A STUDY OF CONDITIONS FOR THE OPTIMUM PRODUCTION OF PR8 INFLUENZA VIRUS IN CHICK EMBRYOS*

By GAIL LORENZ MILLER, Ph.D.

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

(Received for publication, November 3, 1943)

Before detailed studies of the chemical and physical properties of influenza virus can be carried out, it is necessary to obtain the material in relatively pure form and in as large quantities as possible. In an effort to ascertain the optimum conditions for the production of the PR8 strain of influenza virus in chick embryos, investigations have been carried out on the specific rôles of a number of variables which are known to be involved in the relationship between the disease agent and the host. The variables studied include (*a*) the concentration of virus in the inoculum, (*b*) the temperature of incubation of the infected embryos, (*c*) the length of time of incubation of the infected embryos, and (*d*) the age of the embryos at the time of inoculation. The effects of these variables upon such factors as the rate of death of the embryos and the amount and activity of the virus which is produced were observed and are recorded in the present paper.

Materials and Methods

Stock Inoculum.—The frozen, infectious allantoic fluid employed as stock inoculum contained around 100 units of CCA activity per cc. (1, 2) and, when titrated in chick embryos by Hirst's method (3), produced infection at a maximum dilution of 10^{-7}.

Inoculations.—Embryonated White Leghorn eggs were inoculated with 0.1 cc. of suitable inoculum introduced with a hypodermic needle into the extraembryonic fluids through a small hole drilled in the shell above the air sac. The holes were sealed with collodion before further incubation of the eggs.

Measurements of Virus Activity.—On the basis of the assumption that virus infectivity and chicken red blood cell agglutination (CCA) activity are the properties of the same molecule (4), or at least go hand in hand, the relative amounts of virus in different preparations were measured indirectly by means of determinations of CCA titers. The CCA titrations were carried out according to the method of Hirst and Pickels (1), as modified for use in this laboratory (2). Standardization of the apparent titers was not usually carried out in the present work, since, in general, it was not essential to the significance of the results.

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and The Rockefeller Institute for Medical Research.

Nitrogen Determinations.—Nitrogen determinations were carried out by the following modification of the direct nesslerization micro-Kjeldahl method of Koch and McMeekin (5). Aliquots of test solutions containing 0.03 to 0.3 mg. of nitrogen were transferred to micro-Kjeldahl flasks and 0.25 cc. portions of H_2SO_4 digestion mixture, prepared by adding one volume of concentrated H_2SO_4 to 1.2 volumes of water, were added. A glass bead was introduced into each flask and the mixtures were heated over a hot flame until the water was evaporated and the digests became dark in color. Two drops of 30 per cent hydrogen peroxide were then added and the mixtures were digested for 10 minutes longer. The digests were allowed to cool, 10 cc. of water and two drops of gum ghatti solution were added, and finally 2 cc. of Eimer and Amend Nessler's reagent (for aldehydes and ammonia) were added to each flask with swirling of the contents. Readings of color intensities were made within 2 or 3 minutes in the Klett-Summerson photoelectric colorimeter with the use of a No. 54 green filter. The method was standardized with ammonium sulfate and Beer's law was found to be followed within the range of nitrogen indicated. When determinations were made on virus preparations containing phosphate buffer at pH 7, it was necessary that the sample contain not more than 0.1 millimole of the buffer.

Because preparations of virus of varying purity may contain different percentages of nitrogen, estimates of virus protein have, in general, been calculated on the basis of protein nitrogen.

EXPERIMENTAL

The Rôle of Concentration of Virus in Inoculum

Henle and Chambers (6), working with the WS strain of influenza A virus, observed that higher titers of virus were obtained when embryonated eggs were inoculated with low, rather than with high, concentrations of inoculum. In our own preliminary work with PR8 virus, a 10^{-3} dilution of stock inoculum was used for the inoculations. Under these conditions, however, a rapid death and disintegration of the embryos occurred rather often. It was found that this result could be obviated by the use of a lower concentration of virus inoculum, and for routine work a dilution of 10^{-5} proved to be more satisfactory. It was thought of importance, nevertheless, to establish more quantitatively the relative effects of different concentrations of virus inocula.

