A LABILE COMPONENT OF NORMAL SERUM WHICH COMBINES WITH VARIOUS VIRUSES. NEUTRALIZATION OF INFECTIVITY AND INHIBITION OF HEMAGGLUTINATION BY THE COMPONENT

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During an investigation of the etiology of an epidemic of a mild infectious disease, for convenience termed "epidemic myalgia," it was observed that a heat labile component in serum obtained during convalescence neutralized Newcastle disease virus. It was noted that serum stored at 4°C. for more than 2 weeks was no longer able to protect chick embryos from infection with this virus, whereas serum held in the frozen state at −28°C. retained such activity. The importance of this phenomenon seemed considerable on both a practical and theoretical level.

A number of workers have observed that certain thermolabile components of serum, either alone or in conjunction with immune antibodies, may neutralize the infectivity of various viruses. Gordon (1) observed that the addition of fresh guinea pig serum to heated serum of a rabbit immunized with vaccinia virus increased the neutralization titer against vaccinia virus. Douglas and Smith (2) also noted a heat labile component in normal rabbit blood, plasma, and serum which inhibited infection of rabbits with vaccinia virus, and which was not active when sodium citrate was employed as an anticoagulant. That the activity of immune serum against Rous sarcoma virus could be enhanced by the addition of fresh guinea pig serum was pointed out by Mueller (3) who interpreted this to be an activity of complement. More recently Morgan (4) and Whitman (5) both demonstrated that unheated immune rabbit serum neutralized Western equine encephalomyelitis virus in higher dilutions than did the same serum heated at 56°C. for 30 minutes. The addition of fresh, unheated guinea pig serum (4, 5), monkey, or human serum (5) to inactivated immune serum increased neutralization. Morgan (4) believed the increased activity resulting from the addition of normal guinea pig serum to be the result of the enhancement of antibody activity by complement. Leymaster and Ward (6) have demonstrated higher mumps virus neutralization titers when immune sera were not heated. The addition of normal, unheated human or monkey serum also enhanced the neutralization titers of heat-inactivated human sera (7). It has been pointed out recently by Smith and Westwood (8) that unheated sera of guinea pigs neutralized influenza A virus.

It is the purpose of this paper to show that there is present in the serum of normal human beings, guinea pigs, rabbits, or mice a heat labile component which neutralizes the infectivity of a number of viruses and inhibits hemagglu-

tination of chicken erythrocytes by these agents; that the serum component produces these effects by combining with the viruses studied; that calcium ions are necessary for serum component–virus combination to take place; that the labile component is probably a protein or protein complex; that the thermolabile component is not identical with hemolytic complement.

Materials and Methods

Viruses.—The following viruses were employed: influenza A, PR8 strain; influenza B, Lee strain; Newcastle disease; and mumps which will be referred to as PR8, Lee, NDV, and MV, respectively. Each virus was propagated in the allantoic sac of chick embryos 7 to 12 days of age. The methods of cultivation and storage were identical to those previously described (9).

Virus Infectivity and Hemagglutination Titrations.—The methods employed were identical to those used previously (9). Infectivity titrations were carried out with serial tenfold dilutions of infected allantoic fluid inoculated into the allantoic sac of chick embryos of appropriate age. Hemagglutination titrations were performed with serial twofold dilutions in 0.85 per cent NaCl buffered at pH 7.2 (0.01 M phosphate), hereafter referred to as saline, and an equal volume of a 1 per cent suspension of chicken RBC. The results of all titrations are expressed in terms of final concentrations.

Virus Neutralization Titrations.—Twofold dilutions of serum were made in saline, and to each was added a constant quantity of virus (usually 10^2 to 10^3 embryo infectious doses, E.I.D.). After a period of incubation, 0.1 or 0.2 cc. of each mixture was injected intra-allantoically into each of 4 embryos of appropriate age. The hemagglutination titer of each allantoic fluid was determined after incubation at 35°C. for a period dependent upon the virus employed.

Hemagglutination-Inhibition Titrations.—Twofold dilutions of serum were prepared in saline, and to each was added a constant quantity of virus; 16 hemagglutinating units usually was employed. After incubation for 15 or 30 minutes in a water bath at 37°C., an equal volume of a 1 per cent suspension of chicken RBC was added. The titer was taken as the highest dilution in which there was complete inhibition of hemagglutination.

Sera.—Human, guinea pig, rabbit, and mouse sera were employed. Guinea pig and mouse sera were obtained from groups of at least 4 animals and pooled. Blood was obtained from children 3 to 4 years of age with no history of mumps or influenza. All blood specimens were allowed to clot at 4°C. The serum was separated promptly, then stored in 1 to 2 cc. volumes in nitrose cellulose tubes at −28°C.

Complement Titrations.—Titrations were carried out with various guinea pig serum fractions as well as fresh guinea pig serum by employing decreasing quantities of a 1:20 or 1:30 dilution. Two units of anti-sheep hemolysin and a 3 per cent suspension of sheep RBC were employed. All suspensions and dilutions were made in 0.85 per cent NaCl containing 0.1 gm. $MgSO_4$ per liter. Complement titers are expressed as the smallest quantity of serum in which complete hemolysis of the sensitized sheep RBC was brought about.

EXPERIMENTAL

Prevention of Virus Infection of the Chick Embryo by a Heat Labile Component of Serum.—Sera obtained from patients ill with a mild infectious disease were examined for antibodies against various viruses. Preliminary tests showed that unheated sera obtained during the acute phase of the illness did not prevent infection of chick embryos with Newcastle disease virus (NDV), whereas unheated sera obtained during convalescence did prevent such infection. As

it happens, the acute phase sera had been stored at 4°C. for 10 to 18 days before being frozen and stored at —28°C., whereas the convalescent sera were stored at the latter temperature immediately after separation. This question arose: was neutralization of NDV a result of the development of specific antibodies or was it due to the presence of an unstable component of serum other than antibody which was capable of inactivating the virus?

