INFLUENZA

I. The Hemagglutination and Infectivity Titre Curves of PR8 Influenza Virus Cultivated in Embryonated Eggs at Different Temperatures*

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Previous work has shown that while typhus rickettsiae grow freely in the yolk sacs of embryonated eggs incubated at 34° or 37.5°C., their growth is almost completely inhibited when the temperature of incubation is raised to 40°C. (1). The introduction of potassium cyanide in low concentrations, however, resulted in excellent growth of rickettsiae in eggs incubated at 40°C. This work suggested that the determining factor might be the rate of intracellular respiration, rather than the temperature of incubation *per se* (2).

In view of evidence that certain small viruses, in contrast to rickettsiae, grow best when the rate of intracellular metabolism is relatively high (3) it seemed that a study of the influenza virus, by methods previously used for typhus rickettsiae (1, 2, 4), might lead to results of interest. When this study was begun, it was believed that the hemagglutination titre could be used as an index of the rate of multiplication of the virus. It was thought necessary, however, in the initial experiments, to check the titre of hemagglutination by the more laborious process of infectivity titration. This study has brought out anomalous relationships between the hemagglutination and infectivity titre curves.

Material and Methods

Eleven day old eggs of the White Rock variety, incubated with air sacs uppermost, were used throughout these experiments. Previous to injection, all eggs were incubated at 37.5°C. The eggs were then candled and a pencil mark placed at the base of the air space directly over the embryo. This portion of the egg shell was painted with an alcoholic solution of iodine and a hole was made directly above the pencil mark. A 10^{-3} dilution of pooled allantoic fluid from chick embryos infected with an egg-adapted PR8 strain of influenza A virus was used as a source of virus in the majority of experiments. Physiological saline was used as the diluting fluid. Several preliminary experiments showed this solution to work as well as the horse serum broth originally utilized by Hirst (5). Infected allantoic fluids were stored at −40°C.

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One-tenth cc. of the inoculum was injected by inserting the needle of a 1 cc. tuberculin syringe through the previously made hole, at a 45° angle with the long axis of the egg, for a distance of one inch. Eggs containing live embryos were sacrificed at intervals and the allantoic fluids were pipetted off immediately. The hemagglutination titres were carried out as originally described by Hirst (6). Infectivity titres were determined by making tenfold dilutions of the collected fluids in physiological saline and injecting 0.1 cc. of each dilution into the chorioallantoic sacs of 5 eggs. At the end of 48 hours, allantoic fluids were removed from these eggs and evidence of virus growth determined by the ability of the fluids to agglutinate chicken red blood cells. The allantoic fluids used in the determinations of both hemagglutination and infectivity titres were obtained by pooling infected fluids from at least 3 eggs for each time period. The 50 per cent infectivity end points were calculated by the method of Reed and Muench (7).

EXPERIMENTAL RESULTS

Latent Period of Infectivity and Early Growth.—It was necessary to determine whether the latent period of infectivity described by others (8–10) would occur under the conditions of our experiments, and whether it would be affected

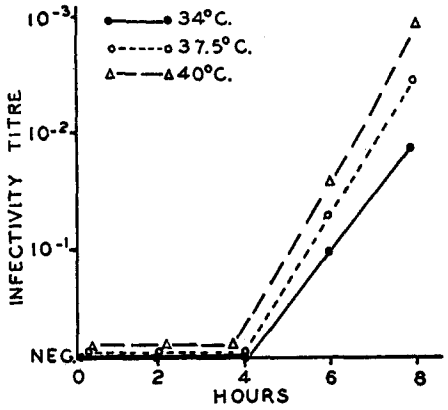

FIG. 1. Latent period of infectivity at different temperatures.

by the temperature of incubation. Three groups of 30 eggs each were inoculated with infected allantoic fluid, and thereafter incubated at 34°, 37.5°, and 40°C. The infectivity titres of pooled fluids removed at 2 hour intervals are shown in Fig. 1. No virus was detectable at the 2nd and 4th hours after inoculation. In subsequent experiments, in which more concentrated inocula were used, the presence of some unadsorbed live virus was demonstrated at these time intervals.

Fig. 2 shows the results of infectivity titre determinations made at intervals of 2 hours, during the initial 24 hour growth period, on pooled allantoic fluids from a series of infected eggs incubated at 37.5°C. It will be noted that in addition to the initial latent period there were three subsequent periods during

which no increase of the infectivity titre occurred. These periods occurred between the 8th and 10th hours, between the 14th and 16th hours, and between the 18th and 22nd hours.

