INFLUENCE OF HEATING AT 120°C ON THE BACTERIAL CELLS
II. "FIELD OF REACTION" OF VI AGGLUTINATION AND O AGGLUTINATION IN THE TESTS CONDUCTED WITH THE SUPERNATANT FLUID ANTIGEN OBTAINED BY HEATING AT 120°C THE BACTERIAL CELLS OF DIFFERENT SPECIES SUSPENDED IN THE SUPERNATANT FLUID OF TYPHOID BACILLI V AND W FORM

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According to the author's previous paper (1952), saline suspensions of typhoid bacilli V form were subjected to heating at 120°C for varying periods of 10, 20, 30 minutes and 1, 2, 3, 5 hours. After centrifugation, the sediments were resuspended in saline solution to run agglutination tests. Starting from the suspension heated for 20 minutes, the reaction became stronger as the periods of heating prolonged, showing the highest Vi agglutination reaction when saline suspensions of typhoid bacilli V form were heated for a period of 3 to 5 hours.

Regarding O agglutination, practically equal titers were observed with the bacterial suspensions heated at 120°C for a period from 10 minutes to 1 hour, thereafter showing a decrease as the heating period prolonged for 2-3 and 5 hours. Then, when heated at 120°C the bacterial cells of different species (paratyphoid bacilli A, B, C, pullorum bacilli, dysentery bacilli, coli bacilli *etc.*) suspended in the supernatant fluid of the heated physiological saline suspension of typhoid bacilli V form react as Vi and O agglutinogens in the same manner as those of the cells of typhoid bacilli, and the above reaction takes place even when the supernatant fluid is diluted to a certain degree. Accordingly, the "field of reaction" of Vi agglutination, O agglutination, Vi precipitation and O precipitation were carefully studied in order to know what amount of Vi and O antigen in the supernatant fluid were destroyed or combined with the bacterial cells of different species by heating at 120°C.

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METHOD

Preparation of pure Vi serum: Serum obtained from the rabbit immunized with the living bacilli of the 17 hours' bouillon culture of *S. typhi* ViI grown at 37°C was diluted 25 times with physiological saline and absorbed twice with the living cells of H901 strain.

Preparation of supernatant fluid: Physiological saline suspensions of the 17 hours' nutrient agar cultures of *S. typhi* ViI, *S. typhi* Ty2, *S. typhi* 6S, *Ballerup* etc.

Table 1. "Field of Reaction" in the agglutination with pure Vi serum

Supernatant fluid and treatment	Supernatant fluid dilution	Pure Vi serum dilution								Control
		50	100	200	400	800	1600	3200	6400	
Ty2 + <i>Sh. flex. var. Y</i> 120°C 2 2/3 hrs.	1: 2	††	††	††	††	††	††	+	(+)	—
	1: 4	††	††	††	††	††	+	(+)	±	—
	1: 8	††	††	††	††	††	+	(+)	±	—
	1: 16	+	+	+	+	+	+	(+)	±	—
	1: 32	±	±	±	±	(+)	(+)	±	±	—
	1: 64	±	±	±	±	±	±	±	±	—
	1:128	—	—	—	—	—	—	—	—	—
Ballerup + <i>Sh. flex. var. Y</i> 120°C 2 2/3 hrs.	1: 2	††	††	††	††	††	+	(+)	±	—
	1: 4	††	††	††	††	††	+	(+)	±	—
	1: 8	††	††	††	††	††	+	(+)	±	—
	1: 16	+	+	+	+	+	+	(+)	±	—
	1: 32	(+)	+	+	+	+	+	(+)	±	—
	1: 64	±	±	±	(+)	(+)	(+)	(+)	±	—
	1:128	—	—	—	—	—	—	—	—	—
6S + <i>Sh. flex. var. Y</i> 120°C 2 2/3 hrs.	1: 2	††	††	††	††	††	††	+	(+)	—
	1: 4	††	††	††	††	††	††	+	(+)	—
	1: 8	+	+	+	+	+	+	+	—	—
	1: 16	±	±	(+)	(+)	(+)	(+)	±	—	—
	1: 32	—	—	—	—	—	—	—	—	—
Ty2 + <i>E. coli</i> 120°C 3 hrs.	1: 2	††	††	††	††	††	††	(+)		—
	1: 4	††	††	††	††	††	††	(+)		—
	1: 8	+	+	+	+	+	+	(+)		—
	1: 16	(+)	(+)	+	+	+	+	(+)		—
	1: 32	±	±	±	±	(+)	(+)	(+)		—
	1: 64	—	—	—	—	—	—	—		—
Ty2 Filtrate + <i>E. coli</i> 120°C 3 hrs.	1: 2	+	+	+	+	+	(+)	(+)		—
	1: 4	+	+	+	+	+	(+)	(+)		—
	1: 8	+	+	+	+	+	(+)	(+)		—
	1: 16	±	±	±	(+)	(+)	(+)	(+)		—
	1: 32	—	—	±	±	±	±	±		—
	1: 64	—	—	—	—	—	—	—		—

