THE EFFECT OF HEAT ON ANTIBODIES.

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(Received for publication, May 4, 1927.)

Although it is well known that at certain temperatures antibodies are destroyed or inactivated, nevertheless with few exceptions detailed experiments covering this point are not readily available. T. Smith and Reagh (1) showed that there were two well defined types of agglutinin for the hog cholera bacillus; one they termed flagellar agglutinin, the other, body agglutinin. Beyer and Reagh (2) were able by a series of experiments to differentiate the flagellar and somatic (body) agglutinins by means of heat; the former was unimpaired when heated at 70°C. for 20 minutes, while the action of the somatic agglutinin was markedly impaired under these conditions. Joos (3) had previously called attention to the fact that when typhoid agglutinin was heated at 60–62°C. for 1 hour, a portion of the agglutinin was destroyed. More recently Orcutt (4) took up the study and showed that a temperature of 70°C. destroyed the somatic agglutinin to a considerable extent and 75°C. rendered it completely inactive. On the other hand, 70°C. failed to affect appreciably the flagellar agglutinin and 75°C. rendered it a little less active. It seems definite that certain types of agglutinin react differently to varying temperatures. The data in regard to the behavior of other types of agglutinins, precipitins, and hemolysins are not so definite.

In addition to a study of the specific effect of the various substances upon their respective antigens, it seemed of further interest to ascertain whether antibody was still capable of combining with its antigen although remaining in insufficient quantities to give visible reactions. It might also be possible that heat so affects some of the serum proteins that they no longer respond in a characteristic manner, thus rendering inoperative the usual physical phenomena used to interpret the results.

Complement fixation seemed to meet these objections. With these points in view, it was decided to study the effect of various temperatures on certain antibodies contained in the blood serum of rabbits.

EXPERIMENTAL.

Rabbits were immunized to various substances and when a sufficiently high titered serum was obtained the animals were bled and the serum collected and stored in the refrigerator. The serum containing agglutinin and hemolysin was

TABLE I.

The Effect of Various Temperatures on Flagellar Agglutinin.

	Dilutions of serum										
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240
Unheated	C*	C	C	C	C	C	+++	++	+	+−	+−
Heated at °C.											
65	C	C	C	C	C	++++	++	+	+	+−	−
70	C	C	C	C	+++	++	+	+	+	+−	−
75	+++	+++	++++	++++	+++	++	+	+	+−	−	−
80	+	+	+	+	+	+−	+−	−	−	−	−
85	+−	+−	+−	−	−	−	−	−	−	−	−
90	+−	+−	+−	−	−	−	−	−	−	−	−

* C indicates complete clumping of the antigen; ++++, marked agglutination without complete clumping of the test fluid; +++, well defined agglutination; ++, less well defined; +, definitely positive; +−, small deposits of clumped bacilli on the bottom of the tube.

diluted in four parts of normal NaCl solution and heated at various temperatures. The precipitin was diluted with equal parts of NaCl solution and then heated.

In all experiments corrected thermometers were used and the various materials heated in a deep water bath in tightly stoppered tubes for 20 minutes. The tests were always made with the same lot of specific antigen. When complement was employed it was of the same lot and of uniform concentration. The appended protocols afford examples of various observations.

Experiment 1.—Agglutinin was prepared by immunizing a rabbit with a motile strain of the hog cholera bacillus. The serum was diluted with four parts of normal

salt solution and distributed in sterile tubes. One part was left unheated, the others were heated for 20 minutes at various temperatures. A portion of the contents of each tube was then tested for "flagellar" agglutinin with the motile strain. Another portion was tested with a non-motile strain of the hog cholera bacillus and a portion of the remainder used in the complement fixation tests further to confirm the findings. The protocols are submitted in Tables I and II.

These experiments substantiate the results of the previous workers. 75°C. for 20 minutes is a critical temperature at which the two agglutinins may be readily separated. The flagellar type resists temperatures considerably higher; even after exposure to 80°C. well de-

TABLE II.

The Effect of Heat on the Agglutinin for the Non-Motile Strain of the Hog Cholera Bacillus.

