THE STABILITY OF BACTERIAL SUSPENSIONS.

IV. The Combination of Antigen and Antibody at Different Hydrogen Ion Concentrations.

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INTRODUCTION.

In a preceding paper (1) it was observed that the agglutinating power of normal and immune serum increased as the pH approached the acid agglutination optimum of the bacterium under test. Several hypothetical explanations of this phenomenon suggest themselves: (*a*) The agglutinating power of antibody might be enhanced by incubation at high C_H (4.0 to 5.0); (*b*) the maximal combination of antigen and antibody might take place at this zone; (*c*) the same amount of antibody in combination might have a greater effect in this range; or (*d*) the other constituents of the serum may have an agglutinating influence in this range. The following experiments show that the first two suggestions are untenable, and that the third hypothesis accounts for the observations.

Method.

The organism used in these experiments was *Bacillus typhosus*, Pfeiffer strain, obtained through the kindness of Dr. Charles Krumwiede of the Department of Health of the City of New York, to whom we are also indebted for the high titer antityphoid agglutinating serum. The serum dilutions were carried out in glycocoll-Na acetate- Na_2HPO_4 buffer mixtures of definite pH, described in the preceding paper (1). The "standard" bacterial suspensions were prepared as described in the preceding paper (1). Suspensions of higher concentration were used for absorption and other tests, and are always referred to the standard suspensions, as follows: A 10 $\times$ *T*

suspension would be one in which the bacteria from 150 cc. of broth were suspended in *15 cc.* of distilled water. A 20 × *T* suspension would be made by suspending the bacteria from 150 cc. of media in *8 cc.* of distilled water, and so on.

Experiment 1. Agglutinating Power of Antityphoid Serum Kept at Various C_H *for 2 Hours at 43°C.*—0.1 cc. quantities of serum>typhoid were diluted with 2.9 cc. of glycocoll-acetate-phosphate buffer mixtures (M/12) of various pH from 9.9 to 2.0. Dilution = 1:30. The tubes containing these dilutions were placed in the water bath at 43°C. for 2 hours. 0.1 cc. of each sample was then diluted with 3.35 cc. of buffer mixture pH 6.0. Colorimetric test showed all of the mixtures to be at or very near to pH 6.0. Dilution now approximately 1:1,000. Each cc. contained 0.001 of immune serum. Each sample was now diluted by 2's with buffer solution pH 6.0 to a point where 3.7×10^{-6} cc. of the original immune serum was present in each tube. 1 cc. of standard 4 times washed typhoid suspension was now added to each series of dilutions and incubated at 43°C. for 14 hours. The results are summarized in Table I.

It will be observed from Table I that over a range from pH 9.9 to 3.9, 43°C. for 2 hours has very little effect on the subsequent agglutinating power of antityphoid serum. It is true that a slight decrease in activity manifests itself in Tubes 4 and 5 (pH 6.0 and 5.0). This difference is probably not adventitious, since subsequent tests confirmed the observation. It is probably due to precipitation of the globulin. A really perceptible deleterious effect is observed, however, in Tubes 9 and 10 (pH 2.7 and 2.2). Here a decrease of eightfold loss of agglutinative power is observed in Tube 9, and sixteenfold in Tube 10. The experiment demonstrates that hydrogen ion concentrations of pH 5.0 and 4.6 do not have an enhancing effect on the agglutinating power of the serum, and the first hypothesis has, therefore, to be rejected.

A second explanation of the increased agglutinating activity of serum at pH 4.5 to 5.0 lies in the idea that the combination between antigen and antibody might be more complete in this zone of hydrogen ion concentration. This would be the assumption, *a priori*, of those who believe that the combination of antigen and antibody is influenced by electrical forces (*2*). Assuming that antibody behaves in a manner similar to serum protein, the former should carry a slight *positive* charge at pH 4.6. The bacterium, on the other hand, is

negatively charged at this point (1). Therefore, if union between bacterium and antibody is a matter of neutralization of opposite charge, it should be more complete at pH 3.5 to 4.5, where the organism and antibody are oppositely charged, than at pH 6.0 or 7.0, where both bacterium and antibody would be negatively charged.

TABLE I.

Agglutinating Power of Antityphoid Serum Kept at Various C_H for 2 Hours at 43°C.