Embryonated eggs, which were brought to 10 days of age at an incubator temperature of 39°C., were divided into four batches of 38 eggs each and were inoculated respectively with 10^{-1}, 10^{-3}, 10^{-5}, and 10^{-7} dilutions of stock inoculum in 0.1 M phosphate buffer at pH 7. The eggs were then incubated at 35°C. and at intervals of 24, 36, and 48 hours they were candled and those containing dead embryos were separated, chilled, and harvested. At the end of the 48-hour period, one half of the eggs which still contained live embryos was harvested while the remaining half was incubated for a further 24 hours before it was harvested. The volume of the harvested fluid and the CCA titer for each test sample were measured, and the CCA activities per test sample were calculated by multiplying the values obtained from these two measurements. The results are summarized in Table I, from which it is

apparent that the highest activities were recovered from embryos inoculated with the 10^{-3} and 10^{-5} dilutions. It may further be noted that the embryos died relatively more rapidly when inoculated with the lower dilutions of virus. Thus, in the cases of the 10^{-3} and 10^{-5} inocula, for example, 84 per cent of the embryos were dead at the end of the 48-hour period of incubation in the former case whereas only 16 per cent

TABLE I

Effects of Different Concentrations of Virus in Inoculum

Concentration of inoculum	Test sample	No. of eggs in test sample	No. of CCA units in test sample	Total No. of CCA units
10^{-1}	24 hr. Dead	9	3,100	
	36 " "	13	5,100	
	48 " "	7	4,000	
	48 " Live	5	0	
	72 " Dead	0	—	
	72 " Live	4	0	12,200
10^{-3}	24 hr. Dead	6	2,160	
	36 " "	12	14,400	
	48 " "	14	30,200	
	48 " Live	3	9,540	
	72 " Dead	1	1,800	
	72 " Live	2	2,840	70,940
10^{-5}	24 hr. Dead	2	0	
	36 " "	2	2,600	
	48 " "	2	1,920	
	48 " Live	16	33,600	
	72 " Dead	4	11,520	
	72 " Live	12	18,740	68,380
10^{-7}	24 hr. Dead	2	0	
	36 " "	1	27	
	48 " "	0	0	
	48 " Live	18	9,120	
	72 " Dead	0	0	
	72 " Live	17	0	9,147

were dead in the latter case. Since contamination of harvested fluids due to the disintegration of the contents of the eggs would be at a minimum in infected eggs which were still alive, it appeared that the use of the 10^{-5} inoculum was preferable to the use of the 10^{-3} inoculum.

In order to test the effect of concentration of inocula as described above but with the limitation of a 36-hour period of incubation, two experiments were carried out in which batches containing 40 to 60 eggs each were inoculated with the four different concentrations of virus used previously. The harvests of all of the eggs in each sepa-

rate batch were pooled and were tested for CCA activity. In the first experiment the CCA activities for each of the fluids from embryos inoculated with the 10^{-1}, 10^{-3}, 10^{-5}, and 10^{-7} dilutions were, respectively, 68, 147, 126, and 15 units per cc. In the second experiment the corresponding values were 85, 218, 213, and 5 units per cc. The data therefore confirmed those obtained in the original experiment, and, from the standpoint of the lower death rate of embryos at lower concentrations of inocula, it was concluded that the 10^{-5} dilution yielded the most satisfactory results.

Henle and Henle have recently demonstrated that the low titers of virus which result from the use of high concentrations of inocula can be attributed to the presence of inactive virus which exerts an inhibiting influence upon the propagation of the virus (7). The inhibiting influence was apparently minimized when low concentrations of inocula were employed. It was observed, in addition, that the effect also could be minimized by the use of an inoculum obtained from infected eggs which had been incubated for only 12 to 18 hours and which therefore contained relatively little inactive virus. It was not apparent from the data, however, whether one of these alternative procedures might give rise to a more favorable production of virus than the other. We have therefore carried out the following additional tests.

