ELECTROPHORETIC STUDY OF ANTIVIRAL SERA

By HILARY KOPROWSKI, M. D., GILBERT RICHMOND, AND DAN H. MOORE, PH.D.

(From the Section of Viral and Rickettsial Research, Lederle Laboratories Division, American Cyanamid Company, Pearl River, and the Electrophoresis Laboratory, College of Physicians and Surgeons, Columbia University, New York)

(Received for publication, January 14, 1947)

Reports of electrophoretic analyses on sera of animals immunized against egg albumin (1) and various bacterial agents or products (2–13) indicate that the antibody activity is associated with the slower moving fractions of the serum, such as γ-globulin in the case of some of the antipneumococcic sera (3, 4, 9, 11), or a new "T" component which migrates between the β- and γ-globulins in the case of hyperimmune sera against *Corynebacterium diphtheriae* (6), *Clostridium tetani* (7, 8, 10), and certain other bacteria (9). Electrophoretic separation of syphilitic sera indicates that the fraction responsible for positive serological reaction migrates between β- and γ-globulins (14–16). In contrast to the extensive studies done on antibacterial sera, limited data are available on electrophoretic analyses of antiviral sera, even though the results with the latter appear to differ, indicating for example that in some antiviral sera antibodies may be recovered from all fractions, including albumin (17–19).

In view of the above information and the fact that the serum fraction containing γ-globulin has been found to be rich in viral antibodies (20), and that the γ-globulin itself has been used successfully to modify or prevent such viral diseases as measles (21–26) and infectious hepatitis (27–29), a more detailed electrophoretic study of antiviral sera seemed to be indicated. It was hoped that such a study would furnish some evidence as to whether the clinical use of γ-globulin in viral infections in immunologically valid, or whether the antiviral property of immune sera is diminished by fractionation into specific components. In addition, the study was planned to include observations on electrophoretic patterns of sera from the same animal, obtained before and after immunization with the virus. By so doing it was hoped to learn whether any changes, similar to those observed with antipneumococcic sera (3, 4, 9, 11), might occur as a result of immunization, although in view of the evidence obtained by Wyckoff and Rhian (19) with anti-influenza horse sera significant changes in electrophoretic patterns seemed improbable.

Materials and Methods

Viruses.—Three strains of viruses were employed. The Nakayama strain of Japanese B encephalitis (Jap. B) virus (30) was obtained through the Office of the Surgeon General of the Army of the United States. Prior to the present experiment the virus had undergone

three mouse brain passages in this laboratory; the number of previous passages is unknown. The V-1938 strain of Venezuelan equine encephalomyelitis (Ven. E.E.) virus (31–32) was obtained from Dr. J. Casals of The Rockefeller Institute for Medical Research, New York City. The number of previous passages of this virus is not known; it was passed through four mouse brain passages in this laboratory.

The strain of Western equine encephalomyelitis (W.E.E.) virus, orignally furnished in 1942 by the Bureau of Animal Industry, Department of Agriculture, Washington, D. C., in form of an infected horse brain, was obtained through the courtesy of Miss Frances Clapp and Miss Dorothy Novotny of the Testing Department of Lederle Laboratories. This strain had been carried through one mouse brain and two guinea pig brain passages.

The technique employed for the inoculation of mice, harvesting of infected brains, etc., has been previously described (33). Freshly removed mouse brains were used as the source of Jap. B virus, whereas a 20 per cent suspension of infected mouse brains kept in dry ice was used as the source of Ven. E.E. and W.E.E. viruses.

Sera.—Immune sera were prepared in adult New Zealand rabbits, in adult Rhode Island Red roosters, and in a guinea pig. Hyprimmune sera against Jap B. virus were prepared in rabbits following the technique previously described (34). Chickens were immunized in the same manner as rabbits except that they were bled four or five times after the immunization schedule was completed in order to collect larger amounts of serum.

Rabbits immunized against Ven. E.E. virus were first injected subcutaneously with 1.0 ml. of formalinized chick embryo vaccine. Seven days later each animal received an intraperitoneal injection of mouse brain suspension containing from one to twenty million mouse intracerebral LD_{50} doses of virulent Ven. E.E virus. Ten days later the animals were bled and the sera thus obtained were employed for electrophoretic analysis.

Immune sera against W.E.E. virus were obtained by immunizing a rabbit and guinea pig. The rabbit was inoculated intraperitoneally with 2 ml. doses of infected mouse brain suspension starting with $10^{-4.0}$ and ending with $10^{-1.0}$ dilution. The animal was exsanguinated 10 days after the last injection. Immunization of the guinea pig was initiated with two subcutaneous injections of 0.5 ml. of formalinized 20 per cent chick embryo suspension. The injections were given 1 week apart and 2 months later were followed by six injections, at 1 month intervals, of 2 ml. of living virus (10 per cent guinea pig brain suspension). One week after the last injection the animal was exsanguinated.

Prior to immunization large quantities of normal serum were collected from each animal and kept frozen in sealed ampoules.

Neutralization Test.—Frozen standardized suspensions of infected mouse brains were used in the neutralization tests. Dilutions containing from 200 to 400 LD_{50} doses of Jap. B virus, 20 to 40 LD_{50} doses of Ven. E.E. or W.E.E. virus were prepared in 10 per cent normal rabbit serum–saline and mixed respectively with an equal volume of aliquots from fivefold to tenfold dilutions in saline of antiserum,[1] or its fraction. The serum–virus mixtures of Jap. B. virus were incubated for 2 hours at 37° C. and then injected intracerebrally in 0.03 ml. amounts in albino Swiss mice 21 to 27 days of age, using six mice for each serum–virus mixture. The serum–virus mixtures of either Ven. E.E. or W.E.E. viruses were incubated for 1 hour at 37° C. and then injected intraperitoneally into mice in 0.03 ml. amounts. Mice used for the test with Ven. E.E. virus were of the same age and strain as those used for the Jap. B. test, whereas mice injected with the W.E.E. virus were of thes ame strain but 16 to 17 days of age.

