OBSERVATIONS ON FLAGELLAR AND SOMATIC AGGLUTINATION.

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In a recent review Hadley (1) presented a critical discussion of the flagellar antigen concept as originally proposed by Smith and Reagh (2) to account for the difference in agglutination between motile and non-motile strains of the hog cholera bacillus. He questioned the validity of the hypothesis on the ground that the particular order of serologic reaction was not limited to bacterial species characterized by the possession of flagella. He suggested that such serologic differences might eventually come to be studied in terms of the presence or absence of certain specific soluble substances wherever their point of origin in the bacterial cell. Some work done by the writer on the agglutinability of deflagellated motile bacteria seemed to have a bearing on the latter suggestion. The work was extended somewhat and is discussed in the present paper.

The initial work of Smith and Reagh (2) was concerned with the agglutinative affinities of two strains of the hog cholera bacillus, one motile, the other a non-motile variant. A "motile" antiserum agglutinated the motile strain in high dilution with a floccular type of clump. Microscopically the clumped bacilli were separated by narrow spaces. It agglutinated the non-motile strain in a lower dilution with granular clumps in which the bacteria were not separated by any appreciable space. A "non-motile" antiserum agglutinated both strains in relatively low dilution with granular clumping. The non-motile strain removed the granulating but not the flocculating agglutinin from "motile" antiserum. The motile strain reduced the granulating agglutinin of the "non-motile" serum. The writers concluded that the agglutinins for the flagella and for the body of the bacilli, at least so far as the large group of pathogenic colon derivatives were concerned, were distinct, not mutally interacting substances.

These observations were subsequently extended by other workers among whom the following may be mentioned. Beyer and Reagh (3), working under Smith,

showed that flagellar antigen and somatic agglutinin were heat-labile at 70°C. while somatic antigen and flagellar agglutinin were heat-stable. Orcutt (4) working with the hog cholera bacillus and Balteanu (5) with the vibrio comma demonstrated the agglutinative and antigenic properties of pure flagellar suspensions. Yokota (6) carried out similar studies on flagellar suspensions from *B. typhosus* and also on the bacteria deflagellated by shaking. Goyle (7) compared the agglutinative relationships of heated and untreated suspensions of *B. typhosus* and *B. enteritidis* and their variants. Arkwright (8) described the microscopic findings with floccular and granular agglutination.

The multiple antigen hypothesis as related to bacteria has also been criticized by Tulloch (9) in an extended series of papers. He pointed out certain irregularities which cannot be cited here.

Direct Agglutination of Whole, Shaken and Heated Antigens by "Whole" Antiserum.

Two species of Salmonella designated *B. paratyphi* Types I and II were employed in the present work.

The strains used were originally cultivated from spleen tissue. They were isolated during the course of a natural epidemic in a guinea pig population (10). The final cultures represented the growth from single colonies replated three times on agar. They were normal smooth strains as to their growth in broth and their colony formation on agar, were actively motile and gave a characteristic agglutination with diagnostic antiserums then in use.

Fresh antiserums against the whole bacteria were prepared in rabbits.

The growth from 18 hour agar cultures was removed with physiological salt solution, centrifuged, the packed bacteria washed once, resuspended in 0.2 per cent formalinized saline and heated to 56°C. for 1 hour. Five intraperitoneal injections at 3 day intervals were given. The rabbits were previously tested for normal or immune agglutinins active for the particular antigens. The two type antiserums, termed "whole," were tested against whole, heated and shaken suspensions of both types of *B. paratyphi*. The suspensions were prepared as follows: The growth from a Blake bottle was removed with 10 cc. of saline, washed once, resuspended in saline and divided into three portions. One was untreated. One was heated to 100°C. for 30 minutes in a water bath, washed once after centrifuging and resuspended in saline. One was shaken for 1 hour in a mechanical shaker, washed twice after centrifuging and resuspended in saline. The three suspensions were finally standardized to equal opacity, 2.4 on the Gates scale. In testing, 0.5 cc. amounts of diluted serum and suspension were mixed. The final dilutions were doubled at each interval from 1:100 through 1:51,200. The

tubes were incubated at 37°C. for 3 hours and read after overnight refrigeration. The limits of agglutination with each serum against the six antigens are given in Table I. The limit of agglutination is defined as the highest dilution showing any macroscopic evidence of clumping after approximately 24 hours.