Two batches of eggs, each represented by forty 10-day old embryos, were inoculated, respectively, with 10^{-1} and 10^{-5} dilutions of a freshly prepared inoculum obtained from infected embryos incubated for just 12 hours. As controls, two similar batches of eggs were inoculated with 10^{-1} and 10^{-5} dilutions of the stock inoculum which was used in the previous studies and which had been obtained from infected eggs incubated for 48 hours. After the test eggs were incubated for 36 hours at 36°C., they were chilled and harvested. The CCA titers were found to be 292 and 294 units per cc., respectively, for the pooled harvests obtained from embryos inoculated with the 10^{-1} and 10^{-5} dilutions of the 12-hour inoculum. Those for the harvests obtained from embryos inoculated with the 10^{-1} and 10^{-5} dilutions of the 48-hour inoculum were, respectively, 111 and 284 CCA units per cc. This experiment was repeated and the results were duplicated. The results thus lend confirmation to the findings of Henle and Henle and in addition demonstrate that essentially the same results are obtained by the use of 10^{-5} dilutions of either the 12-hour or the 48-hour inocula.

The low production of virus which resulted when the 10^{-7} inoculum was used can readily be explained by the fact that the stock inoculum, when titrated in chick embryos, barely produced infection at a dilution of 10^{-7}, as already noted under Materials and methods. It appears quite probable that, in general, optimum results in chick embryos with a given inoculum of PR8 virus may be expected at a concentration of inoculum approximating 100-fold that of the highest dilution which will produce infection.

The Rôle of Temperature of Incubation

Sigurdsson (8) found that about a 100-fold higher titer of vesicular stomatitis virus is produced in infected embryos when they are incubated at a temperature of 35–36°C.

than when incubated at 39–40°C. The titers in this case were based on relative infectivities as determined by subinoculation in additional embryos. It appeared worthwhile to determine whether a similar situation obtained in the case of PR8 influenza virus when titers were followed by means of the CCA test. In a preliminary experiment, 80 eggs brought to 10 days of age at 37°C. were each inoculated with stock inoculum at a 10^{-5} dilution and were divided into two equal batches, one of which was incubated at 35°C. and the other at 37°C. At the end of an incubation period of 40 hours, the eggs were candled and the four batches of eggs representing dead and live embryos at the two temperatures were harvested separately and the fluids tested for agglutination titer. The titers of the fluids from embryos held at 35°C., dead and live, were respectively 260 and 248 units per cc. The corresponding titers of the 37° eggs

TABLE II

Effects of Different Temperatures of Incubation

Test sample		CCA activities of allantoic fluids			
		Temperature of incubation 35°C.		Temperature of incubation 39°C.	
		Individual eggs	Average	Individual eggs	Average
6 hr.	Dead	<4*	<4	<4	<4
12 "	"	<4	<4	<4	<4
24 "	"	<4	<4	24, 52, 52, 86, 258, 276, 288	148
29 "	"	176	176	0, 4, 94, 148, 176, 200, 250, 332	151
46 "	"	8, 18, 568, 568, 662, 904, 1292	574	10, 54, 124, 146, 214, 372	153
72 "	"	36, 84, 162, 294, 392, 414, 530, 564, 800, 860	414	0, 0, 8, 28	9
94 "	"	306, 412	359		
94 "	Live	0, 0, 0, 0, 0, 332, 356, 662	169	0, 0, 0, 4	1

* The numbers represent units of CCA activity per cubic centimeter of allantoic fluid.

were 266 and 246. It was concluded that there was little if any difference in the specific CCA activities of the fluids from eggs incubated at the two temperatures studied. A marked difference in rate of death was noted, however. All except one of the embryos incubated at 37°C. were dead at the time of harvest, whereas in the 35° experiment 14 embryos were still alive.

The experiment was repeated with a greater range of temperatures, namely, 35° and 39°C. In this experiment, the eggs employed were candled at periods of 6, 12, 24, 29, 46, 72, and 94 hours, and those containing dead embryos were transferred to the cold room in order to prevent deterioration. Furthermore, the eggs were harvested individually and the fluid from each egg was titrated for CCA activity. Two batches of 31 eggs, brought to 10 days of age at 39°, were used. The results are shown in Table II, from which it is quite apparent that at the relatively high temperature of 39° a high rate of death occurred but at the same time a lower titer of virus was obtained than at the lower temperature of 35°C.

