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Yellow Fever Virus

II. Factors Affecting the Plaque Neutralization Test

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In studies on the factors and conditions influencing the yellow fever (YF) virus plaque neutralization test, 60 min was found to be the minimal time necessary for equilibration of the virus-antibody complex at 23 and 37 C. Maximal virus titers in the diluent controls and the pre- and postinoculation serum-containing mixtures occurred by the 60-min adsorption time. Serum neutralization indices also seemed to level by this time. Heating (56 C for 30 min) decreased the neutralizing capacity of serum. However, the slope of the neutralization curve was not affected. The addition of native (unheated) "serum factor" in the form of fresh guinea pig or monkey sera partially restored the neutralizing activity lost by heating in some, but not all, sera. Many sera contained nonspecific inhibitors of YF virus infectivity and neutralization. Preliminary studies with ether extraction suggest that these inhibitors are lipid in nature.

In a previous paper, we reported the development of a plaque neutralization (PN) test for measuring yellow fever (YF) virus antibody (16). We also described our evaluation of the PN test with the more conventional mouse neutralization test as a method for determining YF virus neutralizing capacity in sera. While these studies were being performed, we investigated various factors and conditions which might affect the PN test. Certain questions merited scrutiny. Did heat inactivation decrease the capacity of serum to neutralize YF virus as had been previously reported with other arboviruses (3, 7, 8, 11, 12, 18, 19)? If a heat-labile factor was destroyed by heating, could it be restored by the addition of "fresh serum" or complement to the system? To what extent could sera be diluted and still retain sufficient neutralizing capacity? What was the optimal temperature and time for incubation of the virus-antibody mixtures and for adsorption in cell cultures?

The answers to these questions have not only theoretical value, but also have practical importance in making performance of the PN test convenient, reproducible, and reliable. This paper presents the results of our investigations on the factors and conditions affecting the YF virus PN test.

MATERIALS AND METHODS

Virus. The passage history and method of preparation of infectious stock virus used in these studies have been described (16).

Animals. Newborn mice (1 to 3 days old) and weanling mice (16 to 20 g; 24 to 28 days old; Swiss albino, NIH-General Purpose strain) used for the mouse neutralization tests were obtained from the Rodent and Rabbit Production Section, Laboratory Aids Branch, National Institutes of Health (NIH). We used either complete litters (from 6 to 16 newborn mice) or 10 weanling mice at each dilution point.

Indian rhesus monkeys (*Macaca mulatta*) were immunized with YF vaccine either on the day of their arrival at NIH or after 6 weeks of quarantine. Some monkeys were inoculated intramuscularly with 0.5 ml of either commercial RIF (13) virus-contaminated YF vaccine or a RIF virus-free YF vaccine (17), developed in our laboratory. Titer of these vaccines varied from $10^{4.2}$ to $10^{4.8}$ LD_{50}/ml in weanling mice inoculated by the intracerebral route.

Burro no. 64 was inoculated subcutaneously with 10 ml of monkey plasma containing YF virus, Asibi strain ($10^{4.8}$ mouse LD_{50}/ml), and 32 days later was challenged with 20 ml of the same infectious monkey plasma given intravenously. (Inoculations and maintenance of this burro were performed at Fort Detrick under the direction of Stewart McConnell.)

Antisera. Sera used in the neutralization tests were obtained from two sources: monkeys before and 28 days after immunization with YF vaccine and burro no. 64 before and at various stipulated times after the second inoculation of Asibi virus.

Native (unheated) serum was used except in those experiments dealing with the effects of heating (56 C for 30 min). In some experiments in which the possible presence of viral inhibitors was evaluated, sera were treated with anhydrous ether according to the method of Hana and Styk (6).

