A STUDY OF THE NATURAL AND ACQUIRED ANTISHEEP
HEMOLYSINS OF THE RABBIT AS REGARDS
THERMOLABILITY.*

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The so-called natural antibodies found in the sera of many animal species have attracted attention from various points of view, viz., their origin, specificity, relation to natural immunity and particularly their relation to the corresponding acquired antibodies. The point mentioned last is of especial significance in connection with the origin of acquired antibodies and has, therefore, been the subject of a number of investigations. The view is held by some investigators (1) (2) that the identity of these two classes of antibodies has been established, while others (3) do not accept the evidence which has been advanced in favor of their identity, or have pointed out instances wherein a natural antibody is qualitatively different from the corresponding acquired antibody. Thiele and Embleton (4) have recently advanced a theory of the origin of acquired hemolysins which involves the existence of thermolabile and thermostabile hemolysins. Sherman (5) questions the soundness of this theory because he found all hemolysins to be thermostabile. Kolmer (6), on the other hand, concludes that natural hemolysins are less resistant to heat than immune hemolysins.

The purpose of the present paper is to record observations which we have made concerning the effects of different degrees of heat on the natural and immune hemolysins of the rabbit for sheep corpuscles.

EXPERIMENTAL.

Four rabbits (two normal and two immune) were selected for the study on the basis of the hemolytic titers of their sera, a wide range of titer being sought. The serum of one of the normal rabbits had a titer 1-10, the other 1-100; while the serum of one of the immunized rabbits had a titer of 1-2,500 and the other 1-25,000. The point to be de-

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terminated was the rate of loss of amboceptor content of the different sera heated at different temperatures.

Technique.—The corpuscles used in the titrations were collected from the sheep in a solution of sodium citrate and then washed four times in large volumes of salt solution (0.85 per cent. NaCl) and resuspended in salt solution so that the corpuscles were one fourth as concentrated as in the original blood. One tenth of a cubic centimeter of this suspension was taken as a unit of cells. Pooled guinea pig serum was diluted with four parts of salt solution and 0.1 cc. of the diluted serum was used as a unit of complement. The sera to be titrated were heated in a water bath for thirty minutes at varying degrees of temperature (ranging from 56° C. to 84° C.) and then diluted with salt solution in order that graded quantities could be conveniently put in a series of serological tubes. Salt solution was now added to make the volume in each tube 0.8 cc. A unit of cells was then put in each tube and the tubes were placed in a water bath (temperature 37° C.) for one hour. At the end of the incubation period a unit of complement was added and the tubes were returned to the water bath for another hour. The extent of hemolysis was observed at the expiration of the second incubation period and again after the tubes had stood at room temperature for twelve hours. Different degrees of hemolysis are indicated in the protocols by numerals; 4 indicates complete hemolysis, 0 no hemolysis and the intervening figures a corresponding degree of hemolysis. Protocol 1 illustrates the type of titration experiments used throughout the work.

PROTOCOL 1.

Type protocol used in the titration experiments.

Tubes.....	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
	(1-0)*				(1-10)					(1-100)					
Serum...	0.6	0.4	0.2	0.1	0.6	0.4	0.3	0.2	0.1	0.8	0.6	0.4	0.2	0.0	0.0
Salt.....	0.2	0.4	0.6	0.7	0.2	0.4	0.5	0.6	0.7	0.0	0.2	0.4	0.6	0.8	0.9
Cells 1-4	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Incubated 1 hour at 37° C.

Complement 1-5	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0

* The numbers in parenthesis indicate the extent to which the serum was diluted before the different quantities were measured into the various tubes. Thus tubes 1 to 4 contained the indicated quantities of undiluted serum and tubes 5 to 9 serum diluted 1-10 with salt solution, etc.

The effect of different temperatures on immune hemolysins.—The question here was the determination of the rate at which the immune amboceptor disappears from the serum when subjected to heat for 30 minutes through a graded series of temperatures. The two immune sera which have already been mentioned were used for this purpose. The sera are further described under sections (a) and (b), and the results of the experiment are recorded in Tables I and II respectively.

(a) *Antisheep rabbit 266.*—The serum from this rabbit was very potent, the titer being 1-26,000 after inactivation at 56° C. for 30 minutes. Specimens of the serum were kept at six different degrees of temperature (56° C.-84° C.) for 30 minutes and then titrated for hemolytic power. The results are collected in Table I and are represented graphically by Curve D in the text figure.

These curves are based on "1" degree of hemolysis at the 12-hour readings. The temperatures at which the sera were heated are expressed by the ordinates, while the abscissae express the square roots of the hemolytic titers. The curves show the rate of destruction of the hemolysins when heated through a range of temperatures above 56°C. Curve A represents normal rabbit serum, low titer (Table III); Curve B, normal rabbit serum, high titer (Table IV); Curve C, immune hemolysins in normal rabbit serum (Table V); Curve D, immune serum, low titer (Table II); Curve D, immune serum, high titer (Table I).

(b) *Antisheep rabbit 260.*—The serum from this rabbit was about one tenth as potent as the serum from rabbit 266. The results obtained with this serum are given in Table II and Curve C (see text figure).

