A HEMOLYSIN ASSOCIATED WITH THE MUMPS VIRUS* ‡

By HERBERT R. MORGAN, M.D.,§ JOHN F. ENDERS, PH.D., AND PHILIP F. WAGLEY, M.D.||

(From the Research Division of Infectious Diseases of the Children's Hospital, Children's Medical Center, and the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Departments of Medicine and Bacteriology and Immunology, Harvard Medical School, Boston)

(Received for publication, June 4, 1948)

During the course of unpublished experiments by Levens and Enders, hemolysis was observed in suspensions of chicken erythrocytes exposed at 37°C. to amniotic or allantoic fluids derived from chick embryos infected with mumps virus.[1] The results of systematic studies of this hemolytic factor are described in the present communication.

Methods

Preparation of Virus Suspensions.—A strain of mumps virus which had been carried for 42 passages in eggs was used. Infected amniotic fluid was inoculated into the amniotic sac of 7-day-old embryos which were then incubated at 35°C. for 6 to 8 days. At the end of this time the amniotic and allantioc fluids were removed and pooled. Aliquots were placed in glass ampoules, sealed, and stored in the dry ice cabinet until used. Allantoic fluids withdrawn from embryos 48 hours after inoculation by the allantoic route with the PR8 and Lee B strains of influenza virus were also employed in certain experiments.

Titration of Hemolysin.—Serial twofold dilutions of infected amniotic fluids were prepared in isotonic phosphate buffer (pH 7.0–7.2). To 0.5 cc. of each dilution was added an equal volume of a suspension in the buffer of chicken erythrocytes in concentrations of 1 per cent, 2 per cent, or 4 per cent. Only 2 per cent or 4 per cent suspensions of cells were used in most experiments, since they were found to give more uniform results. In the determination of hemagglutination, the procedure of Hirst (1) was employed except in one instance when that of Salk (2) was used. After 1 hour at 4°C. the degree of hemagglutination was noted and the tubes were *gently* agitated to resuspend the agglutinated red cells. They were then incubated for 2 to 4 hours at 37°C. Following incubation, the tubes were again very cautiously agitated and were centrifuged at 1500 R.P.M. The supernatant fluids were poured off, diluted with 9 volumes of distilled water, and their content of hemoglobin determined. The degree of

* Certain of the data described in this paper were presented at a Symposium on Mumps held on May 12, 1948, in Minneapolis, at a meeting of the Society of American Bacteriologists.

‡ Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

§ Senior Fellow in Medical Sciences of the National Research Council.

|| Research Fellow of the American College of Physicians.

[1] The first observations on this hemolytic factor were made with a strain of mumps virus which had been subjected to 35 egg passages. Since then hemolysin has been demonstrated in the amniotic fluid of the 5th egg passage of this same strain and in three other strains isolated directly from the saliva of mumps patients by embryonic inoculation and carried for 4 or 5 passages in eggs.

hemolysis was calculated with reference to a control standard. This standard was prepared with the same volume of cells completely hemolyzed by suspension in distilled water and addition of a drop of dilute ammonium hydroxide. The hemoglobin determinations were made with an Evelyn photoelectric colorimeter using filter 540 (3).

EXPERIMENTAL

Comparison of the Hemolytic and Hemagglutinative Activity of Mumps and Influenza Viruses

Allantoic fluids infected respectively with mumps, PR8, and Lee B influenza viruses were titrated, using a 1 per cent suspension of chicken erythrocytes. Hemagglutination was recorded after 1 hour at 4°C. and hemolysis after 4 hours at 37°C. The results of these titrations are presented in Table I. They show

TABLE I

Hemagglutinative and Hemolytic Activity of Mumps and Influenza Viruses on Chicken Erythrocytes

Infected fluid	Test	Dilutions of fluid									Buffer control
		8	16	32	64	128	256	512	1024	2048	
Mumps	Hemagglutination	3*	3	2	1	1−	0	0	0	0	0
	Hemolysis	48‡	43	29	25	13	0	0	0	0	0
PR8	Hemagglutination	4	4	3	3	2	2	1	1	0	0
	Hemolysis	0	0	0	0	0	0	0	0	0	0
Lee B	Hemagglutination	4	3	2	2	2	1	1	1−	0	0
	Hemolysis	0	0	0	0	0	0	0	0	0	0

* 1 per cent chicken cells. Hirst technique. Figures denote degree of hemagglutination.
‡ Per cent of cells hemolyzed determined colorimetrically.

that fluids infected with mumps virus agglutinate chicken red cells at refrigerator temperatures and, on subsequent incubation at 37°C., hemolyze them to about the same extent. In contrast the influenza viruses readily agglutinate these erythrocytes but produce no hemolysis. In these and other experiments, the titers of hemagglutinin and of hemolysin in preparations of mumps virus were usually of the same order. Occasionally, however, a fluid might exhibit considerable hemagglutinative activity but little hemolytic effect.