The agglutination of the several antigens with reference to the character of the sediment and the limiting dilution was much the same with both antiserums. The whole antigen was agglutinated in high dilution by its homologous serum. The heated and shaken antigens were agglutinated equally through a much lower limit. With the

TABLE I.

Direct Agglutination of Whole, Shaken and Heated Antigens with Type I and Type II Antiserums.

Serum	Antigen	Limiting dilution	Type of agglutination
Type I "whole"	Type I whole	1:25,600	Mixed
	" " shaken	1:3,200	Granular
	" " heated	1:3,200	"
	" II whole	1:800	Atypical granular
	" " shaken	1:800	Granular
	" " heated	1:800	"
Type II "whole"	" II whole	1:25,600	Mixed
	" " shaken	1:3,200	Granular
	" " heated	1:3,200	"
	" I whole	1:200	Atypical granular
	" "shaken	1:200	Granular
	" " heated	1:200	"

antiserums of opposite type the three antigens were agglutinated equally in still lower dilution. The reaction with the homologous whole antigen was predominantly floccular throughout with an admixture of granular clumps in the lower dilutions. The reaction was rapid with the formation of coarse clumps which on settling formed a light feathery sediment. In the lower dilutions the supernatants were clear or nearly so. Microscopically the sediment was composed mainly of large, poorly defined clumps with an open work appearance. With the lower dilutions there were also smaller compact clumps which were more sharply defined. The type of agglutination with the ho-

mologous heated and shaken antigens was granular throughout. The reaction was retarded. The sediment varied from an irregular, wrinkled, compact disc in the lower dilutions to a granular mold fitting the rounded portion of the tube in the higher dilutions. The clumps were not easily broken up on shaking. The supernatants tended to be clear up to the last two dilutions with a graded sediment. With the antigens of the opposite type the agglutination was granular. The macroscopic picture with the heated and shaken antigens was identical with that described above. The character of the sediment with the whole antigens was somewhat different. The Type II antigen formed a compact, even, button-like sediment with a practically clear supernatant. The Type I antigen formed a sediment closely approaching the typical granular form except that the clumps were larger and not as closely packed. Microscopically the clumps were of the granular type in both cases.

Absorption of "Whole" Antiserum with Whole, Shaken and Heated Antigens.

The two "whole" antiserums were absorbed with the three homologous antigens.

In absorbing 0.25 cc. of packed bacteria, 0.1 cc. of serum and 2.4 cc. of 0.2 per cent formalinized saline were employed, an absorbing dose and a serum dilution of approximately 1:10 and 1:25 respectively. The absorbing antigens were prepared as previously described and sedimented for 1 hour in graduated centrifuge tubes to a constant volume. The final antigen-serum mixtures were incubated at 37°C. for 3 hours with frequent shakings. After overnight refrigeration they were centrifuged and the clear supernatants tested. The dilution series previously described was employed. Unabsorbed serum similarly diluted with formalinized saline was subjected to the same incubation and tested with the same antigen suspension. The results were identical with those given in Table I. The limiting agglutination of the three antigens by the absorbed serums is given in Table II.

After absorption with whole antigen both serums continued to give a floccular reaction with the homologous antigen in very low dilution. There was no reaction with the shaken and heated antigens in the lowest dilution employed. Absorbed with shaken and heated antigens both serums agglutinated the homologous whole antigen through dilution 1:12,800, one dilution removed from the limit of agglutina-

tion with the unabsorbed serum. The type of clumping was purely floccular in all cases. The supernatants showed a diffuse turbidity which was graded. There was no agglutination of the shaken and heated antigens in the lowest dilution. The removal of a considerable amount of flocculating agglutinin by the treated antigens was indi-

TABLE II.

