ON THE CHEMICAL NATURE OF THE FACTOR RESPONSIBLE FOR ZONE REACTIONS IN THE TUBE AGGLUTINATION TEST FOR PULLORUM DISEASE IN TURKEYS

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Walter E. Hewes, Jr.

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INTRODUCTION

This work has been done in an attempt to form at least a basis from which further studies on the nature of the factor responsible for zone reactions in tube agglutination tests can be carried out. Aside from giving some insight into the chemical nature of this factor, it is also desired to show some possible methods of removing the factor when conducting routine tube agglutination tests.

The zone, or prozone, phenomenon in various types of serological reactions has been reported on numerous occasions. It was generally assumed by the workers concerned that the zone phenomenon was due to some inhibitive substance which combined with the antigen and thus prevented the formation of the specific antigen-antibody complex. For example, Fenton and Bailey (1926), in their studies on the protective action of pneumococcus antiserum in mice infected with pneumococci, observed zone reactions. They found that in some instances larger doses of antiserum were less effective than smaller doses. They attributed this zone phenomenon to a substance, differing from the protective antibody, that was capable of paralyzing the defensive mechanisms of the host.

The exact nature of the inhibitive factor responsible for zone reactions has been the subject of

much speculation. Eisenberg and Volk (1902) attempted to explain the appearance of the zone phenomenon in agglutination reactions on the basis of Ehrlich's conception of agglutinins in which the agglutinin consisted of a haptophore and a zymophore group. The haptophore group was considered to be responsible for the specific union between the agglutinin and the antigen, and the zymophore group was considered to be responsible for agglutination. These workers assumed that in cases where zone reactions were encountered, the zymophore group was destroyed, while the haptophore group was not affected. They called this modified agglutinin an agglutinoid, and assumed further that the agglutinoid had a greater affinity for the antigen than the agglutinin and could inhibit agglutination.

Zinsser (1923) suggested that the zone phenomenon is due to the presence of a non-specific protein colloid substance which coats the antigen particle, and thus prevents the formation of the antigen-antibody complex.

Another hypothesis has been advanced by Landsteiner (1945) in which he states that the zone phenomenon in agglutination tests are due simply to the physico-chemical laws which govern the absorption of all colloidal substances and that optimum proportions of two colloids are necessary for complete agglutination

in all dilutions. If antigen or antibody is present in excess of these optimum proportions there will be an incomplete agglutination.

In recent studies on the agglutination of red blood cells by anti-Rh sera, Wiener (1944), (1945), Race (1944), Levine and Waller (1946), Hattersby and Fawcett (1947), and Diamond and Denton (1945) have shown the presence of both "complete" and "incomplete" antibodies. The "incomplete" antibodies or "blocking" antibodies, according to Wiener (1945) are univalent and are capable of combining with receptors or individual erythrocytes but will not link receptors of two or more erythrocytes, thus preventing agglutination of the red blood cells.

Diamond and Denton (1945) observed that the zone phenomenon was present only in the serum of individuals who had received repeated antigenic stimuli prior to being tested for Rh agglutination. Individuals who had not received a severe antigenic stimulation before the agglutination tests were made showed no zone reaction. These authors state, "After repeated stimulation of experimental subjects or following multiple pregnancies in some women, inhibitor or "blocking" antibody appears. This may be called the late, mature or complete antibody." ..... "The term 'early, or immature, antibody' would seem to apply to that causing agglutination in saline suspensions, whereas 'late, or mature, antibody'

is more descriptive of the 'blocking' antibody which is recognizable only in albumin or plasma media and is associated with more severe or prolonged sensitization." This concept is in direct opposition to that proposed by Wiener (1945).

Wai and Stafseth (1950) reported that zone reactions in tube agglutination tests are due to the presence of an inhibitive colloidal substance which is adsorbed by the bacterial cell and prevents reaction with agglutinins. These authors state that the colloidal substance is non-specific in its protective action.

MATERIALS AND METHODS

Twelve, four month-old, White Holland turkey hens were obtained from a "pullorum-free" flock. The birds were kept in an indoor pen previously cleaned with hot lye solution. They were kept in this same pen throughout the experiment and were thus maintained under similar conditions of management.

The antigens used in the experimental stimulation of antibody production in the turkeys, and in the tube agglutination test, consisted of three standard strains of Salmonella pullorum obtained from the U. S. Bureau of Animal Industry. These strains, designated as strains 4, 10, and 11, were classified in the Poultry Clinic as S. pullorum on the basis of morphology, motility, and biochemical reactions. They were then identified

serologically by the author according to the method of Edwards and Bruner (1942).

The antigens for the tube agglutination tests were prepared by seeding a layer of nutrient agar (Difco) in 32 ounce, screw-cap bottles with a 24 hour culture of the respective strain followed by incubation at 37°C. for 48 hours. The culture was washed off the agar with a small amount of physiological saline (0.85 per cent NaCl). This suspension was then diluted with physiological saline to an optical density corresponding to tube 2 of a McFarland Nephelometer (McFarland 1907). Phenol was used as a preservative and was added to the suspension to a final concentration of 0.5 per cent. The resulting antigens were adjusted with brom-thymol-blue pH 7.8 with 0.1 N NaOH. The three antigens were designated as 4, 10, and 11 corresponding to the three strains of S. pullorum used in their preparation.

The antigens for experimental stimulation of antibody production in the turkeys were prepared by seeding nutrient agar (Difco) slants with the three respective strains of S. pullorum. After 24 hours incubation at 37°C. the cultures were washed off the slants with a small amount of physiological saline. The suspensions were diluted with physiological saline to an optical density corresponding to tube 1 of a McFarland Nephelometer.

In the experimental work described below, the standard tube agglutination test, using constant amounts of antigen (1 ml.) and two-fold serial dilutions of antiserum, was used in all cases, The serum-antigen mixtures were incubated at 37°C. in all cases unless otherwise designated.

The results of the tests are expressed in terms of degree of agglutination as follows:

Complete agglutination--	+ + + +
Marked agglutination----	+ + +
Moderate agglutination--	+ +
Slight agglutination----	+
No agglutination--------	−

EXPERIMENTAL PROCEDURE

The turkeys were divided into four groups of three birds each, designated Group 4, Group 10, Group 11, and Control Group, the numbers corresponding to the strain of organism used in immunizing the birds.