In this and subsequent tables †, (†), ††, ††, +, (+), ± indicate deminishing degrees of agglutination.

grown at 37°C each in a concentration of 20 billion per cc were heated at 56 to 60°C for 1 hour and centrifuged immediately for the collection of the supernatant fluids.

Treated supernatant antigen (fluid): An equal amount of the physiological saline suspension of the bacterial cells of different species, such as *Sh. flexneri* variant Y, *E. coli communis*, was added to the two-fold dilutions of the above mentioned supernatant fluid. The mixture was autoclaved at 120°C for 2 to 3 hours and the cells obtained by centrifugation were resuspended in physiological saline to serve as antigens.

"FIELD OF REACTION" OF AGGLUTINATION REACTION

The "field of reaction" responded to pure Vi serum was different from those responded to *Ballerup* and 6S serums when the antigens used were prepared by heating the two-fold dilutions of supernatant fluid (up to a 1:512 dilution) at 120°C after suspending the bacterial cells of different species.

a) "Field of Reaction" of Vi Agglutination Reaction with Pure Vi Serum

Supernatant fluids were prepared from the cultures of *Ty2*, 6S, *Ballerup* etc. As shown in Table 1, generally speaking, the lower the dilution of pure Vi serum or the supernatant fluid used, the stronger agglutination resulted regardless of the origin of the supernatant fluid, the "field of reaction" being oblong in shape. But intensity of the reaction became weak in a serum dilution of 1:50 to 1:100. Further, the supernatant fluids of *Ty2* and its filtrate were diluted by two-fold steps up to 1:64. Cells of *E. coli* were placed in each dilution of the above mentioned 2 materials. After the autoclaving treatment at 120°C, the intensity of agglutination was compared each other. As the results of the above tests, the filtrate was found to present weaker reaction than the supernatant fluid, the end titer being 1:32.

b) "Field of Reaction" of Vi Agglutination Reaction with *Ballerup* Serum

Ballerup serum was used in place of pure Vi serum and the findings are shown in Table 2. Namely, the agglutination was positive only up to a dilution of 1:4 or 1:8 in the case the serum was in a dilution of 1:50. Then, the serum in a dilution of 1:1600 to 1:3200 gave a positive agglutination with the treated supernatant antigen in a dilution of 1:64. Thereafter, however, the reaction suddenly became weak. But when the supernatant fluid of *Ballerup* was used, as it contained both free Vi antigen and *Ballerup* O antigen, the "field of reaction" was resembling to that of reaction done with pure Vi serum. This might probably be due to the overlap of "field of reaction" between O agglutination and Vi agglutination. Further, the supernatant fluid of *Ty2* and its filtrate were put into serial two-fold dilutions and the cells of *Sh. flex. var. Y* were suspended in each of the dilutions. After treatment of the heating at 120°C, they were used as antigens. As shown in Table 2, the treated supernatant antigen presented the strongest agglutination. On the other hand, treated filtrate antigen gave a weak positive agglutination as far as a dilution of 1:32 against the serum dilution of 1:1600, and their "fields of reactions" were similar to each other.

