THE FURTHER DIFFERENTIATION OF FLAGELLAR AND SOMATIC AGGLUTININS.*

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In a paper by Theobald Smith and Arthur L. Reagh,[2] read at a meeting of the Congress of American Physicians and Surgeons at Washington, in May, 1903, two different agglutinins were described as occurring in the blood serum of rabbits immunized toward Bacillus choleræ suis *a*.[1]

When a culture of the hog-cholera bacillus belonging to the motile race was exposed to the action of serum from an animal previously inoculated with motile bacilli, it was noticed that large, loose flocculi appeared soon after beginning the experiment, whereas, if serum from a rabbit immunized toward the non-motile bacillus was mixed with this motile culture the clumps appeared fine and powdery to the naked eye and formed quite slowly. It was subsequently demonstrated that the loose flocculi were produced by a flagellar agglutinin, while the fine clumps were attributed to a somatic † (body) agglutinin.

The separation of these substances was effected by means of absorption experiments. A "motile" ‡ serum was first saturated with a non-motile culture, then the mixture was centrifugalized and filtered to remove the bacilli. Serum thus treated was found to have largely lost its power of clumping non-motile bacilli, while its full action with reference to the motile form was retained. This result indicated that the somatic agglutinin had been absorbed by the non-motile bacilli, leaving the flagellar agglutinin in the fluid.

* Received for publication July 2, 1904.

† In the present paper the term *somatic* is used in place of the term *body* employed in a previous paper (2) in referring to non-motile agglutination.

‡ To avoid a circumlocution of the terms, *motile* and *non-motile* are applied to serum where the animal which is the source of such serum has been immunized toward either the motile or the non-motile bacillus, as the case may be.

In a like manner nearly all of the somatic agglutinin was removed from a "non-motile" serum by adding an excess of motile bacilli.

The identity of the somatic agglutinins of "motile" and "non-motile" sera was thus established and also the non-identity of the flagellar and somatic agglutinins co-existing in "motile" serum.

At about the same time with the above-mentioned paper appeared a communication by A. Joos,[3] in which he describes two agglutinins, designated by him α and β, in the serum of animals immunized toward Bacillus typhosus. He also describes two other complementary agglutinable substances of the bacilli themselves, which he calls α and β agglutinogens.

Joos was able to demonstrate these different bodies concerned in typhoid agglutination by virtue of their unlike reactions toward heat, and the different appearance of the clumps to which they gave rise. According to him, a temperature of 60° to 62° C. maintained for an hour was sufficient to destroy the α agglutinable substance and the β agglutinin, while the α agglutinin and the β substance remained unaffected. At ordinary temperatures the α agglutinin showed a specific affinity for the α substance, their union being characterized by the formation of coarse, rapidly forming flocculi. The β agglutinin and the β substance likewise combined, but the clumps to which they gave rise were fine and formed comparatively slowly.

Thus Joos' description of the α and β clumps brings out a striking resemblance between them and the previously described flagellar and somatic clumps, respectively. This suggested the possibility of effecting a further differentiation of the flagellar and somatic bodies by means of heat. It was with this problem in mind that the present investigation was undertaken under the direction of Professor Smith.

OUR OWN EXPERIMENTS (*Method*). — Two cultures of the hog-cholera bacillus were used in our work, both stocks having been previously described. The first was from a

typical motile race, hog-cholera Mass.,[2] and the second from a non-motile race.[4]

Rabbits were inoculated with either heated or living cultures of these bacilli, according to the end in view. Where living cultures were used, care was taken to inject at first only such small doses as would be readily tolerated by the animal, the dose being gradually increased as immunization progressed. With heated cultures, large doses could be injected without causing noticeable symptoms. Where heated cultures were used to determine whether the agglutinins formed were different from those due to living bacilli, it became necessary to guard against injecting any accidentally surviving bacilli, which would multiply within the tissues of the animal and convert the experiment into one made with living bacilli. The following method of procedure was, therefore, adopted in the preparation of such heated cultures.

