NEUTRALIZING AND COMPLEMENT-FIXING ANTIBODY PRODUCTION AND RESISTANCE FOLLOWING VACCINATION IN EXPERIMENTAL ENCEPHALITIS INFECTIONS

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The correlation between neutralizing antibodies and resistance to encephalitis virus infections cannot as yet be stated in general terms. Each individual virus seems endowed with its own characteristics in a particular host. The subject has become of such practical importance, however, especially in evaluating vaccines, that in spite of excellent work already reported, further studies seemed indicated. Consequently experiments were carried out in mice to compare resistance to a test infection with level of (*a*) neutralizing and (*b*) complement-fixing antibodies in the blood at various intervals following vaccination.

Materials and Methods

Virus.—The St. Louis encephalitis virus used in our work is the Strain B 33 which was isolated in 1933; it had been propagated in our laboratory for several years, then sent to Dr. G. O. Broun in St. Louis in 1937, and in 1938 the strain was sent back to us. The strain of W.E.E. virus employed in this study is one (McMillan) which was isolated by us (1) from a fatal case in Canada in 1941.

The viruses are maintained in our laboratory in the form of a 10 per cent mouse brain emulsion stored in dry ice at −76°C., thus reducing the number of mouse passages to a minimum. The St. Louis virus had had a considerable number of passages prior to 1940, when it was propagated by alternating mouse intracerebral passage with storage in glycerine. The W.E.E. strain used in the tests had had only four or five passages.

Mice.—All mice employed in this investigation were of the W-Swiss strain (2). Special care was taken to secure large groups of animals of similar age and weight in order to insure uniformity of results. The mice used for vaccination in the experiment with W.E.E. virus were from 70 to 90 days old at the time of vaccination and weighed from 23 to 28 gm. Those used for the tests with the St. Louis virus were from 60 to 70 days of age when the experiment was started and their weights varied between 20

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and 24 gm. Mice employed for titrations of virus or in the neutralization tests were younger, from 22 to 30 days old, and their weights were from 11 to 13 gm.

A group of mice needs special mention here, namely, those used for intraperitoneal neutralization tests with W.E.E. virus. As shown by Olitsky and Harford (3), very young animals are necessary for that purpose. We used mice between 14 and 17 days of age. However, the supply of such mice was rather limited and in some cases they were not as uniform in size as desired.

Immunization and Vaccines.—The immunization of mice was carried out either with live virus or with inactivated virus. For immunizing with live virus we proceeded as follows: Three mice were infected intracerebrally with the particular virus in 10^{-2} dilution. When prostrate, about 40 hours later in the case of W.E.E. virus and 3 to 4 days in the case of St. Louis virus, their brains were removed, ground up in a mortar, and emulsified in a sufficient amount of buffered distilled water to yield a 10 per cent suspension. This suspension was centrifuged at 2,000 R.P.M. for 10 minutes, the supernatant pipetted off and further diluted in the same diluent to the required strength, and then used for vaccination. The amount of active virus present in the dilutions was determined by intracerebral titration in young mice. The titer of the St. Louis virus was 10^{-7} to 10^{-8} and that of the W.E.E. virus 10^{-8} to 10^{-9}. Mice were vaccinated by means of one subcutaneous injection of 0.5 cc. of the stated dilution.

For immunization with inactivated virus the following procedure was carried out. A batch of thirty mice 25 days of age were inoculated intracrebrally with a 10^{-2} suspension of St. Louis virus and another similar group of mice were inoculated with the same dilution of W.E.E. virus. When sick these animals were sacrificed, their brains removed and weighed and emulsified with the aid of a mechanical blendor in sufficient amount of physiological saline to give a 10 per cent suspension of mouse brain. This suspension was then centrifuged at 2,000 R.P.M. for 10 minutes and the supernatant pipetted off. This supernatant was titrated in young mice by intracerebral inoculation, the titer being 10^{-7} for the St. Louis virus and 10^{-8} for W.E.E. virus. To the 10 per cent supernatant sufficient formalin was added to give a 0.5 per cent concentration of formalin and this material was kept in the ice box in glass-stoppered flasks. The St. Louis vaccine was tested intracerebrally for virulence 14 days later with the result that of eight mice inoculated all survived. The W.E.E. vaccine was likewise tested, 11 days later, and again all mice of eight inoculated survived. Both vaccines were used for vaccination, 15 and 20 days respectively after preparation. Vaccination of mice with the avirulent formolized vaccine was carried out in the following manner.

St. Louis vaccine was given subcutaneously in 0.25 cc. amounts daily for 4 consecutive days, making a total of 1 cc. of vaccine. The W.E.E. vaccine was also administered subcutaneously, but in 0.2 cc. amounts daily for 3 consecutive days, making a total of 0.6 cc. of vaccine. This dosage of equine encephalomyelitis vaccine has been found to give good protection (4).

Resistance Tests.—At intervals following vaccination, groups of vaccinated mice, as well as controls, were tested for resistance by means of an intracerebral test. To that end a fresh suspension of virus was prepared by emulsifying the brains of two or three mice prostrate after intracerebral infection, in a diluent consisting of 10 per cent normal rabbit serum in saline, to a concentration of 10^{-1}. This emulsion was then centri-

fuged at 2,000 R.P.M. for 10 minutes, the supernatant pipetted off, and further diluted in tenfold dilutions. Usually groups of four mice were infected with each dilution. Following infection the mice were observed for a 21 day period and the survivors discarded.

Sera for Neutralization and Complement-Fixation Tests.—On the same days that mice were tested for resistance to infection, additional animals from the same vaccinated groups and from the control group were bled from the heart under ether anesthesia. No separate mice were kept in each group for bleeding purposes, but special care was taken not to bleed and infect an individual mouse on the same day. The bloods from each group were pooled, allowed to stand at room temperature, and centrifuged. Sera were kept in the dry ice box until used.