Table 2. "Field of Reaction" in the agglutination with *Ballerup* serum

Supernatant fluid and treatment	Supernatant fluid dilution	<i>Ballerup</i> serum dilution								Control
		50	100	200	400	800	1600	3200	6400	
<i>Ty2</i> + <i>Sh. flex. var. Y</i> 120°C 2 2/3 hrs.	1: 2	⏏	⏏	⏏	⏏	⏏	⏏	⏏	±	—
	1: 4	⏏	⏏	⏏	⏏	⏏	⏏	⏏	±	—
	1: 8	(+)	+	+	⏏	⏏	⏏	+	±	—
	1: 16	—	±	(+)	⏏	⏏	⏏	+	—	—
	1: 32	—	—	—	(+)	+	+	(+)	—	—
	1: 64	—	—	—	—	±	(+)	(+)	—	—
	1:128	—	—	—	—	—	—	—	—	—
<i>Ballerup</i> + <i>Sh. flex. var. Y</i> 120°C 2 2/3 hrs.	1: 2	⏏	⏏	⏏	⏏	⏏	⏏	⏏	—	—
	1: 4	⏏	⏏	⏏	⏏	⏏	⏏	⏏	—	—
	1: 8	(⏏)	(⏏)	(⏏)	⏏	⏏	⏏	+	—	—
	1: 16	⏏	(⏏)	(⏏)	(⏏)	⏏	⏏	+	—	—
	1: 32	+	+	+	+	+	+	+	—	—
	1: 64	(+)	(+)	(+)	+	+	+	(+)	—	—
	1:128	±	±	±	±	—	—	—	—	—
<i>6S</i> + <i>Sh. flex. var. Y</i> 120°C 2 2/3 hrs.	1: 2	⏏	⏏	⏏	⏏	⏏	⏏	⏏	±	—
	1: 4	⏏	⏏	⏏	⏏	⏏	⏏	⏏	±	—
	1: 8	—	(+)	(+)	⏏	⏏	⏏	+	±	—
	1: 16	—	—	—	±	+	+	(+)	—	—
	1: 32	—	—	—	±	±	(+)	±	—	—
	1: 64	—	—	—	—	—	—	—	—	—
<i>Ty2</i> + <i>Sh. flex. var. Y</i> 120°C 3 hrs.	1: 2	+	⏏	⏏	⏏	⏏	⏏	+		—
	1: 4	+	+	⏏	⏏	⏏	⏏	+		—
	1: 8	—	+	+	+	+	+	(+)		—
	1: 16	—	(+)	(+)	+	+	+	(+)		—
	1: 32	—	—	—	(+)	(+)	+	±		—
	1: 64	—	—	—	—	—	—	—		—
<i>Ty2</i> <i>Filtrate</i> + <i>Sh. flex. var. Y</i> 120°C 3 hrs.	1: 2	(+)	+	+	+	⏏	⏏	(+)		—
	1: 4	—	(+)	+	+	+	+	(+)		—
	1: 8	—	—	±	+	+	+	(+)		—
	1: 16	—	—	±	(+)	(+)	+	(+)		—
	1: 32	—	—	—	±	(+)	+	(+)		—
	1: 64	—	—	—	—	—	—	—		—

c) "Field of Reaction" of Vi Agglutination Reaction with 6S Serum

Similar tests were undertaken employing 6S serum. Findings are shown in Table 3. End titer agglutination of the antigen was observed when the serum was in a dilution of 1:1600 to 1:6400. Generally, the end titers of the treated supernatant antigens were much lower than those responded to the other serums, the supernatant of *Ty2* being 1:32, that of *Ballerup* 1:64 and in the case of 6S it was only 1:16. The agglutination between 6S serum and

