ulcer in rabbits.[4] Recent evidence from this laboratory has shown that both vasoconstriction[5] and venous stasis[6] may aid and abet the ulcer diathesis by producing anoxic areas which then fail to resist the digestive action of the peptic mixture. Inasmuch as the effect of histamine is directly on the parietal cell, in the gastric tubule, the results of these experiments are not to be interpreted as a criticism of the application of vagotomy to the problem of ulcer in man.

Conclusions. 1. Bilateral vagotomy (infra- and supradiaphragmatic) failed to protect against ulcer or erosion (gastric and/or duodenal) produced by chronic histamine action in the dog, cat and rabbit.

5 Baronofsky, I., and Wangensteen, O. H., *Bull. Am. Coll. Surg.*, 1945, **30**, 59.

6 Baronofsky, I., and Wangensteen, O. H., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 234.

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Hemagglutination by Amniotic Fluid from Normal Embryonated Hen's Eggs.*

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During studies on the detection of influenza virus in throat washings[1] an agglutinin for guinea pig erythrocytes was noted in the amniotic fluid from certain inoculated eggs. Chicken red cells were not agglutinated by these fluids. This suggested that the fluids might contain the "O" form of influenza virus described by Burnet,[2] which grows only in the amniotic sac and which agglutinates guinea pig cells to higher titers than chicken cells. Subsequent study of amniotic fluid from uninoculated eggs, however, revealed that normal fluid also contained an agglutinin for guinea pig erythrocytes but not for chicken red cells. The phenomenon was unrelated to and independent of the presence of influenza virus.

The agglutinin was capable of clumping the erythrocytes of a wide variety of animals. It was present in the egg white of non-embryonated eggs and the albumin sac of embryonated eggs, gaining access to the amniotic sac after the establishment of the communication between it and the albumin sac which normally takes place between the 11th and 13th days of incubation.[3] It was associated with the globulin fraction of the egg white, was thermolabile, and was adsorbed to erythrocytes during agglutination. The characteristics of the hemagglutinin are described in this report.

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1 Commission on Acute Respiratory Diseases, to be published.

2 Burnet, F. M., and Bull, D. R., *Australian J. Exp. Biol. and Med. Sc.*, 1943, **21**, 55.

3 Hirota, J., *J. Coll. Sc., Imp. Univ. Japan*, 1894, **6**, 339. Cited in Needham, J., *Chemical Embryology*, London, Cambridge University Press, 1931, vol. 2, p. 1100.

TABLE I.
Agglutination of Erythrocytes of Different Species by Amniotic Fluid from Normal 14- to 17-Day Embryos, Normal Adult Chicken Serum, and Normal 17-Day Embyro Serum.

	Final agglutinative titer		
Erythrocytes	Amniotic fluid pool	Adult chicken serum pool	Chick embryo serum pool
Mouse (Swiss)	32	16	<2
Rabbit	32		
Dog	16	32 or >	<2
Guinea pig	8	32 or >	<2
Cotton rat	8		<2
Horse	8	16	<2
Human Group O	8		
,, ,, A	4		
,, ,, B	4		
,, ,, AB	2		
Chimpanzee	4		
Hamster (Syrian)	4		
Cat	4	8	<2
Mule	4	<2*	<2
Pigeon	4	32 or >	<2
Monkey (Rhesus)	2		
Ferret	<2*		
Sheep	<2*	8	<2
Cow	<2*	2	<2
Toad	<2*	8	<2
Chicken	<2	<2	<2

* Agglutination doubtful in 1:2 dilution.

Materials and Methods. Source of materials. The eggs from which the hemagglutinin was derived all came from New Hampshire Red chickens. Fertile eggs were incubated at 37.7°C (100°F). Egg white, albumin sac contents, allantoic fluid, and amniotic fluid were obtained using sterile technic and were stored at 4°C without preservative. Pools of amniotic fluid were made up of individually tested fluids known to agglutinate guinea pig cells.