Group 4		Group 11	
Turkey	5702	Turkey	5708
Turkey	5703	Turkey	5709
Turkey	5704	Turkey	5710

Group 10		Control Group	
Turkey	5705	Turkey	5711
Turkey	5706	Turkey	5712
Turkey	5707	Turkey	5713

On September 21, 1949 the turkeys in each group were inoculated intravenously with 1.0 ml. of a heat killed (60°C. for 1 hour) saline suspension of the strain of S. pullorum corresponding to the number of the group. The control group was not inoculated. The turkeys subsequently were inoculated intravenously according to the following schedule:

Volume of Inoculation	Days after Initial Inoculation
1	10
2	15
3	20
5	25

The turkeys were tested, by the whole blood plate agglutination method, (Stafseth 1938), using Redigen "Turkey" Antigen and Lederle Polyvalent K Antigen, at 5-day intervals after the final inoculation. At the 15th day there was only a slight agglutination and daily tests were made from this time until 19 days after the final inoculation. At this time, all turkeys showed complete agglutination by the whole blood method. The turkeys were then bled from the brachial vien.

The blood was collected in sterile bottles and allowed to clot at room temperature. After complete clotting, the blood was placed in a refrigerator at 4°C. and the sera was allowed to separate. The sera were decanted into sterile test tubes and stored at 4°C. until used. The sera from the turkeys in Group 4 thus contained anti-strain 4 S. pullorum antibodies, those from Group 10 contained Anti-strain 10 S. pullorum antibodies, those from Group 11 contained anti-strain 11 S. pullorum antibodies, and the sera from the birds in the Control Group contained no S. pullorum antibodies as confirmed by tube agglutination tests.

Since the sera from turkeys in each group showed similar titers, in respect to sera from the three birds in each group, they were pooled. This was done so that a larger number of tests might be conducted on each group of sera.

Chemical and Physical Treatment of Sera.

1. Effect of Phenol.

(a) The antisera were diluted with an equal volume of phenolated saline (2 per cent phenol by volume in 0.85 per cent NaCl solution) giving a final concentration of 1 per cent phenol by volume.

(b) The antisera were diluted with an equal volume of 4 per cent phenolated saline, giving a final concentration of 2 per cent phenol by volume.

These treated sera were placed at 4°C. for 24 hours, and the precipitate that formed was sedimented by centrifugation at 2000 rpm. for 30 minutes. The supernatant fluids were decanted and were used as antisera in the tube agglutination tests. The serum-antigen mixtures were incubated at 37°C. and results were read at 24 and 30 hours.

2. Effect of filtration.

(a) Samples of each group of sera were passed through a No. 3 Seitz EK filter. The filtrates were tested by the tube agglutination test at 37°C. Results were read at 24 and 30 hours.

3. Effect of Freezing (-37°C.)

(a) Samples of antisera were stored at -37°C. for periods of 24, 48, and 96 hours. At the end of the storage period the sera were thawed at room temperature and one sample from each group was tested for agglutinability.

(b) Another sample from each group was centrifuged at 2000 rpm. for 30 minutes.

(c) A third sample from each group was passed through a No. 3 Seitz EK filter.

All treated sera were than tested by the agglutination test at 37°C. Results were read at 24 and 30 hours.

4. Effect of using Bovine Albumin as the Diluent for Bacterial Antigens in the Tube Agglutination Test.

(a) In this experiment the phenolated antigens used for the tube agglutination tests were centrifuged at 2000 rpm. for 1 hour. The supernatant fluids were decanted and the sediments were allowed to drain. The sedimented organisms were resuspended in a 20% bovine albumin solution in distilled water to original volume.

The tube agglutination tests were conducted on all groups of sera using the bovine albumin suspensions as antigen. Incubation was at 37°C., room temperature (approximately 22°C.), and 4°C. with different samples of sera. Results were read at 18, 24, and 30 hours.

5. Effect of Absorption.

(a) Tube agglutination tests were conducted on several samples of each group of sera. Incubation was at 37°C. and at 4°C. with different samples of sera. At the end of 24 hours results were read, and all tubes were centrifuged at 2000 rpm. for 30 minutes. The supernatant fluids were decanted and retested using equal volumes of fresh antigen. These mixtures were all incubated at 37°C. and readings were made at 24 and 30 hours.

6. Effect of Heat

(a) Samples of each group of antisera were heated in a water bath at 51°C. for 1 hour, and other samples were heated at 60°C. for 1 hour. After heating, all sera were centrifuged at 2000 rpm. for 45 minutes, and standard tube agglutination tests were incubated at 37°C. and readings were made at 24 and 30 hours.

Chemical Fractionation of Antisera.

1. In this experiment, representative antisera, one showing a strong zone reaction, one showing no zone reaction, and one showing no agglutination (Control serum), were fractionated by the method of Greenburg (1929). The serum constituents (albumin, globulin, and total protein) were determined photometrically on a Cenco No. 41000 photelometer, using a green filter. This method of determination has been proved to be accurate to within 1 to 3 per cent of the results obtained by the Micro-Kjeldahl method.

2. The above experiment was repeated following absorption of the antisera with homologous antigen at 4°C. and 37°C. The absorption was carried out by mixing equal volumes of antisera and homologous antigen. After the incubation period the serum-antigen mixtures were centrifuged at 2000 rpm. for 1 hour. The supernatant fluids were then fractionated and the serum proteins determined as above.

The method of Greenberg for the determination of serum proteins is as follows:

"1. To prepare the albumin solution place 9.5 ml. of 22.2% Na_2SO_4 solution in a centrifuge tube. Add 0.5 ml. of serum and mix well. Add 3.0 ml. of ethyl ether, stopper the tube and shake vigorously for a few seconds. Stopper the tube loosely and centrifuge at moderate speed for 10 minutes. Pipette 5.0 ml. of the lower albumin solution into a 50 ml. volumetric flask.

2. To determine total protein, dilute 1 ml. of serum with 9.0 ml. of 0.85% NaCl solution and mix. Pipette 2.0 ml. of the diluted serum into another 50 ml. volumetric flask.

3. To each of the flasks add about 35 ml. of distilled water, exactly 4.0 ml. of 10% NaOH solution and 3.0 ml. of phenol reagent (Folin-Ciocalteu, 1929). Dilute to volume with water and mix well. After 5 minutes read in the photelometer using a green filter (about 520 milli-microns)."

Specificity of the Zone Phenomenon

1. To determine the "strain-specificity" of the zone phenomenon, samples of each group of antisera were used in the tube agglutination test with heterologous antigen. Serum from Group 4 was tested with strain 10 antigen, Group 10 serum was tested with strain 11 antigen, and Group 11 serum was tested with

strain 4 antigen.

2. To determine "species-specificity", samples of sera showing zone reactions were tested with Salmonella enteritidis (I, IX, XII); Salmonella gallinarum, (IX, XII); and Salmonella typhimurium var. copenhagen (IV, XII).

RESULTS AND DISCUSSION

The data presented in Tables I -- IX-C represent a summation of the results obtained from several tests conducted with each serum. These data are considered to be representative for each serum.

The preliminary serum titrations, Table I, present an interesting picture in that sera from only Group 11 show a zone of inhibition. All birds in this group present an almost identical agglutination pattern. The zone of inhibition is in practically the same position in the pattern and the end titers are about the same in all three individual sera. The sera from Group 4 and Group 10 show no zones of inhibition and have similar end titers.

