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AGGLUTINATION OF RED BLOOD CELLS OF DIFFERENT ANIMAL SPECIES BY INFLUENZA AND NEWCASTLE DISEASE VIRUSES

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Although data have been published on the agglutinability of red blood cells of different animal species by influenza and related viruses (McClelland & Hare, 1941; Clark & Nagler, 1943), the tests were performed at one fixed virus dilution, thus only giving a rough indication of the degree of susceptibility of the red cells tested. In the course of studies on the mechanism of virus haemagglutination, it was considered that more quantitative data on this point might be desirable. Salk's method (1944) of haemagglutinin titration provides a simple and relatively quantitative technique for this purpose, and has been employed in the present work to study agglutination of red cells from seventeen animal species by one strain each of influenza A, influenza B, swine influenza and Newcastle disease virus (N.D.V.). The same four viruses purified by centrifugation and washing have been similarly tested. Since Curnen & Horsfall (1946) have reported on the effect of heat in unmasking the haemagglutinating property of mouse pneumonia virus, it was decided to test, in parallel with the origin virus, preparations that had been heated at 56 and 70° C. respectively for 30 min. Several unexpected observations were made and have been investigated in greater detail. The results of this study are described in the present paper.

MATERIALS AND METHODS

Viruses, strains and preparation of materials

Influenza A, PR 8 strain; influenza B, Lee strain; swine influenza, Shope's strain; were all egg-adapted. The strain of N.D.V. used was kindly supplied by Dr T. M. Doyle in the form of spleen from an infected fowl, and had since had eight passages in the allantoic sac of chick embryos. All virus materials were prepared by allantoic inoculation of 10-day-old chick embryos. Blood-free allantoic fluid was harvested 48 hr. after inoculation. Bacteriologically sterile samples were pooled, stored at 4° C. and used within a month.

Purification of viruses

The same technique has been applied for the purification of all four viruses. Allantoic fluid virus was first clarified by centrifugation at 3000 r.p.m. for 15 min. on an International Centrifuge. The bulk of the virus was then deposited on a Ivan Sorvall Type SS Superspeed Angle Centrifuge run at 12,000 r.p.m. for 1 hr. The deposited pellet was resuspended in saline and the alternate low-speed and high-speed centrifugations repeated. The second angle centrifuge deposit was resuspended in one-fifth of the original volume of saline. After a final low-speed centrifugation, an opalescent suspension was obtained. Approximately 50% of haemagglutinin contained in the original fluid was recovered.

Red blood cell suspensions

Blood was drawn into an equal volume of 2% trisodium citrate either by venepuncture or by cardiac puncture. The citrated blood was stored at 4° C. up to a maximum of 1 week. Red cells were washed three times and diluted to 1% suspensions on the day of experiment. Rabbit, rat and mouse cells readily autoagglutinate in saline, and were therefore washed and suspended in 3·3% magnesium sulphate solution. Previous experience has shown that the virus haemagglutinin titres for fowl cells, whether suspended in saline or magnesium sulphate, are essentially the same.

Haemagglutination test

The principle of Salk's method was followed. Series of two-fold dilutions of virus in 0·25 c.c., covering the range $\frac{1}{5}$ to $\frac{1}{2560}$, were prepared in 0·8 × 7·0 cm. tubes with rounded bottoms. To each tube 0·25 c.c. of 1% red cell suspension was added. After thorough mixing, the tubes were left to stand upright and undisturbed at room temperature (20–22° C.). A preliminary reading of the pattern of red cells at the bottom of the tubes was taken after 60 min. The final reading was taken after 90 min. The tube showing a ring of red cells surrounded by fine aggregates, arbitrarily designated as 1+, was taken as the titre. Whenever this degree of agglutination was not observed on direct reading, the titre was considered to fall in between two neighbouring dilutions.

