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Direct Isolation of Influenza Virus in Chick Embryos.

GEORGE K. HIRST.

From the Laboratories of the International Health Division of The Rockefeller Foundation, New York.

Primary isolation of influenza virus in chick embryos has become a useful technique in the past several years largely through the work of Burnet on the advantages of inoculation into the amniotic sac,[1] and through the use of hemagglutinins as a means of detecting the virus.[2,3] Recently, Rickard, Thigpen, and Crowley[4] have shown that influenza virus may be isolated by inoculation of untreated throat washings into the allantoic sac. With amniotic inoculation it has been necessary to filter the throat washing to prevent embryonic deaths from bacterial contamination, but in the work to be reported this step has been eliminated through the use of penicillin, and it will be shown that this change makes the chick embryo isolation procedure as sensitive as any now known.

Methods. Throat washings were obtained from influenza A patients in the 1943-44 epidemic, mainly in Army hospitals. The patients gargled with 15 cc of plain broth, which was then kept at a temperature of —72° C until tested. Five methods were used for the isolation or detection of influenza virus in the washings: (1) Penicillin was added to throat washings to a final concentration of 125 units per cc. This mixture was inoculated into the amniotic sacs of 13-day-old chick embryos, 0.2 cc per egg. After incubation at 35° C for 4 days the amniotic fluids were removed and tested for guinea pig and chicken cell hemagglutinins. (2) Throat washings were filtered through 700 mμ collodion membranes and the filtrates were inoculated into the amniotic sac of 13-day-old embryos, which were further treated as in (1). (3) Untreated washings were inoculated into the allantoic sac of 11-day-old chick embryos, and after 2 days' incubation at 37° C the allantoic fluids were removed, tested for hemagglutinins, and passed in series by the allantoic route for 5 passages.[4] (4) Penicillin, in a final concentration of 125 units per cc, was added to throat washings, which were further handled as in (3). (5) Ferrets were tested at 2 weeks for their antibody response to an intranasal inoculation of 1 cc of unfiltered throat washing.

For the amniotic inoculation both the method of Burnet[1] and a method described by Hirst[3] were used with equal success in respect to virus isolations. The former requires the making of a window while the latter does not. Whenever fluids were found that contained hemagglutinins, the material was passed in 10^{-3} dilution by the allantoic route, and 4 agglutinating units of virus (in allantoic fluid) were tested for inhibition by ferret immune sera (Lee and PR8). By this method all the strains isolated were shown to be influenza A. Ferret sera were tested by agglutination inhibition against the PR8 and Lee strains, and all the positive results indicated an infection with a strain of influenza A.

Results. During the past several years a search has been made for a chemical agent which would be bacteriostatic in eggs but which would not affect the viability of influenza virus. Such an agent was found in penicillin, 25 units of which was sufficient to inhibit the growth of bacteria introduced with throat washings into eggs. Parallel *in ovo*[5] titrations of stock strains of influenza virus in plain broth and broth containing 125 units of penicillin per cc showed no effect of the penicillin on the viability of the virus.

The published work on chick embryo iso-

1 Burnet, F. M., *Australian J. Exp. Biol. and Med. Sc.*, 1940, **18**, 353.

2 Burnet, F. M., Beveridge, W. I. B., Bull, D. B., and Clark, E., *Med. J. Australia*, 1942, **2**, 371.

3 Hirst, G. K., *J. Immunol.*, 1942, **45**, 293.

4 Rickard, E. R., Thigpen, M. P., and Crowley, J. H., *J. Immunol.*, 1944, **49**, 263.

5 Hirst, G. K., *J. Immunol.*, 1942, **45**, 285.

TABLE I.
Comparison of Different Methods of Detecting or Isolating Influenza Virus from Throat Washings.

		Method of detection				
	No. of throat washings	Throat washing plus penicillin into amniotic sac	Ferret immune response	Throat washing filtrate into amniotic sac	Throat washing plus penicillin into allantoic sac	Untreated throat washing into allantoic sac
	4	+	+	+	+	+
	4	+	+	+	+	—
	9	+	+	+	—	—
	13	+	+	—	—	—
	3	+	—	—	—	—
	12	—	—	—	—	—
	—	—	—	—	—	—
Total positive	33	33	30	17	8	4

lation of influenza virus shows marked discrepancies in the success of various methods and does not permit a valid comparison,[2-7] because throat washings vary so much in different epidemics. For this reason the 45 throat washings available were each tested for influenza virus by 5 different methods. The results of these tests are given in Table I.

There were 12 completely negative washings as tested by all methods. The inoculation of penicillin-treated washings into the amniotic sac was clearly the most sensitive method of virus detection, and no washings positive by any other method failed to yield virus by this technique on the first egg passage. Three washings yielded virus even though negative antibody responses were obtained in ferrets, a procedure which previously provided the most delicate test. The percentage of positive eggs with different washings varied from 15 to 100, with an average of 65. No throat washings were encountered in which penicillin failed to control bacterial growth. In confirmation of Burnet and Bull[8] it was found that guinea pig hemagglutinins usually (but not always) were present in higher titer than chicken cell agglutinins. In our experience 13-day embryos were vastly superior to 11-day embryos for obtaining positive results. Filtration of throat washings, as has been previously shown[3], removed detectable virus from about half the samples.