There is an indication, therefore, that the antigen used to stimulate antibody production may play some role in the formation of an inhibiting factor.

Table I. Preliminary serum titrations

Source of Antisera	Antigen	Serum Dilutions										
		10	20	40	80	160	320	640	1280	2560	10240	Control
Turkey 5702	4	++++	++++	++++	++++	++++	+++	++	++	+	-	-
Turkey 5703	4	++++	++++	++++	+++	+++	++	++	+	-	-	-
Turkey 5704	4	++++	++++	++++	++++	+++	+++	++	+	+	-	-
Group 4 (pooled)	4	++++	++++	++++	++++	+++	+++	++	+	+	-	-
Turkey 5705	10	++++	++++	+++	+++	+++	++	++	+	+	-	-
Turkey 5706	10	++++	++++	++++	+++	++	+	+	-	-	-	-
Turkey 5707	10	++++	++++	++++	+++	+++	++	++	+	+	-	-
Group 10 (pooled)	10	++++	++++	++++	+++	+++	++	++	+	+	-	-
Turkey 5708	11	-	+	+	++	+	+++	+++	+	+	-	-
Turkey 5709	11	+	+	++	+++	++	+	+	++	+++	+	-
Turkey 5710	11	+	++	+++	+++	++	+	+	++	+	-	-
Control Group (pooled sera)	4,10,11	-	-	-	-	-	-	-	-	-	-	-

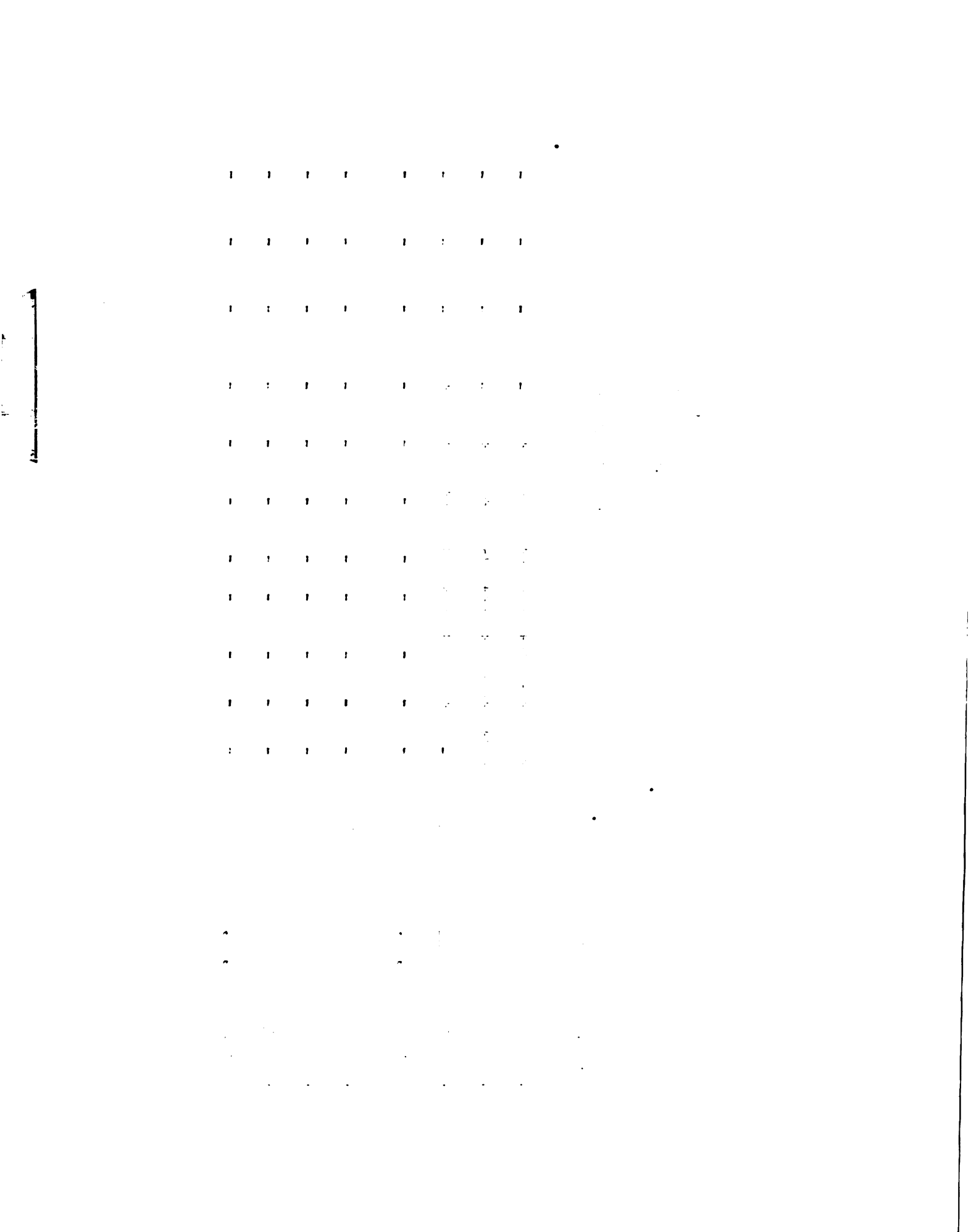
Effect of Phenolation

When pehnol was added to the sera, in a final concentration of 1 per cent, and stored at 4°C. for 24 hours, a precipitate formed. In some cases the precipitate formed immediately, while in others no precipitate was visible by macroscopic observation after several hours. Upon removal of the precipitate by centrifugation, the supernatant fluids revealed, through agglutination tests, a shift in the position of the zone of inhibition and the end titers to the left, Table II. There was a tendency to remove the zone of inhibition, while at the same time there was a reduction in the agglutinin content of the sera.

It is generally agreed, by most workers, that antibodies are present in the various globulin fractions of serum. Since the agglutinin content and the zone of inhibition are both reduced by treatment with 1 per cent phenol, there is an indication that the agglutinins and the inhibitive factor are both related in molecular structure.

Table II. Effect of phenolation and centrifugation

Antisera	Antigen	Conc. of Phenol (%)	Serum Dilutions										
			10	20	40	80	160	320	640	1280	2560	5120	Cont.
Group 4	4	1	++++	+++	+++	+++	++	++	+	-	-	-	-
Group 10	10	1	++++	++++	+++	+++	++	+	+	-	-	-	-
Group 11	11	1	-	+	+++	+++	++	++	+	+	-	-	-
Control	4,10,11	1	-	-	-	-	-	-	-	-	-	-	-
Group 4	4	2	-	-	-	-	-	-	-	-	-	-	-
Group 10	10	2	-	-	-	-	-	-	-	-	-	-	-
Group 11	11	2	-	-	-	-	-	-	-	-	-	-	-
Control	4,10,11	2	-	-	-	-	-	-	-	-	-	-	-



Effect of Filtration

It has been reported by Wai and Stafseth (1950) that zones of inhibition, in agglutination tests with S. pullorum, have been removed by filtration of the sera through a bacterial filter prior to agglutination.

