EXPERIMENTAL STUDIES ON ENCEPHALITIS

IV. Specific Inactivation of Virus by Sera from Persons Exposed to Encephalitis, St. Louis Type, 1933

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With a Note on the Evaluation of the Results of Mouse Tests of Sera

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Experiments on the probable virus nature of the encephalitis prevalent in St. Louis during the summer of 1933 have been reported by Muckenfuss, Armstrong, and McCordock (1), and by Webster and Fite (2, 3). The former workers obtained a virus by inoculating brain tissue from fatal cases into *Macacus rhesus* monkeys; the latter recovered a similar virus by inoculating the brain tissue into special mice. A further step in establishing this virus as the specific cause of the epidemic was the demonstration by Webster and Fite of the inactivation of the virus by human convalescent sera (4). This finding, confirmed by Wooley and Armstrong (5), and Muckenfuss (6), will now be described in detail.

Technique

The effect of serum on the virus was studied by means of the familiar protection test. Virus in suitable dilution was mixed with equal volumes of undiluted serum and injected intracerebrally into mice. The survival time of the injected animals was taken as a measure of the protective action of the sera.

Strain 3 virus (2, 3) was used in all experiments since it proved similar to other strains in every respect tested. Brains from one or two mice prostrate 3 to 5 days following an intracerebral injection of mouse brain virus, were triturated with alundum, diluted one part by weight of brain virus to 50 parts by volume of hormone broth, pH 8.0, centrifuged 10 minutes at 1,000 R.P.M., and the supernatant made up further in serial tenfold dilutions with hormone broth. 0.3 cc. of each chosen dilution of the brain virus was mixed thoroughly with 0.3 cc. of serum

in a test tube. The preparations were incubated 2 hours at 37°C., left at room temperature 2 hours, and then each taken up in a 0.25 cc. tuberculin syringe with 0.25 inch 26 gauge needle and injected intracerebrally in 0.03 cc. amounts into four to six mice lightly anesthetized. Precautions for asepsis were observed throughout. The condition and survival time of the injected mice were recorded for 21 days.

The choice of mice proved a significant factor in maintaining a maximum and similar degree of infectivity of the virus and in reducing irregularities in survival time of mice within and between tests. The unselected Rockefeller Institute stock mice (2) maintained the intracerebral infectivity of the virus at varying levels between 3×10^{-6} and 3×10^{-8} gm. of infected mouse brain and gave irregular survival times. Selected Rockefeller Institute resistant (virus-susceptible mice) (2) and selected Swiss mice proved the animals of choice, since they maintained the infectivity of the virus at about 3×10^{-9} gm. of infected mouse brain and proved most uniform in their response to the virus. All mice were free of intercurrent infection and came from our own breeding stocks. They were 4 to 6 weeks old and weighed 18 to 22 gm. When, on rare occasions, a doubt arose as to whether an injected animal died of encephalitis, brain sections were taken and tissue passed to another animal to establish the diagnosis. To test the uniformity of the mice, four to six were injected with each virus-serum mixture. To test the infectivity of the virus in each experiment, virus plus non-contact serum mixtures were injected into mice in dilutions of 10^{-3} to 10^{-7} inclusive.

Tests were made as soon as possible after withdrawal of blood, since protective bodies against this virus were found to decrease in quantity with *in vitro* age of serum (3). Protective sera diluted beyond 1 to 10 failed to react; hence tests were made at a final serum concentration of 1 to 2. Uncontrolled variations between experiments were checked by testing a greater part of the unknown sera at least twice, the non-contact control sera three to nine times, the doubtful sera two to four times, and some of the protecting sera two to eight times.

The test as now standardized is made with one non-contact and one known protecting serum as controls, together with five to fifteen test sera. The control sera are prepared to give virus dilutions of 10^{-5} to 10^{-7} inclusive, and the test sera to give virus dilutions of 10^{-5} and 10^{-6}. Each dilution of virus-serum mixture is injected intracerebrally into four Swiss mice.

Standardization of Protection Test

The first few protection tests were devoted primarily to standardization of the technique including the proper manipulation and dilution of the virus, determinations of stability of infectivity of the virus and of protective activity of a given serum, variations in effect of different normal non-contact sera, and variability of results between tests. The protocol of Test 3, for example, published elsewhere (7), besides

demonstrating the protective effect of St. Louis convalescent sera, is an instance in which the titre of the virus was limited to the 10^{-4} dilution by the technique employed. Following these early tests, the titre of virus has remained uniform at the 10^{-7} dilution. Again, it was learned that the protective titre of the human convalescent sera decreased with *in vitro* aging in the same manner as in the case of hyperimmune monkey sera (3).

While this standardization of procedure was being accomplished, it appeared that certain sera protected the injected mice fully against 100 killing doses of virus, but that others protected to a considerably less degree. What, then, was to be the basis of evaluating the protective effect of unknown sera?

The problem, a statistical one, was studied by Dr. H. Muench, of the International Health Division of the Rockefeller Foundation, and the results are given below. The data comprised two to nine titrations on each of thirteen normal non-contact sera, and one to six titrations on each of 267 unknown sera. Of these, 184 were classed later as non-protective and 67 as protective.

NOTE ON EVALUATION OF RESULTS OF MOUSE TESTS OF ENCEPHALITIS SERA

In attempting to establish criteria for distinguishing positive (protecting) from negative (non-protecting) sera, the first procedure was to find out what happens to "unprotected" mice that receive virus. For this purpose there was available a set of test results on a series of known normal, non-contact sera which had been used repeatedly as controls for test runs.