The injected mice were observed daily for a period of 15 days and the sick and dead were recorded. In the tables the results of inoculation for any given group of mice are indicated by the survival ratio, in which the numerator represents the number of mice surviving and the

[1] No protein determinations were made on the serum fractions. The dilutions were calculated on volume bases.

denominator the number of mice inoculated (less those that died of non-specific causes). The 50 per cent mortality endpoint titer (35) was calculated in order to obtain a better evaluation of the protective power of the crude serum, or its respective fractions.

Electrophoresis.—Samples of frozen sera were thawed, diluted with buffer, or left undiluted (see below), placed in a viscose casing bag, and dialyzed against 2 liters of buffer for 24 to 48 hours. Two types of buffer solutions were employed; one buffer (2, 36) of pH 7.4–7.6 contained 0.02 M sodium phosphate and 0.15 M NaCl (referred to in the text and tables as "Tiselius buffer") and the other, the so called "Svensson buffer" (37) of pH 7.7 contained 0.008 M NaH_2PO_4 and 0.06 M $Na_2HPO_412H_2O$. Both buffers had the same approximate ionic strength of 0.2.

Rabbit sera were dialyzed in the undiluted state when the electrophoretic separation was carried out in the Tiselius buffer. Chicken sera were diluted with equal volumes of buffer prior to electrophoresis. Sera used for analysis only, and not subsequently separated, were diluted with three parts of buffer before dialysis. Following dialysis the sera were filtered through one layer of Whatman No. 1 filter paper and the resulting filtrates were used for electrophoretic analysis. A Tiselius cell (36) of 11 ml. capacity was employed in the majority of the experiments but occasionally the 2 ml. microcell was used. Each separation experiment lasted from 15 to 16.1 hours following which photographic recording of the magnitude and position of each serum component was made by the Longsworth scanning method (38). The sections of the cell were then segregated and the apparatus was removed from the thermostat bath. Using the photographed patterns as guides, the fractionated samples were carefully removed with a 5 ml. syringe fitted with a 4 inch, blunt-tipped 18 gauge needle. The respective serum fractions were stored at 4° C. and used for neutralization tests within 3 weeks following separation.

EXPERIMENTAL

Electrophoretic Analyses of Normal and Jap. B Immune Rabbit Sera.—Sera from four adult rabbits, taken both before and after immunization against Jap.B virus, were diluted with three parts of Tiselius buffer (pH 7.6) and then dialyzed, filtered, and subjected to electrophoresis. The electrophoretic patterns (38) were projected, magnified, and traced, and the areas under the peaks were measured by a planimeter. The mobilities of the components were calculated on the descending boundaries. The results are summarized in Table I[2] and the electrophoretic patterns are shown in Fig. 1.

It may be observed that only two out of the four sera contained α-globulin, the lack of which has been noted previously (39, 40). The remaining sera contained only three components. The changes occurring in the relative concentration of the protein components in individual rabbit sera were rather inconsistent, varying considerably from one animal to another. For example, the relative concentrations of albumin, β-globulin, and γ-globulin in the sera of rabbits 40-51 and 42-19 remained virtually unchanged after immunization, the minor shifts that occurred in the γ-globulin concentrations being within the limits of experimental error. On the other hand, γ-globulin in the sera of rabbits 37-19 and 42-20 increased considerably after immunization, con-

[2] Both absolute and relative amounts, as deduced from the pattern areas, are given since the total protein concentration changed significantly after immunization.

comitant with a decrease in the albumin concentration. Even in the latter case, however, the differences between the normal and immune serum patterns, as illustrated in Fig. 1, were much less pronounced than those observed after immunization with bacterial antigens (3, 4, 9, 11). Furthermore, it seems doubtful, that the differences in the patterns of the normal and immune sera could be attributed to the appearance of antibodies against Jap.B virus since the immune sera of all four rabbits possessed almost equal strength in neu-

TABLE I

Japanese B Virus

Electrophoretic Analysis of Rabbit Sera before and after Immunization

Rabbit No.	Serum sample	Areas of components*								Mobilities* of components			
		Albumin		α		β		γ		Albumin	α	β	γ
		per cent	*absolute area*	*per cent*	*absolute area*	*per cent*	*absolute area*	*per cent*	*absolute area*				
37-19	N	71.4	404	6.0	34	11.7	66	10.9	62	4.8	3.8	3.0	1.2
	I	61.8	357	6.3	36	10.2	59	21.7	125	4.7	3.6	2.7	0.7
40-51	N	74.6	390			10.5	55	14.9	79	4.85		3.02	0.99
	I	73.2	404			10.7	60	16.1	88	4.93		3.15	1.14
42-19	N	71.9	318			14.9	66	13.2	58	4.75		2.89	1.09
	I	71.8	444			13.0	80	15.2	94	5.46		3.09	1.20
42-20	N	52.8	331	4.9	31	13.5	84	28.8	180	4.82	3.92	2.78	1.32
	I	32.3	201	8.8	55	28.3	176	30.6	191	5.00	4.41	2.93	1.35

N = normal.

I = immune.

* Derived from descending patterns and expressed as square centimeters, per second per volt.

tralization tests against this virus (see below). In addition, the differences in mobilities of the components (Table I) were too small to be considered significant.