Latent Period of Hemagglutination.—Hemagglutination was not demonstrated before the 10th hour after egg inoculation. In Fig. 4, it will be noted that no hemagglutination was detected at 12 hours in the fluids of eggs in the 34°

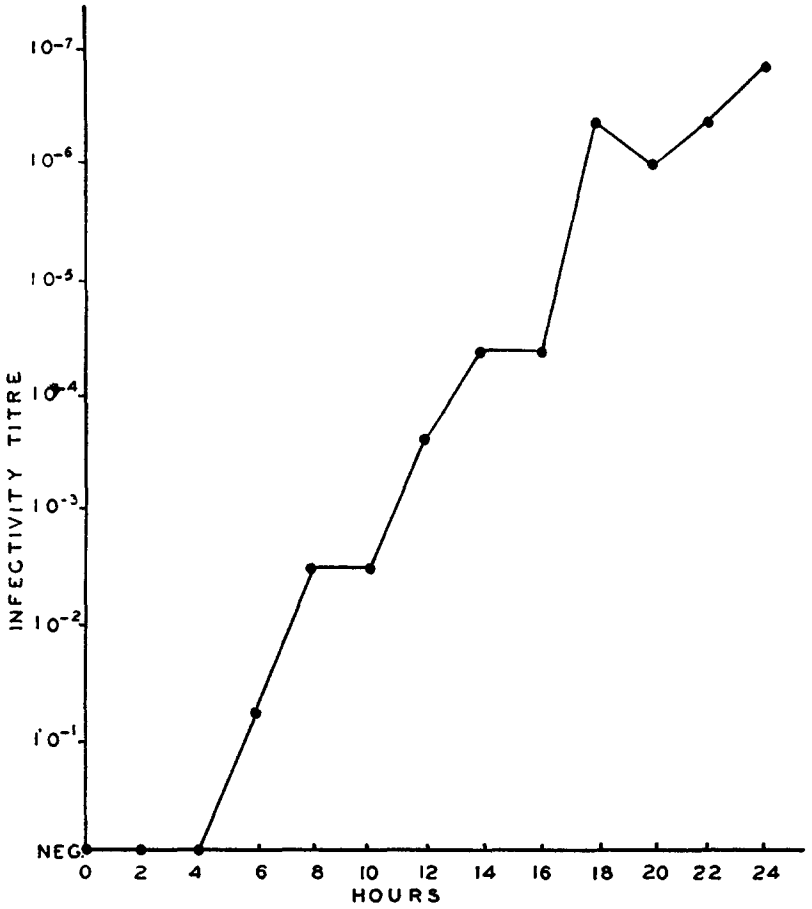

FIG. 2. Cycles of infectivity titre during the first 24 hours of virus multiplication.

and 40°C. groups, although the infectivity titres of these fluids were above 10^{-5}. Some delay in the development of hemagglutinating properties is to be expected, since virus particles must be present in relatively large numbers to cause this phenomenon. This difference of 5 logarithms appears remarkably large, however, and is difficult to explain if one assumes that all infective virus particles possess equal hemagglutinative ability.

Correlation between Infectivity and Hemagglutination.—

Several series of embryonated eggs were incubated at 37.5°C. for 10 days. The eggs in each series were then inoculated with influenza virus, and divided into three groups which were maintained thereafter at temperatures of 34°, 37.5°, and 40°C. Fluids were harvested at various intervals for the determination of infectivity and hemagglutination titres. The