Neutralization Tests.—Neutralization tests were carried out in the following manner. A 10^{-1} suspension of virus was prepared by emulsifying the brain tissue of infected mice in diluent. As diluent, hormone broth (pH 7.2) was used in the first two or three tests; in all subsequent tests 10 per cent rabbit serum in saline was employed instead of broth because of its more satisfactory qualities. The original 10^{-1} suspension of virus was centrifuged at 2,000 R.P.M. for 10 minutes and then further diluted in tenfold dilutions, starting with 1:50, the dilution being carried as far as necessary. Next, 0.3 cc. amounts of serum to be tested were put in small sterile test tubes, and 0.3 cc. of the proper virus dilutions were added. Thus the tubes contained a constant amount of serum and increasing tenfold dilutions of virus from 10^{-2} to 10^{-8} or 10^{-9} as required. From this point on the procedure was different for the St. Louis and W.E.E. viruses. For the neutralization tests with St. Louis virus the serum-virus mixtures were incubated at 37°C. in a water bath for 2 hours and then injected intracerebrally into young mice, four animals being employed for each dilution. With the W.E.E. virus the serum-virus mixtures were injected without incubation, and their virulence was tested in each case by two routes, the intracerebral and intraperitoneal. For the intracerebral neutralization tests, the serum-virus mixtures in 0.03 cc. amounts were injected intracerebrally into mice 22 to 28 days of age, three mice being employed per dilution. For the intraperitoneal neutralization tests (3), the serum-virus mixtures were injected in 0.1 cc. amounts into 14 to 17 day old mice, two animals being employed per dilution. All mice were observed for 21 days and the survivors discarded.

Interpretation of Resistance and Neutralization Tests.—The evaluation of results of resistance and neutralization tests was estimated on the basis of the Reed and Muench method (5). We are aware that in some of the resistance tests the spread of deaths is considerable and irregular, and in those cases the meaning of degree of resistance as represented by the figure arrived at may be vague. In the tables and graphs we have used the notation LD-50 to indicate the 50 per cent lethal dose and it is expressed by the logarithm of the dilution, disregarding the minus sign. The degree of resistance to infection and the degree of protection given by a serum are expressed as *resistance index* and *neutralization index*, indicating the ratio between the LD-50 of the vaccinated mice, or immune serum, and the LD-50 of the control mice, or control serum. With this system the resistance index and the neutralization index of the controls are always 1.

Complement-Fixation Tests.—The determination of complement-fixing antibodies in the sera was done in accordance with the method described in former publications (6).

Antigens were prepared from infected mouse brain tissue by centrifuging a 10 per cent suspension at 2,500 R.P.M. for 30 minutes, freezing and thawing the supernatant four or five times, and then centrifuging in an angle-head centrifuge (7) at about 5,000 R.P.M. for 1 hour.

The sera were inactivated at 60°C. for 20 minutes and used in the tests in twofold dilutions, starting always with undiluted serum. Two units of complement were employed. The first phase of the reaction was incubated in the ice box at 2°C. for 18 hours and the hemolytic system then added, consisting of 0.25 cc. of a 3 per cent suspension of packed sheep cells and 3 M.H.D. of hemolysin, also in a volume of 0.25 cc. The tubes were incubated for ½ hour at 37°C. and then read. The titer of a serum was given as the highest dilution that showed a 2 plus or better fixation. The specificity of the reaction was absolute in all cases, as indicated by the proper controls.

EXPERIMENTAL

The first experiment was designed to compare at various intervals, over a 4 month period of time, the immunity and neutralizing and complement-fixing antibodies of mice vaccinated with virulent or non-virulent W.E.E. virus.

Experiment 1.—784 W-Swiss mice, 70 to 90 days of age and weighing from 23 to 28 gm. at the beginning of the experiment, were employed. These mice were distributed in five groups and treated as follows: group I, 199 mice, were given one subcutaneous injection of 0.5 cc. of virus in a dilution of 10^{-3}; group II, 150 mice, received one subcutaneous injection of 0.5 cc. of virus in a dilution of 10^{-5}; group III, 141 mice, received one subcutaneous injection of 0.5 cc. of virus in a 10^{-7} dilution, and group IV, 164 mice, were given 0.2 cc. subcutaneously of a 10 per cent formolized, avirulent vaccine, each day, for 3 consecutive days, the total amount of vaccine administered being 0.6 cc. Finally, group V, 130 mice, were left untreated and set aside as controls.

The choice of dilution, 10^{-3}, 10^{-5}, and 10^{-7}, was made with the purpose of simulating a severe, moderate, and mild natural infection. As it developed, and the figures indicating the number of deaths following vaccination with live virus show, the group with the 10^{-5} dilution had a higher percentage of deaths than was expected. Following vaccination with formolized material, no mice died; some of them showed a local lesion at the site of inoculation of vaccine that healed in a few days. After injection of live virus, deaths occurred as follows: group I (10^{-3}), out of 199 mice, 41 died (20 per cent); group II (10^{-5}), out of 150 mice, 32 died (21 per cent); group III (10^{-7}), out of 141 mice, 1 died (0.7 per cent).

Following vaccination with either live or inactivated materials, batches of mice were taken from each group at intervals and tested for resistance to intracerebral infection; these animals were also bled to test their blood for neutralizing and complement-fixing antibodies. Such tests were carried out at intervals from the first day of the experiment as follows: 3, 9, 16, 30, 58, and 121 days.

The results of all the tests are given in detail in Tables I, II, and III.

3rd Day Test.—None of the mice showed any resistance, their sera contained no complement-fixing antibodies and, with the exception of group I (10^{-3}), no neutralizing