Slant agar tubes were inoculated and allowed to remain in the thermostat for several days in an inclined position to insure an abundant surface growth. From these cultures a turbid suspension in bouillon was prepared, which was then exposed in a water bath to the required temperature. To avoid the possibility of bacteria accidentally drying above the level of the fluid on the sides of the tube and thereby escaping destruction, test-tubes with glass caps, similar to Pasteur tubes, were used to maintain a saturated atmosphere within the tube during the heating. Great care was taken that no bacteria should be deposited on the sides of the tube during the transfer, with a pipette, of the bouillon suspension. After heating, the suspension was placed in the thermostat for twenty-four hours. During this time any surviving bacilli would multiply in the fresh bouillon used for the suspension, and be detected by inoculating sub-cultures. By this means the possibility of injecting living bacilli was eliminated. This would either have proved fatal to the animals or else have given rise to assumed agglutinins, which, in the later experiments we were endeavoring to exclude. The important points connected with the treatment of the five rabbits which furnished the serum for our tests are shown

in Table I. The blood was drawn either by chloroforming the animal and incising the heart,[1] or by puncturing a marginal ear vein.

TABLE I.

Designation of Animal.	Bacillus Used.	Treatment of Culture.	Dose and Number of Subcutaneous Injections.	Date of Injection and of Drawing Blood.
Rabbit 179..	Non-motile hog-cholera.	Unheated.	1. 0.75 cc. of 1 day bouillon cult. 2. 1 " " 4 " " " 3. 1.5 " " 4 " " " 4. 2 " " 2 " " " Blood drawn.	Oct. 10, 1903 " 31, 1903 Nov. 23, 1903 Dec. 10, 1903 " 30, 1903
Rabbit 181..	Non-motile hog-cholera.	Heated 30 minutes at 60° C.	1. 8 cc. susp. of 8 agar slants. 2. 3 " " " 2 " " 3. 6 " " " 4 " " 4. 5 " " " 6 " " Blood drawn.	Oct. 10, 1903 " 20, 1903 Nov. 9, 1903 Dec. 10, 1903 " 30, 1903
Rabbit 183..	Motile hog-cholera.	Unheated.	1. 0.025 cc. of 1 day bouillon cult. 2. 0.05 " " 2 " " " 3. 0.1 " " 2 " " " Blood drawn.	Oct. 20, 1903 Nov. 9, 1903 Dec. 10, 1903 " 30, 1903
Rabbit 198..	Motile hog-cholera.	Heated one hour at 60° C.	1. 3 cc. susp. of 3 agar slants. 2. " " " " " 3. " " " " " 4. " " " 4 " " Blood drawn.	Feb. 6, 1904 " 13, 1904 " 20, 1904 Mar. 5, 1904 " 9, 1904
Rabbit 199..	Motile hog-cholera.	Heated 20 minutes at 70° C.	1. 3 cc. susp. of 3 agar slants. 2. " " " " " 3. " " " " " 4. " " " 4 " " 5. 6 cc. " " 8 " " Blood drawn.	Feb. 6, 1904 " 13, 1904 " 20, 1904 Mar. 2, 1904 " 17, 1904 " 21, 1904

Our agglutination tests were made according to the macroscopic method described in a former paper.[1] Serum dilutions were first prepared with normal salt solution at twice the strength desired for the final dilution, and were then mixed with equal volumes of twenty-hour bouillon cultures in small test-tubes arranged in series. Each small test-tube received one cubic centimeter of diluted serum and one cubic centimeter of culture fluid. The final serum dilutions ranged from 1 :20 up to 1 :40,000 in some instances. Control tubes were prepared with each set of tests, by substituting normal salt solution for serum. All cases of doubtful clumping were compared with a control.

Dilutions and mixtures of heated cultures and heated sera were made according to exactly the same method as with the unheated fluids. In heating serum at 70° C. it was found necessary to dilute first with salt solution in order to avoid coagulation.

