INACTIVATION OF HAEMAGGLUTININ AND INFECTIVITY OF INFLUENZA AND NEWCASTLE DISEASE VIRUSES BY HEAT AND BY FORMALIN

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Influenza virus, after heating at 56° C. for 30 min., retains its haemagglutinating power, but is no longer demonstrably eluted from fowl red cells (Chu, 1948*a*).* Francis (1947) has reported that normal human serum inhibits to high titre haemagglutination by influenza B virus which has been heated at 56° C. for 30 min., so that when the latter is used as antigen in the routine Hirst test, differentiation between normal and immune sera is no longer possible. Burnet, McCrea & Anderson (1947) demonstrated that active influenza virus can destroy the 'Francis inhibitor'. Influenza virus heated at 56° C. for 30 min., however, is completely inactive in this respect (Chu, 1948*b*).* Heating for 30 min. at 56° C., therefore, changes the influenza virus in such a way that it loses its eluting power, becomes inhibited by 'Francis inhibitor' and can no longer destroy the latter. There is overwhelming evidence in favour of the theory that the virus action on red cells is an enzymic process (Burnet, 1948). In the light of this theory, the 'Francis inhibitor' represents the virus receptor substance in solution, both being the substrate of the virus haemagglutinating 'enzyme'. The last three changes of properties of influenza virus due to heating thus become three different manifestations of a single basic process, namely, that the heated virus, while still capable of combining with its substrate, cannot dissociate itself therefrom, thus failing to complete its 'enzymic' function. Since heating of influenza virus at 56° C. for 30 min. also destroys its infectivity, it seems interesting to investigate how far the loss of 'enzymic' function can be correlated with loss of infectivity. Newcastle disease virus (N.D.V.), when heated at 56° C. for 30 min., suffers large or even complete loss of its haemagglutinating activity (Chu, 1948*a*). In contrast to influenza virus, dissociation of adsorbing and eluting activities of N.D.V. haemagglutinin cannot be achieved by heat. With N.D.V., therefore, a direct correlation of infectivity and haemagglutinin titres was attempted. For comparison, the effect of formalin on these viruses has been similarly investigated.

* After this paper had been prepared, Hirst (*J. Exp. Med.* 87, 315, 1948) independently reported that influenza virus, after heating at 56° C. for various intervals, lost its eluting power from red cells and its ability to destroy 'Francis inhibitor' present in normal rabbit serum.

MATERIALS AND METHODS

The strains of viruses, method of cultivation in fertile eggs and technique of haemagglutination have already been described (Chu, 1948*a*). Both influenza B (Lee) and N.D.V. (Doyle) were prepared as blood-free allantoic fluid from 12-day-old chick embryos inoculated 40–48 hr. previously. N.D.V. fluid maintains its infectivity titre remarkably well over several months at 4° C. Lee fluid tends to flocculate and often loses much of its infectivity when frozen at −20° C. When stored at 4° C., such as in the present experiment, it keeps its titre for at least 3 weeks. All haemagglutinin titrations were performed with 0·5 % red cell suspensions from a single fowl.

Heating of virus preparations

Portions of 2 c.c. of the materials to be heated were placed in rubber-stoppered tubes. The tubes were immersed in a water-bath fitted with a stirrer. The temperature registered by a thermometer in a blank tube was adjusted to ± 0·25° C. of that desired. This temperature was maintained for exactly 15 min., after which the tubes were rapidly cooled down in a cold water-bath.

Formolization

B.D.H. formalin containing 37–41 % of formaldehyde was diluted with saline so that the desired amount was contained in 0·05 c.c. This quantity of diluted formalin was added to 2 c.c. of virus fluid by means of a micro-pipette. The formolized preparations were kept at room temperature (20° C.) for 1 hr. and then at 4° C. for various periods during the experiment.

