CHARACTERIZATION OF THE AGGLUTINATING PRINCIPLE IN CHORIOALLANTOIC FLUID RESPONSIBLE FOR THE CLUMPING OF CERTAIN STRAINS OF STAPHYLOCOCCUS AUREUS¹

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Chorioallantoic fluid from fertile hens' eggs possesses the ability to agglutinate certain strains of *Staphylococcus aureus* when these organisms are grown in the presence of this material (Shrigley, 1945). It has also been found that fluids from eggs infected with the influenza virus (PR8) possess this agglutinating property in a significantly higher degree than do normal fertile egg fluids. It was felt, therefore, that the identification of this principle was of importance, as its nature might shed some light on the effect of the infectious agent on the metabolic economy of the cells involved.

The present article is a more detailed consideration and an extension of observations presented in a preliminary report concerning this phenomenon (Shrigley and Maculla, 1947).

MATERIAL AND METHODS

Most of the observations recorded below have been made on normal chorioallantoic fluids. After comparison of these with the experiments conducted with influenza-virus-infected materials, however, it was concluded that the agglutinating principles in the two fluids differ quantitatively and not qualitatively.

Chorioallantoic fluids were obtained from eggs of hens of the Rhode Island Red breed, the eggs being available from a local hatchery. Following 11 to 12 days' incubation at 103 F in a "humidaire" incubator, the eggs were placed for 2 to 3 hours at 4 C and then harvested in the manner already described (Shrigley, 1945). The material was pooled and stored in 20-ml lots at 4 C until a volume of 500 or 1,000 ml had accumulated. Under these conditions of storage the principle remained active for several months.

The strain of influenza virus (PR8) used was the same as that mentioned in the 1945 report, and in addition an egg-adapted strain of PR8 was employed.³ The methods of inoculating the eggs and the harvesting of the virus fluids were those commonly used in the cultivation of this agent, and have already been

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described in a previous paper. In all cases the presence and potency of the virus were determined by the chicken red blood cell agglutination technique as described by Hirst (1942).

Tests for the bacteria-agglutinating properties of the materials studied were conducted as previously described. Alkaline extract broth (pH 8) was used as a medium and diluent for the various fluids and their fractions under observation. To 1 ml of each of the fluid dilutions, 0.1 ml of an 8- to 10-hour culture of an agglutinable strain of *Staphylococcus aureus* was added and the mixture incubated overnight at 37.5 C. All of the tests for agglutination were made with the Craig strain (Shrigley, 1945). At times the Wood 46 strain was used as a negative control, since these organisms are not agglutinated by the principle in the chorioallantoic fluid.

The studies of the various properties of the agglutinating principle to be described below were made on crude chorioallantoic fluid as well as on fluids that had undergone concentration and partial purification.

TABLE 1

The increase in bacterial agglutination titer of chorioallantoic fluid as a result of concentration by filtration under pressure

	DILUTION OF EGG FLUIDS IN NEGATIVE POWERS OF 2										Control
	2	3	4	5	6	7	11	12	13	14	
<i>Fluid before concentration</i>											
Normal.....	++++	++++	+++	++	+	—					—
Virus-inf.....	++++	++++	++	+	—	—					—
<i>After concentration</i>											
Normal.....	++++	++++	..	..	..	..	++++	++++	++	—	—
Virus-inf.....	++++	++++	..	..	..	..	++	+	—	—	—

RESULTS

Concentration of the agglutinating principle. Of the several different methods tried in an effort to concentrate the staphylococcus-agglutinating principle in the chorioallantoic fluid, two were found to be of practical value. Lyophilization of the crude fluid followed by resuspension of the residue in one-tenth the original volume of sterile distilled water was the technique used for studies on the solubility of the principle in various fat solvents.

The second method of concentration has proved more useful. This consists of filtration of the chorioallantoic fluid under pressure through collodion membranes. Since the agglutinating principle is not dialyzable, it does not pass through this material. Fluids from several harvests were pooled to make 500- to 1,000-ml volumes, and filtration was done in the cold (4 C) under 500-mm pressure of mercury. Following this procedure, which usually took from 24 to 48 hours, the residue was dialyzed overnight in cold running tap water and then dialyzed for another 24 hours in phosphate buffer pH 7.7 at 4 C. Table 1 gives

examples of the degree of concentration of bacterial agglutinating principle that may be achieved by this method. Of the many samples tested, not one showed the presence of the bacterial-agglutinating property in the fluid that passed through the membranes.