Preparation of erythrocytes. Erythrocytes were obtained from citrated blood. They were washed 3 times in buffered saline[4] and packed for 15 minutes at 1500 R.P.M. in the horizontal centrifuge. A 1.5% suspension by volume of packed cells in buffered saline was used throughout except as indicated in the text. Erythrocytes were used within 24 hours of the time they were obtained.

Titration of hemagglutinin. All dilutions were made in buffered saline. To 0.1 ml of the test fluid in an 11 x 75 mm tube was added 0.1 ml of cell suspension. A control included in each test consisted of 0.1 ml of buffered saline mixed with 0.1 ml of cell suspension. After thorough shaking the mixtures were incubated at 4-10°C except as indicated. They were observed at frequent intervals for agglutination. The reciprocal of the highest dilution in which agglutination was observed was recorded as the titer, which was expressed in terms of final dilution.

Experiments and Results. Description of the agglutination. The agglutination was first noted in amniotic fluids from 16-day embryos. Clumping was difficult to discern while the cells were settling, but after complete sedimentation had taken place the cells formed a characteristic pattern on the bottom of the tube. They clung to the entire curve of the bottom, covering it in a uniform, finely granular sheet. Later they slid downward in irregular fashion from the upper and outer edges, the resulting pattern having a stellate configuration with its edges accentuated by the constant accession of cell masses. Eventually the pattern became identical with that of the saline control, in which the cells settled in a homogeneous, compact, circular disc having a regular, sharply demarcated periphery.

This pattern closely resembles that due to

[4] Eagle, H., *The Diagnosis of Syphilis,* St. Louis, C. V. Mosby Co., 1938.

TABLE II.
Distribution of Hemagglutinin for Erythrocytes of Several Species in Normal Embryonated Hen's Eggs by Day of Development.

Day of development of embryo	Amniotic fluid			Allantoic fluid	
	Mouse cells	Guinea pig cells	Chicken cells	Guinea pig cells	Chicken cells
9	N.T.*	0/12	0/12	N.T.	N.T.
11	0/9	0/48	0/48	0/36	0/36
12	3/8	0/48	0/36	0/36	0/36
13	8/8	2/47	0/35	0/24	0/24
14	N.T.	12/41	0/35	0/33	0/33
15	N.T.	19/43	0/36	0/36	0/36
16	10/11	16/44	0/36	0/36	0/36
17	N.T.	23/36	0/36	0/34	0/34

* N.T. = not tested.
Numerator = No. of positive fluids.
Denominator = No. of fluids tested.

influenza viruses A and B,[5,6] vaccinia,[7] and pneumonia virus of mice[8] when they are diluted almost to their agglutinative endpoints, and is indistinguishable from that caused by metallic ions such as Ca++ and Mg++.[9,10] Agglutination caused by these agents and by normal amniotic fluid likewise disappears when the tube is gently shaken and reappears when the cells settle again.

The type of agglutination was the same irrespective of the source of the hemagglutinin, but since there was a considerable variation in viscosity of the fluids tested, the erythrocyte sedimentation rate varied accordingly. For this reason no time interval for reading agglutination was set; instead the settling process was observed frequently until complete. If the cells covered the bottom in a uniform sheet and later assumed a stellate pattern the amniotic fluid was considered to contain hemagglutinin. Patterns corresponding to those of the saline controls were considered negative. Patterns which deviated in any particular from either of the 2 mentioned above were considered doubtful.

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

6 McClelland, L., and Hare, R., *Canad. Pub. Health J.,* 1941, **32**, 530.

7 Nagler, F. P. O., *Med. J. Australia,* 1942, **1**, 281.

8 Mills, K. C., and Dochez, A. R., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 140.

9 Northrup, J. H., and Freund, J., *J. Gen. Physiol.,* 1924, **6**, 603.

10 Landsteiner, K., *The Specificity of Serological* Reactions, Cambridge, Harvard University Press, 1945.

Agglutinability of erythrocytes from different species. A survey of the agglutinability of the erythrocytes of 18 different species was made in order to determine whether a systematic variation in this regard existed. A single positive pool of normal amniotic fluid was used for the experiment. The cells were also tested with normal adult and 17-day embryo chicken serum.

