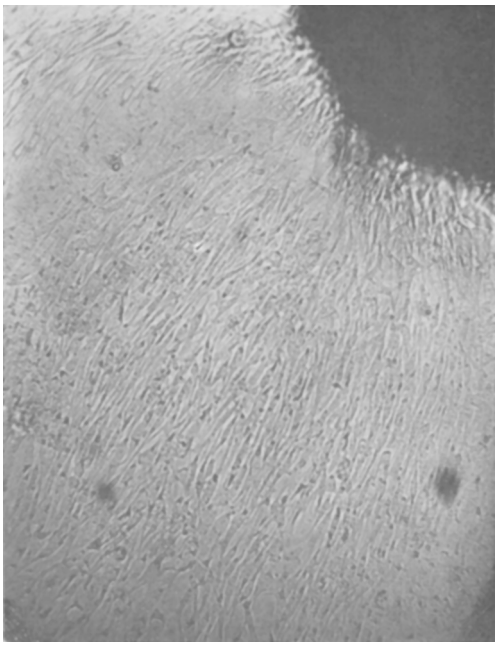

FIG. 1.
Photomicrograph of a living culture of chick embryo heart tissue cultured in embryonic juice and + amniotic fluid (1:1), all obtained from the same embryo.

and the remaining embryonic tissue provides the embryonic juice. The culture medium consists of embryonic juice and amniotic fluid 1:1. The excess of the amniotic fluid may be used for all purposes where saline or other salt mixtures are usually used, *e.g.*, washing of tissue, etc. The growth of cultures in this medium is not inferior to the growth of cultures in other fluid media. The cells are as normal in appearance as cells from a plasma culture (Fig. 1). Amniotic fluid not only replaces but also, in many regards, surpasses Tyrode's and other salt-glucose solutions in that it does not require sterilizing, filtering, or pH-adjusting. This liquid medium method may be recommended for cultures where the primary medium is to be removed for experimental purposes and replaced by other media. It is also convenient for obtaining growth of single cell layers for investigation in dark-field illumination, in phase contrast microscopy and in electron microscopy.

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17230. A Method of Obtaining Influenza Virus Growth Curves in Individual Eggs.*

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Growth curves of influenza virus propagated in the allantoic sac of embryonated eggs have been described by several investigators.[1-6] However, most of the studies reported have been based on results obtained with the pooled allantoic fluids of groups of eggs sacrificed at various intervals of time following inoculation of virus. Relatively little data concerning the characteristics of such curves in individual eggs are available. The use of pooled fluids has yielded valuable information as to the average rate and extent of virus multiplication and, with the aid of special technics, the step-like nature of the curves.[4-6] Growth curves in individual eggs should provide similar information, in addition to presenting a continuous picture of the growth curve in each egg. Recently Hoyle[5] has described growth curves of influenza A virus

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1 Miller, G. L., *J. Exp. Med.*, 1944, **79**, 173.

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3 Ziegler, J. E., Jr., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1944, **79**, 361.

4 Henle, W., Henle, G., and Rosenberg, E. B., *J. Exp. Med.*, 1947, **86**, 423.

5 Hoyle, L., *Brit. J. Exp. Path.*, 1948, **29**, 390.

6 Henle, W., and Rosenberg, E. B., *J. Exp. Med.*, 1949, **89**, 279.

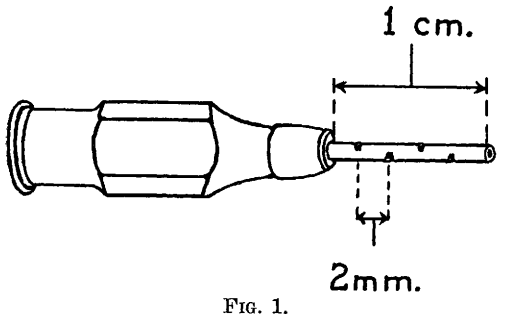

FIG. 1.
Needle devised for aspirating allantoic fluid.

in individual eggs from which allantoic fluid was removed at hourly intervals. However, no details of the technic used for sampling were given by him. The present paper describes a method by which individual growth curves may be obtained.

Materials and methods. Samples of allantoic fluid were aspirated through needles especially devised for this purpose. Aspirating needles were made from ordinary 20 gauge "hypodermic" needles by cutting off the ends and filing 4 holes in the sides of the short lengths of needle remaining. As illustrated in Fig. 1, the length of the needle, from attachment to the hub to the tip, is one centimeter. The holes along the shaft are spaced alternately on opposite sides, 2 mm apart, the first hole being 2 mm from the tip and the fourth 2 mm from the hub. A needle of this type was made readily, in 5 to 10 minutes, with the aid of the flat metal file commonly used to remove the tips of glass ampoules. However, the filing could be done more efficiently by means of a dental drill fitted with a flat cutting disc. During the process of filing, the needle was held firmly by attaching it to a small syringe equipped with a "Luer-Lok" device, and a stylet was inserted before making the side holes.

Needles somewhat similar to those described above have been used previously, especially for flushing the allantoic sac,[7] and for collecting large amounts of allantoic fluid.[8]

[7] Henle, W., and Henle, G., *Am. J. Med. Sci.*, 1944, **207**, 717.

