STUDIES IN AGGLUTINATION

IV. The Agglutination Inhibition Zone

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The prozone* or zone of inhibition of flocculation occasionally observed in the higher concentrations of agglutinating sera has been variously explained. This zone is usually seen in old sera although it is sometimes found in fresh sera. When present it may lead to practical difficulties in the serological differentiation of bacteria unless the tests are carried out to the higher dilutions. In our experience the natural zone phenomenon is extremely rare; in fact, in the course of the last few years we have observed almost no agglutinating sera showing this inhibition zone. In an effort to study this phenomenon it has been necessary, therefore, to take advantage of the well known fact that appropriate heating of agglutinating sera produces such a zone of inhibition. The work herewith reported is limited essentially to observations upon artificially produced inhibition zone sera.

Two chief hypotheses have been presented to explain the agglutination prozone.

The older hypothesis suggested by Eisenberg and Volk (1) and supported by Kraus and Joachim (2) accepts Ehrlich's conception of agglutinin as being made up of an antibody-bacteria binding portion (haptophore) and a flocculating portion (zymophore), and assumes that by heating or aging some of the agglutinin is so modified (agglutinoid) that the clumping component is destroyed without, however, affecting the binding portion. As a result agglutinoid may still unite with the bacteria but does not produce flocculation. In order to explain the inhibition effect in high serum concentrations, it is assumed that the agglutinoid in these concentrations has a greater affinity for the bacteria and is, therefore, bound to them to the exclusion of effective agglutinin. To explain the occurrence of flocculation in the higher dilutions it is assumed that the relative proportion of agglutinoid to agglutinin in these concentrations is such that the former is quanti-

* Owing to the variety of terms used, such as "prezone," "prozone," or "postzone" it has been decided to use the term "inhibition zone."

tatively insufficient to interfere with the effective clumping of the bacteria by the unmodified antibody. The disrepute into which the "haptophore-zymophore" theory of agglutinin structure has fallen has led to the more or less complete discrediting of this explanation.

The second hypothesis is put forward concisely by Zinsser (3) as follows: Agglutinoid zones are analogous to zone phenomena of other antibody reactions, notably the precipitin reaction, and are definitely dependent upon quantitative union between antigen and antibody and have nothing to do with deterioration of antibody by heat or otherwise. In various colloid precipitations in which serum is involved, moderate heating of the serum will strongly reduce its ability to precipitate a suspension. When normal serum is heated it is likely that there is a change in its colloidal state producing a certain amount of colloidal protective property in the serum. In reactions between bacteria and anti-serum it is likely that the antibody carries into union a not inconsiderable amount of active serum constituents. The so-called specific action of agglutinoids is probably due to the fact that the antibody carries into union with the bacteria inactive protein which is colloidally protective by virtue of the heating.

It was with the expectation of producing evidence to substantiate the protective colloid hypothesis that the problem was approached originally in the present investigation. However, as the evidence has accumulated, it has become more and more apparent that the agglutinoid explanation, much modified, more nearly fits the facts, at least as far as the heat induced inhibition zone is concerned.

Methods

Sera Used.—Rabbit and horse agglutinating sera were used. A high titre antityphoid serum (horse) prepared at The Rockefeller Institute in 1917 was obtained from Dr. Chickering. B. melitensis serum was kindly given to us by Dr. Cooper of the New York Board of Health.

Bacterial Strains.—The following organisms were used: B. typhosus, B. melitensis, Type I pneumococcus, and B. dysenteriae, Flexner, Mt. Desert, Shiga, Sonne A and B, and "Y."

Buffers Used.—A glycocoll, sodium phosphate and sodium acetate (G. P. A. mixture) (4) was used in some of the experiments for diluting serum and suspending the bacteria. Most of the experiments were carried out in physiological salt solution.

Washing of Bacteria.—Bacteria were washed at least twice in distilled water in all experiments.

Agglutination Methods.—The macroscopic method was used. Degrees of agglutination are indicated as follows: C., complete, supernatant fluid clear; 3, markedly granular with beginning floculation; 2, same without flocculation; 1, slightly granular; ±, doubtful agglutination; −, no agglutination.

Potential Measurements.—These were made with the Northrop micro-cataphoresis cell (5).