TABLE I

Immunity to Intracerebral Inoculation of W.E.E. Virus Following Vaccination

Time after vaccination	Groups of mice	Mortality of mice injected intracerebrally with W.E.E. virus									Degree of immunity of vaccinated mice	
		Dilution of virus injected intracerebrally									50 per cent lethal dose	Protection index
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}		
days												
	10^{-3}				3/3*	3/3	3/3	2/3			>8.25	2
	10^{-5}				3/3	3/3	3/3	3/3			>8.50	1
3	10^{-7}				3/3	3/3	3/3	3/3			>8.50	1
	Formolized vaccine				3/3	3/3	3/3	3/3			>8.50	1
	Controls					3/3	3/3	3/3	0/3		8.50	
	10^{-3}		4/4	4/4	2/4	1/4	2/4	1/4			5.80	3468
	10^{-5}				3/4	4/4	4/4	2/4			7.80	35
9	10^{-7}				4/4	3/4	4/4	4/4			>8.40	9
	Formolized vaccine		4/4	4/4	4/4	4/4	1/4	1/4			6.84	316
	Controls					4/4	4/4	4/4	3/4	0/4	9.34	
	10^{-3}		0/4	0/4	0/4	0/4	0/4	0/4			2.50	100,000
	10^{-5}				4/4	3/4	1/4	0/4			6.50	10
16	10^{-7}				4/4	4/4	1/4	0/4			6.67	7
	Formolized vaccine			3/4	1/4	3/4	0/4	0/4			5.00	316
	Controls					4/4	2/4	2/4	0/4		7.50	
	10^{-3}	0/4	0/4	1/4	2/4	0/4	0/4				1.88	131,900
	10^{-5}		1/4	2/4	3/4	3/4	0/4	0/4			5.00	100
30	10^{-7}		4/4	4/4	4/4	4/4	1/4	0/4	0/4		6.67	2
	Formolized vaccine	3/4	3/4	4/4	2/4	2/4	1/4	0/4			5.23	59
	Controls			4/4	4/4	4/4	2/4	0/4	0/4		7.00	
	10^{-3}	4/4	3/4	3/4	3/4	4/4	2/4				6.40	7
	10^{-5}			2/2	4/4	2/4	2/4	0/4			6.50	5
58	10^{-7}				4/4	4/4	1/4	1/4			6.84	2
	Formolized vaccine		4/4	4/4	3/4	2/4	2/4	0/4			6.30	8
	Controls					4/4	2/4	1/4	0/4		7.23	
	10^{-3}			4/4	2/4	2/4	2/4	0/4			6.00	100
	10^{-5}				4/4	2/4	3/4	3/4			7.70	2
121	10^{-7}				4/4	4/4	4/4	3/4	1/4		8.50	−3
	Formolized vaccine			4/4	4/4	3/4	3/4	2/4			7.57	3
	Controls					4/4	4/4	2/4	0/4		8.00	

* 3/3 = 3 of 3 mice injected died of W.E.E.

antibodies. Mice in the 10^{-3} group showed some neutralizing antibodies by the intraperitoneal test—a neutralization index (N.I.) of 3,163.

9th Day Test.—Group I mice (10^{-3}) showed good resistance, *i.e.*, a resistance index

TABLE II

Neutralizing Level of Sera from Mice Following Vaccination with W.E.E. Virus

Time after vaccination	Groups of mice	Mortality of mice injected with mixtures of serum and virus														Degree of protection afforded by serum of vaccinated mice			
		Intracerebral test						Intraperitoneal test								By intracerebral test		By intraperitoneal test	
		Dilution of virus in mixture						Dilution of virus in mixture								50 per cent lethal dose	Neutralization index	50 per cent lethal dose	Neutralization index
		10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}				
days																			
	10^{-4}			3/3	0/3	0/3				0/2	0/2	0/2	0/2	0/2			6.50	18	<3.50	3163+
	10^{-5}			3/3	3/3	3/3					2/2	2/2	2/2	2/2			8.50	−6	8.50	−32
	10^{-7}			3/3	2/3	2/3						1/2	2/2	1/2			8.00	−2	7.60	−4
3	Formolized vaccine			3/3	2/3	0/3					1/2	1/2	0/2	1/2			7.25	3	6.00	10
	Controls			3/3	3/3	1/3	0/3					2/2	1/2	0/2			7.75		7.00	
	10^{-4}	3/3	3/3	1/3	0/3	0/3		0/2	0/2	0/2	0/2						5.75	1000	<1.50	3,163,000+
	10^{-5}	3/3	3/3	0/3	0/3	0/3		0/2	0/2	0/2	0/2	0/2	0/2				5.50	1779	<1.50	3,163,000+
	10^{-7}	3/3	3/3	3/3	3/3	3/3	0/3		2/2	2/2	2/2	2/2	2/2	1/2			8.50	1	8.00	1
9	Formolized vaccine	3/3	2/3	1/3	0/3	0/3	0/3	0/2	0/2	0/2	0/2	0/2					5.50	1779	<1.50	3,163,000+
	Controls		3/3	3/3	3/3	3/3	1/3				2/2	2/2	2/2	1/2	0/2		8.75		8.00	
	10^{-4}	3/3	3/3	1/3	0/3	0/3		0/2	0/2	0/2	0/2	0/2					5.75	562	<1.50	10,000,000+
	10^{-5}	3/3	3/3	1/3	0/3	0/3		1/2	0/2	0/2	0/2	0/2					5.75	562	2.00	3,163,000
	10^{-7}		3/3	3/3	0/3	0/3			0/2	0/2	0/2	0/2	0/2	0/2			6.50	100	<2.50	1,000,000+
16	Formolized vaccine	3/3	3/3	2/3	0/3	0/3		1/2	0/2	0/2	0/2	0/2					6.25	178	2.00	3,163,000
	Controls			3/3	3/3	3/3	0/3				2/2	2/2	2/2	2/2	0/2		8.50		8.50	
	10^{-4}	3/3	3/3	0/3	0/3	0/3		0/2	0/2	0/2	0/2	0/2	0/2				5.50	1000	<1.50	10,000,000+
	10^{-5}	3/3	3/3	1/3	0/3	0/3		0/2	0/2	0/2	0/2	0/2	0/2				5.75	562	<1.50	10,000,000+
	10^{-7}	3/3	3/3	3/3	3/3	3/3	1/3	2/2	2/2	2/2	2/2	2/2	2/2	2/2			8.75	−2	8.50	1
30	Formolized vaccine	3/3	3/3	0/3	0/3	0/3		0/2	0/2	0/2	0/2	0/2	0/2	0/2			5.50	1000	<1.50	10,000,000+
	Controls		3/3	3/3	3/3	3/3	0/3				2/2	2/2	2/2	2/2	0/2		8.50		8.50	
	10^{-4}	3/3	3/3	1/3	0/3	0/3		0/2	0/2	0/2	0/2	0/2	0/2				5.75	5624	<1.50	316,300+
	10^{-5}	3/3	3/3	3/3	3/3	0/3		1/2	0/2	0/2	0/2	0/2	0/2				7.50	100	2.00	100,000
	10^{-7}	3/3	3/3	3/3	3/3	3/3		2/2	2/2	2/2	2/2	2/2	2/2	0/2			>8.50	<10	7.50	−3
58	Formolized vaccine	3/3	3/3	2/3	0/3	0/3		0/2	0/2	0/2	0/2	0/2	0/2				6.25	1779	<1.50	316,300+
	Controls		3/3	3/3	3/3	3/3	3/3			2/2	1/2	1/2	2/2	0/2			9.50		7.00	
	10^{-4}	3/3	2/3	1/3	0/3	0/3		0/2	0/2	0/2	0/2	0/2	0/2				5.50	562	<1.50	3,163,000+
	10^{-5}	3/3	3/3	1/3	0/3	0/3		0/2	0/2	0/2	0/2	0/2	0/2				5.75	316	<1.50	3,163,000+
	10^{-7}	3/3	3/3	3/3	3/3	1/3	1/3			2/2	2/2	2/2	2/2	2/2			8.00	2	8.50	−3
121	Formolized vaccine	3/3	3/3	2/3	0/3	0/3	0/3	0/2	0/2	0/2	0/2	0/2	0/2				6.25	100	1.50	3,163,000+
	Controls		3/3	3/3	3/3	2/3	0/3			2/2	2/2	2/2	2/2	1/2	0/2		8.25		8.00	