TABLE I

Neutralization of Newcastle Disease Virus by a Heat Labile Component of Human Serum

Mixture* 0.2 cc. intra-allantoic				Hemagglutination titer allantoic fluids‡				Serum neutralization titer‡
Human serum			NDV virus	A	B	C	D	
Storage 4°C.	Date obtained	Dilution						
days	*1948*		E.I.D.§					
0¶	Aug. 8	1:2	10³	0	0	0	0	
		1:4	"	0	0	0	16	1:5
		1:8	"	32	2	64	4	
		1:16	"	64	32	128	8	
16	" "	1:2	"	128	64	>128	32	0
		1:4	"	>128	128	128	64	
8	" 16	1:2	"	4	0	16	16	0
		1:4	"	16	16	64	16	
0	Sept. 10	1:2	"	0	0	0	0	
		1:4	"	0	4	0	0	1:5
		1:8	"	64	32	32	16	
		1:16	"	128	128	128	64	

* Held at room temperature for 30 minutes before inoculation.
‡ Expressed as the reciprocal.
§ E.I.D. = embryo infectious doses.
¶ Heparinized plasma stored at —28°C.

Human sera and heparinized plasma stored at —28°C., as well as sera stored for varying periods at 4°C. before being frozen, were employed in virus neutralization experiments, performed as described above, with NDV, PR8, Lee, and MV. In each experiment unheated serum and serum heated at 56°C. for 30 minutes were used.

The results of a typical experiment with human serum or heparinized plasma and NDV are presented in detail in Table I. Serum stored at 4°C. for 16 days did not prevent infection with 10³ E.I.D. of virus, whereas plasma obtained simultaneously and stored at —28°C. completely neutralized the agent. Serum obtained later and stored at —28°C. also neutralized the virus. Serum stored at 4°C. for only 8 days did not prevent infection but the hemagglutination

titers of the infected allantoic fluids were reduced. Unheated sera from 5 other patients with a similar disease and from 3 healthy persons were studied with NDV, and corresponding results were obtained; the neutralization titers varied from 1:5 to 1:21 when 10^2 E.I.D. of virus was employed. In each instance serum heated at 56°C. for 30 minutes did not neutralize the virus.

When dilutions of NDV were mixed with unheated human serum, it was found that relatively large amounts of virus were neutralized. The results of one such experiment are presented in Table II, and show that as much as 10^4 E.I.D. of NDV was neutralized by the unheated serum; i.e., the unheated serum had a neutralization index of 10,000 relative to the heated specimen.

Because adult human sera commonly contain specific antibodies against the viruses of influenza A, B, and mumps, they were unsatisfactory for this study. However, sera were obtained[1] from 2 children, 3 and 4 years old, respectively, who had no history of infection with these agents, and studies were carried out

TABLE II

Infectivity Titer of Newcastle Disease Virus in Presence of Unheated and Heat-Inactivated Human Serum

Mixture*		Incubation at 37°C.	Virus infectivity titer	Neutralization index
NDV	Human serum			
0.3 cc.	0.3 cc.	min.		
Serial dilutions	56°C., 30 min.	30	$10^{-7.8}$	
" "	Unheated	"	$10^{-3.7}$	10,000

* 0.1 cc. intra-allantoic per embryo.

in a manner identical to that described above. The results of experiments with these sera and mumps virus are summarized in Table III. The mean neutralization titer of the children's unheated sera was 1:54 when 10^2 E.I.D. of mumps virus was used, whereas the heated sera did not neutralize the virus at a dilution of 1:2. Unfortunately, the children's sera contained heat stable components, probably specific antibodies, which neutralized both influenza A and B viruses, and therefore it was not possible to test for a thermolabile component with these agents.

Inactivation of Various Viruses by Serum of Normal Animals.—In order to learn more about the heat labile component of normal human serum which inactivates NDV and mumps, it seemed advisable to determine: (1) whether a similar component was present in the serum of various laboratory animals, and (2) whether such a component, if present, would inactivate viruses other than NDV and mumps. Serum was obtained from normal guinea pigs, rabbits

and mice, and employed in experiments similar to those described above. The viruses of Newcastle disease, mumps, influenza A, and influenza B were used. The results of these experiments are summarized in Table III, and expressed as the mean of the neutralization titers obtained in at least two experiments. It is evident that a heat labile neutralizing component is present in both guinea pig and rabbit sera. The unheated guinea pig sera neutralized Lee, PR8, and MV, as well as NDV; rabbit sera inactivated NDV in comparable dilutions, but Lee and PR8 were neutralized only in low dilutions of serum; mouse sera did not contain the heat labile component in sufficient quantity to neutralize either NDV or Lee. It should be emphasized that in every instance when normal animal sera were heated at 56°C. for 30 minutes, the capacity to neutralize each of the viruses disappeared.

TABLE III

Neutralization of Newcastle Disease, Influenza A, B, and Mumps Viruses by a Heat Labile Component of Human, Guinea Pig, and Rabbit Sera

Serum	Mean neutralization titers of unheated sera against indicated virus*			
	NDV‡	PR8‡	Lee‡	MV§
Human.....................	1:10	Ab.¶	Ab.¶	1:54
Guinea pig.................	1:19	1:21	1:13	1:48
Rabbit.....................	1:13	1:2	1:4	<1:8
Mouse.....................	<1:2	—	<1:2	—

* In every instance in which a titer is given the heated serum failed to cause any neutralization.

‡ 10³ E.I.D. employed.

§ 10² "   "

¶ Specific antibodies present.

It was found that neutralization of virus infectivity was demonstrable with unheated normal serum when mixtures were injected without preliminary incubation, and the titers obtained were as high as when mixtures were incubated at room temperature for 30 minutes. A twofold increase in titer occurred when serum-virus mixtures were incubated for 30 minutes in a water bath at 37°C. There was no further increase when the period of incubation was increased to 120 minutes at room temperature.

Inhibition of Viral Hemagglutination by a Heat Labile Component of Serum.— In order to investigate more thoroughly neutralization of viruses by a heat labile component of normal serum, it was of importance to attempt to extend the studies by *in vitro* methods. The capacity of unheated serum to inhibit hemagglutination of the viruses used in neutralization experiments was determined.