TECHNICAL CONSIDERATIONS

Besides the usual variables inherent in Salk's test, two points deserve special mention when one is dealing with red cells of different animal species:

(1) Red cells of different animal species sediment at widely different speeds. At the end of 90 min., fowl cells have completely settled to the bottom of the tubes, whereas with goat cells, about half of the height of the cell column still remains in suspension.

by agglutination and an elution phase manifested by redispersion of red cells, leaving the latter altered. Elution is considerably accelerated at 37° C. and delayed at 4° C. At room temperature, therefore, what one observes as haemagglutination is really the net result of balance between these two phases. Evidence will be presented to show that the same viruses may be eluted with widely different ease from the red cells of different animal species. This different speed of elution naturally affects the

Table 1. *Agglutination of red cells of different animal species by allantoic fluid preparations of influenza and Newcastle disease viruses*

		PR 8			Lee			Swine influenza			N.D.V.		
Red cells	Experiment	Unheated	56° C.	70° C.	Unheated	56° C.	70° C.	Unheated	56° C.	70° C.	Unheated	56° C.	70° C.
Fowl	A	640	320	< 1	1280	640	< 1	80	< 10	< 1	1280	< 10	< 1
Fowl	B	160	80	< 1	640	320	< 1	80	< 10	< 1	640	< 10	< 1
Duck	A	640	240	< 10	1280	960	< 10	120	< 10	< 10	480	< 10	< 10
Jackdaw	A	320	160	< 10	640	320	< 10	40	< 10	< 10	320	< 10	< 10
Man	A	640	320	< 1	1280	320	< 1	80	< 10	< 1	160	< 10	< 1
Guinea-pig	A	640	320	< 1	320	160	< 1	80	< 10	< 1	320	< 10	< 1
Guinea-pig	B	80	—	—	240	—	—	40	—	—	240	—	—
Rabbit	A	10	10	< 1	10	< 10	< 1	< 10	< 10	< 1	20	< 10	< 1
Rabbit	B	5	—	—	60	—	—	20	—	—	20	—	—
Rat	A	320	—	—	320	—	—	30	—	—	320	—	—
Rat	B	80	—	—	80	—	—	20	—	—	80	—	—
Mouse	A	20	—	—	< 10	—	—	< 10	—	—	120	—	—
Mouse	B	5	—	—	< 5	—	—	< 5	—	—	30	—	—
Ferret	A	320	120	< 1	320	320	< 1	< 20	< 10	< 1	< 10	< 10	< 1
Ferret	B	80	—	—	80	—	—	5	—	—	< 5	—	—
Dog	A	640	320	< 10	320	160	< 10	80	< 10	< 10	640	< 10	< 10
Cat	A	< 20	—	—	20	—	—	10	—	—	< 20	—	—
Cat	B	< 5	—	—	10	—	—	10	—	—	< 5	—	—
Pig	A	< 10	< 10	< 10	< 10	80	< 10	< 10	< 10	< 10	< 10	< 10	< 10
Pig	B	< 5	—	—	< 5	—	—	< 5	—	—	< 5	—	—
Horse	A	< 10	< 10	< 1	< 10	< 10	< 1	< 10	< 10	< 1	< 10	< 10	< 1
Horse	B	< 5	—	—	< 5	—	—	< 5	—	—	< 5	—	—
Ox	A	< 10	20	< 1	< 10	160	< 1	10	< 10	< 1	< 10	< 10	< 1
Ox	B	< 5	10	< 10	< 5	80	< 10	5	—	—	< 5	—	—
Sheep	A	40	120	< 1	20	240	< 1	10	< 10	< 1	< 10	< 10	< 1
Sheep	B	20	40	< 10	10	120	< 10	10	—	—	< 5	—	—
Goat	A	30	120	< 1	20	120	< 1	10	< 10	< 1	< 10	< 10	< 1
Goat	B	20	40	< 10	20	120	< 10	10	—	—	< 5	—	—
Hedgehog	A	160	—	—	120	—	—	20	—	—	80	—	—

Figures express the reciprocal of titres observed in titration by Salk's method.

Preliminary experiments have shown that more comparable results are obtained if the time of reading is fixed at 90 min. after setting up the test instead of reading at different times after each kind of cell has completely settled. Certain red cells, particularly those which sediment rapidly, tend to slip along the bottom toward the centre, thus obscuring the agglutination pattern. To avoid this difficulty, a preliminary reading at 60 min. is necessary.

(2) As first demonstrated by Hirst (1942*a*) in the course of virus haemagglutination, two distinct phases are involved: an adsorption phase manifested

actual haemagglutinin titres obtained with different red cell systems. The full significance of this phenomenon will be further discussed.