After the small test-tubes had received the different mixtures of serum and culture fluid, they were placed in the incubator at a temperature of 37° C. for about two hours, when they were removed and examined. Full notes were taken of the condition observed in each tube. During the next three hours the tubes received further examination, and at the end of twenty hours a final record was made of any changes which had occurred during the intervening time. Where clumping was well marked the naked-eye examination was chiefly relied upon, but where the clumps were very fine, and in doubtful cases, the hand lens and oil-immersion objective were employed.

The reaction of each immune serum toward the cultures was tested in four different series of experiments, which differed according to the elements heated. In Series 1 the serum and the cultures were not heated. This series, representing normal agglutination, stands in all the tables as a control with which the results obtained by heating the sera and cultures, shown in Series 2, 3, and 4, may be compared. In the second series the serum remained unheated, while the cultures were heated. The third series shows the action of heated serum on unheated cultures, and the fourth shows the result of heating both serum and culture fluid. This arrangement of the tests, which is seen in Table IV. and those following, has been followed throughout the work for the sake of uniformity and to insure correct comparison of results.

In Table II. we have shown in detail how the various tests were carried out and the range of dilutions employed, because in future tables only synopses will be given and the results stated in terms of the limit of agglutination. This limit simply means that beyond the dilution given as still clumping the bacilli, higher dilutions yielded doubtful results or none. This method of pushing the agglutination to the limit was

first used in a former investigation.[1] Independently of Smith and Reagh, Wassermann[6] has also adopted this method of using the limit (Endagglutination). In general, the treated rabbits yielded sera whose agglutinative limits for the motile and the non-motile race were, respectively, 1 : 20,000 and 1 : 200.

TABLE II.

Serum of Rabbit 183 Immunized towards Unheated Motile Hog-cholera Bacilli.			Serum of Rabbit 179 Immunized towards Unheated Non-motile Hog-cholera Bacilli.	
Dilutions.	Motile.	Non-motile.	Motile.	Non-motile.
1 : 20	+ +*	+ +	+ +	+ +
1 : 50	+ +	+ +	+ +	+ +
1 : 100	+ +	+ +	+	+
1 : 200	+ +	+	+	+
1 : 500	+ +	+	o	o
1 : 1,000	+ +	o§		
1 : 2,000	+ +			
1 : 5,000	+ +			
1 : 10,000	+†*			
1 : 20,000	+			
1 : 40,000	+‡			

In Table III. are recapitulated the main characteristics of the clumping of the motile and the non-motile race of hog-cholera bacilli as taken from a previous article,[2] to which the reader is referred for more minute details.

* Nearly complete subsidence in clumps.
† General clumping with partial subsidence.
‡ Trace of clumping.
§ No agglutination.

TABLE III.

Characteristic differences between flagellar and somatic agglutination. (Rabbits' serum.)

Flagellar (Motile).	Somatic (Non-motile).
1. Clumps appear promptly after mixing serum and culture.	1. Clumps appear much more slowly than with flagellar agglutination.
2. Clumps appear as large, loose flocculi, which subside to form a copious whitish, fluffy deposit.	2. Clumps are fine and compact in structure, forming after subsidence a compact, brownish-white deposit of relatively small volume.
3. Limit of agglutination approximately 1 : 20,000.	3. Limit of agglutination approximately 1 : 200.

RESULTS.

The outcome of the various agglutination tests made with serum and cultures, both heated and unheated in the four combinations already mentioned, are synoptically stated in Table IV. and those following.*

* Whole numbers have been employed in these tables in place of fractions, for the sake of simplicity; *e.g.*, two hundred stands for a dilution of 1/200.

TABLE IV.

Serum of Rabbit 183 immunized towards unheated (living) motile bacilli.