Source of complement. Lyophilized pooled guinea

TABLE 1. *Effect of temperature and time of incubation on the plaque and mouse neutralization tests*

Incubation		Time serum obtained[a]	Neutralization tests[b]			
			PN		WMN	
Temp (C)	Time (min)		Titer	NI[c]	Titer	NI
4	30	Pre	6.6[d]		4.3	
		Post	5.4	1.2	4.8	(−0.5)
	60	Pre	6.5		5.4	
		Post	5.5	1.0	4.2	1.2
23	30	Pre	6.5		5.0	
		Post	4.6	1.9	3.5	1.5
	60	Pre	6.4		5.2	
		Post	4.5	1.9	3.4	1.8
37	30	Pre	6.3		5.1	
		Post	4.7	1.6	3.5	1.6
	60	Pre	6.1		4.6	
		Post	4.1	2.0	3.4	1.2

[a] Pre = preinoculation serum from burro no. 64: post = serum obtained from same burro 72 days after first inoculation and 40 days after second inoculation of yellow fever (Asibi strain) virus.

[b] PN = plaque neutralization; WMN = weanling (16 to 20 g; 24 to 28 days old) mouse neutralization.

[c] NI = neutralization index (difference in titer between pre- and postinoculation serum samples) expressed as Dex values.

[d] Decimal exponent (Dex) value (5) as determined by the Kärber method (10).

pig sera (lot 370A51, Hyland Laboratories, Los Angeles, Calif.) was used as a source of complement. At least two ampoules were reconstituted with diluent and pooled prior to use in each experiment. Appropriate dilutions were made in phosphate-buffered saline (PBS; 4) with 0.5% bovine plasma albumin (BPA; obtained as Fraction V of bovine plasma from Armour Pharmaceutical Co., Kankakee, Ill.). In simultaneously performed controls, heated (56 C for 30 min) guinea pig serum which was similarly diluted was used. Control virus titrations were also performed in the presence of heated and unheated guinea pig serum. All manipulations were done immediately prior to performance of the experiment.

Fresh monkey serum was also used as a source of complement. Blood was collected from a rhesus monkey, the clot was allowed to retract at 4 C overnight, and, after centrifugation at 900 × *g* for 30 min at 4 C, the serum was removed and used within 24 hr.

Cell culture. The MA-104 embryonic rhesus monkey kidney cell cultures (Microbiological Associates, Inc., Bethesda, Md.) were prepared by the Cell Biology Section of our laboratory in a routine manner established by them. The method of preparation has been described (16).

Neutralization tests. The plaque and intracranial mouse neutralization tests have been described previously (16). The difference in titer between serum samples taken before and after immunization represents the neutralizing capacity of the serum and will be expressed as neutralization indices in this paper. Mouse LD_{50} end points were determined by the Kärber method (10) and are expressed as decimal exponent (Dex) values (5).

Statistical analysis. Our results on the effect of "serum factor" upon the YF neutralizing capacity of sera were analyzed statistically by Clifford J. Maloney, Biometrics Section, Division of Biologics Standards.

Results

Effect of time and temperature of incubation of the virus-serum mixtures upon the plaque and mouse neutralization tests. Virus-serum mixtures were incubated simultaneously at 4, 23, and 37 C.

TABLE 2. *Effect of temperature and time of incubation of virus-serum mixtures on the plaque neutralization test*

Temp (C)	Incubation time (min)	Titer				NI[b]	
		Pre[a]		Post			
		Expt 1	Expt 2	Expt 1	Expt 2	Expt 1	Expt 2
4	15	6.6[c]		5.3		1.3	
	30	6.5		5.1		1.4	
	60	6.4	5.7	4.9	4.4	1.5	1.3
	90	6.4		4.6		1.8	
	120	6.3	5.8	4.4	4.1	1.9	1.7
	180		5.9		4.1		1.8
	240		5.7		4.1		1.6
23	15	6.4		5.2		1.2	
	30	6.3		4.7		1.6	
	60	6.3	5.7	4.4	3.8	1.9	1.9
	90	6.1		4.4		1.7	
	120	6.2	5.6	4.1	3.4	2.1	2.2
	180		5.6		3.3		2.3
	240		5.5		3.2		2.3
37	15	6.3		4.9		1.4	
	30	6.1		4.0		2.1	
	60	5.9	5.7	3.7	3.2	2.2	2.5
	90	5.9		3.8		2.1	
	120	5.9	5.3	3.5	3.1	2.4	2.2
	180		5.3		3.1		2.2
	240		5.3		2.9		2.3

[a] Pre = preinoculation serum; post = postinoculation serum; experiment 1 was performed with serum from burro no. 64, postinoculation serum obtained 56 days after first inoculation and 24 days after second inoculation of yellow fever (Asibi strain) virus; experiment 2 was performed with serum from monkey no. 23, postinoculation serum obtained 28 days after inoculation of yellow fever vaccine (17D strain).

