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Non-Specific Inhibitors of Influenza Viruses in Normal Sera

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The presence of non-specific inhibitors in immune influenza sera may falsify the antibody pattern as shown by the haemagglutination-inhibition test, and it is consequently often necessary to pre-treat sera in order to inactivate these inhibitors. A number of different methods are in use for this purpose. It was therefore thought useful to compare the efficacy of the various methods with different representative influenza strains and normal sera from eight animal species and acute-phase human sera. Tests were carried out with simple heating at 56°C, the use of receptor-destroying enzyme, the use of potassium periodate, and two methods involving the use of trypsin. No one technique was found to be universally applicable for all types of serum against all strains, and further testing with larger numbers of sera is advocated. It is felt that a combination of potassium periodate and trypsin with a change in concentration and times of action may be found advantageous.

The presence of an inhibitor of haemagglutination by influenza virus in normal sera was pointed out first by Hirst (1942). Further studies (Francis, 1947; McCrea, 1946; Sampaio & Isaacs, 1953; and others) show that there are two types of inhibitor, termed "Francis inhibitor" or "alpha inhibitor" (Smith et al., 1951) and "Chu inhibitor" (Sampaio & Isaacs, 1953) or "beta inhibitor" (Smith et al., 1951). These inhibitors have to be inactivated in assessing antibody patterns in immune sera. Different laboratories use different methods for this purpose (Kaplan & Payne, 1959). It was therefore considered fruitful to compare the efficacy of the various methods using different representative strains and normal sera from several animal species.

MATERIALS AND METHODS

Sera

Sera from nine animal species were used. Normal guinea-pigs, horses, monkeys, dogs, cows, rabbits, sheep and fowls were bled and sera stored at −20°C, For human sera, four acute-phase sera collected during the 1957 pandemic and stored at −20°C were utilized.

Antigens

Five antigens were used:

(1) B/33/52/Bombay (Salunke), an Indian B strain;

(2) FM1, an American A1 strain;

(3) Geetha/A/51/Coonoor, an Indian A strain;

(4) Shope 15, swine influenza strain;

(5) Ramt/A/57/Trivandrum, a 1957 A2 pandemic strain.

These were prepared as described previously (Ananthanarayan, 1958) and stored at −20°C, in lots of 5 ml, in Bijou bottles.

Methods

Inactivation. The following methods were used for inactivation of non-specific inhibitors.

(*a*) *Heating.* Simple heating at 56°C for ½ hour.

(*b*) *RDE.* One volume of serum, mixed with 5 volumes of receptor-destroying enzyme (RDE) (Duphar-freeze-dried), was incubated at 37°C for 16-18 hours and then heated at 56°C for one hour; the volume was made up with normal saline to give a final dilution of 1 : 10.

(*c*) *Potassium periodate.* One volume of serum was mixed with 2 volumes of M/90 KIO_4 and kept at 4°C for 12 hours. Two volumes of 1% glycerol saline were then added and the mixture was heated at 56°C for ½ hour. Five volumes of normal saline were finally added to give a dilution of 1 : 10.

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(*d*) *The "Alabama" method.* One volume serum was mixed with ½ volume trypsin (10 000 USP units/ml) and heated at 56°C for ½ hour; 1½ volumes of M/90 KIO_4 were added and kept on the bench (about 30°C) for 15 minutes; 1½ volumes of 1% glycerol saline were then added and allowed to act for a further 15 minutes on the bench. Normal saline was finally added to give a dilution of 1 : 10. Armour crystalline trypsin was substituted for Bacto-Trypsin 250, as the latter was not available locally. (This is the method of the Communicable Disease Center, Montgomery, Ala., 1958.)

(*e*) *Trypsin.* Sampaio & Isaacs (1953), using Armour crystalline trypsin showed that 8 mg/ml of serum was sufficient to inactivate non-specific inhibitors to A and A1 viruses. They have not given the strength of their trypsin in units. The crystalline trypsin used in this study was obtained from Armour Laboratories Ltd. and was stated to have a strength of 2000 units per mg. The method of Sampaio & Isaacs (1953) was followed, using a concentration of 8 mg/ml of serum.

(*f*) *Control.* A control was included using an unheated sample for each of the sera.

Fowl cells. These were collected from stock fowls aged one to one-and-a-half years and preserved in modified Alsever's solution (Bukantz et al., 1946) for periods up to a week. All the fowls were previously tested and proved to give cells which showed good agglutination with influenza viruses. The stock cells were washed thrice in normal saline, and a 0.5% suspension was prepared using the photoelectric densitometer.

Haemagglutination-inhibition test. This test was performed as described by the WHO Expert Committee on Influenza (1953), but using 8 haemagglutinating doses of virus. The test was performed in plastic plates distributed by the World Influenza Centre and the readings taken after 90-100 minutes at 4°C.