It became apparent that conditions in the first six test runs were widely divergent from those in the seventh and following. The latter group was very uniform. For this reason all findings have been based entirely on the analysis of tests after the sixth run. The results in the normal non-contact serum group are summarized in Table I.

Mortality rates are not well determined by one or two deaths even in a group of about 150, so that the rates at 10^{-4} and 10^{-5} cannot be regarded as very definite. The mortality at 10^{-6} is based on only forty-seven mice and therefore it is quite unreliable. The factors most certain, at least for the first two dilutions, are the average time of death after inoculation and its standard deviation.

The tests were done as routine on groups of four or of six mice. Now the standard deviation of the mean time of death in a sample of six mice at 10^{-4} would be $0.7810/\sqrt{\ }$ or 0.3188 days from the mean value of the total. In other words, a mean time of death of 6.01 days would be twice the standard deviation

above the expected and this would occur accidentally in unprotected mice only some twenty-three times in 1000. Likewise, a mean time of death of 6.33 days would exceed the expected by three times the standard deviation; this could be accidental only thirteen times in 10,000.

For 10^{-5} dilutions and for four-mouse groups, the appropriate values are used. In this way it is possible to arrive at the criteria given in Table II. Here the + value corresponds to twice the standard deviation above the average; ++ is three times. Values as large as the latter or larger are almost certainly not due to chance and the corresponding sera cannot be called "negative."

This provides a criterion of what is not a negative serum. The question of what cannot be positive is still to be answered: it is not known whether positive

TABLE I

Virus dilution	No. of mice	No. dying	Mortality rate	Day of death	
				Average (mean)	Standard deviation
10^{-4}	148	147	0.9932	5.3537	0.7810
10^{-5}	146	144	0.9863	5.9514	0.9953
10^{-6}	47	37	0.7872	6.7027	0.8339

TABLE II

Average Survival Time in Days

Dilution	4 mice		6 mice	
	+	++	+	++
10^{-4}	6.13	6.53	6.01	6.33
10^{-5}	6.95	7.44	6.76	7.17

sera behave so differently that the average time of death is necessarily longer than in the case of negatives.

This cannot be answered from a study of known negative sera. It would be difficult to establish the distribution of longevity among "protected" mice from the results of tests of unknown sera, since these are evidently a mixture of positive and negative. A criterion based on mortality rates might be more definite if mortalities in protected and unprotected mice are sufficiently distinct and can be closely evaluated. In addition, such a criterion would be simpler and easier to apply than one based on length of life.

The problem then is to evaluate the two different mortalities at different virus dilutions and to find the point at which there will be the sharpest difference between them. Here there should be the least overlapping of criteria with consequent throwing of results into an "inconclusive" group.

In essence this is a study of binomial distributions. A group of tests, each test comprising the same number of mice among which there is a constant mortality rate would, in the long run, be distributed in a perfectly stable pattern. For example, 1000 groups of six mice each, with a mouse mortality of 0.9, would have the following most probable distribution:

6 deaths	532
5 "	354
4 "	98
3 "	15
2 "	1

while if the mortality were 0.2 we should expect:

5 deaths	2
4 "	15
3 "	82
2 "	246
1 death	393
0 deaths	262

As a starting point, we may assume that the distribution of test results (by number of mice dying) of any group of unknown sera is made up of a mixture of two such single distributions. One would be the scatter produced by a high mortality in unprotected mice which receive normal serum, which would have a peak at high numbers of deaths. The other, composed of test results in protected mice, would have a peak at a lower mortality. There are three unknown factors to be found: (*a*) the mortality rate of the unprotected group (= negative sera); (*b*) the mortality of the other group (protected mice or positive sera); (*c*) the proportion of each which makes up the entire number of tests; *i.e.*, the number of positive and of negative sera included.

These three factors may be derived from any actual array of test results mathematically in a perfectly straightforward way. Due to sampling variations, the actual distribution of a series of tests would hardly ever be exactly the expected one. For this reason the values we get for the factors are the best values, meaning those which will give the closest approximation to the series we are dealing with since we can hardly expect absolute concurrence.

In Table III is given, as an illustration, an actual distribution; in this case that of 110 tests, using four mice each, at a virus dilution of 10^{-5}. A survivor is a mouse alive on the 21st day after inoculation, when it was dropped from observation. Just below the actual number of tests in each mortality group is the hypothetical number calculated on the basis of the three factors obtained from the actual distribution. It will be seen that the correspondence is very close; the main difference is that there are actually rather more two-death results than might be expected.

If the calculated mortality rates are accurate, it would be expected that any other group of tests at the same dilution would give comparable results. The

third factor (distribution of positives and negatives) would vary from group to group depending on how many positive sera happened to be included in each.

As a matter of fact, the unprotected mortality is very constant. This is illustrated in Table IV which summarizes the values obtained by analyzing different sets of data. Four-mouse and six-mouse groups must be separated for study on a binomial basis. The two different strains of mice were also kept separate in

TABLE III

Results of Four-Mouse Tests at 10^{-5} (Swiss Mice)

No. of mice dying	4	3	2	1	0	Total
No. of tests (actual)	67.0	10.0	7.0	9.0	17.0	110.0
" " " (calculated)	66.5	12.2	3.7	11.1	16.5	110.0

Calculated rates on basis of: 0.9571 mortality in 79 negative tests.
0.1455 " " 31 positive "

TABLE IV

Mortality Rates Derived from Test Results on "Unknown" Sera

Dilution	Mouse strain	No. of tests	Calculated mortality rates		"Unprotected" rates from normal sera (Table I)
			"Protected"	"Unprotected"	
10^{-4}: 6 mice	VS	33	0.6504	1.0	
	Swiss	95	0.4485	0.9943	0.9932
	VS	27	0.1382	0.9841	
4 mice	Swiss	99	0.6954	1.0	
10^{-5}: 6 mice	VS	43	0.1712	0.9717	
	Swiss	92	0.2800	0.9760	0.9863
	VS	27	0.0639	0.9463	
4 mice	Swiss	110	0.1455	0.9571	
10^{-6}: 4 mice	VS	27	0.0739	0.9186	0.7872
	Swiss	67	0.1076	0.9153	

case there should be a difference in their reactions. Known normal sera were excluded.