No systematic variation in agglutinability was apparent (Table I). Mouse and rabbit cells gave the highest agglutinative titer. No apparent relation existed between the agglutinative titer of normal amniotic fluid and that of normal adult chicken serum for erythrocytes of the respective species examined. The red cells of none of the species tested agglutinated in serum from normal 17-day chick embryos.

Effect of temperature of incubation on agglutinative titer. The titer of the hemagglutinin depended on the temperature at which the agglutination reaction was carried out. It was considerably greater at low than at higher temperatures. For example, in a representative experiment the titer of an amniotic fluid pool for mouse erythrocytes was 32 when the reaction was carried out at 8°C, 8 at 30°C, 2 at 37°C, and <2 at 56°C. As has been indicated, incubation of agglutination tests was routinely carried out at 4-10°C.

Relationship between the appearance of hemagglutinin and embryonic development. In order to determine whether the age of the embryos was correlated with the appearance

TABLE III.
Approximate Agglutinin Titers for Mouse and Rabbit Erythrocytes of Fractions of Pooled Egg White and of Pooled Albumin Sacs from 12-Day Embryos.

Fraction		Titer* according to source of hemagglutinin: Egg white		Albumin sac	
% $(NH_4)_2SO_4$ saturation	Precipitate or supernatant fluid	Mouse erythrocytes	Rabbit erythrocytes	Mouse erythrocytes	Rabbit erythrocytes
No fractionation		64	64	96	96
29	ppt.†	6	6	8	8
	super.	16	16	48	48
48	ppt.	64	32	32	32
	super.	<8	<8	<12	<12
100	ppt.	<4	<4	<6	<6
	super.	<8	<8	<12	<12

* Final titer (by weight of original specimen).
† ppt. = precipitate; super. = supernatant fluid.

of the hemagglutinin in the amniotic fluid, and also whether it appeared at any time in the allantoic fluid, embryo fluids were examined daily with mouse, guinea pig, and chicken erythrocytes. Amniotic fluids from embryos incubated less than 12 days failed to cause agglutination (Table II). The agglutinin for mouse cells was first detected on the 12th day, and for guinea pig cells on the 13th day, and was found in the majority of eggs from then on. Chicken cells failed to agglutinate in any fluid.

There was a definite correlation between the viscosity of the amniotic fluid and the presence of hemagglutinin. Thin, watery fluids contained none; thick, viscous fluids usually agglutinated the red cells. This correlation may be considered simply another manifestation of that existing with the degree of embryonic development, since the amniotic fluid becomes thickened when the contents of the albumin sac find their way into it.[3]

No hemagglutinin for any of the 3 species of erythrocytes was detected in the allantoic fluid of embryos of any age, regardless of whether the amniotic fluid contained hemagglutinin.

Nature of the hemagglutinin. The appearance of hemagglutinin in the amniotic but not the allantoic fluid at the approximate time of rupture of the content of the albumin sac into the amnion, and the correlation between increased viscosity of the amniotic fluid and hemagglutinative properties, suggested that the content of the albumin sac was the source of the agglutinin. The albumin sac contains inspissated egg white; therefore, both the white of non-embryonated eggs, and albumin sac contents of 10 11- to 12-day embryos, were examined for the hemagglutinin. It proved to be present in all specimens tested. Thorough mixing of the specimens was essential.

To determine which fraction contained the agglutinin, ammonium sulfate, first in half saturation, then in full saturation, was added to amniotic fluid having a titer of 16 for mouse erythrocytes. The precipitate resultant from half saturation (globulins), when dissolved in 0.85% sodium chloride solution, had a titer of 12, while that resultant from subsequent full saturation (albumins) had a titer of <2.

