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.

O. LAHELLE* AND FRANK L. HORSFALL, JR.

From the Hospital of The Rockefeller Institute for Medical Research, New York City.

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

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2 Smadel, J. E., Viral and Rickettsial Infections of Man, 1948, chap. 3, J. B. Lippincott Co., Philadelphia, Pa.

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

4 Hallauer, C., 4th Internat. Cong. Microbiol., July 20-26, 1947, Copenhagen, 1949, p. 257.

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GDVII strain was obtained from Dr. Max Theiler, I.H.D. Laboratories, The Rockefeller Foundation, New York City. Three FA strains were used; one obtained from Dr. Theiler and 2 obtained from Dr. J. Melnick, Yale University, New Haven, Conn. Three poliomyelitis virus strains were employed; the Brunhilde strain was kindly supplied by Dr. D. Bodian, The Johns Hopkins University, Baltimore, Md., and the Lansing and MEF1 strains were obtained from Dr. P. K. Olitsky, The Rockefeller Institute, New York City. The Brunhilde strain was contained in infected monkey spinal cord. The other viruses were maintained by occasional intracerebral passage in mice. Brains were removed from exsanguinated mice shortly after the appearance of signs indicating infection of the central nervous system. Ten per cent brain suspensions were prepared with 0.01 *M* phosphate buffer at pH 7.2. The suspensions were ground for 2½ minutes in a modified Waring Blendor which was cooled with ice and then were centrifuged for 15 minutes at 7,760 g. The supernates were employed either promptly after preparation or following storage at −70°C, sometimes for as long as 14 days. Virus titrations were performed by the intracerebral technic using serial 10-fold dilutions in 10% normal rabbit serum saline. A group of 5 or 6 mice was used for each dilution and the 50% infectivity end point, LD_{50}, was calculated in the usual manner.

Hemagglutination technic. Hemagglutination titrations were carried out in a manner similar to that employed with influenza virus.[1] Serial 2-fold dilutions of brain suspensions in saline buffered at pH 7.2 and a final concentration of 0.25% human Group O erythrocytes were employed. With the GDVII strain the mixtures were held at 4°C for 2 hours. Readings were recorded in the usual manner and the end point was taken as the highest dilution which gave a 2+ reaction.

Hemagglutination-inhibition technic. Antibody titrations were carried out with serial 2-fold dilutions of inactivated (56°C/30 min.) sera in buffered saline and a constant amount of virus, usually 16 hemagglutinating units. A final concentration of 0.25% RBC

TABLE I.
Hemagglutination with GDVII Strain of Mouse Encephalomyelitis Virus.

Supernate of mouse brain suspension	Human group O erythrocytes, %	Held 2 hr at °C	Final dilution of supernate									
			125	250	500	1000	2000	4000	8000	16,000	32,000	64,000
GDVII strain	0.25	4	4*	4	4	4	4	4	3	1	±	0
,,	0.25	23	0	0	0	0	0					
,, ,,	0.25	37	0	0	0	0	0					
,, ,,	2.5	4	4	4	2	0	0	0	0	0	0	0
,, ,,	1.0	4	4	4	4	3	2	1	0	0	0	0
,, ,,	0.5	4	4	4	4	4	3	2	1	±	0	0
,, ,,	0.25	4	4	4	4	4	4	4	3	1	±	0
FA ,,	0.25	4	0	0	0	0	0					
Poliomyelitis, Lansing	0.25	4	0	0	0	0	0					
,, MEF1	0.25	4	0	0	0	0	0					
Normal, control	0.25	4	0	0	0	0	0					

* Indicates degree of hemagglutination.

was used and readings were made after 2 hours at 4°C. The end point was taken as the highest dilution of serum which completely inhibited hemagglutination.

Immune sera. Through the courtesy of Dr. P. K. Olitsky immune sera against a large number of different neurotropic viruses were made available. In most instances the sera were obtained from rabbits which had been repeatedly injected intraperitoneally or subcutaneously with infected mouse brain. In some instances sera were also obtained from immunized guinea pigs, mice or monkeys. Immune sera and control normal sera usually were stored at –30°C.