Not only are the unprotected mortalities homogeneous within a given dilution; they agree quite well with the mortality rates in known normal sera as given in Table I. The average values of 0.9640 at virus dilutions of 10^{-5} and 0.9160 at 10^{-6} are probably very close to the true mortalities. This permits the statement that, in negative sera, the expected occurrence of surviving mice would be as given in Table V. Unprotected mortality appears to have a fixed value and the

occurrence of five or six survivors in a test group of six mice, for instance, would throw a serum out of all reasonable probability of being negative.

The answer to the search for a protected mortality is nothing like as clear. It appears that there is no such entity, but rather a band of mortalities covering a considerable range which may reflect variations in protective power in different sera. Such spreading is indicated by the wide fluctuations between the calculated values for different groups in the same dilution (Table IV). The number of positive tests in each group is comparatively small and the variations show the effects of sampling from a widely spreading field. Confirmation of this conception of protected mortality as a band instead of a point is found in the quite regular excess of actual over computed results around the 50 per cent mortality point, which is shown in Table III.

But the computed protected mortality must be a "centering constant" of some sort. That is, it must be somewhere around the middle of the band of mortalities, some values being higher and some lower. In that case the lower the computed

TABLE V

Expected Survivors with Negative Sera

Dilution	2 or more	3 or more	4 or more
10^{-5}: 6 mice	176	8	— per 10,000 tests
4 "	74	2	— " 10,000 "
10^{-6}: 6 "	842	97	6 " 10,000 "
4 "	378	23	1 " 10,000 "

value, the less spread is to be expected. If the computed mortality is 0.5, the scatter of actual values might conceivably be quite even from 0 to 1.0. But if its value is 0.1, then it cannot be an average of a series which runs heavily to high values.

From this viewpoint it is possible to discuss results at different virus dilutions and their bearing on setting up criteria.

Dilution 10^{-4}. Negative sera produce scarcely any survivors, but the mortality among protected mice is so high that many positive sera, especially if weakly protective, will show few or no survivors and so cannot be differentiated.

Dilution 10^{-5}. The mortality in negative sera is still high, so that a test running to three or more survivors is quite certainly positive (Table V). Protected mortalities are evidently at a lower level than at 10^{-4}, but it might be expected that a weakly protective serum would show a sufficient number of deaths to throw the results into the possibly negative category.

Dilution 10^{-6}. Although lower, unprotected mortality is still sufficiently high to make the occurrence of three or more survivors indicative of a positive

serum. Protected mortality now centers about a value around 0.1 or less and in all probability seldom is high enough to confuse protective with non-protective sera.

Results at virus dilutions of 10^{-6} are thus undoubtedly the most sensitive and delicate. Dilutions were not carried farther than this: at 10^{-7} protected mortality would be lower but so would unprotected, and there might be less certainty in differentiating between the two than at 10^{-6}. It is impractical to embody dilutions of 10^{-4} in the criterion as the two mortality rates are so nearly alike.

As finally set up, the criterion of positive protection on the basis of mortality is as follows:

TABLE VI

Criterion for Test Results

No. of survivors*	0	1	2	3	4	5	6
10^{-5}: 6 mice	−	−	±	+	+	+	+
4 "	−	−	+	+	+		
10^{-6}: 6 "	−	−	−	+	+	+	+
4 "	−	−	±	+	+		

* At 21 days.

A number of sera, by this criterion, are positive at 10^{-6} but negative at 10^{-5}. This would be expected in the case of weakly protective sera according to the interpretation of the analysis. The reverse result should be found very seldom and it has not, in fact, occurred so far. Confirmation of the conception of such positive-negative results as weak positives is seen in the fact that the same sera are almost invariably outside the range of possible negatives on the basis of average length of life (Table II).

A ± result (Table VI) must be regarded as inconclusive unless the result at another dilution is clearly positive.

The final criterion for use in practise may be established as follows:

+ at 10^{-5} and + at 10^{-6}: ++ (strongly positive)
− at 10^{-5} and + at 10^{-6} } : + (weakly positive)
± at 10^{-5} and + at 10^{-6} }
− at 10^{-5} and ± at 10^{-6}: ± (inconclusive, probably negative)
− at 10^{-5} and − at 10^{-6}: − (negative, no protection)

where the +, ±, and − values are determined from the number of survivors as given in Table VI.

Results with Sera from Persons with No History of Exposure to St. Louis Encephalitis

Sera were collected from September, 1933, to date from healthy non-contacts and from cases of encephalitis and poliomyelitis mostly in

hospitals in eastern and north central states.[1] The cases chosen were believed to have received careful clinical study, a matter of prime importance in determining the specificity of the reaction between serum and virus. The sera from the healthy non-contacts described below, when mixed with virus in dilutions of 10^{-4} to 10^{-6} and injected into mice, gave mortality rates of 99 per cent, 98 per cent, and 78 per cent, respectively, and average survival times in days of 5.3, 5.9, and 6.7 (Table I). The other sera, under similar conditions, gave mortality rates of 99 per cent, 96 per cent, and 91 per cent (Table IV), indicating that practically all mice given these sera plus virus in dilutions as low as 10^{-6} die in 5 to 7 days.