Comparative Activity of the Hemolysin on Chicken, Sheep, and Human Erythrocytes

Four per cent suspensions of freshly obtained chicken, sheep, and human type 0 erythrocytes were prepared in phosphate buffer. Allantoic fluid infected with mumps virus was diluted and its hemolytic titer simultaneously deter-

mined against each type of cell suspension. At the same time the hemagglutinative titer was determined with an 0.25 per cent suspension of the cells of each species. The method of Salk was employed instead of that of Hirst because tests with human erythrocytes are unsatisfactory by the latter method. The results are shown in Table II.

It is apparent that although the erythrocytes of these three species are all susceptible to hemolysis, those of man are less affected than the cells of the sheep or the chicken. The human cells also proved to be less sensitive to hemagglutination by the virus.

TABLE II

Hemolytic Effect of Mumps-Infected Allantoic Fluid on Erythrocytes of Various Species

Species	Test	Cell concentration	Dilutions of fluid								Buffer control
			8	16	32	64	128	256	512	1024	
		per cent									
Chicken	Hemagglutination	0.25	+*	+	+	+	+	±	±	0	0
	Hemolysis	4.	44‡	36	26	12	7	5	1	0	0
Sheep	Hemagglutination	0.25	+	+	+	+	+	+	±	±	0
	Hemolysis	4.	18	18	13	8	5	3	0	0	0
Human	Hemagglutination	0.25	+	+	+	+	±	0	0	0	0
	Hemolysis	4.	22	17	6	1	0	0	0	0	0

* Salk technique. + denotes complete agglutination of cells.

‡ Per cent of cells hemolyzed as determined colorimetrically.

Effect of Physical and Chemical Agents on the Hemolytic Activity of Mumps Virus

Inactivation by Heat.—Fluids infected with mumps virus were placed in sealed glass ampoules and completely immersed in a water bath at various temperatures for varying periods of time. Afterwards they were immediately placed in ice water. The concentrations of hemagglutinin and hemolysin in each specimen were then determined. These, together with that of the starting material, are recorded in Table III.

The data show that the hemagglutinative and hemolytic activity of the virus are destroyed after 30 minutes at 55°C.; both properties are markedly impaired after 30 minutes at 50°C.; but exposure for 10 minutes at this temperature almost completely destroys the hemolytic activity while leaving the hemagglutinin almost intact.

The hemolysin also seems to be less stable at low temperatures. A pool of

infected amniotic fluid stored at −20°C. was thawed and refrozen four times during an interval of 3 weeks. At the end of 3 days after the beginning of the period of storage the concentration of hemolysin had decreased and by the end of 3 weeks no hemolysin could be detected, whereas the hemagglutinin titer remained unchanged.

Effect of Temperature and Time of Incubation on Hemolysis.—To determine the effect of temperature and time of incubation on the degree of hemolysis,

TABLE III

Inactivation of the Hemolytic Activity of Mumps Virus by Heat

Mumps-infected fluid	Temperature	Time	Test	Dilutions of serum									Buffer control
				4	8	16	32	64	128	256	512	1024	
	°C.	*min.*											
	Unheated	—	Hemagglutination	3*	3	3	3	2	2	±	0	0	0
			Hemolysis	29‡	36	29	29	20	10	4	1	0	0
Allantoic	50	30	Hemagglutination	1−	1−	1−	1−	±	0	0	0	0	0
			Hemolysis	5	0	0	0	0	0	0	0	0	0
	55	30	Hemagglutination	0	0	0	0	0	0	0	0	0	0
			Hemolysis	0	0	0	0	0	0	0	0	0	0
	Unheated	—	Hemagglutination	4	4	4	3	2	1	1	±	0	0
			Hemolysis	53	51	46	40	29	20	15	2	0	0
Amniotic	50	10	Hemagglutination	4	4	3	2	1	1	±	0	0	0
			Hemolysis	6	5	5	±	0	0	0	0	0	0
	50	20	Hemagglutination	4	4	3	2	1	1	±	0	0	0
			Hemolysis	5	0	0	0	0	0	0	0	0	0