(R.I.) of 3,468, good neutralizing antibodies both intracerebrally and intraperitoneally, with N.I. of 1,000 and 3,163,000 respectively. The titer of complement-fixing antibodies was high, 1:8 considering that this was not a hyperimmune serum. In group II (10^{-5}), the amount of resistance was doubtful, with a R.I. of 35. Yet neutralizing

antibodies were as high as in the preceding group, namely, N.I. of 1,779 on intracerebral test and 3,163,000 on intraperitoneal test. Complement-fixing antibodies were present in very low titer, only the undiluted serum giving a partial reaction. In group III (10^{-7}), all tests were negative. In group IV, treated with formolized vaccine, the degree of resistance was low, with a R.I. of 316. This group did not show as

TABLE III

Summary of Protection Tests, Neutralization Tests, and Complement-Fixation Tests with W.E.E. Virus

Treatment of mice	Test	Time after vaccination—days:					
		3	9	16	30	58	121
0.5 cc. active virus, 10^{-6} subcutaneously	Resistance: protection index	2	3,468	100,000	131,900	7	100
	Neutralization (icer.): neutralizing index	18	1,000	562	1,000	5,624	562
	Neutralization (iper.): neutralizing index	3,163	3,163,000	10,000,000	10,000,000	316,300+	3,163,000
	Complement-fixation: titer of serum	0	1:8	1:8	1:8	1:8	1:16
0.5 cc. active virus, 10^{-6} subcutaneously	Resistance: protection index	1	35	10	100	5	2
	Neutralization (icer.): neutralizing index	−6	1,779	562	562	100	316
	Neutralization (iper.) neutralizing index	−32	3,163,000	3,163,000	10,000,000	100,000	3,163,000
	Complement-fixation: titer of serum	0	1:1	1:8	1:8	1:8	1:8
0.5 cc. active virus, 10^{-7} subcutaneously	Resistance: protection index	1	9	7	2	2	−3
	Neutralization (icer.): neutralizing index	−2	1	100	−2	10	2
	Neutralization (iper.): neutralizing index	−4	1	1,000,000	1	−3	−3
	Complement-fixation: titer of serum	0	0	0	0	0	0
0.2 cc. formolized vaccine × 3 subcutaneously	Resistance: protection index	1	316	316	59	8	3
	Neutralization (icer.): neutralizing index	3	1,779	178	1,000	1,779	100
	Neutralization (iper.): neutralizing index	10	3,163,000	3,163,000	10,000,000	316,300	3,163,000
	Complement-fixation: titer of serum	0	1:4	1:8	1:8	1:8	1:8

high a degree of resistance as was anticipated in view of the experience of other workers and it remained low during the entire experiment. Yet, the values obtained were well within the significant range. On the other hand, the neutralizing antibodies in this group were very high, with a N.I. of 1,779 on intracerebral test and 3,163,000 on intraperitoneal test. Complement-fixing antibodies were present in fair titer in a dilution of 1:4. This is noteworthy because of the short time that elapsed between time of vaccination and bleeding for test, and also because the material used for vaccination was *avirulent.*

16th Day Test.—In group I (10^{-3}), resistance to intracerebral infection was very high,

with a R.I. of 100,000. Neutralizing antibodies remained at approximately the same level as in the 9th day test, showing a N.I. of 562 for the intracerebral test and 10,000,000 for the intraperitoneal test. Complement-fixing antibodies had a titer of 1:8. In Group II (10^{-5}), immunity was either absent or negligible, with a R.I. of 10, whereas the neutralizing antibodies showed a level as high as that of the preceding group, that is, a N.I. of 562 on intracerebral test and 3,163,000 on intraperitoneal test. The titer of complement-fixing antibodies rose to 1:8. In group III (10^{-7}), there was no immunity. The neutralizing antibodies were positive to the extent of a N.I. of 100 on intracerebral test and 1,000,000 on intraperitoneal test. Probably not much significance should be attached to these figures for neutralizing antibodies since this was the only positive reaction shown at any time by this group. It might be that the development of antibodies in an individual mouse made this particular batch of serum positive. Complement-fixing antibodies were recorded as negative, although a plus-minus reaction occurred in the tube containing undiluted serum. In group IV, treated with formolized vaccine, a significant though low resistance was demonstrated, with a R.I. of 316; by intracerebral test neutralizing antibodies were slightly lower than in groups I and II and there was a N.I. of 178. On intraperitoneal test the level was again 3,163,000; the titer of complement-fixing antibodies was 1:8.

30th Day Test.—Group I (10^{-3}) mice showed good resistance, with a R.I. of 131,900; neutralizing antibodies had a level of 1,000 (N.I.) by intracerebral test, and 10,000,000 by intraperitoneal test. The titer of complement-fixing antibodies was 1:8. Mice in group II (10^{-5}) showed a mild resistance, with a R.I. of 100. Neutralizing antibodies showed a good level, *i.e.*, 562 N.I. on intracerebral test and 10,000,000 on intraperitoneal test. The titer of complement-fixing antibodies was again 1:8. In group III (10^{-7}), resistance, neutralizing and complement-fixing antibodies were all negative. In group IV, treated with formolized vaccine, the amount of resistance was barely significant, with a R.I. of 59. On the contrary, neutralizing antibodies were high, with a N.I. of 1,000 on intracerebral test and 10,000,000 on intraperitoneal test; the complement-fixing antibodies were also high, with a titer of 1:8.