Hemagglutination with GDVII virus. Positive results were obtained in hemagglutination experiments with the GDVII strain when (a) suspensions of infected mouse brain, (b) human Group O erythrocytes, and (c) a reaction temperature of 4°C were employed. The results of typical experiments are shown in Table I. High titers ranging from 1:2,000 to 1:16,000 or more were commonly obtained with 0.25% RBC. In general, the hemagglutination titer was inversely proportional to the concentration of RBC, as is the case also with influenza virus.[7] Hemagglutination occurred only if the mixtures were cold (4°C), disappeared rapidly when cold mixtures were warmed either at room temperature (23°C) or at 37°C, and reappeared when the mixtures were again cooled to 4°C. The reaction developed relatively slowly and, although clear evidence of hemagglutination was present at one hour, more definite agglutination was present at 2 hours. The pattern of agglutinated cells was closely similar to that observed with human RBC and either influenza or mumps virus.

Despite numerous attempts employing a wide range of experimental conditions, it was not possible to obtain evidence of hemagglutination with the FA strain nor with the Lansing, MEF1 or Brunhilde strains of poliomyelitis virus. In addition to human RBC, erythrocytes from the following species were used: monkey, horse, sheep, cat, dog, guinea pig, hamster, mouse and chicken. The GDVII strain was incapable of causing agglutination of any RBC other than those derived from man. Supernates of normal mouse brain suspensions, prepared as described above, did not cause agglutination of human RBC at dilutions greater than 1:4. As is pointed out also in the accompanying paper,[5] erythrocytes of certain species, *e.g.*, hamster, dog, cat and guinea pig, as well as the mouse, commonly showed agglutination when mixed with normal mouse brain suspensions.

The agglutination of human RBC which is caused by GDVII virus in the cold disappears after a few minutes at room temperature. Because of this, both the reaction and readings of titrations are best carried out in the cold room. As is shown below, agglutination is associated with adsorption of the virus to RBC and the dispersal of the agglutinated cells is associated with elution of the virus from them. Successive cycles of adsorption and elution, dependent merely on changes in temperature, can be repeated at will with a single mixture of RBC and GDVII virus. Four such cycles have been carried out.

Hemagglutination-inhibition with immune serum. Agglutination of human RBC with GDVII virus in the cold was prevented by high dilutions of anti-GDVII serum as well as anti-FA serum but not by immune serum against other viruses. The results of typical experiments are shown in Table II. Hemagglutination-inhibition titers ranging from 1:4,000 to 1:16,000 were obtained commonly with anti-GDVII serum and similar high titers were obtained also with anti-FA serum. Normal serum usually caused some non-specific inhibition; with rabbit and guinea pig serum titers of 1:32 or lower were commonly found; with human and mouse serum titers as high as 1:128 were encountered. In some instances heating at 56°C for 30 minutes reduced the degree of non-specific inhibition. It should be emphasized that the injection of mouse brain suspensions into animals other than mice commonly results in the development of antibodies which cause agglutination of human RBC. Usually the agglutination titer of such sera is not greater than 1:128 but in occasional instances it may be considerably higher. With immune serum agglutination of RBC occurs not only at 4°C but also

[7] Whitman, L., *J. Immunol.*, 1947, **56**, 167.

TABLE II.
Inhibition of Hemagglutination with GDVII Virus by Immune Serum.

Serum		GDVII virus units	Held 2 hr at °C	Serum hemagglut. inhibition titer
Immune vs.	Species			
Normal m.br.*	Rabbit	16	4	0†
GDVII m.br.	"	16	4	8000
Normal m.br.	Mouse	16	4	0
FA m.br.	"	16	4	8000
" "	Monkey	16	4	8000
Poliomyelitis (conval.)	"	16	4	0
" Lansing m.br.	Rabbit	16	4	0
Mengo, m.br.	"	16	4	0

* m.br. = Mouse brain.
† O = No inhibition of hemagglutination at 1:32 serum dilution.

at room temperature or at 37°C, as not with GDVII virus. The agglutinins were readily removed from such sera by absorption with 20% human RBC at 4°C. Absorbed immune sera gave hemagglutination-inhibition titers with GDVII virus which were identical with those obtained with unabsorbed sera.

Immune sera against the following viruses were employed in hemagglutination-inhibition experiments with GDVII virus: lymphocytic choriomeningitis, Eastern equine encephalitis, Japanese B encephalitis, St. Louis encephalitis, Russian Far East encephalitis, vesicular stomatitis, West Nile, rabies, herpes simplex, vaccinia, Columbia SK, Columbia MM, Mengo encephalomyelitis, encephalomyocarditis, influenza A (PR8 strain) and PVM. In no instance was significant inhibition of GDVII virus demonstrable with these sera.

