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Studies on Heat-Inactivated Salmonella Antibodies

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STUDIES ON HEAT-INACTIVATED SALMONELLA ANTIBODIES

by

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LIFE

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CHAPTER I

INTRODUCTION AND PURPOSE

It is known that heating destroys the agglutinating ability of the O antibody. Rowe (1952) found that heating at 75 C completely destroyed the agglutinating ability, 70 C considerably reduced it, and 65 C caused a slight reduction. The literature pertaining to this subject has been reviewed adequately by Rowe.

Although the agglutinating ability of the antibody is destroyed by heat, there may still be an antigen-antibody reaction occurring. If this is so, antibody adsorbed onto the cells should be detectable by means of the Coombs Test. That is, the addition of an anti-globulin serum should bring about agglutination. In this work, an anti-rabbit-globulin serum was prepared in chickens in an attempt to detect heat-inactivated antibody. The possibility that heat inactivated antibody could be absorbed onto the cells and prevent agglutination of an unheated serum was also investigated.

The presence of incomplete antibodies in rabbit sera was also investigated by means of the Coombs Test, since this bears indirectly on the problem.

CHAPTER II

HISTORY

The earliest investigation as to what happens to heat-inactivated antibody dates back to Ehrlich (1904) and Eisenberg and Volk (1902). These workers found that heating antisera at or near 65 C caused a prozone and heating at higher temperatures resulted in a complete loss of agglutinating ability. When heat-inactivated serum was added to the antigen, and subsequently, unheated serum added, agglutination did not occur. They concluded that the heat-inactivated antibody had reacted with antigen, thus preventing agglutination with unheated antibody. Ehrlich believed that heating changed the antibody into what he called "agglutinoid", antibody that would react with antigen but would not agglutinate. He visualized the antibody as consisting of two parts, a "haptophore" group and a "zymophore" group. The "haptophore" group combined with antigen, and the "zymophore" group brought about agglutination. He believed that heating destroyed the "zymophore"group, with a resultant loss in agglutinating ability but not in reactive ability.

Kleczkowski (1941) thought that heat caused the antibody to combine with other protein substances in the serum, thus preventing agglutination. Jones (1927) believed that the antibody was actually destroyed by heat, because the heated antibody would not fix complement and would not prevent agglutination by an unheated serum; that is, it would not "block". In another

work (1928), however, he claimed that the heat-inactivated antibody would "block" unheated antibody from reacting with antigen. Felton and Bailey (1926) found that heating destroyed the agglutinating and complement-fixing ability of antipneumococcus sera but not the mouse protective ability.

Pauling's theory (1943) of the effect of heat on antibodies is based on the "lattice hypothesis" of Marrack (1938). The "lattice hypothesis" states that antigen and antibody are multivalent (two or more reactive sites) with respect to each other. Thus, when an antigen and antibody react, they have sites left to react with other antigens and antibodies. In this way, a "lattice framework" is built up, and the visible phenomenon of agglutination is due to the building up of these larger particles. Pauling believed that the antibody was bivalent, and heating destroyed one of the reactive sites, making the antibody univalent. These univalent or "incomplete" antibodies are known to exist in unheated sera, and the pertinent literature on this subject is reviewed very well by Treffers (1952).

Coombs, Mourant, and Race (1945 a) and 1945 c) have devised a method for detecting these incomplete antibodies in human Rh sera. They allow "incomplete" antibodies to react with the antigen, Rh cells. The cells are then washed, and should have incomplete antibody adsorbed on their surfaces. An anti-human serum is added, and after incubation, the cells are found to be agglutinated. A similar phenomenon was noted by Moreschi (1908). He failed to get agglutination with sheep red cells and rabbit sheep-red-cell antibodies. When a goat anti-rabbit serum was added, however, agglutination occurred. Also, Jones (1928) claimed that heat-inactivated rabbit antibodies

could be detected by this method.

Coombs (1945 b) claimed that heating Rh sera at 65-70 C destroyed the complete antibody but not the incomplete antibody. In another work (1945 c) , however, he concluded that, when complete antibody was heat-inactivated, it could be made to agglutinate by means of the Coombs test.

CHAPTER III

MATERIALS AND METHODS

1. Materials

A. Media

1. Brain Heart Infusion, Difco #B37
2. North's Gelatin Agar, Difco #B50
3. Motility Test Medium, Difco #B105
4. Blood Agar
5. Phensal: 5 ml. of 88% Carbolic acid added to 1L. of 0.9% NaCl (to make a 0.5% solution).
6. Formsal: 5 ml. of 37% formaldehyde added to 1L. of 0.9% NaCl (to make a 0.5% solution).

B. Antigens

Stock cultures are kept on Brain Heart infusion semi-solid agar.

1. H Antigen: Stc 57 (Ty H 90lw) from Salmonella Type Culture Collection as distributed from the International Salmonella center.
2. O Antigen: #3180 (Ty O 90lw) from our stock culture collection.

C. Rabbit Antisera

Prepared by the injection of Salmonella typhosa (Ty H 901w).