As can readily be seen by comparing Tables III and I, this work could not be duplicated in the present studies. Filtration of the antisera through a Seitz No. 3 EK filter before agglutination tests resulted in practically no change in the agglutinability of the sera. Similar results were obtained by filtering sera that had been previously heated to 56°C. for 30 minutes.

If, as has been proposed by Zinsser (1923), the zones of inhibition were due to a non-specific colloid coating on the antigen, it might be expected that filtration of the serum would remove this substance and permit complete agglutination. However, since neither the inhibitive factor nor the agglutinin content were affected by filtration, it is further indicative that the two substances are similar.

Table III. Effect of filtration (#3 Seitz filter)

Antisera	Antigen	Serum Dilutions											
		10	20	40	80	160	320	640	1280	2560	5120	10240	Control
Group 4	4	++++	++++	++++	+++	+++	+++	++	++	+	-	-	-
Group 10	10	++++	++++	++++	++++	+++	++	++	+	-	-	-	-
Group 11	11	++	+++	+	+	++++	++++	+++	+++	+	+	-	-
Control	4,10,11	-	-	-	-	-	-	-	-	-	-	-	-

Effect of Freezing

Freezing at -37°C. for as long as 96 hours seems to have very little effect on either the end titers or zone of inhibition of treated sera, Table IV. Filtration and centrifugation of these treated sera did not alter the results.

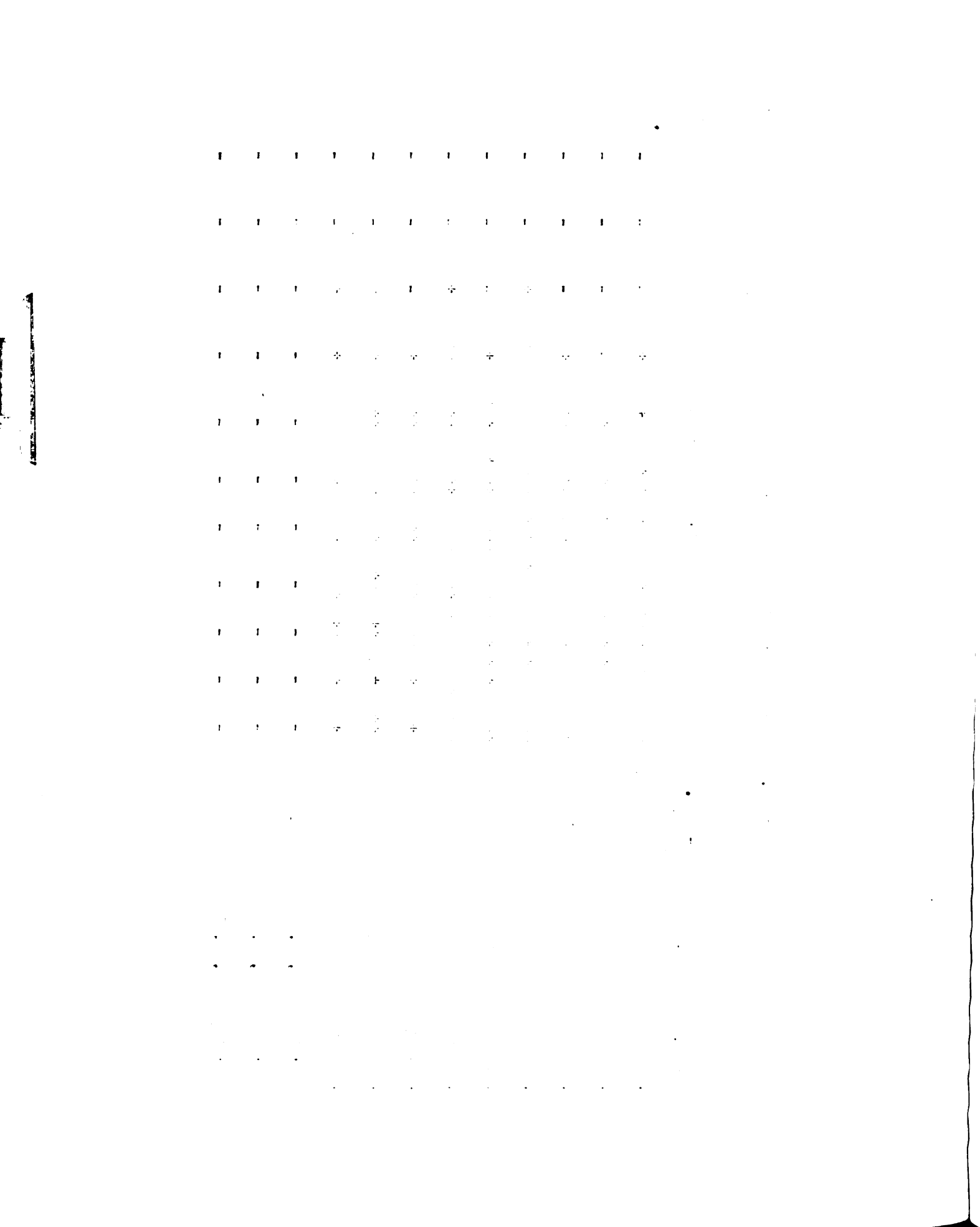
This experiment proved little except that the inhibitive factor is stable and persists after freezing and thawing. The experiment was done on the assumption that the inhibitive factor might be a non-specific colloidal protein of large molecular size which might be inactivated or denatured by freezing and thawing.