* 2 per cent chicken cells. Hirst technique. Figures denote degree of hemagglutination.
‡ Per cent of cells hemolyzed as determined colorimetrically.

amniotic fluids infected with mumps virus were mixed with 4 per cent suspensions of chicken red blood cells and incubated at various temperatures for varying periods. Unless otherwise noted, the suspensions were first placed at 4°C. for 1 hour, shaken, and then incubated at 37°C. Table IV provides a summary of the results obtained in these experiments. They indicate that most of the hemolysis occurs during the 1st hour of incubation at 37°C. This temperature appears to be optimal, since less hemolysis was observed at 25°C. and none at 4°C. during the same interval. These data also show that preliminary chilling for 1 hour at 4°C., which permits marked hemagglutination, is not essential for the subsequent occurrence of hemolysis at 37°C. In fact, it has been found that

when virus and red cells are mixed and maintained at 37°C., conditions which are attended by little observable hemagglutination, maximal hemolysis ensues. These observations taken together suggest that hemagglutination is not an essential factor in the hemolytic process.

Effect of pH on Hemolysis.—The effect of different hydrogen ion concentrations on the activity of the hemolysin was determined by titrations carried out in isotonic phosphate buffers of varying pH values. To allow for the fact that

TABLE IV

Effect of Time and Temperature of Incubation on the Degree of Hemolysis Produced by Mumps Virus

Mumps-infected fluid	Reaction	Temperature	Time	Dilutions of fluid									Buffer control
				4	8	16	32	64	128	256	512	1024	
		°C.	*hrs.*										
Amniotic I	Hemolysis	37	1	19*	16	14	9	7	nd	0	0	0	0
			2	17	19	14	8	7	1.2	0	0	0	0
			3	22	19	15	8	4	2	1	0	0	0
			4	26	19	13	12	8	3	0	0	0	0
Amniotic II	Hemolysis	4	4	0	0	0	0	0	0	0	0	0	0
		25	4	12	10	8	0	0	0	0	0	0	0
		37	4	33	27	19	10	6	4	0	0	0	0
Amniotic III	Hemagglutination	4‡	1	4	4	2	2	1−	1−	0	0	0	0
	Hemolysis	37	2	48	42	30	26	16	8	0	0	0	0
	Hemagglutination	37§	1	0	0	0	0	0	0	0	0	0	0
	Hemolysis	37	2	48	40	35	22	16	5	0	0	0	0

nd, not done.

* Per cent of cells hemolyzed determined colorimetrically. 4 per cent suspension.

‡ 2 per cent cells used for experiments with amniotic fluid III. Hirst's technique. Figures denote degree of hemagglutination.

§ No initial chilling at 4°C. Virus dilutions and red blood cells mixed at 37°C.

red blood cells themselves exert a considerable buffering action, the pH values at which the reaction occurred were determined after incubation for 4 hours at 37°C. The results, presented graphically in Fig. 1, indicate that the hemolytic activity appears to be maximal between pH values of 7.0 and 8.0. Below pH 6.5 or above pH 8.5 little or no activity was observed. In contrast the hemagglutinin was not significantly affected over the range of hydrogen ion concentrations which was employed.

Effect of Composition of Buffer on Hemolysis.—Dilutions of an infected amniotic fluid were prepared in monobasic sodium and phosphate buffer (pH 7.2) containing sodium chloride and in a sodium citrate buffer (pH 7.4). To each

were added chicken red cells diluted in the homologous buffer. The titer and degree of hemolysis observed in each buffer system were essentially the same.