1. #666 glycerol preserved
2. #622 glycerol preserved
3. #663 merthiolate preserved
4. #149 merthiolate preserved
5. #3 no preservative, stored frozen

II. Methods

A. Preparation of Antigen

Stock cultures Ty H901w and Ty 0901w were streaked on blood agar plates. They were incubated at 37 C, the incubation temperature used throughout this work. After 24 hours, smooth, round, well isolated colonies were picked. The H strain was transferred to a tube of motility test medium. The O strain was transferred to a tube of North's gelatin agar. After 24 hours incubation, each strain was transferred to fresh media. Four such transfers were made. Gram stains were made on the original colonies, and both H and O showed Gram-negative rods. A small amount of Brain Heart Infusion broth was added to the last tubes of motility test medium and North's gelatin agar. These were incubated six hours and then poured into bottles containing about 250 c.c. of Brain Heart Infusion broth. Three bottles of the H antigen and three bottles of the O antigen were prepared. They were incubated for twelve hours. Then motility test media were inoculated from each bottle and incubated 24 hours. The spreading of the three H cultures appeared very distinct, whereas the growth of the O cultures appeared as a

thin line. After the motility tubes had been inoculated, an equal volume of formsal was added to the H antigen, and an equal volume of phensal was added to the O antigen. The bottles were stored at 4 C. After 24 hours, sterility tests were made by inoculating Brain Heart Infusion broths. No turbidity appeared in these tubes.

B. Titration Methods

Thirteen X 100 m.m. Pyrex tubes were used throughout. These were placed in racks holding twenty tubes. The water-bath used was manufactured by the Precision Scientific Co., Cat. #6606. It has a nearly uniform temperature in all parts.

Titrations were set up in the following manner: 0.5 c.c. of Phensal was added to each of ten tubes. 0.5 c.c. of serum was added to the first tube and mixed by drawing in and out of the pipette three times. 0.5 c.c. was carried over from the first to the second tube and mixed. This procedure was carried out through nine tubes, and 0.5 c.c. from the ninth tube was discarded. Then, 0.5 c.c. of antigen was added to all tubes. This gives serial dilutions ranging from 1:4 to 1:1024 and a control tube of antigen and saline. Whenever a different dilution of serum was desired in the first tube, a preliminary dilution was made, one-fourth of the desired dilution. Then 0.5 c.c. of the preliminary dilution was added to the first tube and treated in the usual manner. For the O agglutination tests, the tubes were allowed to stand in the water-bath for 18 hours at 51 C. Readings were rated in the following manner:

4, Large, fluffy particles

3: large particles

2: small particles, agglutination throughout

1: slight agglutination; can only be seen clearly in the concave mirror.

C. Heating of Antisera

It was found that seven minutes were required to raise the temperature of the serum to 70 C. Thus, the heating times expressed in the results indicate the total time minus seven minutes.

One c.c. of serum was pipetted into a 13 X 100 m.m. Pyrex test tube. The tube was allowed to remain unstoppered and was placed in the water-bath at the desired temperature. After heating, the tube was stoppered and allowed to cool to room temperature before titrating. The serum that was left over was stored at 4 C. and used in further titrations.

With one exception, none of the sera stored in the ice-box changed in titer. The storage time varied from 1 to 60 days. Serum #3, the unpreserved serum, was the exception. It was heated at 70 C for 25 minutes and went from a titer of 1:2560 to zero. After overnight storage in the icebox, however, the titer was found to be 1:128.

D. Preparation of Chicken Anti-Rabbit Serum

The literature shows that very few workers have employed chickens in immunological studies, and they do not generally give the details of immunization procedure. Stratton (1950) obtained an anti-rabbit serum by injecting hens intramuscularly with 5 ml. of alum-precipitated rabbit serum. Another injection was given thirty days later, and the animals were bled ten

to eighteen days after the second injection. Wolfe (1942) obtained high titered antisera by injecting chickens in the wing vein with sheep, bovine, and buffalo sera. Three injections were given on alternate days, the total dosage varying from 1.75 to 4.3 ml. The chickens were bled from the heart eight to ten days after the last injection. In this work, the procedure of Wolfe was followed. Goodman, Wolfe, and Norton (1951) found that eight per-cent NaCl was optimum for the reaction of chicken precipitin. When testing chicken sera for Salmonella antibodies, it was found in this work that eight per cent NaCl increased the titer one or two tubes.

The antigen used in this work was whole, normal rabbit serum. It was obtained by bleeding rabbits from the heart. The rabbit blood was placed in a flask tilted at a 45 degree angle, so that the clot would form at one side of the flask. After the clot had formed, the flask was tilted in the opposite direction and placed in the 4 C icebox. This allows the serum to drip to the opposite side of the flask. The next day, the serum was poured into serum bottles and kept in the deep-freeze until ready for use.

Two Plymouth Rock roosters, weighing approximately one kg. each, were injected with the rabbit serum. All injections were made in the main branch of the wing vein. Each chicken received three injections of one c.c. each, given on alternate days. Ten days after the last injection, the chickens were bled from the heart, under ether anaesthesia. When attempts were made to obtain serum by the same method as that used for the rabbits, the serum failed to drip from the clot. So, the clot was broken up with an applicator stick and centrifuged. The serum was poured off and stored in the

deep-freeze.

E. Chicken Precipitin Tests

The anti-rabbit precipitin content of the chicken sera was determined by ring precipitin tests. 0.1 c.c. portions of the antigen, normal rabbit serum, were layered over 0.1 c.c. portions of undiluted chicken serum. The antigen was diluted by tens from 1:10 to 1:100,000 with 0.9% NaCl. The control consisted of antigen layered over saline. The titer was taken as the highest dilution of antigen showing a ring at the junction of antigen and antibody. Rings formed within a few minutes, and no change occurred after one hour at room temperature and overnight in the icebox. As shown in Table XIII, each of the two chicken sera had a titer of 1:10,000.