The experiment was repeated with egg white and albumin sac contents, using successively 29, 48, and 100% saturation with ammonium sulfate. Samples of the respective supernatant fluids were dialyzed at 4°C for 2 days against distilled water, then for a few hours against 0.85% sodium chloride solution. The latter was again used as a solvent for the precipitates.

The results indicated that most of the hemagglutinin was associated with the fraction precipitated by 48% ammonium sulfate ("pseudoglobulins") (Table III). To verify the fact that the albumin fraction contained no hemagglutinin, a sample of purified oval-

TABLE IV.
Inactivation by Heat of Fresh Egg White Agglutinin for Mouse Erythrocytes, According to Temperature and Time of Exposure.

	Titer* according to time of exposure					
Temperature (C)	5 min	10 min	30 min	60 min	4 hr	20 hr
37	32	32	32	32	32	32
48	32	32	32	32	16	<4
56	32	32	32	16	8	<4
60†	8	8	4	4	—	—
65†	8	4	<4	<4	—	—

* Titer of the egg white for mouse erythrocytes before heating = 32.
† The egg white solution began to coagulate within 5 minutes at 60 and 65°C.

bumin‡ was tested with mouse, rabbit, and guinea pig erythrocytes. Agglutination did not take place.

Thermolability of the hemagglutinin. As would be expected in view of the properties described above, the hemagglutinin was inactivated by heat. Fresh egg white having an agglutinative titer of 32 for mouse erythrocytes was heated for various periods at different temperatures (Table IV). It was completely inactivated at 48°C in 20 hours. At higher temperatures inactivation proceeded more rapidly. Exposure to 37°C for 20 hours, however, did not decrease the titer.

Adsorption of agglutinin to erythrocytes. To determine whether the agglutinin was adsorbed to erythrocytes, packed mouse red blood cells were added to a positive amniotic fluid pool to make a 2% suspension by volume. The mixture was incubated at 4°C and aliquots were drawn off at intervals after mixing, following which the cells were immediately separated by centrifugalization and the supernatant fluids set aside. The supernatant fluids were then tested simultaneously with mouse erythrocytes to determine the titer of residual hemagglutinin. The titer decreased from 32 to 4 within 30 seconds and was <4 within 10 minutes, indicating adsorption had occurred. Elution did not take place within 24 hours.

The same experiment was done with chicken erythrocytes and the same amniotic fluid pool. The titer remained at 32 for the duration of the experiment, showing that the chicken cells did not adsorb the agglutinin.

To determine whether the agglutinin eluted at 37°C, the same amniotic pool was adsorbed to mouse erythrocytes at 4°C for 10 minutes. The supernatant fluid was removed and found to have a titer of <4. An equal volume of 0.85% sodium chloride solution was added to the erythrocytes and the suspension incubated at 37°C. Elution did not occur within 6 hours.

Discussion. The agglutination of red blood cells by egg white and extraembryonic fluids containing egg white, is not a unique phenomenon. In addition to specific antisera and the hemagglutinins which are present in certain normal sera, numerous other agents which can cause hemagglutination have been described. Ricin, abrin, and crotin, and other substances derived from plants, have this ability.[10] Influenza viruses A and B,[5,6] vaccinia,[7] and the viruses of Newcastle disease,[11] fowl plague,[12] mouse pneumonitis,[8] and mumps[13] agglutinate red cells. Certain inorganic colloidal acids or bases, such as colloidal silicic acid, and basic proteins such as protamines can induce hemagglutination.[10] Numerous metallic ions[9,10] have this property. These hemagglutinating agents, in common with the hemagglutinin in egg white, albumin sac content, and normal amniotic fluid, are selective in their action, failing to agglutinate the

‡ A sample of purified ovalbumin, shown to be homogeneous by means of electrophoretic determinations carried out by Dr. G. R. Cooper, was kindly furnished by Dr. A. R. Taylor, of the Department of Experimental Surgery, Duke University, School of Medicine.