Adsorption and Elution of the Hemagglutinin and the Hemolysin by Chicken Red Blood Cells

To determine whether the hemolytic activity could be adsorbed and eluted from chicken red cells like the hemagglutinin (4), 1 cc. of infected amniotic fluid was added to 5 cc. of 10

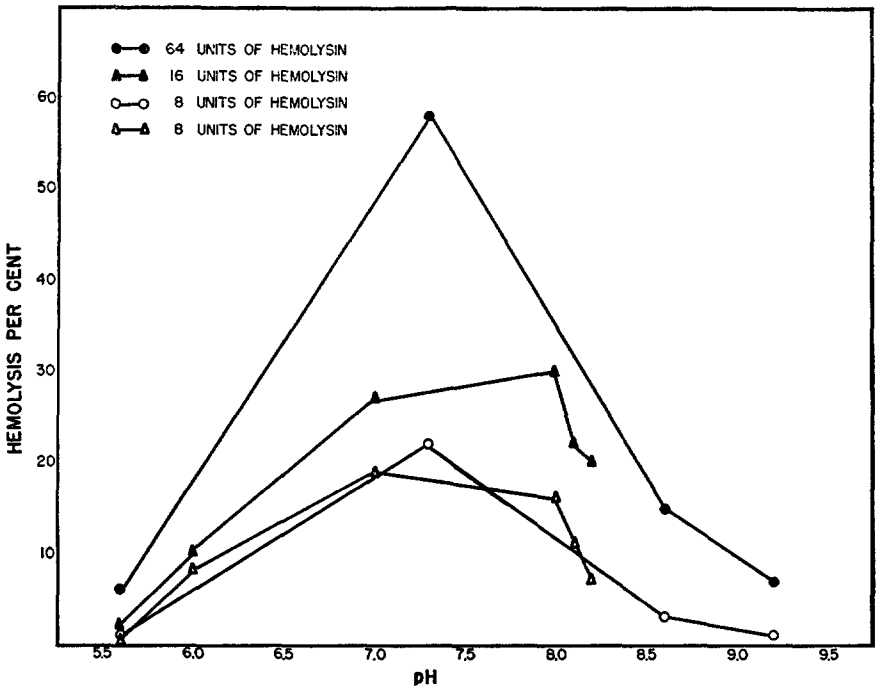

FIG. 1. Effect of pH on the activity of mumps hemolysin. A unit of hemolysin is determined by the ratio: $\frac{\text{Endpoint of hemolytic effect}}{\text{Dilution of fluid used}}$.

per cent chicken cells in saline. The mixture was allowed to stand for 1 hour at 4°C. After removal of the supernatant fluid aliquots of the cells were resuspended in phosphate buffer at pH 6.0 and pH 7.2 respectively and then placed in a water bath for 2 hours at 37°C. These eluates were then separated from the cells by centrifugation and the cells washed twice in buffer (pH 7.2) and resuspended in a concentration of 2 per cent in buffer. The supernatant fluid and eluates were tested for hemolytic activity after dilution in buffer and addition of an equal volume of 2 per cent red cells. To allow for the hemolysis which occurred during elution, the eluate was diluted in buffer, and the concentration of hemoglobin determined in the photoelectric colorimeter. This value was then used to correct the readings obtained after incubation of the eluate with untreated chicken cells. The cells from which the virus was eluted were washed three times in buffer and made up in buffer (pH 7.2) as a 2 per cent suspen-

sion. These cells were then tested for susceptibility to hemolysis by adding to them dilutions of fresh virus suspensions.

TABLE V

The Adsorption and Elution of the Hemagglutinative and Hemolytic Activity of Mumps Virus with Chicken Red Cells

Fluid	Test	Dilutions of fluids*							Buffer control
		12	24	48	96	192	384	768	1526	
Original Amniotic fluid	Hemagglutination	4	3	2	1	1	±	0	0	0
	Hemolysis	46‡	40	29	20	15	2	0	0	0
Supernatant (pH 6.0) after 1 hr. at 4°C.	Hemagglutination	0	0	0	0					0
	Hemolysis	0	0	0	0					0
Eluate 1 (pH 6.0) after 2 hrs. at 37°C.	Hemagglutination	4	2	±	0	0	0	0	0	0
	Hemolysis	15	11	7	2	0	0	0	0	0
Supernatant (pH 7.2)	Hemagglutination	0	0	0	0					0
	Hemolysis	0	0	0	0					0
Eluate 2 (pH 7.2)	Hemagglutination	4	4	1−	0	0	0	0	0	0
	Hemolysis	12	16	14	4	0	0	0	0	0

* In terms of original fluid before addition of adsorbing chicken red cells.

‡ Per cent of hemolyzed cells as determined colorimetrically with 2 per cent cells.