Non-specific haemagglutinins in normal sera. 0.25 ml of 0.5% of the red blood cell suspensions was added to 0.25 ml of each of the serial dilutions of sera in plastic plates, and readings were taken after 90-100 minutes at 4°C. Fowl and guinea-pig cells were used. Guinea-pig cells took a longer time to settle and the patterns were never so sharp.

RESULTS

Guinea-pig sera (Tables 1 and 11)

Non-specific inhibitors were present against all the strains tested. The titres against individual strains in the sera did not show much variation except in the case of the Shope 15 and Ramt strains. Only 5 out of 12 sera showed inhibitors to Shope 15, and that in low titres. Inhibitor against Ramt strain was present in very high titre in all sera and ranged from 3 840 to 163 840. The different techniques varied in their effectiveness in removing the inhibitors against the individual strains. There was also variation in the effectiveness from serum to serum in some cases. Trypsin was uniformally effective in completely removing the inhibitors to all the strains tested except Ramt. RDE was ineffective in removing inhibitors to Ramt strain but effective against B/33 and Geetha. Periodate acted best against Ramt, and the Alabama method against B/33, FM1 and Geetha.

Monkey sera (Tables 2 and 11)

Inhibitors were present against FM1 and Ramt strains in all the sera tested. Seven out of 8 sera had inhibitors to B/33 and Geetha, and 5 to Shope 15. Inhibitor titres to Ramt strain were the highest. Simple heating at 56°C for 30 minutes was the least effective method. The effect of various techniques was irregular and varied from serum to serum and from strain to strain. The Alabama method was the most effective against Ramt strain.

Rabbit sera (Tables 3 and 11)

Except for one serum which had no inhibitor to Shope 15, all the sera had inhibitors to the different strains. The inhibitor titre against Ramt strain was the highest. Simple heating at 56°C for 30 minutes was ineffective in removing the inhibitors. RDE was effective except in the case of Ramt strain. Periodate was effective against FM1, Ramt and Shope 15 strains. The Alabama method was the best in that it was uniformly effective for all the strains in all the sera. Trypsin removed inhibitors against all strains except Ramt.

Calf sera (Tables 4 and 11)

None of the four sera tested had inhibitor to Shope 15. Inhibitor titre against Ramt was very high, though one serum had no inhibitor against the strain. Inhibitors against FM1 and Geetha were low in titre, when present. Simple heating at 56°C for 30 minutes had not much effect on the inhibitors. RDE was irregular in its results. Periodate, trypsin and the Alabama method were active against the inhibitors to FM1. The Alabama method was the most effective of all against Ramt strain.

TABLE 1

HAEMAGGLUTINATION-INHIBITION TITRES WITH GUINEA-PIG SERA

Serial No. of serum	Method	Strain					Serial No. of serum	Method	Strain				
		B/33	FM1	Geetha	Ramt	Shope 15			B/33	FM1	Geetha	Ramt	Shope 15
C'A	Unheated	240	60	120	5 120	0	C	Unheated [b]	160	80	320	20 480	0
	56°C	60	15	60	>20 480	0		56°C[c]	0	0	0	122 880	0
	RDE	0	80	0	>20 480	0		RDE [d]	0	0	0	122 880	0
	KIO_4	0	0	0	0	0		KIO_4[e]	0	0	0	0	0
	Alabama	0	0	0	160	0		Alabama[f]	0	0	0	0	0
	Trypsin	0	0	0	5 120	0		Trypsin	0	0	0	7 680	0
1	Unheated	480	60	480	7 680	0	D	Unheated	480	240	480	40 960	60
	56°C	60	40	60	>20 480	0		56°C	30	30	60	245 760	40
	RDE	0	60	0	>20 480	0		RDE	0	80	0	245 760	80
	KIO_4	0	0	0	0	0		KIO_4	0	0	0	0	0
	Alabama	0	0	0	0	0		Alabama	0	0	15	122 880	20
	Trypsin	0	0	0	15 360	0		Trypsin	0	0	0	81 920	0
2	Unheated	480	80	320	7 680	0	P	Unheated[g]	320	60	80	61 440	0
	56°C[a]	—	—	—	—	—		56°C[h]	30	0	0	61 440	0
	RDE	0	0	0	>20 480	0		RDE[i]	0	0	0	163 840	0
	KIO_4	40	0	20	0	0		KIO_4[j]	120	0	0	0	0
	Alabama	0	0	0	320	0		Alabama [k]	0	0	0	2 560	0
	Trypsin[a]	—	—	—	—	—		Trypsin [l]	0	0	0	5 120	0
3	Unheated	480	120	480	7 680	60	Q	Unheated	240	60	80	81 920	0
	56°C	80	30	80	>20 480	0		56°C	40	30	30	163 840	0
	RDE	0	0	0	>20 480	0		RDE	0	0	0	122 880	0
	KIO_4	0	0	0	0	0		KIO_4	60	0	15	0	0
	Alabama	0	0	0	0	0		Alabama	0	0	0	2 560	0
	Trypsin	0	0	0	>20 480	0		Trypsin	0	0	0	1 920	0
A	Unheated	120	120	240	3 840	30	R	Unheated	480	80	160	163 840	0
	56°C	15	30	40	81 920	30		56°C	60	30	30	61 440	0
	RDE	0	30	0	81 920	60		RDE	0	120	0	122 880	0
	KIO_4	15	0	15	1 280	0		KIO_4	60	10	15	240	0
	Alabama	0	0	0	60	0		Alabama	0	0	0	240	0
	Trypsin	0	0	0	15 360	0		Trypsin	0	0	0	5 120	0
B	Unheated	120	80	240	15 360	30	S	Unheated	320	60	120	163 840	10
	56°C	15	30	40	122 880	15		56°C	60	30	30	40 960	0
	RDE	0	40	0	122 880	80		RDE	0	80	0	122 880	0
	KIO_4	10	10	10	60	0		KIO_4	60	0	15	120	0
	Alabama	0	0	0	320	20		Alabama	0	0	0	120	0
	Trypsin	0	0	0	5 120	0		Trypsin	0	0	0	5 120	0