Measurement of virus elution

The technique has been described in a previous paper (Chu, 1948*a*). The eluting power is expressed

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as the percentage of virus eluted to that adsorbed on the red cells and is calculated as follows:

$$\frac{\text{Titre of eluate}}{\text{Original titre} - (\text{Titre of adsorbed supernatant} + \text{Titre of washing})} \times 100.$$

It must be emphasized that, since the two-fold dilution method of titration is at most accurate to about ± 25 %, only large variations in eluting power can be considered significant.

Haemagglutinin inhibition by 'Francis inhibitor'

Lee virus heated at different temperatures was diluted to contain one complete agglutinating dose in 0·25 c.c. As 'Francis inhibitor', a 0·01 % solution of human blood group 'O' substance* in buffered saline (M/100 phosphate buffer at pH 7·0) was employed. A 1 % human red cell suspension was used as an indicator. 0·25 c.c. of each reagent was used. The red cells were added first to doubling dilutions of inhibitor, followed immediately by one complete agglutinating dose of the virus to be tested. The test was read after incubation at room temperature (20–22° C.) for 90 min. The highest dilution of inhibitor showing complete absence of agglutination was taken as the titre.

'Enzymic' action on 'Francis inhibitor'

To 0·5 c.c. of 0·01 % solution of blood group 'O' substance in buffered saline, an equal volume of virus was added. A drop of toluene was added to each tube which was then stoppered, shaken vigorously and incubated at 37° C. for approximately 18 hr. A control tube in which the virus was replaced by saline was set up under similar conditions. At the end of incubation, all tubes were immersed in a 65° C. water-bath for 30 min. to inactivate the virus haemagglutinin. The amount of residual 'Francis inhibitor' was then titrated against one complete agglutinating dose of Lee virus heated at 56° C. for 30 min. 0·01 % human 'O' substance has a 'Francis inhibitor' titre of 1/32. After overnight incubation with active influenza virus, no more inhibitory activity could be detected at the lowest dilution 1/2, thus over 94 % of 'Francis inhibitor' had been destroyed. When virus heated at 56° C. for 15 min. was used, in place of the active virus, the inhibitor titre remained at 1/32, indicating that no destruction had taken place. It will be seen that when virus heated at temperatures between 50 and 56° C. was used, various grades of residual inhibitor titres were obtained indicating partial destruction. The percentage of 'Francis inhibitor' destroyed by any virus preparation in 18 hr. is calculated as follows:

$$\frac{\text{Original inhibitor titre} - \text{Residual inhibitor titre}}{\text{Original inhibitor titre}} \times 100.$$

* Purified from human ovarian cyst mucoid and kindly supplied by Dr W. T. J. Morgan of the Lister Institute.

Infectivity titration in ovo

Ten-fold dilutions of virus were prepared in heart infusion broth. As a rule, two eggs were used for each dilution, 9-day-old embryos being used for N.D.V. and 11-day-old embryos for Lee virus. 0·1 c.c. of material was inoculated into the allantoic sac. The eggs were then incubated at 37° C. for 3 days, during which they were candled daily and any containing dead embryos were opened. After 3 days, all the remaining eggs were chilled at − 20° C. for 30 min. The allantoic fluid was pipetted out from the air sac end and tested for sterility on blood agar. Any lesion on the embryos was noted. Haemagglutination test was done by mixing 2 drops of allantoic fluid with 0·25 c.c. of 0·5 % fowl red cells. In case of any doubt, a full-scale haemagglutinin titration was carried out and the specificity of haemagglutination checked by inhibition test with a specific antiserum. This is taken as the most reliable single criterion for infection. Most embryos inoculated with low dilutions of N.D.V. die within 48 hr., while those inoculated with high dilutions may survive up to 72 hr. by which time all infected embryos show severe haemorrhagic lesions and a positive haemagglutination is always obtained. The Lee strain of influenza B virus is only lethal in low dilutions. In order to avoid the possibility of auto-interference, particularly in partially inactivated preparations, all titrations covered at least two different dilutions and undiluted virus was not used. The number of eggs employed does not justify statistical evaluations, but for the present purpose when only detection of gross difference is desired, uniform and satisfactory results seem to have been obtained.

