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Physical-Chemical Factors in Agglutination of Sheep Erythrocytes by Influenza Virus.*

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The literature contains conflicting data concerning the agglutination of sheep erythrocytes by influenza virus. McLelland and Hare[1] failed to obtain agglutination with either of the A or B strains used in their tests; Burnet[2] and Clark and Nagler[3] obtained agglutination with some influenza virus strains but not with others.

The data included in the present paper show that agglutination of sheep erythrocytes by the WS strain[4] of influenza virus is dependent upon physical-chemical factors; when the requirements are satisfied, sheep cells yield essentially the same results as chicken cells in tests for the presence of influenza virus, and for the demonstration of influenza virus antibodies by the agglutination-inhibition test. The WS strain seemed particularly well suited to studies concerning the effects of physical-chemical factors on agglutination of sheep erythrocytes by influenza virus because this strain has been found by others not to cause agglutination of sheep cells under the usual test conditions.

Materials and Methods. The stock virus suspensions consisted of pooled allantoic fluid obtained from 30 to 40 eggs containing 13- to 14-days-old embryos, and which had been inoculated beneath the chorio-allantoic membrane 2 days previously, with an egg adapted strain. The normal allantoic fluid was pooled from eggs containing embryos of the same age as those from which the infected fluid was obtained. The erythrocyte suspensions were prepared in saline after thorough washing, from citrated sheep blood which contained 0.04% formalin for bacteriostatic purposes. McIlvaine's phosphate-citric acid buffer[5] was used in all tests; it seemed to be superior to phosphate buffers for the present purposes.

All tests for hemagglutination were made after the method described by Hirst,[6] in 10 x 75mm precipitin tubes. The total volume of ingredients was 0.6 ml. The degrees of agglutination were read on the basis of the cell patterns on the bottoms of the tubes, after the tests had been at room temperature sufficiently long (about 2 hours) for the erythrocytes to completely settle; they are recorded as no agglutination (0), partial agglutination (+), and complete agglutination (++).

Results. Table I shows the results of an experiment made to determine the effect of pH upon agglutination of sheep erythrocytes by different concentrations of WS-infected, and normal allantoic fluids. Test mixtures consisted of equal volumes (0.2ml) of allantoic fluid (infected or normal), buffer, and ¼% suspension of sheep erythrocytes.

In tests (Table I) with 1-100 dilution of infected allantoic fluid the virus caused agglutination of sheep erythrocytes over most of the pH range tested. However, in tests with other concentrations of infected allantoic fluid, agglutination depended upon pH, and to some extent upon the concentration of al-

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3 Clark, E., and Nagler, F. P. O., *Aust. J. Biol. and Med. Sc.*, 1943, **21**, 103.

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5 Clark, W. M., *The Determination of Hydrogen Ions,* Baltimore, Williams and Wilkins Co., 1928, p. 214.

6 Hirst, G. K., *Science,* 1941, **94**, 22.

TABLE I.

Influence of pH upon Agglutination of Sheep Erythrocytes by Different Concentrations of Infected and of Uninfected Allantoic Fluids.

Initial dilution of allantoic fluid		pH of test mixtures																	
		Saline	8.0	7.8	7.6	7.4	7.2	7.0	6.8	6.6	6.4	6.2	6.0	5.8	5.6	5.4	5.2	5.0	4.8	4.6
Undil.	A	+	+	+	+	+	0	0	+	+	+	+	+	+	+	+	+	+	+	+
	B		+	+	0	0	0	0	0	0	0	0	0	0	0	0	0	P	P	H
1-5	A	0	0	0	0	0	0	0	0	0	+	+	+	+	+	+	+	+	+	0
	B		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	H
1-25	A	0	0	0	0	0	0	+	0	+	+	+	+	+	+	+	+	+	+	+
	B		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+
1-50	A	0	+	+	0	0	0	0	0	+	+	+	+	+	+	+	+	+	0	+
	B		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	+
1-100	A	0	+	+	+	+	+	+	+	+	+	+	+	+	+	+	0	0	+	+
	B		0	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0	+	+
1-200	A	0	0	+	+	+	+	+	0	0	+	+	+	+	+	0	0	0	+	+
	B		0	0	+	+	0	0	0	0	0	0	0	0	0	0	0	0	+	+
1-400	A	0	0	0	+	0	0	0	0	+	+	+	+	+	0	0	0	0	+	+
	B		0	0	+	+	0	0	0	0	0	0	0	0	0	0	0	0	+	+
No virus			0	0	+	+	+	0	0	0	0	0	0	0	0	0	0	+	+	+
No allantoic fluid			0	0	+	+	+	0	0	0	0	0	0	0	0	0	0	+	+	+