11 Burnet, F. M., *Australian J. Exp. Biol. and Med. Sc.*, 1942, **20**, 81.

12 Lush, D., *J. Comp. Path. and Therap.*, 1943, **53**, 157.

13 Levens, J. H., and Enders, J. F., *Science*, 1945, **102**, 117.

erythrocytes of certain species, and agglutinating those of others at different titers.

The resemblance in some respects of the behavior of normal amniotic fluid hemagglutinin to that of influenza virus is striking and assumes practical importance because of the value of the amniotic route of inoculation for the primary isolation of influenza virus.[1,14,15] Embryos of that degree of development in which the probability of finding normal amniotic fluid hemagglutinin is greatest are best for the isolation of influenza virus. Burnet[2] has described a form of influenza virus, the "O" form, which grows well only in the amniotic sac and which agglutinates guinea pig cells better than chicken cells. It is readily apparent that the normal amniotic fluid agglutinin, which agglutinates guinea pig cells but not chicken cells, could be mistaken for the "O" form of influenza virus if the identification were allowed to depend on the demonstration of hemagglutination in undiluted fluid and were not verified by other procedures. The phenomenon described by Burnet, however, was not due to normal amniotic fluid agglutinin. The titer of "O" influenza virus was usually higher than that of normal amniotic fluid agglutinin; in most instances there was concurrent agglutination of both guinea pig and chicken cells by "O" strains; and the strains were identified as influenza virus by serological methods. Attention should also be called to the fact that human erythrocytes of all blood groups are agglutinated by the normal amniotic fluid factor. Human erythrocytes have been routinely used for the agglutination reaction with influenza virus in some instances.[16]

It appears that the hemagglutinin, which is associated with the globulin fraction of egg white, gains access to the amniotic fluid after the rupture of the albumin sac into the amniotic sac. This accounts for the fact that amniotic fluid is free from hemagglutinin until the 12th day of development, and that those fluids containing the hemagglutinin are of increased viscosity. The thermolability of the agglutinin is consonant with the fact that it is associated with the globulin fraction. The agglutinin is adsorbed to mouse erythrocytes, which are agglutinated by it, but is not adsorbed to chicken erythrocytes, which are not agglutinated by it.

The agglutination of certain strains of *Staphylococcus aureus* by allantoic fluid from normal 10-day embryonated hen's eggs has been reported by Shrigley.[17,18] In the earlier report[17] he mentions that a limited number of observations suggest that amniotic fluid may also cause the reaction, but that albumin does not. This phenomenon therefore does not appear to bear any relationship to the hemagglutination reaction.

Summary. A hemagglutinin appears in the amniotic fluid of normal chick embryos between the 11th and 13th days of incubation. The erythrocytes of 13 of 18 species tested are agglutinated. Mouse and rabbit cells are agglutinated to the highest titer. Guinea pig cells are also agglutinated, but chicken cells are not. Hemagglutination occurs best in the cold.

The hemagglutinin is associated with the globulin fraction of egg white. It gains access to the amniotic fluid when the content of the albumin sac ruptures into the amniotic sac between the 11th and 13th days of development of the embryo.

The hemagglutinin is thermolabile. It is adsorbed by mouse but not by chicken erythrocytes.

14 Burnet, F. M., *Australian J. Exp. Biol. and Med. Sc.*, 1940, **18**, 353.

15 Hirst, G. K., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 155.

16 Personnel of Naval Laboratory Research Unit No. 1, *U. S. Naval Med. Bull.*, 1943, **41**, 114.

17 Shrigley, E. W., *Science*, 1945, **102**, 64.

18 Shrigley, E. W., *Yale J. Biol. and Med.*, 1945, **18**, 81.

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