Serum.	Cultures.	Agglutination.	
		Motile Bacilli.	Non-motile Bacilli.
Series 1. Unheated.	Unheated (living).	20,000	200
Series 2. Unheated.	Heated 1 hr. at 60° C.	20,000	100
" "	" 20 min. " 70° C.	100	100
" "	" " " 75° C.	100	50
Series 3. Heated 1 hr. at 60° C.	Unheated (living).	20,000	200
" " " " 65° C.	" "	> 1,000	—
" " 20 min. " 70° C.	" "	10,000	0
Series 4. Heated 1 hr. at 60° C.	Heated 1 hr. at 60° C.	2,000	100
" " " " 65° C.	" " " 65° C.	< 100	—
" " 20 min. " 70° C.	" 20 min. " 70° C.	0	0

In Series 1, the control series of Table IV., are given the agglutination limits for the action of unheated immune serum upon living bacilli. In Series 2, 3, and 4 of the same table is shown the effect of heat on the different elements concerned in agglutination.

In our preliminary experiments the serum and cultures were subjected to a temperature of 60° to 62° C. for an hour. This was the heat that Joos found best for demonstrating his α and β bodies. It was soon learned, however, that the agglutinins and agglutinable substances of hog-cholera bacilli were not impaired by this temperature, and higher degrees of heat were tried up to 75° C. Finally it was decided that a temperature of 70° C. maintained for twenty minutes was, on the whole, most advantageous for our problem, and the decisive tests were, accordingly, all made with this temperature.

How can the different results obtained by heating serum and bacilli at 70° C., as exhibited in Series 2, 3, and 4 of the tables, be explained? If we assume the parallelism to exist between typhoid agglutination, as described by Joos, and

hog-cholera agglutination, we should expect the effect of heat on the different elements to be as follows:

ACTION OF HEAT UPON AGGLUTININS AND AGGLUTINABLE SUBSTANCES.

1. Not impaired by heat:
   - *a.* Flagellar agglutinin, corresponding to *a* agglutinin of Joos.
   - *b.* Somatic agglutinable substance, corresponding to *β* agglutinogen of Joos.
2. Impaired by heat:
   - *c.* Flagellar agglutinable substance, corresponding to *a* agglutinogen of Joos.
   - *d.* Somatic agglutinin, corresponding to *β* agglutinin of Joos.

Applying this hypothesis to Series 2 of Table IV., we should expect that but one element, the flagellar substance, would be impaired. This would leave nothing with which the flagellar agglutinin could unite, and no flagellar agglutination would occur, but there ought to be a somatic clumping for both cultures. The actual results agree with the prediction, the table showing a somatic agglutination of 100 for both cultures, and no flagellar clumping.

Comparing this result with the control series, it will be seen that the amount of somatic substance has undergone a reduction. Series 1 shows a somatic agglutination of 200; Series 2 shows 100.

If we compare the results at 70° C. with those at 60° in this same series, we shall see that where the former temperature yielded no flagellar agglutination, the latter gave a full flagellar agglutination of 20,000, proving in this case that 60° C. does not cause any perceptible impairment of the flagellar substance, and is, therefore, too low to be of service in our work. The results of heating at 75° C. are similar to those where 70° is employed, excepting that the limit in the case of the non-motile bacillus is low (50), showing a too great

reduction in the amount of somatic substance. On this account 75° C. was considered to be too high.

In Series 3 of the same table, where only the serum is heated, nothing would be impaired but the somatic agglutinin, according to our theory. In this case, then, we should predict a high flagellar agglutination. The table shows a flagellar agglutination of 10,000, with no somatic clumping, thus agreeing exactly with the hypothesis. This also indicates that there has been a considerable reduction in the amount of flagellar agglutinin, the control series showing a limit of 20,000 as contrasted with 10,000 of Series 3.

Series 4 shows the action of heated serum upon heated cultures. We should expect here an absence of clumping for both cultures, as the somatic agglutinin and flagellar substance would be impaired, leaving nothing with which the unimpaired elements could unite. Here also the results are in accord with the theory, and we get no clumping of either motile or non-motile bacilli. Where a temperature of 60° was employed in this series we find a flagellar agglutination of 2,000 and a somatic agglutination of 100, as contrasted with the complete absence of clumping at 70°, thus furnishing additional proof that 60° C. will not differentiate the flagellar and somatic bodies.

