ON THE MECHANISM OF OPSONIN AND BACTERIOTROPIN ACTION

IV. The Isoelectric Points of Certain Sensitized Antigens

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The behavior of antibodies in an electric field was early studied by Field and Teague[1] and Teague and Buxton.[2] They concluded that the antibodies migrated to the cathode and were therefore electropositive. Using similar apparatus Maver and Falk[3] have since reproduced these results and shown them to have been due to failure to take account of electroendosmotic streaming through the agar gels used. Eliminating this error, Maver and Falk have found that diphtheria antitoxin migrated to the anode at pH 6.0 or more alkaline reactions, and to the cathode at pH 4.6 or more acid reactions. Landsteiner and Pauli[4] showed that hemagglutinins behaved in an electric field as ampholytes.

Michaelis and Davidsohn,[5] using macrocataphoresis, found the isoelectric point of typhoid agglutinin to be between hydrogen ion concentrations of 1.0 and 5.1 $\times$ 10^{-6}. This range agreed with the isoelectric range for serum globulins as earlier determined by Rona and Michaelis;[6] their mean value of 3.6 $\times$ 10^{-6} (pH = 5.4) for serum globulin is usually cited as the isoelectric point of globulin. Von Szent-Györgyi[7] confirmed the value for the isoelectric point of typhoid agglutinin found by Michaelis and Davidsohn.

The isoelectric point of typhoid agglutinin was reinvestigated by Ottenberg and Stenbuck,[8] using both agglutinins in immune serum and relatively pure agglutinins dissociated from the sensitized bacteria. They obtained values for the isoelectric point between pH 4.4 and

pH 4.6. However Ottenberg and Stenbuck used citrate buffers. Michaelis and Rona[9] showed that the isoelectric point of a protein could be shifted toward the acid side by almost a full pH unit by polyvalent anions such as citrate. The effect was later given theoretical treatment by Michaelis.[10] The shift of the isoelectric point toward the acid side by citrate has been confirmed by one of us, using animal membranes,[11] and in this present study using sensitized bacteria and serum globulin. The discrepancy between the isoelectric point of typhoid agglutinin as found by Ottenberg and Stenbuck and by earlier investigators is thus certainly due in whole or in part to the use by the former of citrate buffers.

Study of the cataphoretic behavior of antibody when combined with its corresponding antigen was undertaken by Coulter.[12] He reported the isoelectric point of sheep red blood cells to be at about pH 4.6 and their optimum for acid flocculation to be at about pH 4.75. After heavy sensitization with immune serum the agglutination optimum was shifted to about pH 5.3. Coulter reported no corresponding shift of the isoelectric point, however, with serum sensitization. However, the description of his technique suggests that the erythrocytes used in the cataphoresis experiments were only lightly sensitized. It will be apparent from the graphs in this paper that submaximal sensitization may readily lead to attributing too alkaline an isoelectric point to the sensitizing substance.*

Northrop and De Kruif,[13] in a study which gave the first adequate experimental basis for analysis of the mechanism of agglutination, showed the isoelectric point of typhoid bacilli to be progressively shifted toward the alkaline side by increasing concentrations of sensitizing serum. Their curves (Fig. 3) show sensitization with no greater concentration of serum than 1:150, however, and undoubtedly the isoelectric point of 4.7 attributed to the sensitized bacteria is on the acid side of that which would have been reached with maximal sensitization.

* Dr. Coulter has informed one of us that in later, unpublished work he found the cataphoretic isoelectric point of more strongly sensitized sheep erythrocytes to have been shifted to about pH 5.3. In our own work sensitization with our best immune serum shifted the isoelectric point of sheep erythrocytes to pH 5.7; this will be reported in detail in a later paper.

Shibley[14] found that colon bacilli sensitized with homologous agglutinating serum in 1:20 dilution were isoelectric at about pH 5.3. The cataphoretic velocity of colon bacilli and pneumococci so sensitized varied with respect to pH and molar concentration of the suspending buffers almost precisely like denatured serum globulin.

EXPERIMENTAL

Bacteria Used.—An avian tubercle bacillus, *Mycobacterium avium* (Arloing strain), and the turtle bacillus, *Mycobacterium chelonei*. These were grown on glycerol-agar slants and suspended in 0.85 per cent NaCl solution. Both microorganisms were acid-fast.

Sensitization.—The bacteria were sensitized with homologous rabbit immune serum or plasma. Plasma was obtained by drawing blood into test tubes containing a few crystals of K-oxalate; the plasma was centrifugalized and the supernatant used. One volume of bacterial suspension was added to one volume of each serum or plasma dilution; the abscissae on the graphs are the serum or plasma dilutions after mixing. The mixtures were kept in the ice-box over night. They were centrifugalized, the supernatant fluid decanted, the sediment resuspended in excess of 0.85 per cent NaCl solution, again centrifugalized, decanted and resuspended in fresh NaCl solution. One or two drops of these suspensions of sensitized, washed bacteria were added to each 3 cc. of buffer solution.

