17307. A Chemical Method for the Detection of Virus Infection of the Chick Embryo.

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Chemical or physical differences in the allantoic fluid of normal and virus-infected chick embryos have been emphasized by few investigators. McLean *et al.*[1] mentioned differences in the pH of normal and influenza virus-infected allantoic fluids. Parodi and his collaborators[2] reported a slower decline in pH and an increase in volume of the allantoic fluid from embryos infected with influenza virus. In studies with their common cold virus (MR-1) Atlas and Hottle[3] noted high absorption peaks with dialyzed infected allantoic fluid which were attributed to protein. Such peaks were sometimes observed with normal fluid. In view of such observations, it appeared possible that there might be sufficient chemical difference in virus-infected and normal allantoic fluid to permit the development of a chemical test for virus infection of the chick embryo.

Early in the course of a systematic investigation of the properties of infected and normal allantoic fluid, it was discovered that allantoic fluid from embryos infected with influenza virus contained appreciably greater quantities of protein. Accordingly, a simple quantitative method for the determination of protein in allantoic fluid was devised and subsequenty utilized in studies of infection of the allantoic sac with various viruses.

Materials and methods. Allantoic fluid. Allantoic fluids used in turbidity determinations were carefully harvested from 10-12-day-old White Leghorn embryos previously chilled for 12-18 hours at 4°C. Grossly bloody fluids and those inadvertently contaminated by yolk were discarded. Groups of 5-6 embryos were employed.

Viruses. The PR8 and Lee strains of influenza virus and a strain of Newcastle disease virus adapted to the allantoic sac by serial passage were used. The Habel strain of mumps virus, adapted in this laboratory to the allantoic sac, was also utilized. Semliki Forest virus (SFV) in the form of desiccated mouse brain (110th passage) was obtained through the courtesy of Dr. K. C. Smithburn, who had demonstrated[4] rapid multiplication of the virus in the chick embryo. This mouse brain suspension killed embryos within 24-36 hours when injected into the allantoic sac. Subsequent passages were made with allantoic fluid.

Control materials. Allantoic fluids, hereinafter referred to as normal, were obtained from embryos inoculated with normal or heated (65°C for 30 minutes) allantoic fluid diluted 1:10 to 1:1,000 in 0.85% sodium chloride solution buffered to pH 7.2 with phosphate. The same diluent was used for virus inocula.

Turbidity determination. One cc of 10% trichloracetic acid is added to 1 cc of allantoic fluid in a soft glass test tube measuring 100 × 10 mm. Reagents are measured with ordinary serologic pipettes. Two to 5 minutes after the addition of acid, turbidity readings are determined in a Klett-Summerson colorimeter. An adapter for the small test tube and a blue filter (peak transmittance, 420 mμ) are employed. Mixing of reagents is accomplished by inversion of the colorimeter tube. A blank of 10% trichloracetic acid is used for preliminary setting of the zero reading of the colorimeter. In the instrument used in this study a 0.03% suspension of barium sulfate gives a reading of 70. Turbidity is expressed directly in the units comprising the scale of the colorimeter, these units being directly proportional to optical density. Fifteen serial determinations of

[1] McLean, I. W., Jr., Cooper, G. K., Taylor, A. R., Beard, D., and Beard, J. W., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 192.

[2] Parodi, A. S., Lajmanovich, S., Pennimpede, F., and Mittelman, N., *J. Immunol.*, 1948, **58**, 109.

[3] Atlas, L., and Hottle, G., *Science*, 1948, **108**, 743.

[4] Smithburn, K. C., *J. Immunol.*, 1946, **52**, 309.