Effect of Using 20% Bovine Albumin as Diluent for the Antigens in Tube Agglutination Tests

It was reported by Diamond and Denton (1947) that certain Rh Antisera, showing no agglutination of human erythrocytes suspended in saline, did show high agglutination titers when the erythrocytes were suspended in human plasma. They further demonstrated that other substances could be used in place of plasma. Bovine albumin in concentrations of 20 and 30 per cent seemed to give the best results and there were no non-specific effects observed. From their observations they concluded that the inhibition of agglutination in saline suspensions of erythrocytes was due to the presence of "complete" antibodies. The "complete"

Table IV. Effect of freezing and centrifugation

Antisera	Antigen	Storage at -37°C. (Hours)	Serum Dilutions										
			10	20	40	80	160	320	640	1280	2560	5120	Cont.
Group 4	4	24	+++	+++	++++	++++	+++	+++	++	+	-	-	-
Group 4	4	48	++++	++++	++++	+++	+++	++	+	-	-	-	-
Group 4	4	96	++++	+++	++++	++++	+++	++	++	+	-	-	-
Group 10	10	24	++++	++++	++++	++++	++++	++++	+++	++	+	-	-
Group 10	10	48	++++	++++	++++	++++	++++	++++	+++	+	-	-	-
Group 10	10	96	++++	++++	++++	++++	++++	+++	++	++	+	-	-
Group 11	11	24	+	+	+++	+++	++	++	++	+	-	-	-
Group 11	11	48	++	+	++	++	++++	++	++	+	+	-	-
Group 11	11	96	+	+	++	+++	+++	++	++	+	+	-	-
Control	4,10,11	24	-	-	-	-	-	-	-	-	-	-	-
Control	4,10,11	48	-	-	-	-	-	-	-	-	-	-	-
Control	4,10,11	96	-	-	-	-	-	-	-	-	-	-	-



antibodies had the ability to react with the erythrocytes but lacked the ability to cause agglutination. The results obtained by these workers would seem to indicate support for the zymophore-haptophore concept. The "complete" antibodies apparently have a greater affinity for the antigen and thus prevent reaction of the "incomplete" antibodies with the antigen. The rather unusual conclusions expressed by these workers were discussed in the Introduction of this thesis. The author wishes to re-emphasize the fact that these conclusions are contrary to the more widely regarded concept in which the "incomplete" antibody is univalent and acts to inhibit agglutination.

These workers also observed that the "incomplete" antibodies were only present in serum from individuals who had received the antigenic stimulation a relatively short time prior to being tested. Serum from individuals receiving the antigenic stimulus years before being tested were found to show a high incidence of "complete" antibodies and showed inhibition of agglutination. They concluded from this observation that during the process of formation, antibodies pass through various phases. After they are completely formed, the antibodies are capable of reacting with the antigen but are incapable of causing agglutination. These workers did not attempt to explain why the presence of human blood plasma or bovine albumin prevented the

"complete" antibodies from inhibiting the action of the "incomplete" antibodies.

Table V shows that the use of 20% bovine albumin as a diluent for the bacterial antigens produced results similar to those of Diamond and Denton. The zone of inhibition was removed completely and the end titers were increased slightly.

Due to the lack of a sufficient supply of turkey blood plasma, this experiment does not include the use of bacterial antigens suspended in turkey blood plasma.

The results obtained in this experiment show only that the presence of a relatively high concentration of a colloidal protein solution, such as bovine albumin, prevents the inhibition of agglutination. An explanation of this phenomenon is not offered.

The fact that zones of inhibition of agglutination can be removed by using bovine albumin in place of 0.85% NaCl as diluent for bacterial antigens affords a practical solution to the problem presented by the occurence of false negatives in routine tube agglutination tests employing only a few serum dilutions.

Table V. Effect of using 20% bovine albumin as diluent for the antigens in tube agglutination tests

Antisera	Antigen	Temp. of Incubation	Serum Dilutions										
			10	20	40	80	160	320	640	1280	2560	5120	Cont.
Group 4	4	37°C.	++++	++++	++++	+++	+++	++	++	+	+	-	-
Group 4	4	4°C.	++++	++++	+++	+++	++	++	++	+	+	-	-
Group 10	10	37°C.	++++	++++	++++	++++	+++	+++	+++	++	++	+	-
Group 10	10	4°C.	++++	++++	++++	++++	++++	+++	+++	++	++	+	-
Group 11	11	37°C.	++++	++++	++++	++++	+++	+++	+++	++	++	+	-
Group 11	11	4°C.	++++	++++	++++	++++	++++	+++	++	++	++	+	-
Control	4,10,11	37°C.	-	-	-	-	-	-	-	-	-	-	-
Control	4,10,11	4°C.	-	-	-	-	-	-	-	-	-	-	-

The Effect of Absorption with Homologous Antigen

In 1948, Jamil and Stafseth demonstrated the removal of zones by retitration of absorbed sera after removal of bacterial cells by centrifugation.

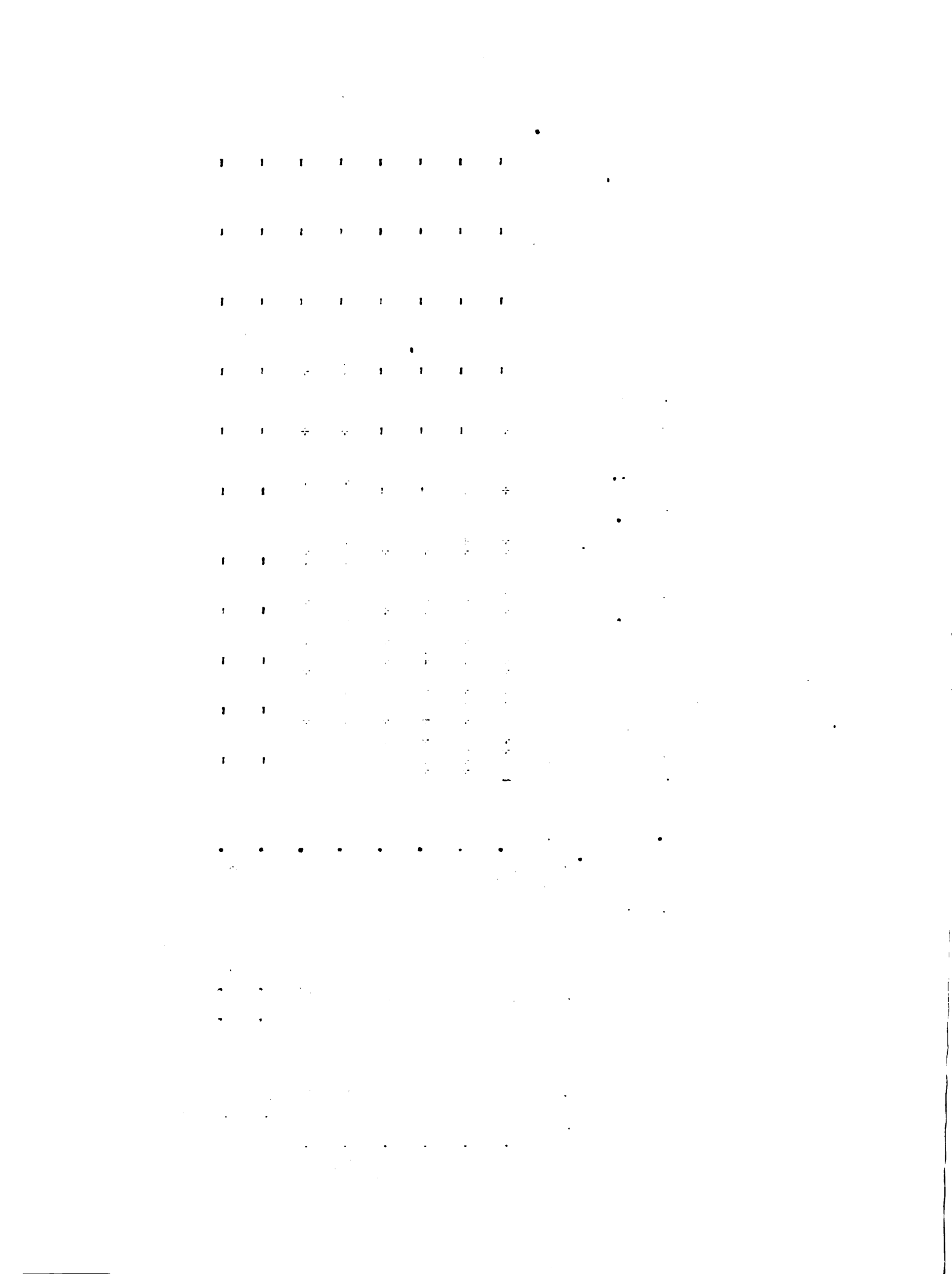
The data in Table VI show that similar results were obtained in this experiment. The temperature at which absorption was carried out had no effect on the results. The inhibitive factor was completely removed by absorption to the bacterial cells. In the tubes originally showing zone reactions, a high agglutinin content was demonstrated by retitration with fresh antigen.