	Dilutions of serum								
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560
Unheated	C	C	C	C	+++	++	++	+	−
Heated for 20 min. at °C.									
65	+	+	+	+	+	+	+	−	−
70	+	+	+	+	+	+−	−	−	−
75	−	−	−	−	−	−	−	−	−
80	−	−	−	−	−	−	−	−	−

fined agglutination occurs when the heated serum and antigen are mixed. At temperatures still higher it is possible to show that agglutinin still persists. It will be noted in Table I that in the lower dilutions the reaction has been interpreted as +−. If the small deposits found on the bottom and sides of the tube are examined microscopically it is found that they are composed of clumped masses of bacilli. If this experiment is repeated as a microscopic agglutination test, comparable results are obtained. The bacilli lose their motility and form small, loose clumps.

It might be argued that the antibody still remained in the serum, particularly in the case of the somatic agglutinin, but through some physical or other change in the globulin was incapable of reacting

with its antigen in the characteristic manner. As an additional control, to 10 volumes of the heated antiserum, 1 volume of fresh normal rabbit

TABLE III.

The Persistence of Antibody in Flagellar Agglutinin Exposed to Various Temperatures.

Treatment of agglutinin	Antigen	Complement	Antibody	Amboceptor	Hemolysis
Unheated	+	+	+	+	0*
	+	0	+	+	0
	+	+	−	+	C
	−	+	+	+	C
Heated at 70°C. for 20 min.	+	+	+	+	0
	+	−	+	+	0
	+	+	−	+	C
	−	+	+	+	C
Heated at 75°C. for 20 min.	+	+	+	+	+−
	+	−	+	+	0
	+	+	−	+	C
	−	+	+	+	C
Heated at 80°C. for 20 min.	+	+	+	+	+
	+	−	+	+	0
	+	+	−	+	C
	−	+	+	+	C
Heated at 85°C. for 20 min.	+	+	+	+	++
	+	−	+	+	0
	+	+	−	+	C
	−	+	+	+	C
Heated at 90°C. for 20 min.	+	+	+	+	+++
	+	−	+	+	0
	+	+	−	+	C
	−	+	+	+	C

* 0 indicates no hemolysis; C, complete; the plus signs, gradations from a very strong reaction (++++) to barely perceptible hemolysis (+−).

serum was added and the mixture tested. The addition of fresh normal rabbit serum failed to activate the inactivated antibody.

The resistance of the flagellar agglutinin to heat seemed so remarkable that a further control procedure seemed desirable. If it were

TABLE IV.

The Effect of Heat on Cow Serum Precipitin.

	Dilutions of antigen								
	1:100	1:200	1:400	1:800	1:1,600	1:3,200	1:6,400	1:12,800	1:25,600
Unheated	+++*	+++	+++	+++	+++	+++	++	+	+−
Heated for 20 min. at °C.									
60	+++	+++	++	++	++	++	+	+	+−
65	++	+	+	+	+	+	+	+−	−
70	+−	+−	+−	+−	+−	+−	+−	+−	−
75	−	−	−	−	−	−	−	−	−
80	−	−	−	−	−	−	−	−	−

* Precipitation has been recorded as follows: +++, the maximum; ++, less; +, weaker but well precipitated; +−, a trace of deposit.

TABLE V.

The Effect of Heat on the Complement-Binding Properties of Precipitin.

Treatment of precipitin	Antigen	Complement	Precipitin	Amboceptor	Hemolysis
Unheated	+	+	+	+	0
	+	−	+	+	0
	+	+	−	+	C
	−	+	+	+	C
Heated at 70°C. for 20 min.	+	+	+	+	0
	+	−	+	+	0
	+	+	−	+	C
	−	+	+	+	C
Heated at 75°C. for 20 min.	+	+	+	+	++++
	+	−	+	+	0
	+	+	−	+	C
	−	+	+	+	C
Heated at 80°C. for 20 min.	+	+	+	+	C
	+	−	+	+	0
	+	+	−	+	C
	−	+	+	+	C

TABLE VI.

The Effect of Heat on Hemolysin.