Electrophoretic Separation of Protein Components of Normal Rabbit Sera.— Since the immune sera, prior to testing for neutralizing capacity, had been diluted with buffer, dialyzed, etc., it seemed imperative to determine whether normal sera treated in like manner would acquire non-specific neutralizing properties. Four samples of sera from normal rabbits were subjected to electrophoretic separation and the resulting fractions tested against Jap.B and Ven.E.E. viruses in neutralization tests.

It is seen from the results presented in Table II that in no instance did any of the serum specimens acquire virus-neutralizing properties because of elec-

trophoresis. Even the sensitive test performed against 6 LD_{50} doses of Ven.E.E. virus failed to reveal any virucidal properties in the fractionated serum. Furthermore, no difference was observed between the Tiselius and Svensson

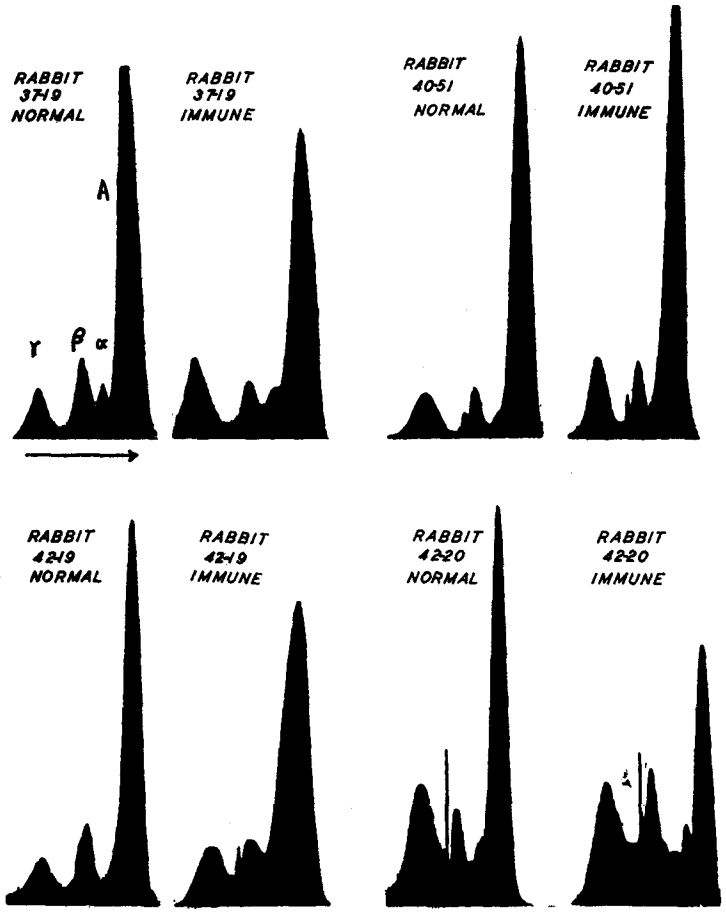

FIG. 1. Electrophoresis patterns of rabbit sera before and after immunization with Japanese B virus.

buffers even though the latter contained more than two and one-half times as much sodium phosphate.

Electrophoretic Fractionation of Jap. B Immune Rabbit Sera.—Two rabbits were immunized with infected mouse brain tissue and one with infected chick embryo tissue. Immune serum samples obtained from these animals were separated electrophoretically and the respective fractions were then examined for ability to neutralize Jap.B virus. The results are presented in Table III.

By comparing the neutralizing power of the "original" (prior to electrophore-

sis) serum sample from rabbit 40-51 with that of the "middle" unseparated portion (obtained after electrophoresis), it becomes apparent that electrophoresis, *per se*, changed the neutralizing power of the serum very little. The

TABLE II

Neutralization Tests with Electrophoretically Separated Fractions of Normal Rabbit Sera

Rabbit No.	Serum dilution	Buffer		Tested against		Fraction of serum tested	Survival ratio of mice inoculated with virus and undiluted serum
		pH	Molarity	Virus	LD_{50} doses		
42-96	Undiluted	7.7	0.1	Jap. B	91	Original*	0/6
						Middle‡	0/6
						Albumin	0/6
						Albumin + β-globulin	0/6
						β- + γ-globulin	1/6
						γ-globulin	0/6
42-88	1:2	7.7	0.1	Jap. B	315	Middle‡	0/5
						Albumin	0/6
						Albumin + β-globulin	0/5
						β- + γ-globulin	0/6
						γ-globulin	0/5
42-92	Undiluted	7.6	0.02+S§	Jap. B	400	Middle‡	0/5
						Albumin	0/5
						Albumin + β-globulin	0/5
						β- + γ-globulin	0/5
						γ-globulin	0/5
42-93	Undiluted	7.6	0.02+S§	Ven. E.E.	6	Middle‡	0/5
						Albumin	0/5
						Albumin + β-globulin	0/5
						β- + γ-globulin	0/5
						γ-globulin	0/5

* Serum sample after dialysis but before electrophoretic fractionation.

‡ Refers to the middle portion of the cell containing unseparated components.

