ON HEAT-SENSITIVE ANTICOMPLEMENTARY BODIES IN HUMAN BLOOD SERUM. 1

BY HANS ZINSSER, M.D., AND W. C. JOHNSON, M.D.

(From the Laboratory of St. Luke’s Hospital, New York.)

INTRODUCTION.

It has long been well known that normal human and animal blood sera may, under certain conditions, contain elements which exert antihemolytic activity when added to mixtures of red blood cells, hemolytic serum (amboceptor), and complement. The work of most of the writers who have studied these bodies has revealed that normal sera, showing no evidence of antihemolytic action originally, may develop this property when subjected to heat ranging from 50° to 56° C. Such has been the experience of P. Th. Müller (1), Ehrlich and Sachs (3), Noguchi (8), and others. Once formed, moreover, this class of antihemolysins has been found, by some workers, to possess no inconsiderable degree of thermostability, Noguchi finding that 90° C. were necessary to destroy completely the inhibitory properties of such sera.

There is still another class of antihemolytic substances, however, which may appear in normal sera that have not been subjected to heat. Müller mentions the occurrence of such bodies in rabbit and guinea-pig sera studied by him, and attributes their inhibiting properties to direct anticomplementary action. Noguchi, who has studied them in greater detail in connection with his recent work on the “Serum Diagnosis of Syphilis,” states that such bodies are not present in fresh human serum, but develop gradually as the serum is preserved, and may be eliminated by heating to a temperature of 54° C. for thirty minutes.

There appear to be two distinct antihemolytic bodies, therefore, which may occur in normal sera. One of these is found in sera after heating, and is relatively thermostable. The other develops in sera on standing, and is destroyed by temperatures which are ordinarily employed for serum inactivation, 54° to 56° C.

The development of specific antibacteriolytic and antihemolytic substances in immune sera previously treated with bacteria or red blood cells, respectively, as described by Pfeiffer and Friedberger (7), and later by Sachs, may, we believe, be ignored, since Gay (5) has satisfactorily shown that the phenomena observed by Sachs did not occur when the corpuscles originally added to the serum had been sufficiently washed. Gay showed that the antihemolytic action observed by Sachs was due to complement absorption by means of specific precipitates formed between the immune serum and the alien serum carried into the mixtures with the insufficiently washed corpuscles.

The thermostable antihemolytic bodies referred to above have been extensively studied. That their inhibitory action depends upon a directly anticomplementary

1Received for publication, September 6, 1910.
mechanism, all workers agree. As to the nature of the substances themselves, however, there is much discrepancy of opinion. Müller believed that the antilytic elements were probably present in the native serum but were temporarily masked by hemolytic elements simultaneously present. When the action of the latter had been eliminated by heating, the action of the former became evident.

Ehrlich and Sachs suggest that the application of heat serves, in some cases at least (dog serum heated to 51° C.), to transform the complement into a complementoid with a higher avidity for sensitized corpuscles than that possessed by the complement itself.

Sachs, who attributed the anticomplementary action to complement deviation by free amboceptors of many varieties, assumed to be present in the serum, has apparently been conclusively answered by Gay. His view, moreover, is opposed to present experience, which seems to indicate that complement is not bound by amboceptor uncombined with its specific antigen.

Noguchi, whose studies of the thermostable antihemolysins of heated sera have been extensive, has come to the conclusion that these bodies are set free in sera as the latter are subjected to heat, more and more being liberated as the temperature is raised above 50° C. At 90° C. he believes that these bodies are either inactivated by conversion into more stable compounds or are masked by the formation of new bodies, "auxilysins," which aid hemolysis. He finds that the inhibitory substances are anticomplementary, active against alien as well as against homologous complement, and that they may be removed from the serum by digestion with red blood corpuscles, the corpuscles becoming thereby more resistant to hemolytic influence. Finally, he states that the antihemolysin, which he names "protectin," may be obtained from such sera as lipoids, or together with these, by ether extraction.