[b] NI = neutralization index (difference in titer between pre- and postinoculation serum samples) expressed as Dex values.

[c] Decimal exponent (Dex) value (5) as determined by the Kärber method (10).

Samples of each mixture were removed after 30 and 60 min and were inoculated into cell cultures and weanling mice (Table 1). The neutralization indices in the two systems were similar except for the results obtained at 4 C after 30 min when the titer of the mixture containing the preimmunization serum in mice appears questionable. The most favorable temperatures for achieving neutralization, especially for the PN test, appeared to be 23 and 37 C.

In an attempt to clarify the effect of time and temperature, additional experiments were performed with serum from burro no. 64 obtained at a different time from that used in the experiment described above and serum from a monkey immunized with YF vaccine (Table 2). When burro serum was used (experiment 1, Table 2), the neutralization indices appeared to increase with an increase in time and temperature of incubation. When monkey serum was used (experiment 2, Table 2), the neutralization indices were highest at 23 and 37 C and appeared to increase with an increase in time of incubation. The value obtained at 37 C after 60 min appears to be spuriously high.

The convenience and availability of space at room temperature (23 C) prompted us to select this temperature (for 1 hr) as the most practical for subsequent experiments.

Effect of adsorption time on the PN test. After incubation of the virus-serum mixture at room temperature for 1 hr, 0.2 ml of each mixture was inoculated into each culture bottle of MA-104 cells. After different periods of adsorption at 37 C, two bottles at each dilution were removed and overlaid. Table 3 shows that maximal virus titers in the diluent control and pre- and postinoculation serum-containing mixtures occurred by the 60-min adsorption time. The neutralization indices also seemed to level by this time. The data from experiments 1 and 3 (Table 3) suggest that higher neutralization indices occurred at the 15- and 30-min adsorption time. Lower neutralization indices which occurred afterwards could have resulted from dissociation of virus/antibody complexes prior to reaching a state of equilibrium.

Effect of dilution of serum on the PN test. Serial twofold dilutions of unheated serum in PBS with 0.5% BPA were added to equal amounts of serial 10-fold dilutions of virus in the same diluent. Incubation proceeded as described for the PN test (6). Figure 1 shows what effect dilution of the serum had upon the neutralizing capacity of two different sera obtained from monkeys immunized with YF vaccine. From a dilution of 1:2 and higher, there appeared to be a linear relationship between serum dilution and amount of virus neutralized. The degree of neutralization by the undiluted serum appeared to be less than what could have been expected by extrapolating (dotted line, Fig. 1a) from the data of the higher dilutions. It is also possible that the correct slope of neutralization is reflected by the solid line connecting the undiluted and 1:2 dilution points and that further dilution removed some accessory substance required for neutralization.

Figure 1b shows the data obtained with three other sera which were used before and after heating (56 C for 30 min). The results were similar to that shown in Fig. 1a. In addition, although heat-

TABLE 3. *Effect of adsorption time on the plaque neutralization test*

Time (min)	Titer									LNI[c]		
	Control[a]			Pre[b]			Post[b]					
	1[d]	2	3	1	2	3	1	2	3	1	2	3
15	6.1[e]					5.8			2.4			3.4
30	6.4	6.1	6.1	5.7		6.1	4.0		2.9	1.7		3.2
60	6.7	6.5	6.3	5.8	5.8	6.2	4.5	3.3	3.5	1.3	2.5	2.7
90	6.8	6.6		5.8	6.0	6.3	4.6	3.3	3.6	1.2	2.7	2.7
120	6.8	6.7		5.8	6.2	6.5	4.6	3.3	3.7	1.2	2.9	2.8
150	6.8											
180	6.7											

[a] Diluent control.
[b] Pre = preinoculation serum; post = postinoculation serum.
[c] Log neutralization index.
[d] Experiment number. No. 1 performed with postinoculation burro (no. 64) serum obtained 56 days after first inoculation and 24 days after second inoculation of YF (Asibi) virus; no. 2 and 3 performed with sera from monkeys inoculated with YF vaccine.
[e] Dex value (5).