The experiments were carried out with two different methods: (1) the hemag-glutination-inhibition technique with dilutions of serum, as described above, and (2) undiluted serum and virus were mixed, incubated for 15 or 30 minutes at 37°C., and then the hemagglutination titer of the mixture was determined. Most experiments *in vitro* were carried out with Lee virus, but PR8, MV, and NDV were also employed.

For purposes of clarity the results of experiments with each of the *in vitro* inhibition methods are presented in Tables IV and V. Table IV shows the

TABLE IV

Inhibition of Hemagglutination with Lee Virus by a Heat Labile Component in Guinea Pig Serum

Mixture*		Hemagglutination with serum dilutions							Hemagglutination-inhibition titer
Guinea pig serum dilutions	Lee virus	4	8	16	32	64	128	256	
Unheated..................	4 units‡	0	0	0	0	±	2	3	1:64
56°C., 30 min..............	" "	2	2	3	3	3	3	3	0

* Incubated at 37°C. for 30 minutes before addition of 1 per cent RBC.
‡ Final concentration.

TABLE V

Combination of Lee Virus and a Thermolabile Component in Guinea Pig Serum

Mixture*		Hemagglutination with dilutions of mixture										Hemagglutination titer of mixture
Guinea pig serum undiluted	Lee virus	4	8	16	32	64	128	256	512	1024	2048	
Unheated..............	1024 units	0	0	0	0	0	0	0	0	0	0	0
56°C., 30 min..........	" "	4	4	4	3	3	3	3	3	2	0	1:1024

* Incubated for 30 minutes at 37°C. before dilution and addition of 1 per cent RBC.

inhibition of hemagglutination of chicken RBC by a small amount of Lee virus in the presence of dilutions of unheated guinea pig serum. The same serum heated at 56°C. for 30 minutes showed no inhibition. Table V points out the extent of inhibition obtained with undiluted guinea pig serum and a large amount of Lee virus. The unheated serum completely inhibited hemagglutination on dilution of the mixture, whereas the heated serum did not.

Table VI shows a summary of the results obtained in similar *in vitro* inhibition experiments with normal human, guinea pig, and mouse sera and four viruses: NDV, Lee, PR8, and MV. Because of the presence of heat stable inhibitors, rabbit sera were unsatisfactory in these experiments and human sera could only be employed with NDV. It will be noted that both guinea pig and

mouse sera contain a heat labile component which inhibits hemagglutination of each of the viruses tested, and that unheated human serum inhibits hemagglutination of NDV, whereas heated serum does not. Certain guinea pig and mouse sera in low dilutions also contain a stable inhibitor of viral hemagglutination which is not inactivated by heating at 56°C. The stable inhibitor is most readily demonstrated when Lee or PR8 which has been heated at 56°C. for 30 minutes is employed (10).

It was found that inhibition was demonstrable immediately after mixing virus and unheated serum; that the extent of inhibition increased to a maximum value after incubation of the mixture for 15 minutes at 37°C.; that incubation for as long as 3 hours at 37°C. neither increased the degree of inhibition

TABLE VI

Inhibition of Hemagglutination with Newcastle Disease, Influenza A, B, and Mumps Viruses by a Heat Labile Component of Human, Guinea Pig, and Mouse Sera

Serum	Hemagglutination-inhibition titer vs.			
	NDV*	PR8*	Lee*	MV*
Human—unheated	1:16	—	—	—
" —heated‡	<1:4	—	—	—
Guinea pig—unheated	1:64	1:128	1:64	1:128
" " —heated‡	<1:8	1:8	<1:4	1:8
Mouse—unheated	1:64	1:64	1:256	1:64
" —heated‡	<1:8	<1:8	<1:8	<1:8

* Final concentration of 4 hemagglutinating units employed.
‡ 56°C. for 30 minutes.

nor brought about separation of the heat labile serum component and virus. It is of interest that not only does virus combine with serum component so that hemagglutination is not brought about by virus, but also virus inactivated by this component does not alter "virus receptors" of erythrocytes; fresh virus is adsorbed by cells treated for long periods with the virus-serum component combination.

Quantitative Factors Concerned with Neutralization and Hemagglutination-Inhibition Titrations.—The relationship between the quantities of virus and serum employed in *in vivo* and *in vitro* titrations of the heat labile serum component was studied.

The experiments were carried out with the Lee virus and a fresh pool of normal guinea pig serum. Hemagglutination-inhibition titrations were carried out with varying quantities of virus. In order to reduce dilution errors to a minimum, a single series of serum dilutions was prepared and aliquots of each dilution were tested against different quantities of virus to give

final concentrations of 1, 2, 4, and 8 hemagglutinating units. Following incubation of the mixtures at 37°C. for 30 minutes, a 1 per cent suspension of chicken RBC was added.

Virus neutralization titrations were carried out as described above. A single series of serum dilutions was used, and an equal volume of Lee virus varying in quantity from 10^2 to 10^5 E.I.D. was added to an aliquot of each dilution.
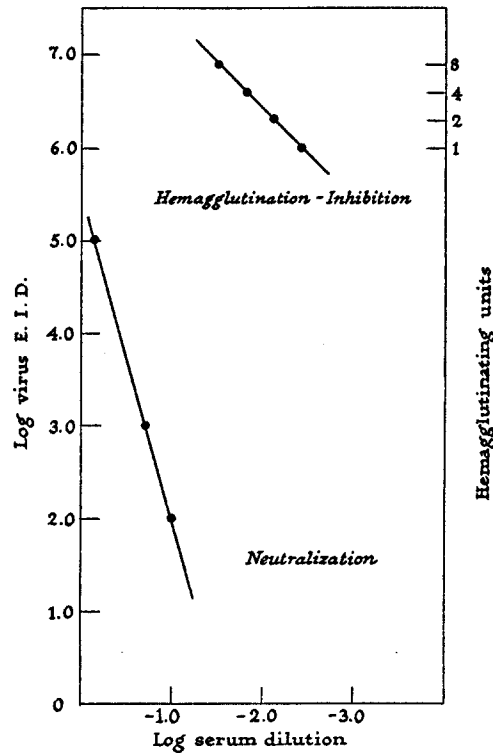

FIG. 1. Linear relationship between the quantity of Lee virus and the neutralization titer as well as the hemagglutination-inhibition titer of unheated guinea pig serum. In neutralization titrations 50 per cent end points were determined with at least 5 serum dilutions. Each serum-virus mixture was inoculated in a group of 4 chick embryos.