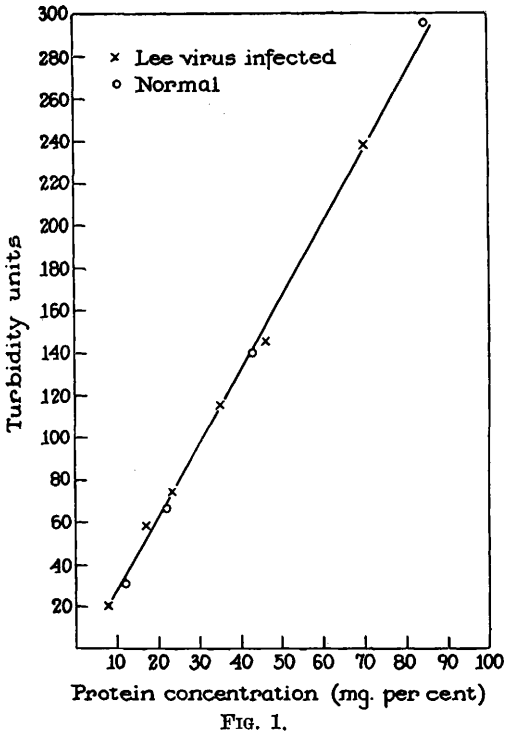

FIG. 1.

Relation between turbidity and protein concentration in allantoic fluid. Turbidity was produced with 10% trichloracetic acid. Protein N_2 was determined by micro-Kjeldahl.

turbidity, using the same allantoic fluid pool, showed an experimental error of ± 2.8% for the procedure as described above.

Experimental. Studies of dialyzed allantoic fluids revealed a substance precipitable by 10% trichloracetic acid to be present in both normal and influenza virus-infected fluids and greatly increased in infected fluids. Similarly, micro-Kjeldahl determinations of total nitrogen demonstrated a higher concentration of nitrogen in fluid from infected embryos. The linear relation of the turbidity with trichloracetic acid and protein concentration of both normal and infected allantoic fluids may be seen in Fig. 1 in which the turbidities of varying dilutions of concentrated dialyzed allantoic fluids are plotted against the protein concentrations of the fluids as determined by the micro-Kjeldahl method. This relationship was found to hold with concentrations up to 500 turbidity units. However, specimens giving readings above 300 were diluted and re-examined because of difficulties experienced in obtained accurate scale readings in the higher range. A similar straight line relationship between serum protein concentration and turbidity produced by trichloracetic acid has been established within certain limits of concentration by Chow *et al.*[5]

Further study of the acid-precipitable substance in allantoic fluid has indicated its protein nature. Dialysis in cellophane against 0.85% saline did not reduce the concentration of the substance. The characteristic biuret, xanthoproteic, and ninhydrin color reactions were given by dialyzed allantoic fluids. Precipitates were formed in dialyzed and non-dialyzed fluids by the addition of 95% ethyl alcohol or ammonium sulfate. The antigenicity of the substance, which is discussed below, is further evidence for its protein nature.

Fractionation of dialyzed allantoic fluids by half and full saturation with ammonium sulfate was performed. The protein concentration of these fractions was then determined by micro-Kjeldahl analysis after redialysis against saline. The albumin-globulin ratios in normal and infected fluids did not

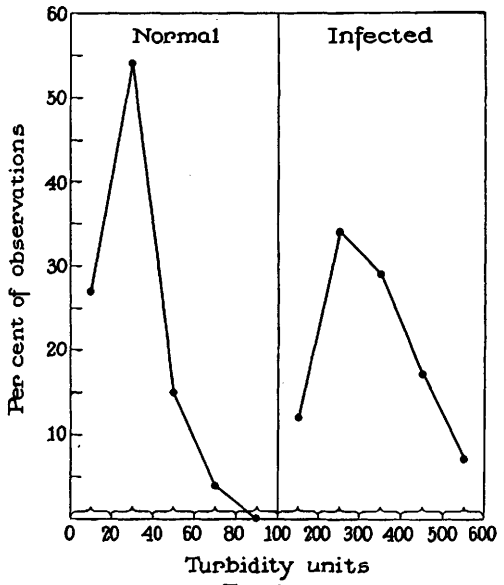

FIG. 2.

Frequency distribution of turbidity values with normal and Lee virus infected allantoic fluids.

[5] Chow, B. F., Hall, L., Duffy, B. J., and Alper, C., *J. Lab. and Clin. Med.*, 1948, **33**, 1440.

TABLE I.
Increase in Allantoic Fluid Protein with Various Viruses.