At the lower concentrations there was a closer correlation between the degree of concentration of the fluid and the increase in bacterial agglutinin titer. On the other hand, for the more highly concentrated material it appeared that some of the activity was lost during the concentration process. This is illustrated below:

<i>Number of times concentrated</i>	<i>Number of times titer increased</i>
5	4
9	8
10	4.5
17	8
50	32
60	16
100	35
150	64

When attempts were made to concentrate larger volumes of fluid, a longer period of time was required for the concentration and therefore it is possible that more loss of activity was incurred because of the instability of the principle under these conditions.

The amount of protein nitrogen in the dialyzed concentrate varied with the degree of concentration achieved and with the amount of protein present in the original fluid. Of this original protein it was found that only a small fraction possessed the agglutinating activity. The nitrogen values in the concentrated material varied from 0.12 ± 0.04 mg per ml after a concentration of 10 times, to 0.97 ± 0.13 mg per ml after a concentration of 100 times.

In spite of the increase in the agglutination titer of the chorioallantoic fluid as measured by the Craig strain, at no time did the concentrated material agglutinate the nonagglutinable Wood 46 organisms. On the other hand, it was of interest to note that occasionally fluids that would not agglutinate Craig at a dilution of 1:4 would do so after being concentrated 10 times.

Defining the Properties of the Agglutinating Principle

Solubility in various solvents. The methods used in this study involved several procedures. Attempts were first made to extract the agglutinating principle by treating crude lyophilized material with acetone and alcohol. On the other hand, ether, being immiscible with chorioallantoic fluid, was added directly to the crude liquid. Another method consisted of applying the various cooled fat solvents to the chilled residues obtained from crude fluids that had been allowed to dry by evaporation at 37 C. Solvents were allowed to stand in contact with their respective samples for 10 minutes before separation. Following separation, all fractions were evaporated at 4 C before resuspension of the residues. The residues, both soluble and insoluble, were resuspended in sterile distilled water

if the fluids had originally been lyophilized, and in alkaline nutrient broth if they had been dried by evaporation. All extractions were made in the cold.

Observations made on highly concentrated and purified preparations of the agglutinating principle confirm in every detail those made with the crude material. The findings are consistent with the fact that the agglutinating principle is not soluble in chloroform, alcohol, ether, or acetone under the conditions of these studies.

The effect of heat. Although it has been reported that the agglutinating principle is thermolabile (Shrigley, 1945), it was considered of interest to examine more fully the rates of inactivation of this material at various temperatures. For this study 2-ml samples of crude fluid were placed in each of three tubes and all placed in a thermostatically controlled water bath. At various time intervals, depending on the temperature being studied, the tubes were removed and the contents titrated for agglutinating activity. The time periods between sampling

TABLE 2

The mean velocity constants of heat of inactivation of the agglutinating principle at various temperatures

	TEMPERATURES, C						
	46	45	44	43	42	41	40
Mean velocity constants = k	0.41	0.11	0.048	0.030	0.020	0.010	0.010
Number of observations used.....	2	5	5	5	9	4	6
k_I/k_{40} *	41.0	11.0	4.8	3.0	2.0	1.0	1.0
$\log k_I/k_{40}$	1.61	1.04	0.68	0.48	0.30	0.00	0.00

*Ratio of velocity constant for a particular temperature to the velocity constant at 40 C.

were shorter, from necessity, for the higher than for the lower temperatures. No attempt was made to keep the pH constant during these studies. It was felt that the observations on inactivation at higher temperatures possessed a larger intrinsic error than those involving less heat, since inactivation took place so promptly that it was possible the temperature of the fluid in the test tube did not reflect the true temperature of the water bath. For lower temperatures this was not a factor since at 45 C and less no samples were taken before 5 minutes after contact of the fluid with bath temperature. The temperatures studied ranged from 40 C to 46 C.

On the whole, the results from different samples of egg fluid varied considerably as to their rate of inactivation. However, it was found consistently that the agglutinating principle was quickly destroyed at 46 C and slowly inactivated at 40 C. On the other hand, the percentage of activity at various times during exposure to these temperatures was not always uniform. For example, some fluids were reduced in strength at the particular temperature in question but never became completely inactivated during the time studied. The causes of this variation may have been several. Possibly, as stated in earlier studies, the original quantity of agglutinating principle present in the crude material was a

factor in its destruction. Support for this suggestion was seen in that, in general, the concentrated, purified material seemed more resistant to heat than the crude fluid. Further, the experimental error in determining the quantity of agglutinating principle remaining after heating is an exceedingly important variable. By the method of titration used here it was arbitrarily considered that this error was as much as plus or minus one power of 2, the serial dilutions being by halves. Attempts were made to control these variables and data were obtained which, though not large in number, suggested a definite trend.