TABLE VII

Criteria for Protective Serum

Dilution of virus	Average survival time		No. of survivors	
	4 mice tests	6 mice tests	4 mice tests	6 mice tests
	days	*days*		
10^{-4}	6.53	6.33	—	—
10^{-5}	7.44	7.17	2	3
10^{-6}	—	—	3	3

Healthy Non-Contacts.—Sera from thirteen adults working in medical institutions in or near New York have been tested one to eight times with negative results (Table VII). Protocols of repeated tests with three of these sera are given in Table VIII, showing the uniformity of results subsequent to Test 14. In

[1] The authors are grateful for the generous cooperation of physicians and hospital authorities in calling our attention to cases of encephalitis, collecting and sending sera, and supplying clinical data. Mentioning all these collaborators by name is regarded as impractical. Most of the sera from the cases of chronic encephalitis with Parkinson sequelae were sent by Dr. David Marine, Montefiore Hospital, New York; Dr. C. H. Andrewes, National Institute for Medical Research, London; Dr. E. A. Carmichael, National Hospital Queen Square, London; Drs. Josephine B. Neal and Frederick Tilney, Neurological Institute, New York; and Dr. M. W. Raynor, Bloomingdale Hospital, White Plains, N. Y. Professors R. Inada and K. Kakinuma sent sera from twelve cases of Japanese B encephalitis, and Professor I. Takaki sent sera from three similar cases plus samples of hyperimmune sera and virus A and B in glycerine. Dr. G. F. Kempf forwarded sera from cases of meningoencephalopathy in Indianapolis.

TABLE VIII

Repeated Tests with Three Sera from Healthy Non-Contacts

Serum	Test	Date	Virus-serum dilution				
			10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
		1933					
C	13	Nov. 23	4, 5, 6, 7	5, 5, 5, 6	5, 6, 6, S		
	14	" 29	4, 4, 4, 6	4, 5, 6, 7	6, 6, 9, S		
	16	Dec. 20		4, 5, 5, 5, 6, 6	5, 5, 5, 6, *, *		
		1934					
	28	Apr. 25		5, 5, 5, 6, 6, 6	6, 6, 6, 6, 7, 7		
	43	Dec. 4		6, 6, 6, 6	6, 6, 6, 7	7, 7, 7, 8	8, S, S, S
		1935					
	45	Jan. 18		5, 5, 5, 6	6, 6, 6, 7	6, 6, 7, 8	6, 6, 11, 12
	47	Mar. 7		5, 5, 6, 6	6, 6, 6, 6	5, 7, 7, 7	8, 8, S, S
	48	" 12		5, 5, 5, 5	5, 5, 5, 6, 6, 7	6, 6, 7, 7, 7, 9	7, 8, 8, 9
	49	May 8		6, 6, 6, 6	5, 6, 6, 7	6, 6, 7, 7	7, 8, S, S
	50	" 24		5, 5, 6, 6	6, 6, 7, 7	7, 7, 9, 9	8, 10, S, S
	51	June 5		5, 6, 6, 6	5, 6, 7, 7	5, 6, 6, 7	6, 6, 7, 7
	52	" 10		*, 5, 5, 6	5, 5, 6, 6	5, 6, 6, 7	6, 7, 8, 8
		1934					
J	28	Apr. 25		5, 6, 6, 6, 6, 6	6, 6, 6, 8, 8, 8		
	36	Oct. 10		5, 5, 5, 6, 6, *	5, 5, 5, 6, 6, 6		
	39	" 25		4, 5, 5, 5	4, 5, 5, 7	6, 6, 7, 7	
		" 30		4, 6, 6, 8	6, 6, 6, 6		
		1935					
	46	Jan. 31		5, 6, 6, 6	5, 6, 6, 6	6, 7, 8, 8	6, 8, 9, S
		1933					
H	12	Nov. 16	4, 5, 5, 6	5, 5, 6, 6	5, 6, 6, 6	5, 5, 7, 9	
		1934					
	28	Apr. 25		6, 6, 6, 6, 6, 7	6, 6, 6, 6, 7, 7		
	33	Sept. 25		5, 5, 5, 5, 5, 5	5, 5, 5, 6, 6, 9		
	41	Nov. 12		5, 5, 5, 5	5, 5, 7, 7	7, 7, 7, 7	7, 7, 7, 7

S = mouse remained well 21 days.
Blank spaces indicate dilution not tested.
* = mouse died from trauma.

contrast to these findings reported in 1933 (4), Wooley and Armstrong state (1934) (5) that eleven of 113 sera (9.7 per cent) from individuals with no special contact with cases gave strong or moderate protection. This discrepancy will be discussed later.

Chronic Lethargic Encephalitis with Parkinson Sequelae.—Webster and Fite reported in 1933 (4) that sera from sixteen cases of lethargic encephalitis with Parkinson sequelae failed to protect mice against the virus. Similar results were reported by Levaditi, Schoen, and Levaditi (1934) (8) on sera from four cases. Wooley and Armstrong, however, state (1934) (5) that sera of four of twenty-nine (13.7 per cent) tested cases showed strong protection.

We have now studied a total of twenty-seven sera from typical cases. The results are negative. Nine cases gave a history of onset following an attack of influenza in 1918 to 1920. All have had Parkinson sequelae for 1 to 15 years.

Typical, Acute Encephalitis (Economo).—The negative effect of sera from the twenty-seven chronic cases of outspoken encephalitis of the Economo type raised the question of whether sera from the same disease in the more acute stages would show protective substances against the St. Louis virus. Realizing that a clinical diagnosis of this disease in the acute stages is often difficult, an effort was made to obtain sera from both typical and atypical cases.