Agglutination Limits with Type I and Type II Antiserums after Absorption with Whole, Shaken and Heated Antigens.

Serum	Absorbing antigen	Agglutination after absorption	Antigen agglutinated
Type I whole	Type I whole	1:400	Whole
		<1:100	Shaken
		<1:100	Heated
	Type I shaken	1:12,800	Whole
		<1:100	Shaken
		<1:100	Heated
	Type I heated	1:12,800	Whole
		<1:100	Shaken
		<1:100	Heated
Type II whole	Type II whole	1:200	Whole
		<1:100	Shaken
		<1:100	Heated
	Type II shaken	1:12,800	Whole
		<1:100	Shaken
		<1:100	Heated
	Type II heated	1:12,800	Whole
		<1:100	Shaken
		<1:100	Heated

cated. Further evidence bearing on the absorption of flocculating agglutinin by the treated antigens will be presented in another paper.

Agglutination with Shaken and Heated Antiserums before and after Absorption.

The immunizing properties of the shaken and heated suspensions of the motile bacteria were determined.

The suspensions were prepared as described. The treated and washed bacteria were sedimented in the centrifuge, resuspended in 1 per cent formalinized saline and kept for 3 days in the refrigerator. They were resedimented, washed twice and resuspended in 0.2 per cent formalinized saline. It was necessary to kill the shaken bacteria and the above method was chosen in preference to heat at 56–58°C. on successive days. To keep the suspensions uniform both were subjected to the same conditions although the heated suspension (100°C. for 30 minutes) was obviously inactivated. Subcultures made on consecutive days were sterile. A rabbit was immunized intraperitoneally with each suspension. Five injections at 3 day intervals were given with the Type I antigens, four injections with the Type II. 7 days after the last injection the animals were bled and killed. At autopsy no focal lesions were detected in any of the usual sites: spleen, liver or lymphoid tissue of the intestinal tract. There were traces of exudate on one or another of the visceral organs. Walled-off accumulations of purulent material along the cecum were commonly observed. Cultures from spleen, liver, purulent material and cecal feces failed to show *B. paratyphi*. The agglutinin content of the several antiserums was determined by direct agglutination and by absorption employing whole, shaken and heated antigens of the same type. The limits of agglutination of the shaken and heated antiserums before and after absorption are given in Table III.

The shaken and heated antiserums agglutinated the three homologous antigens equally. The limiting dilutions were higher with the Type I serums as might be expected in view of the longer immunization. In both cases the clumping of the shaken and heated antigens was typically granular. The sediment with the whole antigens was granular microscopically. Macroscopically the sediment with each type was identical in appearance with that described previously for whole antigen agglutinated by serum of opposite type.

The absorption tests gave further evidence for the absence of flocculating agglutinin. Absorption with any one antigen removed most of the agglutinin for all three suspensions. Irregularities in the titer were noted but had no bearing on the presence or absence of flocculating agglutinin. In general the reduction of agglutinin was a little less complete than that previously noted with the whole antiserums. Although the shaken and heated antigens were able to remove some flocculating agglutinin from whole antiserum, there was no indication that they were able to produce the same upon injection in rabbits.

TABLE III.

Agglutination Limits with Shaken and Heated Serums of Both Types before and after Absorption.

