, and supported the suspicion that the virus present in the NIH control vaccine was more closely related to the mouse-passage line than to the egg-passage line first used as the test antigen. The absence of any neutralizing effect against the F198 M70 TC717 E101 line of the PR8 strain indicates still greater differences between this agent and the PR8 virus in the vaccine.

These results are examples of the high degree of specificity of the antibody response to a single inoculation of influenza virus in experimental animals,[12] and of the variations in antigenic[13] and other properties[14] that can occur in different passage-lines of the same strain. The spotty results obtained with the F198 M593 E64 antigen suggests that antibody to this virus was induced by the inoculation of the 1:10 to 1:640 dilutions of the NIH control vaccine, but that the actual level of antibody for this line was much lower than for the F198 M720 E2 line. In this experiment there was no demonstrable crossing with the F198 M70 TC717 E101 line, although in other studies the relationships between all three have been demonstrated.

The results of this experiment emphasize the need for maintaining the identity of the test virus and the virus in the vaccine in any

10 Hirst, G. K., *J. Exp. Med.*, 1942, **76**, 195.

11 Francis, T., Jr., and Salk, J. E., *Science*, 1942, **96**, 499.

12 Magill, T. P., and Francis, T., Jr., *Brit. J. Exp. Path.*, 1938, **19**, 273.

13 Francis, T., Jr., PROC. SOC. EXP. BIOL. AND MED., in press.

14 Salk, J. E., PROC. SOC. EXP. BIOL. AND MED., 1946, **63**, 134, 140.

TABLE I.

Results of Test for Antibody in Serum of Mice Inoculated with Serial Dilutions of 3 Different Preparations Containing the PR8 Strain (F198 M593 E60) of Influenza Virus Type A.

Dilutions of vaccine inoculated	Serum obtained from immunized mice (groups of 10)	Vaccine preparations 1	2	3
	64			0 0 0 0
	128			0 0 0 0
	256	0 0 0 0	0 0 0 0	0+++
	512	0 0 0 0	0 0 0 0	++++
	1024	0 0 0 0	0 0 0+	
	2048	0 0 0 0	++++	
	4096	0 0 0 0	++++	
	8192	++++		
	16000	++++		
	Normal serum plus virus	++++		

The symbols, + or 0, indicate the presence or absence of influenza virus infection in each of 4 embryos inoculated with the same serum-virus mixture. Evidence of infection was the presence of hemagglutinin in the allantoic fluid. The serum-virus mixture was made up of equal volumes of undiluted serum and 10^{-5} dilution of virus. The result of the virus titration was:

10^{-6}	10^{-7}	10^{-8}	10^{-9}
+++++ 0	++++++	++ 0 0 0 0	0 0 0 0 0 0

TABLE II.

Effect of the Quantity of Virus, Used in the Neutralization Test Upon the Titer of Neutralizing Antibody in Serum.

*Serum	Dilution	†Virus dilutions used in neutralization test 10^{-4}	10^{-5}	10^{-6}	10^{-7}	
Low titer	8	0 0 0 0	0 0 0 0			
	16	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	
	32	0 0 0+	0 0 0+	0 0 0 0	0 0 0 0	†Virus
	64	++++	++++	0 0++	0 0 0+	titration
	128			++++	0+++	10^{-4} ++++
						10^{-5} ++++
High titer	128	0 0 0 0	0 0 0 0			10^{-6} ++++
	256	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	10^{-7} ++++
	512	++++	0 0++	0 0 0 0	0 0 0 0	10^{-8} + 0 0 0
	1024	++++	++++	++++	0 0 0+	10^{-9} 0 0 0 0
	2048			++++	0 0 0+	
Normal control	2	++++	++++	++++	++++	

Symbols same as in Table I.

* Low titer serum was pool from 10 mice inoculated 2 weeks earlier with 0.5 cc of 1:1024 dilution of allantoic fluid containing PR8 strain of influenza virus Type A.

High titer serum was from similar group inoculated with a 1:4 dilution of the same fluid.

† Virus used was in allantoic fluid from chick embryos infected with the same strain of virus used for immunization of mice, *i.e.*, PR8 (F198 M593 E64).

method employing active immunization for the estimation of the quantity of antigenic substance in a vaccine. Otherwise, the factor of qualitative differences among different lines of the same strain tend to confuse and obscure the quantitative estimate which the test is designed to provide. For this reason it is not known to what extent the data shown in Table V represent a correct evaluation of the antigenic extinction titer of any of the strains in the vaccine tested.