§ S = 0.15 M sodium chloride.

serum fraction containing albumin alone showed no neutralizing effect, while that containing albumin+β-globulin likewise showed negligible neutralizing values (rabbit 40-51). In contrast, the neutralizing power of serum fractions containing γ-globulin was in general equal to, or greater than, that of the unseparated serum specimens. In order to determine whether any virus-neutralizing activity was associated with the β-globulin, an immune serum specimen from rabbit 37-19 was subjected to electrophoretic separation and

TABLE III

Neutralization Tests with Whole and Fractionated Japanese B Virus Immune Rabbit Sera

Rabbit No.	Serum dilution	Buffer		Serum fraction tested	Survival ratio of mice injected with virus and dilutions of serum					Minimal protective titer of serum*
		pH	Molarity		1:2	1:20	1:200	1:2000	1:200000	
37-19	Undiluted	7.7	0.1	Middle‡		5/5	5/5	2/5		1:1350
				Albumin	0/5	0/5				<1:2
				Albumin + α- + β-globulin	3/5	0/5				1:3
				β- + γ-globulin	5/5	5/5	4/4	0/4		1:630
				γ-globulin	5/5	4/4	5/5	3/5		>1:2000
	Undiluted	7.4	0.02+S‡	Middle‡			4/6	0/6	0/6	1:350
				Albumin	0/6	0/6	0/6			<1:2
				Albumin + α- + β-globulin	0/6	0/6	0/6			<1:2
				β- + γ-globulin		6/6	5/6	0/5	0/6	1:500
				γ-globulin		5/6	4/4	0/6	0/6	1:470
	Undiluted	7.4	0.02+S‡	β- + γ-globulin		4/4	4/5	2/5	0/5	1:960
				β-globulin	2/4	0/5	0/5	0/5		1:2
				γ-globulin		5/5	5/5	2/5	0/5	1:1350
40-51	1:2	7.7	0.1	Original‡		4/4	5/5	2/5	0/4	1:1350
				Middle‡		4/4	5/5	0/5	0/5	1:630
				Albumin	0/5	0/4	0/5			<1:2
				Albumin + β-globulin	5/5	2/5	0/5			1:13
				β- + γ-globulin	5/5	5/5	4/5	0/5		1:470
				γ-globulin		3/4	4/4	0/5	0/5	1:470
	Undiluted	7.6	0.02+S‡	Middle‡			5/5	3/4	0/5	1:4280
				Albumin	0/5	0/5				<1:2
				Albumin + β-globulin	4/5	1/5	0/5	0/5		1:6
				β- + γ-globulin	4/4	5/5	1/5	0/5		1:85
				γ-globulin		5/5	3/5	0/5	0/5	1:300
40-47§	Undiluted	7.6	0.02+S‡	Middle‡		5/5	0/6	0/6	0/6	1:60
				Albumin	0/6	0/6	0/6			<1:2
				Albumin + β-globulin	0/6	0/6	0/6			<1:2
				β-+ γ-globulin		6/6	0/6	0/6	0/6	1:60
				γ-globulin		6/6	0/6	0/6	0/6	1:60

* Calculated according to the Reed and Muench formula.

‡ See legend, Table II.

§ Immunized with infected chick embryo suspension instead of mouse brain suspension.

the fraction containing β- and γ-globulins was further separated in the 2 ml. Tiselius cell. The two resulting electrophoretically homogeneous fractions, representing β- and γ-globulins respectively, were then used in a neutralization test. The results of the test, presented in Table III, indicate that the neutralizing activity of the serum for Jap.B virus was confined almost entirely to the γ-globulin fraction.

Electrophoretic Separation of Anti-Jap. B Chicken Sera.—Since the electrophoretic patterns of chicken sera differ quite markedly from other animal sera (40, 41) the separation of an immune rooster serum (No. 6-20) is illustrated by a diagram, as presented in Fig. 2. In this diagram the components are classified according to their mobilities and are numbered from 1 to 6 in the order of decreasing mobility. Samples of components 1, 2, and 3 were taken

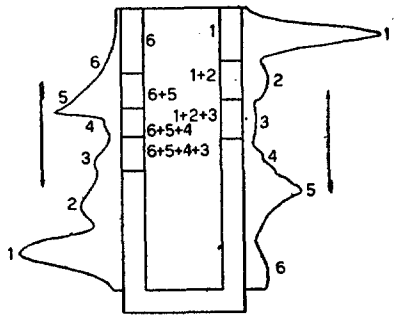

FIG. 2. Schematically illustrated electrophoresis cell indicating the position of the components of immune rooster serum (No. 6-20) and the manner in which they were recovered for neutralization tests. (See Table IV.)

from the ascending leg of the cell and samples of components 6, 5, 4, and also 3 from the descending leg.

The results of the neutralization tests performed with the electrophoretically separated fractions of three chicken sera are presented in Table IV. It may be observed that the results obtained with fractions of chicken sera 6-07 and 6-20 differ slightly from those obtained with fractions of serum 6-08 even though the neutralizing titer of serum 6-20 was markedly less than the other two. However, certain characteristics seem to be common to all three separations. Thus, components 1 and 6[3], representing the fastest and slowest moving components respectively, were devoid of any demonstrable virus-

[3] In a previous communication (40) containing data on cockerel serum, component 6, which is indeterminant in the descending pattern (see Fig. 2), was omitted and what is here labeled as component 5 was previously designated as 6. We now believe that component 5 is a better designation for this globulin fraction since it has a mobility appreciably greater than γ-globulin of other species (see Table 1 in reference 40).

neutralizing activity. Component 6 represents, in all probability, the salt boundary (42) whereas component 1 corresponds to the albumin fraction of other animal sera. Likewise those fractions containing components 1 plus 2,