In cross-immunity experiments Theiler and Gard[6] demonstrated an immunological relationship between GDVII and FA viruses. It appears of considerable interest that the results of hemagglutination-inhibition experiments indicate not only that antibody specifically directed against GDVII virus is present in high titer in immune sera but also show clear evidence of a close antigenic relationship to FA virus.

Adsorption and elution of GDVII virus. When mixtures of human RBC and GDVII virus were held at 4°C, the virus was adsorbed rapidly by the erythrocytes and sedimented with them on light centrifugation. When the sedimented RBC were resuspended in buffered saline and held at 4°C, elution of the virus did not occur. However, when the resuspended RBC were warmed to 37°C, elution of the virus occurred very rapidly and maximum titers were obtained in the supernate within 5 to 10 minutes. Results of typical experiments are shown in Fig. 1. As would be expected, the concentrations of RBC employed affected the extent to which the virus was adsorbed at 4°C but did not have any striking effect on the rate or degree of elution at 37°C. Mixtures held at 4°C for as long as 24 hours showed no significant elution of the virus from RBC.

That hemagglutination with GDVII virus is caused by the virus particle itself and not by a component separable from the virus is

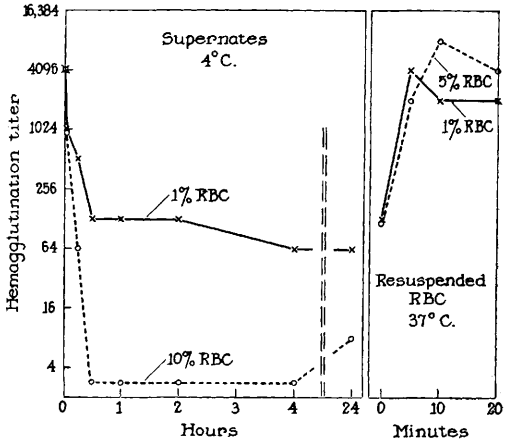

FIG. 1.

Adsorption of GDVII virus on human Group O RBC at 4°C and elution at 37°C. Hemagglutination titer of supernates from mixtures of virus and RBC is plotted against time mixtures were held at 4°C. Hemagglutination titer of supernates from resuspended RBC is plotted against time such erythrocytes were held at 37°C.

TABLE III.
Adsorption on and Elution from Human RBC of GDVII Virus.

Material tested	Hemagglutination titer* at 4°C vs. human RBC	Virus infectivity titer* in mice I.C., LD_{50}
GDVII m.br. suspension	10,240	$10^{-8.1}$
Supernate after adsorption with 10% RBC, 1 hr at 4°C	160	$10^{-5.6}$
Supernate of resuspended RBC in saline, 15 min. at 37°C	10,240	$10^{-7.8}$

* Titers are expressed in terms of final dilution of brain material.

indicated by the results shown in Table III.

Adsorption of a suspension with human RBC at 4°C resulted in reductions in the hemagglutination and virus infectivity titers of the supernate which were of similar degree. Moreover, on warming the resuspended RBC at 37°C, similar increases in both titers occurred indicating that elution of the virus was effected at the higher temperature.

Properties of hemagglutination component. The hemagglutination titer of GDVII mouse brain suspension was not diminished by storage at 4°C for 43 days. Heating crude suspensions in saline at 56°C for 30 minutes caused marked loss, *i.e.* 99%, of hemagglutinating capacity. On the other hand, suspensions prepared from brain material extracted by methanol in the cold showed only a 2- to 4-fold reduction in titer on similar heating. Moreover, such suspensions showed hemagglutination titers of 1:1,000 after heating at 65°C for 30 minutes. Centrifugation at 7,760 g for 30 minutes did not reduce the hemagglutination titer of suspensions. Filtration through Seitz-EK pads caused an 8-fold reduction in the titer of the filtrate. Suspensions buffered at pH values from 4.8 to 8.3 gave similar titers. The amount of virus adsorbed by human RBC at 4°C was not significantly affected by the pH of the mixture within this range. Furthermore, elution of virus from RBC at 37°C was complete when erythrocytes were resuspended in buffer of pH 4.8 to 8.

Concentration of GDVII virus. By resuspension of RBC with adsorbed virus in small volumes of buffered saline and warming the suspension to 37°C, it was possible to achieve considerable concentration (10 times or more) of the virus in the eluate. In most instances the increase in titer obtained was as great as or greater than would have been expected in terms of the volumes of eluate employed. Either high or low titer suspensions as well as suspensions which had been diluted before adsorption yielded satisfactory results in concentration experiments of this kind.