The allantoic route of inoculation was markedly less sensitive than any other method, yielding only 4 positive results with untreated washings; but it gave somewhat better results when penicillin was added to the inoculum. With the latter group all the positive results were obtained on the first passage. It is possible that the bacterial contamination that always occurs on the inoculation of untreated washings may have interfered (in 4 instances) with the propagation of the virus, since virus was detected in these washings by allantoic inoculation in the presence of penicillin. Allantoic takes occurred only with throat washings which also gave positive filtrates by the amniotic route, and it is fair to assume that the success of the allantoic method depends on the presence of a relatively high concentration of virus in the inoculum.

Acute and convalescent sera of the individuals from whom washings were obtained were tested in 35 instances. In 25 cases both serological and throat-washing results were positive, and in 7 cases both were negative. Two patients gave a positive serological response and negative throat washings, while 1 individual had a negative serological response but the washings did yield virus. There was agreement in the results of the 2 tests in 32 of 35 instances.

In summary, the allantoic route of inoculation was found to be advantageous for the chick embryo isolation of influenza virus chiefly because of its technical simplicity; however, the method fails to detect virus in many

6 Beveridge, W. I. B., Burnet, F. M., and Williams, S. E., *Australian J. Exp. Biol. and Med. Sc.*, 1944, **22**, 1.

7 Andrewes, C. H., and Glover, R. E., *Lancet*, 1944, **2**, 104.

8 Burnet, F. M., and Bull, D. B., *Australian J. Exp. Biol. and Med. Sc.*, 1943, **21**, 231.

known positive washings. While technically more difficult, the inoculation of penicillin and throat washing into the amniotic sac proved to be a highly satisfactory method of detecting influenza A virus and was the most sensitive of present-day methods in this experiment.

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Influence of Protein and Thiamine Intake on Pathologic and Weight Changes Produced by Atabrine.*

HENRY SIEGEL, CHARLES W. MUSHETT, AND GLADYS A. EMERSON.
From the Merck Institute for Therapeutic Research, Rahway, N.J.

The oral administration of atabrine produces necrosis and replacement fibrosis of the liver and myocardium in the rat.[1-3] A recent report[4] indicates that the toxic effects of atabrine upon the liver, as measured by an increase in plasma fibrinogen, can be largely prevented by a high protein-low fat diet. The present investigation was undertaken to determine the effect of the protein and thiamine content of the diet upon the hepatic and myocardial lesions in the rat which result from atabrine administration. The concomitant effect upon growth was also studied.

Experimental. Effect of Protein Intake. Young male rats of the Carworth strain were segregated into 8 groups of like average weight (123 g) and were maintained on high or low protein diets. In addition several of the groups received atabrine as indicated in Fig. 1. Four rats were employed in each of the control groups and 20 rats in each of the groups receiving atabrine.

The two experimental diets used had the following composition:

Low Protein—vitamin free casein, 6; dextrose, 78; hydrogenated vegetable fat, 8; cod liver oil, 2; mazola, 1; dried beef liver, 1; salt mixture USP XI No. 1, 4; supplemented with 0.8 mg each of thiamine, riboflavin, and pyridoxine; 8.0 mg each of nicotinamide and calcium pantothenate and 100 mg of choline chloride per 100 g of diet.

High Protein—This diet was identical with the low protein diet except that 24% of the dextrose was replaced by 24% casein, thus raising the casein content to 30% and lowering that of the dextrose to 54%.

The rats were allowed to equilibrate on these diets for a period of one week prior to dosing with atabrine. All animals receiving the drug were dosed daily by stomach tube with 45 mg per kg (5% of the L.D. 50). The experiment was terminated after 48 days (41 days of atabrine administration), at which time the rats on the low protein diet were in a debilitated condition.

At autopsy the degree of hepatic damage was estimated grossly by the amount of the entire liver which was necrotic. The heart, after fixation in 4% formaldehyde solution, was cut in half by frontal section. Histologic sections were made parallel to the cut surfaces of both halves and stained with hematoxylin-eosin. The degree of myocardial damage was graded as slight or moderate. Rats with "slight" involvement had only an occasional focus of necrosis; in those with "moderate" involvement several areas of the myocardium were affected.

Atabrine administration had a marked depressing effect upon weight as evidenced by the difference in the growth of rats in Groups

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1 Wright, C. I., and Lillie, R. D., *Public Health Rep.*, 1943, **58**, 1242.

2 Fitzhugh, O. G., and Nelson, A. A., *Fed. Proc.*, 1944, **3**, 72.

3 Siegel, H., and Mushett, C. W., *Arch. Path.*, 1944, **38**, 63.

4 Scudi, J. V., and Hamlin, M. T., *J. Pharm. and Exp. Therap.*, 1944, **80**, 150.