Tube.	pH at which serum was kept for 2 hrs. at 43°C.	Serum diluted from various pH to pH 6.0; 43°C., 14 hrs., + typhoid suspension. Cc. of serum per cc. of solution.*								
		1×10^{-3}*	5×10^{-4}	2.5×10^{-4}	1.2×10^{-4}	6×10^{-5}	3×10^{-5}	1.5×10^{-5}	7×10^{-6}	3.5×10^{-6}
1	9.9	C.	C.	C.	C.	C.	++	+	Tr.	Tr.
2	9.0	C.	C.	C.	C.	C.	++	+	−	−
3	7.5	C.	C.	C.	C.	C.	++	+	Tr.	−
4	6.0	C.	C.	C.	C.	++	+	Tr.	−	−
5	5.0	C.	C.	C.	C.	++	++	+	Tr.	−
6	4.6	C.	C.	C.	C.	C.	++	+	Tr.	−
7	3.9	C.	C.	C.	C.	C.	++	+	+	Tr.
8	3.3	C.	C.	C.	C.	++	++	+	+	Tr.
9	2.7	C.	C.	++	+	−	−	−	−	−
10	2.2	C.	++	+	−	−	−	−	−	−
11	Control serum undiluted; 43°C.; 2 hrs.	C.	C.	C.	C.	C.	++	+	−	−

*Represents concentration of serum $\left(i.e. \frac{1}{\text{dilution}} = \text{cubic centimeter of serum per cubic centimeter of solution}\right)$. Subsequent addition of equal volume of suspension divides this concentration by 2.

The degree of combination is best studied by absorption experiments. Since the absorption of antibody is carried out with concentrated suspensions, it was considered necessary to determine the relationship existing between concentration of the microbic suspension and the amount of serum necessary to cause complete agglutination.

Experiment 2. Relation between Concentration of Bacterial Suspension and Complete Agglutinating Dose of Typhoid Immune Serum.—As described above, the standard suspension used in these typhoid agglutination experiments is pre-

pared by suspending the 4 times washed organisms from 150 cc. of a 16 hour broth culture in 160 cc. of distilled water. Five suspensions were now prepared. Washed organisms from 150 cc. of broth were suspended in 8, 16, 32, 64, and 160 cc. of distilled water, thus securing suspensions of 20, 10, 5, and 2.5 times the concentration of the original. 1 cc. of each of these suspensions was now tested against a series of dilutions of antityphoid serum. Glycocoll-acetate- phosphate buffer mixture, pH 6.3, was used as diluent. Incubation at 43°C. for 14 hours. "Complete agglutination" was considered to take place when the supernatant fluid was water-clear, no whorl of suspended bacteria being visible when the tube was agitated before a strong beam of light. "++" was read when a heavy sediment was present. with a still cloudy supernatant fluid showing a distinct whorl of unagglutinated organisms. The results are summarized in Table II.

TABLE II.

Relation between Concentration of Suspensions and Complete Agglutinating Dose of Antityphoid Serum.

Concentration of *T* suspension.	Concentration of antityphoid serum × 10^{-5}=					
	150	75	37.5	18	9	4.5
20 times.	C.	++	+	+	Tr.	Tr.
10 "	C.	C.	++	++	++	+
5 "	C.	C.	C.	++	++	+
2.5 "	C.	C.	C.	C.	++	++
1	C.	C.	C.	C.	C.	++

Table II demonstrates that within the limits of suspension concentration studied in this experiment, the amount of serum necessary to agglutinate varies directly with the concentration of the suspension. The results show that at least 16 times less serum is required to agglutinate completely the standard suspension than to cause the same result in the suspension of 20 times the standard. There is reason to believe that the result would have been the same had the suspension varied in exactly the same proportion as the serum dilution, that is to say 16 times. It is therefore justifiable to calculate the agglutinating dose for a suspension 20 times the standard from a result gained from titration of serum on the standard suspension, and *vice versa*.

Experiment 3. Combination of Antigen and Antibody at Various Hydrogen Ion Concentrations.—0.1 cc. quantities of antityphoid serum were diluted 1:30 with glycocoll-acetate-phosphate buffers of pH 7.5, 5.2, 3.9, and 3.3. 3.0 cc. of serum so diluted were then mixed with equal volume of typhoid suspension, 10

times the standard concentration. Parallel control tubes were set up with 3.0 cc., 1:30 serum + 3.0 cc. of distilled water. The eight tubes were incubated at 43°C. for 2 hours, centrifuged at 3,000 R.P.M. for 30 minutes, and the supernatant fluids diluted to 0.001 cc. (1:1,000) with buffer pH 6.0. This brought

TABLE III.