Since higher titers of virus were obtained at 35° than at 39°, it was of interest to

test the results obtained at a still lower temperature. Accordingly, three batches of 58 inoculated embryos each were incubated for 36 hours at approximate temperatures of 33°, 36°, and 39°C., respectively. The final corresponding CCA titers of the harvested allantoic fluids were 100, 209, and 154. In a duplicate experiment in which each test sample was represented by 28 embryos, final titers of 73, 279, and 45 were obtained. It is obvious from these data that the low temperature of 33° as well as the high temperature of 39° caused a low production of virus.

The results of the temperature studies as a whole indicate that an optimum production of virus is obtained at incubation temperatures of 35° to 37°C. and that significantly lower titers are obtained at temperatures outside this range. From the standpoint of maintaining the death rate at a minimum, it appears that the most satisfactory results are obtainable at 35°C.

The Rôle of Length of Time of Incubation

In early studies on the preparation of influenza virus, it was observed that at times pale-colored and only slightly opalescent pools of infected allantoic fluids were obtained, whereas at other times the allantoic fluids were orange-red in color and more densely opalescent. When the virus was isolated from the fluids by means of ultracentrifugation, the final product obtained from darkly colored harvests usually possessed low CCA activities calculated on the basis of units per milligram of protein. The darkly colored harvests resulted from the hemolysis and general disintegration of embryos which had been killed by the influenza virus. It appeared that this difficulty might be avoided if the rate of death could be minimized and also if eggs containing dead embryos were harvested as soon as possible after the death of the embryos. At the same time, it was important to allow a period of incubation long enough to assure the maximum possible production of influenza virus. It has been demonstrated in the preceding sections that the rate of death of embryos can be minimized by the proper choice of dilution of inoculum and of temperature of incubation, and it will also be shown in the following section that the age of embryo has a marked influence upon the rate of death. The effect of the length of time of incubation upon the CCA titer of the harvested allantoic fluids is illustrated by the results of experiments summarized in Table III. On the basis of these data and those previously shown in Table II, periods of incubation of 36 to 48 hours, depending upon the age of the embryos, were chosen as roughly optimum, since it appeared that under these conditions the virus reproduction was near a maximum, yet the number of deaths was not too great.

The Rôle of Age of Embryos at Time of Inoculation

Different investigators studying influenza virus in the chick embryo have employed embryos varying in age from 9 to 12 days (3, 4, 6, 9–12).

Sigurdsson, in studies with vesicular stomatitis virus, found that 7-day old embryos were much more susceptible to infection than 10-day embryos (8). In order to determine whether a similar relationship existed between the age of embryo and the susceptibility to influenza virus, four batches of 23 to 31 embryos each, brought to respective ages of 9, 10, 11, and 12 days at 37°C., were inoculated with a 10^{-5} dilution of stock PR8 virus and incubated at 37°. At the end of 48 hours, the eggs were candled and the numbers of dead embryos were noted. The percentages of dead embryos were found to be 91, 72, 46, and 12 per cent, respectively, for the 9, 10, 11, and 12-day embryos. It was concluded from these data that young embryos are highly susceptible to death from influenza and that, from the standpoint of minimizing the numbers of deaths for the isolation of influenza virus from infected embryos, the use of the older embryos was strongly favored.

TABLE III

Effects of Different Lengths of Time of Incubation

Experiment No.	Age of embryo	No. of eggs in test sample	Period of incubation	Rate of death	Average CCA titer
	days		*hrs.*	*per cent*	*units per cc.*
1	9	40	24	8	70
		40	36	28	146
		120	48	81	162
2	9	25	24	17	80
		75	42	87	112
3	10	25	24	3	90
		25	42	36	138
		51	65	71	156

Knight has shown recently that high molecular weight substances lacking infectivity or CCA activity are elaborated in appreciable amounts by uninoculated chick embryos, and, furthermore, that the amount of these substances which is formed increases markedly with the age of the embryo (13). It appeared, therefore, that such substances would be present as contaminants in preparations of ultracentrifugally prepared virus and should be reflected in a variation in the specific CCA activities of virus samples prepared from infected embryos of different ages. Accordingly, experiments were designed to establish the relationship of age of embryo to the specific activity of purified virus. In order that the results obtained with young embryos could be compared directly with those obtained with the more mature embryos, two age groups were tested simultaneously in each experiment. It was also possible in the same experiments to test the effect of age of embryo on the total yield of virus.