EXPERIMENTS AND RESULTS

Haemagglutination by allantoic fluid viruses

The haemagglutinin titres of allantoic fluid preparations of PR 8, Lee, swine influenza and N.D.V. against red cells of seventeen animal species are shown in Exp. A of Table 1. The titres of unheated viruses, and viruses heated at 56 or 70° C. for 30 min. are shown under their respective columns. The result

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of a separate experiment with different batches of virus preparation and red cells from different individual animals is shown under Exp. B. The data can be best appraised by comparing the titres against different red cell systems with the fowl titre which may be taken as a standard. The following observations may be noted:

(1) The range and relative degree of agglutination of red cells of different species by the three influenza viruses are in general very similar. Red cells of fowl, duck, jackdaw, man, guinea-pig, rat, ferret and dog were agglutinated to high titre. Those of rabbit, mouse, cat, pig, ox, sheep and goat were either not agglutinated at the dilutions tested or agglutinated to low titre only. As compared with the influenza viruses, N.D.V. is chiefly characterized by its relatively low human and ferret titres and high mouse titre. Tests with N.D.V. and ox or sheep cells showed a 'zone phenomenon' with irregular weak agglutination in one or two tubes of intermediate dilutions. These findings were at first disregarded, and the titres against these cells are registered as negative in Table 1. Their true significance was only realized on further investigation, and will be discussed later.

(2) With the exceptions to be mentioned, heating of PR 8 and Lee at 56° C. for 30 min. only slightly reduced their titres for most cells. The same amount of heating, on the other hand, completely inactivated the haemagglutinin of swine influenza and N.D.V. Heating at 70° C. for 30 min. abolished the haemagglutinating activity of all four viruses.

(3) With respect to ox, sheep, goat and pig cells, heating of PR 8 and more particularly of Lee at 56° C. for 30 min., far from reducing their titres, actually enhanced them by two- to twelve-fold. The heated haemagglutinin is specific, since it was inhibited by the homologous, but not by the heterologous type-specific horse anti-influenza serum.

Haemagglutination by purified viruses

The result of haemagglutinin titrations of purified viruses is shown in Table 2. The purified viruses were prepared from the same batch of allantoic fluid viruses used in Exp. B of Table 1 and the same blood specimens were used. The fowl titres of purified viruses had been purposely adjusted to the same as those of the original allantoic fluid viruses. Comparison, therefore, should be made of the data in Table 2 with those under Exp. B of Table 1.

Table 2. *Agglutination of red cells of different animal species by purified influenza and Newcastle disease viruses*

	PR 8			Lee			Swine influenza			N.D.V.		
Red cells	Unheated	56° C.	70° C.	Unheated	56° C.	70° C.	Unheated	56° C.	70° C.	Unheated	56° C.	70° C.
Fowl	160	120	< 40	640	480	< 160	80	< 80	< 80	640	< 80	< 80
Man	80	80	< 40	640	640	< 160	80	< 80	< 40	160	< 80	< 40
Guinea-pig	80	80	< 40	320	320	< 160	80	< 80	< 40	320	< 80	< 40
Rabbit	< 40	< 40	< 40	< 160	< 160	< 160	80	< 80	< 40	< 80	< 80	< 40
Rat	40	40	< 40	160	160	< 160	80	< 80	< 40	160	< 80	< 40
Mouse	< 40	< 40	< 40	< 160	< 160	< 160	< 80	< 80	< 40	80	< 80	< 40
Ferret	40	80	< 40	160	< 160	< 80	< 80	< 80	< 40	160	< 80	< 40
Cat	< 40	< 40	< 40	< 160	< 160	< 160	< 80	< 80	< 40	80	< 80	< 40
Pig	< 40	< 40	< 40	240	320	< 80	< 40	< 40	< 40	640	< 80	< 40
Horse	< 20	< 20	< 20	< 80	< 80	< 80	< 40	< 40	< 40	160	< 40	< 40
Ox	< 20	60	< 20	< 80	320	< 80	< 40	< 40	< 40	480	< 40	< 40
Sheep	120	120	< 20	< 80	320	< 80	40	< 40	< 40	240	< 40	< 40
Goat	160	160	< 20	< 80	320	< 80	40	< 40	< 40	240	< 40	< 40

In the case of influenza viruses, the purified preparations behaved in the same general way as the allantoic fluid preparations. Notable variations were the relatively high sheep and goat titres of purified PR 8 and a similar high pig titre of purified Lee virus. After heating at 56° C. for 30 min., the haemagglutinin titres for ox, sheep and goat cells were enhanced in the same way as with allantoic fluid viruses.