Combination of Antigen and Antibody at Various Hydrogen Ion Concentrations.

pH at which absorption took place.	Supernatant fluids after absorption. Concentration in buffer pH 6.0 (cc. × 10^{-5}).							Agglutinating doses remaining.*	Agglutinating doses absorbed.
	100	50	25	12	6	3	1.5		
pH 7.5 Absorbed with 10 × *T* suspension.	++	−	−	−	−	−	−	<3	>50
pH 7.5 Control.	C.	C.	C.	C.	C.	++	++	55	
pH 5.2 Absorbed with 10 × *T* suspension.	++	+	−	−	−	−	−	<3	>50
pH 5.2 Control.	C.	C.	C.	C.	C.	++	++	55	
pH 3.9 Absorbed with 10 × *T* suspension.	C.	C.	C.	++	+	Tr.	−	14	41
pH 3.9 Control.	C.	C.	C.	C.	C.	++	+	55	
pH 3.3 Absorbed with 10 × *T* suspension.	C.	C.	C.	C.	++	Tr.	−	27	27
pH 3.3 Control.	C.	C.	C.	C.	C.	++	+	55	

* Agglutinating doses calculated for three volumes of 10 times standard suspension, since this was the concentration used in the absorbing suspension.

all supernatant fluids to a common pH. These neutralized diluted supernatant fluids were now diluted with pH 6.0 buffer by 2's to 0.000015 cc. and 1 cc. of standard suspension added to each tube. Incubation for 14 hours at 43°C. The result is given in Table III.

The experiment summarized in Table III shows that at pH 3.9 and more especially at pH 3.3, much less immune body is absorbed than at pH 5.2 and 7.5. There is no sensible difference between the amount absorbed at the latter two hydrogen ion concentrations. In both cases at least 94 per cent of the agglutinating substance was removed. The removal, however, was not complete in any case. Now, the agglutinating power of immune serum is distinctly higher at pH 5.2 than at pH 7.5 (1, 3). Therefore, it is evident that this increased effectiveness cannot be ascribed to a more complete quantitative union at 5.2 than at 7.5.

This experiment was subsequently elaborated and modified in certain respects. In the first place it is desirable, for constant results, to use a more concentrated absorbing suspension. Again the reaction is better conducted at room temperature than at 43°C., since at lower pH (3.3 to 3.0) the destructive effect of temperature on immune body begins to be a marked one. Finally, it seemed wise to conduct the experiment at a greater number of C_H, thus filling in the gaps existing in Experiment 3.

Experiment 4. Combination of Antigen and Antibody at Various Hydrogen Ion Concentrations.—2.0 cc. quantities of antityphoid horse serum, diluted 1:50 in buffer mixtures, pH 7.5, 5.2, 4.7, 3.9, 3.3, 3.0, and 2.7 were mixed with 2.0 cc. amounts of *B. typhosus* suspension 20 times the concentration of the standard (organisms from 150 cc. broth suspended in 8 cc. distilled water). Similar amounts of immune serum diluted with buffers of same pH were mixed with equal amounts of distilled water. These latter served as controls. All tubes were allowed to stand at room temperature (18°C.) for 11 hours.

The tubes with the immune serum *B. typhosus* mixtures were then centrifuged at 3,000 R.P.M. for 30 minutes. The supernatant fluids, together with the controls (immune serum + distilled water) were now diluted 10 times with buffer pH 6.5, bringing all mixtures to that pH.

These dilutions, equivalent to 0.001 cc. of original serum, were diluted by 2's to 0.000007 cc. of the original; 1 cc. of standard 4 times washed distilled water suspension was added to each tube, and all were incubated at 43°C. for 14 hours.

The smallest amount causing complete agglutination was taken as the criterion for the agglutinating unit. The agglutinating units recorded after the last incubation (supernatant fluids + standard suspension) were calculated to the agglutinating units required for the absorbing suspension (20 times the standard). This was done by dividing by 40 (since twice the volume of suspension was used in the adsorption experiment), in accordance with Experiment 2.

The results are given in Table IV.

TABLE IV.