The inhibitive factor apparently has a greater affinity for the antigen than do the agglutinins. This experiment also proves that the prozones are not due to the absence of optimum proportions of antigen and antibody.

The removal of prozones by absorption supports several of the theories presented in the Introduction of this work, and no definite conclusions may be offered as to the nature of the inhibitive factor.

Table VI. Retitration of antisera following absorption with homologous antigen at 4°C. and 37°C. for 24 hours

Antisera	Antigen	Temp. of Absorption Incubation	Serum Dilutions										
			10	20	40	80	160	320	640	1280	2560	5120	Cent.
Group 4	4	37°C.	++++	++++	++++	+++	++	+	+	-	-	-	-
Group 4	4	4°C.	++++	++++	+++	++	++	+	-	-	-	-	-
Group 10	10	37°C.	++++	++++	+++	++	+	-	-	-	-	-	-
Group 10	10	4°C.	++++	++++	+++	+	+	-	-	-	-	-	-
Group 11	11	37°C.	++++	++++	++++	++++	+++	++	++	++	-	-	-
Group 11	11	4°C.	++++	++++	++++	+++	+++	++	+	+	-	-	-
Control	4,10,11	37°C.	-	-	-	-	-	-	-	-	-	-	-
Control	4,10,11	4°C.	-	-	-	-	-	-	-	-	-	-	-



Effect of Heat

The effect of heat on prozones in sera was one of the first phenomena reported having a bearing on this subject. Dreyer and Jex-Blake (1906) demonstrated that by heating "normal" antisera at 70°-75°C. for a few minutes, an intermediate zone of inhibition was produced. They attributed this to the fact that the heat probably produced a substance which was capable of impeding agglutination. Shibley (1929) made further studies on the production of zones by heat treatment of antisera. He was unable to demonstrate zones of inhibition in unheated sera. Sera heated at 60°-70°C. showed zones of inhibition in the lower dilutions while agglutination occured at the higher dilutions. He also demonstrated that heating the sera at 76°C. produced no zones but did lower the end titer of the sera tested.

Spencer (1930) was able to demonstrate that, with sera heated at 56°C. for periods varying from 10 to 60 minutes, the zone of inhibition widened in proportion to the time of heating. Corpron, Bivens and Stafseth (1947) made similar observations. The end titers were decreased in the heated sera.

The following experiment was performed to substantiate these data, since the determination of the heat on zones of inhibition is a necessary and help-

ful step in determining the chemical nature of the inhibitive substance. The results shown in Table VII correlate with those obtained by other workers.

Sera showing no zones when unheated, show a definite zone of inhibition when heated at 51°C. for 1 hour. Sera showing zones when unheated, show a definite widening of the zones when heated at 51°C. for 1 hour. Heating at 60°C. for 1 hour completely inactivated the agglutinins in all sera tested.

There are several possible explanations for this phenomenon. The most reasonable one seems to be that some of the agglutinins are partially denatured by heat. These altered agglutinins seem to have the ability to react with the antigen but do not cause agglutination. The zones produced by heat seem to occur in the higher serum dilutions. That they do not completely inhibit agglutination can be explained by the dilution factor in that the numbers of unaltered agglutinins exceed the numbers of altered agglutinins.

Since some sera show zones of inhibition prior to any heat treatment, it is reasonable to assume that the inhibitive factor is similar to that produced by heating these sera. This seems to indicate, therefore, that the inhibitive factor is a partially formed agglutinin, as in the case where zones are found without treating the sera, or a partially denatured agglutinin

Table VII. Effect of Heat and centrifugation

Antisera	Antigen	Temp. of Heating for 1 hr.	Serum Dilutions										
			10	20	40	80	160	320	640	1280	2560	5120	Cont.
Group 4	4	51°C.	-	+	+	++	+++	++	+	-	-	-	-
Group 4	4	60°C.	-	-	-	-	-	-	-	-	-	-	-
Group 10	10	51°C.	-	+	+	++++	++	+++	++	+	-	-	-
Group 10	10	60°C.	-	-	-	-	-	-	-	-	-	-	-
Group 11	11	51°C.	-	-	-	+	-	-	+	+++	+++	+	-
Group 11	11	60°C.	-	-	-	-	-	-	-	-	-	-	-
Control	4,10,11	51°C.	-	-	-	-	-	-	-	-	-	-	-
Control	4,10,11	60°C.	-	-	-	-	-	-	-	-	-	-	-

as in the case where heat treatment is required to produce the zones.