Table 3. "Field of Reaction" in the agglutination with 6S serum

Supernatant fluid and treatment	Supernatant fluid dilution	6S serum dilution								Control
		50	100	200	400	800	1600	3200	6400	
<i>Ty2</i> + <i>Sh. flex. var. Y</i> 120°C 2 2/3 hrs.	1: 2	++	++	++	##	##	##	++	+	—
	1: 4	+	++	++	##	##	##	++	+	—
	1: 8	—	(+)	+	+	+	++	++	+	—
	1: 16	—	—	+	+	+	+	+	+	—
	1: 32	—	—	—	—	±	±	(+)	(+)	—
	1: 64	—	—	—	—	—	—	—	—	—
<i>Ballerup</i> + <i>Sh. flex. var. Y</i> 120°C 2 2/3 hrs.	1: 2	+	+	++	(##)	(##)	(##)	+	+	—
	1: 4	+	+	++	++	++	++	+	+	—
	1: 8	—	±	+	+	++	++	+	+	—
	1: 16	—	—	(+)	+	+	+	+	+	—
	1: 32	—	—	—	±	±	±	+	+	—
	1: 64	—	—	—	—	—	—	—	(+)	—
	1:128	—	—	—	—	—	—	—	—	—
<i>6S</i> + <i>Sh. flex. var. Y</i> 120°C 2 2/3 hrs.	1: 2	++	(##)	##	##	##	##	++	+	—
	1: 4	(+)	+	++	++	++	++	++	+	—
	1: 8	—	—	+	+	+	+	+	+	—
	1: 16	—	—	—	—	—	±	±	(+)	—
	1: 32	—	—	—	—	—	—	—	—	—

treated 6S supernatant antigen, contrary to the similar findings observed in the case of *Ballerup*, failed to present the oblong "field of reaction" but showed a characteristic triangular figure which might probably be due to the absence of O antigen.

d) "Field of Reaction" of Vi Agglutination Reaction with
ViOH (ViI) Serum

The same agglutinogens were used with *ViI* serum. Findings are shown in Table 4. Namely, even with the treated *Ty2* supernatant antigen positive agglutination was observed only up to a dilution of 1:8 provided *ViI* serum used was in a dilution of 1:50. While, when the serum was in a dilution of 1:1600, the agglutination was positive as far as the dilution of 1:32. This finding might be due to the fact that the serum used was prepared by immunization with *ViI* strain in which the development of O antigen was poor. With the treated supernatant antigens of *Ballerup* and 6S, no agglutination took place provided *ViI* serum used was in a dilution of 1:50. And when the serum dilutions was 1:100, slight agglutination began to appear. The serum dilutions of 1:1600 to 1:3200 gave the highest, the treated *Ballerup* supernatant antigen being 1:64 and 6S 1:16.

The immune serums hitherto used were *Ballerup* (Vi antibody) and 6S (Vi, H antibodies) and the antigens were the treated supernatant antigens of

Table 4. "Field of Reaction" in the agglutination with ViI serum

Supernatant fluid and treatment	Supernatant fluid dilution	ViI serum dilution								Control
		50	100	200	400	800	1600	3200	6400	
<i>Ty2</i> + <i>Sh. flex. var. Y</i> 120°C 2 2/3 hrs.	1: 2	+	+	+	±	±	±	+	(+)	—
	1: 4	(+)	+	+	±	±	±	+	(+)	—
	1: 8	±	(+)	+	+	+	+	+	±	—
	1: 16	—	±	(+)	+	+	+	(+)	—	—
	1: 32	—	—	±	±	±	(+)	±	—	—
	1: 64	—	—	—	—	—	—	—	—	—
<i>Ballerup</i> + <i>Sh. flex. var. Y</i> 120°C 2 2/3 hrs.	1: 2	—	(+)	±	±	±	±	+	(+)	—
	1: 4	—	(+)	±	±	±	±	+	(+)	—
	1: 8	—	—	(+)	+	+	+	+	(+)	—
	1: 16	—	—	(+)	+	+	+	+	(+)	—
	1: 32	—	—	—	(+)	(+)	(+)	(+)	±	—
	1: 64	—	—	—	—	±	(+)	(+)	±	—
<i>6S</i> + <i>Sh. flex. var. Y</i> 120°C 2 2/3 hrs.	1: 2	—	(+)	+	±	±	±	+	+	—
	1: 4	—	—	+	+	+	+	+	+	—
	1: 8	—	—	±	+	+	+	+	(+)	—
	1: 16	—	—	—	—	—	±	±	±	—
	1: 32	—	—	—	—	—	—	—	—	—
	1: 128	—	—	—	—	—	—	±	—	—

Ty2 (ViOH), *Ballerup* (Vi) and *6S* (ViH). And, as the result of the experiments stated above, it was found that in either case of the agglutination between ViOH immune serum and treated Vi supernatant antigen or between Vi immune serum and treated ViOH supernatant antigen, characteristic "field of agglutination reaction" was observed.

