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A New Method for Concentrating Influenza Virus from Allantoic Fluid.

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In a recent communication from this laboratory[1] a number of different influenza vaccines were described, and their relative antigenicity in man was compared. The highest mean post-vaccination antibody levels were obtained with those preparations which because of concentration contained the greatest amounts of virus. The concentrated virus was obtained from allantoic fluid by centrifugation at 11,000 rpm for 2 hours. In the present paper another method of virus concentration is described, together with the data showing the antigenic potency of such concentrates when given to man.

Preparation of Virus Suspensions. All of the virus suspensions used, both for concentration experiments and for testing of sera, were prepared from the allantoic fluid of eggs infected with either the PR8 strain of influenza A virus[2] or the Lee strain of influenza B virus.[3] The method of preparation used was described in the previous report on the series of vaccinations.[1]

Virus titrations in mice, measurements of red cell agglutination titer, and agglutination inhibition antibody titrations in human sera were all done as described in the previous report.[1] The only change in the method was that the degree of agglutination in the red cell tests was determined by means of a photoelectric cell rather than by comparison with visual standards. In order that the antibody levels of the human sera in this series might be compared directly with those previously reported, the same standard ferret immune sera for each virus (PR8 and Lee) which had been used as controls in the former tests were again titrated. Seven duplicate titrations of each standard serum were distributed throughout the tests as a check on the uniformity of the technic.

We repeatedly observed that when allantoic fluid from 13-day-old chick embryos, both normal and infected, was frozen and thawed,

[1] Hirst, G. K., Rickard, E. R., Whitman, L., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1942, **75**, 495.

[2] Francis, T., Jr., *Science*, 1934, **80**, 457.

[3] Francis, T., Jr., *Science*, 1940, **92**, 405.

a fluffy white precipitate appeared in the suspension. This precipitate usually went back into solution when the fluid was warmed to 37°C. The amount of precipitate varied but was of the order of 0.2 to 0.5 mg per cc of fluid and consisted of both protein material and amorphous urates.

In addition, another type of precipitate occurred in allantoic fluid after prolonged storage at −10° or at 4°C. This precipitate was insoluble even when warmed above 37°C; it consisted mainly of urates. This insoluble precipitate did not possess the properties which will be described for the soluble sediment.

A suspension of PR8 virus in allantoic fluid, which had been stored for several weeks at −72°C, was thawed, and the precipitate which formed was removed by centrifugation at low speed (1000 rpm) before the temperature of the fluid had risen appreciably. The supernatant fluid was removed and the sediment resuspended in one-tenth the original volume, the supernatant fluid being used as diluent. Samples of this suspension were taken immediately after thawing. Together with the supernatant fluid and the resuspended sediment they were tested in mice for lethal virus titer and *in vitro* for agglutination titer. The results recorded in the first part of Table I show that approximately 90% of the virus originally present in the suspension was removed with the precipitate and that a large part of the removed virus could be accounted for by the titer obtained with the concentrated sediment. The *in vitro* and *in vivo* tests gave corresponding results. This experiment has been repeated a number of times, and 90 to 95% of the virus was usually carried down with the sediment, as judged from *in vitro* titrations.

Similar attempts to concentrate the virus with suspensions of the insoluble type of precipitate failed, and amorphous urates did not show the phenomenon. The factor responsible for bringing down the virus was soluble at 37°C.

When the same technic was applied to the concentration of the Lee strain, the results varied and usually only about one-half of the total virus was removed with the precipitate. It was found, however, that when a recently thawed suspension was changed from the usual pH of 8.0 to 6.6 or 6.7, a higher proportion of the virus was adsorbed on the precipitate. Separation of the sediment by low speed centrifugation at a pH of 6.7 usually removed 75% of the virus originally present. Reducing the pH to this level did not detectably destroy any of the virus activity. The results of concentrating a Lee virus suspension at pH 6.7 are given in Table I.

In order to test the antigenic potency of influenza virus concen-

TABLE I.
Concentration of Influenza Virus on the Precipitate of Freshly Thawed Allantoic Fluid.

Strain		Fluid before concentration	Suspension of precipitate concentrated 10 ×	Supernatant after removal of precipitate
PR8	50% mouse mortality titer	$10^{-4.1}$	$10^{-4.8}$	$10^{-3.2}$
	Agglutination titer	275	2900	26
Lee	50% mouse mortality titer	$10^{-3.6}$	$10^{-4.6}$	$10^{-2.7}$
	Agglutination titer	64	1024	14

trated in this way, the following vaccine was prepared and given to human beings.

Preparation of Vaccine 66. One liter of fluid containing PR8 virus was carefully thawed, and the precipitate which formed was removed by centrifugation at 1000 rpm for 10 minutes. The temperature of the fluid did not rise appreciably above 0° during this process. The sediment was resuspended in 100 cc of the supernatant fluid and formalin added to a final concentration of 0.4% formaldehyde. The suspension stood overnight at 4°C. One liter of fluid containing Lee virus was similarly treated, except that the pH was adjusted to 6.7 by the addition of acetic acid, before the precipitate was removed. Care was taken to keep the temperature of the suspension close to 0°C until the sediment and supernatant were separated. The precipitate was resuspended in 100 cc of supernatant and formaldehyde added to a final concentration of 0.4%. After 18 hours the two concentrates were combined and homogenized in a Waring mixer for 3 minutes. The mixed suspension was distributed in ampoules in 25 cc amounts, frozen, and dried.[4] Each ampoule was rehydrated with 50 cc of saline just before use. Each person received a subcutaneous injection of 2 cc, which contained 20 mg of dried material. On rehydration much of the dried material proved to be insoluble but formed a fine suspension which was administered without difficulty.