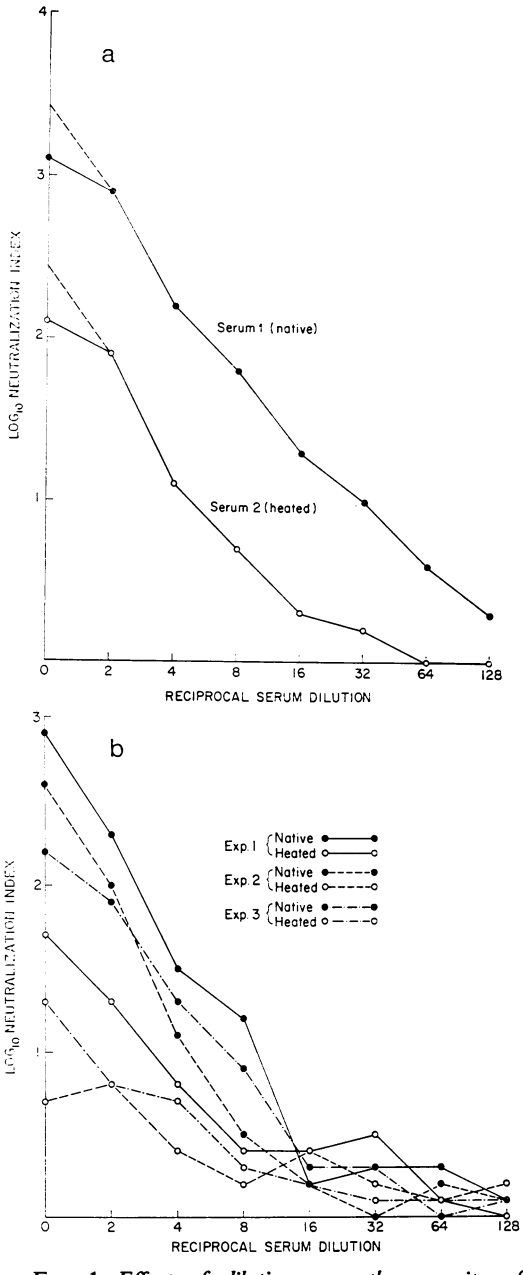

FIG. 1. *Effect of dilution upon the capacity of serum to neutralize yellow fever virus.*

ing lowered the neutralizing capacity of the serum, it did not greatly affect the slope of neutralization.

Presence in serum of an inhibitor to plaquing of YF virus; possible presence of an inhibitor to neutralization. Our data were examined to determine what effect "normal" monkey serum had upon the growth of YF virus in cell culture and mouse

TABLE 4. *Comparison of titer of yellow fever (YF) virus between diluent and serum-containing mixtures*

No. of preinoculation sera per test	YF virus titer in presence of		Difference in titer between diluent and serum
	Diluent	Serum	
	PFU titer		
4	4.6[a]	4.1–4.5[b]	−.5 to −.1[c]
4	3.9	3.2–3.9	−.7 to 0
4	3.5	3.0–3.2	−.5 to −.3
4	6.4	6.2–6.4	−.2 to 0
4	6.4	6.2–6.3	−.2 to −.1
4	6.7	6.5–6.7	−.2 to 0
8	6.5	5.7–6.1	−.8 to −.4
8	6.6	6.1–6.4	−.5 to −.2
7	6.7	5.9–6.3	−.8 to −.4
	LD_{50} titer		
4	5.5[d]	4.8–5.9	−.7 to +.4
8	5.3[e]	5.3–6.5	0 to +1.2
8	4.9[e]	5.3–6.2	+.4 to +1.3
7	6.0[e]	5.5–6.1	−.5 to +.1