Purified N.D.V., on the other hand, acquired high agglutination titre against ferret, cat, pig, horse, ox, sheep and goat cells, none of which was agglutinated in one-fifth dilution of allantoic fluid N.D.V. The agglutination is specific since it was specifically inhibited by fowl anti-N.D.V. serum.

Summarizing the above findings, PR 8 and Lee viruses after heating at 56° C. for 30 min. show an enhancement of haemagglutinin titre against ox, sheep, goat and pig cells. The same enhancement has been obtained, particularly in the case of Lee virus, by heating the purified virus. It appears, therefore, that heating has changed the virus itself. Purified N.D.V. agglutinates red cells from a number of animals which are not agglutinated by the original allantoic fluid virus. Thus, the change appears to be due to the elimination of an inhibitor present in the allantoic fluid. To study the mechanisms of these changes, further experiments were carried out.

Observations on the course of reactions of fowl and ox red cells with Lee virus and Newcastle disease virus allantoic fluids at 4 and 20° C.

In this experiment, parallel tests were set up at 4 and 20° C. and readings were taken after 30, 60 and 90 min. The protocols and result are presented in Table 3. At 4° C., both fowl and ox cells were agglutinated to the indicated titres independent of whether the reading was taken after 30, 60 or 90 min. At 20° C., agglutination of fowl cells can be clearly read in 30 min. when the titres observed were substantially the same as those read after 90 min. The patterns of ox cells, which settle more slowly, were barely visible in 30 min. with the aid of a magnifying glass, but could be read by naked eye after 60 min. There was a continuous change of red cell patterns. Detectable agglutination with both Lee and N.D.V. began at the low dilution end in 30 min. At 60 min., all influenza tubes appeared negative. Similarly, by that time, the N.D.V.-agglutinated cells at the low dilution end had slipped down and appeared unagglutinated, but definite agglutination appeared in intermediate dilutions. Finally, after 90 min., the ox cells in all influenza as well as N.D.V. tubes had settled in unagglutinated patterns. These observations indicate that ox cells are actually agglutinated by allantoic fluid preparations of both Lee and N.D.V., but at room temperature elution takes place so rapidly that no reading can be taken within a convenient time.

Elution of Lee and Newcastle disease virus from fowl and ox red cells

To series of doubling dilutions of allantoic fluid Lee virus, either unheated or heated at 56° C. for 30 min., and of N.D.V., either allantoic or purified, 1% fowl or ox cells was added. Agglutination was read after 90 min. The tubes were then left at room temperature overnight and thoroughly shaken up on the next morning. A second reading was taken. Except tubes containing heated Lee virus, the cells in all other tubes settled in unagglutinated patterns owing to elution of viruses. From the latter tubes, the supernatant fluid was pipetted off and discarded. A sufficiently large dose (four complete agglutinating doses) of either heated Lee virus or purified N.D.V., as the case may be, was added to the red cells. The tubes were shaken and again read after 90 min. The second dose of virus acts as a 'challenge'. If the cells have been altered by their first contact with virus, they are no longer agglutinated by the challenge dose. The protocols and result of this experiment are shown in Table 4. The data indicate that ox cells, in the presence of unheated Lee or allantoic fluid N.D.V., although not visibly

Table 3. *Agglutination of fowl and ox red cells by Lee virus and Newcastle disease virus allantoic fluid at 4 and 20° C., read at different time intervals*

Virus	Red cells	Temperature (° C.)	Time (min.)	Virus dilutions: $\frac{1}{5}$	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{320}$	$\frac{1}{640}$	$\frac{1}{1280}$	$\frac{1}{2560}$	Saline
Lee, allantoic	Fowl	4	30, 60 or 90	++	++	++	++	++	++	++	+±	±	−	−
		20	30, 60 or 90	++	++	++	++	++	++	++	+	±	−	−
	Ox	4	30, 60 or 90	++	++	++	++	++	−	−	−	−	−	−
		20	30	++	++	++	−	−	−	?	?	?	?	?
			60	±	±	−	−	−	−	−	−	−	−	−
			90	−	−	−	−	−	−	−	−	−	−	−
N.D.V., allantoic	Fowl	4	30, 60 or 90	++	++	++	++	++	++	+	−	−	−	−
		20	30, 60 or 90	++	++	++	++	++	++	++	+	±	−	−
	Ox	4	30, 60 or 90	++	++	++	++	++	+	−	−	−	−	−
		20	30	±	++	?	?	?	?	?	±	−	−	−
			60	−	±	+	+	++	++	±	−	−	−	−
			90	−	−	−	±	+	+	−	−	−	−	−

++ Complete agglutination. ± Doubtful agglutination.
+± Nearly complete agglutination. − No agglutination.
\+ Partial agglutination. ? Unreadable.