Cells	Test	Dilutions of virus								Buffer control
		2	4	8	16	32	64	128	256	
Untreated	Hemagglutination	4*	4	2	2	1	±	0	0	0
	Hemolysis	51‡	45	43	35	30	14	6	0	0
Eluted 1	Hemagglutination	±	±	0	0	0	0	0	0	0
	Hemolysis	0	0	0	0	0	0	0	0	0
Eluted 2	Hemagglutination	0	0	0	0	0	0	0	0	0
	Hemolysis	0	0	0	0	0	0	0	0	0

* 2 per cent suspension of cells. Hirst technique. Figures denote degree of hemagglutination.

‡ Per cent of cells hemolyzed as determined colorimetrically.

The results of these experiments are presented in Table V. It is clear from them that the hemagglutinative and hemolytic activities of mumps virus are completely adsorbed on chicken erythrocytes at 4°C. and may subsequently

be released in part at least by elution at 37°C. Moreover, the cells from which the virus was eluted are no longer susceptible either to the hemagglutinative or hemolytic action of the virus. The cause of our failure to recover all the activity in the eluates is probably to be attributed to incomplete elution after the comparatively short interval of 2 hours.

Neutralization of the Hemolysin by Immune Serum

The action of mumps immune sera on the hemolysin was investigated.

Acute phase and convalescent sera were obtained from a patient with parotitis and a monkey in which the disease was produced experimentally. These sera, heated for ½ hour at 56°C., were diluted in 0.5 cc. amounts of buffer (pH 7.2) containing diluted infected amniotic fluid (1:16) with a hemolytic titer of 1:128. The mixtures were placed at 4°C. overnight, and 0.5 cc. of 4 per cent suspension of red cells was added to each on the following morning. They were then placed at 4°C. for 1 hour, shaken gently, and incubated for 4 hours at 37°C. As a control on the possible hemolytic action of the sera, dilutions of serum in buffer were prepared and an equal volume of 4 per cent red cells was added. These controls were treated in the same manner as the virus-serum-red cell mixtures. At the end of the incubation period, the degree of hemolysis was determined on the supernatant fluid by means of the photoelectric colorimeter.

TABLE VI

Inhibition of the Hemolytic Activity of Mumps Virus by Immune Sera

Species	Serum	Dilutions of serum										Buffer control	Serum control
		8	16	32	64	128	256	512	1024	2048	4096		
Monkey	Normal	3*	5	23	23	27	27	27				29	0
	Convalescent	0	0	0	0	0	0	0	5	10	27	30	0
Man	Acute	8*	13	17	22	28	31					31	0
	Convalescent	0	0	4	7	13	16	15	33	33	34	31	0

* Per cent of cells hemolyzed determined colorimetrically with 4 per cent cells.

The results are presented in Table VI. They show that the hemolysis of chicken red cells is inhibited by the sera of man and monkey convalescent from mumps. In contrast, normal monkey serum and serum taken during the early stage of the disease in man had only a slight inhibiting effect. These findings suggest that the hemolytic activity of amniotic fluid infected with mumps virus is due to a factor specifically produced by this agent.

Effect of Mumps Virus on the Fragility of Human and Chicken Erythrocytes

In view of the foregoing observations, it became of interest to see whether the adsorption of mumps virus on human and chicken cells would affect their fragility.

Red cells were recovered from defibrinated human blood. Chicken cells which had been recently collected in Alsever's solution were used. A 5 per cent solution of human albumin in saline was added to minimize the effect of handling on cell fragility. The cells were then washed three times in this mixture and a 6 per cent suspension of human or chicken red cells was prepared in the albumin-saline. To 40 ml. of the suspension of each of the two types of cells, 3 ml. of allantoic fluid infected with the virus of mumps was added. To serve as controls on the possible mechanical effects of hemagglutination, 3 ml. of PR8 and of Lee B influenza viruses were added to 40 ml. portions of these cell suspensions. To the same volumes of the suspensions of human and of chicken erythrocytes, 3 ml. of normal allantoic fluid was added to detect any non-specific action of this material. The virus-cell mixtures were allowed to stand at 25°C. for ½ hour when the supernatant fluids were removed and tested for the presence of virus by means of the hemagglutination reaction. The results showed that almost all the mumps and influenza viruses had been adsorbed to the cells. The chicken cells were sedimented and then resuspended in fresh albumin-saline, whereas the human cells were resuspended in the original supernatant fluid during the subsequent elution at 37°C. for 1 hour. The eluates were then removed and the cells were washed twice in albumin-saline. Hemagglutination tests on the eluate showed that most of the virus in all instances had been released from the cells. Some of these cells were then shown to be agglutinated by the addition of fresh homologous virus. The residue of the cells was used to determine the osmotic fragility by methods which have been described (3).