[a] Dex value (5) of PFU/0.2 ml of inoculum.
[b] Range of titer of serum-containing mixtures.
[c] Minus values represent decrease and plus values represent increase in titer of serum-containing mixture when compared with diluent control.
[d] Dex value (5) as calculated by the Kärber method (10); weanling mice receiving 0.03 ml of inoculum by the intracerebral route.
[e] Same as *d* except that newborn mice were used.

systems. Table 4 reveals that in MA-104 cell culture the plaque-forming unit (PFU) titer of YF virus was almost always less in the presence of preimmunization monkey serum than in the corresponding diluent (PBS with 0.5% BPA) control. In the in vivo mouse system the titer of serum-containing mixtures tended to be slightly higher than that of the diluent control. When the PFU titer was lower in the presence of serum, the plaques themselves usually were smaller than those in the diluent control bottles.

In addition, our studies of the effect of serum dilution on the PN test suggest that serum might contain an inhibitor to neutralization. A study is currently being performed to determine the nature of this inhibitor. A preliminary experiment demonstrated the presence of an inhibitor in the lipid fraction, as had been postulated by Casals and Olitsky (2). We have been using the ether extraction method of Hana and Styk (6) to remove the lipid fraction from sera prior to using sera in the hemagglutination-inhibition test. In our hands, this method does not result in loss of any of the three major classes of immunoglobulins, IgG,

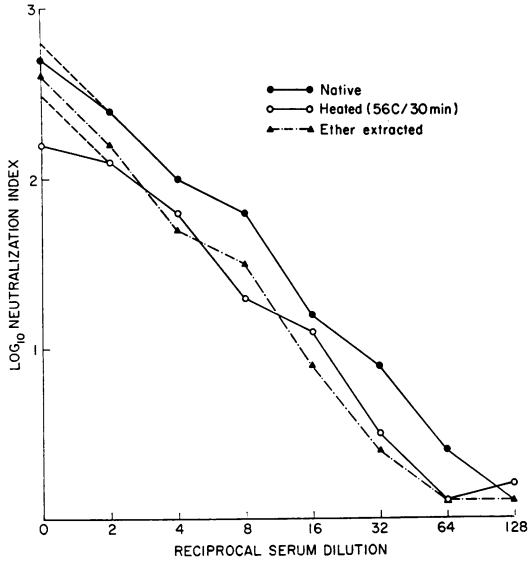

FIG. 2. *Effect of heat (56 C, 30 min) and ether extraction upon the capacity of serum to neutralize yellow fever virus.*

IgA, or IgM (*unpublished observations*). Figure 2 is representative of our results. The slope of neutralization of ether-extracted serum closely approximates that for unheated serum, except for the mixture containing undiluted serum where the degree of neutralization is what might have been expected by extrapolating from the mixtures containing serum diluted 1:2 and greater.

Effect of heating (56 C for 30 min) upon the neutralizing capacity of sera. Samples of pre- and postimmunization sera obtained from monkeys immunized with YF vaccine were heated (56 C for 30 min). The PN and weanling mouse neutralization tests were performed simultaneously on the same virus-serum mixtures with the use of both unheated and heated samples of the same sera. We concluded from our results (Table 5) that (i) the PN test was more sensitive than the weanling mouse neutralization test for determining YF virus neutralizing activity in unheated sera and (ii) heating appeared to reduce the neutralizing capacity of sera to a "baseline" value which was almost the same in both the PN and weanling mouse neutralization tests. This reduction in the neutralization index was greater for the PN test, probably because of the higher values obtained with the unheated sera when this test was used. Occasionally, the neutralization index was greater with the heated serum when the weanling mouse neutralization test was used (Table 5, monkeys 10 and 11). This might have been due to complement-like accessory factors present in vivo.

TABLE 5. *Effect of heating on the neutralizing capacity of sera*

Monkey no.	Neutralization indices[a]					
	PNT[b]			WMNT[b]		
	Unheated	Heated[c]	Dex decrease	Unheated	Heated	Dex decrease
9	3.9[d]	2.7	1.2	2.3[e]	2.7	(+0.4)[f]
10	3.8	2.6	1.2	2.2	2.9	(+0.7)
11	4.0	2.7	1.3	3.7	3.0	0.7
12	4.2	2.9	1.3	2.9	2.8	0.1
910	3.1	1.5	1.6	1.9	1.1	0.8
917	3.3	1.1	2.2	2.3	0.8	1.5
918	3.7	1.6	2.1	2.6	1.5	1.1
920	3.5	1.7	1.8	3.4	1.8	1.6
924	1.7	0.9	0.8	1.1	1.0	0.1

[a] Neutralization index = difference in titer between serum samples taken before and after immunization.