Table 2 shows the mean velocity constants for fluids heated at various temperatures. These constants were obtained by substituting in the equation⁴ describing the rate of a first-order reaction the value of the highest dilution of the heated fluid which gave agglutination of staphylococci and by contrasting it with the highest dilution of unheated fluid which caused clumping. All observations presented are on crude chorioallantoic fluid.

Figure 1 represents the relation between the logarithm of k_1/k_{40} for the various temperatures studied: k_1 is the velocity constant for the specific temperature under consideration, and k_{40} represents a similar expression for 40 C. From these determinations it is possible to obtain an approximation of the amount of heat energy necessary to inactivate the agglutinating principle. This figure was obtained from the following relationship:

$$E_A = \frac{4.58 T_1 T_2}{T_2 - T_1} \text{Log } k_2/k_1$$

where E_A represents the activation energy of the inactivation process, T_1 and T_2 the absolute temperatures studied, and k_1 and k_2 the respective velocity constants. The 4.58 is the product of the gas constant, 1.987, and the conversion factor, 2.303 (Höber, 1945). E_A was found in these experiments to be of the order of 120 kilocalories per mole.⁵

The effect of ultraviolet light. Freshly harvested, crude chorioallantoic fluid was exposed to the ultraviolet light from a mercury-arc tube at a distance of 14 cm. Three samples from the treated fluid were removed and tested, one at 30, one at 45, and one at 60 minutes. There was no evidence of any destruction of the agglutinating principle even after the 60-minute exposure.

Attempts at purification of the agglutinating principle. Pooled normal chorioallantoic fluids as well as those that were influenza-virus-infected were concentrated by ultrafiltration as described above and then subjected in the cold to fractional precipitation with ammonium sulfate. During this fractionation the pH

⁴ The authors acknowledge with thanks the help of Dr. Ernest C. Pollard in pointing out this approach to the problem of heat inactivation and in giving aid with the calculations. The velocity constants were obtained by solving for k in the equation $I/I_0 = e^{-kt}$ in which I equals the titer of the fluid after heating for a time t at a specific temperature, and I_0 is the titer of the fluid before heating, and e is the base of the Napierian system of logarithms.

⁵ For example, from the line in figure 1 we may read directly the values for $\text{Log } k_1/k_{40}$ at 46 C, and at 41 C. Substituting in the equation above we get $E_A = \left(\frac{(4.58)(319)(314)}{5} \right)$ (1.37-0.07). Solving this one gets E_A to equal 120 kilocalories per mole.

was held at 7.7. The agglutinating activity appeared to be precipitated maximally when the concentration of ammonium sulfate was at $\frac{1}{4}$ saturation. A minimal amount of the activity was occasionally present in the precipitate that was obtained when the ammonium sulfate concentration was brought to $\frac{1}{2}$ saturation. Precipitates that formed by the addition of higher concentrations of the salt did not yield any agglutinating activity. The properties of the original normal fluids and those of their various fractions may be seen in figure 2.

Figure 1. The ratio of the mean velocity constants of inactivation of the agglutinating principle at various temperatures with respect to k_{40} . The mean activation energy of inactivation equals 120 kilocalories per mole.

Although most of the observations on the chemical properties of the agglutinating substance were made on normal chorioallantoic fluid, there were also some data obtained from the study of influenza-virus-infected material. As indicated by the chicken red blood cell agglutination technique, the majority of the virus was precipitated from the concentrate by the conditions of $\frac{1}{4}$ and $\frac{1}{2}$ ammonium sulfate saturation. At $\frac{3}{4}$ ammonium sulfate saturation only a small amount of the agent was precipitated; none was recovered at complete saturation.