TABLE IV

Neutralization Tests with Fractionated Japanese B Virus Immune Chicken Sera

Chicken No.	Serum fraction* tested	Survival ratio of mice injected with virus and dilutions of serum					Minimal protective titer of serum‡
		1:2	1:10	1:50	1:250	1:1250	
6-07	Middle§	6/6	6/6	6/6	2/6		1:170
	1	0/6	0/6	0/6	0/6		<1:2
	1 + 2	0/6	0/6	0/6	0/6		<1:2
	1 + 2 + 3	6/6	1/6	0/6	0/6		1:5
	6	0/6	0/6	0/6	0/6		<1:2
	6 + 5	6/6	5/6	2/6	0/6		1:30
	6 + 5 + 4	6/6	6/6	6/6	3/6	0/6	1:250
	6 + 5 + 4 + 3	6/6	6/6	4/6	5/6	0/6	1:350
6-08	Middle§	6/6	6/6	3/6	4/6	2/6	1:360
	1	0/6	1/6	0/6	0/6		<1:2
	1 + 2	6/6	0/6	0/6	0/6		1:5
	1 + 2 + 3	5/6	6/6	4/6	0/6		1:340
	6	1/6	0/6	0/6	0/6		<1:2
	6 + 5	6/6	5/6	3/6	0/6		1:40
	6 + 5 + 4	6/6	6/6	5/6	1/6	0/6	1:110
	6 + 5 + 4 + 3	5/6	4/6	5/6	1/6	0/6	1:70
6-20	Middle§	5/5	5/5	1/5	0/4		1:28
	1	0/5	0/4	0/4	0/4		<1:2
	1 + 2	0/5	0/5	0/5	0/5		<1:2
	1 + 2 + 3	0/5	0/5	0/5	0/5		<1:2
	6	0/5	0/5	0/5	0/5		<1:2
	6 + 5	2/5	0/5	0/5	0/5		<1:2
	6 + 5 + 4	4/5	3/5	0/5	0/5		1:10
	6 + 5 + 4 + 3	4/4	5/5	0/5	0/5		1:22

* All sera were diluted 1:2 and separated in Tiselius phosphate-sodium chloride buffer, pH 7.6.

‡ Calculated according to the Reed and Muench formula.

§ See legend, Table II.

or 6 plus 5, showed relatively little neutralizing power. It is somewhat difficult to generalize on the limited data available, but it is seen that in sera 6-07 and 6-20 the fractions that show definite neutralizing titer all contain component 4. It should be pointed out that the component boundaries are not sharply defined and there is some intermixing of the individual components into their adjacent ones. For example, in recovering the fraction labeled 6 + 5 it is

TABLE V

Neutralization Tests with Whole and Fractionated Venezuelan E.E. Virus Immune Rabbit Sera

Rabbit No.	Serum fraction* tested	Survival ratio of mice injected with virus and dilutions of serum						Minimal protective titer of serum‡
		1:2	1:20 **1:10**	1:200 **1:50**	1:2000 **1:250**	1:20000 **1:1250**	1:200000 **1:6250**	
41-54	Middle§		5/5	2/4	2/5	0/5		1:540
	Albumin	0/5	0/5	0/5				<1:2
	Albumin + β-globulin	4/5	2/5	0/5	0/5			1:10
	β- + α-globulin		5/5	4/5	2/5	1/5		1:1390
	γ-globulin		5/5	1/5	1/5	1/5		1:150
32-21	Original§			3/5	3/5	1/5	0/5	1:2000
	Middle§			3/5	2/5	0/5	2/5	1:1420
	Albumin	1/5	1/5	0/5				<1:2
	Albumin + α- + β-globulin	1/5	0/5	0/5				<1:2
	β- + γ-globulin		3/5	3/5	1/5	1/5	0/5	1:280
	γ-globulin		4/5	2/5	2/5	0/5	0/5	1:200
37-73	Middle§		**4/4**	**6/6**	**2/6**	**0/6**	**0/6**	1:170
	Albumin	**0/6**	**0/6**	**0/6**				<1:2
	Albumin + β-globulin	**2/6**	**2/6**	**0/6**	**0/6**			1:2
	β- + γ-globulin	**6/6**	**2/6**	**6/6**	**6/6**	**1/6**	**1/6**	1:430
	γ-globulin		**3/6**	**3/6**	**0/6**	**0/6**	**0/6**	1:20
37-72	Middle§		**6/6**	**5/6**	**5/6**	**1/6**	**1/6**	1:560
	Albumin	**0/6**	**2/6**	**0/6**				<1:2
	Albumin + β-globulin	**3/6**	**0/6**	**0/6**	**0/6**			1:2
	β- + γ-globulin	**6/6**	**6/6**	**4/6**	**6/6**	**0/6**	**1/6**	1:490
	γ-globulin		**5/6**	**5/6**	**1/6**	**0/6**	**1/6**	1:110
Pool 41-56 41-57 41-58	Middle§	**6/6**	**6/6**	**5/6**	**6/6**	**5/6**	**1/6**	1:2380
	Albumin	**2/6**	**0/6**	**0/6**	**0/6**	**0/6**		<1:2
	Albumin + α-globulin	**2/6**	**0/6**	**0/6**	**0/6**	**0/6**		<1:2
	Albumin + α- + β-globulin	**4/6**	**4/6**	**2/6**	**1/6**	**1/6**	**0/6**	1:20
	β- + γ-globulin	**6/6**	**5/6**	**5/6**	**3/6**	**4/6**	**2/6**	1:880
	β-globulin		**5/6**	**4/6**	**0/6**	**0/6**	**0/6**	1:60
	γ-globulin	**6/6**	**6/6**	**6/6**	**4/6**	**0/6**	**3/6**	1:610

Figures in bold-faced type refer to fivefold dilutions.

* Undiluted sera separated in Tiselius phosphate-sodium chloride buffer at pH 7.6.

‡ Calculated according to the Reed and Muench formula.