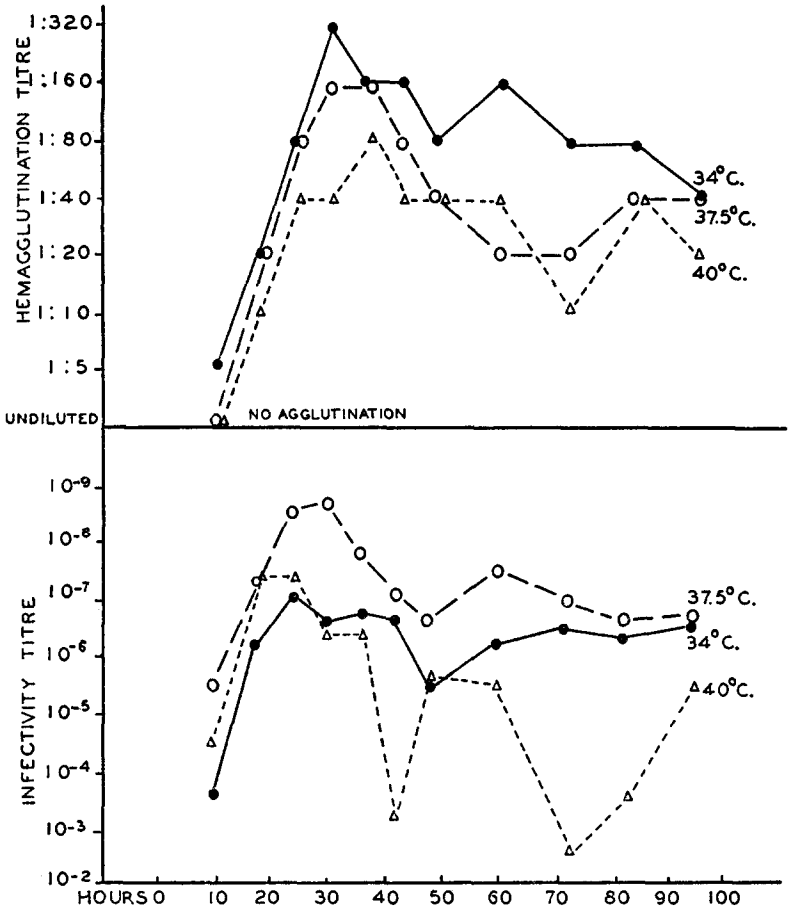

FIG. 3. Comparison of hemagglutination and infectivity titre curves at different temperatures. Experiment 1.

results obtained in two such experiments are shown graphically in Figs. 3 and 4. These two experiments were essentially identical except that in the experiment represented by Fig. 3 the inoculum for each egg was 0.1 cc. of a 10^{-3} dilution of infected allantoic fluid, while in the experiment represented by Fig. 4 each egg received 0.1 cc. of a 10^{-5} dilution of the infected fluid. It should also be noted that fluids were tested at shorter intervals in the experiment depicted in Fig. 3.

In the first place, it will be noted that in each of these experiments the hemagglutination titre reached its highest peak and was best sustained in

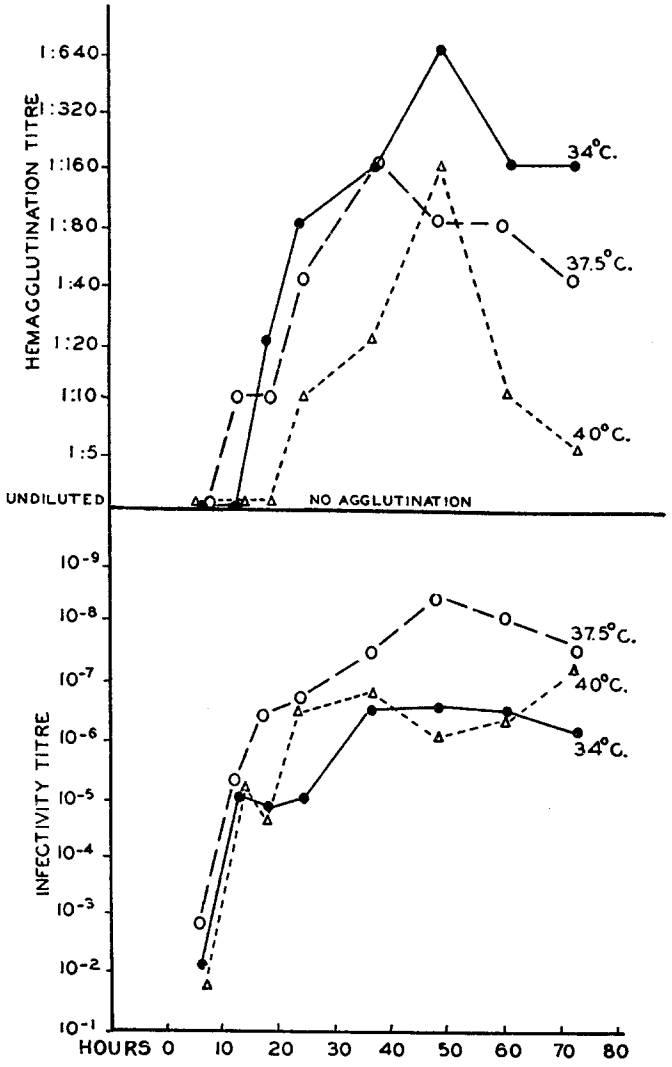

FIG. 4. Comparison of hemagglutination and infectivity titre curves at different temperatures. Experiment 2.