The effect of proteolytic enzymes on the agglutinating principle. In an effort to determine further the nature of the agglutinating principle, the effect of crystal-

line trypsin⁶ on the potency of the material was studied. As a control, the activity of the enzyme was tested by measuring its ability to hydrolyze fibrinogen. The tests were conducted at pH 7.7 by adding an equal volume of trypsin, diluted 1:1,000 by weight in phosphate buffer, to 0.5 ml of a solution of the concentrated agglutinating principle and incubating the mixture at 37 C. Samples were taken and titrations made immediately after mixing enzyme and substrate, and further samples were removed for study at 5- and 10-minute intervals for 1 hour. The

Figure 2. The summary of some properties of normal and influenza-virus-infected chorio-allantoic fluids and their fractions.

final dilution of the trypsin in these tests was 1:2,000. The enzyme was not inactivated in the various samples before they were removed for titration of the agglutinating principle, since it was feared that if this were done the agglutinating principle might also be damaged. Further, it was considered that since the dilution of the trypsin in the first tube of the titration was 1:8,000, it would be

⁶ It is a pleasure to acknowledge the generosity of Doctor Downie, of the Department of Surgery, Yale University School of Medicine, in furnishing us with crystalline trypsin.

very unlikely that the inhibiting effect observed would be the result of the enzyme acting directly on the bacteria themselves rather than on the agglutinating principle. Studies were made on crude as well as purified agglutinating principle and, on the whole, the results were in agreement. The data, however, were conclusive only when the concentrated, relatively pure principle was used.

It was found that crystalline trypsin in a concentration of 1:2,000 destroyed the agglutinating principle almost immediately on mixing, and there was definitely no ability on the part of the fluid to clump staphylococci after 5 minutes of contact with the enzyme at 37 C. The controls, which consisted of heated enzyme plus concentrated principle, and of concentrated principle alone, showed no diminution of titer during the whole experiment. Further controls were also made using crystalline enzyme plus staphylococci, and no agglutination was observed here. The studies undertaken with pepsin did not yield clear-cut results. Since crude enzyme preparations only were available, the activity of this enzyme was not studied in detail.

Migration of principle in an electric field. For the past year attempts have been made to concentrate the agglutinating principle sufficiently so that it might be studied in the Tiselius apparatus. We have reported observations on crude chorioallantoic fluid which showed that the principle migrated to the positive electrode (Shrigley and Maculla, 1947). However, the boundaries indicated, as one might expect, that the material was not homogeneous. In our apparatus it is necessary to have at least 5 mg of total protein in a volume of 5 ml in order to get a boundary. We have never been able to meet these requirements by our methods of concentration and purification.

Spectrophotometric studies of the agglutinating principle. Figure 3 illustrates the ultraviolet absorption curves of concentrated influenza-virus-infected chorioallantoic fluid, as well as the absorption spectra of various fractions from concentrated normal fluids precipitated by different amounts of ammonium sulfate. All samples were dialyzed in pH 7.7 phosphate buffer and then diluted so that they contained 0.03 mg of nitrogen per ml before testing. It can be seen that in the 275- to 280-m μ region the absorption by the concentrated, unfractionated virus-infected material dropped from a plateau which extended from 255 m μ . The absorption of the respective resuspended precipitates obtained from the $\frac{1}{2}$ and $\frac{3}{4}$ ammonium sulfate saturation of concentrated normal chorioallantoic fluid possessed similar characteristics. Special attention should be directed to the absorption curve of the resuspended precipitate recovered by the $\frac{1}{2}$ saturation with ammonium sulfate of normal, concentrated fluid. This fraction possessed the greatest amount of bacteria-agglutinating activity. From figure 3 it may be seen that there is a definite absorption in the 260-m μ region and a minimum at about 245 to 250 m μ . Although not all resuspended precipitates from this fraction showed this pattern exactly (figure 4), the majority gave a comparable picture.

Fractions of the normal chorioallantoic fluids obtained from the $\frac{1}{2}$ saturated ammonium sulfate solutions that had for one reason or another lost their ability

Figure 3. The ultraviolet absorption curves of concentrated virus-infected chorioallantoic fluid and various ammonium-sulfate-precipitated fractions of concentrated normal chorioallantoic fluid. All fluids were adjusted to 0.03 mg of N per ml. Dialysis was against pH 7.7 phosphate buffer.

Figure 4. The ultraviolet absorption curves for the fraction of normal concentrated chorioallantoic fluid precipitated by the addition of $\frac{1}{4}$ saturation of ammonium sulfate, and the effect of heating this fraction at 56 C for 30 minutes. All fluids were adjusted to 0.03 mg of N per ml. Dialysis was against pH 7.7 phosphate buffer.

to agglutinate staphylococci, were also studied spectrophotometrically. The absorption curves in the ultraviolet of these inactive samples differed from the spectra of the active ones. The outstanding alterations seen were the increase in absorption in the 245- to 250-m μ region and the decrease in the 260-m μ area. The former change is most clearly demonstrated in the experiment, illustrated in figure 4, in which the active fraction had been inactivated by heating to 56 C for 30 minutes. Other samples that had been allowed to stand at 4 C for several days, if undiluted, retained their original absorptive properties. On the other hand, if diluted with phosphate buffer pH 7.7 so that they contained 0.03 mg of nitrogen per ml and then allowed to stand at 4 C, the agglutinating activity decreased and there was alteration in the absorption patterns. Figure 4 is also presented to show that the absorption spectra of all $\frac{1}{2}$ -saturated fractions were not uniform. This is interpreted as an indication that the precipitates obtained by this procedure are not always composed of the same materials even though this fraction does always contain the agglutinating principle. Finally the supernatant of the $\frac{3}{4}$ fraction possessed almost no material that absorbed light in the ultraviolet region.