Batches of embryonated eggs, brought to 7 to 12 days of age, in some instances at 37° and in other instances at 39°, were inoculated with a 10^{-5} dilution of stock inoculum and were incubated for 36 to 40 hours at 35°. The infected eggs were then chilled, harvested, and small aliquots of the allantoic fluids were set aside for measurements of CCA titers. The virus present in the residual portions of allantoic fluids was isolated by centrifugation of the fluids for periods of ½ hour at 24,000 R.P.M. (14). The pellets which were obtained were taken up in 0.1 M phosphate buffer at pH 7, and following clarification at low speed were then subjected to a second high-speed centrifugation. Nitrogen determinations and CCA titrations were carried out on the final preparations. Parallel with these tests, equally sized batches of uninoculated embryonated eggs were carried through the same incubation and isolation process. In this way, indirect estimates of the amounts of inactive heavy components present in the various test samples were made.

TABLE IV

Effect of Age of Embryos at Time of Inoculation

Experiment No.	Age of embryo	Total No. of eggs	CCA activity of harvested fluid	Yield of virus protein N from infectious allantoic fluid	Yield of heavy material N from normal allantoic fluid	CCA activity of purified virus
	days		*units per cc.*	*mg. per cc.*	*mg. per cc.*	*units per mg. N*
1	7 at 37°	40	22	0.00024	0.000067	10,100*
	10 " 37°	40	274	0.0035	0.00033	33,700
2	8 at 37°	100	52	0.00081	0.00027	22,400
	11 " 37°	100	528	0.0082	0.00103	42,900
3	7 at 39°	88	34	0.0012	0.00074	37,800
	10 " 39°	100	146	0.0061	0.00058	36,800
4	9 at 39°	124	206	0.0059	0.00099	33,900
	12 " 39°	80	210	0.019	0.024	9,650

* The CCA activities presented in this column are standardized as described elsewhere (2).

The final results are presented in Table IV. From the data for the CCA activities of the harvested fluids, it is evident that the amounts of virus in the 7- and 8-day embryos were significantly lower than those for the older embryos. The CCA titers of the various fluids were paralleled quite well by the amounts of protein nitrogen which were isolated by differential centrifugation. The amounts of normal heavy material obtained in the control experiments also increased with age of embryo but, as a rule, were much lower than the corresponding yields of virus. In the cases of the 7- and 8-day embryos, the yields of normal heavy material were on the average about 40 per cent of those of virus, whereas in the cases of the 10- and 11-day embryos the yields of normal material were only about 10 per cent of those of virus. If it were assumed that the impurities present in the purified virus preparations arose only from

the presence of normal heavy material, it might be concluded that virus preparations have been obtained which are approximately 90 per cent pure. Further studies are being carried out in this laboratory to test this possibility.

From the data for the CCA activity of the purified virus preparations, shown in the last column of Table IV, it may be noted that relatively low activities were obtained in two out of three cases with the 7- and 8-day embryos and also in the case of the oldest embryos, namely, the 12-day embryos. The low titers may be explained, in part at least, by the relatively higher proportions of normal heavy materials in these preparations, due in the former cases to the small amount of virus and in the latter case to the relatively large amount of normal heavy material present. Consistently favorable results, both from the standpoint of yield and of specific activity of purified virus, were obtained, on the other hand, with embryos brought to 10 to 11 days of age at 37° or 9 to 10 days of age at 39°.

DISCUSSION

The rôles of concentration of virus in inoculum, temperature of incubation of infected embryos, period of incubation of infected embryos, and age of embryos at time of inoculation are illustrated by the results of the typical experiments described above. It should be pointed out, however, that these results can best be interpreted to indicate tendencies, and that an exact duplication of results under a given set of conditions cannot be expected at every trial. This is due to an uncontrolled factor, namely, the inherent variation in the behaviors of individual eggs and of different batches of eggs. In order that significant results may be obtained, therefore, sufficient numbers of embryos must be employed in the tests and the results must be checked in replicate experiments. These precautions have been taken in the studies described above. It may be of value, however, to discuss some examples of the types of variation which may be encountered.