0 = No agglutination; + = partial aggl.; ++ = complete aggl; A = WS infected allantoic fluid; B = uninfected allantoic fluid; P = partial hemolysis; H = complete hemolysis.

lantoic fluid. Agglutination was caused by all dilutions of infected fluid, between pH 5.6 and pH 6.4; but in the more alkaline pH range the agglutination of sheep cells by virus was inhibited by substances present in the allantoic fluid, the degree of inhibition decreasing with decrease in concentration of allantoic fluid.

Buffer, in the absence of normal and infected allantoic fluids caused agglutination of the sheep cells between pH 4.6 and 5.0 and between pH 7.4 and 7.6; also, some agglutination was caused by concentrated normal and infected allantoic fluid at pH 7.8-8.0.

The combined effects of agglutination by virus, allantoic fluid and buffer (pH) on the one hand, and inhibition by allantoic fluid on the other, would render sheep cell tests unreliable over a large portion of the pH range tested. Nevertheless, at the pH range 5.8-6.0, sheep erythrocytes were so readily agglutinated by the virus that it seems likely that in a system adjusted to that pH, sheep erythrocytes should be satisfactory for influenza virus tests. It is to be noted, however, that concentration of cells is a very important factor. The best results are obtained with suspensions containing between ¼% and ½% of packed cells; very little agglutination is obtained with suspensions of 2% or more.

Table II compares sheep with chicken erythrocytes in tests to determine the virus content of allantoic fluid. The test mixtures contained equal volumes (0.2 ml), in the one instance of allantoic fluid (infected or uninfected), saline and ½% chicken cell suspension; and in the other instance, allantoic fluid, buffer pH 5.8, and ¼% sheep cell suspension.

It is clear (Table II) that the hemagglutinating effect of the infected allantoic fluid was approximately the same in tests with sheep cells as in tests with chicken cells.

Table III summarizes the results of an agglutination-inhibition test of known influenza virus antiserums versus the WS strain of virus. The pooled serums were from ferrets which had been repeatedly inoculated intranasally, in the one instance with strains of the virus of influenza A, and in the other instance

TABLE II.
Comparison of Chicken with Sheep Erythrocytes for the Determination of Virus Content of Allantoic Fluid.

Erythrocyte suspension		2-fold dilution of fluid 1	2	3	4	5	6	7	8	9	10
Chicken	A	++	++	++	++	++	++	++	+	0	0
	B	0	0	0	0	0	0	0	0	0	0
Sheep	A	++	++	++	++	++	++	++	+	0	0
	B	0	0	0	0	0	0	0	0	0	0

A = WS infected allantoic fluid; B = Uninfected allantoic fluid.
The systems in the case of sheep cell tests contained pH 5.8 buffer, those in the chicken cell tests contained saline.

TABLE III.
Comparison of Chicken with Sheep Erythrocytes for the Determination of Influenza Virus Antibodies by the Agglutination-inhibition Test.

Erythrocyte suspension	Ferret serum	Initial 2-fold dilution of serum 3	4	5	6	7	8	9	10	11	12
Chicken	infl. A	0	0	0	0	0	0	0	++	++	++
	infl. B	0	+	++	++	++	++	++	++	++	++
Sheep	infl. A	++	+	0	0	0	0	+	++	++	++
	infl. B	++	++	++	++	++	++	++	++	++	++

Titration of Virus Suspension Used in Test.