[a] Not done [b] Zone dilutions 10 and 20.

[c] ? Zone against B/33 & Shope & Geetha, Zone in 10 against FM1, and Zone in 10 and 20 against Ramt.

[d] Zone in 10 against FM1, Zone in 10 and 20 against Ramt, and ? Zone in 10 against Shope 15.

[e] Zone in 10 against Ramt. [f] Zone in 10 throughout.

[g] Zone in 10 against all except Shope 15. [h] Zone in 10 against Ramt and B/33.

[i] Tendency to zone in 10 against Shope and zone in 10 against Ramt. [j] Zone in 10 and 20 against B/33.

[k] Zone in 10 against Ramt. [l] Zone in 10 and 20 against Ramt.

TABLE 2
HAEMAGGLUTINATION-INHIBITION TITRES WITH MONKEY SERA

Serial No. of serum	Method	Strain				
		B/33	FM1	Geetha	Ramt	Shope 15
3	Unheated	40	120	240	2 560	40
	56°C	30	120	240	2 560	0
	RDE	0	0	0	0	0
	KIO_4	0	0	0	80	0
	Alabama	0	0	0	0	0
	Trypsin	0	80	40	1 280	0
4	Unheated	60	60	640	1 280	0
	56°C	60	80	120	2 560	0
	RDE	120	0	60	0	0
	KIO_4	60	0	40	30	0
	Alabama	0	0	0	120	0
	Trypsin	0	0	0	640	0
7	Unheated	160	160	640	2 560	80
	56°C	60	240	320	5 120	60
	RDE	0	0	0	40	0
	KIO_4	0	0	0	120	0
	Alabama	0	60	0	0	0
	Trypsin	20	120	160	1 280	0
14	Unheated	0	60	0	>5 120	0
	56°C	0	0	0	>5 120	0
	RDE	0	80	0	>5 120	0
	KIO_4	0	0	0	0	0
	Alabama	0	0	0	0	0
	Trypsin	0	0	0	2 560	0
01	Unheated	480	160	320	480	60
	56°C	40	120	80	480	30
	RDE	0	0	0	0	0
	KIO_4	30	0	0	0	0
	Alabama	0	0	0	0	0
	Trypsin	0	60	15	160	0
02	Unheated	240	120	120	240	0
	56°C	160	60	80	240	0
	RDE	240	0	60	0	0
	KIO_4	160	0	40	40	0
	Alabama	0	0	0	0	0
	Trypsin [a]	0	0	0	80	0
03	Unheated	240	160	240	480	60
	56°C	60	120	60	480	20
	RDE	0	0	0	0	0
	KIO_4	60	0	0	0	0
	Alabama	0	0	0	0	0
	Trypsin	0	80	30	240	0
04	Unheated	60	60	30	160	30
	56°C	15	60	0	240	0
	RDE	0	0	0	0	0
	KIO_4	0	0	0	0	0
	Alabama	0	0	0	0	0
	Trypsin	0	20	0	80	0

[a] Zone in dilution 10 against Ramt strain.

TABLE 3
HAEMAGGLUTINATION-INHIBITION TITRES WITH RABBIT SERA

Serial No. of serum	Method	Strain				
		B/33	FM1	Geetha	Ramt	Shope 15
1	Unheated	160	40	60	1 280	40
	56°C	120	30	60	1 280	30
	RDE	0	0	0	60	0
	KIO_4	80	0	30	0	0
	Alabama	0	0	0	0	0
	Trypsin	0	0	0	240	0
2	Unheated	160	30	60	1 920	0
	56°C	120	30	60	1 920	0
	RDE	0	0	0	160	0
	KIO_4	60	0	15	0	0
	Alabama	0	0	0	0	0
	Trypsin	0	0	0	640	0
3	Unheated	120	30	60	1 280	20
	56°C	120	30	60	1 280	15
	RDE	0	0	0	80	0
	KIO_4	30	0	15	0	0
	Alabama	0	0	0	0	0
	Trypsin	0	0	0	320	0
4	Unheated	120	40	60	1 280	30
	56°C	80	30	40	1 280	0
	RDE	0	0	0	120	0
	KIO_4	60	0	20	0	0
	Alabama	0	0	0	0	0
	Trypsin	0	0	0	640	0