58th Day Test.—In group I (10^{-3}), resistance to infection was negative, but neutralizing antibodies by both intracerebral and intraperitoneal test remained high, with N.I. of 5,624 and 316,300 plus respectively. The titer of complement-fixing antibodies remained unchanged, 1:8. In group II (10^{-5}), no resistance was detected. On intracerebral test neutralizing antibodies had a level of 100 (N.I.) and on intraperitoneal test the N.I. was 100,000. The titer of the complement-fixing antibodies was 1:8. In group III (10^{-7}), all results were negative. In group IV, treated with formolized vaccine, resistance to intracerebral infection was negative; neutralizing antibodies were positive with a level of 1,779 (N.I.) by intracerebral test, and 316,300 by intraperitoneal test. The titer of complement-fixing antibodies was 1:8.

121st Day Test.—In group I (10^{-3}) the amount of resistance in this last test was found to be 100 (R.I.). Neutralizing antibodies were at about the same level as they had been in most of the other tests, that is, 562 (N.I.) by intracerebral test and 3,163,000 by intraperitoneal test. The titer of complement-fixing antibodies did not show any tendency to fall off at this stage, remaining at 1:16. In group II (10^{-5}) resistance to infection was negative. The level of neutralizing antibodies was 316 (N.I.) by intracerebral test and 3,163,000 by intraperitoneal test. The titer of complement-fix-

ing antibodies was 1:8. In group III (10^{-7}), all results were again negative. In group IV (formolized vaccine), no resistance was present. Neutralizing antibodies by intracerebral test had the lowest level except in the test on the 3rd day, when the N.I.

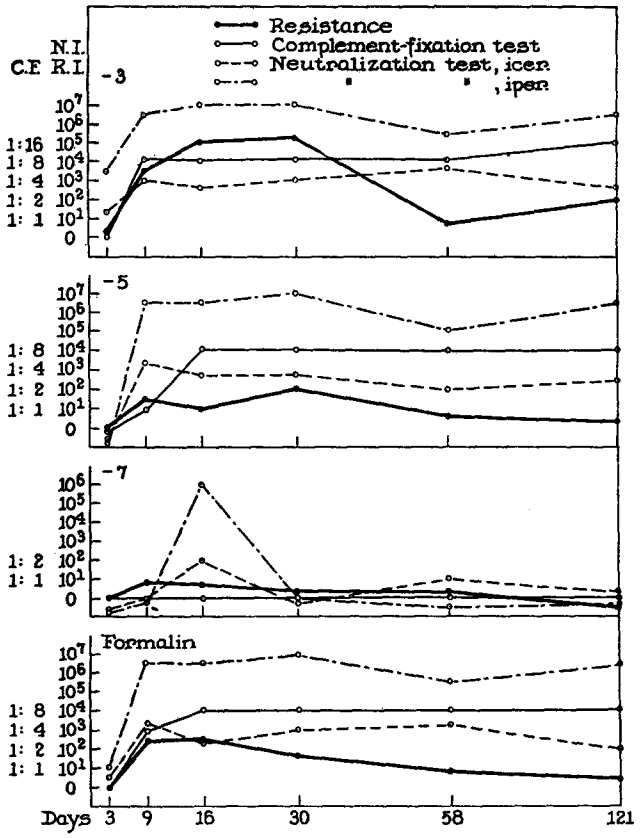

FIG. 1. Correlation between resistance to infection, complement-fixing and neutralizing antibodies in mice vaccinated with W.E.E. virus. C.F. = titer of complement-fixing antibodies. R.I. (resistance index) = degree of resistance (M.L.D.) shown by mice infected intracerebrally. N.I. (neutralization index) = degree of protection (M.L.D.) given by the immune sera. −3, −5, −7, and Formalin indicate the four different groups of vaccinated mice included in the experiment.

was 100. Tested intraperitoneally the level was 3,163,000. Complement-fixing antibodies were present to a titer of 1:8.

The results of this experiment are presented graphically in Fig. 1 and will be discussed together with those of the next experiment.

The second experiment was designed along the lines of the first—this time with St. Louis encephalitis virus—to compare at various intervals over a period

of 9 months the immunity, and neutralizing and complement-fixing antibodies in mice following vaccination with virulent or non-virulent virus.

Experiment 2.—740 W-Swiss mice, 60 to 75 days of age and weighing from 20 to 24 gm. at the beginning of the experiment, were employed. These mice were divided into four groups and treated as follows. Group I, 196 mice, were given one subcutaneous injection of 0.5 cc. of live virus in a dilution of 10^{-2}; group II, 193 mice, received one injection of live virus subcutaneously, 0.5 cc. in a dilution of 10^{-4}; group III, 199 mice, received one subcutaneous injection of 0.25 cc. of a 10 per cent formolized avirulent St. Louis vaccine daily, for 4 consecutive days, the total amount of vaccine given being then 1 cc. Finally, group IV, 152 mice, were set aside as untreated controls.

Following vaccination no mice died in any of the different groups. At intervals after vaccination mice selected from each group were tested for resistance by intracerebral injection of St. Louis virus and bled so that their sera might be tested for neutralizing and complement-fixing antibodies. Tests were carried out on the following days from the beginning of the experiment: 3, 7, 14, 31, 59, 91, 203, and 266.

The detailed results are given in Tables IV, V, and VI.

3rd Day Test.—None of the mice had developed any resistance, neutralizing or complement-fixing antibodies by the 3rd day.

7th Day Test.—Mice in group I (10^{-2}) showed a low but definite resistance, with a R.I. of 145. Neutralizing antibodies were negative and complement-fixing antibodies positive, with a titer of 1:4. Mice in group II (10^{-4}) had developed no resistance, neutralizing or complement-fixing antibodies. Mice in group III (formolized vaccine) had no resistance or neutralizing antibodies; the complement-fixing antibodies were recorded as negative, although a partial fixation occurred with the undiluted serum.

14th Day Test.—Mice in group I (10^{-2}) had a high degree of resistance with an index of 1,000,000. On the other hand, neutralizing antibodies were negative since the N.I. was 7 or less. The complement-fixing antibodies had a titer of 1:2. Group II (10^{-4}) showed no significant resistance or neutralizing antibodies, but some complement-fixing antibodies were present to a titer of 1:1. The group with the formolized vaccine was negative in all tests.