EXPERIMENTS AND RESULTS

Heat inactivation of Newcastle disease virus

Aliquots of N.D.V. allantoic fluid were heated at temperatures of 54, 56 and 58° C. for 15 min. The heated preparations and unheated control were titrated for haemagglutination and for infectivity *in ovo* (Table 1). The result indicates that a moderate but progressive drop of both haemagglutinin and infectivity titre occurred after heating at 54 and 56° C. After heating at 58° C., haemagglutination could no longer be detected; at the same time, the infectivity had been reduced at least 10^6 times. Heated N.D.V., if causing haemagglutination at all, appeared to be eluted from fowl red cells as usual.

Formolized N.D.V.

Aliquots of N.D.V. allantoic fluid with added formalin in 0·2 and 0·5 % concentrations were stored at 4° C. Haemagglutinin titrations were carried out after 18 hr. and again after 3 and 6 days. Infectivity was tested after 18 hr. and 6 days (Table 2). It will be seen that even 0·5 % formalin had little effect on the haemagglutinin titre in 18 hr., although

the infectivity dropped from 10^{-9} to negative at 10^{-6}, which was the lowest dilution tested. Both 0·2 and 0·5 % formalin caused a slow and progressive deterioration of haemagglutinin titre so that only 1–2 % of the original activity was left in 6 days. The 2° C. intervals, for 15 min. The heated virus and unheated control were tested for:

(*a*) Haemagglutinin titre against 0·5 % fowl red cells.

(*b*) Eluting power from fowl red cells.

Table 1. *The haemagglutinin and infectivity of Newcastle disease virus heated at various temperatures*

N.D.V.	Haemagglutinin titre	Infectivity* 10^{-1}	10^{-3}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Unheated	960	—	—	—	—	2/2	3/3	2/2	0/2
Heated to:									
54° C. for 15 min.	320	—	—	—	2/2	3/4	1/2	—	—
56° C. for 15 min.	40	—	—	2/2	2/2	1/2	0/2	—	—
58° C. for 15 min.	< 1	2/2	0/2	0/2	—	0/2	—	—	—

* Expressed as no. of eggs infected/no. of eggs tested.

Table 2. *The haemagglutinin and infectivity of formolized Newcastle disease virus*

Time after addition of formalin	Concentration of formalin (%)	Haemagglutinin titre	Infectivity 10^{-1}	10^{-3}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
18 hr.	0	1280	—	—	—	—	2/2	1/2	0/2
	0·2	1280	—	—	—	—	—	—	—
	0·5	640	—	—	0/2	0/2	0/2	0/2	0/2
3 days	0	1280	—	—	—	—	—	—	—
	0·2	40	—	—	—	—	—	—	—
	0·5	20	—	—	—	—	—	—	—
6 days	0	960	—	—	—	—	2/2	2/2	—
	0·2	20	0/2	0/2	—	—	—	—	—
	0·5	10	0/2	0/2	—	—	—	—	—

Table 3. *Properties of Lee virus heated at different temperatures*

Lee virus	Haem-agglutinin titre	Elution from fowl red cells (%)*	Inhibition by 'Francis inhibitor'†	Inhibitor destroyed (%)‡	Infectivity.§ Virus dilution 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Unheated	960	76	< 1	> 93	—	—	—	4/4	2/4	0/4
Heated to:										
46° C. for 15 min.	960	74	< 1	> 93	—	—	—	—	—	—
48° C. for 15 min.	960	70	< 1	> 93	—	—	—	2/2	1/2	0/2
50° C. for 15 min.	960	4·3	2	88	—	—	2/2	1/2	0/2	0/2
52° C. for 15 min.	960	1	4	62	—	2/2	1/2	0/2	0/2	—
54° C. for 15 min.	960	1	16	0	1/2	0/2	0/2	—	—	—

* Percentage of virus eluted to virus adsorbed by fowl red cells. For conditions of experiment and calculation, see text.