the eggs incubated at 34°C., while the infectivity titre was highest and best sustained in the eggs incubated at 37.5°C. This inverse relationship between hemagglutination and infectivity was noted consistently in all four experiments

of this type which were carried out. In two experiments, the infectivity titre curves of the eggs incubated at 40°C. were of the "picket-fence" type shown in Fig. 3. Since the time intervals are usually longer in the graph shown in Fig. 4, some of these irregularities may have been missed.

In general, the best correspondence between infectivity and hemagglutination titres occurred in the eggs incubated at 37.5°C., and the poorest correspondence occurred in eggs in the 40°C. group. Remarkable instances of disagreement between the two tests for viral activity is, however, to be seen at all temperatures when certain portions of the two curves are compared. At 37.5°C. (Fig. 4) there is a rise in infectivity from $10^{-7.5}$ to $10^{-8.5}$ between the 36th and 48th hours, while the hemagglutination decreased from 1:160 to 1:80. A similar situation (Fig. 3) is seen at 37.5°C. between the 48th and 60th hours. At 34°C., we find a rise in hemagglutination titre from 1:160 to 1:640 between the 36th and 48th hours without any observed change in the infectivity titres (Fig. 4). At 40°C. (Fig. 4) an increase in hemagglutination titre from 1:20 to 1:160 occurred simultaneously with a drop in infectivity titre which probably was significant.[1] Moreover, the apparent peak of infectivity titre occurred at 70 hours, when the hemagglutination titre had fallen from a peak of 1:160 to the low point of 1:5. These discrepancies lie far beyond the range of experimental error.

DISCUSSION

In spite of much study, the relation between the agents responsible for infectivity and hemagglutination by the influenza virus is still far from clear. It is generally accepted that both phenomena may be caused by the same particle (12), and the hemagglutination titre has been used widely in chemotherapeutic studies as an index of virus multiplication. Hemagglutination is known to be caused by inactivated virus as well as live virus (13), and it is obvious that hemagglutination does not occur until the concentration of virus particles is much higher than that required to give a positive infectivity test. Our experimental data indicate, however, that the infective virus present after about 12 hours of multiplication in the egg is often strangely lacking in hemagglutinative ability, and that at later time intervals there are extraordinary discrepancies between infectivity and hemagglutination.

The exact stage of disintegration of influenza virus particles at which the hemagglutinating property is lost has not been determined. The possibility exists that this stage might be arrived at more rapidly at higher temperatures of incubation. This may partially explain the fact that incubation at 34°C. invariably resulted in the highest titres of hemagglutination, although the infectivity titres were far below those developed at 37.5°C.

[1] On the basis of observations reported by Knight (11) differences of 0.62 logarithmic unit may be regarded as significant.

Friedewald and Pickels (14), by means of high speed centrifugation, have shown differences in the behavior of the particles causing hemagglutination and those causing infection. Inactive virus, produced by heating or exposure to ultraviolet light, was found by the Henles (15) to interfere with hemagglutination, but not with infectivity, when mixed with active virus before inoculation into eggs.

Another variable has come into the picture with the discovery that agents which inhibit hemagglutination are present in a variety of biological materials (16–21), including allantoic fluid (22). De Burgh and his coworkers (19) have shown that such inhibitors are inactivated on incubation at 37°C. Hardy and Horsfall (23) have shown that virus bound by allantoic fluid inhibitor is still infective. Since virus bound by inhibitor does not cause hemagglutination, they point out that "hemagglutination may not reflect accurately the total concentration of virus in allantoic fluid." The situation apparently is still further complicated by the presence in allantoic fluid of altered inhibitor which cannot recombine with virus. The paradoxical results shown in Figs. 3 and 4 may be caused by different rates of adsorption and alteration of this allantoic fluid inhibitor at different temperatures of incubation and at various time intervals of viral growth.