Virus 10^{-3} dilution inoculated	Time after inoculation, days	No. of eggs	Allantoic fluid turbidity†	
			Range	Mean
Lee	2	41	118-540	316
SFV*	1-1½	17	40-300	119
NDV	2	20	52-260	104
PR8	2	12	30-152	87
MV	4-5	12	56-160	84
Control	2	100	8-75	29
"	4-5	10	21-50	33

* 10^{-1} to 10^{-3} dilutions used.
† Turbidity developed with 10% trichloracetic acid.

differ materially, being 7.3/1 in normal and 8/1 in infected fluids. The addition of trichloracetic acid to fractions obtained by ammonium sulfate precipitation produced turbidity equivalent to the protein nitrogen concentration. Ultraviolet absorption curves obtained with the Beckman spectrophotometer disclosed minima of 252 and 290 mμ and maxima of 265 mμ with both dialyzed normal and infected fluids of equal protein concentration (40 mg %). Absorption in this range is characteristic of proteins.

Electrophoretic studies* were made of dialyzed normal and infected allantoic fluids. Normal fluid was concentrated 23-fold, and infected fluid 9-fold, resulting in protein concentrations of 375 and 300 mg %, respectively. These concentrations proved insufficient for accurate analysis of mobilities or sharp delineations of peaks; however, the rate of boundary migration and the degree of boundary spreading were consistent with what would be expected with protein solutions containing several components. With both normal and infected fluids at least 3 peaks were clearly discernible.

Comparison of normal and infected allantoic fluid protein concentrations. Studies of uninfected embryos demonstrated low concentrations of protein in the allantoic fluid as measured by turbidity produced with trichloracetic acid. In embryos of 10-12 days of age turbidity values varied from 8 to 75, representing protein concentrations of 2.4 to 23 mg % with an average value of 8.7 mg %. The frequency distribution of turbidity readings of allantoic fluids from 100 normal embryos is charted in Fig. 2 and compared with the range of turbidity (*i.e.* protein concentration) found in fluids from 41 embryos infected with the Lee strain of influenza virus. Turbidity readings of the infected allantoic fluids ranged from 118 to 540 (*c.f.* Table I), indicating concentrations of 35.4 to 162 mg % of protein.

Lee-infected allantoic fluid was subjected to differential centrifugation to determine to what extent sedimentable substances contributed to turbidity produced with trichloracetic acid. Low speed centrifugation (3,000 r.p.m. for 10 minutes) caused a 12% reduction in the turbidity observed initially, suggesting the presence of considerable cellular material. Total cell counts disclosed an average of 560 cells/cu mm in Lee-infected allantoic fluid contrasted with an average of 93 cells/cu mm in normal fluid. Further centrifugation at 37,900 g for 30 minutes resulted in a further reduction of the turbidity produced with acid amounting to 8% of the turbidity originally present. Thus, Lee virus, itself, contributes little, if any, to the turbidity of infected allantoic fluid, as this amount of centrifugation leaves less than 1% of the virus in the supernate.

Source of protein in infected allantoic fluid. It appeared likely that the increased protein in the allantoic fluid of virus-infected embryos was attributable to host reaction and perhaps to destruction of host tissue, especially in

* Electrophoretic analysis was kindly carried out by Dr. Gertrude E. Perlmann, The Rockefeller Institute, New York City.

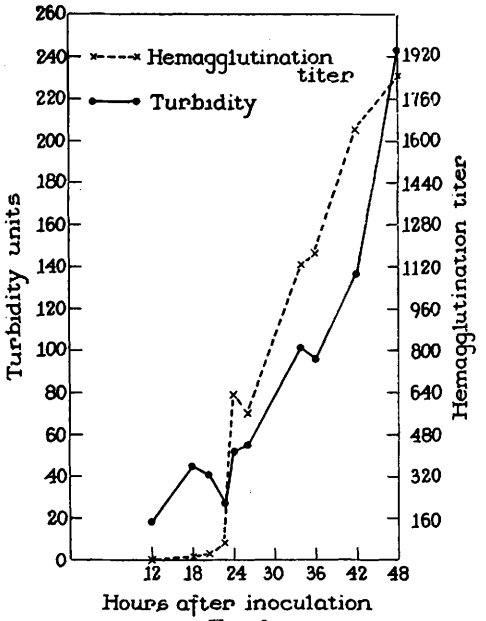

FIG. 3.
Temporal relation between increase in turbidity and Lee virus concentration in allantoic fluid.