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agglutinated, nevertheless became refractory to a challenge dose which agglutinated normal ox cells. It may also be noted that heated Lee virus failed to elute from either fowl or ox cells. From other experiments not detailed here, it has been shown that, as compared with allantoic fluid virus, purified N.D.V. is eluted more slowly from fowl cells.

fluid, which had been heated at 56° C. for 30 min., was mixed with an equal amount of 5% fowl red cell suspension. A similar set-up with unheated virus served as a control. After 30 min., at 4° C., the red cells which were strongly agglutinated in both tubes were quickly separated, washed once in 5 c.c. of chilled saline and finally resuspended in 1 c.c. of

Table 4. *Agglutination, elution and destruction of virus receptor of fowl and ox red cells by viruses*

Virus	Red cells	Exp.	Virus dilutions $\frac{1}{5}$	$\frac{1}{10}$	$\frac{1}{20}$	$\frac{1}{40}$	$\frac{1}{80}$	$\frac{1}{160}$	$\frac{1}{320}$	$\frac{1}{640}$	$\frac{1}{1280}$	$\frac{1}{2560}$	Saline
Lee, allantoic	Fowl	*a*	++	++	++	++	++	++	++	+	±	−	−
		b	+	±	±	±	±	±	−	−	−	−	−
		c_1	+	±	±	±	±	±	+	++	++	++	++
	Ox	*a*	−	−	−	−	−	−	−	−	−	−	−
		b	−	−	−	−	−	−	−	−	−	−	−
		c_1	−	−	−	−	−	+	++	++	++	++	++
Lee, allantoic heated at 56° C. for 30 min.	Fowl	*a*	++	++	++	++	++	++	++	+	±	−	−
		b	++	++	++	++	++	++	++	±	−	−	−
	Ox	*a*	++	++	++	+±	±	−	−	−	−	−	−
		b	++	++	++	+	±	−	−	−	−	−	−
N.D.V., allantoic	Fowl	*a*	++	++	++	++	++	++	++	+	±	−	−
		b	±	−	−	±	−	−	−	−	±	−	−
		c_2	±	−	−	−	−	−	±	+	++	++	++
	Ox	*a*	−	−	−	±	+	+	−	−	−	−	−
		b	−	−	−	−	−	−	−	−	−	−	−
		c_2	−	−	−	−	−	−	+	++	++	++	++
N.D.V., purified	Fowl	*a*	++	++	++	++	++	++	++	+	±	−	−
		b	−	−	−	−	−	−	−	−	±	±	−
		c_2	H	H	−	−	±	±	±	±	++	++	++
	Ox	*a*	++	++	++	++	++	++	±	−	−	−	−
		b	−	−	−	−	−	−	−	−	−	−	−
		c_2	−	−	−	−	−	−	+±	++	++	++	++

Experiments: H Partially haemolysed; *a* agglutination read after 90 min. at 20° C.; *b* readings taken after standing at 20° C. for 18 hr. and shaken up; c_1 readings after the addition of a challenge dose of heated Lee virus; c_2 readings after the addition of a challenge dose of purified N.D.V.

Table 5. *Elution of heated and unheated Lee virus from fowl red cells*

Virus	Haemagglutinin titre: Original	Supernatant after adsorption with red cells	Washing, cold saline	Eluate 37° C.
Lee, unheated	960	80	< 10	640
Lee, heated at 56° C. for 30 min.	640	30	< 10	5

Measurement of elution of Lee virus from fowl red cells

It appears that the essential difference between native and heated Lee virus is the failure of the latter to be eluted from red cells. An experiment was therefore performed to measure, in each case, the amount of virus adsorbed by a fixed quantity of fowl red cells and the amount subsequently eluted therefrom. For this purpose, 1 c.c. of Lee virus allantoic saline and incubated at 37° C. for 2 hr. The red cells which had adsorbed the unheated virus became rapidly redispersed, whereas cells which had adsorbed the heated virus remained firmly agglutinated. The supernatant fluids after adsorption, the washings and the eluates were titrated by haemagglutination test (Table 5). All titres are expressed in terms of dilution of the original allantoic fluid. In confirmation of direct observations made from the agglutination reaction, heated Lee virus was

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normally adsorbed by fowl red cells but not demonstrably eluted therefrom.