On the Specificity of the Zone Reaction

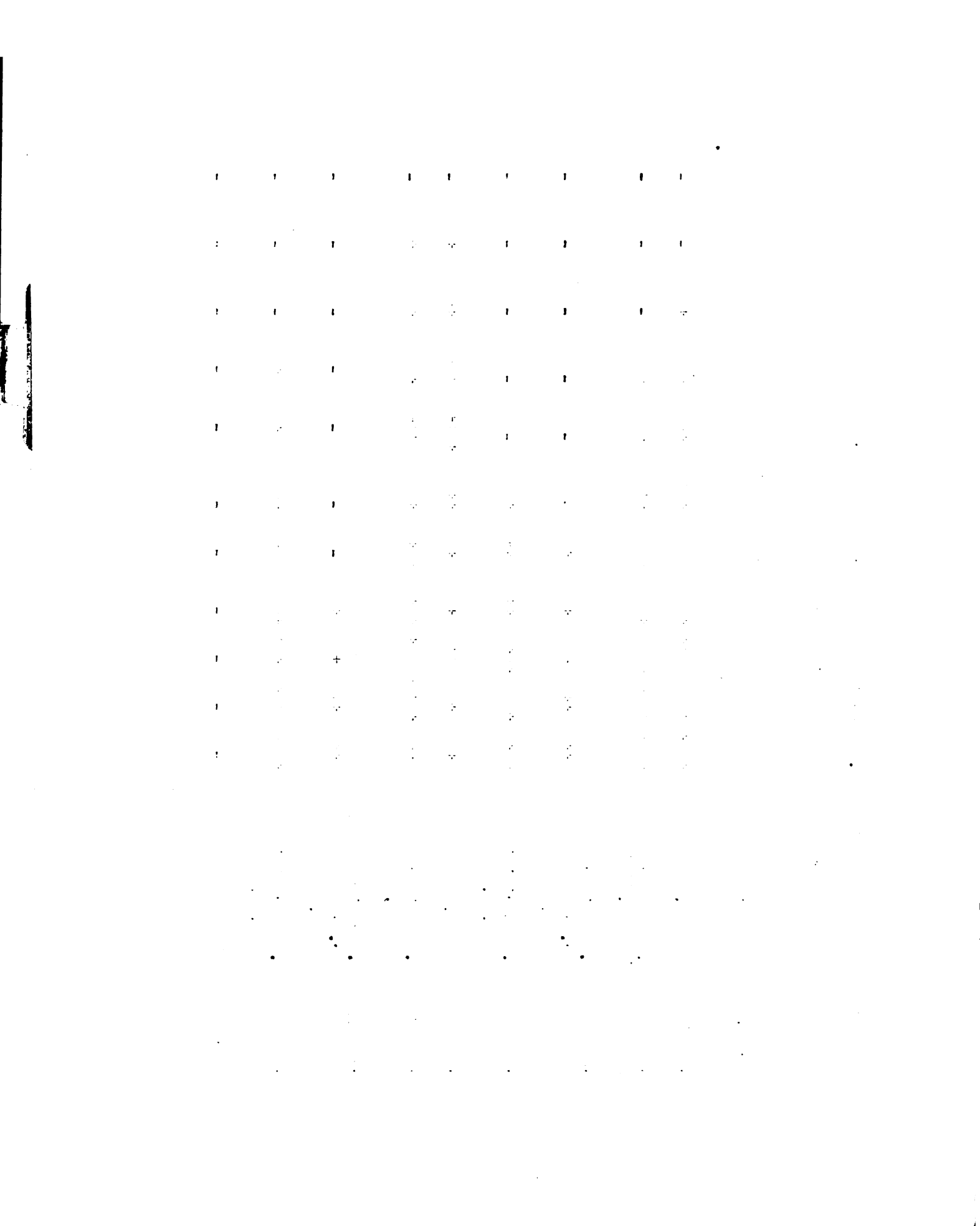
It has been reported by Zinsser (1923), Landsteiner (1942) and Wai and Stafseth (1950) that prozones are due to a non-specific agent.

The following experiment was performed in an effort to determine the specificity with respect to antigenic structure. As shown in Table VIII, the inhibitive factor was specific for the antigen used. Although some inhibition is shown when Group 11 serum reacts with S. gallinarum, the relationship between S. gallinarum and S. pullorum is so close that inhibition is to be expected. However, when S. enteritidis was used as antigen, there was no inhibition. The same result was obtained using S. typhimurium var. copenhagen as antigen, although the agglutination was much lower. This is to be expected since only the XII somatic antigen is present as the common factor.

It may be concluded from this that the inhibitive factor is specific for the antigen used to stimulate antibody production. This is further indication that the inhibitive factor is similar in molecular structure to the agglutinins.

Table VIII. Reaction of antisera with heterologous antigen to show specificity of the zone reaction

Antisera	Antigen	Serum Dilutions										
		10	20	40	80	160	320	640	1280	2560	5120	Cent.
Group 4	10 (IX,XII)	++++	++++	++++	++++	+++	+++	++	+	+	-	-
Group 4	S. gallinarum (IX,XII)	++++	++++	++++	++++	+++	++	+	+	-	-	-
Group 4	S. typhimurium var. copenhagen (IV,XII)	++	++	+	+	+	-	-	-	-	-	-
Group 4	S. enteritidis (I,IX,XII)	+++	+++	+++	++	++	+	-	-	-	-	-
Group 11	4 (IX,XII)	+	+	++	+	+	++	++++	+++	++	+	-
Group 11	S. gallinarum (IX,XII)	++	+++	++++	+++	+++	++	+++	+++	++	+	-
Group 11	S. typhimurium var. copenhagen (IV,XII)	++	++	+	+	-	-	-	-	-	-	-
Group 11	S. enteritidis (I,IX,XII)	++++	++++	+++	+++	++	++	+	+	-	-	-
Control	All of the above	-	-	-	-	-	-	-	-	-	-	-



Chemical Fractionation of Antisera

The most significant differences in Tables IX-A, IX-B, and IX-C are found in the globulin fractions of the sera. The sera showing no zone reaction (Group 4) had a relatively high serum globulin content, and after absorption with homologous antigen this serum globulin was greatly reduced. However, even after absorption, the concentration of the globulin fraction was sufficient to give an inverted albumin-globulin ratio. The normal A/G ratio is about 1.5.

In Table IX-B the antisera showing a strong zone reaction (Group 11) had low globulin content. Absorption with homologous antigen lowered the globulin content to about the same extent (0.4 grams%) as in the Group 4 sera. The albumin content was somewhat higher in the Group 11 sera than in the Group 4 and Control sera, but it is not lowered after the absorption process. The albumin fraction was not, therefore, responsible for the zone reactions since the zone was removed by absorption,but the albumin fraction was not altered. This indicated that the agglutinins found in the globulin fraction are responsible for an increase in serum globulins. The occurence of zone reactions seems to indicate the presence of partial or incomplete agglutinins as evidenced

by the low globulin content of such sera.

Summation of the results of the several experiments indicate that zones of inhibition in tube agglutination tests for pullorum disease in turkeys were found to be caused by the presence of partial agglutinins. The partial agglutinins were specific with regard to serological activity and had a greater affinity for bacterial antigens than did "normal" agglutinins. The partial agglutinins did not have the ability to cause agglutination but they did react with the antigens.

It is realized that the evidence presented here is not complete, and further work should be undertaken along this line. It is suggested that electrophoretic analysis of zone antisera and further chemical fractionation studies be carried out.