Horse sera (Tables 5 and 11)

Except for one serum, the sera had no inhibitors to B/33, Geetha and Shope 15 strains. Inhibitors to Ramt and FM1 strains were present in all; the titre against the former was uniformly high. Periodate and the Alabama method were equally good in removing inhibitors to Ramt strain; RDE and trypsin were ineffective with this strain. Periodate, the Alabama method and trypsin were effective in destroying the inhibitor to FM1 strain.

TABLE 4

HAEMAGGLUTINATION-INHIBITION TITRES WITH CALF SERA

Serial No. of serum	Method	Strain				
		B/33	FM1	Geetha	Ramt	Shope 15
1	Unheated	30	20	15	3 840	0
	56°C	15	15	0	3 840	0
	RDE	0	80	0	5 120	0
	KIO_4	0	0	0	30	0
	Alabama	0	0	0	0	0
	Trypsin	0	0	0	320	0
2	Unheated [a]	2 560	0	160	0	0
	56°C [b]	1 920	0	0	0	0
	RDE [c]	80→2 560	0	80→640	0	0
	KIO_4 [d]	80→1 280	0	0	0	0
	Alabama	0	0	0	0	0
	Trypsin	0	0	0	0	0
3	Unheated	0	30	0	>5 120	0
	56°C	0	15	0	>5 120	0
	RDE	0	120	0	>5 120	0
	KIO_4	0	0	0	0	0
	Alabama	0	0	0	0	0
	Trypsin	0	0	0	1 920	0
4	Unheated	0	30	0	>5 120	0
	56°C	0	0	0	>5 120	0
	RDE	0	120	0	>5 120	0
	KIO_4	0	0	0	30	0
	Alabama	0	0	0	15	0
	Trypsin	0	0	0	1 280	0

[a] Zone against B/33 up to dilution 80; against Geetha 50 % inhibition in dilutions 80 and 160 but no inhibition either above or below.

[b] Zone against B/33 up to 80, no inhibition to Geetha up to 80; 25 % inhibition dilutions 80 and 160; above 160 no inhibition.

[c] No inhibition to B/33 up to dilution 80. Inhibition from 80 to 2 560, but no inhibition beyond.

[d] No inhibition to B/33 in dilution 40. Inhibition in dilutions 80 → 1 280. No inhibition above 1 280. Against Geetha 25 % inhibition in dilutions 40 and 80 only. No inhibition in other dilutions.

Sheep sera (Tables 6 and 11)

None of the sera tested had inhibitors to Shope 15. The titre to Ramt strain was low and was similar to that against the other three strains. The Alabama method and trypsin were uniformly able to remove inhibitors to all the strains. Periodate was able to remove inhibitors to Ramt strain in all the sera.

TABLE 5

HAEMAGGLUTINATION-INHIBITION TITRES WITH HORSE SERA

Serial No. of serum	Method	Strain				
		B/33	FM1	Geetha	Ramt	Shope 15
1	Unheated	0	40	0	>5 120	0
	56°C	0	0	0	>5 120	0
	RDE	0	160	0	>5 120	0
	KIO_4	0	0	0	30	0
	Alabama	0	0	0	30	0
	Trypsin	0	0	0	2 560	0
2	Unheated	240	80	60	2 560	160
	56°C	30	60	30	2 560	40
	RDE [a]	0	0	0	480	0
	KIO_4	0	0	0	0	0
	Alabama	0	0	0	0	0
	Trypsin [b]	0	0	0	960	0
3	Unheated	0	30	0	>5 120	0
	56°C	0	0	0	>5 120	0
	RDE	0	160	0	>5 120	0
	KIO_4	0	0	0	0	0
	Alabama	0	0	0	0	0
	Trypsin	0	0	0	1 920	0
4	Unheated	0	30	0	5 120	0
	56°C	0	0	0	5 120	0
	RDE	0	60	0	5 120	0
	KIO_4	0	0	0	20	0
	Alabama	0	0	0	15	0
	Trypsin	0	0	0	1 920	0

[a] Zone in dilution 10 against Ramt.

[b] Zone ? dilutions 10 and 20 against Ramt and ? dilution 10 against FM1.