	Dilutions of hemolysin										
	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	1:10,240
Unheated	C	C	C	C	C	++++	++++	++++	++++	+++	++
Heated at °C.											
65	C	C	C	C	++++	++	+	+	+−	−	−
70	C	C	C	C	+++	+−	−	−	−	−	−
75	C	++++	++	+−	−	−	−	−	−	−	−
80	+	−	−	−	−	−	−	−	−	−	−
85	−	−	−	−	−	−	−	−	−	−	−
90	−	−	−	−	−	−	−	−	−	−	−

possible to show that sufficient antibody remained in heated serum to inhibit complement, the experiment would be further substantiated. With this in view, the whole series was tested, but as the sample exposed to 65°C. behaved like the unheated mixture the results are not given. The antigen consisted of actively motile hog cholera bacilli in normal NaCl solution. The complement was fresh guinea pig serum. The washed red cells of the sheep and the specific amboceptor were employed. These substances were always used in the same concentrations. The antiserum consisted of 0.02 cc. of the serum diluted 1:4 with salt solution. The results are given in Table III.

TABLE VII.

The Effect of Heat on Red Cell Agglutinin.

	Dilutions of agglutinin					
	1:10	1:20	1:40	1:80	1:160	1:320
Unheated	+++*	+++	+++	+++	++	−
Heated at °C.						
65	+++	+++	+++	+++	M+	−
70	+++	+++	+	M−	M−	M−
75	++	++	M−	M−	M−	M−
80	M−	M−	−	−	−	−
85	M−	M−	−	−	−	−

* Agglutination in this table has been recorded as follows: +++, maximum; ++, less strong; +, clumps definite to the naked eye; M +, the presence of microscopic clumps; M −, the absence of microscopic clumps.

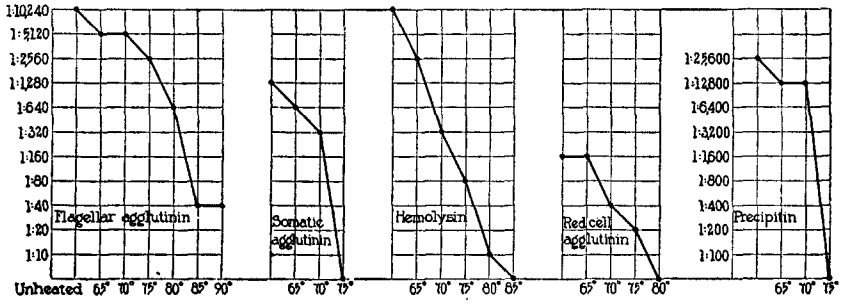
It is apparent from the results given in Table III that sufficient flagellar agglutinin, heated to 70°C. for 20 minutes, remains to divert completely the complement. As the temperature is increased less of the complement is diverted, although the inhibition is strong even after 80°C. for 20 minutes. Less of the complement is fixed when the serum is heated to 85°C. and 90°C., although it is apparent that sufficient antibody still remains to influence the intensity of the hemolysis. It can be said that the findings recorded in Table I are confirmed by the results of complement fixation.

It seemed of interest to test various other antibodies under the

same conditions. With this in view, a cow serum precipitin, and anti-sheep hemolysin and red cell agglutinin were subjected to the same method of procedure. In the instance of precipitin the serum was diluted with an equal part of NaCl solution, the hemolysin and hemagglutinin were diluted 1:4.

The effect of heat on precipitin and the persistence of sufficient antibody to bind complement are recorded in Tables IV and V.

It is obvious that the visibility of the precipitin reaction is destroyed when the precipitin is heated to 75°C. for 20 minutes. Complement fixation tests confirm this observation. It might be argued that the comparison between precipitin and the other antibodies is hardly a fair one since the serum mixtures were more concentrated in the



TEXT-FIG. 1. The effect of heat on the various antibodies. Logarithmic curves based on the data given in the tables.

former cases. Such is not the case, since precipitin diluted 1:4 and heated gave the same results in complement fixation tests as that diluted 1:1 and treated in the same manner.

The evidence of the effect of heat on hemolysis and red cell agglutinin is given in Tables VI and VII.

With the increase in temperature the hemolytic titer declines. 65°C. for 20 minutes has an appreciable effect, and increase to 75°C. materially affects the antibody, and only a trace remains after heating to 80°C. A similar result is obtained with the red cell agglutinin except that no agglutinin can be demonstrated in the serum held at 75°C. for 20 minutes.

DISCUSSION AND SUMMARY.

It is possible by means of curves to depict graphically the behavior of the various antibodies under various conditions. Logarithmic curves based on the data presented in the tables are submitted in Text-fig. 1.