It could be said, therefore, the reaction of Vi agglutination was inhibited when the serum used was in a low dilution and following the advance of serum dilution, the treated supernatant antigen of no much higher dilution presented a positive agglutination giving the highest titers against the serum in a dilution of 1:1600 to 1:3200. The agglutination reaction with pure Vi serum, however, did not have such a "field of reaction" and showed a field of oblong in shape. This fact might be due to liberation of O antigen into the serum while undergoing the processes of absorption. Determination of the O precipitin content of this pure Vi serum using *O901* serum (1:5) presented a positive reaction up to a dilution of 1:64.

e) *Vi Agglutination Reaction with Ballerup and 6S Serum Treated by H901*

The "field of agglutination reaction" between pure Vi serum and treated supernatant antigen was found to be oblong as stated above, while the same agglutination reaction between the treated supernatant antigen and the serum

such as *Ballerup* or 6S became triangular in shape. Since this difference was considered to be probably due to liberation of O antigen into the serum while undergoing the processes of absorption in the preparation of pure Vi serum, exactly the same processes were applied on *Ballerup* serum and 6S serum by using H901. Agglutination tests were carried out with those two kinds of sera employing the supernatant fluid of Ty2 treated by heating at 120°C after mixing the cells of *Sh. flex. var. Y*. The findings are shown in Table 5. The

Table 5. "Field of Reaction" in the agglutination with treated *Ballerup* and 6S serums

Supernatant fluid and treatment	Supernatant fluid dilution	Treated <i>Ballerup</i> serum dilution								Control
		50	100	200	400	800	1600	3200	6400	
Ty2 + <i>Sh. flex. var. Y</i> 120°C 2 2/3 hrs.	1: 2	++	(##)	##	##	##	##	+	(+)	—
	1: 4	++	(##)	##	##	##	##	+	±	—
	1: 8	+	+	+	+	+	+	+	—	—
	1: 16	(+)	+	+	+	+	+	(+)	—	—
	1: 32	±	±	(+)	(+)	(+)	(+)	±	—	—
	1: 64	±	±	±	±	(+)	(+)	±	—	—
	1:128	—	—	—	—	—	—	—	—	—
Treated 6S serum dilution										
Ty2 + <i>Sh. flex. var. Y</i> 120°C 2 2/3 hrs.	1: 2	++	++	++	++	++	++	(+)	—	—
	1: 4	++	++	++	++	++	++	(+)	—	—
	1: 8	++	++	++	++	++	+	(+)	—	—
	1: 16	(+)	+	+	++	+	+	(+)	—	—
	1: 32	(+)	(+)	(+)	(+)	(+)	(+)	±	—	—
	1: 64	(+)	(+)	(+)	±	±	±	±	—	—
	1:128	—	—	—	—	—	—	—	—	—

findings of untreated *Ballerup* serum and 6S serum to serve as controls are presented in the Tables 2 and 3.

Untreated *Ballerup* serum when in a dilution of 1:1600, presented the end titer agglutination of the antigen (1:64) but it was only 1:8 when the serum was in a dilution of 1:50. On the other hand, when the same antigen was used with the treated serum, the "field of reaction" became almost oblong in shape. The findings with 6S serum were mostly the same. Thus it was concluded that the difference observed between the treated and untreated serum was due to the presence of free O antigen liberated into the treated serums.