The antihemolysins occurring in unheated sera have been less extensively studied, and it was these bodies particularly to which the attention of the writers was directed.

Occurrence of the Thermolabile Antihemolytic Substances.—The development of antihemolytic substances in unheated human blood sera was noticed by the writers, as it had been by Noguchi, in the course of work with complement-fixation tests. It was observed, also, that the inhibitory bodies did not occur in fresh, active sera, but developed as the sera were preserved at room or refrigerator temperatures, and that, as stated by Noguchi, heating to 56° C. for fifteen to thirty minutes served to eliminate the antilytic powers. After a number of such sera had been studied, it was deemed desirable to ascertain the speed of development of these bodies in normal human serum (animal sera were not investigated), and a series of sixteen normal specimens were examined for their inhibitory powers at intervals of three to five days.
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In these experiments .2 cubic centimeter of the serum to be examined was added to .1 cubic centimeter of fresh guinea-pig complement, the volume was brought up to 3 centimeters with salt solution, and the mixture was allowed to stand in a water bath at 40°C. for thirty minutes. At the end of this time .1 cubic centimeter of a 5 per cent. emulsion of red blood cells sensitized with two units of amboceptor were added, the total volume being brought to 5 cubic centimeters. The usual controls were made.

By such experiments it was found that some sera, kept at refrigerator temperature (about 10°C.), would become distinctly antilytic as early as the fifth day, and many completely inhibited hemolysis at the end of two weeks. In the case of two sera not in this particular series, complete inhibitory power, for the quantities given, appeared as early as the fifth day. Other sera exhibited no trace of antihemolytic power after four and five weeks. These sera were examined at the same time for complement, and in every case where no antihemolytic substances had developed, free complement could be detected.

It is probable that these thermolabile bodies develop in most, if not in all, human sera on standing, appearing as the complement disappears, and that they are originally either not present or are masked in their action as long as traces of complement remain.

Two of the sera in which the antihemolytic power developed most rapidly and strongly were taken from patients suffering from chronic nephritis with symptoms of uremia. As this seemed significant, in view of the paper of Neisser and Döring (2), who have claimed the presence of antihemolytic substances in uremic sera, a number of tests were made with sera from similar cases and from two cases of eclampsia. No evidence was obtained, however, which justified any conclusion regarding a definite relationship between the clinical condition and these substances.

In an experiment to determine the temperature at which the inhibitory action was eliminated, sera (after dilution)2 were heated to 90°C. It was found that most of the antihemolytic action (roughly speaking, about four-fifths) had disappeared. The fact

2 In all our experiments the volumes of the mixtures were brought to five cubic centimeters with salt solution to avoid errors resulting from the inhibitory influence of high concentrations of serum, which, as shown by Bordet and Gay (10), oppose hemolysis.
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that a trace still remained would tend to show either that the ther-
mosable and thermostable antilytic bodies may be present at the
same time in the same serum or that the latter may be developed
in a serum that contained the former.

Quantitative Titration of the Thermolabile Antihemolytic Body
in Some Sera.—The strength of the antihemolytic power developed
in some of the human sera, may be seen in the following titration,
in which the serum employed was about three weeks old:

<table>
<thead>
<tr>
<th>Quantity of Serum</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2 c.c. serum &quot;W&quot;</td>
<td>No hemolysis.</td>
</tr>
<tr>
<td>0.1 c.c. serum &quot;W&quot;</td>
<td>No hemolysis.</td>
</tr>
<tr>
<td>0.05 c.c. serum &quot;W&quot;</td>
<td>Very slight hemolysis.</td>
</tr>
<tr>
<td>0.04 c.c. serum &quot;W&quot;</td>
<td>Slight hemolysis.</td>
</tr>
<tr>
<td>0.02 c.c. serum &quot;W&quot;</td>
<td>Considerable hemolysis, but incomplete.</td>
</tr>
<tr>
<td>0.005 c.c. serum &quot;W&quot;</td>
<td>Hemolysis complete.</td>
</tr>
<tr>
<td>0.0025 c.c. serum &quot;W&quot;</td>
<td>Hemolysis complete.</td>
</tr>
</tbody>
</table>

It will be seen, therefore, that quantities as small as 0.005 cubic
centimeter of this serum exerted considerable inhibiting action.