5. *Influence of age of mice upon the results of the antigenic extinction test.* It was of interest to determine to what extent age or size of mice influenced the results of the antigenic extinction test. Accordingly, 2 groups of mice of different ages were obtained from the same breeder. The weight of the young mice averaged 10 g and the older mice weighed about 26 g each. Groups of

TABLE III.

Results of Egg-Infectivity Titrations of Different Strains of Virus Used as Antigen in the Virus Neutralization Tests; to Illustrate the Degree of Stability of Infectivity of the Preparation when Stored at 4°C for Different Lengths of Time.

Strain Passage Date of preparation	Date of test	Infectivity titrations: Dilutions 10-5	10-6	10-7	10-8	10-9	10-10
PR8 (11-28-46)	12-15-46		6/6	6/6	6/6	0/6	
F198	1- 2-47		6/6	6/6	5/6	0/6	
M593	31		6/6	6/6	3/6	0/6	
E64	2-13		6/6	6/6	4/6	1/6	
	27		6/6	6/6	2/6	0/6	
	3-14		6/6	6/6	2/6	0/6	
	4-11		4/4	2/4	0/4	0/4	
	21		4/4	2/4	0/4	0/4	
	5- 5		4/4	2/4	0/4	0/4	
PR8 (12-21-46)	12-27-46		4/4	4/4	4/4	3/4	1/4
F198	1-23-47		4/4	4/4	4/4	2/4	0/4
M70	31		6/6	6/6	5/6	0/6	0/6
TC717	3- 3		6/6	6/6	3/6	0/6	0/6
E101	5- 5		4/4	4/4	3/4	0/4	0/4
PR8 (2-17-47)	2-19-47		4/4	4/4	3/4	1/4	
F198	3-10		4/4	2/4	1/4	0/4	
M720	31		4/4	2/4	0/4	0/4	
E2	4-21		4/4	3/4	0/4	0/4	
	5- 5		3/4	0/4	0/4	0/4	
Weiss (12-9-46)	12-24-46	6/6	6/6	6/6	3/6	0/6	
F3	1- 8-47	6/6	6/6	4/5	3/6	0/6	
M32	2- 5	6/6	6/6	4/6	0/6	0/6	
E58	4- 3	4/4	0/4	0/4	0/4		
	21	2/4	1/4	0/4	0/4		
E59 (4-28-47)	28	4/4	4/4	1/4	1/4	0/4	
Lee (11-28-47)	12- 4-46	6/6	6/6	5/6	0/6	0/6	
F8	19	4/4	4/4	3/4	0/4	0/4	
M137	1-20-47	5/6	1/6	0/6	0/6		
E109	2-10	2/6	1/6	0/6	0/6		
E110 (2-12-47)	14	4/4	4/4	4/4	2/4	0/4	
	3-21	5/6	3/6	0/6	0/6		
	4-11	4/4	0/4	0/4	0/4		
	30	4/4	2/4	0/4	0/4		
E111 (4-28-47)	5- 5	4/4	4/4	1/4	0/4		

Denominator = No. of eggs inoculated.
Numerator = No. of eggs infected as indicated by presence of hemagglutinin in allantoic fluid.

10 mice were given one intraperitoneal inoculation of 0.5 cc of the respective 2-fold dilutions of the Provisional Control Vaccine of the National Institute of Health (Lot F2). Two weeks later all mice were bled and the sera tested for the presence of neutralizing antibody. The results are summarized in Table VI. The data for the old mice are the same as those shown in Table V. It is of interest that the antigenic extinction titration done in the older mice gave somewhat higher values than the test done in the younger mice. The earlier comments with regard to differences in results observed when different strains or passage-lines were used as antigen in the neutralization test apply to the present findings as well. Unfortunately, sufficient serum from the young mice was not available to permit further studies of the height to which antibody titers were elevated in the old and young mice inoculated with corresponding dilutions of the vaccine.

Summary. A procedure applicable to the standardization of influenza virus vaccines has

TABLE IV.

Influence of the Interval Between Inoculation and Bleeding of Mice upon Antibody Titer (as Measured by Virus-Neutralization in Chick Embryos and by Red Cell Agglutination-Inhibition).

Interval between inoculation* and bleeding (days)	Results of Virus-Neutralization Test† in Chick Embryos. Serum dilutions: 4	16	64	256	1024	4096
7	0 0 0 0	0+++	++++	++++	++++	++++
10	0 0 0 0	0 0 0 0	++++	++++	++++	++++
14	0 0 0 0	0 0 0 0	0 0 0+	0+++	++++	++++
21	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0++	++++
Normal	++++					

(Symbols as in Table I.)