"FIELD OF REACTION" OF O AGGLUTINATION REACTION

Antigens used were the same as those used under "a" of the previous chapter. The findings of the agglutination reactions carried out between those antigens and O901 serum are shown in Table 6. The reactions were stronger

Table 6. "Field of Reaction" in the agglutination with *O901* serum

Supernatant fluid and treatment	Supernatant fluid dilution	<i>O901</i> serum dilution								Control
		50	100	200	400	800	1600	3200	6400	
<i>Ty2</i> + <i>E. coli</i> 120°C 3 hrs.	1: 16	++	++	++	++	++	+	(+)	±	—
	1: 32	+	+	+	+	+	(+)	(+)	—	—
	1: 64	—	—	—	—	—	—	—	—	—
<i>Ty2</i> Filtrate + <i>E. coli</i> 120°C 3 hrs.	1: 16	++	++	++	++	++	+	(+)	±	—
	1: 32	+	+	+	+	+	(+)	(+)	—	—
	1: 64	±	(+)	(+)	—	—	—	—	—	—
	1:128	—	—	—	—	—	—	—	—	—
<i>H901</i> + <i>Sh. flex. var. Y</i> 120°C 3 1/2 hrs.	1: 2	##	##	##	++	+	(+)	—	—	—
	1: 4	++	++	++	+	(+)	—	—	—	—
	1: 8	+	+	+	+	(+)	—	—	—	—
	1: 16	+	+	+	(+)	(+)	—	—	—	—
	1: 32	(+)	(+)	(+)	±	—	—	—	—	—
<i>Ballerup</i> + <i>Sh. flex. var. Y</i> 120°C 2 2/3 hrs.	1: 2	—	—	—	—	—	—	—	—	—
	1: 4	—	—	—	—	—	—	—	—	—
	1: 8	—	—	—	—	—	—	—	—	—
<i>6S</i> + <i>Sh. flex. var. Y</i> 120°C 2 2/3 hrs.	1: 2	—	—	—	—	—	—	—	—	—
	1: 4	—	—	—	—	—	—	—	—	—
	1: 8	—	—	—	—	—	—	—	—	—

the lower the dilution of the supernatant antigen was and the lower the dilution of the serum was and lowering in the agglutination titer was observed following the advance in the dilution of the supernatant antigen. Since O precipitinogen is heat labile (*cf*: precipitation reaction) the "field of O agglutination reaction" should be oblong in shape. Further, as the strains *Ballerup* and *6S* possess no O antigen, the supernatant fluids treated by heating at 120°C after mixing the bacterial cells of different species develops no O agglutination as shown in Table 6.

Next, the agglutination reaction of the treated supernatant antigens of *H901* (prepared by heating at 60°C and 100°C for 1 hour) presented a "field of reaction" of oblong in shape in most cases. Generally low agglutination titers observed in this case might probably be due to the prolonged treatment of heating at 120°C for such a long period of 3 1/2 hours. The supernatant fluid of *Ty2* and its filtrate through Chamberland L 3 were heated at 120°C after mixing the cells of *E. coli*. The "field of reaction" of the agglutination responded to those 2 kinds of antigens were mostly oblong in shape.

"FIELD OF REACTION" AND INTENSITY OF AGGLUTINATION RESPONDED
TO THE TWICE-TREATED SUPERNATANT ANTIGEN

The supernatant fluids of *ViI* and *Ballerup* treated by heating at 120°C after

[illegible]

But as for the supernatant dilution, the end titer of 1:16 to 1:32 was observed against the serum dilution of 1:1600 which was characteristic to Vi agglutination.

c) *O Agglutination Reaction Conducted with O901 Serum and Twice-Treated Supernatant Antigens of Ty2 and H901*

The twice-treated Vi supernatant antigen was the same as that used above. As shown in Table 9, 2 times of heating at 120°C resulted in marked weakening of the agglutinability. Even the control treated supernatant antigen presented positive agglutination only up to a supernatant dilution of 1:16. Twice-treated supernatant by adding the bacterial cells of different species presented positive agglutination only up to a dilution of 1:4.