EXPERIMENTAL RESULTS

Preparation of Inhibition Zone Serum.—When an agglutinating serum is appropriately heated inhibition of flocculation is obtained in the higher concentrations.

A typical heat inhibition zone is shown in Table 1. This is a rabbit antidysenteric serum (Shiga) diluted 1:10 in normal salt and heated for ten minutes at 67°C. In all cases heating of prozone serum was carried out in 1:5–1:10 dilution. Undiluted, or greater concentrations of serum than the above are unsatisfactory for heating as they become turbid and are difficult to work with. It will be observed that no agglutination occurs in the dilutions 1/20 to 1/80 but that it is present above this.

TABLE 1

The Effect upon Agglutination of Heating B. dysenteric (Shiga) Serum at 67°C. for 10 Minutes: a Typical Heat Inhibition Zone Serum

	Agglutination									
	Dilutions of serum									
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	Control
Serum unheated (control).....	C	C	C	C	C	C	C	C	—	—
Serum heated at 67°.....	—	—	—	C	C	C	C	C	—	—

It is important to note that the upper agglutinative titre is practically the same as that of the unheated control serum.

In order to produce this zone phenomenon in different sera, exposure to varying temperatures for the various sera is required.

The minimal point, using short periods of exposure (6–10 minutes), ranged from 62°C., in a Type I pneumococcus serum, to 76°C. for a B. typhoid serum. In some sera we were unable to produce an inhibition zone when using the shorter periods of heating. As the heating point is raised, a level is reached when all agglutination is abolished. This point also is quite variable for the different sera. In our experiments the range was from 64° to 78°C.

The time factor is an important consideration. If sera are heated over prolonged periods at temperatures lower than those noted above, it is possible to produce an inhibition zone.

For example, in the case of a Shiga serum, in which the inhibition zone was obtained by heating for 10 minutes at 66°C., a comparable inhibition zone was produced in 4 hours at 59°C. Table 2 shows the comparison in detail. It will be noted from this table that prolonged heating at this lower temperature (24 hours) has an effect similar to that of the higher temperatures, as noted above, of abolishing all agglutinin. When the time factor is studied for the higher temperatures it is found that this effect is more rapid, being more or less proportional to the temperature. For example, when the serum is heated at 68°C., the inhibition zone begins to appear in 1 minute, is complete in 6 minutes, and practical abolition of all agglutination occurs in 90 minutes.

TABLE 2

The Effect upon Agglutination of Heating Shiga Serum at 59° for 1 to 48 Hours. Shiga Serum Heated at 66° for 10 Minutes Is Shown for Comparison

Time of heating	Agglutination									
	Dilutions of serum									
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	Control
Unheated	C	C	C	C	C	C	C	C	C–	–
30 minutes	C	C	C	C	C	C	C	C	C–	–
1 hour	3	C	C	C	C	C	C	C	C–	–
2 hours	2	C	C	C	C	C	C	C	C–	–
4 hours	–	–	3	C	C	C	C	C	2	–
8 hours	–	–	2	C	C	C	C	C	3	–
24 hours	–	–	–	–	–	–	±	±	–	– –
Heated at 66° 10 minutes	–	–	±	C	C	C	C	C	C	–

An interesting demonstration of the effect of varying the heating levels is shown in Table 3.

Separate portions of Shiga serum were heated at temperatures ranging from 55° to 80°C., for 10 minutes. At 63° there is a beginning appearance of the inhibition zone. The zone then widens to reach a peak at 66–69°. Above this the zone narrows to disappear at 72°. Coincident with this narrowing and its loss there is a corresponding drop in the agglutinative titre. At 76° all agglutination disappears. This experiment was done in physiological salt solution; when repeated in a buffer mixture of pH 7.0, similar results were obtained.

A comparable result, though far less marked, was obtained with one other serum. The more usual finding is the appearance of an

inhibition zone immediately succeeded by disappearance of all agglutination as the temperature is further increased.