The results of these experiments are presented graphically in Fig. 1. There is a linear relationship between the quantity of virus used and the dilution of unheated serum required to inactivate it both *in vivo* and *in vitro*. However, it is important to note that for hemagglutination-inhibition the slope of the line is 1, *i.e.* a twofold decrease in the quantity of virus employed causes a twofold increase in the serum dilution end point, whereas for virus neutralization the slope of the line is much steeper, *i.e.* 3.8, indicating that comparatively large variations in the quantity of virus are required to alter significantly the neutralization titer. These quantitative virus-serum component relationships are

not remarkably different from those which have been found in similar experiments with virus and specific antibody (11, 12).

Inactivation of Serum Component.—One of the distinguishing properties of the normal serum component under study is its lability to heat and upon storage at 4°C. The rate of inactivation of the component during storage at 4°C. and on heating at 56°C. was investigated.

The results of an experiment on heat inactivation of the component in guinea pig serum are presented in Fig. 2. The time of heating in a 56°C. water bath is plotted against the hemagglutination-inhibition titer determined with Lee virus. About 75 per cent of the component was inactivated in 10 minutes, and complete inactivation had occurred after 30 minutes at this temperature.
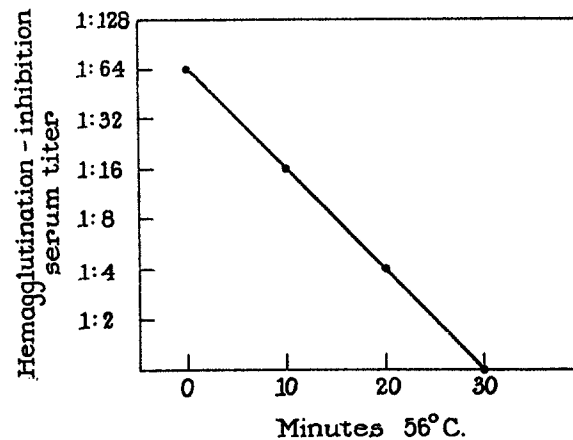

FIG. 2. Relation between the time of heating at 56°C. and the degree of inactivation of guinea pig serum component. Hemagglutination-inhibition titrations were carried out with 4 units of Lee virus.

When guinea pig serum was stored at 4°C., the capacity to neutralize Lee virus in the chick embryo was completely inactivated in 18 hours, but the capacity to inhibit hemagglutination by this virus did not begin to diminish until 7 days of storage, and the inhibitory property was still present, although in very low titer, after 3 weeks. As was pointed out above, the component in human serum which prevents the infection of chick embryos with NDV is inactivated slowly at 4°C. When human serum was tested at regular intervals after storage at 4°C., it was found that inactivation of the neutralizing component commenced after 7 days and was complete after 15 days.

Mechanism of Inactivation of Virus by Serum Component.—The data presented above suggested that virus inactivation by the heat labile serum component was a result of either: (1) stable combination between serum component and virus, or (2) destruction of virus by the serum component, perhaps

enzymatically. That the component acted directly on the virus and not on the host cells was indicated by the finding that a mixture of virus and serum showed no hemagglutination when diluted, and that there was a quantitative relationship between virus and serum component in both neutralization and hemagglutination-inhibition experiments. Attempts were made to obtain further data concerning the mechanism of viral inactivation by the component.

Undiluted fresh guinea pig serum was mixed with an equal volume of NDV diluted 10^{-1} in sterile normal horse serum. A similar mixture of serum which had been heated at 56°C. for 30 minutes and NDV was prepared. Following incubation of the mixtures at 37°C. for 30 minutes each was initially diluted 1:5 following which serial tenfold dilutions were made in broth, and infectivity titrations were carried out in embryos as described above.

TABLE VII

Inactivation of Newcastle Disease Virus by a Thermolabile Component of Guinea Pig Serum

Mixture held at 37°C., 30 min.		Constituents of inoculum final dilutions		Embryo infectivity score	Virus infectivity titration end point E.I.D.$_{50}$
Guinea pig serum	NDV dilution	Serum	NDV		
				positive/total	
		10^{-5}	10^{-6}	4/4	
Heated*	10^{-1}	10^{-6}	10^{-7}	4/4	
		10^{-7}	10^{-8}	4/4	$10^{-8.5}$
		10^{-8}	10^{-9}	0/4	
		10^{-3}	10^{-4}	4/4	
Unheated	"	10^{-4}	10^{-5}	3/4	$10^{-5.5}$
		10^{-5}	10^{-6}	1/4	
		10^{-6}	10^{-7}	0/4	

* 56°C. for 30 minutes.

The results of this experiment are shown in Table VII. The infectivity titer of NDV which had been mixed with unheated serum was 1,000 times less than that of the virus which had been mixed with heated serum. It will be noted that at a virus dilution of 10^{-7} the serum dilution was 10^{-6}, and yet each embryo was protected from infection with the mixture containing unheated serum. These results indicate that inactivation of NDV by the heat labile component is a result of an effect of the component on the virus itself, and serve to substantiate the results obtained with unheated serum-virus mixtures by the hemagglutination technique.

In order to obtain more direct evidence bearing on the question: is virus inactivation a result of combination with the serum component or a result of enzymatic inactivation by it, the following experiment was carried out.