Eight typical acute cases were tested and found to be negative. All gave histories of lethargy, ptosis or diplopia, tremors, and mononuclear pleocytosis of the spinal fluid. Two had beginning mask facies. Blood was drawn for testing 2, 2, 4, 6, 10, 12, 16, and 26 weeks after onset.

Atypical Primary Encephalitis.—Sixty-nine cases of atypical primary encephalitis were also tested with negative results. Symptoms and signs varied widely but were of such a nature in each case as to warrant a clinical impression of "encephalitis." Three cases occurred in 1932, twenty-eight in the autumn of 1933, thirty in 1934, and eight in 1935. Sera for testing were obtained 2 to 52 weeks after onset of illness. Twenty-four were from New York, two from New Jersey, six from Connecticut, two from Massachusetts, one from Pennsylvania, five from Maryland, four from Virginia, one each from Florida and Alabama, five from Illinois, four from Missouri, one from Indiana, eleven from Ohio, and two from California.

Japanese B Encephalitis.—We found (1934) (9) that sera from fifteen cases of Japanese B encephalitis did not protect against the St. Louis virus. These results were surprising in view of the reported similarity in epidemiological and clinical features of the Japanese and St. Louis diseases (10).

Sera were received from three persons supposed to have had the disease in August, 1924, aged 50, 51, and 60 years, and from nine persons convalescent from the August, 1933, epidemic, aged 17, 17, 20, 26, 33, 46, 53, 62, and 65 years. In these cases fever subsided 6 to 12 days after onset of symptoms. Sera were likewise received from three additional persons convalescent from the 1933 epidemic. Blood specimens were drawn from the 1924 cases about 10 years after onset and from the 1934 case about 4 months after onset. Specimens were tested after

about 6 weeks' aging *in vitro*. Each serum was tested twice with negative results. Kodama has recently confirmed these findings using Strain 3 virus and serum from convalescents in Japan (13). Further negative tests on sera from animals immunized with Takaki's B and A viruses (11), and futile attempts to establish the B virus from glycerinized material are reported below.

Postinfectious Encephalitis.—Sera from ten cases of postinfectious encephalitis were tested and found negative. Three cases were encephalitis following herpes zoster; two additional cases of herpes zoster without encephalitis were found negative. Four cases were post-measles encephalitis, two post-pertussis encephalitis, and one encephalitis complicating varicella.

Meningoencephalopathy, Indianapolis.—Sera from two convalescents from meningoencephalopathy at Indianapolis described by Kempf, Gilman, and Zerfas (12) did not protect against the virus (4).

Australian "X" Disease.—Serum from a case reported to have had "X" disease was found negative.

Poliomyelitis, Los Angeles, 1934.—Sera from eleven cases of poliomyelitis in Los Angeles, 1934, were obtained 4 weeks and 8 months after onset of symptoms. All were negative.

In summary, none of the 156 tested sera from persons believed to have had no exposure to St. Louis encephalitis has shown protective antibodies against the virus. Wooley and Armstrong's series of similar cases (*a*) lethargic encephalitis, (*b*) unclassified encephalitis, acute meningoencephalitis, epidemic meningoencephalopathy, poliomyelitis, traumatic encephalitis, postinfectious encephalitis, Jacksonian epilepsy, (*c*) other diseases not neural, and (*d*) normal controls with no special contact, gave positives at the rates of 13.7 per cent of 29, 11.4 per cent of 34, 13.1 per cent of 99, 9.7 per cent of 113, respectively, or 11 per cent of the total 275.

Results with Sera from Animals Immunized with Known Viruses

The protection test was used not only to determine the specificity of the reaction between serum and virus but to discover a serological relationship between the St. Louis and other known viruses. Sera from immunized animals known to protect against the homologous virus[2] were tested against the St. Louis virus. The results were nega-

[2] The herpes sera were supplied by Dr. Margaret Holden, and the Japanese encephalitis A and B sera by Professor I. Takaki. Dr. C. TenBroeck sent us the equine encephalomyelitis sera and carried out the protection tests with the encephalomyelitis virus. We are indebted to Dr. P. J. du Toit, Pretoria, South

tive, indicating no serological relation between this and the following viruses: herpes, Japanese B and A, poliomyelitis, equine encephalomyelitis, vesicular stomatitis, louping ill, blue tongue, and fox encephalitis.[2]

Herpes.—Sera from six rabbits immunized with the E. L. 1 Perdrau strain were tested with negative results.

Japanese Encephalitis A and B.—One specimen each of anti-A and anti-B goat serum was received for testing. The anti-A serum run on two occasions did not protect against the St. Louis virus. The anti-B serum contained preservative and gave some protection against St. Louis encephalitis, louping ill, and yellow fever viruses. When injected without virus into mice, it induced transient convulsions.

Besides sera, specimens of virus A and virus B were received. One lot of B virus in rabbit brain preserved in glycerin was received Feb. 8, 1934, and injected into six white-face mice and three 800 gm. rabbits intracerebrally, intracutaneously, and intracorneally. The animals remained well. Later, the material was injected into three 800 gm. rabbits intracorotidly, into three subdurally, into three intracerebrally, into two intratesticularly, and into two intravenously. All remained well. A second lot of B virus and a specimen of A virus were received May 11, 1934, and each injected subdurally, intracerebrally, intracarotidly, and intratesticularly into a total of twenty-five 800 gm. rabbits. No virus could be demonstrated.

Poliomyelitis.—Sera from two *Macacus rhesus* monkeys immunized by repeated injections of the M. V. virus did not protect against the St. Louis virus.