[8] Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, D., and Beard, J. W., *J. Immunol.*, 1945, **50**, 291.

Preparatory to inoculation of virus, each egg was candled and a mark was made on the side of the shell in the vicinity of the embryo, directly over an area of chorio-allantoic membrane where no large blood vessels were seen. A hole of about the same size as a cross section of the needle was then drilled through the shell at the designated mark. The hole used for inoculation of virus, as a rule, was used for subsequent aspirations. After inoculation and after each aspiration the holes were sealed with a drop of paraffin-bee's wax mixture. Prior to aspiration the eggs were candled to determine viability; the wax seals were removed with a hot metal spatula and the areas about the holes cleansed with alcohol. After attachment to a syringe of suitable capacity the needle was slowly inserted to the hilt and suction applied by withdrawing the plunger. At each aspiration exactly 0.2 cc of allantoic fluid was withdrawn and added to 0.8 cc of 0.85% NaCl. Serial two-fold dilutions were then made in saline in 0.5 cc amounts. To each tube 0.25 cc of 1% chicken RBC was added and readings were made after the cells had settled out, on standing at room temperature for about an hour. The end-point was taken as the highest dilution of allantoic fluid in which a pattern characteristic of complete or ++++ agglutination occurred. The PR8 strain of influenza A virus was used exclusively.

Experimental. Groups of 10 to 20 embryonated hens' eggs, on the tenth day of incubation, were inoculated with influenza virus via the allantoic sac. As a rule, the inoculum consisted of approximately 100 ID_{50} in a volume of 0.05 cc. The eggs were subsequently incubated at 37°C. Chilling was not done, and the eggs were removed from the incubator only for the brief periods of time required for each aspiration. When small inocula of virus were used, the first aspiration was done at 18 hours after inoculation because preliminary experiments had shown that hemagglutinins usually could not be demonstrated before that time. Repeated aspirations were then done at arbitrarily chosen intervals as long as the embryos survived.

In Fig. 2 are shown the curves, representing

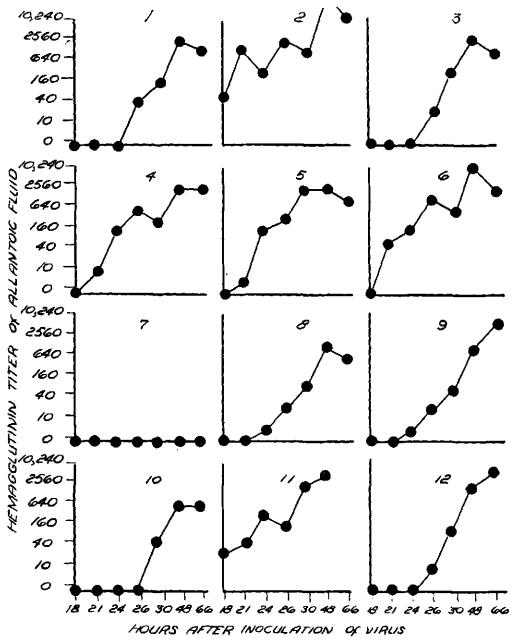

FIG. 2.

Growth curves, based on hemagglutination titers, of the PR 8 strain of influenza virus in individual eggs. Results are those obtained in a single experiment, in which each of 12 eggs received an inoculum of approximately 100 ID_{50} of virus.

the serial hemagglutination titers in individual eggs, obtained in an experiment in which each of a group of 12 eggs received a standard inoculum of about 100 ID_{50} of virus. While some of the curves are quite similar, others show obvious differences with respect to time of appearance of detectable hemagglutinin, slope of curve and maximum titer of hemagglutinin. Moreover, as previously demonstrated by Henle[4,6] and Hoyle,[5] several of the eggs, especially numbers 2, 4, 6 and 11, show step-like increases in the amount of virus. The fact that egg number 7 never became positive may have been due to failure to inoculate it; however, in other similar experiments, an occasional egg has shown an even longer lag phase, hemagglutinins first making their appearance some 90 hours or more after inoculation. All embryos in this experiment were alive 48 hours after inoculation of virus but when candled just prior to the 66 hour aspiration all except number 10 were found to be dead. In most similar experiments a rather high mortality has occurred somewhat earlier.

Comment. The method described is technically easy; relatively small numbers of eggs are required, and the growth curves obtained are individual curves rather than those based on group sampling. Furthermore, this method provides a suitable way of studying the differences between curves in untreated eggs and those in eggs treated with various substances which inhibit the multiplication of influenza virus. However, a high mortality rate usually occurs and about 50% of the eggs die before satisfactory growth curves are obtained. Mortality varies considerably from one experiment to another and apparently depends upon a number of factors which have not been investigated fully. Only a small percentage of the eggs become contaminated with bacteria. In most instances aspiration yields clear allantoic fluid, but occasionally bloody fluid or yolk is obtained. A higher mortality and greater difficulty in aspiration are most likely to be encountered with eggs which have been incubated for only 10 days or less.

Summary. With the aid of an easily constructed needle a method has been devised for obtaining growth curves of influenza virus in individual embryonated eggs.

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