TABLE 6

HAEMAGGLUTINATION-INHIBITION TITRES WITH SHEEP SERA

Serial No. of serum	Method	Strain				
		B/33	FM1	Geetha	Ramt	Shope 15
1	Unheated [a]	240	30	240	120	0
	56°C [b]	20	30	30	80	0
	RDE	120	20	120	30	0
	KIO_4 [c]	120	0	60	0	0
	Alabama	0	0	0	0	0
	Trypsin	0	0	0	0	0
2	Unheated [d]	160	60	120	80	0
	56°C [e]	60	0	40	40	0
	RDE [c]	60	0	80	0	0
	KIO_4 [c]	60	0	30	0	0
	Alabama	0	0	0	0	0
	Trypsin	0	0	0	0	0
3	Unheated [b]	160	40	120	120	0
	56°C [b]	40	20	60	60	0
	RDE [f]	120	20	160	0	0
	KIO_4 [c]	120	0	60	0	0
	Alabama	0	0	0	0	0
	Trypsin	0	0	0	0	0
4	Unheated [b]	160	40	120	120	0
	56°C [e]	30	0	40	60	0
	RDE [c]	120	0	120	0	0
	KIO_4 [c]	120	0	40	0	0
	Alabama	0	0	0	0	0
	Trypsin	0	0	0	0	0

[a] Zone in dilution 10 against B/33 and Ramt.
[b] Zone in dilution 10 against all strains except Shope 15.
[c] Zone in dilution 10 against B/33 and Geetha.
[d] Zone in dilution 10 against B/33 and Ramt and in dilution 20 against FM1 and Geetha.
[e] Zone in dilution 10 against B/33, Geetha and Ramt.
[f] Zone in dilution 10 against B/33.

TABLE 7

HAEMAGGLUTINATION-INHIBITION TITRES WITH FOWL SERA

Serial No. of serum	Method	Strain				
		B/33	FM1	Geetha	Ramt	Shope 15
1	Unheated [a]	0	0	0	60	0
	56°C [a]	0	0	0	240	0
	RDE	0	0	0	0	0
	KIO_4	0	0	0	0	0
	Alabama	0	0	0	0	0
	Trypsin	0	0	0	0	0
2	Unheated	0	0	0	30	0
	56°C	0	0	0	240	0
	RDE	0	0	0	0	0
	KIO_4	0	0	0	0	0
	Alabama	0	0	0	0	0
	Trypsin	0	0	0	0	0
3	Unheated	30	0	0	30	0
	56°C	30	0	0	240	0
	RDE	0	0	0	0	0
	KIO_4	30	0	0	0	0
	Alabama	20	0	0	0	0
	Trypsin	0	0	0	0	0
4	Unheated [a]	0	0	0	60	0
	56°C [a]	0	0	0	480	0
	RDE	0	0	0	0	0
	KIO_4 [b]	0	0	0	0	0
	Alabama	0	0	0	0	0
	Trypsin	0	0	0	0	0

[a] Zone in dilution 10 against Ramt.
[b] Tendency to zone in dilutions 20-160.

Fowl sera (Tables 7 and 11)

Non-specific inhibitors were absent to FM1, Geetha and Shope 15 strains, and in three out of four sera to B/33. The titre of inhibitors to Ramt strain was low. Simple heating at 56°C for 30 minutes was unable to destroy the inhibitors when present (Menon, 1953). The inhibitor against Ramt strain showed a significant increase in titre after heating. RDE, periodate, trypsin and the Alabama method were all equally effective in removing the inhibitors to Ramt strain.

Human sera (Table 8)

RDE, periodate and the Alabama technique were able to remove completely all the inhibitors to Ramt strain but trypsin could not.

Dog sera (Tables 9 and 11)

The inhibitor titre was uniformly high against Ramt strain but was low and varied from serum to serum against other strains. Four out of the 8 sera tested did not have any inhibitor against strains other than Ramt. RDE was able to remove inhibitors against B/33 and Shope 15. Periodate was effective against B/33, Geetha and Shope 15; the Alabama method against B/33 and Geetha; and trypsin against B/33, FM1, Geetha and Shope 15. RDE was the most effective against inhibitors to Ramt strain.

TABLE 8
HAEMAGGLUTINATION-INHIBITION TITRES WITH HUMAN SERA

Serial No. of serum	Method	Strain				
		B/33	FM1	Geetha	Ramt	Shope 15
B	Unheated	30	120	60	120	30
	56°C	30	120	30	120	15
	RDE	15	80	15	0	0
	KIO_4	15	60	15	0	0
	Alabama	15	80	20	0	0
	Trypsin	15	60	30	20	0
K	Unheated	120	240	120	240	30
	56°C	60	120	120	240	40
	RDE	60	120	60	0	0
	KIO_4	60	80	60	0	30
	Alabama	60	120	120	0	0
	Trypsin [a]	40	120	80	30	40
T	Unheated	120	160	120	480	0
	56°C	40	160	80	480	0
	RDE	60	120	60	0	0
	KIO_4	30	80	60	0	0
	Alabama	30	120	60	0	0
	Trypsin	15	120	60	120	0
V	Unheated	60	120	40	480	30
	56°C	0	120	0	640	0
	RDE	30	60	15	0	0
	KIO_4	15	60	0	0	0
	Alabama	0	60	0	0	0
	Trypsin	0	80	0	120	0

[a] Zone in dilution 10 against all strains.