The findings noted in Table 3 strongly support the hypothesis that the inhibition zone is caused by modification of agglutinin. If one makes the assumption that such heat modified agglutinin (a) still

TABLE 3

Effect of Heating Shiga Serum at Various Temperatures for 10 Minutes

Temperature of heating	Agglutination									
	Dilutions of serum									Controls
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
Unheated	C	C	C	C	C	C	C	C−	−	−
55°	C	C	C	C	C	C	C	C−	−	−
56	C	C	C	C	C	C	C	C−	−	−
57	C	C	C	C	C	C	C	C−	−	−
58	C	C	C	C	C	C	C	C−	−	−
59	C	C	C	C	C	C	C	C−	−	−
60	C	C	C	C	C	C	C	C−	−	−
61	C	C	C	C	C	C	C	C−	−	−
62	C	C	C	C	C	C	C	C−	−	−
63	C	C	C	C	C	C	C	C−	−	−
64	±	±	C	C	C	C	C	C−	−	−
65	−	−	C−	C	C	C	C	C−	−	−
66	−	−	−	−	C	C	C	C−	−	−
67	−	−	−	−	C	C	C	C−	−	−
68	−	−	−	−	C	C	C	C−	−	−
69	−	−	−	−	C	C	C	C−	−	−
70	−	−	C−	C	C	C	C−	±	−	−
71	C−	C−	C	C	C	C	±	±	−	−
72	C	C	C	C	C	C−	−	−	−	−
73	C	C	C	C	C	C−	−	−	−	−
74	C	C	C	C	C	C−	−	−	−	−
76	−	−	−	−	−	−	−	−	−	−

retains its binding power although when union has taken place the agglutinin-bacteria complex fails to clump and (b) has a greater affinity for the bacteria than unchanged agglutinin, it follows that the total amount of binding agglutinin in the inhibition zone (*e.g.*, at 66°) will be equal to that of the unheated serum, *i.e.*, the agglutinative titre of the two sera will be the same. This is the fact. Now, when

the heating level is further raised, it will be seen that the inhibition zone is reduced and then disappears. If we attempt to explain this upon the assumption that this higher heating further modifies the modified agglutinin so that it now loses its binding power, we should expect that the total agglutinative titre would fall off correspondingly. This also is the experimental fact. Such evidence is indirect rather than direct proof. It is believed that the experimental findings presented below will give more direct support to the correctness of this view.

Relationship of pH.—Most of the experiments reported were done in physiological salt solution, of pH's varying from 6.0 to 7.0. That the H ion concentration might be a factor was considered probable. Accordingly, its relationship to the production of the heat inhibition zone was tested at pH's ranging from 5.4 to 7.0. Table 4 shows the results. It will be seen that, within the limits of the experiment, a wider prozone is obtained at the higher pH's. This is essentially the range in which the experiments with salt solution as diluent were carried out.

Specificity of the Inhibition Zone.—In the colloidal protective theory of the inhibition zone it is assumed that heating of the serum changes serum protein so that it has protective properties, and the so-called specific action of agglutinoid is considered to be due to the carrying over of such inactive protein into the union between antibody and organism. This explanation is usually offered to overcome the stumbling block presented by the older experiments in which it has been shown that absorption of agglutinoid is possible in the inhibition zone. If colloidally protective inactive protein (*i.e.*, non-agglutinin) is present in an inhibition zone serum it should follow that it might exert an inhibitory effect when added to another, heterologous, serum. Accordingly a suitable inhibition zone serum was obtained by heating an antityphoid serum and this was added, in small to large proportions, to an active, unheated B. melitensis serum. When the latter was now tested against its homologous organisms, no interference with agglutination in any concentration was noted. Such findings support the contention that the phenomenon is specific.

In order further to test this hypothesis a similar experiment was performed with very closely related organisms.