[b] PNT = plaque neutralization test; WMNT = weanling mouse neutralization test. Tests were simultaneously performed on the same virus serum mixtures.

[c] Samples of both pre- and postimmunization sera were heated at 56 C for 30 min.

[d] Dex value (5) as calculated from direct plaque counts.

[e] Dex value as calculated by the Kärber method (10).

[f] Positive values represent an increase NI in the heated serum when compared with the unheated.

Effect of the addition of "serum factor" upon the YF virus neutralizing capacity of sera. The results of the previous experiments prompted us to determine whether the loss of neutralizing activity caused by heating could be restored by the addition of complement or complement-like factors. Table 6 shows the results of this study. Lyophilized pooled guinea pig serum was used as the source of "fresh serum" factors in 17 tests; fresh monkey serum was used in 3. Neutralization tests were performed with diluent containing 0.5, 1, 2, or 10% serum factor. Diluent plus either unheated or heated serum factor served as a guide to the reproducibility of the tests, since it was not expected that the addition of serum factor would influence the virus titer.

These experiments confirmed our previous observation concerning the effect of heating upon the neutralizing capacity of serum. Table 7 essentially summarizes the data presented in longer form in Table 6. Comparison of the native antiserum plus diluent with the heated antiserum plus diluent controls shows that heating the serum decreased

the neutralizing capacity of that serum in 17 of 20 tests (A1, Table 7). Exceptions were the PN test (experiment 3a, Table 6), when heating resulted in a slight increase in the neutralizing capacity of YF immune burro serum; the newborn mouse neutralization test (experiment 5b), when heating resulted in a slight increase in neutralizing capacity of YF immune monkey serum; and the PN test (experiment 11a), when heating resulted in essentially no change in the neutralization index. In each instance, another neutralization test performed with the same virus-serum mixture showed the decreased neutralizing capacity of the heated serum (experiments 3b, 5a, and 11b). When compared with native antiserum (Table 7), the loss of neutralizing capacity caused by heating was not restored by native (unheated) serum factor (A4, Table 7). However, a comparison with heated antiserum shows that the addition of native serum factor could partially restore this loss (B1, Table 7, and Table 6). The effects of native serum factor upon the neutralizing capacity of native YF anti-

TABLE 6. *Effect of the addition of "serum factor" upon the yellow fever (YF) virus neutralizing capacity of sera*