31st Day Test.—Mice in the 10^{-2} group still showed high resistance with an index of 426,000. The level of neutralizing antibodies remained unchanged and below significant value; the titer of complement-fixing antibodies was 1:4. Group II (10^{-4}) had developed complement-fixing antibodies in a titer of 1:2 but neither resistance nor neutralizing antibodies. The formolized vaccine group had developed complement-fixing antibodies in fair titer (1:4) but no resistance or neutralizing antibodies by this time.

59th Day Test.—The resistance shown by the mice in the 10^{-2} group was very low and barely significant (R.I. of 45). Neutralizing antibodies which in previous tests had remained below a N.I. of 10 now acquired a level of 46 N.I., which, although within the doubtful range, was the highest yet obtained in this group. This neutralization test was repeated and then it gave a N.I. of 129 which must be regarded as definitely positive. The titer of complement-fixing antibodies rose to 1:16. This test was also repeated and the same titer (1:16) was obtained a second time. Group II (10^{-4})

TABLE IV

Immunity to Intracerebral Inoculation of St. Louis Encephalitis Virus Following Vaccination

Time after vaccination	Groups of mice	Mortality of mice injected intracerebrally with St. Louis encephalitis virus								50 per cent lethal dose	Protection index
		Dilution of virus injected intracerebrally									
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}		
days											
3	10^{-2}				3/3*	3/3	2/3	0/3		7.25	2
	10^{-4}				3/3	3/3	1/3	0/3		6.75	6
	Formolized vaccine				3/3	3/3	2/3	0/3		7.25	2
	Controls				3/3	3/3	2/3	1/3		7.50	
7	10^{-2}	4/4	2/4	2/4	2/4	3/4	2/4			5.34	145
	10^{-4}	4/4	3/4	4/4	3/4	4/4	2/4			6.60	8
	Formolized vaccine			4/4	4/4	4/4	4/4	1/4		7.67	−1
	Controls				4/4	4/4	4/4	0/4	0/4	7.50	
14	10^{-2}	0/4	0/4	1/4	0/4	2/4	1/4			2.00	1,000,000
	10^{-4}		4/4	4/4	4/4	4/4	2/4			7.00	10
	Formolized vaccine			4/4	4/4	4/4	4/4			7.50	3
	Controls				4/4	4/4	4/4	2/4	0/4	8.00	
31	10^{-2}	3/4	0/4	1/4	0/4	2/4	1/4			2.87	426,600
	10^{-4}			4/4	4/4	4/4	4/4	0/4		7.50	10
	Formolized vaccine			4/4	4/4	4/4	4/4	1/3		7.75	6
	Controls				4/4	4/4	4/4	4/4	0/4	8.50	
59	10^{-2}	4/4	2/4	3/4	3/4	2/4	1/4	1/4	0/4	5.58	45
	10^{-4}			4/4	4/4	4/4	1/4	0/4	0/4	6.67	4
	Formolized vaccine			4/4	4/4	4/4	2/4	0/4	0/4	7.00	2
	Controls				4/4	4/4	2/4	1/4	0/4	7.23	
91	10^{-2}		3/4	1/4	1/4	3/4	1/4			4.53	501
	10^{-4}		4/4	4/4	3/4	2/4	0/4	0/4		5.77	29
	Formolized vaccine		4/4	4/4	4/4	4/4	2/4	0/4		7.00	2
	Controls				4/4	4/4	2/4	1/4	0/4	7.23	
203	10^{-2}		4/4	4/4	4/4	3/4	2/4	0/4		6.77	4
	10^{-4}		4/4	4/4	4/4	3/4	4/4	0/4		7.38	−1
	Formolized vaccine		4/4	4/4	4/4	4/4	4/4	1/4		7.67	−2
	Controls			4/4	4/4	4/4	3/4	0/4	0/4	7.34	
266	10^{-2}			3/4	4/4	3/4	2/4	0/4		6.56	28
	10^{-4}			4/4	4/4	4/4	3/4	1/4		7.50	3
	Formolized vaccine			4/4	4/4	4/4	3/4	0/4		7.34	5
	Controls				4/4	4/4	4/4	2/4	0/4	8.00	

* 3/3 = 3 of 3 mice injected died of St. Louis encephalitis.

TABLE V

Neutralizing Level of Sera from Mice Following Vaccination with St. Louis Encephalitis Virus

Time after vaccination	Groups of mice	Mortality of mice injected intracerebrally with mixtures of serum and virus							Degree of protection afforded by serum of vaccinated mice	
		Dilution of virus in mixture							50 per cent lethal dose	Neutralization index
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}		
days										
3	10^{-2}	4/4	4/4	4/4	1/4	0/4			5.67	4
	10^{-4}	4/4	4/4	4/4	1/4	0/4			5.67	4
	Formolized vaccine	4/4	4/4	4/4	2/4	1/4			6.23	1
	Controls		4/4	4/4	2/4	1/3	0/4		6.25	
7	10^{-2}	4/4	4/4	3/4	0/4	0/4			5.34	1
	10^{-4}	4/4	4/4	4/4	0/4	0/4			5.50	1
	Formolized vaccine	4/4	4/4	3/4	0/4	0/4			5.34	1
	Controls		4/4	4/4	0/4	0/4	0/4		5.50	
14	10^{-2}	4/4	4/4	4/4	4/4				>6.50	<7
	10^{-4}	4/4	4/4	4/4	4/4				>6.50	<7
	Formolized vaccine	4/4	4/4	4/4	4/4				>6.50	<7
	Controls		4/4	4/4	4/4	3/4	0/4		7.34	
31	10^{-2}	4/4	4/4	4/4	4/4				>6.50	<7
	10^{-4}	4/4	4/4	4/4	4/4				>6.50	<7
	Formolized vaccine	4/4	4/4	4/4	4/4				>6.50	<7
	Controls		4/4	4/4	4/4	3/4	0/4		7.34	
59	10^{-2}	4/4	4/4	3/4	0/4	0/4	0/4		5.34	46
	10^{-4}	4/4	4/4	4/4	2/4	0/4	1/4		6.22	6
	Formolized vaccine	4/4	4/4	4/4	3/4	0/4	0/4		6.34	5
	Controls		4/4	4/4	4/4	2/4	0/4	0/4	7.00	
91	10^{-2}	4/4	4/4	4/4	1/4	0/4	0/4		5.67	32
	10^{-4}	4/4	4/4	4/4	3/4	0/4	0/4		6.34	7
	Formolized vaccine	4/4	4/4	4/4	4/4	0/4	1/4		6.57	4
	Controls		4/4	4/4	3/4	3/4	0/4	0/4	7.17	
203	10^{-2}	4/4	4/4	4/4	4/4	1/4	0/4		6.67	5
	10^{-4}	4/4	4/4	4/4	3/4	0/4	0/4		6.34	10
	Formolized vaccine	4/4	4/4	3/4	2/4	0/4	0/4		5.77	37
	Controls		4/4	4/4	4/4	3/4	0/4	0/4	7.34	
266	10^{-2}		4/4	4/4	3/4	2/4	0/4		6.77	8
	10^{-4}		4/4	4/4	4/4	0/4	2/4		6.75	8
	Formolized vaccine		4/4	4/4	3/4	2/4	0/4		6.77	8
	Controls				4/4	4/4	1/4	0/4	7.67	