To 0.8 cc. of fresh guinea pig serum was added 0.8 cc. of Lee-infected allantoic fluid. After incubation at 37°C. for 30 minutes, an aliquot of 0.8 cc. was removed, and to the remainder

of the mixture was added another 0.8 cc. of Lee virus. The mixture was incubated for 30 minutes. This procedure was repeated a third time, and with each aliquot obtained the amount of residual serum component was determined in the following manner: twofold dilutions of each aliquot were made in saline, to each dilution was added an equal volume of Lee virus, and mixtures were incubated at 37°C. for 30 minutes. The hemagglutination titer of each mixture then was determined in the usual manner. Appropriate unheated and heated serum controls were included.

The results of this experiment are presented graphically in Fig. 3 in which the hemagglutination titer of each mixture is plotted against the final concen-
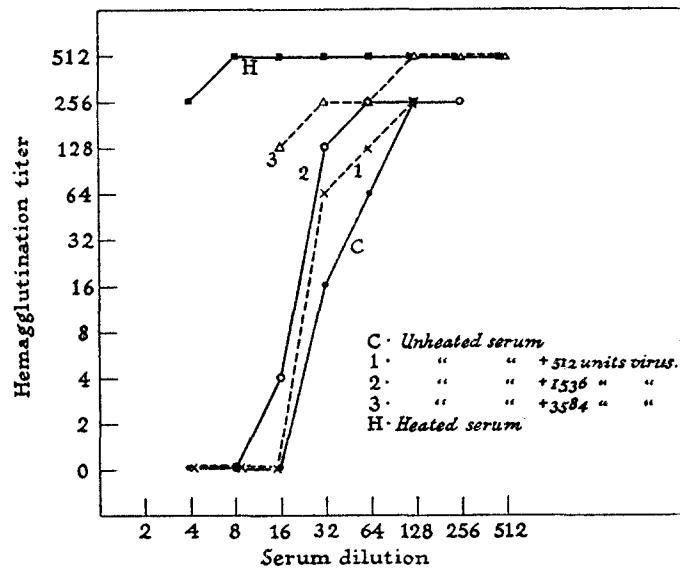

FIG. 3. Effect of adding increasing quantities of Lee virus to unheated guinea pig serum upon the capacity of the serum to cause inhibition of hemagglutination by Lee virus. After the serum had been mixed with the amounts of virus indicated, serial dilutions of the mixtures were prepared, and 512 units of fresh Lee virus was added to each dilution. The hemagglutination titer of each mixture is plotted against the dilution of serum present.

tration of serum present. The control unheated serum and the serum to which only 1 volume of virus had been added completely inhibited 512 hemagglutinating units of Lee virus at a dilution of 1:16, whereas the serum to which 2 volumes of virus had been added inhibited hemagglutination by 128 units of virus at an equivalent dilution. Serum which had been mixed with 3 volumes of virus inhibited only 4 hemagglutinating units of virus at a dilution of 1:16.

These results clearly indicate that Lee virus combines with the heat labile serum component, and prevents the component from combining with viral particles which are added subsequently. When sufficient virus is added to unheated serum, the combining capacity of the component is saturated.

Effect of Various Electrolytes on Serum Component-Virus Combination.—Certain electrolytes have been shown to play an important rôle in various virus-RBC (13-15) and virus-tissue component combinations (15) as well as in many serological reactions (16, 17). The effect of some electrolytes upon heat labile serum component–virus combination was studied.

The electrolyte solutions employed were: 0.85 per cent NaCl buffered at pH 7.2 (0.01 M phosphate), 0.85 per cent NaCl, 0.1 per cent CaCl₂ in 0.85 per cent NaCl, 0.01 per cent MgSO₄ in 0.85 per cent NaCl, 2.5 per cent sodium citrate, and 1.0 per cent sodium oxalate. Hemagglutination-inhibition titrations were carried out using each of these solutions as diluent with guinea pig serum. A final concentration of 4 units of Lee virus was employed.

The results of typical experiments are summarized in Table VIII. Sodium citrate or oxalate completely prevented virus-serum component combination,

TABLE VIII

Effect of Various Electrolytes on Serum Component-Virus Combination

Guinea pig serum dilutions	Diluent	Lee virus	Hemagglutination-inhibition titer*
Unheated	0.85 per cent NaCl; 0.01 M phosphate	4 units	64
"	" " " "	" "	64
"	2.5 " " sodium citrate	" "	<8
"	1.0 " " " oxalate	" "	<8
"	0.1 " " CaCl₂ in 0.85 per cent NaCl	" "	64
"	0.01 " " MgSO₄ " " " " "	" "	64
Heated‡	0.85 " " NaCl; 0.01 M phosphate	" "	<8

* Expressed as the reciprocal.
‡ 56°C. for 30 minutes.

which suggested that calcium or magnesium ions were necessary for serum component–virus combination to take place. It will be noted that there was no difference in the hemagglutination-inhibition titers when 0.85 per cent NaCl was the diluent and phosphate, calcium, or magnesium ions were present.

To determine whether calcium or magnesium ions were necessary for the reaction, guinea pig serum was dialyzed at 4°C. for 16 hours against 1500 volumes of either 0.85 per cent NaCl or 0.1 per cent CaCl₂ in 0.85 per cent NaCl. Following dialysis the hemagglutination-inhibition titers of the serum specimens were determined. The dialyzed serum was diluted in CaCl₂ and MgSO₄ solutions as well as phosphate-buffered saline. The results are summarized in Table IX. Serum dialyzed against saline appeared to lose 75 per cent of its inhibitory activity, but full activity was restored upon the addition of calcium ions although not upon the addition of magnesium ions. Dialysis against a 0.1 per cent CaCl₂ solution caused no loss of inhibitory activity.

Dissociation of Serum Component-Virus Combination.—Combination of the viruses of influenza A and B, mumps, and Newcastle disease with heat-resistant inhibitors present in human, rabbit, or ferret sera (18) as well as normal allantoic fluid (19) or egg white (20) is not stable, and spontaneous dissociation with inactivation of such inhibitors occurs after relatively short periods of incubation (21). However, dissociation of virus and the heat labile serum component does not occur on incubation; 24 hours after preparing a mixture, virus was still not demonstrable by the hemagglutination technique.