Several other workers have obtained results which are inconsistent with the view that there is a constant quantitative correlation between infectivity and hemagglutination. Burnet *et al.* (24) found that the change from the original (O) phase of the virus to the egg-adapted (D) phase was accompanied by a change from a low to a high F/G ratio (F being the hemagglutination titre for fowl cells and G the titre for guinea pig cells). He conceives of virus adaptation to a new host as resulting from the "selective survival of spontaneously occurring discontinuous mutants." Unfavorable conditions resulting from development at temperatures outside of the optimum range conceivably might favor such mutations.

Wang (25) has shown that mouse-adapted influenza virus gave higher peaks of hemagglutination titre than the unadapted virus, although the infectivity titre peaks reached by the two strains of virus in eggs were about the same. Moreover, the mouse-adapted strain showed infectivity titres of $10^{-6.7}$ to 10^{-7} at 12 hours, when no hemagglutination activity could be detected. During the next 12 hours, the hemagglutination titre rose to 1:1250, while the infectivity titre remained practically constant.

Henle (26) and Gard and von Magnus (27) have been led by their experimental observations to postulate the existence of an immature, non-infectious form of the influenza virus in the early stages of intracellular multiplication. This form causes hemagglutination and complement fixation, in spite of its lack of infectivity. Hoyle (9) on the other hand believed that his evidence indicated the presence of an intracellular phase of the virus which, like that suggested by the Henles, is non-infective and causes complement fixation, but does not agglutinate red blood cells. He suggests that this may be identical with the complement-fixing soluble antigen previously described, and that it may be a nucleoprotein of relatively small size. He also suggests that the extracellular, infective, red cell–agglutinating phase (the larger virus particle) may be formed from the intracellular, non-infective phase by the addition of other components by the living host cells.

Influenza A virus has been shown to reach high hemagglutination titres when

cultivated at 39°C., while a strain of influenza B virus was scarcely detectable by hemagglutination when grown at this temperature (28). McLean and his coworkers (29), using the Lee strain of influenza virus B, obtained the highest titres for both infectivity and hemagglutination by cultivation at 35°C. Incubation at 39°C. resulted in much lower infectivity and hemagglutination titres, and the conclusion was drawn that this temperature was unsuitable for the cultivation of this strain of virus.

Miller (30) obtained the "best concentration of influenza PR8 virus," as measured by hemagglutination, by cultivation at 35°C. (after preliminary incubation of the eggs for 10 to 11 days at 37°C. or for 9 to 10 days at 39°C.). It will be noted that our results also show the highest hemagglutination titre of the PR8 strain in eggs cultivated at a low temperature (34°C.) but that we found the infectivity titre to be relatively low at this temperature.

It seems difficult at present to decide on a satisfactory criterion for multiplication of the influenza virus. In working with rickettsiae, the rate of multiplication has been roughly estimated by counting the organisms in film preparations of the infected membranes (1). Undoubtedly some dead organisms are counted by this method, but in general results have been satisfactory. On the basis of our observations, and in view of the many variables involved, it seems dangerous to rely on reduction in hemagglutination titre as an indication of suppression of the growth of influenza virus. Although this test may be valuable as a preliminary screening method for compounds with possible antiviral activity, it is obviously not suitable for metabolic studies.

It would be desirable to know what morphological forms the virus assumes and what biological properties it acquires or loses as it passes through stages of relative variation in infectivity and hemagglutinative ability. It seems possible that some of these alterations might be correlated with important modifications in immunizing ability.

SUMMARY

Groups of embryonated eggs infected with the PR8 strain of influenza virus A were incubated at 34°, 37.5°, and 40°C. At frequent intervals, for periods ranging up to 96 hours, pooled allantoic fluids were tested simultaneously for infectivity and hemagglutination.

After about 12 hours of virus growth, fluids often showed infectivity titres greater than 10^{-5}, but were incapable of causing hemagglutination. At later time intervals, marked disagreement between the two tests for viral activity was noted at all temperatures, but most strikingly at 40°C.

Hemagglutination titres were highest and best sustained in eggs incubated at 34°C., while incubation at 37.5°C. resulted in the highest and best sustained infectivity titres.

Hemagglutination titre determinations do not reflect accurately the rate of influenza virus multiplication.

Possible reasons for the lack of correspondence between hemagglutination and infectivity are discussed.

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