In the experiment summarized in Table I, it was apparent that high concentrations of virus in inocula brought about a relatively more rapid death of the embryos. In one of the replicate experiments this tendency was duplicated, but in the other replicate experiment the embryos proved quite resistant to the virus and very few died even with high concentrations of inocula. A variation in resistance of different batches of eggs to the virus is thus exemplified.

The variation in amount of virus produced in different eggs infected with the same inoculum is demonstrated by the data shown in Table II, where the CCA activities of fluids from individual embryos can be seen to vary from 4 or fewer units per cc. to 1292 units per cc. From these results, the dangers involved in carrying out quantitative tests with individual embryos are clearly apparent.

The average yield of allantoic fluid per egg was found to be 4.5 to 5.0 cc. Occasionally, however, somewhat lower yields and also yields as high as 7 to 9 cc. per egg were obtained.

The pellets obtained from the high-speed centrifugation of infectious allantoic fluids were usually relatively small and in these cases were found to be readily dispersed in 0.1 M phosphate buffer at pH 7. Such preparations of virus protein were found to possess high specific CCA activities, provided the original allantoic fluids contained a high activity. All of the virus preparations described in Table IV, except that from the 12-day embryos, were characterized by small pellets and ease of dispersion. On the other hand, pellets obtained on high-speed centrifugation may occasionally be large in size and in this case they are usually difficultly and incompletely dispersed in 0.1 M phosphate buffer. Furthermore, such preparations are found to possess a relatively low specific CCA activity.

It is apparent from the variability described above that a preparation of influenza virus of highest purity cannot be assured absolutely even under optimum controlled conditions. The relative purity of each preparation must therefore be tested by a comparison of its specific activity with that of another preparation of virus employed as an arbitrary standard (2).

SUMMARY

In order to determine the conditions for the optimum production of PR8 influenza virus in chick embryos, a study has been made of the rôles of concentration of virus in the inoculum, temperature of incubation of infected embryos, length of time of incubation of infected embryos, and age of embryos at the time of inoculation. Relative amounts of virus in different preparations were measured indirectly by means of determinations of chicken red blood cell agglutination titers. Frozen infectious allantoic fluid which produced infection in chick embryos at a maximum dilution of 10^{-7} was employed as a stock inoculum. Best results were obtained with an amount of stock inoculum of 0.1 cc. of a 10^{-5} dilution, a temperature of incubation of 35°C., a length of time of incubation of 36 to 48 hours, and with embryos brought to 10 or 11 days of age at 37°C. or 9 or 10 days of age at 39°. An uncontrolled factor arising from inherent variations in the properties of different embryos and different batches of embryos was discussed.

BIBLIOGRAPHY

1. Hirst, G. K., and Pickels, E. G., *J. Immunol.*, 1942, **45**, 273.
2. Miller, G. L., and Stanley, W. M., *J. Exp. Med.*, 1944, **79**, 185.
3. Hirst, G. K., *J. Immunol.*, 1942, **45**, 285.
4. Hirst, G. K., *J. Exp. Med.*, 1942, **75**, 49.
5. Koch, F. C., and McMeekin, T. L., *J. Am. Chem. Soc.*, 1924, **46**, 2066.

6. Henle, W., and Chambers, L. A., *Proc. Soc. Exp. Biol. and Med.*, 1941, **46,** 713.
7. Henle, W., and Henle, G., *Science*, 1943, **98,** 87.
8. Sigurdsson, B., *J. Exp. Med.*, 1943, **78,** 17.
9. McClelland, L., and Hare, R., *Canad. Pub. Health J.*, 1941, **32,** 530.
10. Hare, R., McClelland, L., and Morgan, J., *Canad. Pub. Health J.*, 1942, **33,** 325.
11. Burnet, F. M., and Bull, D. R., *Australian J. Exp. Biol. and Med. Sc.*, 1943, **21,** 55.
12. Taylor, A. R., Sharp, D. G., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1943, **47,** 261.
13. Knight, C. A., data to be published.
14. Stanley, W. M., *J. Exp. Med.*, 1944, **79,** in press.