Because calcium ions appear necessary for combination between the heat labile serum component and virus, it seemed possible that the addition of sodium citrate to a serum component-virus mixture would remove the available

TABLE IX

Effect of Dialysis and Addition of Calcium on Activity of Thermolabile Serum Component

Serum dialyzed against*	Period dialysis at 4°C.	Dialyzed serum diluted in	Hemagglu- tination- inhibition titer‡
	hrs.		
None	—	Phosphate buffered 0.85 per cent NaCl	64
0.85 per cent NaCl	16	" " " " " "	16
" " " "	"	0.01 per cent MgSO₄ in 0.85 per cent NaCl	16
" " " "	"	0.1 " " CaCl₂ " " " " "	128
0.1 " " CaCl₂ in 0.85 per cent NaCl	"	Phosphate buffered " " " "	64

* Dialyzed against 1500 volumes.

‡ 4 hemagglutinating units of Lee virus employed.

calcium ions and release the virus from combination. To test this hypothesis the following experiments were carried out.

To unheated guinea pig serum was added an equal volume of Lee virus-infected allantoic fluid, and the mixture was incubated at 37°C. for 30 minutes. An aliquot was diluted in saline, and a second aliquot was diluted in 2.5 per cent sodium citrate. The dilutions were held for 15 minutes at 37°C. and the hemagglutination titers were then determined. Heated serum was treated in an identical manner.

The results of three experiments with different Lee virus preparations are shown in Table X. It will be noted that release of virus from combination with the heat labile serum component in the presence of sodium citrate was not consistent, and was of varying degree. The largest amount of virus that was released was only about 25 per cent of that added to the serum. In many experiments it was not possible to show that any virus was released from combination by citrate. In no instance in which PR8 was used was it possible to demonstrate release of virus once combination had taken place, but with mumps virus release of about 1 per cent was demonstrable.

The effect of prolonged incubation of mixtures of unheated serum and virus was investigated to determine whether this would cause more complete release of virus.

Mixtures of unheated guinea pig serum and Lee virus were heated at 56°C. for 30 minutes, and also kept at room temperature for 24 hours, and the hemagglutination titers determined before and after heating at 56°C. The mixtures were diluted in either saline or 2.5 per cent sodium citrate solutions.

The results of a typical experiment are shown in Table XI. When mixtures were heated at 56°C. for 30 minutes in order to inactivate the heat labile com-

TABLE X

Effect of Removal of Calcium Ions by Citrate on Serum Component-Virus Combination

Mixture		Incuba-tion at 37°C.	Diluent	Hemagglu-tination titer of mixture
Guinea pig serum*	Lee virus prepara-tions‡			
	No.	*min.*		
Unheated	1	30	NaCl	0
"	"	"	2.5 per cent sodium citrate	0
56°C., 30 min.	"	"	" " " " "	1024
Unheated	2	"	NaCl	0
"	"	"	2.5 per cent sodium citrate	128
56°C., 30 min.	"	"	" " " " "	1024
Unheated	3	"	NaCl	0
"	"	"	2.5 per cent sodium citrate	1024
56°C., 30 min.	"	"	" " " " "	4096

* Undiluted.
‡ Undiluted allantoic fluid.

ponent, virus was not demonstrable even when the mixture was diluted in sodium citrate solution. However, after the mixture had remained at room temperature for 24 hours and was diluted in citrate, about 6 per cent of the virus was released; when heated at 56°C. and diluted in saline, less than 1 per cent was released; but, on dilution in citrate, more dissociation occurred and about 12 per cent of the virus was demonstrable. These results indicate that sponteneous dissociation of virus-serum component combination does not occur; that some release of virus can be accomplished by appropriate procedures; that virus is not irreversibly inactivated by the serum component.

Effect of Various Chemical Reagents on the Heat Labile Serum Component.— To obtain some idea as to the nature of the serum component, the effect of various chemical reagents was studied.

TABLE XI

Partial Dissociation of Combination between Serum Component and Virus upon Prolonged Incubation and Removal of Calcium Ions by Citrate

Mixture		Mixture held at			Mixture diluted in	Hemag-glutina-tion titer of mixture
Guinea pig serum	Virus	1	2	3		
Unheated	Lee	37°C., 15 min.	—	—	NaCl	0
"	"	" " "	—	—	2.5 per cent sodium citrate	0
"	"	" " "	56°C., 30 min.	—	NaCl	0
"	"	" " "	" " "	—	2.5 per cent sodium citrate	0
"	"	" " "	25°C., 24 hrs.	—	NaCl	0
"	"	" " "	" " "	—	2.5 per cent sodium citrate	128
"	"	" " "	" " "	56°C., 30 min.	NaCl	16
"	"	" " "	" " "	" " "	2.5 per cent sodium citrate	256
56°C., 30 min.	"	" " "	" " "	" " "	" "	2048

TABLE XII

Effect of Various Procedures on the Activity of the Heat Labile Component in Guinea Pig Serum

Guinea pig serum treated with:	Conditions of treatment	Hemagglu-tination-inhibition titer* of serum	Embryo infectivity score‡
			positive/total
Boiled trypsin 2 mg. per cc.	37°C., 120 min.	1:64	0/4
Trypsin 2 mg. per cc.	" " "	1:32	4/4
Lithium periodate 0.1 M.	" " "	1:64	0/4
30 per cent methyl alcohol	4°C., 60 min.	"	—
90 " " ethyl "	23°C., 15 min.	0	—
(NH₄)₂SO₄, 1/2 saturated (globulin)	23°C.	0	4/4
" , saturated (albumin)	"	0	4/4
" , globulin + albumin	—	0	4/4

* 4 hemagglutinating units of Lee virus employed.

‡ 10³ E.I.D. Lee virus used.