DISCUSSION

Because it was observed that the staphylococcus-agglutinating principle in the chorioallantoic fluid of hens' eggs was of greater potency in influenza-virus-infected material than in normal fluid (Shrigley, 1945), studies have been made in an attempt to characterize more fully the properties of the principle. It was hoped that a better knowledge of these properties might contribute something to an understanding of the complex processes of virus infection. Although the chemical nature of this substance responsible for the clumping of some strains of staphylococci is not known, its properties as studied are considered below.

The size of the molecule is such that it does not pass through a collodion membrane. The agglutinating principle is not soluble in chloroform, alcohol, ether, or acetone. It is destroyed rapidly by heating to 46 C and decreased in potency at 40 C. The energy involved in this process is of the order of 120 kilocalories per mole. Pollard and Forro (1949) report that the activation energy for the inactivation of T_1 bacteriophage is 73.6 kilocalories per mole, whereas Kunitz (1948) has shown it to be 57 kilocalories per mole for crystalline soybean trypsin inhibitor protein. Eyring and Stearn (1939) have presented inactivation energies for egg albumin and hemoglobin when these substances are subjected to a variety of catalytic conditions.

Ultraviolet light does not alter the activity of the agglutinating principle under the conditions of our experiments. The active substance was precipitated from concentrated dialyzed chorioallantoic fluid by $\frac{1}{2}$ saturation with ammonium sulfate. Although the biuret test for protein was negative in this fraction, it is conceivable that some protein was there, since this test is not very sensitive. Like the concentrated fluid before separation, the $\frac{1}{2}$ saturation fraction contained no carbohydrate as shown by a negative Molisch test; no urates, as the murexide test was negative and the absorption at 290 m μ was minimal; and

only a suggestion of the presence of phenolic OH groups, since the phenol reagent test yielded only a faintly positive reaction. Crystalline trypsin destroyed the agglutinating principle almost immediately on contact.

It is obvious from our studies in general and from the spectrophotometric examinations in particular that this principle varies in quantity from sample to sample. Further, the material obtained by $\frac{1}{4}$ ammonium sulfate saturation of the concentrated, dialyzed chorioallantoic fluid is not homogeneous and the quantity of associated proteins are not the same from one sample to the next. However, from the configuration of the ultraviolet absorption curve of the $\frac{1}{4}$ -saturation fraction, the compound responsible for agglutination is so constituted that when active its absorption at $245\text{ m}\mu$ is less than after it has been inactivated. In addition, the absorption in the $260\text{-m}\mu$ region, together with the action of trypsin on the principle, suggests that the principle may be protein in nature. From the observations above on the heat of inactivation, one might say that the agglutinating principle in chorioallantoic fluid is a relatively unstable molecule at temperatures over 41°C . It is not soluble in lipid solvents.

How the agglutinating principle acts, and why it is greater in influenza-virus-infected fluid, are questions that cannot be answered at present. Further, there is no explanation as to why only approximately 46 per cent of the strains of *Staphylococcus aureus* are agglutinated by this substance. The solution of all of these problems must await further investigation.

A comparison of the properties of this material with those of lysozyme emphasizes that the agglutinating principle is not related to the latter (Fleming, 1932-1933; Alderton, Ward, and Ferold, 1945). Lysozyme is considerably more stable to heat and acts best at neutrality, its activity being reduced both on the acid and alkaline side.

SUMMARY AND CONCLUSIONS

The properties of the principle in chorioallantoic fluid that agglutinates *Staphylococcus aureus* strains have been studied. This principle is not soluble in the common fat solvents, nor does it contain polysaccharide. It does contain nitrogen, and from its ultraviolet absorption curves, as well as from the fact that it is promptly destroyed by trypsin, one may conclude that the principle is protein in nature. The molecule is labile at temperatures over 41°C and requires an activation energy of 120 kilocalories per mole to inactivate its biological effect.

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