Serum	Direct agglutination	Antigen agglutinated	Absorbing antigen	Agglutination after absorption	Antigen agglutinated
Type I shaken	1:6,400	Type I whole	Type I whole	1:200	Type I whole
				1:200	" " shaken
				1:200	" " heated
	1:6,400	Type I shaken	Type I shaken	1:200	" " whole
				1:200	" " shaken
				1:200	" " heated
	1:6,400	Type I heated	Type I heated	1:200	" " whole
				1:200	" " shaken
				1:200	" " heated
Type I heated	1:6,400	Type I whole	Type I whole	1:200	Type I whole
				1:200	" " shaken
				1:100	" " heated
	1:6,400	Type I shaken	Type I shaken	1:200	" " whole
				1:200	" " shaken
				1:200	" " heated
	1:6,400	Type I heated	Type I heated	1:200	" " whole
				1:200	" " shaken
				1:100	" " heated
Type II shaken	1:3,200	Type II whole	Type II whole	<1:100	Type II whole
				<1:100	" " shaken
				<1:100	" " heated
	1:3,200	Type II shaken	Type II shaken	1:100	" " whole
				1:100	" " shaken
				1:100	" " heated
	1:3,200	Type II heated	Type II heated	1:100	" " whole
				1:100	" " shaken
				1:100	" " heated
Type II heated	1:3,200	Type II whole	Type II whole	1:200	Type II whole
				1:200	" " shaken
				1:200	" " heated
	1:3,200	Type II shaken	Type II shaken	1:200	" " whole
				1:200	" " shaken
				1:200	" " heated
	1:3,200	Type II heated	Type II heated	1:100	" " whole
				1:100	" " shaken
				1:100	" " heated

Agglutination of Untreated, Shaken and Heated Suspensions of a Non-Motile Bacterium.

The agglutination tests were repeated with a culture of *Staphylococcus aureus* and its homologous antiserum to afford a basis for comparing the agglutinative affinities of a non-motile organism. The staphylococcus was recently isolated from a skin abscess and was a normal, smooth strain. A rabbit antiserum against the whole antigen was prepared. The same procedures outlined for the motile bacteria were followed in immunization, preparation of antigens and performance of the agglutination tests. The limits of agglutination before and after absorption with whole and heated antigens are given in Table IV.

TABLE IV.

Limits of Agglutination with Staphylococcus Antiserum before and after Absorption

Serum	Direct agglutination	Antigen agglutinated	Absorbing antigen	Agglutination after absorption	Antigen agglutinated
Staphylococcus whole	1:1,600 G*	Whole	Whole	1:50 G	Whole
			"	1:50 G	Heated
	1:1,600 G	Heated	Heated	1:50 G	Whole
			"	1:50 G	Heated

* Granular clumping.

The shaken antigen showed the same limiting dilution upon direct agglutination and was not employed in absorption.

The staphylococcus antiserum agglutinated whole and heated antigens to the same titer limit. The reactions were retarded, the limiting dilutions were relatively low and the clumping was typically granular both macroscopically and microscopically. The titer of the serum, 1:1,600 after five injections, was not noticeably increased by three additional injections. The agglutination of whole and heated antigens by the "non-motile" antiserum was identical in character with that of heated and shaken antigens by the "motile" antiserums. The whole and heated staphylococcus antigens possessed approximately the same absorptive capacity for agglutinin as indicated by partial absorption. Reciprocal absorption with a "heated" staphylococcus antiserum was not made.

The Origin of Soluble Material Extracted by Heat from the Motile Bacteria.

Some additional experiments bearing on the production of soluble material from the motile bacteria may be cited. A considerable decrease was noted in the volume of packed bacteria after heating to 100°C. for 30 minutes. Goyle (7) had previously called attention to the decreased opacity of heated bacterial emulsions. The supernatants from such heated suspensions after sedimentation of the bacteria were distinctly milky in appearance and when mixed with "whole" antiserum gave a precipitate in moderate dilution. Specific soluble material precipitable with immune serum had evidently been extracted from the bacteria at the temperature employed. Happold (11) has recently described a precipitinogen present in the filtrates of steamed broth cultures of *B. aertrycke* (mutton).

Comparative tests were made on the supernatants from heated whole suspensions and heated shaken suspensions to determine the effect of the presence of flagella on the amount of extracted soluble material. Filtrates of the growth from 18 hour agar cultures of the two motile bacteria were included.

Blake bottles were inoculated with 2.5–3 cc. of 6 hour broth cultures of the bacteria and incubated at 37°C. for 18–24 hours. The growth from each bottle was removed with 5 cc. or so of saline, transferred to a graduated tube and centrifuged for 1 hour. The supernatant was withdrawn and the packed bacteria washed once with 8 cc. of saline. Both supernatants were saved and later filtered through medium Berkefeld candles. For the final suspensions 1 cc. of saline was added for each 0.1 cc. of packed bacteria and the cells resuspended. One suspension of each type was shaken for 1 hour. The packed bacteria, after centrifuging, were washed three times and finally resuspended in saline according to the same ratio. The untreated and the shaken suspensions of each type were then heated to 100°C. for 1 hour in a water bath. After centrifuging the supernatants were removed and filtered through Berkefeld filters.