Table 9. O agglutination reaction conducted with O901 serum and twice-treated supernatant fluids antigens of Ty2 and H901

Supernatant fluid and treatment	Supernatant fluid dilution	O901 serum dilution								Control
		50	100	200	400	800	1600	3200	6400	
<i>Ty2</i> 120°C 3 hrs. + <i>Sh. flex. var. Y</i> 120°C 3 hrs.	1: 2	+	+	+	(+)	(+)	±	—	—	—
	1: 4	(+)	(+)	(+)	(+)	(+)	±	—	—	—
	1: 8	(+)	(+)	(+)	(+)	(+)	±	—	—	—
	1: 16	±	±	±	±	±	±	—	—	—
	1: 32	—	—	—	—	—	—	—	—	—
<i>Ty2</i> + <i>Sh. flex. var. Y</i> 120°C 3 hrs. centrifuged + <i>Sh. flex. var. Y</i> 120°C 3 hrs.	1: 4	(+)	(+)	(+)	±	±	±	—	—	—
	1: 8	—	—	—	—	—	—	—	—	—
	1: 16	—	—	—	—	—	—	—	—	—
<i>H901</i> 120°C 3 hrs. + <i>Sh. flex. var. Y</i> 120°C 3 hrs.	1: 2	+	+	+	(+)	(+)	(+)	—	—	—
	1: 4	(+)	(+)	(+)	(+)	±	±	—	—	—
	1: 8	(+)	(+)	±	±	±	±	—	—	—
	1: 16	±	±	±	±	±	—	—	—	—
	1: 32	—	—	—	—	—	—	—	—	—
<i>H901</i> + <i>Sh. flex. var. Y</i> 120°C 3 hrs. centrifuged + <i>Sh. flex. var. Y</i> 120°C 3 hrs.	1: 4	(+)	(+)	(+)	(+)	±	—	—	—	—
	1: 8	—	—	—	—	—	—	—	—	—
	1: 16	—	—	—	—	—	—	—	—	—

Similar findings were obtained from treated *H901* supernatant fluid antigen. O antigen being different from Vi antigen, the treatment of heating at 120°C repeated 2 times resulted in a marked weakening of the agglutinability which might be partly due to the absorption of free O antigen to the bacterial cells of

different species but might also due to the weakness in the resistance of free O antigen to the influence of heating. This can easily be understood in comparison with the findings of a single treatment of heating at 120°C for 3 hours shown in Table 6. As to the "field of reaction", fields characteristic to the respective antigen were observed even after the treatment repeated 2 times.

PRECIPITATION REACTION

Same supernatant fluids as those used in the previous experiment were used in the precipitation tests in order to know their "fields of reaction". Namely, the ordinary supernatant fluids of *Ty2* and *ViI* without any treatment, the same merely heated at 120°C for 2 2/3 hours and the supernatant fluids obtained by centrifugation of the supernatant fluids in the serial two-fold dilutions up to 1:1024 and heated at 120°C after mixing with an equal quantity of the bacterial cells of different species suspended in physiological saline solution were used as precipitinogen putting them into serial dilutions up to 1:2048. The serums used were 1:25 to 1:1600 dilutions of *Ballerup*, 1:10 to 200 of *O901*. The method of precipitation reaction was ring test and reaction was observed for 3 hours.

a) *Precipitation Reaction with Ballerup Serum*

The supernatant fluids without any particular treatment presented a strong reaction but low dilutions of the supernatant fluids (up to 1:8) failed to react with the serum in a dilution of 1:1600. The supernatant fluids in a dilution of 1:16 to 1:256 presented a weakly positive precipitation, while the supernatant fluids in a still higher dilution presented no reaction. The supernatant fluids treated by heating at 120°C presented the reaction of the same intensity when they were in low dilutions, but the reaction became weak when they were in high dilutions. The findings of the supernatant fluids treated by heating at 120°C after mixing with the bacterial cells of different species showed weak reaction against the serum in a dilution of 1:800. The findings of the same experiments repeated with *Ballerup* serum and *6S* serum were mostly the same as shown in Table 10.

b) *Precipitation Reaction with O901 Serum*

The supernatant fluids without treatment presented a positive precipitation up to a dilution of 1:128 against a serum dilution of 1:10. Following the advance in the serum dilution, the intensity of the reaction became weak, becoming negative against the serum dilution of 1:200. When the supernatant fluids were treated by heating at 120°C, positive precipitation was observed only up to a serum dilution of 1:25. In addition, when the supernatant fluids were treated by heating at 120°C, after mixing the bacterial cells of different species, the precipitation was positive only against the serum in a dilution of 1:10 and the intensity of the reaction became weak. This finding was quite different from that observed with *Vi* antigen. Namely, the O precipitinogen was shown

to be highly heat labile.