view of the increased number of cells in infected fluid. Evidence cited above demonstrated that the virus itself did not contribute to the turbidity produced with acid, and further studies have shown no direct relation between virus and protein concentrations. Moreover, experiments with Lee virus demonstrated a difference in the rate of virus multiplication and the increase in allantoic fluid turbidity as is shown graphically in Fig. 3. Corroboration of this difference was obtained in experiments in which the time required to reach maximal virus concentration was varied by the use of inocula of differing dilutions of virus. The results are presented in Table II.

The protein of infected allantoic fluid did not differ immunologically from the protein normally present in allantoic fluid. Sera from rabbits injected intravenously with dialyzed infected or normal allantoic fluid contained antibodies capable of forming precipitates with concentrated allantoic fluid from either infected or normal embryos. These antibodies were absorbed from either antiserum by normal or infected allantoic fluid antigen, as well as by a suspension of normal chorioallantoic membrane (C.A.M.). These data are summarized in Table III.

Viruses which cause an increase in allantoic fluid protein. The Lee and PR8 strains of influenza virus, Semliki Forest virus, Newcastle disease virus, and mumps virus consistently caused an increase in allantoic fluid protein during the course of infection of the allantoic sac. A correlation may be drawn between the toxicity of the viruses studied and the degree of protein increase. Lee and Newcastle disease viruses, which killed embryos after 48 hours, and Semliki Forest virus, which killed even sooner, caused significantly more turbidity in allantoic fluid than did the considerably less toxic PR8 and mumps strains, as is shown in Table I.

Nonviral causes of protein increase in allantoic fluid. The production of the turbidity reaction by 5 different viruses is evidence of its non-specificity. It is obvious that any reaction dependent upon response or destruction of host tissue may be induced by chemical or physical agents as well as infectious ones. Thus, it was found that injection of 0.1 cc quantities of broth or serum might increase the allantoic fluid protein of embryos beyond the low concentrations usually seen. Such increases were greater than would be anticipated on the basis of the amount of protein injected, demonstrating an actual reaction of the embryonic tissue to the material introduced.

When broth was injected in control embryos, mean turbidity values were almost double (*i.e.* 48 units) those observed in saline inoculated embryos, and occasional fluids exceeded 100 turbidity units. The injection of undiluted allantoic fluid occasioned similar non-specific response, although it was of lesser degree (mean turbidity, 38 units), and only 10% of individual fluids exceeded the upper limit of 75 turbidity units observed in normal embryos (Table I).

The effect of bacterial infection of the chick embryo was not systematically studied, but examination of bacterially contaminated allantoic fluids disclosed increased turbidity, *i.e.* more than 75 units, in only 2 of 16 instances. In any event, such fluids are unsuitable for virus study, and are customarily discarded.

In normal embryos incubated for more

TABLE II.
Relation of Increase in Allantoic Fluid Turbidity to Extent of Multiplication of Lee Virus.

Virus dilution inoculated	Time after inoculation: 24 hr Hem. titer*	24 hr Turb.†	36 hr Hem. titer	36 hr Turb.	48 hr Hem. titer	48 hr Turb.
10^{-3}	1:636	53	1:1331	96	1:1843	243
10^{-5}	0	28	1:1536	93	1:2048	218
10^{-7}	0	21	1:65	42	1:1229	145

* Mean hemagglutination titer of allantoic fluids.
† Mean turbidity value of allantoic fluids.

TABLE III.
Absorption Experiments with Antisera Against Normal and Infected Allantoic Fluid Protein.

Rabbit serum	Absorbed with	Precipitin titer* Normal all. fl. prot.	Precipitin titer* Infected all. fl. prot.
Anti normal all. fl. prot.	—	1:250	1:250
" " " " "	Infected all. fl. prot.	0	0
Anti infected all. fl. prot.	—	1:250	1:250
" " " " "	Normal all. fl. prot.	0	0
" " " " "	Normal C.A.M.	0	0

* Highest dilution of antigen which gave a positive reaction with serum diluted 1:2.