§ See legend, Table II.

probable that appreciable quantities of component 4 would be included in the sample. This would explain the apparent neutralizing activity of fraction 6 + 5 in serum 6-07. Similarly, inclusion of component 4 may account for

the high neutralizing titer of fraction 1 + 2 + 3 in serum 6-08, especially since the electrophoretically determined boundaries of this latter serum were much less distinct than those of sera 6-07 and 6-20.

Electrophoretic Separation of Anti-Ven.E.E. Rabbit Sera.—Four immune serum samples obtained from rabbits immunized against Ven.E.E. virus were separated electrophoretically. Three additional immune rabbit sera (Nos. 41-56, 41-57, 41-58) were pooled and after a preliminary electrophoretic separation, the fraction containing both the β- and γ-globulins was subjected to electrophoresis in a 2 ml. microcell. The separated β- and γ-globulins were tested for their virus-neutralizing activity.

TABLE VI

Neutralization Tests with Whole and Fractionated Western E.E. Virus Immune Sera

Animal	Serum	Serum fraction* tested	Survival ratio of mice injected with virus and dilutions of serum						Minimal protective titer of serum‡
			1:2	1:10	1:50	1:250	1:1250	1:6250	
Guinea pig	W.E.E. immune	Middle§	6/6	6/6	4/4	4/5	2/6	0/5	1:690
		Albumin	0/5	0/6	0/6	0/6	0/6		<1:2
		Albumin + α-globulin	1/6	0/6	0/4	0/5	0/6		<1:2
		Albumin + α- + β-globulin	5/6	4/6	3/6	1/5	0/6		1:30
		α- + β- + γ-globulin		5/6	5/6	4/6	0/6	2/6	1:360
		β- + γ-globulin		4/5	5/6	5/6	0/6	0/6	1:350
		γ-globulin		4/6	2/4	0/6	0/6	0/6	1:25
Rabbit	W.E.E. immune	Original§			4/5	6/6	4/6	0/6	1:1500
		Middle§			6/6	4/5	2/5	0/6	1:750
		Albumin	0/4	0/6	0/6	0/6	0/5		<1:2
		Albumin + α-globulin	0/6	0/6	0/6	0/6	0/6		<1:2
		Albumin + α- + β-globulin	5/6	6/6	3/6	0/6	0/6		1:40
		α- + β- + γ-globulin		3/3	6/6	4/5	6/6		>1:1250
		β- + γ-globulin		6/6	6/6	4/5	5/5		>1:1250
		γ-globulin		5/5	2/2	4/6	0/6		1:370
Rabbit 42-92	Normal	Middle§	0/5						<1:2
		Albumin	0/6						<1:2
		γ-globulin	0/6						<1:2

* Undiluted sera separated in Tiselius phosphate-sodium chloride buffer at pH 7.6.

‡ Calculated according to the Reed and Muench formula.

§ See legend, Table II.

The results of the Ven.E.E. virus neutralization test with fractions of the four individual sera and the serum pool are presented in Table V. A comparison of the results obtained with the "original" and "middle" fractions of rabbit serum 32-21 again indicates that electrophoresis, *per se*, affected the neutralizing power of the serum to a negligible degree only.

The albumin fractions failed to reveal any demonstrable virus-neutralizing ability. Similarly, the fractions containing albumin + α- + β-globulin were either inactive or nearly so. Again, as was true for the Jap.B antisera, most of the virus-neutralizing activity was associated with the serum fraction containing β- and γ-globulin components, although in serum 37-73 the neutralizing effect exerted by the γ-globulin component was very small in proportion to that of the β- + γ-fraction. However, the results obtained with the individual β- and γ-components of the serum pool No. 41-56, 41-57, and 41-58 indicate that the neutralizing power of the latter fraction was almost equal to that of the unseparated portion, whereas the neutralizing ability of the β-globulin component was inferior to that of γ-globulin, though proportionally greater than the corresponding fractions of the Jap.B antisera.

Electrophoretic Separation of Anti-W.E.E. Sera.—Sera obtained from one rabbit and one guinea pig were submitted to electrophoresis and the resulting fractions were tested by a neutralization test against W.E.E. virus.

The results of the test are presented in Table VI. In this instance the α-globulin was present in both sera. Again there was no neutralizing effect of the serum fractions containing either albumin or albumin +α-globulin. Fractions containing albumin + α- + β-globulin had a very low neutralizing effect. Most of the virus-neutralizing activity was associated with the serum fractions containing β- and γ-globulins. (The high protective titer of α- + β- + γ-fraction must be ascribed to the presence of β- and γ- globulins, since the fraction containing albumin + α-globulin showed no neutralizing activity. Since no attempt was made to separate the β- and γ-globulin fraction into the two components, it is difficult to ascertain whether the virus-neutralizing ability was associated mostly with the γ-globulin component as in the case of Jap.B antisera, or with both β- and γ-globulin as in the case of Ven.E.E. antisera. The fact, however, that the neutralizing titers of the γ-globulin fractions were consistently lower in comparison with those of the β- + γ-fractions and that albumin + α- + β-globulins showed some neutralizing power in both sera, indicates that both β- and γ-globulins contain neutralizing antibodies.

DISCUSSION

In these experiments two different methods and three representative viral agents were employed to immunize rabbits. In the case of Jap.B and W.E.E. viruses the animals were hyperimmunized by multiple injections of living virus, whereas the rabbits immunized with the Ven.E.E. virus received two injections only, one of killed virus vaccine and the other of a challenge dose of living virus. In addition antisera prepared by immunizing chickens against Jap.B virus and a guinea pig against W.E.E. virus were obtained for comparison of results with sera from different animal species.