Equine Encephalomyelitis.—Sera from a rabbit immunized with the western strain and from a rabbit and horse immunized with the eastern strain were reported negative by Cox and Fite (14). Later tests have now been made with sera from a guinea pig immunized to the western strain and from a horse immunized to the eastern strain. These likewise showed no protective effect against the St. Louis virus. Here it should also be stated that sera from monkeys immunized with the St. Louis virus failed to protect guinea pigs against the two strains of encephalomyelitis virus (14).

Vesicular Stomatitis.—Serum from a rabbit immunized with the New Jersey strain and one from a rabbit immunized with the Indiana strain were reported negative by Cox and Fite (14). Serum from a monkey immunized with the St. Louis virus failed to protect guinea pigs against the vesicular stomatitis strains (14).

Louping Ill.—Serum from a horse convalescent from an experimental infection of louping ill showed no protective activity against the virus. In addition, sera

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Africa, for the louping ill and blue tongue sera, and to Dr. R. G. Green for the fox encephalitis sera.

from four persons showing specific protective substances against the louping ill virus (15) failed to protect against the St. Louis virus. Finally, anti-St. Louis monkey serum did not protect mice against the louping ill virus.

Blue Tongue.—Serum from sheep immunized with the routine passage virus said to be antigenically similar to all other known strains did not protect against the St. Louis virus.

Fox Encephalitis.—Sera from a normal fox and a fox immunized with fox encephalitis virus were negative.

Results with Sera from Persons with History of Exposure to the St. Louis Type of Encephalitis[3]

St. Louis Cases, 1933.—Sera from thirty-six cases on the encephalitis wards of the St. Louis City Isolation Hospital during the epidemic in August and September, 1933, were obtained for study. Eight cases diagnosed as not encephalitis of the prevailing type showed no protective properties in their sera. The remaining twenty-eight cases presented the clinical picture characteristic of the majority of cases of the epidemic (10); namely, high incidence among adults (17, 18, 22, 23, 28, 30, 31, 33, 36, 37, 38, 40, 49, 49, 50, 53, 55, 55, 60, 62, 64, 68, 75, and 75 years), systemic reactions including fever, headache, and vomiting, stiff neck and tongue tremors, and a lymphoid cell pleocytosis of spinal fluid. 82.5 per cent of the twenty-eight cases showed protective properties against the virus (Table IX). The negative cases were aged 23, 36, 40, 49, and 75 years. If the cases are grouped according to whether the first bleeding was made before or after the 14th day from onset, the seventeen sera drawn on the 14th day or later all protect, while of the eleven drawn less than 14 days from onset, only six, 54.5 per cent protect.

The negative effect of the "early" sera is not understood. The

[3] Sera from St. Louis cases of encephalitis, 1933, were obtained through the courtesy of Drs. R. S. Muckenfuss, J. Eschenbrenner, Jr., and S. Weisman. Dr. P. F. Stookey sent us sera from cases of encephalitis of the St. Louis and other types occurring in Kansas City, and brain tissue from fatal cases. Dr. H. D. McIntyre forwarded sera from sixteen cases of encephalitis in Cincinnati, 1933 and 1934. Sera from five cases of encephalitis in Paris, 1932, were sent by Dr. W. E. Conklin; later, sera from fifty cases of encephalitis of the St. Louis type in Paris, Danville, and Canton, Illinois, were sent by Dr. W. H. Tucker through the courtesy of Dr. F. J. Jirka. The Indiana State Board of Health kindly supplied sera from five cases of the disease.

view was taken in an earlier report that insufficient time had elapsed for the development of antibodies (4), but this is now untenable since second bleedings from these same cases 4 months after onset likewise gave negative results. Such explanations as inability of certain persons to elaborate demonstrable antibodies or incorrect diagnoses

TABLE IX

Sera Tested against Encephalitis Virus (St. Louis Type)

Diagnosis	No. tested	No. positive	Per cent positive
Healthy non-contacts	13	0	0
Chronic encephalitis Economo	27	0	0
Acute " "	8	0	0
Atypical, suspected encephalitis	69	0	0
Japanese B encephalitis	15	0	0
Postinfectious encephalitis	10	0	0
Meningoencephalopathy, Indianapolis	2	0	0
Australian "X" disease	1	0	0
Poliomyelitis, Los Angeles, 1934	11	0	0
Immunized animals	21	0	0
St. Louis not encephalitis, 1933	8	0	0
" " encephalitis, 1933	28	23	82.5
Kansas City encephalitis, 1933	4	3	75.0
Cincinnati " 1933	1	1	100.0
New York " 1933	2	2	100.0
Paris, Ill. " 1932	12	11	91.6
" " " 1934	9	6	66.6
Danville, Ill. " 1934	21	14	66.6
Canton, Ill. " 1934	6	3	50.0
Indiana " 1934	5	5	100.0
Cincinnati " 1934	4	2	50.0
California " 1934	3	1	33.3

appear most probable, even though the negative reactors were concentrated in the less than 14 day group.

These results are not in complete agreement with those of Muckenfuss (6). Fifteen of the thirty-four sera were tested by both workers with agreement in the case of eleven and in the case of four, positive results by Muckenfuss and negative by ourselves, a disagreement of 26.6 per cent. We tested two of the disputed sera three times on two specimens; the other two, once only.

Kansas City Cases, 1933.—Encephalitis, so conspicuous in St. Louis during August and September, 1933, was present at the same time in Kansas City. Here, however, fewer cases were recorded and the clinical pictures were more varied. From one of two tested fatal cases, a virus was recovered similar to that obtained from fatal cases in St. Louis (2). At the same time sera from four outspoken cases were tested against the St. Louis virus and three found positive (4).