Zone phenomena in sera

Zone phenomena were noticed in some sera. Some showed a tendency to zoning and a few had double zones. Zoning was seen in the sera of calf (1/4), dog (7/8), fowl (2/4), guinea-pig (2/12), horse (1/4), human (1/4) and sheep (4/4).

Non-specific haemagglutinins in sera

We also tested the non-specific haemagglutinins in the sera against both fowl and guinea-pig cells, since they have been reported by other workers recently (Kaplan & Payne, 1959). The results are shown in Table 10. Dog sera contain thermolabile lysins to fowl and guinea-pig cells.

DISCUSSION

Hirst (1942) showed that the non-specific inhibitors of influenza viruses could be partially destroyed by heating the serum to 56°C for 30 minutes and pointed out the need for taking this into consideration in estimating antibody titres in immune sera. He found (1948) that these inhibitors could be destroyed by low concentrations of sodium periodate. McCrea (1946) showed that heating at 62°C for 15-20 minutes rapidly destroyed the non-specific inhibitors in rabbit sera without affecting the antibodies; but Hirst (1948) found that the inhibitor was very stable at high temperatures, resisting 100°C for 15 minutes.

Burnet et al., (1947) demonstrated that the alpha-inhibitor could be destroyed by the receptor destroying enzyme of *V. cholerae*. Van der Veen & Mulder (1950) showed that crude extracts of *V. cholerae* inactivate the non-specific inhibitors in normal ferret serum. Isaacs & Bozzo (1951) found that normal ferret sera contained mainly the "Francis inhibitor", readily destroyable by purified RDE. Chu (1951) demonstrated that crude *V. cholerae* extracts, but not RDE, inactivated the beta-inhibitor in normal mouse serum. Magill & Jotz (1952) found that crude *V. cholerae* extracts inactivated normal rabbit serum inhibitor. Sampaio & Isaacs (1953) showed that crystalline trypsin was highly active in

TABLE 9

HAEMAGGLUTINATION-INHIBITION TITRES WITH DOG SERA

Serial No. of serum	Method	Strain					Serial No. of serum	Method	Strain				
		B/33	FM1	Geetha	Ramt	Shope 15			B/33	FM1	Geetha	Ramt	Shope 15
1	Unheated [a]	60	240	20	960	60	5	Unheated [m]	0	40	0	240	0
	56°C [b]	30	120	0	960	60		56°C	0	0	0	320	0
	RDE	0	20	0	60	0		RDE	0	0	0	0	0
	KIO_4 [c]	0	60	0	480	0		KIO_4 [n]	0	0	0	80	0
	Alabama [d]	0	80	0	480	60		Alabama [o]	0	0	0	60	0
	Trypsin [e]	0	0	0	240	0		Trypsin	0	0	0	0	0
2	Unheated [f]	0	0	0	640	0	6	Unheated [k]	0	0	0	240	0
	56°C [f]	0	0	0	640	0		56°C [k]	0	0	0	480	0
	RDE	0	0	0	0	0		RDE	0	0	0	0	0
	KIO_4 [g]	0	0	0	0	0		KIO_4 [k]	0	0	0	240	0
	Alabama [h]	0	0	0	0	0		Alabama [j]	0	0	0	0	0
	Trypsin [h]	0	0	0	0	0		Trypsin	0	0	0	0	0
3	Unheated	30	60	0	640	0	7	Unheated	20	40	10	240	0
	56°C	0	60	0	960	0		56°C	0	40	0	240	0
	RDE	0	0	0	0	0		RDE	0	0	0	0	0
	KIO_4	0	0	0	0	0		KIO_4	0	0	0	30	0
	Alabama	0	0	0	0	0		Alabama	0	0	0	20	0
	Trypsin [i]	0	0	0	0	0		Trypsin	0	0	0	60	0
4	Unheated [j]	0	0	0	960	0	8	Unheated [p]	0	0	0	240	0
	56°C [j]	0	0	0	960	0		56°C [n]	0	0	0	480	0
	RDE	0	0	0	0	0		RDE	0	0	0	0	0
	KIO_4 [k]	0	0	0	240	0		KIO_4 [n]	0	0	0	120	0
	Alabama [j]	0	0	0	160	0		Alabama	0	0	0	0	0
	Trypsin [l]	0	0	0	0	0		Trypsin [q]	0	0	0	0	0