TABLE 4

Relationship of pH to Zone of Inhibition in Heat Produced Inhibition Zone Serum. Antityphoid Serum Used

pH	T° of heating	Agglutination											
		Dilutions of the serum											
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	1:20480	Controls
7.0	70	–	–	–	–	–	±	C	C	C	C	C–	–
	71	–	–	–	–	–	±	C	C	C	C	C–	–
	72	–	–	–	–	–	±	C	C	C	C	C–	–
	73	–	–	–	–	–	±	C	C	C	C	C–	–
	74	–	–	–	–	–	±	C	C	C	C	C–	–
	75	–	–	–	–	–	±	C	C	C	C	C–	–
6.6	70	–	–	–	–	3	C–	C	C	C	C	±	–
	71	–	–	–	–	3	C–	C	C	C	C	±	–
	72	–	–	–	–	3	C–	C	C	C	C	C–	–
	73	–	–	–	–	3	C–	C	C	C	C	C–	–
	74	–	–	–	–	3	C–	C	C	C	C	C–	–
	75	–	–	–	–	±	3	C	C	C	C	C–	–
6.2	70	–	–	–	–	3	C	C	C	C	C	C–	–
	71	–	–	–	–	3	C	C	C	C	C	C–	–
	72	–	–	–	–	3	C	C	C	C	C	C–	–
	73	–	–	–	–	3	C	C	C	C	C	C–	–
	74	–	–	±	3	C	C	C	C	C	C	±	–
	75	–	–	2	3	C	C	C	C	C	C	C	–
5.8	70	–	–	2	C–	C	C	C	C	C	C–	±	–
	71	–	±	2	C	C	C	C	C	C	C–	±	–
	72	–	2	2	C	C	C	C	C	C	±	±	–
	73	–	2	C	C	C	C	C	C	C	±	±	–
	74	C	C	C	C	C	C	C	C–	±	±	–	–
	75	C	C	C	C	C	C	C	C–	±	–	–	–
5.4	70	–	–	2	C–	C	C	C	C	C	C	±	–
	71	2	C–	C	C	C	C	C	C	C	C–	±	–
	72	2	C–	C	C	C	C	C	C	C	C	±	–
	73	C–	C	C	C	C	C	C	C	C	±	±	–
	74	C	C	C	C	C	C	C	C	±	±	–	–
	75	C	C	C	C	C	C	C	C	±	±	–	–

Shiga serum, diluted 1:9 in normal saline, was heated 10 minutes at 66.5°C. and an inhibition zone obtained. To 0.9 cc. of this Shiga inhibition zone serum, 0.1 cc. each of active (*i.e.*, unheated) Shiga, Flexner, Mt. Desert, Sonne B and "Y"

dysenteric serum was added. This gave proportions of inhibition zone serum to unheated serum of a little more than 1:1. These 1:10 dilutions of the different sera, each with its added component of inhibition zone serum, were now diluted out by halves and homologous organisms were added to each. The results of the experiment are shown in Table 5.

TABLE 5

Effect of Addition of Shiga Inhibition Zone Serum to Unheated Shiga, Flexner, Mt. Desert, Sonne "B" and "Y" Dysenteric Sera

The inhibition zone serum was added in an effort to produce a non-specific inhibition zone.

Serum	Organism	Agglutination										
		Dilutions of the sera										Controls
		1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	
Shiga, unheated	Shiga	C	C	C	C	C	C	C	C	C−	−	−
Shiga, heated	"	−	−	−	C	C	C	C	C	C−	−	−
Shiga, unheated + heated	"	−	−	−	−	C	C	C	C	C	±	−
Shiga, unheated	Flexner	C	C	−	−	−	−	−	−	−	−	−
Flexner, unheated + Shiga, heated	"	C	C	C	C	C	C	C	C	C−	−	−
Shiga, unheated	Mt. Desert	C	C	−	−	−	−	−	−	−	−	−
Mt. Desert + Shiga, heated	"	C	C	C	C	C	C	C	C	C−	−	−
Shiga, unheated	Sonne "B"	±	−	−	−	−	−	−	−	−	−	−
Sonne B, unheated + Shiga, heated	"	C	C	C	C	C	C	C	C	±	−	−
Shiga, unheated	"Y"	±	−	−	−	−	−	−	−	−	−	−
"Y", unheated + Shiga, heated	"	C	C	C	C	C	C	C	C−	−	−	−

It will be seen that in the case of the unheated Shiga serum, the addition of Shiga inhibition zone serum produces a definite zone of inhibition in its higher concentrations; the zone is a little wider here, probably because of the slightly higher proportion of heated to unheated serum as pointed out above. It is worthy of note that the total agglutinative titre is slightly increased, a finding to be expected if the agglutinoid hypothesis is correct. The highly specific nature of the inhibition zone is demonstrated by the failure of the addition

of the Shiga inhibition zone serum to exert any inhibitory effect upon the agglutination of the heterologous organisms by their respective sera.