In order to determine whether the electrophoretic procedure changed the properties of the serum, comparative neutralization tests were made of both normal and immune serum specimens before and after (middle portion) electrophoresis. The results obtained clearly indicated that the normal sera did not acquire any antiviral properties (see Table II) and that the immune sera did not lose neutralizing power because of electrophoresis.

On the basis of the preliminary electrophoretic studies of Jap.B immune rabbit sera (Table I, Fig. 1) it can be concluded that the absolute concentration of the virus-neutralizing antibody was very low, even in high titer sera. This was indicated by the lack of significant and consistent changes in the serum after immunization. It is possible, of course, that if a greater number of serum patterns were examined electrophoretically the increase in the concentration of γ-globulin, which occurred in two out of four sera tested in the present experiment, would gain some statistical significance. The experiments of other workers with influenza (19) and Western equine encephalomyelitis viruses (17), however, failed to disclose any significant difference between the electrophoretic patterns of virus-immune and normal sera.

The results of electrophoretic separation of immune sera and of subsequent tests on the various fractions for the presence of virus-neutralizing bodies were similar in all species of animals tested. In no instance was the virus-neutralizing activity associated with the fastest moving component, *i.e.* albumin in rabbit and guinea pig sera, and component 1 in chicken sera. Neither did the next fastest moving fractions, *i.e.* albumin + α-globulin in the rabbit and guinea pig sera, nor did fraction 1 + 2 in chicken sera show any significant degree of virus-neutralizing ability.

In general, it seemed that the viral antibodies were in greater concentration in the slower moving serum fractions although certain differences were observed between the different groups of antiviral sera. The γ-globulin component of the Jap.B immune rabbit serum (Table III) seemed to possess a neutralizing titer as high as, and at times even higher, than that of the β- + γ-fraction. This occurred despite the fact that the γ-globulin fraction was diluted with buffer in the separation procedure and thus was in a more dilute state of protein concentration than the γ-globulin component in the fraction labeled β- + γ-globulin. Separate tests performed on the β- and γ-globulin components, obtained by reseparation of the β- and γ-globulin fractions, indicated that the virus-neutralizing activity was centered almost entirely within the mobility range of the γ-globulin component with only a trace remaining in the β-globulin component.

On the other hand, the virus-neutralizing titer (Tables V and VI) of the γ-globulin fraction of the Ven.E.E. immune rabbit and W.E.E. immune rabbit and guinea pig sera was usually lower than that of the homologous β- + γ-fraction and on separation of the anti-Ven.E.E. serum-pool into β- and

γ-globulins, the antibodies, although appearing in a much higher concentration in the latter component, were still present in the β-globulin component in a concentration relatively greater than was found in the case of Jap.B immune sera. The above mentioned differences might have been due to the fact that rabbits immunized against the Ven.E.E. virus received only two immunizing injections and the resulting sera, which showed neutralizing titers relatively lower than the Jap.B sera, contained an antibody which had a broader range of mobility. On the other hand, the Jap.B hyperimmune sera, with relatively higher neutralizing titers than the Ven.E.E. sera, contained an antibody with a narrower range of mobility, the latter being limited almost entirely to the γ-globulin component. Yet, against this hypothesis is the fact that the results obtained with the anti-W.E.E. sera, which were prepared by hyperimmunizing animals in a manner similar to that used for anti-Jap.B sera, paralleled the results obtained with anti-Ven.E.E. sera and not those with anti-Jap.B sera. Thus, it must be assumed that the differences in results should be ascribed to the fact that the Jap.B antibody in immune rabbit serum has a slightly different mobility (γ-globulin) from the Ven.E.E., and W.E.E. antibodies, the mobility of the latter two antibodies falling between that of β- and γ-globulins.

Although the electrophoretic patterns of rabbit sera cannot be compared directly to the patterns of chicken sera, the results of neutralization tests on fractions of the latter sera indicate that the Jap.B virus antibody has its mobility center in component 4 (Fig. 2), which has a mobility of approximately 2.3×10^{-5} cm.^{2}/volt/sec. This is appreciably greater than the mobility of rabbit γ-globulin.

The results herein reported appear at variance with those obtained by other workers (17, 18) who showed that antibodies against W.E.E. virus are present in all electrophoretically separated immune rabbit sera fractions, including the albumin. This discrepancy may be due to the fact that the rabbits immunized against W.E.E. virus in the experimental work cited above (17) were subjected to a more prolonged and intensive immunizing procedure than were the animals in the experiments described herein. However, it should be pointed out that in the present work carried out with three serologically distinct neurotropic viruses, including W.E.E. virus, in no instance were the antibodies associated with the electrophoretically faster moving components of the serum. On the contrary the antibodies were consistently present in the slower moving γ- and/or β-globulin components.

SUMMARY

1. Sera of animals immunized against Japanese B encephalitis, Venezuelan equine encephalomyelitis, and Western equine encephalomyelitis viruses were fractionated by electrophoresis.

2. Electrophoretic patterns of rabbit sera before and after immunization against Japanese B virus showed no consistent change traceable to antibody formation.

3. To determine the antibody content, the electrophoretic fractions of the respective sera were mixed in varying dilutions with infected mouse brain suspensions, and the neutralizing titers of the fractions were compared.

4. In all instances serum fractions containing γ-globulin were protective, whereas in no case did serum albumin show any virus-neutralizing activity. The Japanese B encephalitis antibody appeared to be associated entirely with the γ-globulin. The Venezuelan and Western equine encephalomyelitis antibodies were associated with the β- and γ-globulins and probably possessed an average electrophoretic mobility between that of β- and γ-globulins.