† Dilution of 0·01 % human 'O' substance which inhibits one agglutinating dose of each of the virus preparations heated at different temperatures.

‡ Percentage of inhibitor destroyed by virus in 18 hr. For calculation, see text.

§ Expressed as no. of eggs infected/no. of eggs tested.

decline in infectivity, however, was much more rapid. Formolized N.D.V., when causing haemagglutination at all, was eluted from fowl red cells as usual.

Heat inactivation of influenza virus

Samples of Lee virus allantoic fluid were heated at temperatures ranging from 46 to 54° C., varying at

(*c*) Inhibition by 'Francis inhibitor' present in purified human 'O' substance.

(*d*) Ability to destroy 'Francis inhibitor' present in purified human 'O' substance.

(*e*) Infectivity *in ovo*.

The result of this experiment is shown in Table 3. It will be seen that heating up to 48° C. for 15 min. had no effect on any one of the various properties of

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Lee virus as detected by the above tests. At 50° C., there was a moderate drop in eluting power, accompanied by a similar fall in infectivity. After being heated at 52° C. or higher, the virus was no longer significantly eluted from fowl red cells. At the same time, it became increasingly inhibited by 'Francis inhibitor', its 'enzymic' action on 'Francis inhibitor' became increasingly impaired and there was a steady decline in infectivity. In no case, however, did heating affect the haemagglutinin titre itself.

Formolized influenza virus

Samples of Lee virus allantoic fluid containing 0·05, 0·1, 0·2 or 0·5% formalin, and kept at 4° C. were tested after 18 hr., 3, 6 and 8 days. The result obtained after 18 hr. and 8 days is shown in Table 4. The data indicate that as little as 0·05% formalin completely inactivated the infectivity of Lee virus in 18 hr., but neither its eluting power from fowl red cells nor its 'enzymic' function on 'Francis inhibitor' was in any way impaired. The haemagglutinin of formolized Lee virus was not inhibited by 'Francis inhibitor' present in 0·01% purified human 'O' substance. Only the highest formalin concentration tested, i.e. 0·5%, had any direct effect on the haemagglutinin itself, a slow deterioration being observed over a period of 8 days. In the last case, it is interesting to note that even though the haemagglutinin titre had been reduced to 1/20, the residual haemagglutinin was still capable of elution from fowl red cells.

Table 4. *Properties of formolized Lee virus*

Time after addition of formalin	Concentration of formalin (%)	Haem-agglutinin titre	Elution from fowl red cells (%)	Inhibition by 'Francis inhibitor'	Inhibitor destroyed (%)	Infectivity. Virus dilution 10^{-1}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
18 hr.	0	640	100	< 1	> 93	—	—	2/2	2/2	1/2
	0·05	640	100	< 1	> 93	0/2	0/2	—	—	—
	0·1	960	70	< 1	> 93	0/2	0/2	—	—	—
	0·2	960	70	< 1	> 93	—	—	—	—	—
	0·5	640	100	< 1	88	—	—	—	—	—
8 days	0	1280	58	—	—	—	—	—	—	—
	0·05	1280	58	—	—	—	—	—	—	—
	0·1	960	71	—	—	—	—	—	—	—
	0·2	960	71	—	—	—	—	—	—	—
	0·5	20	50	—	—	—	—	—	—	—