Curves C and D obtained with the two immune sera of widely different titers are directly comparable. It is seen that the curves are parallel beyond 66° C. and that the hemolysins practically disappear at 76° C. Between 60° C. and 66° C. there is a marked difference showing that the rate of destruction is greatest between these two temperatures for the serum of the higher titer.

TABLE I.

The effect of different temperatures on immune serum, rabbit 266.

Temperature of inactivation.	Titer of the serum at 1 hour.	Titer of the serum at 12 hours.
56° C.	35,000†	50,000
60° C.	30,000	41,000
63° C.	8,000	11,000
66° C.	1,700	2,300
70° C. (1-2)*	730	1,015
73° C. (1-2)	400	600
76° C. (1-2)	50	70
84° C. (1-4)	6	0

TABLE II.

The effect of different temperatures on immune serum, rabbit 260.

Temperature of inactivation.	Titer of the serum at 1 hour.	Titer of the serum at 12 hours.
Unheated	5,000†	6,700
56° C.	3,300	3,300
60° C.	2,200	2,500
63° C.	1,540	1,666
66° C.	1,000	1,000
70° C. (1-2)*	333	400
73° C. (1-2)	250	260
76° C. (1-2)	10	20
80° C. (1-4)	8	13

The effect of different temperatures on natural hemolysins.

(a) *Normal rabbit serum, low titer.*—After inactivation at 56° C. this serum failed to cause complete hemolysis in dilution above 1-10. The effect of higher degrees of temperature on this serum is shown in Table II. It is seen that the hemolysins were completely destroyed at 64° C. Curve A (see text figure) gives a graphic representation of the rate at which the hemolysins were destroyed.

(b) *Normal rabbit serum high titer.*—This serum caused complete hemolysis in a dilution of 1-50 after inactivation at 56° C. The effects of higher temperatures are shown in Table IV. It is seen that a temperature of 70° C. does not completely destroy the hemolysins of this serum (see Curve B).

A study of the foregoing tables shows that all the sera, both normal and immune, contained thermostabile hemolysins, i.e., hemolysins that were not destroyed by being heated for 30 minutes at 56° C. It is

* The figures in parentheses indicate the dilution of the serum before heating.

† These numbers represent the number of hemolytic units per cc. of serum.

TABLE III.
The effect of different temperatures on natural hemolysins, serum of low titer.

Tubes	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Serum	(1-0)* 0.6	0.4	0.3	0.1	(1-10) 0.5	0.5	0.4	0.3	0.2	0.1	(1-100) 0.6	0.5	0.4	0.3	0.2	0
1 hour reading:																
Active, no complement	4	4	4	1-	0	0	0	0	0	0	0	0	0	0	0	0
Unheated	4	4	4	4	1	1	1	1	1	0	0	0	0	0	0	0
56° C.†	4	4	4	4	1	1	1	1	1	0	0	0	0	0	0	0
60° C.	3	3	3	3	1	1	1	1	0	0	0	0	0	0	0	0
62° C.	3	3	3	1	1	1	0	0	0	0	0	0	0	0	0	0
64° C.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12 hour reading:																
Active, no complement	4	4	4	2	1-	0	0	0	0	0	0	0	0	0	0	0
Unheated	4	4	4	4	2	2	2	2	1	1	1-	0	0	0	0	0
56° C.	4	4	4	4	2	2	2	2	1	1	1-	0	0	0	0	0
60° C.	3	3	3	3	3	3	3	3	1	1	1	0	0	0	0	0
62° C.	3	3	3	3	1	0	0	0	0	0	0	0	0	0	0	0
64° C.	1-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

* The figures in parentheses indicate the extent to which the serum was diluted before the quantities indicated were added to the tubes. Thus, tubes 1-4 received undiluted serum and tubes 5-10 received serum diluted 1-10 with salt solution, etc.

† The heated serum was reactivated with a unit of complement, 0.1 cc. of guinea pig serum diluted 1-5 with salt solution.

seen that in one of the normal sera (the one of low titer, Curve *A*) the hemolysins were completely destroyed by a temperature of 64° C., while complete destruction of the hemolysins was not effected in the other normal serum (the one of high titer, Curve *B*) until a temperature of 74° C. was reached. The two immune sera, being of widely different titers, show that the number of units of hemolysin destroyed within certain temperature limits (60° C.–66° C.) depends upon the concentration of the antibody in the serum (Curves *C* and *D*), but that in each case a certain residue of antibody remains which requires practically the same amount of heating to destroy completely. A comparison of the four curves indicates that the thermal death point of the sera (complete destruction of the hemolysins) is a function of the hemolytic potency of the sera rather than the kind (natural or immune) of hemolysin present. This point will be referred to again.

The effect of heat on diluted immune hemolysins.—The foregoing experiments having indicated that the degree of heat necessary to effect complete destruction of the hemolysins in a given serum depends upon the concentration rather than the kind (natural or immune) of hemolysin present, it was desirable to test this point further by testing the effect of heat on two sera of the same potency, one containing natural hemolysins and the other immune hemolysins. For this purpose an artificial immune serum was prepared in such a way that it would have the same titer as a given normal serum. The normal serum, which has already been discussed (Table IV), contained 100 hemolytic units per cc. The immune serum was prepared by two methods and designated Solutions *A* and *B* respectively.