The final solutions prepared according to the above methods all had a milky, opalescent appearance. There was no deposit upon standing. The culture supernatants were perfectly clear but showed a yellowish tinge. The volume of packed bacteria before heating was approximately 1 cc. with Type I, 0.7 cc. with Type II. After heating, the volumes were 0.7 cc. and 0.4 cc. respectively. The suspensions were centrifuged for 1 hour at the same speed in both cases. The culture and suspension filtrates were tested with "whole" antiserum of the same type. Only one culture filtrate was employed with the Type II *B. paratyphi.*

In testing, the antigens were diluted in series and the serum kept constant, 0.5 cc. amounts of the former and 0.1 cc. amounts of the latter being employed. The tubes were incubated at 37°C. for 1 hour, followed by overnight refrigeration. The final readings are given in Table V. The first culture filtrates and the suspension filtrates showed a ring reaction with beginning precipitation in the lowest dilutions after 1 hour. Upon standing there were decreasing amounts of a granular sediment, indicated in the table by plus signs. The highest dilutions showed a distinct clouding of the supernatant but no sediment. No bacteria were detected upon microscopic examination.

TABLE V.

Precipitin Tests with Culture Filtrates and Heated Suspension Filtrates of the Two Salmonella Types.

Antigen	Serum	Serum dilutions								
		1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280
Type I	Type I									
1st culture filtrate	whole	+	+	±	Cl.*	S.cl.*	−	−	−	−
2nd " "		S.cl.	−	−	−	−	−	−	−	−
Heated whole suspension		++	++	+	+	±	±	Cl.	S.cl.	−
" shaken "		++	++	+	+	±	±	Cl.	S.cl.	−
Type II	Type II									
1st culture filtrate	whole	+	±	±	S.cl.	−	−	−	−	−
Heated whole suspension		++	++	++	+	±	±	Cl.	S.cl.	−
" shaken "		++	++	++	+	±	±	Cl.	S.cl.	−

* Cl. = cloudy supernatant; S.cl. = slightly cloudy supernatant.

The above tests indicate the presence of small amounts of soluble precipitate material in the diluted culture filtrates of both Salmonella species. The amount was greatly reduced by one washing as indicated by the reaction with the second Type I culture filtrate. Similar material was extracted from the bacteria by heat and was present in considerably greater amount in the diluting medium. The supernatant from the heated whole bacteria reacted quantitatively the same as that from the heated shaken bacteria. The amount of soluble material present in the fluid as a result of flagellar disintegration was evidently too small to affect the titer. It may be said, too, that the

agglutinability of the bacteria was not greatly influenced by the loss of the somatic constituents extracted by heat.

DISCUSSION.

The experimental work on agglutination reported in the previous section is in no sense original. The general conformation of the observations to the early flagellar hypothesis of Smith and Reagh (2) and its extension by others is the main reason for its presentation. The observations bear out the altered agglutinability of motile bacteria following shaking and heating at 100°C. Both treatments result in deflagellation of the bacteria. With the former the flagella are mechanically broken off and subsequently removed by washing. With the latter a disintegration of the flagella occurs and the soluble products are removed upon washing. Tulloch (9) has objected to the application of heat for demonstrating separate antigens on the ground that the physical nature of the bacterial cell as a whole may undergo a change resulting in altered agglutinability. The results following deflagellation by shaking are less easily disposed of in this way. No marked change in the physical state of the cell is apparent, aside from the removal of one morphological structure, the flagellum. The shaken bacteria are viable and capable of normal growth upon transfer to media. In common with the heated bacteria, however, they are incapable, in the present case, of producing flocculating agglutinin upon injection in rabbits. Admittedly there is lack of agreement concerning the true antigenic nature of deflagellated bacteria. In some instances at least the reported ability of treated bacteria to produce flocculating agglutinin appears referable to the presence of flagellar material in the suspension used for immunization.