Fresh guinea pig serum was treated with: (1) crystalline trypsin,[2] 2 mg. per cc.; (2) 0.1 M lithium periodate; (3) saturated and half-saturated ammonium sulfate; (4) 30 per cent methyl alcohol in the cold; and (5) 90 per cent ethyl alcohol. Virus hemagglutination-inhibition and neutralization titrations were then carried out with the various serum preparations and fractions.

[2] Obtained through the courtesy of Dr. M. Kunitz, The Rockefeller Institute, Princeton, New Jersey.

The results of a number of experiments are summarized in Table XII. It is of interest that neither the precipitates obtained with one-half saturated or saturated ammonium sulfate, nor a mixture of the two, contained demonstrable serum component. Trypsin inactivated the capacity of the serum component to prevent viral infection, but caused only a twofold reduction in the hemagglutination-inhibition titer. The greater quantity of serum necessary to neutralize virus infectivity as compared with the serum dilution which inhibits hemagglutination by virus, as illustrated in Fig. 1, serves as an explanation of this apparent discrepancy. Periodate did not alter the capacity of the serum component to combine with virus, and thus inhibition of both infection and hemagglutination was obtained. It will be noted that the component was not inactivated by precipitation with 30 per cent methyl alcohol in the cold, but following precipitation with ethyl alcohol at room temperature, the activity of the component was destroyed.

Relationship of Heat Labile Serum Component to Complement.—That the heat labile component of serum might be identical to complement or one of its components appeared possible. As has been pointed out, others (1, 3, 4, 6, 7) have suggested that complement may play a rôle in the neutralization of some viruses by specific antibodies. Evidence bearing on this possibility was sought by three different procedures: (a) the study of serum from which single components of complement had been removed by chemical treatment; (b) the study of serum from which complement had been removed by an antigen-antibody precipitate; and (c) the study of serum from which most of the heat labile component had been removed by combination with virus.

1. Effect of "Incomplete" Complement on Lee Virus.—

Different fractions of guinea pig serum obtained through the courtesy of Dr. Michael Heidelberger, College of Physicians and Surgeons, Columbia University, were employed: (a) "endpiece" represented a fraction from which midpiece (C_1) had been removed as well as some of C_3, but all of C_2 and most of C_4 were present; and (b) "midpiece" consisted of a fraction from which endpiece (C_2) and some of C_4 had been removed, but all of C_1 and most of C_3 remained. In addition, similar endpiece and midpiece fractions were prepared from guinea pig serum in this laboratory in the following manner. Six cc. of guinea pig serum was dialyzed in a rocker against 750 cc. of phosphate buffer pH 5.4, ionic strength 0.02, for 6 hours at 4°C.; a change of buffer was made after 3 hours. The material was then centrifuged at 8,000 R.P.M. for 10 minutes. The supernate was decanted, neutralized immediately, and brought to isotonicity with NaCl. This fraction is termed "endpiece." The precipitate was broken up and well mixed with 6 cc. of phosphate buffer, and then was recentrifuged. The final precipitate was resuspended in 6 cc. 0.85 per cent NaCl containing 0.1 per cent $CaCl_2$. This fraction is termed "midpiece."

The results of an experiment with endpiece and midpiece fractions and Lee virus are presented in Table XIII. It will be noted that neither endpiece nor midpiece prevented viral infection, whereas a mixture of the two fractions neu-

tralized virus infectivity in 3 of 4 embryos. It should be emphasized that these fractions contain numerous constituents in addition to components of com-

TABLE XIII

Effect of Guinea Pig Complement Fractions on Infectivity of Lee Virus

Mixture		Incu-bation at 37°C.	Hemagglutination titers allantoic fluids*				Embryo infectivity score
Guinea pig serum fraction	Lee virus		A	B	C	D	
	E.I.D.	*min.*					*positive/total*
Whole serum	10^2	30	0	0	0	0	0/4
" " 56°C., 30 min.	"	"	>128	>128	>128	>128	4/4
"Endpiece" (C_2, C_3, C_4)	"	"	"	"	"	"	4/4
"Midpiece" (C_1, C_3, C_4)	"	"	"	"	"	"	4/4
"Endpiece + midpiece" fractions	"	"	0	0	32	0	1/4
" + " " 56°C., 30 min.	"	"	>128	>128	>128	>128	4/4

* Removed after incubation of embryos for 2 days at 35°C.

TABLE XIV

Effect of Removal of Complement by an Antigen-Antibody Precipitate on the Serum Component

Mixture				Incuba-tion at 37°C.	Results of serum titrations			
Guinea pig serum	Strepto-coccus MG immune rabbit serum*	Strepto-coccus MG polysac-charide	NaCl		Comple-ment titer‡	Hemagglu-tination-inhibition titer§	Virus neutralization¶	
							Serum dilution	Embryo infectivity score
cc.	*cc.*	*mg.*	*cc.*	*min.*	*cc.*			*positive/total*
0.45	0.1	0	0.35	30	<0.004	1:64	1:4	0/4
							1:8	1/4
							1:16	4/4
0.45	0.1	0.035	0	30	>0.20	1:32	1:4	4/4

* Diluted 1:2 and heated 65°C. for 30 minutes.

‡ Quantity of guinea pig serum required to hemolyze completely a 3 per cent suspension of sensitized sheep RBC.

§ 4 hemagglutinating units of Lee virus used.

¶ 10^2 E.I.D. Lee virus employed.

plement, and that a mixture of endpiece and midpiece fractions is in effect a reconstituted serum. Neither the endpiece nor the midpiece fraction alone had complementary activity, but a mixture of the two was effective in lysing sensitized sheep RBC. Results of hemagglutination-inhibition titrations with

these preparations were irregular, and could not be interpreted satisfactorily due to the fact that heat stable inhibitors were present.

2. Effect of Removing Complement with an Antigen-Antibody Precipitate on the Heat Labile Serum Component.—

To fresh guinea pig serum were added inactivated antistreptococcus MG rabbit serum and capsular polysaccharide of streptococcus MG. It is known that the polysaccharide-antibody precipitate which forms in such a mixture binds complement (22). The mixture was held at 37°C. for 30 minutes, following which it was centrifuged. The precipitate was discarded, and the supernate employed in hemagglutination-inhibition, neutralization, and complement titrations.

