An Hemagglutination Test for Titration of Antibodies to Polioviruses.* (23675)

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It was shown by Bautista, *et al.*(1) that polioviruses were adsorbed to certain human and animal erythrocytes, but that hemagglutination did not occur either in the presence or absence of homologous immune serum. The treatment of red cells with dilute solutions of tannic acid renders these cells capable of adsorbing proteins, and agglutinable in the homologous antiprotein serum(2). In a further extension of this technic, McKenna(3) demonstrated that bovine serum albumin and bovine gamma globulin could be coupled to formalinized erythrocytes, and antibodies to either antigen measured by an hemagglutination pattern.

This report shows that antibodies to polioviruses, which may be considered as protein antigens(4), can be measured by a similar technic.

Materials and methods. Antigen preparations. Seitz-filtered polioviruses, Type I (Mahoney), Type II (MEF-1) and Type III (Saukett) (monkey kidney tissue culture source), were used throughout these studies. The post-filtration infectious titers of these viruses were $10^{6.0}$, $10^{6.8}$ and $10^{6.8}$ $TCID_{50}$/ml respectively(5). Control monkey kidney cell antigens (MKTCF) were prepared by rapidly freezing and thawing uninfected cell sheets followed by Seitz-filtration. The antigens were concentrated approximately 10-fold by dialysis against 10-20 volumes of a 20% aqueous polyvinylpyrrolidone (PVP) solution at 4°C for 48 hours. Neutralization tests for antibody were performed by use of the metabolic inhibition test against 100 $TCID_{50}$ of virus(6). *Immune Rabbit Sera.* Twenty albino rabbits each weighing about 3 kg were divided into 4 groups of 5 animals each. Each group received either 1.0 ml of viable monotypic poliovirus or 1 ml of control antigen intravenously every other day until 5 ml had been injected. Sera obtained one week after the last injection were pooled according to virus type or control and were stored at –40°C. Five other rabbits received 1 ml of trivalent polio vaccine at weekly intervals for three weeks, and sera were obtained 28 days after the first injection. *Immune monkey and human sera.* Both immune monkey and human sera were taken randomly from freezer stocks. Neutralization tests had been carried out previously on all these sera. The monkey sera had been prepared by the intramuscular injection of rhesus monkeys with 1 ml of polio vaccine at weekly intervals for 3 weeks. Immune sera were secured 28 days after the first injection. The immune human sera were taken from individuals who had received one or more inoculations of polio vaccine. *Sheep erythrocytes.* Washed sheep red blood cells were formalinized according to the technic described elsewhere(3). *Experimental.* A 2.5% suspension of formalinized sheep red blood cells was incubated at 37°C for 20 minutes with a freshly prepared 1:20,000 dilution of tannic acid in 0.9% saline. The cells were washed once with saline, centrifuged at 1500 rpm for 5 minutes at 5°C and reconstituted to a 2.5% suspension with saline. Two volumes of these cells then were added to one volume of antigen and one-half volume of 0.15 M phosphate buffered saline at pH 6.4. After incubation at 37°C for 2 hours, the cells were washed once with 1.0% normal rabbit serum in 0.9% saline, and reconstituted to volume with the same diluent. Before freezing the cells at –70°C, infectious titers were determined for the supernates after sensitization of the cells. The initial viral infectious titers, those following PVP concentration, and those of the supernates are

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TABLE I. Infectious Titers before and after 10-Fold PVP Concentration and of Supernates after Sensitization of Red Cells.

	Type I	Type II	Type III
Initial	6.0	6.8	6.5
After PVP	7.4	8.0	8.0
" sensitization	4.5	5.8	6.0

Titers expressed as $\log_{10}$ $TCID_{50}$/ml.

presented in Table I. It may be seen that over 99% of the virus had disappeared, presumably due to adsorption to the red cells. The viral particles spontaneously eluted from the red cells after standing 2-3 days at 5°C. However, the cells remained agglutinable after storage for 6 weeks in the frozen state. Two-fold dilutions of antisera were made in 0.5 ml amounts in 1.0% normal rabbit serum in saline and 0.15 ml of thawed, sensitized cells was added to each of the serum dilutions. The mixtures were shaken and immediately placed at 1-4°C for 12-18 hours. Hemagglutination titers (HA) were recorded as the highest dilution of antiserum causing agglutination of the cells. All antisera were inactivated at 56°C for 30 minutes, but preliminary work indicated that inactivation was not necessary.