Combination of Antigen and Antibody at Various Hydrogen Ion Concentrations.

pH	Tube.	Mixture.	Total agglutinating units present.*	Units remaining after 11 hrs. at 18° C.	Units absorbed.
7.5	1	Antityphoid serum absorbed with concentrated suspension.	16	< 1.3	>14.7
	2	Antityphoid serum + distilled water. Control.	16	16.0	0
5.2	3	Antityphoid serum absorbed with concentrated suspension.	16	< 1.3	>14.7
	4	Antityphoid serum + distilled water. Control.	16	16.0	0
4.7	5	Antityphoid serum absorbed with concentrated suspension.	16	< 1.3	>14.7
	6	Antityphoid serum + distilled water. Control.	16	16.0	0
3.9	7	Antityphoid serum absorbed with concentrated suspension.	16	1.3	14.7
	8	Antityphoid serum + distilled water. Control.	16	16.0	0
3.3	9	Antityphoid serum absorbed with concentrated suspension.	16	2.0	14.0
	10	Antityphoid serum + distilled water. Control.	16	16.0	0
3.0	11	Antityphoid serum absorbed with concentrated suspension.	16	8.2	7.8
	12	Antityphoid serum + distilled water. Control.	16	16.0	0
2.7	13	Antityphoid serum absorbed with concentrated suspension.	16	8.2	7.8
	14	Antityphoid serum + distilled water. Control.	16	16.0	0

* Calculated for 2 cc. of 20 times the standard suspension.

Control experiments on the effect of time showed that the combination was almost instantaneous, in agreement with results on the potential (1). The results therefore are not due merely to a different speed of combination but to a difference in the final stage.

Table IV confirms the conclusion drawn from Experiment 3; *i.e.*, that there is no demonstrable difference between the amount of immune body absorbed at pH 4.7 and that taken up at 5.2 and 7.5.

Relation of the Results of the Absorption and Agglutination Experiments.

The experiments described in the preceding paper (1) show that the amount of immune serum or of protein required to agglutinate bacteria depends entirely on the pH at which the experiment is performed. The nearer the pH is to the acid agglutination zone of the organism in question, the smaller the amount of serum required to agglutinate. This result has also been obtained by Michaelis and Davidsohn (3) and by Eggerth and Bellows (4). The present experiments show that this result cannot be ascribed to an increase in the amount of antibody combined in this region nor to any increase in the amount of immune body. These results also show that the combination between organism and immune body is independent of the charge, since the amount of immune body combined at pH 4 to 5, where the organism is negative and the immune body positive, is the same as in the range between 5 and 8, where both are negative. The influence of salt on the combination of antibody and organism also contradicts the idea that the combination is caused by opposite electric charges. If this were the case the combination should be greatest when the charges were greatest. Salt, however, decreases the charge but increases the combination. The same fact is brought out by Loeb's (7) experiments with collodion treated with gelatin. The collodion is always negative yet will become coated with alkaline gelatin solutions in which the gelatin is also negative.

The present experiments could be explained by assuming that the immune body was always *positive* and that the reversal found by Michaelis (and which was confirmed in the course of this work) was due to the fact that the immune body was carried along with the globulins as is the case with pepsin (Pekelharing and Ringer (5)). This explanation, however, could not account for the experiment shown in Fig. 3 of the preceding paper (1), which shows that at a pH of 3.0, where the organisms themselves are positive, the addition of serum *increases* this positive charge. It would seem to be improbable

that an increase in a positive charge could be caused by combination with a substance having a negative charge.

The experiments may be simply accounted for if it be supposed that the antibody or protein forms a film on the surface of the organism and is held by the ordinary forces of valence. This mechanism has been found by Langmuir (6) to exist in the case of oil films on water. The portion of the surface that is covered would then acquire the characteristics of the added substance. It is evident from this point of view that the smaller the charge on the original particle, the smaller the amount of the surface which would have to be covered in order for the particle to have the charge of the added substance; in other words, the less the amount of substance necessary to agglutinate. This is the experimental fact. This is clearly shown in Fig. 1 in which the charge on the organisms, the amount of serum to agglutinate, and the amount of antibody combined, are plotted against the pH. The figure shows that there is no relation between the amount of antibody in combination and the amount required to agglutinate, but a very close connection between the amount required to agglutinate and the charge carried by the organism.

The independence of the agglutination and sign of charge is also shown in Table V. In this experiment the amount of serum necessary to agglutinate organisms in NaCl in which they are negative is compared to the amount of serum required to agglutinate the same suspension in Cu acetate in which they carry an equal positive charge.