Solution A.—Normal rabbit serum was freshly collected and rendered free of hemolysins by adsorption at 4° C. with an excess of washed sheep corpuscles. The corpuscles were removed from the serum by centrifugalization and the desired amount of a highly potent immune serum was added. The artificial serum had a titer of 1–100 after inactivation at 56° C. for 30 minutes.

Solution B.—This preparation was the same as Solution *A* with the exception that salt solution instead of normal rabbit serum was used to dilute—the immune serum. The titer was the same as that of Solution *A*.

The effect of different degrees of heat on the hemolytic titers of both of these solutions was determined, the plan of the experiment being the same as in previous experiments. The results are given in Tables V and VI respectively.

TABLE V.
The effect of different temperatures on immune hemolysins, artificial serum A.

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It is seen from Tables V and VI that immune hemolysins diluted with salt solution are only slightly more susceptible to the action of heat than when diluted with normal serum. It is of particular interest to note that the normal hemolytic serum (Table IV) and the artificial immune serum resist destruction by heat through practically the same range of temperatures (compare Curves *B* and *B'* in the text figure). It is further indicated therefore, that the rate at which hemolysins are destroyed by heat, as well as the temperature necessary to destroy completely the amboceptor content of a given serum, depends upon the quantity rather than the quality of the hemolysins.

TABLE VII.

The effect of salt solution on the thermolability of immune hemolysins.

Diluent.	Temperature.	1 hour, °.	12 hours, °.
Salt	56° C.	25,000	33,000
Serum	56° C.	25,000	33,000
Salt	60° C.	25,000	25,000
Serum	60° C.	25,000	25,000
Salt	63° C.	8,333	8,333
Serum	63° C.	8,333	8,333
Salt	66° C.	625	625
Serum	66° C.	625	625
Salt	70° C.	500	500
Serum	70° C.	500	500

The relative thermolability of hemolysins in the presence and absence of other serum constituents.—In the foregoing experiments, as will have been seen, it was necessary to dilute the hemolytic sera before testing the effects of the higher temperatures on them because undiluted serum forms a firm coagulum at these temperatures. Physiological salt solution was used as the diluent. The propriety of this procedure was questioned at the time. A casual experiment showed, however, that there was no great difference in the heat susceptibility of hemolysins when diluted with salt solution and when in undiluted serum. Since this result was not in agreement with what one would be inclined to conclude *a priori* and since the conclusions to be drawn from other experiments (see Table VI) are based upon the assumption that dilution with salt solution does not affect the thermolability of immune hemolysins, it was deemed advisable to subject this point to more exact tests.

An immune antish sheep rabbit serum of very high titer was chosen for this experiment. Two preparations were made with the immune serum: (1) 1 cc. of the serum was added to 49 cc. of salt solution; (2) 1 cc. of the hemolytic serum was added to 49 cc. of normal rabbit serum from which the natural hemolysins had been removed by specific adsorption. The two preparations were heated in 3 cc. quantities at different temperatures for 30 minutes and the hemolytic titers determined. The results are given in Table VII.

Table VII shows that the thermolability of hemolysins is in no way affected by the presence or absence of other serum constituents. The first preparation contained 98 per cent. of salt solution during the heating process, while in the second preparation the salt solution was replaced by normal rabbit serum. The hemolytic titers of the preparations were exactly the same.

DISCUSSION.

The results of the experiments recorded in this paper seem to support the contention that natural hemolysins are qualitatively the same as the corresponding acquired or immune hemolysins. It has been shown that two specimens of normal serum containing different quantities of hemolysins for a particular corpuscle will, on being heated at different temperatures, give the impression that the antibodies of one specimen are more sensitive to heat than those of the other. However, the serum containing the more sensitive antibodies is found to be the one which had the lowest original titer. It would follow, therefore, that a serum of very low titer might be entirely inactive (not contain an appreciable amount of antibody) after inactivation at 56° C. for 30 minutes, and thus give rise to the impression that only thermolabile antibodies were present, while a more potent serum of the same kind would give the opposite result. A consideration of normal sera alone leads to the conclusion that the amount of heat necessary permanently to inactivate a serum depends in part upon the quantity of antibody present.

In comparing normal and immune sera, it was found that natural and acquired hemolysins are equally resistant to heat. The comparison was made possible by obtaining normal and immune sera of the same potency. The immune serum was prepared artificially, but it was shown by experiment that its sensitivity to heat was not thus affected. It seems fair to conclude, therefore, that the apparent relative thermolability of the natural hemolysins, as compared with acquired

hemolysins, is due to a quantitative rather than a qualitative difference.

SUMMARY.

1. The natural hemolysins of the rabbit for sheep corpuscles are as heat resistant as the corresponding immune hemolysins.

2. The apparent difference in the thermolability of natural and immune hemolysins is due to the relative potency of the sera, the more potent serum apparently being more resistant to heat.

3. Hemolysins are just as resistant to heat when in salt solution as in undiluted serum.

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