Also used in these experiments, was a chicken anti-rabbit serum obtained from the Wisconsin Alumni Research Foundation. It was found to have a titer of 1:20,000, when using 0.9 per cent saline.

In these precipitin tests, it was found that rabbit antiserum, heated at 71° for forty minutes, (and no longer reacting as an antibody) was as good an antigen as normal rabbit serum.

F. Coombs Tests

The usual procedure was as follows: The O antigen and rabbit serum were set up as for an ordinary agglutination test. They were allowed to react in the 51 C water-bath for one hour or twenty-four hours ("sensitization period"). The tubes were then centrifuged for fifteen minutes at 4,000 R.P.M. The supernatant was discarded, and the cells were resuspended in 1 c.c. of 0.9 per cent NaCl. Three such centrifugations were carried out, after which,

the cells were resuspended in 0.5 c.c. of saline, and 0.5 c.c. of chicken antiserum was added to each tube. Controls consisted of "unsensitized" antigen plus chicken serum and, when the chicken serum was serially diluted, "sensitized" antigen alone. Then, the tubes were incubated at 51 C for 18 hours and read in the usual manner.

When heat-inactivated sera were tested, the titer after heating was determined, and then serial dilutions past the titer were allowed to sensitize antigen. In the final stage of the test, chicken serum was added in a dilution of 1:8 (final). The control consisted of 1:8 chicken serum and "unsensitized" antigen. Then, the test was repeated with certain variations. Instead of serial dilutions past the titer, a dilution between the titer before and after heating was chosen. The antigen-antibody test was then set up in the usual manner, but with each tube containing this dilution of rabbit serum. In the final stage of the test, chicken serum was added in serial dilutions ranging from 1:4 to 1:1024 (final). The control was the same as the other tubes, but without chicken serum.

When unheated sera were tested, several serial dilutions past the titer were allowed to react with antigen. The test was carried out in the usual manner with 1:8 chicken serum. Then, the test was varied as with heated serum. A dilution three or four tubes past the titer was chosen. 0.5 c.c. portions of antigen were added to 0.5 c.c. portions of this dilution of rabbit serum. After the cells were washed in the usual manner, serial dilutions of chicken serum were added (1:4 to 1:1024).

It was found necessary to absorb Salmonella antibodies from the

chicken serum. This was done by allowing the chicken serum to react with Salmonella antigen for one hour at 51° C. The serum and cells were centrifuged for fifteen minutes at 3,000 R.P.M., and the cells were discarded. This procedure removed all visible traces of Salmonella antibody.

G. Experiments on the Prozone

Serum #3, the unpreserved serum, was heated at 65 C for 25 minutes. It was found to have a prozone of four tubes, i.e. 1:4 to 1:32. Whether this is due to excess antibody or a modified antibody, the prozone, theoretically, should agglutinate in the Coombs test. To test the validity of all the negative results, it was decided to try the Coombs test on the prozone. This would show that the chicken anti-rabbit serum could cause agglutination, and could do so under the conditions used throughout all the experiments.

The four tubes in the prozone were centrifuged and washed in the usual manner. The cells were resuspended in 0.5 c.c. of 0.9 per cent saline, and no agglutination could be observed at this time. To each tube, was added 0.5 c.c. of a 1:8 (final) dilution of chicken serum. Another set of tubes was run simultaneously as a control. These received no chicken serum. The tubes were then incubated at 51 C for eighteen hours and read in the usual manner.

H. Blocking Tests

Serum #663 was heated at 70 C for thirty minutes and dropped from a titer of 1:640 to 1:64. Serial dilutions ranging from 1:128 to 1:1024 were allowed to "sensitize" the antigen for one hour at 51 C. Then, the cells were centrifuged at 4,000 R.P.M. for twenty minutes. The supernatant was

discarded, and cells were resuspended in 0.5 c.c. of phensal. 0.5 c.c. of a 1:50 dilution of unheated #663 was added to each tube, giving a final dilution of 1:100. As a control, 0.5 c.c. of the 1:50 unheated serum was added to 0.5 c.c. of "unsentitized" antigen. The tubes were then incubated at 51 C for eighteen hours and read in the usual manner. The test was repeated using a 1:320 dilution of unheated #663 in place of the 1:1000.

CHAPTER IV

RESULTS

I. Coombs Tests

A. Heat-Inactivated Sera

The addition of a chicken anti-rabbit serum to cells which had been treated with heat-inactivated antibody did not bring about agglutination.

Serum #666 had been inactivated from a titer of 1:1280 to 1:64. No antibody could be detected by the Coombs method in dilutions past 1:64. This was true whether sensitization of the cells was carried out for one hour at room temperature, one hour at 51 C, 24 hours at 51 C, or one hour at 37 C plus sixteen hours at room temperature. Also, when cells were treated with 1:260 dilutions of heated #666, no agglutination occurred upon the addition of dilutions of chicken serum ranging from 1:4 to 1:1024.

Serum #149 (titer of 1:32) was completely inactivated by heating at 65 C. No antibody could be detected in this serum by the Coombs method. Also, serial dilutions of chicken serum ranging from 1:4 to 1:1024 could not bring about agglutination when tested against the 1:16 dilutions of Serum #149.

Serum #663 was inactivated from 1:640 to 1:20. Dilutions past 1:20 were tested with 1:8 chicken serum. Also, 1:256 dilutions were tested with

serial dilutions of chicken serum. In both cases, agglutination failed to occur.

Serum #622, which had a titer of 1:256, was completely inactivated by heating at 70 C for forty minutes. A 1:8 dilution of chicken serum failed to detect any antibody, as did serial dilutions of chicken serum tested with 1:64 dilutions of #622.