c) *Precipitation Reaction of the Twice-Treated Supernatant Fluids*

The same twice-treated Vi supernatant fluid used in the agglutination reaction was used again in the precipitation reaction with *Ballerup* serum. The supernatant fluid heated only at 120°C used as control presented weaker precipitation when compared with the supernatant fluid without heating, but the titers of the serum and the supernatant fluid were mostly the same. In the case of the twice-treated supernatant fluid by mixing the bacterial cells, the intensity of the reaction became still more weak.

In view of the above findings, it can be said that the free Vi antigen is heat stable while free O antigen is heat labile. As to the "field of reaction," Vi precipitation was mostly oblong in shape while O precipitation was triangular showing lower titer of the supernatant following the advance in the serum dilution. On comparison of the intensity of agglutination reaction and precipitation reaction with treated supernatant fluid, O agglutination was positive, in most cases, as far as O precipitation was positive, but in the case of Vi agglutination it was only positive when the antigen dilution of the precipitation reaction was as high as 1:16 to 1:32.

SUMMARY

1. The "field of reaction" of agglutination reaction with pure Vi serum was studied employing the treated supernatant antigens of *Ty2*, *ViI*, *6S*, *Ballerup* etc. and it was found to be oblong in shape.

2. The findings of agglutination reaction between the treated supernatant antigens of *Ty2*, *ViI*, *6S*, *Ballerup* etc. and *Ballerup* serum and *6S* serum were fairly different from the findings of the reaction between the same antigens and pure Vi serum. When the serum dilution was low, there appeared the inhibition of the agglutination, and following the advance in the serum dilution so much higher dilution of the treated supernatant antigen began to show positive reaction. The end titer of treated supernatant antigen was obtained when the serum used was in a dilution of 1:1600 to 1:3200. Since the antigen and antibody which could react each other in this case were Vi antigen and Vi antibody, this "field of reaction" was considered to be Vi agglutination.

3. The treated supernatant antigens of *Ballerup* and *6S* were used to agglutination test with *ViI* (ViOH) serum and the same "field of reaction" was observed, in this case also the antigen and antibody which could react each other were Vi antigen and Vi antibody.

4. The same absorption processes applied on *ViI* serum for the preparation of pure Vi serum were given on *Ballerup* and *6S* serums. The agglutination reaction observed between the above treated serums and the treated supernatant antigen of *Ty2* showed the weakening or disappearance of the inhibition observed when the serums were in a low dilution.

5. The bacterial cells of twice-treated supernatant fluid (treated twice by heating at 120°C after mixing the bacterial cells of different species) were still to have weak Vi agglutinability. On the contrary, when the supernatant was treated by heating at 120°C without mixing bacterial cells for the first time and then heated again at 120°C after mixing bacterial cells, it showed the agglutination reaction of the same intensity as that observed with the supernatant treated once at 120°C after mixing bacterial cells. And their "field of reaction" was characteristic to Vi agglutination. In the case of O agglutination, a single treatment at 120°C resulted in remarkable weakening of O agglutinability.

6. As for Vi precipitation reaction, the heating of the supernatant fluid at 120°C caused the weakening of the precipitation reaction but the titers of the supernatant and serum remained practically unchanged with the "field of reaction" oblong in shape.

As to O precipitation reaction, the titer of the supernatant became low following the advance in the serum dilution. The influence of heating was extremely severe. When the supernatant fluid was treated at 120°C after mixing the bacterial cells of different species, the reaction was positive only up to a serum dilution as low as 1:10.

On comparison of the intensity of agglutination reaction and precipitation reaction with treated supernatant antigen, O agglutination was positive, in most cases, as far as O precipitation was positive, but in the case of Vi agglutination it was only positive when the antigen dilution of the precipitation reaction was as high as 1:16 to 1:32.

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