Serum "W," employed in the above experiment, had the strongest
antihemolytic action of any in our possession. As early as the
fifth day, 0.1 cubic centimeter of it sufficed to prevent completely
the hemolysis of one cubic centimeter of sensitized corpuscles.

Does the Antihemolytic Action Bear Any Relationship to Sub-
stances Derived from the Red Corpuscles?—Since a number of the
sera which showed antilytic properties were observed to be tinged
with blood pigment, owing to accidents during separation, one of the
thoughts first suggested was that the antilytic bodies might be de-
rivatives of red blood cells, given up to the serum during its separa-
tion from the clot. This seemed the more likely because of the
observations of Landsteiner and von Eisler (4), Muir (9), and
others, that certain red cell extractives did actually exert antilytic
action. A considerable number of experiments were made by the
writers with this point in view. For the sake of brevity, however,
these experiments will be omitted. It is sufficient to say that they
showed that both the shadows and products of hemolyzed red cells,
when added to a hemolytic system, lent slight antilytic action to sera.
otherwise not antilytic. These substances exerted, also, a slightly inhibitory action upon hemolysis when added without the presence of human serum. In these respects the experiments confirmed the observations of other writers. In no case, however, did this antilytic action amount to more than a definite slowing of the hemolytic reaction, or, at most, to an extremely slight permanent inhibition. It was impossible, therefore, to attribute the very marked and often complete inhibitory action of some sera, often perfectly clear, to accidental extractions from the clot. For this reason, this course of experimentation was not carried further and is not enlarged upon. It was of use only in again calling attention to the necessity for caution when doing complement fixation tests with sera containing any considerable amount of blood pigment.

**MECHANISM OF THE ACTION OF THE THERMOLABILE ANTIHEMOLYTIC BODY.**

In speaking of the thermolabile antihemolytic substance, Noguchi stated that its action was anticomplementary. As it seemed to us desirable to verify this, a number of experiments were made.

In one of these a mixture consisting of two units of anti-sheep amboceptor (1/660 cubic centimeter serum in 1 cubic centimeter salt solution), 0.2 cubic centimeter of inhibiting serum "W," and 3 cubic centimeters salt solution, was incubated for one hour at 40°C, and then after the addition of 1 cubic centimeter of a 5 per cent. emulsion of sheep corpuscles, the whole was incubated for one hour at 40°C, and separated by centrifugalization into (1) sediment of red blood cells (subsequently washed three times with salt solution), and (2) supernatant fluid.

To the washed red cell sediment were added 0.1 cubic centimeter of complement and 5 cubic centimeters salt solution. On exposing the mixture to 40°C for half an hour, the hemolysis was complete. The hemolysis was also complete in the controls. To 3 cubic centimeters of the supernatant fluid (see (2) above) were added 0.1 cubic centimeter complement and 2 cubic centimeters sensitized corpuscles (1 cubic centimeter corpuscles to which two units of amboceptor had been added). On exposing this mixture to 40°C no hemolysis occurred.

The above and similar experiments showed that the exposure of amboceptor to the inhibiting substance did not prevent amboceptor from becoming attached to the red cells subsequently added. They indicated, furthermore, that exposure of the "sensitized" cells to
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This antilytic substance did not deprive these cells of the power of subsequently absorbing complement. Neither the different receptors of the amboceptor, nor the complex of amboceptor and red cell were, therefore, in any way altered by the antilytic body, this remaining active in the supernatant fluid. The possibility of explaining the antilytic action as due to the presence of complementoid, was also disposed of by these experiments.

It remained to show that there was no reaction between the antilytic body and the unsensitized blood corpuscle rendering the latter resistant to the subsequent influence of amboceptor and complement.