TABLE IV.
Prevention of Protein Increase in Allantoic Fluid by Virus Antiserum.

Inoculum: Serum	Inoculum: Lee virus dilution	Mean hemagglutination titer of allantoic fluids	Mean turbidity value of allantoic fluids
—	10^{-6}	1:1877	150
Normal 1:100	"	1:2046	93
" 1:500	"	1:2253	188
Anti Lee 1:100	"	0	25
" " 1:500	"	1:2	19
" " 1:100	—	—	20
" " 1:500	—	—	42

than 12 days, sharp, capricious increases in the allantoic fluid protein may occur, making such embryos unsuitable for use with the present method. Fluids grossly contaminated by blood introduced at the time of harvest also proved useless because of the presence of extraneous serum protein.

Prevention of protein increase by virus antiserum. Neutralization of Lee virus by specific immune rabbit serum prevented the increase in allantoic fluid protein which occurred following the injection of virus and normal serum or virus alone. The results are summarized in Table IV. It will be seen that, in the dilutions indicated, inactivated rabbit serum *per se* caused no undue elevation of mean turbidity. In other experiments dilutions of rabbit serum as low as 1:40 were used without increase in allantoic fluid protein. This experiment affords definitive evidence that the protein increase which follows introduction of virus into the allantoic sac is a corollary of virus multiplication.

Discussion. The study of many animal viruses and attempts to recover new viruses have been handicapped by the lack of simple

in vitro technics comparable to the hemagglutination reaction. The method outlined in the present communication has the virtue of simplicity, and in theory may be of value in the detection of any virus capable of multiplication in the cells of the allantoic sac of the chick embryo. Because the method is dependent upon the vagaries of host response, it is unsuitable for the direct measurement of virus concentration. However, both specificity and quantitation may be obtained by the employment of immune serum. It is conceivable that a virus might be recovered and identified immunologically as the etiological agent of a disease solely by chemical evidence of infection of the chick embryo.

Summary. A method is described for the detection of virus infection of the allantoic sac of the chick embryo. The method is dependent upon the increased concentration of protein in infected allantoic fluid. Protein concentration is measured by determining the degree of turbidity produced upon the addition of 10% trichloracetic acid to allantoic fluid.

While this article was in press, Polson and Dent (*Nature*, 1949, **164**, 233) described increases in the protein concentration of allantoic fluid from eggs infected with lumpy skin disease virus or blue tongue virus.

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17308. Hemagglutination with the GDVII Strain of Mouse Encephalomyelitis Virus.

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The capacity of certain viruses to cause agglutination of erythrocytes[1] has permitted the development of *in vitro* procedures which have greatly facilitated investigative and diagnostic work with these agents. Although at least 10 different animal viruses are known to cause hemagglutination (pertinent data have been summarized recently),[2] there is almost no evidence indicating that any of the neurotropic viruses possesses a similar capacity. Recently, however, Bremer and Mutsaars[3] stated that the Lansing strain of poliomyelitis virus caused agglutination of sheep RBC, and Hallauer[4] stated that Columbia SK and Columbia MM viruses also agglutinated sheep RBC. We have been unable to confirm the results reported for the Lansing strain, as too have other workers.[5] However, in the accompanying paper Olitsky and Yager[5] have confirmed and extended the results reported for SK and MM viruses.

The present study was concerned chiefly with the GDVII strain of mouse encephalomyelitis virus as well as with the FA strain.[6] In addition, the Lansing, MEF1, and Brunhilde strains of poliomyelitis virus were investigated. It will be demonstrated that the GDVII strain causes agglutination of human RBC at 4°C, but not at 23 or 37°C; that such hemagglutination is inhibited by homologous immune serum, and by anti-FA virus serum, but not by antiserum against other viruses. No evidence of hemagglutination could be obtained with the FA strain nor with any of the strains of poliomyelitis virus which were employed.

Materials and methods. Viruses. The

* Aided by a Fellowship from the National Foundation for Infantile Paralysis.

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3 Bremer, A., and Mutsaars, W., *Compt. rend. Soc. Biol.*, 1948, **142**, 1192.

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5 Olitsky, P. K., and Yager, R. H., accompanying paper.

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