Recently it was reported[8] that GDVII virus could be purified considerably by precipitation with 25 to 30% methanol in the cold. In the present study numerous attempts were made to concentrate the virus by means of such a procedure. In all instances the virus titer was determined by the hemagglutination technic. It was found that 52% methanol mixtures held at 4°C for 3 hours yielded better results than mixtures at other methanol concentrations. Despite the use of a large variety of experimental conditions which included variations in pH, ionic strength, amount of centrifugation before and after the addition of methanol, as well as extraction of brain material with organic solvents, it was not possible to obtain consistent results. In some experiments 10-fold or greater concentration was achieved but the results were not sufficiently reproducible to make the procedure valuable. Moreover, after concentration by methanol precipitation, the virus appeared to be unstable and hemagglutination titers decreased rapidly on storage of concentrated material at 4°C.

Failure of FA virus to cause hemagglutination. The infectivity titer of GDVII virus is

[8] Brumfield, H. P., Stulberg, C. S., and Halvorson, H. O., PROC. SOC. EXP. BIOL. AND MED., 1948, **68**, 410.

definitely higher than that of FA virus. With the strains employed in this study, GDVII gave LD_{50} titers of the order of 10^{-8} or more while FA gave titers of the order of 10^{-6} or less. On the assumption that the infectivity titer is proportional to the virus concentration, it seemed possible that the failure to demonstrate hemagglutination with FA might be attributable to a relatively low concentration of the agent in infected brain tissue. Because of the numerous similar properties of the 2 viruses[6] and the close antigenic relationship disclosed in the hemagglutination-inhibition experiments described above, it appeared desirable to determine if FA shared with GDVII the capacity to cause hemagglutination of human RBC in the cold.

Attempts to concentrate FA virus, as was feasible with GDVII, by adsorption on human RBC at 4°C and elution in a small volume of diluent at 37°C, were uniformly unsucessful. In no instance was hemagglutination demonstrable with the eluates despite the use of erythrocytes derived from numerous species. Moreover, the results of infectivity titrations indicated that FA virus was not adsorbed by human RBC under the conditions employed; supernates of virus-RBC mixtures held at 4°C showed no reduction in infectivity titer. It appears, therefore, that despite similarities relative to numerous properties FA virus and GDVII virus do not give similar reactions with human erythrocytes *in vitro*.

Discussion. That certain neurotropic viruses possess the capacity to agglutinate in the cold erythrocytes deriving from certain animal species appears evident from the results of this study and that described in the accompanying paper.[5] By means of the hemagglutination reaction with human RBC at 4°C it is possible to estimate *in vitro* the concentration of GDVII virus in a suspension of infected mouse brain. As is the case with other animal viruses which cause hemagglutination, relatively high concentrations are required before positive results are obtained; with GDVII of the order of 10^4 mouse infectious doses of virus correspond to one hemagglutinating unit. The available evidence suggests that the infective virus particle is itself responsible for hemagglutination with this agent. By means of the hemagglutination-inhibition technic, also carried out with human RBC at 4°C, the concentration of antibodies in immune serum specifically directed against the virus can be estimated *in vitro*. Evidence obtained in hemagglutination-inhibition experiments indicates that GDVII virus is immunologically closely related to FA virus, but is not related to any other of the numerous agents tested.

Despite numerous attempts with a wide variety of experimental conditions, it was not possible to demonstrate hemagglutination with FA virus. Moreover, with the Lansing, MEF1 and Brunhilde strains of poliomyelitis virus no evidence was obtained indicative of a capacity to combine with erythrocytes. It may be pertinent that neither FA virus nor poliomyelitis virus reaches high titers in infected central nervous system tissue and it is possible that the failure to show hemagglutination with these agents is attributable to insufficient concentration. On the other hand, qualitative factors also may be of critical importance and it seems possible that with erythrocytes from still other species and with different experimental conditions positive results might be obtained.

The dependence of hemagglutination with GDVII virus and of adsorption of the agent by human RBC upon a low temperature, *i.e.* 4°C, appears to be unique. With the exception of the neurotropic viruses discussed in the accompanying paper,[5] other viruses which cause hemagglutination show no such temperature effect.

Summary. Suspensions of mouse brain infected with the GDVII strain of mouse encephalomyelitis virus cause agglutination of human Group O RBC at 4°C. Anti-GDVII virus serum inhibits hemagglutination by the agent as also does anti-FA virus serum. GDVII virus is adsorbed by human RBC at 4°C and rapidly elutes from them at 37°C. Three strains of poliomyelitis virus failed to show any evidence of hemagglutination.

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