Fig. 2 presents representative curves of the results. The data show that exposure to mumps virus caused a definite increase in fragility of human and chicken erythrocytes. On the other hand, adsorption and elution of influenza virus brought about no detectable effect. This latter finding is of especial significance in view of the fact that the influenza virus is known to inactivate the hemagglutinin receptors of the chicken red cell for mumps virus (5).

DISCUSSION

The observations on the hemolysin of the mumps virus which we have presented reveal a property exhibited by many bacteria but, in so far as we are aware, one hitherto undescribed among viruses. The experiments have been carried out with precautions to eliminate any hemolysis which might be caused by non-specific chemical or physical factors. This fact together with the findings that normal egg fluids or those obtained from embryos infected with PR8 or Lee B influenza viruses did not exert a hemolytic effect under the same conditions show that the hemolysin present in fluids infected with mumps virus is a specific product of this agent. From the results, two inferences have been drawn: (1) the hemolysin is not identical with the hemagglutinative property; (2) the hemolysin in its behavior is in many respects analogous to an enzyme.

Hemolytic activity can be experimentally distinguished from the hemagglutinative capacity of infected fluid by the inactivating effect of moderate heating, reaction temperature, and the pH range of activity. On the other hand, the two factors behave essentially alike in respect to their adsorption and elution with red cells and their inhibition by specific immune serum. It cannot therefore be now asserted that these two factors represent distinct constituents or