[a] Zone in dilution 10 against all strains.
[b] Zone in dilution 10 against all strains except Geetha.
[c] Zone in dilution 10 against Geetha and Ramt and up to dilution 80 against Shope 15.
[d] Zone in dilution 10 against FM1, Ramt and Shope 15.
[e] Zone in dilutions 20, 40 and 80 against FM1, up to dilution 40 against Ramt, and up to dilution 160 against Shope 15.
[f] Zone in dilution 10 against FM1 and up to dilution 40 against Ramt.
[g] Tendency to zone up to dilution 160 against Ramt.
[h] Tendency to zone against Ramt in dilution 10.
[i] Tendency to zone up to dilution 80 against Ramt.
[j] Zone up to dilution 40 against Ramt.
[k] Zone up to dilution 80 against Ramt.
[l] Zone in dilution 10 against Ramt.
[m] Lysins to fowl cells in dilution 10.
[n] Zone in dilution 10 against Ramt.
[o] Partial zone in dilutions 40 and 80 and in dilution 1 280 against Ramt.
[p] Lysins to fowl cells in dilution 10; zone to dilution 40 against Ramt.
[q] Tendency to zone against Ramt in dilutions 20 and 40.

TABLE 10
NON-SPECIFIC AGGLUTINATION BY SERA

Serial No. of serum		Fowl cells	Guinea-pig cells [a]
Guinea-pig	1 to 3	0	0
	A	10	0
	B	0	0
	C	20	0
	D	0	0
	P	10	0
	Q. R & S	0	0
Calf	1, 3 & 4	0	0
	2	30	120
Sheep	1 to 4	15	60
Horse	1 & 3	0	0
	2	30	60
	4	15	0
Monkey	01	10	30
	02	0	40
	03	0	0
	04	10	40
Human	B	0	30
	K	0	240
	T	0	240
	V	0	240
Rabbit	1 to 4	0	0
Fowl	1	0	60
	2 & 3	0	15
	4	0	240
Dog	1	20	20
	2 [b]	60	30
	3	10	15
	4 [b]	40	30

[a] The fact that guinea-pig cells do not form a good button in plastic plates has been taken into account in reading the results. Only unequivocal results have been recorded.
[b] Lysin in dilution 10 to both types of cell.

destroying Chu and Francis inhibitors in normal ferret, fowl, rabbit, guinea-pig and mouse sera in conditions where no significant effect on specific antibody was observed. They used pooled sera from each species. They concluded that " nonspecific and specific inhibitory activities are not so readily distinguished in human as in animal sera and further investigations will be required before trypsin treatment can be safely applied to them ". It is worth recalling that Svedmyr (1949) found 5%-25% of the activity of the inhibitor of influenza viruses present in normal allantoic fluid was resistant to trypsin treatment.

With the advent of the pandemic strains in 1957, RDE, which had been considered very effective in inactivating non-specific inhibitors, was found inadequate. The pandemic strains fell mainly into two types, one highly sensitive to non-specific inhibitors (avid) and the other poorly sensitive (non-avid). The non-specific inhibitors to the former strains in normal guinea-pig sera could not be completely destroyed by treatment with Duphar RDE (Ananthanarayan, 1958). The non-specific inhibitors were not only resistant to simple heating (56°C for 30 minutes) but their titre increased more than eightfold. Potassium periodate was recommended as the best in destroying all non-specific inhibitors to the pandemic strains in animal sera (K. E. Jensen—unpublished data, 1957). Burnet (1951) has observed that the action of periodate on inhibitors is not uniform, qualitatively or quantitatively. Lundback (1949) suggested that human sera contained two or more inhibitory factors, one of which was destroyed by periodate. Thus our techniques for the removal of non-specific inhibitors have been progressively changing as they were found inadequate.

The inhibitor content has been shown to vary from animal to animal and to be very high in rabbit and ferret sera but low in horse sera (Hirst, 1948). Inhibitors to influenza viruses have also been demonstrated in urine, various tissue extracts, apple pectin, etc. (Burnet, 1951).

This study has shown that there is a difference in the inhibitors both qualitatively and quantitatively in different animal species, as revealed by the nature of their response to destruction by different techniques. Some species do not have any inhibitors to some strains—for instance, calf, sheep and fowl sera to Shope 15, and fowl sera to Geetha and FM1. Within the species individual sera do not possess

TABLE 11
INHIBITOR DESTRUCTION IN VARIOUS ANIMAL SERA [a]