Globulins.—Euglobulin was obtained from immune sera by simple dilution with distilled water and chilling, or preferably by dilution with fifteen volumes of distilled water and acidification drop by drop with N/10 hydrochloric or acetic acid. The precipitate formed by slight acidification was thrown down by centrifugalization, and the supernatant decanted; the sediment was resuspended in excess of distilled water; again thrown down, decanted, and resuspended in distilled water. A few drops of the latter suspension were added to dilute buffer solutions for study by cataphoresis. Euglobulin and pseudoglobulin were also prepared by $(NH_4)_2SO_4$ fractionation as described elsewhere.[15] The euglobulin and pseudoglobulin solutions prepared by $(NH_4)_2SO_4$ fractionation were evaporated to dryness in Petrie dishes; the protein was subsequently scraped from the bottom of the dish and suspended in distilled water. A few drops of these suspensions were added to dilute buffers for cataphoresis.

Buffers.—Walpole's acetate, Sørensen's phosphate and McIlvaine's citrate-phosphate buffers were made up according to the tables given by Clark.[16] They were either used in the original strength, *i.e.*, for Walpole's M/5, for Sørensen's M/15, for McIlvaine's M/5 to M/10, or diluted with nine volumes of distilled water added to each volume of buffer. In the extreme acid range phthalate-HCl or KCl-HCl mixtures or dilute HCl were used. The pH of the buffers or buffer dilutions used were checked colorimetrically.

Cataphoresis.—The Northrop-Kunitz microcataphoresis cell as modified by

Kunitz[17] was used with a dark field condenser and a Bausch and Lomb, 8 mm., 0.50 n.a., 21 × objective. Three readings at the lower and three at the upper "stationary level,"[18] *i.e.*, at 0.21 and 0.79 of the inside depth of the cell, were made for each suspension. Radio B-batteries were used; the applied potential was 135 volts, giving a gradient through the cell of about 7 volts per centimeter.

Estimations of Isoelectric Point.—Buffer series were made up so that the successive members differed by 0.4 pH in the case of Walpole's and Sørensen's, by 0.6 pH with McIlvaine's. Cataphoresis determinations were made upon a drop or two of bacterial or globulin suspension in 3 cc. of buffer, until two successive buffers were found in one of which the particles migrated toward the anode, in the other toward the cathode. The isoelectric point was then estimated by interpolation. It will appear later that the important comparisons to be made in this study were between the isoelectric points in acetate or phosphate buffers of strongly sensitized bacteria and of globulin precipitated chemically from the sensitizing serum. These comparisons, we believe, were usually valid to within 0.1 pH. When comparing results of different experiments absolute errors greater than 0.1 pH were no doubt sometimes introduced by inexactitude in determining the pH of the buffers, by changes in the cataphoresis cell due to deposit on its walls of substances from the test suspensions, and to errors in focussing.

The type of result obtained is shown in Fig. 1. The unsensitized bacillus is negatively charged except at very acid reactions. Sensitization with increasing concentrations of homologous immune serum shifts the isoelectric point* progressively toward the alkaline side until it reaches and passes beyond the isoelectric point of globulin precipitated by acidification or salted out of the same immune serum. In the experiments graphed the concentration of immune serum required to bring the microorganisms to the isoelectric point of serum globulin, represented by the horizontal lines at pH 5.2, was between 1 part serum in 64 and 1 part serum in 16 of diluent. At concentrations of immune serum of 1 part in 16 or higher the isoelectric point of the sensitized bacteria was *definitely higher than that of the globulin.*

The progressive change in cataphoretic properties of the bacteria with sensitization may also be followed by determining the cataphoretic velocity at a constant pH. In buffers of pH 5.2 the anodal migration velocity of the bacteria is progressively reduced by sensitization with increasing strengths of immune serum, and in the highest

* "Isoelectric point" is in this paper used in the original empirical sense of Hardy as the hydrogen ion concentration at which no migration in an electric field occurs.

serum concentrations migration becomes cathodal. Quantitatively parallel to these changes in cataphoretic properties other changes regularly occur with sensitization. The bacteria cohere more strongly (positive resuspension and interface reactions), they show increasing resistance to passage through a phase boundary from aqueous to oil phase (positive interface reaction), they become agglutinated, and in