The mechanism of film formation will also account for the results of Arkwright (8) who found that *Bacterium coli*, sensitized with washings from *Bacillus typhosus*, could be agglutinated by anti-*Bacillus typhosus* serum. It was found that the same experiment could be performed with egg albumin. *Bacillus typhosus*, in the presence of egg albumin, could be agglutinated with anti-egg albumin serum.

These experiments show, therefore, that the combination between organism and agglutinin (or protein) is not *due to* opposite electric charges, but that the effect on the charge is the *result* of the combination.

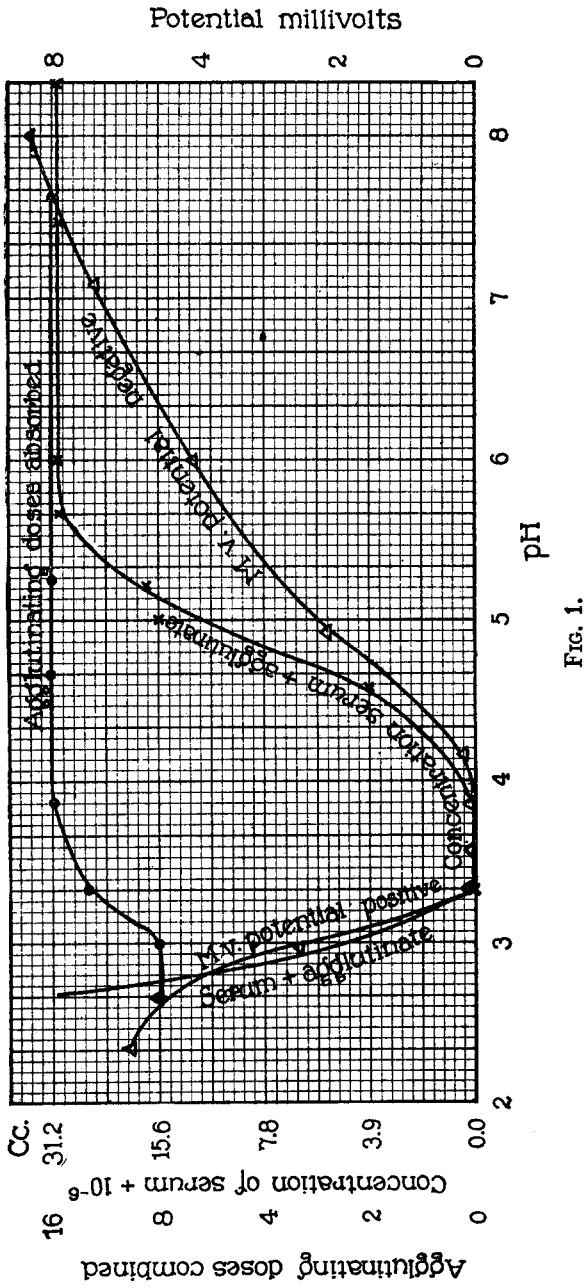

FIG. 1.

TABLE V.

Precipitation with Immune Serum in the Presence of Copper Acetate or Sodium Chloride.

20 cc. one-half suspension + 40 cc. copper acetate or sodium chloride of concentration noted.

2 cc. resulting suspension + 0.5 cc. antityphosus rabbit serum.

Final concentration of salt.	Copper acetate 0.10 N.	Sodium chloride 0.02 N.
Migration velocity under potential gradient of 1 volt per cm., μ *per sec.*	+0.50	−0.56
Potential millivolts.	+6.5	−7.0
Concentration of immune serum.	**Agglutination after 20 hrs.**	
1:4 } Immediate precipitation of copper	C.	C.
1:12 } hydroxide and serum protein.	C.	C.
1:36 }	C.	C.
1:108	C.	C.
1:324	C.	C.
1:976	+++	C.
1:2,700	+++	C.
1:8,100	++	+++
1:2,500	++	+++
1:7,500	++	+
1:22,000	+	+
0	±	−

SUMMARY.

1. The amount of immune body required to agglutinate a suspension of *Bacillus typhosus* increases in direct proportion to the concentration of the suspension.

2. The amount of immune body combined with the organisms is constant from pH 9 to pH 3.7. Below the latter value the amount in combination is decreased.

3. The addition of immune serum to a suspension of *Bacillus typhosus* at a pH of 2.5 increases the positive charge of the organisms.

These results are contradictory to the idea that the combination is caused by a difference in the sign of the charge carried by the immune body and the organism. They agree with the assumption that the immune body forms a film on the surface of the organism and that the effect on the charge is the result of this film.

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