Erythrocyte suspension	Initial 2-fold dilution 1	2	3	4	5	6	7
Chicken	++	++	++	+	0	0	0
Sheep	++	++	++	++	0	0	0

The systems in the case of the sheep cell tests contained pH 5.8 buffer, those in the chicken cell tests contained saline.

with strains of influenza B. The test mixtures in the case of the sheep cell tests contained equal volumes (0.2 ml) serum dilution, WS allantoic fluid diluted 1:32 with pH 5.8 buffer, and ¼% sheep erythrocytes. The tests with chicken cells differed in that the WS allantoic fluid was diluted 1:32 with saline, and the chicken cell suspension was ½%.

It is clear from the data (Table III) that the inhibitory effect of influenza A antiserums on agglutination of erythrocytes by the WS strain was detectable with the sheep erythrocytes as readily as with chicken cells. The inhibitory effect of the serum in the dilutions 2^{-4} to 2^{-9} in both instances must have been specific, because it was not obtained with influenza B virus antiserums. It is interesting that the non-specific effect of the serums (dilutions 2^{-3} and 2^{-4}) differed in the 2 tests. In the case of tests with chicken cells, the serum caused inhibition of the virus hemagglutination. In the sheep cell tests, however, the serum itself agglutinated the erythrocytes.

Summary. The data indicate that agglutination of sheep erythrocytes by influenza virus (WS strain) is influenced by physical-chemical factors within the test systems, and especially by the hydrogen-ion concentration. Over a relatively wide pH range, agglutination of sheep cells is inhibited by substances present in allantoic fluid, normal and infected. The allantoic fluid inhibition is ineffective in the pH range 5.8 to 6.0. When the test systems are adjusted with pH 5.8 buffer, and ½% to ¼% cell suspensions are employed, sheep erythrocytes may be used with essentially the same results as obtained with chicken cells, for the determination of influ-

enza virus content of allantoic fluids, and for the demonstration of antibodies by the agglutination-inhibition test. The data are significant because of the bearing they have upon the mechanism of virus hemagglutination.

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Widespread Distribution of Poliomyelitis in Households Attacked by the Disease.*

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Among patients admitted to the University of Kansas Hospitals because of poliomyelitis in 1946 it was clear that intercurrent illness had occurred in additional family members. Several households were selected for study on the basis that other illnesses had occurred at a time when poliomyelitis attacked a member of the group. Each member was sampled to determine whether poliomyelitis virus could be found in the oropharyngeal exudate and intestinal effluvia. Results obtained in a study of 4 households are reported here.

Material and methods. Throat Swabs: Cotton pledgets on applicator sticks were used to sample exudate from the oropharynx of each person. Pledgets moistened with exudate were stored at -70°C in sterile individual screw-capped bottles containing 1 cc of distilled water. In the preparation of each specimen and its inoculation into monkeys we have used methods described in earlier papers.[1,2,3]

Stool Specimens: The manner of collection of stool samples has been described.[4] No more than 12 hours elapsed between collection of each specimen and storage in a dry ice chest. The method of preparation and inoculation of monkeys with each specimen has also been described.[5,6] Each stool sample was inoculated into a monkey using the intraperitoneal and intranasal portals.

Monkeys: Rhesus *(Macaca mulatta)* and cynomolgus *(Macaca irus)* monkeys were used. A positive test in this study indicates that the monkey developed paralysis. In addition lesions consistent with those observed in poliomyelitis were observed in tissues from the spinal cord and elsewhere in the cerebral axis.

Results. The results of tests for poliomyelitis virus in oropharyngeal exudate and feces derived from each member in each of 4 households appear in Table I.

Twenty people living in these 4 households were studied. Sixteen had poliomyelitis virus in their intestinal discharges, and among these 16, 7 had virus in the oropharynx. Of 12 children in these families, each had poliomyelitis virus in the stool specimen; 5 had virus in the oropharynx. Considering the 8 adults in these households, 4 had poliomyelitis virus in respective stool samples. Among these 4, virus was detected in the throat of 2 fathers. Poliomyelitis virus was not found in the remaining 4 parents.

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