Serum #3, having a titer of 1:2560, was completely inactivated by heating at 70 C for 25 minutes. After one week's storage in the icebox, a titer of 1:128 was found. The Coombs test was carried out on serial dilutions past 1:128. No antibody could be detected.

B. Unheated Sera

All the sera were tested in the unheated state also. In each case, several dilutions past the titer were tested with 1:8 chicken serum. Serial dilutions of chicken serum ranging from 1:4 to 1:1024 were tested with 1:4000 dilutions of Serum #666 (titer of 1:1280). In no case did agglutination occur.

C. Prozone Experiments

As can be seen from Table X, strong agglutination occurred in the prozone upon the addition of 1:8 chicken serum. The tubes receiving no chicken serum, however, remained negative. It was found that the chicken serum would work in this case when diluted up to 1:128.

II. Blocking Tests

Serum #663 was inactivated from 1:640 to 1:64. Dilutions ranging from 1:128 to 1:1024 failed to block the agglutination of 1:100 unheated

#663. These dilutions also failed to block the agglutination of 1:320 unheated serum. Since 1:320 is just one tube below the original titer, this shows that the heat-inactivated serum doesn't even have a slight blocking ability.

CHAPTER V

DISCUSSIONS AND CONCLUSIONS

I. Heat-Inactivated Antibody Does Not React With Antigen.

The results show that heat-inactivated rabbit antibody gives no visible reaction by either the Coombs test or blocking tests. When the Coombs test was applied to the prosone, however, very strong agglutination occurred. This shows that, if heat-inactivated antibody had been adsorbed onto the cells, the method and the anti-globulin serum used would have detected it.

Certain workers have concluded that heat-inactivated antibody can still react with antigen. Jones (1928), for example, claimed that heat-inactivated antibodies could be detected in rabbit Salmonella and Brucella sera by means of blocking and anti-globulin tests. His conclusions, however, were based on titer differences of one or two tubes, which can hardly be considered significant. Coombs, et. al. (1945 b) believed that heat-inactivated antibody could be detected by means of the anti-globulin test. He heated a human Rh serum at 65 C for ten minutes. After this, the serum would no longer agglutinate by the ordinary method, but agglutination occurred when the Coombs test was applied. The evidence in the literature, however, suggests that the Coombs test was detecting a heat-stable incomplete antibody. To understand this, it will be necessary to review the results of other workers who studied the Coombs test:

Morgan and Schutze (1946) applied the Coombs test to human Shigella and Salmonella anti sera and found that the titers by the Coombs method were larger than those obtained by the ordinary agglutination test. The degree of amplification was not the same for all the samples tested and varied from two to 64 times. They believed that these differences in amplification occurred because the serum contained complete and incomplete antibody in different proportions. Thus, a serum containing eight times as much incomplete antibody as complete antibody would be amplified eight times by the Coombs test, i.e. three tubes. Stewart and McKeever (1950) also favored this view. Working with human Salmonella antisera, they found a wide variety of titer amplifications by the Coombs test. They believed these amplifications were given by incomplete antibody, and the differences in amplifications were due to different ratios of incomplete to complete antibody. This is demonstrated more clearly in the work of Jones and Wilson (1951). Out of nine human Brucella antisera tested, they found three that were not amplified at all by the Coombs method, while the others were amplified from two to 128 times. Presumably, the three sera showing no increase in titer did not contain any incomplete antibody, or the amount was less than the amount of complete antibody. Thus, the evidence indicates that the increase in titer by the Coombs test is due to the presence of an incomplete or"univalent"antibody. It also indicates that sera contain varying proportions of incomplete and complete antibody.

Another important fact to consider is that the incomplete antibody is heat-stable. Morgan and Schutze (1946) heated one of their Shigella anti-

sera for twenty minutes at 65 C. Before heating, the serum had an agglutination titer of 1:50 and a Coombs titer of 1:800. After heating, the ordinary agglutination test was negative, but the Coombs titer stayed approximately the same. From this experiment, it can be concluded that the 1:800 titer was given by incomplete antibody, and this could still react in a Coombs test after heating. The complete antibody, on the other hand, was probably destroyed by the heating. There is a possibility, however, that heating changed the complete to incomplete antibody, and it could still react in a Coombs test. If this change actually occurred, however, it would be hidden because of the higher proportion of incomplete to complete antibody.

Coombs, et.al. (1945 c) found that the incomplete antibody was even resistant to 70 C. He also found that heating could not change complete to incomplete antibody. In another work, however, (1945 b) he takes the opposite view; that heat-inactivated antibody will react in a Coombs test. He heated an Rh serum which he claimed contained only complete antibody. After heating, the serum showed no visible reaction in the ordinary agglutination test but was made to agglutinate by means of the Coombs test. Now, although he said that this serum contained only complete antibody, there may have been incomplete antibody present in a smaller amount than complete antibody. Heating this serum, then, would destroy the complete antibody, leaving incomplete antibody to react in the Coombs test. It seems safe to conclude this, since the rest of the evidence, including Coombs' own experiments, indicates that the incomplete antibody is heat-stable, and complete antibody cannot be changed to incomplete antibody by heating.

Further evidence of the heat-stability of the incomplete antibody is offered in the work of Diamond and Abelson (1945). They found that heating at 56 C for fifteen hours inactivated the anti-Rh agglutinins but left the incomplete antibodies relatively unaffected.