TABLE XV

Effect of Removal of Serum Component by Virus on Complement Titer

Mixture				Results of serum titrations			
Guinea pig serum	Lee virus allantoic fluid heated 65°C. for 30 min.	Normal allantoic fluid	Incubation at 37°C.	Complement titer*	Hemagglu-tination-inhibition titer‡	Virus neutralization§	
						Serum dilution	Embryo infectivity score
cc.	*cc.*	*cc.*	*min.*	*cc.*			*positive/total*
0.7	0	0.7	15	0.004	1:320	1:4	0/4
						1:8	2/4
						1:16	3/4
0.3	0	1.2	"	0.005	1:320	—	—
0.7	0.7	0	"	0.004	1:80	1:4	4/4
0.3	1.2	0	"	"	<1:20	—	—

* Quantity of serum which completely lysed sensitized sheep RBC.
‡ 4 hemagglutinating units of Lee virus used.
§ 10^2 E.I.D. Lee virus employed.

The results of a typical experiment are presented in Table XIV. The precipitate resulting from the interaction of streptococcus MG polysaccharide and specific antibodies against it not only removed complement but also removed the serum component which neutralized Lee virus infectivity. However, as indicated by the results of hemagglutination-inhibition titrations, all the serum component was not removed. On this basis the reduction in the concentration of the component was only twofold, whereas the reduction in complement concentration was at least 50-fold.

3. Effect of Virus-Serum Component Combination upon Complement Titer.—

One and 4 volumes,respectively, of Lee virus-infected allantoic fluid heated at 65°C. for 30 minutes were added to aliquots of guinea pig serum. Such treated virus is not infectious,

and does not cause hemagglutination. Similar mixtures of serum and normal allantoic fluid were prepared. Each mixture was held at 37°C. for 30 minutes, and then complement, hemagglutination-inhibition, and neutralization titrations were carried out.

The results of two experiments are summarized in Table XV. The concentration of complement was undiminished even when 4 volumes of virus was mixed with serum, but the concentration of the serum component, as determined by hemagglutination-inhibition, was below the amount which could be measured by this technique representing at least a 16-fold reduction. Similarly, when equal volumes of serum and virus were mixed, there was a definite reduction in the concentration of the serum component as determined both by neutralization and hemagglutination-inhibition without any reduction in the complement titer. These results indicate that the heat labile serum component is not identical to complement.

DISCUSSION

That the serum of human beings, guinea pigs, rabbits, and mice contains a thermolabile component which inactivates influenza A and B, mumps, and Newcastle disease viruses on combining with them is indicated clearly by the evidence presented. The lability of this component when stored at 4°C. and on heating, in addition to the finding that its activity is diminished by crystalline trypsin and destroyed by ethyl alcohol, suggests that it may be an unstable protein or protein complex. The serum component–virus combination is stable and dissimilar to that which occurs between heat-resistant inhibitors of viral hemagglutination present in the serum of rabbits and ferrets (18) as well as in normal allantoic fluid (19) and egg white (20). The latter inhibitors are partially or completely inacitvated by virus and the virus is then released, whereas with the heat labile serum component spontaneous release of virus does not occur. Some release of virus may be achieved by the addition of sodium citrate to the mixture which probably is a result of the removal of calcium ions necessary for combination. A further and most important difference lies in the fact that with but rare exceptions (23, 24) inactivated serum of normal animals does not neutralize the infectivity of the viruses employed in this study.

Certain properties of the heat labile serum component are similar to those of complement, and certain workers (1, 3, 4, 6, 7) have attributed their results with other viruses as due to complement activity. Because the thermolabile component is not removed with complement in like amount by an antigen-antibody precipitate, and because complement is not removed by a quantity of virus sufficient to combine with all demonstrable serum component, it is unlikely that the two are identical.

The fact that there is present in human and animal serum a thermolabile component which can combine with any of the four viruses studied, and

can neutralize the infectivity of these agents, makes it hazardous to employ unheated sera in virus neutralization tests. Differences in the manner in which sera are handled as to separation from the clot, as well as temperature and duration of storage, may markedly affect the results. As example, sera stored at 4°C. for 2 to 4 weeks will usually not contain active component, whereas sera stored for only a few days at this temperature or held for long periods at −28°C. may neutralize 10^3 to 10^4 E.I.D. of virus. Under such circumstances the results might be taken as evidence that specific neutralizing antibodies had developed when this was not the case. Likewise, the addition of unheated normal serum to heat-inactivated immune serum may yield peculiar results which may be difficult to interpret.

It may be that the serum component affects attempts to recover certain viruses from the blood. For example, only rarely has mumps virus been recovered from the blood of a patient (25), despite the evidence that viremia is present in a considerable proportion of patients. It seems probable that unless the quantity of virus present in the blood were relatively large, 10^4 to 10^5 E.I.D. per cc., the heat labile component would prevent infection of susceptible animals. Brooksby (26) has pointed out that the virus of hoof-and-mouth disease is inactivated much more rapidly in defibrinated than in citrated guinea pig blood because calcium ions are needed for inactivation. Such inactivation may be a result of combination of virus with the serum component.

SUMMARY

A labile component present in the serum of human beings, guinea pigs, and rabbits neutralizes the infectivity of mumps, Newcastle disease, influenza A and B viruses. The labile component of these sera and of mouse serum also inhibits hemagglutination of chicken RBC by these viruses. The component is inactivated by heating at 56°C. for 30 minutes and upon storage at 4°C. for periods longer than 2 weeks. The virus-neutralizing and hemagglutination-inhibiting properties result from serum component–virus combination in the presence of calcium. The combination is stable, and does not undergo spontaneous dissociation. Partial separation of virus can be brought about by heating mixtures held for 24 hours or by removal of calcium ions with sodium citrate. The labile serum component appears to be distinct from hemolytic complement.

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