One exception to the flagellar hypothesis was noted with the present work. The shaken and heated suspensions were able to absorb a certain amount of flocculating agglutinin from "whole" antiserum. It is suggested that the anomalous reaction may be attributed to a non-specific adsorption of antibody. Regarding the reaction in an animal host as the more exacting criterion it appears that the motile bacteria have lost in immunizing ability with the loss of flagella. This in turn implies that the substance of the flagella embraces a specific antigen.

The objections of Hadley (1) to such an assumption have been previously noted. He intimates that serological differences similar to those noted with whole and deflagellated bacteria may be explained by the presence of soluble specific substances disregarding particular morphological elements.

A small amount of soluble material precipitable with immune serum was found present in culture medium supporting the growth of the motile bacteria. The suspensions used for the production of "whole" antiserum were carefully washed and the amount of such material reduced to a practically negligible quantity. The resulting antiserum, however, contained both granulating and flocculating agglutinin. If a soluble substance were responsible for the appearance of flocculating agglutinin the bulk of it must have been produced from the bacteria after their introduction into the animal host. The "shaken" and "heated" antiserums contained only granulating agglutinin. Hence if a soluble substance is to be regarded as the antigen leading to the production of flocculating agglutinin it seems necessary to associate it with the flagella. Orcutt (4) has shown that pure flagellar suspensions (hog cholera bacillus) give a floccular agglutination with "whole" or flagellar antiserum. If the suspension is heated to 70°C. for 30 minutes the flagella are broken up. The floccular agglutinability is lost but on animal injection the heated suspension produces flocculating agglutinin. Apparently the flagella go into solution. With our experiments such heated suspensions in moderate dilution (1:80) gave a granular precipitate with "whole" antiserum. One might regard this reaction as the analytical production of soluble specific material which was antigenic in the sense that it could still produce antibody but which because of a change in the physical state of its precursor, the flagellum, showed altered agglutinability. Injected bacteria must eventually undergo disintegration probably with the production of similar soluble materials. Viewed in this light the suggestion of Hadley seems plausible with this modification, however, that the soluble substance be limited as an antigen to the flagella.

Hadley (1) has also questioned the purity of the flagellar suspensions used by Orcutt (4) and Balteanu (5). He suggests that the heat-labile factor present in such suspensions may equally well represent

soluble substances from the bacteria themselves. We have shown that the bulk of soluble precipitable material extracted *in vitro* from motile bacteria or produced *in vivo* in smaller amounts during growth originates in that portion of the bacterial cell which as an antigen gives rise to granular agglutinin. In combination with "whole" antiserum this material gives a precipitate which is likewise granular in nature. Its presence in flagellar suspensions could not be held accountable for the floccular reaction which occurs with "whole" or flagellar antiserums.

SUMMARY.

Whole, shaken and heated suspensions of two Salmonella species were compared as to agglutinability, absorptive capacity and antigenic properties. The results were in general agreement with the flagellar antigen concept of Smith and Reagh. The removal of flagella by shaking or heating (100°C.) resulted in altered agglutinability manifested by failure to give a floccular reaction with "whole" antiserum. The deflagellated bacteria were able to absorb some flocculating agglutinin from that serum. They were unable, however, to produce flocculating agglutinin upon injection in rabbits.

Untreated, shaken and heated suspensions of a non-motile bacterium (*Staphylococcus*) showed no differences with respect to agglutinability or absorptive capacity.

Soluble precipitable material was found present in small amount in culture filtrates of the motile bacteria and in greater concentration in filtrates of heated suspensions. The bulk of the soluble material was of somatic origin and was not appreciably increased by the presence of flagella. It was possible, however, to demonstrate soluble material in heated flagellar suspensions. The relation of such soluble substances to floccular agglutination and the production of flocculating agglutinin as suggested by Hadley is discussed.

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