Felton and Bailty (1926) found that heat-inactivated antipneumococcus sera could still protect mice. Eisenberg and Volk (1902) and Ehrlich (1904) claimed that heat-inactivated antibody, when added to its corresponding antigen, would prevent agglutination of the unheated serum. In these cases, too, the results may have been due to incomplete antibody that was already present in the sera.

II. Incomplete Antibodies

The results suggest that the rabbit does not produce incomplete or univalent antibodies against Salmonella.

When the Coombs test is applied to human sera, it is found that the titers are amplified, and the degree of amplification is different in each sample. If the incomplete antibodies are present in a higher proportion than the agglutinating antibodies, this would account for the increase in titer by the Coombs method. Some human sera show no agglutination at all, until the Coombs method is applied: Coombs, et.al. (1945 a), Schuhardt, Woodfin and Knolle (1951), Wagner and Kuhns (1953). The Coombs test seems to be the best means available for demonstrating incomplete antibodies.

In this work, no amplification of titer was found when the Coombs test was applied. The Prozone, however, agglutinated strongly in the Coombs test. In view of the findings of other workers, it would be difficult to

explain this without admitting that there were no incomplete antibodies present. Now, if incomplete antibodies were present in smaller amounts than agglutinating antibodies, heat-inactivation should reveal their presence, since the incomplete antibody is resistant to 70 C (Coombs, et.al. 1945 c). In none of the sera tested, could incomplete antibody be detected after heating.

These experiments do not show, necessarily, that the rabbit is unable to produce incomplete antibody against Salmonella. Its capacity for doing so may be poor, and some samples of rabbit Salmonella antiserum might show this type of antibody.

Morgan and Schutze (1946) claimed that rabbit Shigella sera were amplified up to 32 times by the Coombs method, indicating the presence of incomplete antibody. In their work, however, they used a poor immunization procedure which produced sera with extremely low titers. Possibly, incomplete antibodies may be produced more readily under such circumstances, but so far, no experiments have been devised to demonstrate the effect of immunization procedure on incomplete antibody production. The evidence of other workers, however, suggests that the rabbit has a generally poor capacity for producing incomplete antibody.

Coca and Kelly (1921) found an antiserum against Hemophilus influenzae that would not agglutinate but would block agglutination. This seems to be the only report of a rabbit serum containing only incomplete antibody. This is found very often, however, in human sera. Sherman, Mensel and Seebohm (1950) found that the rabbit would produce incomplete antibody against alum-

precipitated egg albumin but not against the redissolved egg albumin. Heidelberger and Kendall (1935) also found incomplete rabbit antibodies against egg albumin. Kabat and Benacerraf (1949) found them against ovalbumin. The incomplete antibody in these cases, however, constituted only twenty to thirty per cent of the total antibody. In most of the human sera tested, incomplete antibody has been found to exist in larger amounts than complete antibody.

It is interesting to speculate upon the poor capacity of the rabbit to produce incomplete antibodies. Many workers think the incomplete antibody is the one involved in hypersensitivity, because the antibody in these cases usually will not give a precipitate with antigen. Kabat and Benacerraf (1949) and Sherman, et.al. (1950) showed that incomplete antibody alone could passively sensitize as well as a mixture of complete and incomplete. No one, however, has shown definitely that precipitating antibody by itself can passively sensitize. Sherman, et.al. believed that the incomplete antibody is the one involved in sensitization. This would suggest, then, that an animal having a poor capacity to produce incomplete antibodies could not be sensitized readily, and the rabbit actually is difficult to sensitize (Treffers, 1952). This, however, is highly speculative. The exact role of the incomplete antibody in hypersensitivity has not been determined yet.

III. Suggestions for Further Studies

It would be interesting to determine whether there actually is any correlation between incomplete antibody production and hypersensitivity. Man, in whom hypersensitivity is common, has been shown to produce incomplete antibodies very well (Morgan and Schutze, 1946) (Stewart and McKeever, 1950)

(Jones and Wilson, 1951). Buxton (1952) showed that chickens are also good producers of incomplete antibody, and Makinodan, Wolfe, Goodman, and Ruth (1952) showed that chickens are easily sensitized. Rabbits, on the other hand, are difficult to sensitize, and the observations reported here indicate that they are poor producers of incomplete antibody. Following this line of thought, experiments may show that the guinea pig, which is very easily sensitized, produces incomplete antibodies well. Also, the rat, which is refractory, may be unable to produce incomplete antibodies.

It would also be interesting to determine the exact role of complete antibody alone and incomplete antibody alone in passive sensitization and in protective immunity. Also, the production of incomplete antibody under controlled conditions needs to be worked out.

These antibodies can be differentiated very easily by heat-inactivation, if it should be confirmed that complete antibody is destroyed by heat. Other workers have shown that incomplete antibody is heat-stable. This method would be much simpler, as well as more convincing than the serial addition of antigen method used by Kabat and Benacerraf (1949) and Sherman, et. al. (1950).

APPENDIX

GUINEA PIG PRECIPITIN FORMATION

It was thought that an anti-rabbit-globulin serum could be produced in a common laboratory animal, the guinea pig. Inspection of the literature revealed that various workers were unable to produce such an antibody.

Bordet (1899) gave guinea pigs two injections of defibrinated rabbit blood. When the guinea pig serum was mixed with rabbit serum, no cloud was produced. Hamburger (1902) also obtained negative results. Gengou (1902) gave six 4-5 c.c. injections of rabbit serum. When the guinea pig serum was mixed with rabbit serum, it gave an opalescence but no real precipitate. Furth (1925) claims to have obtained guinea-pig precipitins for heated rabbit serum by the injection of heated rabbit serum. Colwell and Youmans (1941) showed that the guinea pig has, in general, a poor capacity for producing precipitins.