Ohio Case, 1933.—A white male, aged 41, contracted encephalitis August 30, in Cincinnati, of a type similar to the St. Louis disease. His serum, collected and tested 1 year later, October, 1934, neutralized the virus.

New York Cases, 1933.—Recognition of cases of the St. Louis type of encephalitis naturally became more difficult in places remote from the epidemic. Tests were made, therefore, on sera from various forms of atypical encephalitis, sixteen in all, from September to the end of December, 1933. Of these, only two were considered clinically as possible cases of the St. Louis disease.

No. 47. M. S., a white male, aged 27, was admitted to St. Luke's Hospital, Dr. Frissell's service, on Sept. 9, 1933, with a 3 day history of nausea, vomiting, fever, malaise, generalized throbbing headache, and no history of having been to St. Louis or having come in contact with anyone from St. Louis. On admission his temperature was 101°F. There was a slight leucocytosis, 10,900, 80 per cent polymorphonuclears. The Wassermann reaction was negative. The other findings were chiefly neurological and consisted of absent abdominal reflexes, hyperactive tendon reflexes, medium coarse nystagmus, soreness but no stiffness of neck, a suggestive Kernig, and weakness of flexion of right arm. Spinal fluid, pressure 240; cells 144 (lymphoid cells 96 per cent), globulin +, protein 82, sugar 58. Within a week's time all symptoms and signs had returned to normal, save for hyperactivity of tendon reflexes and persistence of nystagmus. The cell count of the spinal fluid had dropped to 50 in 3 weeks, and to 15 in 6 weeks.

Dr. Frissell called this case to our attention as possible St. Louis encephalitis and tests on sera drawn 2 and 6 months after onset, ten in all, were strongly positive (4), (Table X).

No. 63. B., a white female, aged 39. After 3 weeks' vacation in Kentucky, returned to New York with severe headache. She was seen by Dr. H. T. Chickering on Sept. 12, 1933. Her temperature was 103°F. She complained of severe headache, different from any other, accompanied by chills. This condition cleared up rapidly and she was discharged as well on Sept. 25.

Dr. Chickering mentioned this case as possible mild encephalitis of the St. Louis type. Two tests on serum drawn 11 weeks after onset were positive (4), (Table X).

Paris, Illinois, Cases, 1932.—In searching for a possible relationship between the 1933 St. Louis and previous outbreaks of encephalitis, mention has been made of protection tests run on sera from cases of Economo encephalitis with onset following influenza in 1918, and meningoencephalopathy in Indianapolis. The results were negative. The case of the outbreak of encephalitis in Paris, Illinois, in 1932, however, was different. Encephalitis appeared in Paris, Illinois, a

TABLE X

Positive Protection of Sera from New York, 1933, Cases against the St. Louis Virus

Serum No.	Test No	Date of test	Virus-serum dilution				Diagnosis
			10^{-3}	10^{-4}	10^{-5}	10^{-6}	
		1933					
47	8	Nov. 2	6, 7, S, S	6, S, S, S	S, S, S, S	S, S, S, S	Acute encephalitis (St. Louis?)
46	8	" 2	4, 4, 4, 6	5, 5, 5, 5	5, 5, 6, 6	6, 6, 7, 9	Chronic encephalitis (Parkinson)
47	12	" 16	5, 6, 7, 7	7, 7, 9, 10	S, S, S, S	S, S, S, S	Acute encephalitis (St. Louis?)
H			4, 5, 5, 6	5, 5, 6, 6	5, 6, 6, 6	5, 5, 7, 9	Normal non-contact
63	13	Nov. 23	5, 6, 6, 7	6, 7, 7, 7	S, S, S, S	S, S, S, S	Acute encephalitis (St. Louis?)
58			5, 5, 5, 7	5, 5, 6, 8	5, 5, 6, 6	6, 7, 9, 10	Chronic encephalitis (Parkinson)

S = mice remained well 21 days.

community of about 9000 persons, in July and August, 1932 (16, 10). Twenty-seven persons were affected, aged 33 to 80, with only three under 50. The mortality was close to 50 per cent, but convalescents were relatively free of sequelae. The outstanding symptoms recorded were headache, fever, nausea and vomiting, diplopia, and delirium or stupor. Facies were set, neck rigid, and tongue tremorous. Sera from five convalescents drawn 15 months after onset were tested against the St. Louis virus. Four of the five gave definite protection (4), (Table XI). Later, February, 1935, $2\frac{1}{2}$ years after the outbreak,