Virus titrations in mice on the tenfold concentrated suspensions before formalinization gave a 50% mortality titer of 10^{-6} for the PR8 suspension and $10^{-4.7}$ for the Lee suspension. The corresponding agglutination titers were 4000 and 2000, respectively. There was no detectable active virus in the finished vaccine.

This preparation was given to 100 individuals. Each person received the concentrate from 5 cc of PR8 and 5 cc of Lee allantoic fluid, and the number of 50% mouse mortality doses injected is

[4] Bauer, J. H., and Pickels, E. G., *J. Exp. Med.*, 1940, **71**, 83.

TABLE II.
Antibody Response of Human Beings to Injections of Influenza Viruses Prepared from Allantoic Fluid.

Strain	Vaccine No.	No. of 50% mouse mortality doses per person	Mean pre-vaccination antibody titer	Mean 2-wk post-vaccination antibody titer	No. individuals tested
PR8	55	362,000	168	461	140
	57	3,620,000	163	935	120
	66	10,000,000	114	1300	100
Lee	55	10,000	87	276	140
	57	40,000	84	443	120
	66	500,000	81	680	100

Vaccine 55, viruses unconcentrated and active.
Vaccine 57, PR8 virus concentrated tenfold and Lee virus concentrated fourfold by centrifugation. Viruses active.
Vaccine 66, viruses concentrated tenfold on precipitate; 0.4% formaldehyde added.

shown in Table II. The geometric mean of the pre- and postvaccination antibody levels is also given in this table. For purposes of direct comparison, similar data are given on Vaccines 55 and 57, which were described in the previous report.[1] Vaccine 55 contained unconcentrated active virus in allantoic fluid, while Vaccine 57 was made by concentrating the virus from infected fluid by high speed centrifugation (11,000 rpm for 2 hours). The virus in Vaccine 57 was also active.

From Table II it may be seen that the antibody levels following the injection of Vaccine 66 were higher for both the PR8 and the Lee strain than the corresponding mean values obtained with Vaccine 57. These higher levels were doubtless due in part to the larger amount of virus in Vaccine 66. One may safely conclude, however, that the two preparations elicited antigenic responses which were at least of the same order of magnitude, in spite of different methods of virus concentration and the fact that Vaccine 57 contained active virus while Vaccine 66 was formalinized. Vaccine 55 was included to emphasize again the wide disparity in the results obtained with concentrated and unconcentrated preparations. These results fortified our earlier conclusions[1] that the amount of virus injected is the most important single factor in determining the antibody level obtained and that inactivation by formaldehyde does not have a significantly adverse effect on the antigenicity of influenza virus for man.

The formation of a precipitate in freshly thawed allantoic fluid has been described, and it has been shown that under certain conditions a considerable amount of influenza virus may be adsorbed on this precipitate. This phenomenon provides a method for concentrating the virus from allantoic fluid on a large scale which is not

technically difficult and can be done with ordinary laboratory equipment. PR8 and Lee viruses were concentrated from allantoic fluid by this method, and human beings were inoculated with a formalinized concentrate. The mean antibody level of the subjects 2 weeks after vaccination was at least as high as that which followed the injection of similar amounts of active virus concentrated by centrifugation.

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Use of Biotin for Stimulating Growth of Nerve Tissue and Other Cells *in vitro*.

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A rapidly increasing number of data show that biotin is of widespread metabolic significance for a wide range of living things, and the fact that this vitamin markedly increases the growth rate of yeast cells and bacteria indicates that it might have similar effects on other plant or animal cells. Our present limited knowledge about many of the viruses, particularly those of the neurotropic group, is due in large part to the difficulties encountered in trying to grow them *in vitro*. In view of the important relationship between the metabolic state of cells and the ability of viruses to infect them,[1] experiments were devised to test the effects of biotin on various cell types, especially nerve tissue, in an effort to apply the data thus obtained for initiating or improving the growth of viruses and rickettsiae *in vitro*.

The method used for studying the effects of biotin on living cells was a slight modification of a technic previously described, using hanging drop cultures.[2] A piece of tissue was removed, preferably from the midline of an embryo, and divided into two equally-sized (and usually, bilateral) halves. One piece was explanted into a clot made up of plasma, embryonic extract, and biotin in Tyrode solution. A control culture was made of the other half in a clot consisting of the same proportions of plasma, extract, and plain Tyrode solution. Since the biotin (obtained from the S. M. A. Corporation) was furnished in alcoholic solution, it was necessary to add an

1 Plotz, H., *C. R. Soc. Biol.*, 1937, **125**, 603, 719.

2 Hamilton, H. L., *J. Exp. Zool.*, 1941, **88**, 275.