To determine this point, 0.2 cubic centimeter of antilytic serum “W,” 1 cubic centimeter of washed sheep corpuscles (5 per cent. emulsion in salt solution), and 2 cubic centimeters salt solution were mixed, left for one hour at 40° C., and then centrifugalized. The sediment containing the red cells was washed four times in salt solution and then to it were added one unit of amboceptor, 0.1 cubic centimeter of complement, and 4 cubic centimeters of salt solution. On incubating at 40° C. complete hemolysis took place in half an hour (controls hemolyzed in the same length of time). This showed that the corpuscles had not been rendered less sensitive to lysis by exposure to the inhibiting serum. The supernatant fluid from the centrifugalization, containing the 0.2 cubic centimeter of the antilytic serum, was then tested and found to have lost none of its inhibiting power.

This seemed conclusive in excluding the direct action of the antilytic substance upon the corpuscles. Nevertheless, as Noguchi had found that the thermostable antilytic bodies could be absorbed by blood corpuscles if allowed to digest with them at 20° C. for about eighteen hours, it seemed desirable to determine whether the thermolabile body studied by us would be similarly absorbed. To test this question, the following experiments were made on two separate occasions, each time in triplicate.

One cubic centimeter of the antilytic serum was mixed with 4 cubic centimeters of a 5 per cent. suspension of washed corpuscles, and this mixture was left at 18° to 20° C., in one case for twenty, in another for twenty-four hours. At the end of this time the fluid and corpuscles were separated by centrifugalization. The corpuscles were then thoroughly washed, and they and the supernatant fluid were tested as in the preceding experiment.

The result showed (1) that the corpuscles had not absorbed the antilytic principle, for they had not become more resistant to lysis, and (2) that the supernatant liquid had retained its antilytic property undiminished.
As we had thus eliminated the action of the antilytic body upon the corpuscles, the amboceptor, or the corpuscle-amboceptor complex, its action upon the complement remained as the only possibility. This has been proved in a considerable number of experiments which consisted in exposing complement to the antilytic serum for varying periods, and then adding sensitized corpuscles. Complete inhibition of hemolysis has been the uniform result of such a procedure. That the anticomplementary action of the body in question is almost instantaneous, or, at least, more rapid than the attachment of complement to the antigen-amboceptor complex, was shown by the fact that complete inhibition resulted when antilytic serum, complement, and sensitized corpuscles were simultaneously poured into a test tube and immediately incubated.

It was also ascertained that this anticomplementary action could be overcome by gradually increasing the relative amount of complement in the mixtures. Thus, 0.2 cubic centimeter of the antilytic serum "W" completely inhibited hemolysis in the presence of 0.2, 0.4 and 0.6 cubic centimeter of fresh guinea-pig complement, when the cells employed were sensitized with two units of amboceptor. Eight-tenths cubic centimeter of complement, however, caused considerable hemolysis in such mixtures, and one cubic centimeter produced almost complete lysis.

The foregoing experiments thus show, in perfect agreement with the statements of Noguchi, that the mechanism of the action of the thermolabile antihemolytic body is an anticomplementary one.

It seemed of interest to make sure that this anticomplementary action was direct, and not indirect, in the sense that it might depend, (a) upon the absorption of complement by one or a number of antigen-amboceptor complexes preexisting in the serum, or (b) upon complement absorption by a precipitin reaction occurring between the antilytic serum and either the guinea-pig serum of the complement or the rabbit serum of the amboceptor.

The first supposition, namely, that the complement is deviated or absorbed by antigen-amboceptor complexes preexistent in the serum, is difficult to approach experimentally. It seems unlikely, however, that this supposition is correct, for in the experiments cited above, in which the antilytic body, sensitized corpus-
cles, and complement, in excess of the minimum quantity for complete lysis, were mixed simultaneously, absolutely no hemolysis took place. It does not seem reasonable to believe that in such a case all the complement should immediately be taken up by the pre-existing antigen-antibody complex, leaving none available for the hemolytic system. Furthermore, it must be remembered that the sera containing the antilytic principle were not inactivated and, when fresh, contained some excess complement. If antigen-antibody combinations had been present, they would, in these cases, be already supplied with complement, and their reaction complexes would be complete. Moreover, the thermolability of the inhibitory substances (which become inactive on heating to 56°C.), is also against this view.