Absorption Experiments.—The findings of Eisenberg and Volk that absorption of agglutinin (and agglutinoid) occurs in the inhibition zone has been the chief argument in favor of their explanation of the inhibition zone. In order to confirm the work of these investigators the following experiment was carried out.

TABLE 6

Effect of Absorption of Shiga Inhibition Zone Serum with Varying Dosage of Organisms upon Subsequent Agglutination

Dosage of absorbing organisms per cc.	Agglutination after centrifugalization									
	Dilutions of sera									
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	Controls
1 billion	—	—	C—	C	C	C	C	C—	—	—
2 "	—	—	C—	C	C	C	C	C—	—	—
4 "	—	C—	C—	C	C	C	—	—	—	—
8 "	—	C	C	C	C	—	—	—	—	—
*16 "	C	•	•	•	•	•	•	•	•	•
32 "	C	C	C	C	—	—	—	—	—	—
64 "	C	C	C	C	—	—	—	—	—	—
128 "	C	C	C	C	—	—	—	—	—	—
Controls										
Shiga serum, unheated.....	C	C	C	C	C	C	C	C	C—	—
Shiga inhibition zone serum (heated).....	—	—	C—	C	C	C	C	C	C—	—

* Remainder of this experiment lost.

Shiga serum was heated 10 minutes at 67° to produce an inhibition zone. To this serum Shiga organisms were now added in increasing amounts so that the concentrations of the bacteria ranged from 1 billion to 128 billion per cc. The mixtures were then incubated 2 hours in a 37° waterbath and left over night in the icebox. The next day the bacteria were thrown down and the supernatant fluid tested for its agglutinin content. The concentration of the bacteria in this test being 1 billion per cc. The results are shown in Table 6.

It is at once apparent that the findings are in striking agreement with the results reported by Eisenberg and Vook. When the serum

is absorbed with the smaller number of bacteria (1–8 bil./cc.) there is reduction of the inhibition zone and loss of agglutinative titre which is proportional to the concentration of the organisms. Above this, effective agglutinin is absorbed. In other words, it would seem that when the bacteria are relatively fewer in number there is preferential absorption in, or of, the prozone (agglutinoid). This strongly supports the hypothesis that there is present in the inhibition zone serum agglutinin which is so modified that it binds but does not clump bacteria and which has a greater affinity for the organisms than has unmodified agglutinin.

TABLE 7

Effect upon Agglutination, in a Shiga Inhibition Zone Serum, of Varying the Quantity of Bacteria Used for the Test

Number of bacteria per cc.	Agglutination																
	Dilution of the serum																
	1:20	1:40	1:60	1:80	1:100	1:120	1:140	1:160	1:180	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	Controls
16 billion	C	C	C	C	C	C	C	C	C	C	C	C	–	–	–	–	–
8 "	–	C	C	C	C	C	C	C	C	C	C	C	C	–	–	–	–
4 "	–	–	C	C	C	C	C	C	C	C	C	C	C	–	–	–	–
2 "	–	–	–	–	–	–	C	C	C	C	C	C	C	3	–	–	–
1 "	–	–	–	–	–	–	–	–	–	–	C	C	C	C	–	–	–
½ "	–	–	–	–	–	–	–	–	–	–	–	C	C	C	3	–	–
¼ "	–	–	–	–	–	–	–	–	–	–	–	–	C	C	3	–	–
*⅛ "	–	–	–	–	–	–	–	–	–	–	–	–	C	C	3	–	–

* Below this point the suspensions were too light to be read.

In an effort to explain the fact that in an inhibition zone serum, bacteria fail to agglutinate in the low dilutions, but do so in the higher dilutions, the assumption has been made that the relative proportion of modified to unmodified agglutinin is such, in the higher dilutions, that the former is quantitatively insufficient to interfere with effective clumping by the latter. It seemed that this hypothesis should be susceptible of experimental verification. The next experiment (Table 7) seems to supply the necessary proof. If one accepts the assumption that in the lower dilutions of an inhibition zone serum (a) there is present a mixture of agglutinin and agglutinoid (the latter being suffi-

cient in quantity to become an effective factor) and (b) that the agglutinoid has a greater affinity for the bacteria, it follows of necessity, in view of the fact that the agglutinoid must be limited in amount, that when bacteria are added in excess of the number required to unite with all the agglutinoid, unmodified agglutinin will become effective and clumping must result. From Table 7, it will be seen that this is the experimental fact.