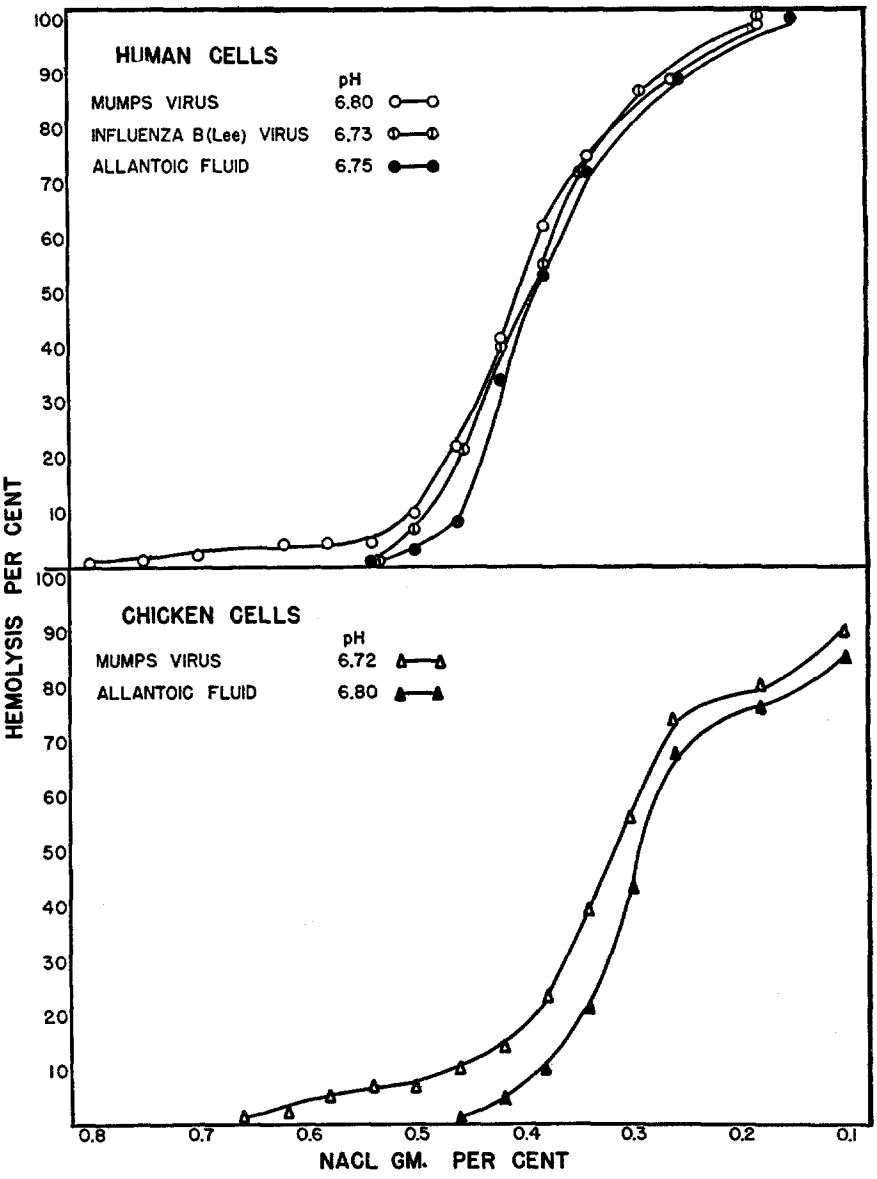

FIG. 2. Effect of the adsorption and elution of mumps and influenza (Lee B) virus on the osmotic fragility of human and chicken erythrocytes.

products of the virus. The determination of their exact relationship must await further experimentation carried out, for example, by differential ultrafiltration and centrifugation. In addition, studies to determine whether

the same cell receptor is involved in both reactions, might also contribute to the elucidation of this problem.[2] If through such experimentation, these two properties are shown to be manifestations of a single constituent, *i.e.* the infective particle, then it is possible that the concentration of hemolysin may afford a more accurate index of infectivity, for our findings indicate that the activity of the hemolysin is more easily impaired than that of the hemagglutinin.

Several features of the hemolysin resemble those of enzymes, such as inactivation by gentle heating, increase in activity correlated with the passage of time, increase of hemolytic effect within the temperature range of 4 to 37°C., the effect of hydrogen ion concentration on the degree of hemolysis. Furthermore, as shown by the results of elution experiments, the hemolysin is not exhausted during hemolysis but retains its characteristic activity when added to fresh cells. In this respect, of course, it behaves like the hemagglutinin. It may in this connection be pointed out that Burnet (6) has described an enzyme-like effect of influenza virus on gastric mucin and Bovarnick and de Burgh (7) have shown that this agent may inactivate comparatively large amounts of the red cell receptor substance without itself undergoing loss of activity.

SUMMARY

1. A factor capable of causing the hemolysis of the erythrocytes of man, chicken, and sheep occurs in the amniotic and allantoic fluids of chick embryos infected with the virus of mumps.

2. The hemolysin has not been found in normal fluids or in those infected with PR8 or Lee B strains of influenza virus.

3. The hemolysin is definitely inhibited by the serum of man and monkey convalescent from mumps, but only slightly by the serum of the acute phase.

4. The hemolysin is almost completely inactivated at 50°C. after 10 minutes. It exhibits maximal activity at 37°C. and is completely inactive at 4°C. A pH range from about 7.0 to 8.0 allows for maximum activity.

5. Adsorption and elution of the hemolysin with red blood cells has been demonstrated. After elution of the hemolysin, the red blood cells exhibit an increased osmotic fragility. Similar treatment of red cells with influenza virus did not alter this property.

6. The relationship of the hemolysin to the hemagglutinin and the enzyme-like behavior of the former have been discussed.

The authors gratefully acknowledge the technical assistance of Mrs. Virginia Peaslee.

[2] Since the completion of the experiments described in this paper, it has been determined that chicken erythrocytes, treated respectively with PR8 and Lee B influenza viruses in such a manner as to render them inagglutinable by mumps virus, were not hemolyzed by fluids infected with this agent. These fluids, however, exhibited marked hemolytic activity on suspensions of untreated cells. This finding might be interpreted as indicating fixation of the mumps hemagglutinin and hemolysin by the same cell receptor.

BIBLIOGRAPHY

1. Hirst, G. K., *J. Exp. Med.*, 1942, **75,** 49.
2. Salk, J. E., *J. Immunol.*, 1944, **49,** 87.
3. Shen, S. C., and Ham, T. H., *New England J. Med.*, 1943, **229,** 701.
4. Hirst, G. K., *J. Exp. Med.*, 1942, **76,** 195.
5. Burnet, F. M., McCrea, J. F., and Stone, J. D., *Brit. J. Exp. Path.*, 1946, **27,** 228.
6. Burnet, F. M., *Lancet*, 1948, **1,** 7.
7. Bovarnick, M., and de Burgh, P. M., *Science*, 1947, **105,** 550.