DISCUSSION

Although heat and formalin have been extensively used for attenuation or inactivation of viruses both as a laboratory procedure and as methods of vaccine preparation, little is known of their exact mode of action, i.e. the specific components of the virus particle upon which they act to produce the inactivating result. Since both are general protein denaturing agents, their action can hardly be expected to be specific. It is equally well known, however, that different biologically active substances, including enzymes, differ quantitatively in their susceptibility to heat and formalin. It should not, therefore, be impossible to study certain selective properties of viruses at certain selected temperature ranges. The real difficulty appears to lie in the fact that in the vast majority of cases, the only property by which viruses can be recognized is their infectivity for susceptible hosts. The discovery of the haemagglutinating property of some animal viruses provides a powerful tool for such studies. Henle & Henle (1947) have, for instance, compared the effect of graded doses of ultra-violet light on the various properties of influenza viruses which have been arranged in the following order of increasing resistance to ultra-violet irradiation: infectivity, toxicity, inhibitory effect on chick embryonic development and interference, haemagglutination including adsorption-elution mechanism, and lastly antigenicity. Their result indicates that the abilities of influenza virus to haemagglutinate and to be adsorbed by the cells of the allantoic sac are destroyed by the same amount of irradiation. The effect of heat and formalin on influenza haemagglutinin was first investigated by Hirst (1942), who found that the haemagglutinin is little affected by heating at 56° C. and only destroyed by formalin in concentrations higher than 1%. The present study indicates that the adsorbing and eluting phases of influenza haemagglutinin can be dissociated by heating at certain critical temperatures. Although most experiments have been carried out with the Lee strain, preliminary experiments with PR 8 virus have demonstrated an essentially similar behaviour. The heated influenza virus can still combine with the virus receptor substance, whether on the red cells as

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receptor or in solution as 'Francis inhibitor', but cannot elute therefrom, thus failing to complete its 'enzymic' function. Furthermore, when heated at increasing temperatures, the progressive loss of 'enzymic' function by Lee virus is accompanied by a parallel fall in infectivity. The critical time-temperature for complete inactivation of both activities appears to be slightly above 54° C. for 15 min. The haemagglutinin itself, which is responsible for the adsorbing phase, is much more heat resistant and, according to Salk (1946), complete destruction only occurs after heating at 61·5° C. for varying periods, depending on the virus strains tested.

In the case of N.D.V., no such dissociation of phases has been achieved by heating. Heat destruction of haemagglutinin is accompanied by a parallel loss of infectivity. The critical time-temperature for complete inactivation appears to be a little over 58° C. for 15 min.

The parallel destruction by heat of the infectivity and of the 'enzymic' function of two viruses at two different critical temperatures appears to be more than chance coincidence. It suggests that heat destroys the infectivity of these viruses primarily by abolishing their haemagglutinating 'enzyme'. This is also in accord with the evidence obtained by Stone (1947) and Burnet (1948), who showed that the destruction of receptor on the susceptible tissue cells appears to be an essential step in the initiation of infection by influenza virus. This, of course, does not mean that the haemagglutinating 'enzyme' is the only essential component for virus infectivity. Formolized influenza and N.D.V and, according to Henle & Henle (1947), irradiated influenza virus may rapidly become non-infective, while their haemagglutinating 'enzyme' is still intact. Formalin thus appears to destroy primarily some other virus components which are equally essential for infectivity. The existence of at least partially independent components in viruses, all essential for infectivity, if confirmed, will further illustrate the complex nature of animal viruses like influenza and may also help to explain the phenomenon of virus interference.

SUMMARY

Progressive heat inactivation of N.D.V. at 54–58° C. for 15 min. is accompanied by parallel destruction of its haemagglutinating activity. Progressive heat inactivation of Lee virus at 50–54° C. for 15 min. is accompanied by parallel loss of eluting power and 'enzymic' action on 'Francis inhibitor'. Formalin in low concentrations rapidly inactivates both N.D.V. and Lee virus without appreciable impairment of their haemagglutinating activity or 'enzymic' function. Higher formalin concentrations acting over several days may, however, produce a slow deterioration of the haemagglutinin of both viruses. It is suggested that heat destroys the infectivity of N.D.V. and influenza virus primarily by abolishing their haemagglutinating 'enzyme'. The possibility of the existence of independent virus components, all of which are essential for full infectivity of these viruses, is discussed.

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