The second suggestion, namely, that complement was deviated by precipitin reactions taking place between the antilytic serum, on the one hand, and rabbit serum or guinea-pig serum, on the other, was disproved by the following experiments:

(a) A precipitin reaction between the inhibiting serum and the rabbit serum of the amboceptor was excluded by sensitizing corpuscles and subsequently washing them very thoroughly (four times) in salt solution. The subsequent addition of antilytic serum and complement resulted in complete inhibition, showing that after the removal of all traces of rabbit serum no diminution of the anticomplementary phenomenon had taken place.

(b) The possibility of a precipitin reaction between the guinea-pig serum of the complement and the antilytic serum was eliminated by the use of complement from other sources. Thus, in the following mixtures:

1. Inhibiting serum ....... 0.2 c.c.  
   Human complement .... 0.2 c.c.  
   Salt solution ........... 3.0 c.c.  
   Left at 40°C. for 15 minutes.

2. Inhibiting serum ....... 0.2 c.c.  
   Rabbit complement ..... 0.2 c.c.  
   Salt solution ........... 3.0 c.c.  
   Left at 40°C. for 15 minutes.

3. Inhibiting serum ....... 0.2 c.c.  
   Guinea-pig complement... 0.2 c.c.  
   Salt solution ........... 3.0 c.c.  
   Left at 40°C. for 15 minutes.
The subsequent addition of sensitized corpuscles resulted in complete inhibition, while control tubes with similar varieties and quantities of complement showed hemolysis. There was one exception to this, which we did not follow in detail. One antilytic serum did not seem to oppose the action of dog complement. We mention this, in passing, without trying to explain it.

It seems reasonable, from the foregoing facts, then, to conclude that the inhibiting action of our serum was due to direct anticomplementary action, using the term merely as differentiating this action from the indirect modes of eliminating complement considered above.

**CHEMICAL NATURE OF THE THERMOLABILE ANTICOMPLEMENTARY BODY.**

Our first attempt toward the identification of this anticomplementary body with any of the serum components, was based upon Noguchi's success in identifying the thermostable anticomplementary body with the serum lipoids. In our tests, serum diluted ten times with salt solution was shaken for fifteen minutes with ether. As the ether did not readily separate from such a mixture, centrifugation was now employed, and the supernatant ether was carefully pipetted away. Re-extraction with ether in the same way was twice repeated. The serum residue was now examined for inhibiting powers, the quantities employed being similar to those which inhibited completely in the original serum. Such experiments showed that absolutely no loss of anticomplementary action had been sustained by ether extraction. It seemed unlikely, therefore, that the serum lipoids, at least those extractable with ether by this rather crude method, should account for the anticomplementary action of our serum.

In order to ascertain whether any of the dialyzable components of the serum were responsible for the anticomplementary action, ten cubic centimeters of diluted anticomplementary serum were dialyzed through parchment for forty-eight hours against frequently changed distilled water. Again, the serum residue which became turbid, probably by the precipitation of globulin in this process, showed absolutely no loss of its antilytic power.
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It remained to examine the serum proteins. Five cubic centimeters of the serum were diluted with forty-five cubic centimeters of salt solution, and an equal bulk (fifty cubic centimeters) of a saturated ammonium sulphate solution was added. The precipitated globulins were removed by repeated filtration through a triple thickness of fine filter paper. The filtrate was dialyzed against running water to remove the ammonium sulphate. The substances on the filter paper were scraped off, redissolved in an 0.85 per cent. solution of sodium chloride, and reprecipitated with ammonium sulphate. Finally, they were dried and rapidly rinsed with cold water to remove gross particles of ammonium sulphate. Allowing for losses during this process, the bulk of the globulin precipitate was taken up in thirty cubic centimeters of an 0.85 per cent. salt solution. This solution was tested in varying quantities roughly representing from 0.1 to 0.2 cubic centimeter of the original serum. Complete inhibition of hemolysis resulted. The filtrate after forty-eight hours of dialysis still contained traces of ammonium sulphate, but possessed absolutely no anticomplementary action. These tests were controlled with ammonium sulphate solutions in order to preclude the possibility of hemolysis due to the ammonium sulphate alone, and with ammonium sulphate solutions plus the original antilytic serum, to preclude the possibility of interference with the inhibiting principle on the part of the ammonium sulphate.