It was thought that widely spaced injections might achieve the desired results. Consequently, three medium sized guinea pigs were injected intraperitoneally with normal rabbit serum, according to the following schedule:

First day: 2 c.c.

Sixth day: 3 c.c.

Eleventh day: 4 c.c.

Twelve days after the last injection, the guinea pigs were bled from the

heart under ether anaesthesia. The blood was allowed to clot at room temperature. Then, it was stored in the icebox overnight, after which, the serum was poured off and centrifuged. The serum was stored unpreserved in the deep freeze.

Ring precipitin tests were carried out with normal rabbit serum. The rabbit serum was diluted 1:10, 1:100 and 1:1,000 with 0.9% NaCl. 0.1 c.c. of each dilution was layered over 0.1 c.c. of undiluted guinea pig serum. The control consisted of saline layered over guinea pig serum. In further tests, the antigen was diluted from 1:4 to 1:64.

As shown in Table XIV, the guinea pigs produced a slight amount of precipitin. Although no rings could be observed after the tubes had stood for one hour at room temperature, overnight storage in the ice-box revealed opalescent rings above the junction. The tubes were stored in the icebox for another 24 hours, after which, a large amount of precipitate could be seen in all the tubes showing rings. The serum from one guinea pig was too cloudy to show rings, but precipitate could be seen after 48 hours storage in the ice-box. By this method, the antigen dilution titers were found to be 1:8, 1:15, and 1:32. The tubes were read again after being stored eight more days in the icebox, and precipitate was noted even in the 1:100 dilution. When used in the Coombs test, the guinea pigs' serum gave negative results.

The failure of previous workers to obtain anti-rabbit precipitins in the guinea pig may have been due solely to their method of carrying out the precipitin test. If they had read their tubes after 48 hours storage in the icebox, they probably would have seen a precipitate. Also, the widely

spaced injections used in this work may have been responsible for the development of precipitins, although very small amounts were produced. Apparently, the extremely low titers are due to the close relationship of the rabbit and the guinea pig, as well as the poor capacity of the guinea pig to produce precipitins.

SUMMARY

1. The heat inactivated antibody in rabbit serum can no longer react with antigen.

2. The rabbit has little or no capacity for producing incomplete antibodies against Salmonella.

3. Heat inactivation should be able to differentiate incomplete and complete antibody.

4. Antibody in the prozone can be made to agglutinate by means of the Coombs test.

5. The chicken produces anti-rabbit-globulin antibodies very well.

6. Guinea pigs can be made to produce a very small amount of antibody against rabbit serum.

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TABLE I

Test For Salmonella Antibodies in Chicken Serum

Description	Run	Reciprocal Dilutions of Chicken Serum									
		4	8	16	32	64	128	256	512	1024	C
Prepared chicken serum; ordinary agglutination; 0.9 % saline	1	1	0	0	0	0	0	0	0	0	0
" "	2	1	0	0	0	0	0	0	0	0	0
Chicken serum added to washed cells	1	2	1	0	0	0	0	0	0	0	0
" "	2	3	1	0	0	0	0	0	0	0	0
" " 8 % saline	1	4	3	1	1	0	0	0	0	0	0
" "	2	3	2	1	0	0	0	0	0	0	0
Absorbed chicken serum added to washed cells; 8% saline	1	0	0	0	0	0	0	0	0	0	0
" "	2	0	0	0	0	0	0	0	0	0	0
Commercial chicken serum added to washed cells; 8% saline	1	0	0	0	0	0	0	0	0	0	0
" "	2	0	0	0	0	0	0	0	0	0	0

4,3,2,1 = Relative amounts of agglutination

TABLE II

Anti Clubulin Tests With Salmonella Serum #666

Description	Run	Reciprocal Dilutions of Rabbit Serum									
		40	80	160	320	640	1280	2560	5120	10240	C
Unheated	1	4	4	3	3	2	2	0	0	0	0
"	2	4	3	3	3	2	2	0	0	0	0
		4	8	16	32	64	128	256	512	1024	C
Heated 30 min. at 72 C	1	3	3	2	2	1	0	0	0	0	0
" "	2	4	3	3	3	1	0	0	0	0	0
Cells sensitized with heated serum 40 min. at room temp.; 1:8 chicken serum	1	3	3	2	2	1	0	0	0	0	0
" "	2	4	3	2	2	1	0	0	0	0	0
" sensitized 1 hr. at 51 C	1	4	3	2	2	1	1	0	0	0	0
" "	2	4	3	2	2	1	0	0	0	0	0
" sensitized 1 hr. at 37 C plus 16 hrs. at room temp.	1	4	3	2	2	1	1	0	0	0	0
" "	2	4	3	2	2	1	1	0	0	0	0
" sensitized 24 hrs. at 51 C	1	4	4	3	2	1	1	0	0	0	0
" "	2	4	3	3	2	1	1	0	0	0	0

4,3,2,1, = Relative amounts of agglutination

TABLE II (Continued)