did not reveal any significant resistance or neutralizing antibodies, while the titer of complement-fixing antibodies was 1:8. The formolized vaccine group had no resistance or neutralizing antibodies but complement-fixing antibodies were positive, with a titer of 1:16.

91st Day Test.—Group I (10^{-2}): Although the R.I. was higher than in the preceding test (501), yet this degree of resistance could not be considered as very high. Neutralizing antibodies in the blood were low, with a level of 32 which places it in the doubtful range. The titer of complement-fixing antibodies was 1:8. Group II

TABLE VI

Summary of Protection Tests, Neuralization Tests, and Complement-Fixation Tests with St. Louis Encephalitis Virus

Treatment of mice	Test	Time after vaccination—days:							
		3	7	14	31	59	91	203	266
0.5 cc. active virus, 10^{-2} subcutaneously	Resistance: protection index	2	145	1,000,000	426,000	45	501	4	28
	Neutralization: neutralizing index	4	1	<7	<7	46	32	5	8
	Complement-fixation: titer of serum	0	1:4	1:2	1:4	1:16	1:8	1:2	1:16
0.5 cc. active virus, 10^{-4} subcutaneously	Resistance: protection index	6	8	10	10	4	29	−1	3
	Neutralization: neutralizing index	4	1	<7	<7	6	7	10	8
	Complement-fixation: titer of serum	0	0	1:1	1:2	1:8	1:8	0	1:4
0.25 cc. formolized vaccine × 4 subcutaneously	Resistance: protection index	2	−1	3	6	2	2	−2	5
	Neutralization: neutralizing index	1	1	<7	<7	5	4	37	8
	Complement-fixation: titer of serum	0	1:1?	0	1:4	1:16	1:4	0	1:4

(10^{-4}) mice showed a doubtful resistance to intracerebral infection (R.I. of 29). Neutralizing antibodies were negative and complement-fixing antibodies positive, with a titer of 1:8. The formolized vaccine group was negative for resistance and neutralizing antibodies; the complement-fixing antibody titer was 1:4.

203rd Day Test.—In the 10^{-2} group resistance and neutralizing antibodies were negative, while the complement-fixing antibodies had a low titer (1:2). In the 10^{-4} group resistance and antibodies were negative. In the formolized vaccine group, the level of neutralizing antibodies was doubtful (N.I. 37); resistance and complement-fixing antibodies were negative.

266th Day Test.—Resistance to infection and neutralizing antibodies were either negligible or negative in all groups. On the other hand, complement-fixing antibodies

were still present in each group. In mice of the 10^{-2} group the titer was particularly high, 1:16.

Fig. 2 presents a graphic summary of the experiment with St. Louis encephalitis virus.

The results of both experiments may be summarized as follows. In the test with W.E.E. virus, the three groups of mice injected subcutaneously with the large and the intermediate doses of live virus, and the formolized vaccine,

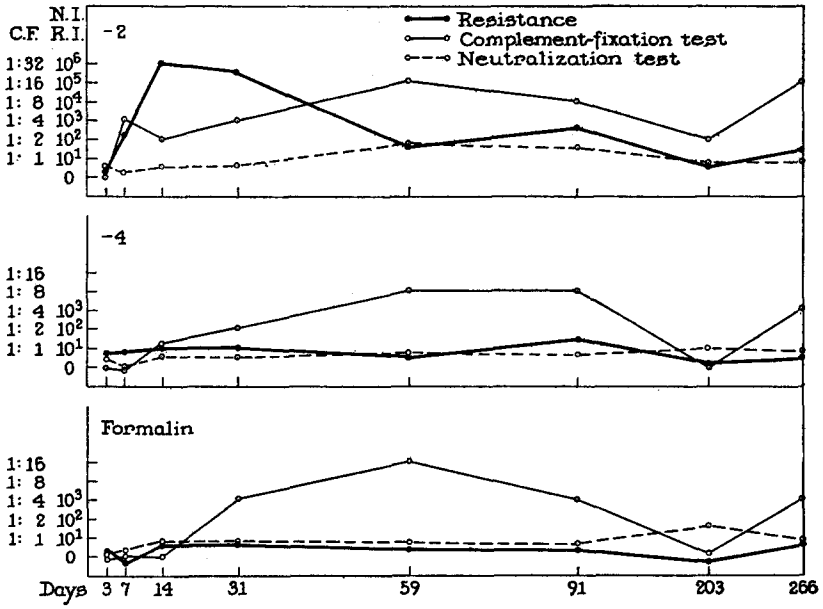

FIG. 2. Correlation between resistance to infection, complement-fixing and neutralizing antibodies in mice vaccinated with St. Louis encephalitis virus. C.F., R.I., N.I., the same as in Fig. 1. −2, −4, and Formalin indicate the three different groups of vaccinated mice included in the experiment.

all showed the same general relationship between intracerebral resistance and antibodies. Of mice receiving the live virus, 20 per cent died, in contrast to none among those receiving the formolized vaccine. Intracerebral resistance developed within 9 to 16 days and reached a maximum by 30 days. The titer of intracerebral resistance was highest in mice receiving the greatest amount of virulent virus, next in those receiving formolized vaccine, and least in those receiving the moderate dose of live virus. Intracerebral resistance in the three groups of mice decreased sharply within 58 days. Neutralizing antibodies behaved alike in all three groups. They reached a similarly high maximum level as soon as or sooner than intracerebral resistance and persisted undiminished

throughout the 4 month period of the experiment. Complement-fixing antibodies developed and persisted in like manner except that their time of development seemed to parallel resistance slightly more closely than that of neutralizing antibodies. On the other hand, mice injected subcutaneously with the smallest dose of live virus, 10^{-7} dilution, fatal to 0.7 per cent, developed little if any resistance. Neutralizing antibodies were detected at 16 days but not thereafter, and complement-fixing antibodies remained negative.