In general it is evident that antibody destruction goes on gradually as the temperature is increased. Thus 65°C. for 20 minutes diminishes the activity of all the antibodies with the exception of red cell agglutinin, and in this case although the final titer was the same evidently some of the antibody was inactivated, since the reaction was weaker in the higher dilutions. It can, then, be said that 65°C. for 20 minutes appreciably affects the activity of all the antibodies tested. When the temperature is increased to 70°C. more marked differences are apparent. Here both types of the bacterial agglutinin and the precipitin are fairly stable when compared with hemolysin and red cell agglutinin. In both instances there is a sharp decline in the activity of the antibody. 75°C., however, is even a more critical temperature since at this point the somatic bacterial agglutinin and the precipitin are completely inactivated. The hemolysin and hemagglutinin behave alike. The flagellar agglutinin is the most resistant of the group to this temperature. When the temperature is increased to 80°C. the red cell agglutinin is completely inactivated, but sufficient hemolysin still remains to give a slight reaction at the lowest dilution. A further increase to 85°C. completely destroyed the hemolysin but left a definite amount of flagellar agglutinin; in fact, 90°C. for 20 minutes did not completely destroy this substance, since well defined clumps in the lower serum dilutions could be detected on microscopic examination. In this respect, then, the observations of Beyer and Reagh and Orcutt that there is a well defined difference between the two agglutinins for the hog cholera bacillus have been confirmed. However, each substance tested, with perhaps two exceptions, differs in its behavior to heat. It is of interest to point out the similarities in the reaction of somatic agglutinin and precipitin. Both are diminished when heated to 65°C.; 70°C. further affects the agglutinin, but not the precipitin; 75°C. completely inactivates both.

The assumption that the substances are apparently destroyed when they cease to react visibly with their respective antigens seems well founded since they cannot be reactivated with normal serum and no longer react to divert complement when combined in a hemolytic system.

It might be of interest to mention briefly other experiments in which the temperature was kept constant and the time varied. Thus temperatures of 50–55°C. and 60°C. maintained for 8 hours had no effect on antibody. 60°C. for 4 days failed to alter materially the flagellar agglutinin, although the same temperature for 24 hours inactivated the somatic agglutinin and the cow serum precipitin. Hemolysin deteriorates slowly at 60°C., so that after 4 days the serum, which originally reacted at a dilution of 1:10,240, only titered 1:160. The red cell agglutinin was about as resistant as the hemolysin in that a little still remained at the end of the test period. The experiments while incomplete add further proof that the somatic agglutinin and the precipitin are the least resistant to heat, while the flagellar agglutinin is on the whole comparatively stable.

A final experiment was performed to determine, if possible, at what temperature rabbit serum globulin was inactivated. With this in view, globulin was obtained by precipitation with ammonium sulfate, and a series of guinea pigs and chickens received several intraperitoneal injections. In no instance was a globulin precipitin obtained. By immunizing fowls in a similar manner with small quantities of rabbit serum good precipitin was obtained. The diluted rabbit sera heated at various temperatures for 20 minutes were tested for their antigenic activity with rabbit serum precipitin. It was found that diluted serum heated to 90°C. for 20 minutes reacted to about the same antigenic level as that not heated. Even boiling for 20 minutes failed to reduce greatly its antigenic properties. Paradoxically the visibility of the reaction was more intense with the antigen heated at the higher temperatures. The phenomenon was altogether so opposed to the usual conceptions of the inactivation of antigens that the subject will be gone into with more detail in a later communication. Although it is not possible to show definitely in the experiments that the globulin is or is not inactivated at certain temperatures, nevertheless it appears

probable that certain of the antibodies are destroyed at temperatures below that capable of greatly altering globulin.

It must be recognized that comparisons cannot be made between similar antibodies in the serum of different species, since somatic agglutinin in rabbit serum resisted 70°C. for 20 minutes, although the same agglutinin in cow serum was destroyed at 65°C.

BIBLIOGRAPHY.

1. Smith, T., and Reagh, A. L., *J. Med. Research*, 1903–04, x, 89.
2. Beyer, H. G., and Reagh, A. L., *J. Med. Research*, 1904, xii, 313.
3. Joos, A., *Centr. Bakt., 1. Abt., Orig.*, 1903, xxxiii, 762.
4. Orcutt, M. L., *J. Exp. Med.*, 1924, xl, 627.