It appeared from these experiments that the antilytic principle had been removed from the serum with the globulins.

It now seemed desirable to ascertain whether the antilytic factor of the globulins obtained from the serum was thermolabile, as was the antilytic principle in the original serum.

To determine this point, 1 cubic centimeter of the globulin solution (representing approximately 0.1 cubic centimeter of the original serum) was placed in each of four test tubes, and in each of four others was placed 1 cubic centimeter of the original antilytic serum, after this had been diluted ten times with salt solution. Each of the tubes containing globulin was paired with one containing the antilytic serum, and the pairs were subjected to heat for twenty minutes, one to 50°C, another to 56°C, another to 60°C, and another to 70°C. To each tube was then added 0.1 cubic centimeter of fresh guinea pig complement and 2 cubic centimeters of salt solution. The mixtures were allowed to stand at 40°C for thirty minutes and sensitized corpuscles were then added.
The result showed that there was a complete parallelism between the globulin solution and the original antilytic serum, neither being affected by 50° C., but the antilytic action of both being removed by temperatures between 56° C. and 60° C. It thus appeared that the thermolabile substance could be removed from the antilytic serum with the globulins, and that the globulins, precipitated from such serum and redissolved, displayed anticomplementary and heat-sensitive properties exactly like those of the original serum.

CONCLUSIONS.

The work recorded above has served to corroborate the observations of other writers, notably of Noguchi, that in many human sera, if not in all, there develop, on standing, anticomplementary bodies which are delicately thermolabile, being inactivated by heating to 56° C. for fifteen to twenty minutes. It has appeared that these bodies may occasionally be present in sera which, after heating, may develop the thermostable anticomplementary body referred to by other authors. It has become evident also that the speed of development of the thermolabile anticomplementary body in different sera is subject to much variation. The thermolabile body appears as the complement disappears. The question arises whether the thermolabile anticomplementary body may not be originally present in the serum, temporarily masked by the native complement. This would seem improbable from the fact that titration has shown that 0.1 and 0.2 cubic centimeter of the inhibiting serum will often inhibit as much as 0.6 cubic centimeter of fresh guinea-pig complement, a quantity superior to the amount of complement present originally in the antihemolytic serum. It seems, therefore, that the anticomplementary body must actually be formed in the serum during the period of preservation.

The thermolabile antihemolytic body studied by us is like the thermostable body investigated by Noguchi, in that it inhibits alien as well as homologous complement; it is unlike the thermostable body, however, in that it cannot be absorbed from serum by digestion with red cells, nor does it render the treated cells more resistant to hemolysis.

The thermolabile body may be removed from inhibiting sera by
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precipitating the globulins. A solution of the globulins then manifests a thermolabile anticomplementary action.

No relationship between a clinical condition and the appearance of these bodies in the sera, has been found.

As a practical result these studies have shown, as have those of Noguchi, that the complement fixation tests should never be made with certain sera which have been preserved for some time without inactivation.

BIBLIOGRAPHY.


*A thorough review of the literature of the subject may be found in Noguchi's article, The Thermolabile Anticomplementary Constituents of the Blood, Jour. Exper. Med., 1906, viii, 726.