TABLE XI

Protection Tests on Sera from Cases in Paris, Illinois, 1932 and 1934

Serum	Test	Virus-serum dilution				Result
		10^{-4}	10^{-5}	10^{-6}	10^{-7}	
C-non-contact	47	5, 5, 6, 6	6, 6, 6, 6	5, 7, 7, 7	8, 8, S, S	0
Monkey immune		6, 6, 8, 8	6, 7, 8, S	S, S, S, S	S, S, S, S	+
246 Paris '31		4, 5, 5, 5	6, 6, 7, 9	6, 7, 7, 9		0
242 " '32		9, 10, S, S	S, S, S, S	S, S, S, S		++
248 " '32		9, 9, 9, S	S, S, S, S	S, S, S, S		++
247 " '32		7, 9, 9, 9	S, S, S, S	S, S, S, S		++
241 " '32		5, 5, 8, S	6, 7, 9, 9	S, S, S, S		+
239 " '34		6, 6, 6, 7	6, 8, 9, S	S, S, S, S		+
244 " '34		6, 7, 7, 8	6, 6, 7, 7	S, S, S, S		+
240 " '34		6, 7, 8, 8	5, 7, 9, 9	10, S, S, S		+
243 " '34		6, 6, 6, 7	6, 6, 9, 9	6, 7, 7, 8		0
245 " '34		4, 5, 5, 6	6, 6, 6, 6	6, 6, 6, 6		0
C-non-contact	48	5, 5, 5, 5	5, 5, 5, 6, 6, 7	6, 6, 7, 7, 7, 9	7, 8, 8, 9	0
251 Paris '32		6, 8, 8, 8	S, S, S, S, S, S	S, S, S, S, S, S		++
250 " '32		5, 7, 8, 8	6, 9, S, S, S, S	S, S, S, S, S, S		++
257 " '32		7, 8, 8, 9	9, 9, 9, S, S, S	S, S, S, S, S, S		+
252 " '32		5, 5, 8, 8	5, 6, 7, 7, 8, 8	6, S, S, S, S, S		+
253 " '32		5, 5, 6, 7	6, 6, 6, 7, 8, 8	9, S, S, S, S, S		+
256 " '32		5, 5, 5, 5	5, 6, 6, 7, 8, 8	8, 8, 8, 8, 8, S		0
258 " '34		7, 8, 8, 8	9, S, S, S, S, S	S, S, S, S, S, S		++
249 " '34		5, 7, 8, 9	9, S, S, S, S, S	8, S, S, S, S, S		++
255 " '34		6, 7, 7, 8	7, 8, 8, 8, 8, 8	7, S, S, S, S, S		+
254 " '34		6, 6, 8, 8	6, 7, 7, 9, 9, 9	7, 8, 8, S, S, S		+

S = mice remained well 21 days.

Blank spaces indicate dilution not tested.

additional sera were obtained from ten of the cases, including three of the five previously tested. Nine of the ten or eleven of the total twelve (91.6 per cent) were positive (Table XI). Wooley and Armstrong found ten of eleven (90.9 per cent) positive (5).

Illinois, Ohio, and Indiana Cases, 1934.—The reappearance of encephalitis in the north central states during the summer of 1934 afforded another opportunity to study the specificity of the serum reaction. Cases clinically resembling the St. Louis type were occurring in localized outbreaks in Illinois and sporadically in Ohio and Indiana.

Paris, Illinois.—Nine convalescents, aged 10, 15, 33, 35, 38, 44, 68, 70, and 76 years, were bled 5 to 6 months after onset of symptoms and their sera tested against the St. Louis virus. Six of the nine specimens, 66.6 per cent, gave definite protection. The negative cases were aged 10, 38, and 70 years. If the 10 year old case is omitted, the percentage of positives becomes 75.

Danville, Illinois.—Twenty-one convalescents, aged 9, 10, 15, 16, 19, 20, 22, 26, 26, 27, 30, 30, 31, 31, 32, 33, 36, 38, 40, 59, and 60 years respectively, were tested 2 months after onset of symptoms. Fourteen of the twenty-one, 66.6 per cent, showed protective properties in their sera. The negative cases were aged 9, 10, 15, 16, 22, 38, and 40 years respectively. If the four cases aged 16 years or less are omitted in the count, the ratio of positive reactors is increased to fourteen of seventeen, or 82.5 per cent.

Canton, Illinois.—Sera were obtained from six convalescents aged 7, 12, 51, 58, 75, and 80 years. Those aged 58, 75, and 80 years protected (50 per cent). If the 7 and 12 year cases are omitted from the count, the ratio of positives becomes three of four (75 per cent).

In summary, of thirty-six Illinois 1934 cases tested, seven were aged 16 years or less and did not protect; of the twenty-nine remaining however, twenty-three (79.3 per cent) were positive.

Indiana Cases.—Sera from five cases drawn at least 4 weeks after onset were tested and found to protect against the virus.

Ohio Cases.—Sera from two of four typical cases drawn 1 to 3 months after onset neutralized the virus.

California Cases, 1934.—Sera from one of three cases protected against the St. Louis virus.

DISCUSSION

The specificity of the encephalitis protection test is indicated by the present work and by reports of Wooley and Armstrong and Muck-

enfuss. The latter workers, however, record a 10 to 30 per cent incidence respectively of positive reactors among groups of persons with no special exposure to the St. Louis disease. This discrepancy is due either to differences in batches of sera tested, or more probably to differences in technique and criteria for testing. Our procedure renders it unlikely that a negative serum would be called positive but admits the possibility of a few weakly positive sera being called negative. Be that as it may, the specificity of the serum-virus reaction is further evidence that this virus is the specific agent responsible for the human disease, and finally, that the virus is different and the encephalitis in human beings is serologically distinct from others previously described.

Knowledge that antibodies persist for $2\frac{1}{2}$ years in the blood of convalescents is an aid in searching for an endemic focus and in mapping out the time and space spread of the virus. Thus far, we know that the disease appeared in Paris, Illinois, in 1932, and was present in 1933 and 1934 in the north central states and New York.

CONCLUSIONS

1. A protection test for measuring serological protective properties against the encephalitis (St. Louis type) virus is described.

2. Normal non-contact sera and sera from persons supposed to have had no exposure to the disease do not protect against the virus. 82.5 per cent of sera from tested St. Louis encephalitis convalescents and at least 66 per cent of sera from tested persons thought to have had the disease do show protective properties.

3. The protective activity of sera is maintained for at least $2\frac{1}{2}$ years after onset of the disease. *In vitro* aging of serum decreases its activity.

4. Protection tests indicate that the virus was present as early as 1932 in Paris, Illinois, spread through the north central states and reached New York in 1933, and was again active in the north central states in 1934.

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