Interval (days)	Results of Agglutination-Inhibition Test‡. Serum dilutions: 8	16	32	64	128	256	512	1024
7	0	0	0	0	0	0	+	+
10	0	0	0	0	0	0	+	+
14	0	0	0	0	0	0	+	+
21	0	0	0	0	0	0	+	+
Normal	+	+	+	+	+	+	+	+

* Mice inoculated intraperitoneally with 0.5 cc of 1:25 dilution of formalinized allantoic fluid containing PR8 strain of influenza virus Type A(F198 M593 E60).

† For the neutralization test in chick embryos 1000 50% egg-infecting doses were used.

‡ For the agglutination-inhibition test, 4 hemagglutinating units of virus were used.

TABLE V.

Antigenic Extinction Titration of N.I.H. Provisional Control Vaccine (Lot F2).

Serum obtained from immunized mice (Groups of 10)	Strain and passage line of virus used as antigen for detection of neutralizing antibodies in serum of immunized mice. PR8: F198 M593 E64	PR8: F198 M720 E2	PR8: F198 M70 TC717 E101	Weiss: F3 M32 E58	Lee: F8 M137 E110
Dilutions of vaccine inoculated					
10	0 0 0+	0 0 0 0	++++	0 0 0 0	0 0 0 0
20	0 0 0 0	0 0 0 0	++++	0 0 0 0	0 0 0 0
40	0 0 0 0	0 0 0 0	++++	0 0 0 0	0 0 0+
80	0 0++	0 0 0 0	++++	++++	0 0 0+
160	0 0 0 0	0 0 0 0	++++	0+++	++++
320	0 0 0+	0 0 0 0		++++	++++
640	0 0++	0 0 0 0		++++	
1280	++++	0 0 0 0			
2560	++++	++++			
5120	++++	++++			
Normal controls	++++	++++	++++	++++	++++

Symbols same as in Table I.

been described. The total time required for completion of the test can be as little as 16 days. The procedure, referred to as the antigenic extinction titration, determines the highest dilution of antigen capable of inducing the formation of demonstrable amount of virus-neutralizing antibody in the pooled serum of groups of 10 mice. The test for neutralizing antibody is carried out *in ovo* and may, therefore, be used in those instances in which the strain of virus that may be included in a vaccine has been adapted only to the chick embryo and is not pathogenic for mice.

TABLE VI.
Comparison of Results of Antigenic Extinction Titration of N.I.H. Control Vaccine in Young and Old Mice.

Dilutions of vaccine inoculated	Challenge Virus*					
	PR8 F198 M720 E2		PR8 F198 M593 E64		Weiss F3 M32 E58	
	Serum from		Serum from		Serum from	
	Old mice	Young mice	Old mice	Young mice	Old mice	Young mice
10	0 0 0 0	0 0 0 0	0 0 0+	0 0 0 0	0 0 0 0	0 0 0 0
20	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	+++ +
40	0 0 0 0	0 0 0 0	0 0 0 0	0 0+ +	0 0 0 0	0+ + +
80	0 0 0 0	0 0 0 0	0 0+ +	+ + + +	+ + + +	+ + + +
160	0 0 0 0	0 0 0 0	0 0 0 0	+ + + +	0+ + +	+ + + +
320	0 0 0 0	0 0 0 0	0 0 0+	+ + + +	+ + + +	+ + + +
640	0 0 0 0	0 0 0 0	0 0+ +	0+ + +	+ + + +	+ + + +
1280	0 0 0 0	0+ + +	+ + + +	+ + + +	+ + + +	+ + + +
2560	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
5120	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +
Controls	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +

Symbols same as Table I.
* Approximately 1000 50% egg-infecting doses of each strain were used.

The use of chick embryos rather than mice as the indicator medium for virus neutralization offers the advantages of completion in 2 days rather than 10 days, applicability to egg-adapted strains and sharpness of results, as well as being less costly.

The importance of maintaining the identity of virus in vaccine and virus used as test antigen for measuring the antibody response induced by immunization has been illustrated and discussed.

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Experimental Infection of Domestic Animals with Japanese B Encephalitis Virus.*

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During the summer of 1946 a series of experiments were carried out on Okinawa with the general purpose of investigating the part that domestic animals might play in the epidemiology of Japanese B encephalitis.[1] Workers from Naval Medical Research Unit Number 2 had presented evidence that horses might have such a role.[2] It appeared desirable to obtain additional evidence on this point and also to study the other domestic animals which were common on the island during the epidemic of 1945. The experiments were designed (1) to determine how long virus persisted in the blood of experimentally infected animals, (2) to determine whether the virus was pathogenic for any of the species studied and (3) to study the time of appearance and increase of neutralizing and complement-fix-

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