In short, we find in this experiment a fair, qualitative parallelism between intracerebral resistance and antibodies, with numerous slight, but generally consistent discrepancies. Thus, (1) neutralizing antibodies appear and reach maximum level sooner than resistance. (2) This maximum level of neutralizing antibodies is approximately the same, regardless of whether intracerebral resistance is high, medium, or low. (3) Neutralizing antibodies persist relatively unchanged in high level over long periods of time while intracerebral resistance is decreasing and even when it is no longer detectable. (4) Neutralizing antibodies may be found in instances in which the dose of virus has been too small to produce resistance. Complement-fixing antibodies, on the other hand, tend to appear somewhat more in conjunction with resistance but, once present, last much longer.

In the test with St. Louis encephalitis virus, mice injected subcutaneously with the large dose of virus, 10^{-2} dilution, remained well and developed high intracerebral resistance which reached a maximum at 14 days, declined sharply at 59 days, remained at this low level at 91 days, but became negligible at 203 and 266 days. Neutralizing antibodies first appeared in significant level at 59 days, when resistance showed its initial sharp drop. They remained at this same approximate level at 91 days but became negligible thereafter. Complement-fixing antibodies developed with resistance but did not reach maximum titer before 59 days, at which time resistance had decreased. They persisted at high levels throughout the 266 days of the experiment. The groups of mice injected subcutaneously with the small dose of virulent virus, 10^{-4} dilution, or with the formolized vaccine, remained well and reacted in the same general manner. No significant resistance developed at any time during the experiment, nor did neutralizing antibodies appear in significant titer. Complement-fixing antibodies, however, developed and persisted in about the same manner as in the first group, although at a slightly lower level. In short, little correlation was found in this experiment between resistance and antibody formation. Neutralizing antibodies appeared only in mice which had developed resistance but in them not until resistance had begun to decrease. Their level was low and their duration varied. Complement-fixing antibodies, on the other hand, developed regularly and persisted at high levels in all groups, whether or not resistance was demonstrable.

DISCUSSION

The trend of the data presented here differs but little from that of those already published (4, 8). Our finding of maximum levels of neutralizing antibodies in spite of different titers of intracerebral resistance should, perhaps, have been scrutinized further by testing diluted sera according to the procedure of Schlesinger, Morgan, and Olitsky (9). Still, the fact remains that antibodies persisted after resistance became negligible and antibodies appeared in instances in which intracerebral resistance was entirely lacking, as Morgan already had found with E. E. E. virus (10). Results of the experiments with St. Louis encephalitis virus differed quantitatively from previous results (8) in that more virus was required to produce resistance and antibodies appeared less readily after 12 weeks, and the phenomenon of falling resistance in the face of rising antibodies, though present, was less pronounced. This difference, we have reason to believe, is due to the present use of virus with a history of many intracerebral mouse passages rather than of freshly isolated strains (11). All in all, we believe that these and other reported experiments fail to warrant the assumption that if antibodies are found, intracerebral resistance must be present too.

Now if the slightest uncertainty obtains in these mouse tests as to the reliability of antibodies—neutralizing or complement-fixing—as an index of immunity, it becomes advisable, in our opinion, to be cautious in applying the antibody test—carried out with undiluted sera—as a criterion of resistance following vaccination of populations in the field. The mouse tests, though well controlled, are artificial: such studies would be of greater value if made in a more natural host under similar controlled conditions. Moreover, in the mouse experiments, the highly artificial intracerebral portal of entry was necessary, but in a more suitable experiment with a natural host, a more natural route might perhaps be used. In short, when faced with controlled though artificial experimental data which themselves are not convincing, one might well approach the problem of field testing of vaccines with caution and regard the presence of antibodies merely as evidence that some specific reaction has occurred, unless actual protection is proved.

SUMMARY

In mice vaccinated subcutaneously with different doses of virulent W.E.E. virus or with formolized vaccine, neutralizing and complement-fixing antibodies paralleled resistance to some extent yet appeared in groups in which resistance remained undetectable, persisted at a similar maximum level in spite of different titers of resistance, and after resistance had become negligible.

In mice vaccinated subcutaneously with different doses of virulent St. Louis encephalitis virus or with formolized vaccine, neutralizing and complement-

fixing antibodies bore little relation to resistance. Neutralizing antibodies appeared only in the group showing resistance but not until resistance was diminishing. Complement-fixing antibodies developed equally well in groups with or without resistance.

BIBLIOGRAPHY

1. Casals, J., and Palacios, R., *Science*, 1941, **94,** 330.
2. Webster, L. T., *J. Exp. Med.*, 1939, **70,** 87.
3. Olitsky, P. K., and Harford, C. G., *J. Exp. Med.*, 1938, **68,** 779.
4. Morgan, I. M., and Olitsky, P. K., *J. Immunol.*, 1941, **42,** 445.
5. Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27,** 493.
6. Casals, J., and Palacios, R., *J. Exp. Med.*, 1941, **74,** 409.
7. Pickels, E. G., *Rev. Scient. Instr.*, 1942, **13,** 93.
8. Hodes, H. L., and Webster, L. T., *J. Exp. Med.*, 1938, **68,** 263.
9. Schlesinger, R. W., Morgan, I. M., and Olitsky, P. K., *J. Am. Med. Assn.*, 1942, **119,** 618.
10. Morgan, I. M., *J. Exp. Med.*, 1941, **74,** 115.
11. Sabin, A. B., assisted by Duffy, C. E., Warren, J., Ward, R., Peck, J. L., and Ruchman, I., *J. Am. Med. Assn.*, 1943, **122,** 477.