When the concentration of bacteria per cc. is 16 billions the condition of excess organisms (over the agglutinoid-binding requirement) is present and agglutination takes place. As the number of organisms drops below this "agglutinoid saturation" point, the inhibition zone appears; and, as the organisms per cc. continue downward their number falls below this saturation level in succeeding dilutions so that with 1/4 billion per cc. in the 1/400 dilution of the serum it falls below such a point and the inhibition zone is present.

These findings have some practical value. Errors in serological diagnostic work due to an inhibition zone may be lessened by having the bacteria in relatively high concentration in setting up agglutination tests. And, conversely, when one is studying the zone phenomenon, the inhibition zone is more readily obtained by keeping the dose of organisms low.

The following experiment was performed in a further effort to test the possible importance, in producing the inhibition zone, of some non-specific, colloidally protective, serum constituent.

Shiga serum was heated and an inhibition zone obtained. The serum was then absorbed by its homologous organisms. This serum now freed of agglutinin and agglutinoid was added to an active unheated serum to see whether there remained in it any non-specific inhibitory factor.

It will be seen from Table 8 that the absorbed serum produces no inhibition zone.

The converse of the experiment above was carried out next, as follows:

Shiga inhibition zone serum was prepared and bacteria in relatively low concentration were added to a 1:20 dilution. They were allowed to incubate for 2 hours in the 56° water bath. The organisms, which had remained unagglutinated (*i.e.*, were stable in suspension), were now centrifuged. They came down in a rather heavy, fluffy mass, more like normally sensitized than unsensitized organisms. The

supernatant fluid was discarded and the bacteria resuspended. Many clumps had appeared following centrifugalization. These were allowed to settle out and the comparatively smooth suspension remaining was tested for agglutinability by addition to active unheated serum. Table 9 shows the result.

TABLE 8

Effect upon Agglutination in a Shiga Unheated Serum of the Addition to This Serum of Absorbed Inhibition Zone Serum

Sera	Agglutination										
	Dilution of the serum										
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	Controls
Unheated serum (control).....	C	C	C	C	C	C	C	C	C	—	—
Heated inhibition zone serum ..	—	—	—	—	—	C	C	C	C	—	—
Absorbed inhibition zone serum.	3	—	—	—	—	—	—	—	—	—	—
Unheated active serum + absorbed inhibition zone serum..	3	C	C	C	C	C	C	C	C	—	—

TABLE 9

Effect upon Agglutination of Bacteria, by Active Unheated Serum, of Previous Sensitization of the Bacteria in an Inhibition Zone Serum of 1:20 Dilution

Sera	Agglutination										
	Dilutions of serum										
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	1:10240	Controls
Unheated active serum + unsensitized bacteria.....	C	C	C	C	C	C	C	C	C	—	—
Heated inhibition zone serum + unsensitized bacteria.....	—	—	—	—	C	C	C	C	C	—	—
Active unheated serum + inhibition zone sensitized bacteria	—	—	—	—	—	—	—	—	—	—	—

It will be observed that the organisms sensitized in the inhibition zone are not agglutinated by active serum. In other words, they are saturated with binding, but non-flocculating, modified agglutinin and are, therefore, insusceptible of agglutination by the super-added active serum. Jones (6) working with sera which had been heated to a

higher level than ours, so that practically all agglutinin effect was lost has recently obtained comparable results.

Filtrability Experiments.—In the case of one of our antityphoid heat induced inhibition zone sera (heating was at 74.5° for 12 minutes) it was observed that there was faint opalescence. Attempts were made to centrifuge out the turbidity, without success. Passage through a Berkefeld N filter was then carried out with complete clearing. When this serum was now tested for its agglutinative properties it was found that the inhibition effect had been entirely removed. Table 10 shows the results.