TABLE II. HA Titers* of the Same Immune Rabbit Sera with Cells Sensitized at Different Times.

Serum prepared to	Serum titers with cells sensitized to I	II	III	MKTCF†
Type I	1280	160	320	40
	1280	80	320	40
	1280	160	640	20
	1280	40	160	80
	1280	640	640	160
GMT‡	1280	128	384	56
Type II	160	2560	80	40
	160	1280	160	40
	80	1280	160	10
	160	2560	160	80
	160	2560	80	80
GMT	144	2048	128	40
Type III	80	80	1280	20
	80	160	1280	40
	640	80	1280	40
	40	40	320	10
GMT	117	80	960	25

Titers expressed as reciprocal of serum dilution.
* HA = Hemagglutinating titers.
† Uninfected monkey kidney tissue culture fluid.
‡ GMT = Geometric mean titer.

Results. The homotypic titer in each instance was appreciably higher than the heterotypic titers when concentrated antigens were used (Table II). These titers represent repeat titrations of the same antisera with cells sensitized at different times. The neutralizing titers of these sera were 1000, 2000 and 640 for Types I, II and III respectively. The 5 rabbits that received the trivalent polio vaccine had the titers shown in Table III, while

TABLE III. HA Titers in 5 Rabbits Given Trivalent Polio Vaccine.

Rabbit No.	Type I	Type II	Type III	MKTCF
256	640	160	320	10
257	"	640	160	<10
258	320	320	320	10
259	640	160	80	"
260	160	320	160	"
GMT	420	280	180	10

Titers expressed as reciprocal of serum dilution.

the nonspecific agglutination was in all cases 1:10 or less. It is possible that the lower nonspecific titers seen in Table III were due to the fewer number of injections. Several weeks later these sera were pooled for neutralization tests. The titers for the pools were as follows: Type I, HA 160, neut. 120; Type II, HA 80, neut. 130; Type III, HA 160, neut. 130.

A comparison of hemagglutinating titers with neutralizing titers in immune human sera is presented in Table IV. It may be seen that sera No. 1, 2 and 3 gave no evidence of HA titers, despite appreciable neutralizing titers. The reason for this lack of agglutination is not evident since all the titrations were made with a single cell lot sensitized to each antigen. A similar comparison in immune monkey sera is shown in Table V.

Discussion. Preliminary experiments using unconcentrated antigens produced low HA titers, but the reaction lacked specificity. By using antigens concentrated about 10-fold with PVP, the homotypic titers were approximately 10 times higher than the heterotypic titers, and about 20-50-fold higher than the uninfected tissue control. Since no soluble component has been demonstrated in or from polioviruses, one can only surmise that the

TABLE IV. Comparison of HA and Neutralizing Titers of Immune Human Sera.

Serum No.	Type I		Type II		Type III		MKTCF
	HA	Neut.*	HA	Neut.	HA	Neut.	HA
1	<10	80	<10	80	<10	68	<10
2	"	40	"	1280	"	136	"
3	"	416	"	1088	"	1664	"
4	10	<10	40	68	640	2560	"
5	40	160	10	13	40	272	"
6	80	104	640	640	<10	20	"
7	160	40	1280	2560	1280	2560	"
8	"	640	"	1280	160	80	10
9	320	416	"	2176	1280	2560	<10
10	640	2560	160	208	2560	"	10
11	"	2176	1280	832	10	<10	<10
12	"	832	640	1280	160	640	"
13	"	"	1280	2560	1280	2560	10
14	"	1664	"	1280	320	1280	"
15	"	1280	640	1664	640	2176	<10

* Neutralizing titers expressed as geometric means of triplicate titrations.
Titers expressed as reciprocal of serum dilution.

entire viral particle became attached to the red cells.

Approximately a 2-5% antigenic cross has been observed with some lots of purified virus when measured by a 50% CF test(7); the average cross as measured by HA was about 12%. This increased percentage may be due to the increased sensitivity of the HA over the CF test, since it has been shown that the HA test is about 35 times as sensitive as the 50% CF test using a bovine gamma globulin-anti-BGG rabbit serum system (unpublished data).

TABLE V. Comparison of HA and Neutralizing Titers of Immune Monkey Sera.