Anti Globulin Tests With Serum #666

Description	Run	Reciprocal Dilutions of Rabbit Serum									
		2560	5120	10240	20480	40960	81920	162840	325680	651360	C
Cells sensitised with unheated serum 40 min. at room temp.	1	0	0	0	0	0	0	0	0	0	0
" "	2	0	0	0	0	0	0	0	0	0	0
" sensitised 1 hr. at 51 C	1	0	0	0	0	0	0	0	0	0	0
" "	2	0	0	0	0	0	0	0	0	0	0
" sensitised 24 hrs. at 51 C	1	0	0	0	0	0	0	0	0	0	0
" "	2	0	0	0	0	0	0	0	0	0	0

TABLE III

Titration of Anti Globulin Activity of Chicken Serum With Serum #666

Description	Run	Reciprocal Dilutions of Chicken Serum									
		4	8	16	32	64	128	256	512	1024	C
Cells sensitized with 1:4000 dilutions of #666 for 40 min. at room temp.	1	0	0	0	0	0	0	0	0	0	0
" "	2	0	0	0	0	0	0	0	0	0	0
" sensitized 1 hr. at 51 C	1	0	0	0	0	0	0	0	0	0	0
" "	2	0	0	0	0	0	0	0	0	0	0
Cells sensitized with 1:160 dilutions of heated #666 for 1 hr. at 51 C	1	0	0	0	0	0	0	0	0	0	0
" "	2	0	0	0	0	0	0	0	0	0	0
" sensitised 24 hrs. at 51 C	1	0	0	0	0	0	0	0	0	0	0
" "	2	0	0	0	0	0	0	0	0	0	0

TABLE IV

Anti Globulin Tests With Serum #149

Description	Run	Reciprocal Dilutions of Rabbit Serum									
		4	8	16	32	64	128	256	512	1024	C
Unheated	1	4	3	1	1	0	0	0	0	0	0
"	2	4	3	2	1	0	0	0	0	0	0
Heated for 30 min. at 65 C	1	0	0	0	0	0	0	0	0	0	0
" "	2	0	0	0	0	0	0	0	0	0	0
Cells sensitized with heated #149 for 1 hr. at 51 C; 1:8	1	0	0	0	0	0	0	0	0	0	0
" "	2	0	0	0	0	0	0	0	0	0	0
" sensitized for 24 hrs. at 51 C	1	0	0	0	0	0	0	0	0	0	0
" "	2	0	0	0	0	0	0	0	0	0	0
		64	128	256	512	1024	2048	4096	8192	16382	C
Cells sensitized with unheated serum 1 hr. at 51 C	1	1	0	0	0	0	0	0	0	0	0
" "	2	1	0	0	0	0	0	0	0	0	0
" sensitized 24 hrs. at 51 C	1	1	0	0	0	0	0	0	0	0	0
" "	2	1	0	0	0	0	0	0	0	0	0

4,3,2,1 = Relative amounts of agglutination

TABLE V

Titration of Anti Globulin Activity of Chicken Serum With Serum #149

Description	Run	Reciprocal Dilutions of Chicken Serum									
		4	8	16	32	64	128	256	512	1024	C
Cells sensitized with 1:16 dilutions of heated serum for 1 hr. at 51 C	1	0	0	0	0	0	0	0	0	0	0
" "	2	0	0	0	0	0	0	0	0	0	0
" sensitized 24 hrs.	1	0	0	0	0	0	0	0	0	0	0
" "	2	0	0	0	0	0	0	0	0	0	0

TABLE VI

Anti Globulin Tests With Serum #622

Description	Run	Reciprocal Dilutions of Rabbit Serum									
		4	8	16	32	64	128	256	512	1024	C
Unheated	1	4	3	3	3	2	2	1	0	0	0
"	2	4	4	4	3	2	2	1	0	0	0
Heated 40 min. at 70 C	1	2	0	0	0	0	0	0	0	0	0
" "	2	0	0	0	0	0	0	0	0	0	0
Cells sensitized with heated serum 1 hr. at 51 C - 1:8 chicken serum -	1	0	0	0	0	0	0	0	0	0	0
" "	2	0	0	0	0	0	0	0	0	0	0

		256	512	1024	2048	4096	8192	16384	32768	65536	C
Cells sensitized with unheated serum for 1 hr. at 51 C	1	1	0	0	0	0	0	0	0	0	0
". "	2	1	0	0	0	0	0	0	0	0	0

4,3,2,1 = Relative amounts of agglutination

TABLE VII

Titration of Anti Globulin Activity of Chicken Serum With Serum #622

Description	Run	Reciprocal Dilutions of Chicken Serum									
		4	8	16	32	64	128	256	512	1024	C
Cells sensitized with 1:64 dilutions of heated serum for 1 hr. at 51 C	1	0	0	0	0	0	0	0	0	0	0
" "	2	0	0	0	0	0	0	0	0	0	0

TABLE VIII

Anti Globulin Tests With Serum #663

Description	Run	Reciprocal Dilutions of Rabbit Serum									
		10	20	40	80	160	320	640	1280	2560	C
Unheated	1	4	4	4	4	4	3	2	0	0	0
"	2	4	4	3	3	2	2	1	0	0	0
Heated 45 min. at 71 C	1	1	1	0	0	0	0	0	0	0	0
" "	2	2	1	0	0	0	0	0	0	0	0

4,3,2,1 = Relative amounts of agglutination

TABLE VIII (Continued)