Berkefeld filtration has apparently completely removed the agglutinoid effect. Accompanying this disappearance there is a propor-

TABLE 10

Effect of Berkefeld Filtration of Inhibition Zone Serum upon Subsequent Agglutination

Sera	Agglutination									
	Dilution of the serum									
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120	Controls
Unheated typhoid serum	C	C	C	C	C	C	C	C	C	—
Heated inhibition zone serum	—	—	—	—	—	C	C	C	C	—
Filtered inhibition zone serum	C	C	C	C	C	C	—	—	—	—

tional drop in the agglutinative titre. From this experiment it is apparent that heat modification of the agglutinin has made it particulate. The fact that upon its removal there is a corresponding drop in agglutinative titre is strong evidence in favor of the fact that this particulate, modified agglutinin still retains its binding power.

This serum was the only one showing perceptible opacity upon heating for the inhibition effect. It was, in fact, the serum which required the highest heating level to produce the zone effect. However, in one of our clear inhibition zone sera, in which filtration showed no effect upon the inhibition or the titre, the addition of kaolin produced identical results as to abolition of the prozone and proportional reduction of titre; kaolin treatment of the unheated serum showed no

effect upon titre. That this is not simple adsorption was demonstrated in an experiment in which treatment of an inhibition zone serum (typhoid) with a heavy dose of heterologous organisms (B. dysenteriae) failed to remove the inhibitory factor.

Cataphoresis Experiments.—It was felt that a study of the charge upon the bacteria when they were sensitized with inhibition zone

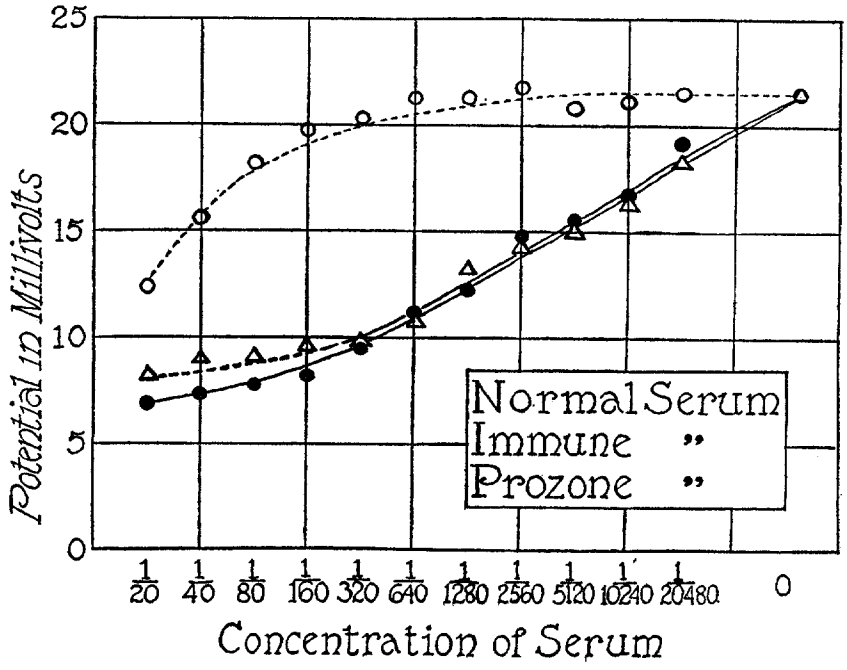

FIG. 1. Effect of unheated agglutinative, and heated agglutinative (inhibition zone) typhoid serum, and of normal serum upon P.D. and agglutination of B. typhosus. Solid line indicates agglutination; broken line, no agglutination. Experiment in G. P. A. buffer mixture, pH 7.0.

serum might be instructive. Accordingly observations were made upon the P.D. of bacteria treated in varying dilutions with normal serum, unheated agglutinating serum, and inhibition zone serum. The results are shown in Fig. 1.

It will be noted from the chart that the charge on the bacteria in the zone of non-flocculation closely approximates that of those which

are sensitized with untreated serum. This is strong evidence that there is union between organism and antibody although there is no clumping. Mudd and his co-workers (7) in their extensive studies of antibody reactions have recently reported similar findings in their observations upon the charge on bacteria in the prozone.