Table V. shows the action of a "non-motile" serum on both cultures. The serum of Rabbit 179, being produced by non-motile bacteria, can contain only somatic agglutinin; consequently nothing but somatic agglutination appears in the present table, and the limit never exceeds two hundred.

TABLE V.

Serum of Rabbit 179 immunized towards unheated (living) non-motile bacilli.

Serum.	Cultures.	Agglutination.	
		Motile bacilli.	Non-motile bacilli.
Series 1. Unheated.	Unheated (living).	200	200
Series 2. Unheated.	Heated 1 hr. at 60° C.	50	100
" "	" 20 min. at 70° C.	50	100
" "	" " " 75° C.	50	100
Series 3. Heated 1 hr. at 60° C.	Unheated (living).	200	200
" " " at 65° C.	" "	—	0
" " 20 min. at 70° C.	" "	0	0
Series 4. Heated 1 hr. at 60° C.	Heated 1 hr. at 60° C.	50	50
" " " " 65° C.	" " " 65° C.	—	0
" " 20 min. at 70° C.	" 20 min. at 70° C.	0	0

In Series 2 we get an agglutination for both bacilli, for the reason that their somatic substance is not impaired by the heat, and can thus unite with the somatic agglutinin of the serum. Here, also, as in the previous instance, we find that there has been a reduction in the amount of agglutinable substance, fifty and one hundred appearing in this series instead of two hundred, as seen in the control.

Series 3 of Table V. shows entire absence of clumping as we should expect, because the somatic agglutinin has been impaired, leaving nothing with which the somatic substance of the bacilli could unite.

The somatic agglutinin is, likewise, impaired in Series 4, and here also we get no clumping.

In Tables VI., VII., and VIII. are given the results of using the serum of rabbits immunized with cultures heated at 60° and 70° C. These will be referred to again in a subsequent section, as they deal with another problem. Here we simply wish to point out that the serum obtained from them acts

like the serum obtained from animals immunized toward living bacilli, and the tests simply confirm the preceding results as to the impairment of flagellar substance and somatic agglutinin at 70° C. From the foregoing analysis it is clear that while the temperature of 70° C. causes some reduction in the amount of the flagellar and somatic bodies, it is, nevertheless, a means of differentiating these substances, and also that the temperature of 60° C., which according to Joos is sufficient for separating the corresponding α and β bodies of typhoid, is too low to employ with the hog-cholera agglutinins.

DO BACILLI RENDERED NONAGGLUTINABLE BY HEAT PRODUCE AGGLUTININS?

It has already been shown that bacilli heated at 60° C. are, in general, agglutinated like unheated bacilli, and that animals treated with them produce agglutinins in the normal way. It has also been demonstrated that a temperature of 70° C. renders inagglutinable the flagellar, but not the somatic substance of the bacilli. In order to test the effect of heat on the production of agglutinins, three rabbits were inoculated (Table I.) with heated cultures as follows:

No. 181 with non-motile bacilli heated at 60° C., No. 198 with motile bacilli heated at 60° C., and No. 199 with motile bacilli heated at 70° C.

According to our earlier results,* we should expect the serum from the first two animals to show normal agglutination, which is actually the case (see Tables VI. and VII.). With the third animal's serum it was thought that, inasmuch as the flagellar substance was impaired by the temperature of 70°, to which it had been subjected, it might not give rise to a flagellar agglutinin. However, the tests showed a flagellar agglutination of 20,000, quite as high as when living bacilli were injected. This result proves that, while the

* In former papers[1], [2] it has already been mentioned that both flagellar and somatic agglutinins are easily produced by immunizing with bacilli heated at 60° C. Many of the animals referred to in these papers were treated with heated bacilli. In undertaking the present work, it was thought best, in deference to Joos' results, to repeat the immunization with bacilli heated at 60° C.

power of clumping is lost, owing to the impairment of the flagellar substance of the motile bacilli at 70° C., this temperature does not, to any appreciable extent, affect the power of the bacilli to generate agglutinins in the animal body.

TABLE VI.

Serum of Rabbit 181 immunized towards non-motile bacilli heated 30 minutes at 60° C.