5. Normal rabbit serum similarly separated electrophoretically showed no neutralizing properties.

6. Chickens, whose electrophoretic serum pattern is markedly different from that of rabbits, were also immunized against the Japanese B encephalitis virus. Their antisera were electrophoretically fractionated and similarly subjected to neutralization tests. The specific neutralizing capacity of chicken serum was considerably lower than that of rabbit serum and no neutralizing activity was found in the fractions containing the faster moving components. The antibody appeared to be associated with component 4 which had a mobility of approximately 2.3×10^{-5} cm.^{2}/volt/sec.

BIBLIOGRAPHY

1. Tiselius, A., *Biochem. J.*, 1937, **31,** 1464.
2. Tiselius A., and Kabat, E. A., *J. Exp. Med.*, 1939, **67,** 119.
3. Moore, D. H., van der Scheer, J., and Wyckoff, R. W. G., *Science*, 1939, **90,** 357.
4. Moore, D. H., van der Scheer, J., and Wyckoff, R. W. G., *J. Immunol.*, 1940, **38,** 221.
5. Kekwick, R. A., and Record, B. R , *Brit. J. Exp. Path.*, 1941, **22,** 29.
6. Pappenheimer, A. M., Jr , Lundgren, H. P., and Williams, J. W., *J. Exp. Med.*, 1940, **71,** 247.
7. Van der Scheer, J., and Wyckoff, R. W. G., *Science*, 1940, **91,** 485.
8. Van der Scheer, J., and Wyckoff, R. W. G., *Proc. Soc. Exp. Biol. and Med.*, 1940, **43,** 427.
9. Van der Scheer, J., Wyckoff, R. W. G., and Clarke, F. H, *J. Immunol.*, 1940, **39,** 65.
10. Van der Scheer, J., Wyckoff, R.W.G., and Clarke, F. H., *J. Immunol.*, 1941, **40,** 173.
11. Van der Scheer, J., Bohnel, E., Clarke, F. H., and Wyckoff, R. W. G., *J. Immunol.*, 1942, **44,** 165.
12. Longsworth, L. G., Curtis, R. M., and Pembroke, R. H., Jr., *J. Clin. Inv.*, 1945, **24,** 46.
13. Stern, K. G., and Reiner, M., *Yale J. Exp. Biol. and Med.*, 1946, **19,** 67.

14. Cooper, J. A., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57,** 248.
15. Cooper J. A., *J. Inv. Dermat.*, 1945, **6,** 109.
16. Davis, B. D., Moore, D. H., Kabat, E. A., and Harris, A., *J. Immunol.*, 1945, **50,** 1.
17. Morgan, I., *J. Immunol.*, 1945, **50,** 359.
18. Olitsky, P. K., and Longsworth, L. G., as quoted by Olitsky, P. K., and Casals, J., *Bull. New York Acad. Med.*, 1945, **21,** 356.
19. Wyckoff, R. W. G., and Rhian, M., *J. Immunol.*,1945, **51,** 359.
20. Enders, J. F., *J. Clin. Inv.*,1944, **23,** 510.
21. Greenbert, M., Frant, S., and Rutstein, D. D., *J. Am. Med. Assn.*, 1944, **126,** 944.
22. Stokes, J., Jr., Maris, E. P., and Gellis, S. S., *J. Clin. Inv.*, 1944, **23,** 531.
23. Ordman, C. W., Jennings, C. G., and Janeway, C. A., *J. Clin. Inv.*, 1944, **23,** 541.
24. Janeway, C. A., *J. Am. Med. Assn.*, 1944, **126,** 674.
25. Janeway, C. A., *Bull. New York Acad. Med.*, 1945, **21,** 202.
26. Anderson, S. G., and Ket, W. M., *Med. J. Australia*, 1946, **2,** 196.
27. Stokes, J., Jr., and Neefe, J. R., *J. Am. Med. Assn.*, 1945, **127,** 144.
28. Gellis, S. S., Stokes, J., Jr., Brother, G. M., Hall, W. M., Gilmore, H. R., Beyer, E., and Morrisey, R. A., *J. Am. Med. Assn.*, 1945, **128,** 1002.
29. Gellis, S. S., Stokes, J., Jr., Forster, H. W., Jr., Brother, G. M., and Hall, W. M., *J. Am. Med. Assn.*, 1945, **128,** 1158.
30. Kasahara, S., Ueda, M., Okamoto, Y., Yoshida, S., Hamano, R., and Yamada, R., *Kitasato Arch. Exp. Med.*, 1936, **13,** 48.
31. Kubes, V., and Rios, F. A., *Science*, 1939, **90,** 20.
32. Beck, C. E., and Wyckoff, R. W. G., *Science*, 1938, **88,** 530.
33. Lennette, E. H., and Koprowski, H., *J. Immunol.*, 1944, **49,** 375.
34. Koprowski, H., and Cox, H. R., *J. Immunol.*, 1946, **52,** 171.
35. Reed, L. J. and Muench, H., *Am. J. Hyg.*, 1938, **27,** 493.
36. Tiselius, A., *Tr. Faraday Soc.*, 1937, **33,** 524.
37. Svensson, H., *J. Biol. Chem.*, 1941, **139,** 805.
38. Longsworth, L. G., and MacInnes, D. A., *Chem. Rev.*, 1939, **24,** 271.
39. Sharp, D. G., Taylor, A. R., Beard, D., and Beard, J. W., *J. Immunol.*, 1942, **44,** 115.
40. Moore, D. H., *J. Biol. Chem.*,1945, **161,** 21.
41. Deutsch, H. F., and Goodloe, M. B., *J. Biol. Chem.*, 1945, **161,** 1.
42. Longsworth, L. G., and MacInnes, D. A., *Chem. Rev.*,1939 **24,** 271.