Anti Globulin Tests With Serum #663

Description	Run	Reciprocal Dilutions of Rabbit Serum									
		10	20	40	80	160	320	640	1280	2560	C
Cells sensitised with heated serum 1 hr. at 51 C; 1:8 chicken serum	1			0	0	0	0	0	0	0	0
" "	2			0	0	0	0	0	0	0	0
		4	8	16	32	64	128	256	512	1024	C
Heated 30 min. at 65 C	1	0	0	1	3	2	1	0	0	0	0
" "	2	0	0	2	3	2	1	0	0	0	0
Cells sensitised with heated serum 1 hr. at 51 C; 1:8 C.S.	1							0	0	0	0
" "	2							0	0	0	0
		320	640	1280	2560	5120	10240	20480	40960	81020	C
Cells sensitised with unheated serum 1 hr. at 51 C	1	2	1	0	0	0	0	0	0	0	0
" "	2	2	1	0	0	0	0	0	0	0	0

4,3,2,1 = Relative amounts of agglutination

TABLE IX

Titration of Anti Globulin Activity of Chicken Serum With Serum #663

Description	Run	Reciprocal Dilutions of Chicken Serum									
		4	8	16	32	64	128	256	512	1024	C
Cells sensitized with 1:256 dilutions of #663 (71 C - 45 min.) for 1 hr. at 51 C	1	0	0	0	0	0	0	0	0	0	0
" "	2	0	0	0	0	0	0	0	0	0	0
" sensitized for 24 hrs. at 51 C	1	0	0	0	0	0	0	0	0	0	0
" "	2	0	0	0	0	0	0	0	0	0	0

TABLE X

Anti Globulin Tests on the Prozone of Serum #3

Description	Run	Reciprocal Dilutions of Rabbit Serum									
		20	40	80	160	320	640	1280	2560	5120	C
Unheated	1	1	2	4	4	4	4	2	1	1	0
"	2	1	2	4	4	4	4	2	1	0	0
		4	8	16	32	64	128	256	512	1024	C
Heated 30 min. at 65 C	1	0	0	0	1	3	4	4	1	0	0
" "	2	0	0	0	0	1	4	4	3	1	0
						Ag. & Sal.		Ag. & C.S.			
Cells sensitized with heated serum 1 hr. at 51 C	1	0	0	1	1	0		0			
" sensitized 2½ hrs. at 51 C	1	0	0	0	0	0		0			
" "	2	0	0	0	0	0		0			
" "; 1:8 chicken serum	1	4	4	4	4	0		0			
" "	2	4	4	4	4	0		0			

4,3,2,1 = Relative amounts of agglutination

TABLE XI

Anti Globulin Tests With Serum #3

Description	Run	Reciprocal Dilutions of Rabbit Serum									
		4	8	16	32	64	128	256	512	1024	C
Heated for 25 min. at 70 C	1	0	0	0	0	0	0	0	0	0	0
Heated serum stored for 24 hrs.	1	2	3	3	3	1	1	0	0	0	0
" " ; 1:8 chicken serum	1							0	0	0	0

TABLE XII

Titration of Anti-Globulin Activity of Chicken Serum With Prozone of Serum #3

Description	Run	Reciprocal Dilutions of Chicken Serum									
		4	8	16	32	64	128	256	512	1024	C
Cells sensitized 24 hrs. with 1:8 dilutions of heated Serum #3 (65 C)	1	4	4	3	3	1	1	0	0	0	0
" "	2	4	4	3	3	2	1	0	0	0	0

4,3,2,1 = Relative amounts of agglutination

TABLE XIII

Chicken Precipitin Tests

Description	Run	Reciprocal Dilutions of Rabbit Serum; 0.9% Saline					
		10	100	1,000	10,000	100,000	C
Chicken Serum I	1	+	+	+	+	0	0
" "	2	+	+	+	+	+	0
Chicken Serum II	1	+	+	+	+	0	0
" "	2	+	+	+	+	0	0
				10,000	20,000	100,000	C
Pooled Serum	1			+	+	0	0
" "	2			+	+	0	0
Commercial Chicken Serum	1			+	+	0	0
" "	2			+	+	0	0
		Rabbit Serum Heated at 71° C. for 40 min.					
				10,000	20,000	100,000	C
Pooled Serum	1			+	+	0	0
" "	2			+	+	0	0
Commercial Chicken Serum	1			+	+	0	0
" "	2			+	+	0	0

TABLE XIV

Guinea Pig Precipitin Tests

Description	Run	Reciprocal Dilutions of Rabbit Serum; 0.9% Saline						
		4	8	16	32	64	128	C
Guinea Pig I	1	+	+	+	0	0	0	0
" "	2	+	+	+	+	0	0	0
Guinea Pig II	1	+	+	0	0	0	0	0
" "	2	+	+	0	0	0	0	0
Guinea Pig III	1	+	+	+	0	0	0	0
" "	2	+	+	+	0	0	0	0

TABLE XV

Blocking Tests With Serum #663

Descriptions	Run	Reciprocal Dilutions of Heated Serum (Titers = 1:64					
		128	256	512	1024	2048	C
Cells sens. with heated serum 1 hr. at 51C; 1:100 unheated serum added	1	4	4	4	4	4	4
" "	2	4	4	4	4	4	4
" 24 hrs. at 51 C	1	4	4	4	4	4	4
" "	2	4	4	4	4	4	4
Cells sens. with heated serum 1 hr. at 51 C; 1:320 unheated serum	1	2	2	2	2	2	2
" "	2	2	2	2	2	2	2
" 24 hrs. at 51 C	1	2	2	2	2	2	2
" "	2	2	2	2	2	2	2

4,3,2,1 = Relative amounts of agglutination

APPROVAL SHEET

The thesis submitted by William G. Scotese has been read and approved by three members of the faculty of the Stritch School of Medicine, Loyola University.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form, and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

January 13, 1954
Date

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