Presence of inhibitors in normal allantoic fluid

The evidence indicated above suggests the presence in N.D.V. allantoic fluid of a haemagglutinin inhibitor which hastens virus elution. A less active and not necessarily similar inhibitor may also be present in influenza fluid as suggested by the higher titres obtained with purified viruses against certain red cells. Experiments have been carried out to investigate if normal allantoic fluid from 12-day-old chick embryos also possesses inhibitory activity. Parallel haemagglutination tests were performed using either saline or normal allantoic fluid as diluent. The result obtained, as shown in Table 6, indicates that weak but definite inhibitory activity of normal allantoic fluid can be detected against both Lee and N.D.V. A batch of normal allantoic fluid was titrated against one complete agglutinating dose of virus and found to show an inhibitor titre of 1/20 against heated Lee virus and of only 1/1 against unheated virus. This normal allantoic fluid inhibitor is, however, evidently of a different nature from that present in N.D.V. fluid, since it inhibits agglutination of fowl and ox cells alike, at 20° C. as well as at 4° C., and is much more active against heated influenza virus. The inhibitor itself is not affected by heating at 56° C. for 30 min. Since influenza virus prepared as such is by necessity a form of allantoic fluid, one would expect that after heating at 56° C. for 30 min., its haemagglutinin titre would be much reduced or even completely masked by this inhibitor. Such, however, is not the case, as influenza fluid heated at 56° C. for 30 min. loses little of its haemagglutinating power. Francis (1947) has described a similar inhibitor in normal human serum. Burnet (1947) has shown that the 'Francis inhibitor' is destroyed by active influenza virus. For reasons which will be given elsewhere (Chu, 1948*b*), it has been shown that the normal allantoic fluid inhibitor is closely similar to the 'Francis inhibitor'. It becomes evident, therefore, that virus-infected fluid does not contain this inhibitor, conceivably because any present has been destroyed by the virus during the process of infection.

Table 6. *Inhibition of influenza and Newcastle disease virus haemagglutination by normal allantoic fluid*

		Fowl cells		Ox cells	
Virus	Diluent	4° C.	20° C.	4° C.	20° C.
Lee, unheated	Saline	960	640	120	< 10
	Allantoic	120	120	10	< 10
Lee, heated at 56° C. for 30 min.	Saline	960	640	160	80
	Allantoic	< 10	< 10	< 10	< 10
N.D.V., purified	Saline	320	640	240	320
	Allantoic	160	160	80	80

Reaction of heated Lee virus with ox cells

As shown in a previous experiment, Lee virus heated at 56° C. for 30 min. is not appreciably eluted from either fowl or ox red cells at room temperature. Further experience has revealed, however, that when ox cells agglutinated by heated Lee virus were removed to a 37° C. water-bath, the red cells were promptly redispersed and settled in unagglutinated pattern. When such tubes were shaken and put back to 4° C., the cells reagglutinated. The agglutination and redispersion can be repeated a number of times. If, however, after the cells had settled unagglutinated at 37° C., the supernatant fluid was pipetted off and replaced with saline and the tubes again incubated at 4° C., no reagglutination took place. Such cells, furthermore, can still be agglutinated by a challenge dose of heated virus, indicating that the virus receptor has not been destroyed. These findings indicate that the heated influenza virus can bind reversibly with ox cells as the temperature of incubation is varied. In effect, it acts in very much the same way as a 'cold agglutinin'. Thus, although heated influenza virus is also eluted from ox cells at 37° C., the elution differs from that of the active virus in one important respect, namely, that the virus receptor is not destroyed in the process.

DISCUSSION

The foregoing experiments appear to furnish a reasonable explanation of the anomalous observation that heated influenza viruses and purified N.D.V. acquire an enhanced haemagglutination titre against red cells of certain animal species. It is well known that different viruses are eluted at different rates from the same red cells. Thus, with the strains used in the present study, N.D.V. is eluted most rapidly from fowl cells, followed by Lee, PR 8 and swine influenza in the order of decreasing rate of elution. Experiments have been presented to show that the same virus may also be eluted at enormously different rates from red cells of different animal species. In the case of fowl red cells, elution is relatively slow so that agglutination can be