Serum.	Cultures.	Agglutination.	
		Motile bacilli.	Non-motile bacilli.
Series 1. Unheated.	Unheated (living).	50	100
Series 2. Unheated.	Heated 1 hr. at 60° C.	50	50
" "	" 20 min. at 70° C.	50	50
Series 3. Heated 1 hr. at 60° C.	Unheated (living).	20	50
" " 20 min. at 70° C.	" "	0	0
Series 4. Heated 1 hr. at 60° C.	Heated 1 hr. at 60° C.	20	20
" " 20 min. at 70° C.	" 20 min. at 70° C.	0	0

TABLE VII.

Serum of Rabbit 198 immunized towards motile bacilli heated 1 hour at 60° C.

Serum.	Cultures.	Agglutination.	
		Motile bacilli.	Non-motile bacilli.
Series 1. Unheated.	Unheated (living).	20,000	200
Series 2. Unheated.	Heated 20 min. at 70° C.	100+	100
Series 3. Heated 20 min. at 70° C.	Unheated (living).	20,000	0
Series 4. Heated 20 min at 70° C.	Heated 20 min. at 70° C.	0	0

TABLE VIII.

Serum of Rabbit 199 immunized towards motile bacilli heated 20 minutes at 70° C.

Serum.	Cultures.	Agglutination.	
		Motile bacilli.	Non-motile bacilli.
Series 1. Unheated.	Unheated (living).	20,000	200
Series 2. Unheated.	Heated 20 min. at 70° C.	100+	100+
Series 3. Heated 20 min. at 70° C.	Unheated (living).	20,000	0
Series 4. Heated 20 min. at 70° C.	Heated 20 min. at 70° C.	0	0

This result harmonizes with the determinations of Eisenberg and Volk,[5] who found that when bacteria were modified by heat or acids so that they are no longer agglutinated, these modified bacteria are still capable of combining with and abstracting agglutinin. This behavior they refer to the existence of a stable haptophore and an unstable agglutinophore group in the bacteria. Two similar groups, a combining and an agglutinating group, were demonstrated in the agglutinin of the blood serum.

Wassermann [6] contributed additional demonstrative experiments, and also immunized animals with agglutinable substance modified with dilute acids. In his cases agglutinins appeared, as in the experiments above described.

More recently Kirstein [7] succeeded in producing agglutinins in animals, with bacteria which had either been heated at 80° C. or exposed to dilute acids, dried and triturated.

Opposed to these concordant results are those of Joos[3] who states that when (typhoid) bacilli heated at 60° to 62° C. are injected into animals, their blood contains only β agglutinin and not a trace of α agglutinin. Evidently there is some error involved in Joos' experiments.

In our own work the maximum temperature we have applied was not sufficient to destroy the agglutinophore group

of somatic agglutinable substance, but the same group in the flagellar substance was destroyed or rendered inactive. The proof that inactivated somatic substance can still produce agglutinins has, therefore, not yet been made, but it is highly probable that it also is constructed like the flagellar substance, in which case we should have at least four groups in the bacilli, one pair for the somatic, the other for the flagellar substance. Similarly, four groups exist in the serum belonging to the two agglutinins.

CONCLUSIONS.

1. The flagellar and somatic agglutinins and agglutinable substances of the hog-cholera bacillus may be differentiated by heat.

2. A temperature of 70° C. acting for at least twenty minutes impairs the somatic agglutinin of the serum and the flagellar agglutinable substance of the hog-cholera bacillus. It leaves nearly intact the flagellar agglutinin of the serum and the somatic agglutinable substance of the bacilli. The impairment is manifested by the absence of clumping, and is referable to a destruction of the agglutinophore group of these bodies.

3. A higher temperature is required to differentiate the flagellar and somatic bodies of the hog-cholera bacillus than is necessary (Joos) in separating the corresponding substances of the typhoid bacillus.

4. The temperature of 70° C., which is sufficient to destroy the agglutinating power of motile hog-cholera bacilli, does not affect their power of generating the flagellar agglutinin in the animal body.

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