Nature and total number of sera tested	Strain	56°C	RDE	KIO_4	Alabama	Trypsin
Guinea-pig (12 sera)	B/33	1/12	12/12	5/12	12/12	11/11
	FM1	2/12	5/12	10/12	12/12	11/11
	Geetha	3/12	12/12	6/12	11/12	11/11
	Ramt	0/12	0/12	8/12	3/12	0/11
	Shope 15	2/5	1/5	5/5	3/5	5/5
Calf (4 sera)	B/33	0/2	1/2	1/2	2/2	2/2
	FM1	1/3	0/3	3/3	3/3	3/3
	Geetha	1/2	1/2	1/2	2/2	1/2
	Ramt	0/3	0/3	1/3	2/3	0/3
	Shope 15 [b]	—	—	—	—	—
Dog (8 sera)	B/33	2/3	3/3	3/3	3/3	3/3
	FM1	1/4	3/4	3/4	3/4	4/4
	Geetha	2/2	2/2	2/2	2/2	2/2
	Ramt	0/8	7/8	2/8	4/8	6/8
	Shope 15	0/1	1/1	1/1	0/1	1/1
Fowl (4 sera)	B/33	0/1	1/1	0/1	0/1	1/1
	FM1 [b]	—	—	—	—	—
	Geetha [b]	—	—	—	—	—
	Ramt	0/4	4/4	4/4	4/4	4/4
	Shope 15 [b]	—	—	—	—	—
Horse (4 sera)	B/33	0/1	1/1	1/1	1/1	1/1
	FM1	3/4	1/4	4/4	4/4	4/4
	Geetha	0/1	1/1	1/1	1/1	1/1
	Ramt	0/4	0/4	2/4	2/4	0/4
	Shope 15	0/1	1/1	1/1	1/1	1/1
Monkey (8 sera)	B/33	0/7	5/7	3/7	7/7	6/7
	FM1	1/8	7/8	8/8	7/8	3/8
	Geetha	1/7	5/7	5/7	7/7	3/7
	Ramt	0/8	6/8	4/8	7/8	0/8
	Shope 15	2/5	5/5	5/5	5/5	5/5
Rabbit (4 sera)	B/33	0/4	4/4	0/4	4/4	4/4
	FM1	0/4	4/4	4/4	4/4	4/4
	Geetha	0/4	4/4	0/4	4/4	4/4
	Ramt	0/4	0/4	4/4	4/4	0/4
	Shope 15	1/3	3/3	3/3	3/3	3/3
Sheep (4 sera)	B/33	0/4	0/4	0/4	4/4	4/4
	FM1	2/4	2/4	4/4	4/4	4/4
	Geetha	0/4	0/4	0/4	4/4	4/4
	Ramt	0/4	3/4	4/4	4/4	4/4
	Shope 15 [b]	—	—	—	—	—

[a] Expressed as the number of sera in which inhibitors are destroyed over the total number of sera with inhibitors.
[b] No inhibitors in any of the sera.

inhibitors to some strains. No single technique is universally applicable for the different types of sera when tested against all the strains. It is therefore suggested that different techniques should be tested against larger numbers of normal sera in different species of animals against various strains. It is felt that a combination of potassium periodate and trypsin with a change in concentration and times of action may be found to be the most suitable.

Inactivation of the inhibitor by periodate has been taken as evidence that carbohydrate forms an essential part of it (Svedmyr, 1949), and the action of trypsin as indicating the existence in it of a protein component (Hirst, 1948). The inhibitors thus belong to the class of mucopolysaccharides and mucoproteins. Their chemistry and structure are complex and have not been finally and fully worked out. The electrophoretic studies so far undertaken (Tyrrel, 1954; Levy et al., 1959) also show that there are differences in inhibitors to different strains in the same serum and that the inhibitor pattern to the same strain varies in different species of sera.

RÉSUMÉ

La présence d'inhibiteurs non spécifiques dans les sérums contenant des anticorps contre le virus grippal peut fausser les résultats du test de l'hémagglutination, considéré comme révélant le schéma des anticorps sériques. Il est donc souvent nécessaire d'éliminer ces inhibiteurs avant de procéder à cette épreuve.

Plusieurs méthodes de destruction des inhibiteurs ont été successivement mises au point au cours des quinze dernières années, chacune devant pallier les défauts et les insuffisances de la précédente: traitement par la chaleur, le periodate de sodium ou de potassium, le RDE extrait des cultures de vibrion cholérique, et la trypsine. C'est ainsi que, lors de la pandémie de 1957/58, la destruction des inhibiteurs par le RDE, considéré jusqu'alors comme généralement efficace, s'est montrée insuffisante dans le cas des souches dites « avides », très sensibles aux inhibiteurs non spécifiques. Dans certains cas, la chaleur non seulement ne suffisait pas à les éliminer, mais elle augmentait leur taux de huit fois.

Dans l'étude dont cet article rend compte qui portait sur les sérums de 8 espèces d'animaux et des sérums humains, il a été confirmé que la teneur en inhibiteurs variait d'un animal à l'autre. Il a été démontré en outre qu'il existe des différences qualitatives et quantitatives entre inhibiteurs, ainsi que le révèle leur résistance variable à la destruction. Certaines espèces sont dépourvues d'inhibiteurs vis-à-vis de certaines souches de virus (le sérum de poule n'en a pas pour la souche FM1, par exemple). En conclusion, les auteurs estiment qu'une même technique n'est pas applicable aux sérums correspondant à toutes les souches de virus grippal. Ils proposent que les diverses techniques soient étudiées sur un grand nombre de sérums normaux de diverses espèces animales, par rapport à plusieurs souches de virus. Il semble que l'on puisse conseiller une combinaison des traitements au periodate et à la trypsine, en faisant varier la concentration et la durée d'action des réactifs.

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