conveniently observed and measured. In the case of a different class of red cells, notably those of ox, sheep, goat and pig, elution takes place hand in hand with adsorption at room temperature so that no agglutination or only weak agglutination may be observed. Heating of influenza virus at 56° C. for 30 min. destroys its eluting power so that those red cells which are only weakly agglutinated by the unheated virus can be strongly agglutinated by the heated virus. In a similar way, purified N.D.V. owes its increased agglutinating power toward certain red cells to the fact that it is more slowly eluted than the allantoic fluid virus. A substance appears to be present in N.D.V.-infected fluid which favours elution of virus. This substance is probably identical with the N.D.V. allantoic fluid inhibitor described by Cunha, Weil, Beard, Taylor, Sharp & Beard (1947). Florman (1947) observed a zone phenomenon in the agglutination of fowl red cells by N.D.V. at room temperature but not at 4° C. An examination of his data suggests that he was in fact dealing with an elution-hastening factor present in N.D.V. fluid. Much evidence has accumulated in favour of the hypothesis that the action of influenza and N.D.V. on red cells may be enzyme-like (Hirst, 1942*a*; Burnet, 1948; Chu, 1948*a*). Since the N.D.V. allantoic factor appears to hasten elution, and thus accelerates rather than inhibits the virus action on red cells, it might be more appropriately called an 'activator' rather than 'inhibitor'. The inhibitor found in normal allantoic fluid belongs to an entirely different category. It is not present in virus-infected fluid, is much more active on heated influenza virus and probably acts by combining with the latter. This normal allantoic fluid inhibitor is closely similar to the 'Francis inhibitor' and both may represent freed virus receptor substance.

The virus haemagglutination reaction, which has gained both theoretical and practical importance, is so simple in performance that its complicated basic mechanism can be easily overlooked. Its analogy with haemagglutination by antibody is more superficial than real. The basic mechanism of virus haemagglutination has already been briefly alluded to. The visible manifestation, namely, the agglutination of red cells, particularly in its quantitative aspect, is influenced by a large number of factors, among which are the red cell concentration (Miller & Stanley, 1944; Salk, 1944), the temperature (Miller & Stanley, 1944; Salk, 1944), pH of the medium (Miller & Stanley, 1944; Magill & Sugg, 1947), time of reading, and the presence of extraneous factors which either inhibit or modify the reaction (Beveridge & Lind, 1946; Cunha *et al.* 1947). The present study illustrates that many of these factors affect different red cells differently so that a given set of conditions suitable for one red cell system may not suit others. Haemagglutinin titrations under certain standard conditions, such as performed here, can at best present a partial picture of the true susceptibility of different red cells. Different strains of influenza virus (Clark & Nagler, 1943), and even the same strains of virus at different stages of host adaptation (Burnet & Bull, 1943) have been reported to exhibit differences in their range and relative titres of red cells agglutinated. How much of this variation is due to intrinsic differences among the viruses remains to be determined. Bearing in mind these inherent variables in the testing technique, it seems that neither biological relationship between the animal species, nor their susceptibility to infection by any particular virus bears any clear correlation with the agglutinability of their red cells by these viruses. Furthermore, there are indications which suggest that the virus receptors on any particular red cells toward different viruses, or on different animal red cells toward any particular virus, may not be exactly identical. This point will be more fully dealt with in a separate paper (Chu, 1948*b*).

Since red cells may react with viruses without giving rise to visible agglutination, and since bacterial enzymes possessing similar action on red cells to that of influenza virus by themselves do not cause visible agglutination (Chu, 1948*a*), one may wonder whether viruses other than those causing haemagglutination will yet be found to react with red cells, though no directly visible effect may be observed.

The fact that influenza virus heated at 56° C. for 30 min. loses its eluting power is of theoretical interest, since this amount of heating also inactivates this virus. Experiments correlating the heat destruction of eluting power and infectivity of influenza virus will be reported separately.

SUMMARY

The agglutination of red cells of seventeen animal species by influenza and Newcastle disease viruses has been quantitatively investigated. The peculiar behaviour of one group of red cells, illustrated by ox, toward influenza virus heated for 30 min. at 56° C. and toward purified N.D.V. has been studied in detail. It is concluded that heated influenza virus acquires an apparently increased titre against ox cells because it has lost its eluting power. Similarly, purified N.D.V. is less readily eluted from red cells, due to the elimination of an 'elution-hastening factor' present in infected fluid. The present study illustrates the importance of careful adjustment of all possible variable conditions for individual viruses and individual red cell systems.

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