Serum No.	Type I		Type II		Type III	
	HA	Neut.*	HA	Neut.	HA	Neut.
Normal	2	<2	2	<2	<2	<2
1	<2	4	<2	23	4	2
2	2	10	8	13	<2	2
3	4	3	8	4	8	4
4	8	3	64	208	16	10
5	8	10	32	23	<2	8
6	10	9	20	16	<10	2
7	16	10	<2	2	<2	8
8	20	36	80	12	40	26
9	20	49	80	128	<20	10
10	80	8	320	320	<2	8
11	"	12	"	332	<10	9
12	"	32	160	16	"	2
13	"	49	320	64	160	97
14	"	64	"	102	"	128
15	"	256	80	416	40	51
16	"	1331	320	24	160	256
17	160	128	"	49	40	140

* Neutralizing titers expressed as geometric means of triplicate titrations.
Titers expressed as reciprocal of serum dilution.

The basic technic as described by Boyden (2), with the formalin modification developed in this laboratory, has proven quite useful to us. However, many factors can influence the outcome of any given titration. The scheme depends on protein adsorption, and the sensitized cells may at times spontaneously agglutinate in high protein concentrations. In this particular instance, 4 of the 15 human sera tested agglutinated cells sensitized to MKTCF to a titer of 1:10. Since all of these sera were from people who had received polio vaccine, the agglutinating titer to MKTCF may have been due to anti-monkey antibodies in these sera. This HA to monkey kidney material is seen also in Tables II and III. However, normal monkey sera showed no agglutination with cells sensitized to any of the 3 viral types. All non-dialyzable material, including kidney proteins, are concentrated by the PVP procedure. It would not be expected that monkeys would form antibodies regularly to homologous kidney proteins with the immunizing schedule used here. It is therefore felt that, when using sera other than monkey, the test is not reliable for the detection of antibodies to polioviruses in serum dilutions <1:10. Chicken serum was unsuitable for assay purposes since spontaneous agglutination of the sensitized cells occurred.

When cells were sensitized to purified Type III virus(7) and monovalent immune rabbit sera were titrated with these cells, homotypic titers averaged 1:320 while the heterotypic

TABLE VI. Molecules of Antigen Used for Sensitization.

Antigen	γ/ml	Molecular wt	Molecules per ml
Ovalbumin	5×10^3	4×10^4	7.5×10^{16}
Human γ-globulin	100	1.6×10^5	3.8×10^{14}
Polio I	13	6.7×10^6	1.2×10^{12}
II	5	*Idem*	4.5×10^{11}
III	5	"	*Idem*

Type I, 64 CF units/ml; Type II, 25 CF units/ml; Type III, 25 CF units/ml.

titers were 1:40. Nonspecific titers were <1:10. These results suggest that cross reactions were not lessened when purified virus was used to sensitize the cells, but nonspecific titers were lessened.

Data showing the number of molecules used to sensitize the cells are given in Table VI. These calculations were based on the relationship of 5 CF unit/γ of viral antigen (J. Charney, personal communication) while the amounts for the ovalbumin and gamma globulin were taken from Boyden(2). It becomes evident that the higher the molecular weight of the antigen, the fewer number of molecules are required for sensitization.

Statistically, the overall correlation between HA and neutralizing titers was fairly good ($r = 0.75$). However, a statistical analysis is an inadequate criterion when sera do not show any HA titers despite appreciable neutralizing antibody. It should be emphasized that if a serum has no HA titer, this does not mean that the serum contains no neutralizing antibodies (Table V, sera 1, 2 and 3).

Summary. An hemagglutination technic for the titration of antibodies to polioviruses has been described. The HA titers showed approximately a 75% correlation with neutralizing antibody. The virus-sensitized cells did not remain agglutinable for more than 2 days at 5°C, but have proven stable for at least 6 weeks when kept frozen. An antigenic cross among viral types of 12% was observed. Purified virus lessened the nonspecific HA, but not the percentage of cross reactions.

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Relationship of Mental and Emotional Stress to Serum Cholesterol Levels.* (23676)

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With the increased incidence of coronary heart disease there has been much speculation concerning a possible cause-and-effect relationship between stress, cholesterol-lipid-lipoprotein metabolism and coronary atherosclerosis(1,2,8). In considering the pathogenesis of a disease such as atherosclerosis or myocardial infarction one must recognize the possibility of direct as well as indirect effects of such factors as stress, diet, sex, age, exercise, etc. This is particularly evident in the case of stress because of its varied nature and the wide spectrum of bodily responses attributed to it(3). That certain forms of physiological stress can elevate serum cholesterol has been demonstrated. In a study of 55 male subjects Kuhl, *et al.*(4) found that brief immer-

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