Expt no.[a]	Concn of serum factor	Factor source	Source of YF antiserum	Type of NT[b]	Conditions of YF antiserum and "serum factor" mixtures used in neutralization tests[c]								
					Diluent +		Native +			Heated +			
					NSF[c] 1[d]	HSF[e] 2	Diluent 3	NSF 4	HSF 5	Diluent 6	NSF 7	HSF 8
1a	0.5[e]	GpS[f]	Burro	PN[b]	0.1[g]	0[g]	0.4[h]	1.9	2.1	0.8	1.1	0.8
1b		GpS	Burro	NBN[b]	0.1	0.3	1.8	1.5	2.0	1.1	1.1	1.4
2a		GpS	Monkey	PN	+0.1	+0.2	2.3	2.4	2.4	1.0	1.2	1.2
2b		GpS	Burro	NBN	+0.2	0	2.2	1.7	1.6	1.1	0.7	0.8
3a	1.0	GpS	Burro	PN	0.1	0.2	0.9	1.7	1.8	1.1	1.5	1.1
3b		GpS	Burro	WMN	+0.3	0	1.3	1.1	0.9	1.0	0.7	0.4
4a		GpS	Monkey	PN	0.2	0.3	2.7	2.9	2.9	1.9	2.3	1.6
4b		Monkey[f]	Monkey	PN	0.1	0.1	2.7	2.5	2.6	1.9	2.2	2.0
5a	2.0	GpS	Monkey	PN	0.2	0.4	1.5	1.3	1.2	0.8	0.4	0.7
5b		GpS	Monkey	NBN	+0.1	+0.3	0.3	1.3	1.0	0.5	0.8	0.6
6		GpS	Monkey	NBN	+0.4	0.6	2.9	2.7	2.4	1.5	2.1	1.6
7		GpS	Monkey	PN	0.1	0.1	2.0	1.7	1.6	0.9	0.9	0.6
8a	10	GpS	Monkey	PN	0.1	0	1.1	1.1	0.9	0.4	0.9	0.5
8b		Monkey	Monkey	PN	0.1	+0.1	1.1	1.2	0.9	0.4	0.8	0.7
9a		GpS	Monkey	PN	0	+0.1	2.9	2.9	2.9	2.6	2.8	2.5
9b		Monkey	Monkey	PN	+0.1	0	2.9	2.9	2.6	2.6	2.8	2.3
10a	0.5	GpS	Monkey	PN	0.1	0	2.6	2.8	2.6	2.3	2.5	2.1
10b	1.0	GpS	Monkey	PN	0	+0.1	2.4	2.7	2.6	2.1	2.4	2.1
11a	2.0	GpS	Monkey	PN	+0.1	+0.1	2.8	2.7	2.7	2.7	2.0	1.9
11b	10	GpS	Monkey	PN	0.1	0	2.8	2.8	2.7	2.4	2.6	2.5

[a] Each experiment is designated by a different number; "a" and "b" parts with the same experiment number were performed simultaneously with the same serum-virus mixtures.

[b] NT = neutralization test; PN = plaque neutralization; NBN = newborn mouse neutralization; WMN = weanling mouse neutralization.

[c] Mixtures of diluent, native (unheated) and heated (56 C/30 minutes) YF antiserum, and native serum factor (NSF) and heated serum factor (HSF).

[d] Treatment number referred to in text.

[e] Percentage of "serum factor" contained in the 0.5 BPA/PBS diluent.

[f] GpS = guinea pig serum; monkey = monkey serum.

[g] Diluent plus either NSF or HSF served as a guide to the reproducibility of the test since it was not expected that the addition of "serum factor" would influence the virus titer. Values represent the difference in virus titers between mixtures prepared in the beginning and end of the experiment. Positive value means a titer increase.

[h] Neutralization index expressed as Dex value (5) as calculated from direct plaque count or by the Kärber method (10) in the mouse neutralization tests.

TABLE 7. *Summary of effect of "serum factor" upon the yellow fever virus neutralizing capacity of sera*

Test conditions[a]	No. of tests[b]		
	Unchanged	Increased	Decreased
A. Comparison with native antiserum + diluent			
1. Heated + diluent			
PN[c]	1	2	12
MN	0	1	4
Total	1	3	16
2. Native + NSF			
PN	7	5	3
MN	0	1	4
Total	7	6	7
3. Native + HSF			
PN	6	4	5
MN	0	2	3
Total	6	6	8
4. Heated + NSF			
PN	4	2	9
MN	0	1	4
Total	4	3	13
5. Heated + HSF			
PN	0	1	14
MN	0	1	4
Total	0	2	18
B. Comparison with heated antiserum + diluent			
1. Heated + NSF			
PN	1	12	2
MN	1	2	2
Total	2	14	4
2. Heated + HSF			
PN	8	2	5
MN	2	1	2
Total	10	3	7

[a] Mixtures of diluent, native (unheated) and heated (56 C, 30 min) YF antiserum, and native serum factor (NSF) and heated serum factor (HSF).

[b] Number of tests showing a neutralization index that is unchanged, increased or decreased when compared to the respective native (A) or heated (B) YF antiserum controls. Test results were considered unchanged when the titers were not different by more than 0.1 Dex (5).

[c] PN = plaque neutralization and MN = mouse neutralization tests.

serum (A2, Table 7), of heated serum factor upon native YF antiserum (A3, Table 7), and of heated serum factor upon heated YF antiserum (B2, Table 7) were variable.

We concluded from these experiments that tests to determine YF virus neutralizing activity were best performed with unheated serum without the addition of serum factor.