Recently, working with pneumococcus Type III antibody solution, prepared essentially according to the method of Felton,* we have obtained a small though definite inhibition zone after 5 minutes heating at 68°. This antibody solution is almost entirely free of serum protein other than serum globulin, 90 per cent being removed in its preparation (9). This finding is further evidence, although indirect, in favor of the importance, in the production of the inhibition zone, of modified agglutinin (antibody globulin).

DISCUSSION

From the foregoing experiments two points appear clear. (1) Inhibition of flocculation in the inhibition zone is highly specific and this specificity is bound up closely with the presence of agglutinin. (2) The effect is dependent upon modification of the agglutinin, and, agglutinin, so modified, although deficient in flocculating power, has a greater affinity for bacteria than unmodified antibody.

The specificity of the phenomenon is striking. At no time have we been able to demonstrate it to be due to the effect of any inactive (non-antibody) serum constituent which may have become protective as a result of heating. Removal, from an inhibition zone serum, of modified agglutinin (agglutinoid), by specific absorption, Berkefeld filtration, or adsorption with kaolin, has been regularly accompanied by the loss of its inhibitory effect. Hence it has seemed a reasonable conclusion that the inhibitory factor is agglutinoid and not a non-specific, inactive, serum constituent.

It has seemed clear from the experiments reported above that the older observations are correct, namely, that modified agglutinin (agglutinoid) has a greater affinity for bacteria than does unheated antibody. The evidence is striking in the selective absorption experiments above, tables 6–9. To attempt to explain the greater

* The antibody solution was kindly given to us by Dr. Heidelberger and Dr. Kendall who have been using it in the studies of the precipitin reaction.

affinity of agglutinoid for bacteria is not possible in view of our ignorance of antibody chemical structure. A parallel exists in the greater avidity of diphtheria toxoid, than toxin, for antitoxin.

That sensitization by unmodified agglutinin and agglutinoid are very similar is brought out by many of the findings above and especially by the P.D. determinations. However, although selective specific sensitization of bacteria by agglutinoid seems to take place, flocculation does not follow. The use of the "haptophore-zymophore" hypothesis in explanation of this failure of clumping is not possible as there is no evidence for such agglutinin structure. However, an attempt may be made to explain this non-flocculability of the agglutinoid-bacterial complex on the basis of previous studies of the mechanism of bacterial agglutination.

In this work (8) it has been shown that sensitization of bacteria by agglutinative sera is selective coating by antibody globulin, that by such film formation at their surface, the bacteria take on the characteristics of particles of denatured globulin and that subsequent agglutination of the coated bacteria follows the laws governing the flocculation of particles of denatured protein by electrolytes. For effective selective coating of bacteria by agglutinin it would seem essential that the antibody be intact. Modification of the agglutinin to agglutinoid by heating may well alter the agglutinin-globulin complex sufficiently to interfere with adequate film formation at the bacterial surface. By virtue of this faulty coating the agglutinoid-sensitized particles will fail to take on the characteristics of denatured globulin and flocculation will not occur.

The problem of the rare appearance of an inhibition zone in fresh sera remains unexplained. It has been shown above that sera are variably heat sensitive with reference to the production of a prozone. It is possible that an occasional serum may be heat sensitive at a lower level than that noted above (59°), that is, within the limits of ordinary experiments, 56° or less. In support of such a suggested explanation we have observed one serum (pneumococcus, Type I, horse) in which an inhibition zone appeared when incubation was at 56° but not at 37°. It is of interest in this connection that Heidelberger and Kendall (9) in studying the precipitin reaction have observed flocculation at ice-box temperatures in mixtures which showed no precipitation at room

temperature. Whenever natural inhibition zone serum is available further investigation will be carried forward.

It is probable that the inhibition zone occasionally observed in old sera is due to some modification of agglutinin comparable to that obtained when serum is heated, *i.e.*, agglutinoid is formed.

CONCLUSIONS

1. The agglutination inhibition zone, artificially produced by heating, has been studied.

2. The phenomenon is specific and is dependent upon the presence in the inhibition zone serum of altered agglutinin (agglutinoid).

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