Table IX-A. Chemical fractionation of antisera, showing no zone reaction, before and after absorption with homologous antigen

Antisera	Antigen	Temp. of Absorption Incubation	Serum Dilutions										
			10	20	40	80	160	320	640	1280	2560	5120	Cont.
Group 4	4	unabsorbed	++++	++++	++++	++++	+++	+++	++	+	+	-	-
Group 4	4	absorbed at 37°C. 24 hr.	++++	++++	++++	+++	++	+	+	-	-	-	-
Group 4	4	absorbed at 4°C. 3 days	++++	++++	+++	++	++	+	-	-	-	-	-

Antisera	Treatment	Serum Protein Fractions			
		Total Proteins (grams %)	Albumin (grams %)	Globulin (grams %)	A/G Ratio
Group 4	unabsorbed	5.30	2.30	3.00	0.77
Group 4	absorbed at 37°C. 24 hrs.	4.95	2.30	2.65	0.88
Group 4	absorbed at 4°C. 3 days	4.80	2.30	2.50	0.92

Table IX-B. Chemical fractionation of antisera, showing strong zone reaction, before and after absorption with homologous antigen

Antisera	Antigen	Temp. of Absorption Incubation	Serum Dilutions										
			10	20	40	80	160	320	640	1280	2560	5120	Cont.
Group 11	11	unabsorbed	-	+	+	++	+	+	+++	+++	+	+	-
Group 11	11	absorbed at 37°C. 24 hrs.	++++	++++	++++	+++	+++	++	+	-	-	-	-
Group 11	11	absorbed at 4°C. 3 days	++++	++++	++++	+++	++	+	-	-	-	-	-

Antisera	Treatment	Serum Protein Fractions (grams %)			
		Total Protein	Albumin	Globulin	A/G Ratio
Group 11	unabsorbed	4.95	2.80	1.70	1.64
Group 11	absorbed at 37°C. 24 hrs.	4.25	2.90	1.35	2.07
Group 11	absorbed at 4°C. 3 days	4.30	2.90	1.40	2.07

Table IX-C. Chemical fractionation of antisera, showing no agglutination, before and after absorption with polyvalent antigen

Antisera	Antigen	Temp. of Absorption Incubation	Serum Dilutions										
			10	20	40	80	160	320	640	1280	2560	5120	Cont.
Control	4,10,11	unabsorbed	-	-	-	-	-	-	-	-	-	-	-
Control	4,10,11	absorbed at 37°C. 24 hrs.	-	-	-	-	-	-	-	-	-	-	-
Control	4,10,11	absorbed at 4°C. 3 days	-	-	-	-	-	-	-	-	-	-	-

Antisera	Treatment	Serum Protein Fractions			
		Total Protein (grams %)	Albumin (grams %)	Globulin (grams %)	A/G Ratio
Control	unabsorbed	5.00	2.90	2.10	1.38
Control	absorbed at 37°C. 24 hrs.	5.10	2.80	2.30	1.20
Control	absorbed at 4°C. 3 days	5.00	2.80	2.20	1.27

SUMMARY

An attempt has been made to determine the chemical nature of the factor responsible for zone reactions in tube agglutination tests for pullorum disease in turkeys.

S. pullorum antisera were prepared by immunizing three groups of turkeys with saline suspensions of different strains of S. pullorum.

These antisera were treated by various chemical and physical methods and fractionated by chemical means.

The results obtained were as follows:

1. Addition of phenol, 0.1 per cent final concentration, to samples of antisera had a tendency to remove zones of inhibition and to reduce the end titers of all sera tested.

2. Filtration of antisera, before and after heating, using a No. 3 Seitz EK Filter, did not alter the zones.

3. Freezing the antisera at -37°C. for varying periods of time and thawing at room temperature had no effect on the zones.

4. Using 20% bovine albumin as the diluent for bacterial antigens in tube agglutination tests resulted in the removal of zones of inhibition.

5. Absorption of antisera with homologous antigens resulted in the removal of zones.

6. Heating the sera at 51°C. for 1 hour produced an increase in the width of the zones and pro-

duced zones in sera that showed no zones before heating. Heating the sera at 60°C. for 1 hour inactivated all agglutinins.

7. All chemical fractionation of antisera showed that sera giving no zone reactions had a higher globulin content than did sera exhibiting strong zone reactions.

These results indicate that the inhibitive factor is a "partial" agglutinin with a greater affinity for the bacterial antigen than the affinity of the normal agglutinins.

BIBLIOGRAPHY

Corpron, R. Bivins, J. A., and Stafseth, H. J. 1947. Pullorum disease studies in turkeys. I. Poultry Sc. 26: 340-351.

Diamond, L. K., and Denton, R. L. 1945. Rh agglutination in various media with particular reference to the value of albumin. J. Lab. and Clin. Med. 30: 821-830.

Dreyer, G. and Jex-Blake, A. J. 1906. On the agglutination of bacteria. J. Path. Bact. 11:1.

Edwards, P. R. and Bruner, D. W. 1942. Serological identification of salmonella cultures. University of Kentucky Agriculture Experiment Station, Circular 54.

Eisenberg, P. and Volk, R. Quoted by Zinsser, H. 1923. Infection and resistance. 3rd. ed. The MacMillen Co., New York.

Felton, L. D. and Bailey, G. H. 1926. Biologic significance of the soluble specific substance of pneumococci. J. Infec. Dis. 38: 131.

Greenburg, 1929. A method for the determination of serum proteins. J. Biol. Chem. 82: 545.

Hattersby, P. C. and Fawcett, M. L. 1947. The prozone phenomenon in Rh blocking serums. Am. J. Clin. Path. 17: 695.

Jamil, M. and Stafseth, H. J. 1948. Pullorum disease studies in turkeys. II. Zone reactions in the tube agglutination test. Poultry Sc. 28: 571-576.

Landsteiner, M. 1945. The specificity of serological reactions. Harvard University Press, Boston, Mass.

Levine, P. and Waller, R. K. 1946. On the blocking antibody and the zone phenomenon in human anti-Rh sera. Science 103: 389.

McFarland, J. 1907. The nephelometer. J. Amer. Med. Assn. 49: 1176.

Race, R. R. 1944. An "incomplete" antibody in human serum. Nature 153: 771.

Shibley, G. S. 1929. Studies in agglutination. IV. The agglutination inhibition zone. J. Expt. Med. 50: 825.

Stafseth, H. J. 1938. The whole blood agglutination test for pullorum disease. Vet. Med. 33:1.

Wai, W. Y. and Stafseth, H. J. 1950. Pullorum disease studies in turkeys, III. Studies on the bactericidal and agglutination properties of serum and plasma of normal and pullorum infected turkeys. In press.

Wiener, A. S. 1944. A new test (blocking test) for Rh sensitization. Proc. Soc. Exptl. Biol. Med. 56: 173.

Wiener, A. S. 1945. The Rh blood types and some of their applications. Amer. J. Clin. Path. 15: 106.

Zinsser, H. 1923. Infection and resistance. 3rd ed. The MacMillen Co., New York.

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