The data in Table 6 were analyzed statistically. A comparison of the PN with the mouse neutralization tests revealed that there was no difference ($P \lesssim 0.05$) except at the 1% serum factor concentration (Table 6, experiment 3), and this was the only experiment where the weanling mouse neutralization test was used.

A *t* test comparing pooled mean variances of treatments 6 and 8 to treatment 7 indicates that the difference of 0.18 of the mean is significant at the 1% serum factor concentration. Although treatment 7 does differ distinctly from treatments 6 and 8, it does not reach the level of treatment 4. The ratio of treatment 7 to 6 and 8 is twofold and of treatment 4 to 7 is threefold.

Concerning concentration of serum factor, an enhancing effect occurred at the 1% strength ($P \lesssim 0.01$), and this effect was maintained but not increased at the higher concentrations.

DISCUSSION

In previous studies (15) of the conditions affecting YF virus neutralization, the white mouse was used to measure YF virus infectivity. The development of a convenient and reproducible plaque assay for YF virus infectivity provided a quicker and more accurate means of studying the factors influencing the neutralization of YF virus. From our experiments with both the mouse and plaque methods, we concluded that factors influencing YF virus neutralization were quite similar in both systems.

The minimal time necessary for equilibration or stabilization of the virus-antibody complex was 60 min at 23 and 37 C. The convenience and availability of space at room temperature (23 C) prompted us to select this temperature for 60 min as the most practical for incubation of the virus-antibody mixture.

Another factor (not mentioned in the Results section) influencing the virus-antibody mixture is the *p*H of the diluent (*unpublished observation*). The addition of BPA decreases the *p*H of the PBS toward the acid range, necessitating readjustment of the *p*H to 7.2 to 7.4. YF virus is rapidly inactivated by acid *p*H but remains stable at neutral or slightly alkaline *p*H during the 60-min incubation time required for the test.

We did not perform experiments to evaluate the effect of different temperatures for virus adsorption. We arbitrarily chose 37 C because we found this temperature suitable for the many different viruses with which we work (*unpublished observations*).

Because maximal virus titers occurred after 60

min of adsorption, this time was selected as standard for infectivity plaque assay and the plaque neutralization test.

The inhibitory effect of serum lipids on hemagglutination by arboviruses is well known. The role of nonspecific substances inhibiting virus infectivity and neutralization has not been well defined. Smithburn (15) and Bugher (1) considered the most important sources of variability in the YF virus mouse protection test to be inherent to sera from different animal species. Koprowski (9) and Bugher (1) found that serum from many animals contained virucidal substances to arboviruses. In addition, Koprowski (9) discovered that the presence of these substances was transitory. We feel that this may be related to the nature and time of the food intake with respect to the time of obtaining the serum.

Casals and Olitsky (2) were able to get plaque reduction with St. Louis, Russian spring-summer, and Japanese B encephalitis viruses by the addition of the fatty layer extracted from serum. Our experience is quite similar. Native serum reduced not only the number but also the size of YF virus plaques. One possibility is that the inhibitor to plaque production (Table 4) represents antibody to either YF or other group B viruses and the detection of these specific neutralizing substances reflects the greater sensitivity of the plaque system for measuring antibody (16). Of course, another possibility is that some nonspecific inhibitor(s) is present. This latter alternative is supported by the fact that ether extraction of serum, which removes lipids without altering the concentration of immunoglobulins, also removes the inhibitor to virus growth.

Table 4 shows that YF virus titers in the presence of some preimmunization sera were decreased when compared with diluent controls.

Perhaps our observations that not all sera contain inhibitors may be related to the transitory nature of these inhibitors, as previously noted by Koprowski (9).

Our data suggest the presence of substances which inhibit the neutralization of YF virus. Figures 1 and 2 show that not all sera contain the neutralizing inhibitor at the zero dilution. Preliminary studies reveal that this inhibitor is present in the serum lipid fraction and can be removed by treating the sera with ether according to the method described by Hana and Styk (6) without affecting antibody titer (*unpublished observation*).

Further studies are currently being performed to elucidate the nature of the inhibitors of YF virus infectivity and neutralization.

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