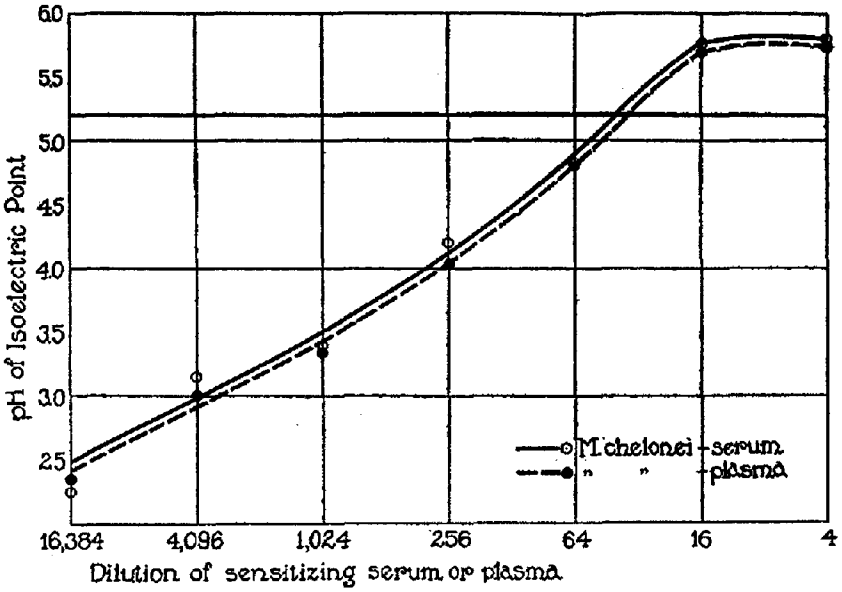

FIG. 1. Progressive change of the isoelectric point of an acid-fast bacterium with sensitization. The isoelectric point of the unsensitized bacterium is below pH 2.5. After exposure to homologous immune serum or plasma in progressively increasing concentrations the isoelectric point shifts to pH 5.7. The horizontal line at pH 5.2 is the mean value for seven determinations of the isoelectric point of serum euglobulin.

the presence of leucocytes, they are phagocyted. Abundant evidence has been submitted by us[15,19] to show that these changes are all a result of the deposit on the bacterial surface of a substance or substances present in the globulin fractions of immune serum. The combination of immune protein with antigen is mediated by specific chemical affinities; the results, *i.e.*, agglutination, the altered surface properties

TABLE I

Agglutination, Changes in Cohesion and Wetting Properties in Experiment Whose Isoelectric Points are Plotted in Fig. I

	Reactions	Dilutions of sensitizing serum or plasma						
		1:4	1:16	1:64	1:256	1:1,024	1:4,096	NaCl control
M. chelonei sensitized with anti-chelonei Serum 2637 and washed	Agglutination	++++	++++	++++	++++ to +++	+++	0	0
	Resuspension	++++	+++	+	++	tr.	tr.	0
	Interface	++++	+++	+ (weak)	0			0
M. chelonei sensitized with anti-chelonei Plasma 2637 and washed	Agglutination	+++	+++	+++	+++	++	0	0
	Resuspension	++++	++	+++	+	tr.	0	0
	Interface	++++	+++ to ++	0	0			0

and phagocytosis, are all dependent upon the properties of the substances from the serum combined with and deposited upon the antigen surface.

In Table I are given the agglutination results and changes in cohesion and wetting properties in the experiment whose isoelectric points are plotted in Fig. I.

The antibody-protein against acid-fast bacteria is found chiefly in the euglobulin, to a less extent in the pseudoglobulin fraction.[15] The isoelectric points of the euglobulin precipitated by dilution and acidification from immune sera used in this study are given in Table II.

TABLE II

Isoelectric Points of Euglobulin Samples

Experiment No.	Method of preparation	Serum	Globulin	Isoelectric point with acetate or phosphate
1	Dilution and acidification	AntiArloing 35	Unheated	5.2
2	Dilution and acidification	Antichelonei 2637	"	5.25
3	Dilution and acidification	Antichelonei 39	"	5.3
4	Dilution and acidification	AntiArloing 35	"	5.2
5	Dilution and acidification	Antichelonei 2637, heated 56° for 30 min.	"	5.0
6	Dilution and acidification	Antichelonei 3293, heated 56° for 30 min.	"	5.1
7	Dilution and acidification	AntiArloing 35, heated 56° for 30 min.	"	5.4
8	Dilution and chilling	AntiArloing 2226	Heated 97°–98° for 10 min.	5.15
9	Dilution and chilling	Antichelonei 2213 and 2228	Heated 100° for 15 min.	5.15
10	Dilution and acidification	Antichelonei 2637, heated 56° for 30 min.	Heated 100° for 15 min.	4.55
11	Dilution and acidification	Normal Serum 3373	Unheated	5.05
11	Dilution and acidification	" " "	Heated 87°–92° for 15 min.	5.1
12	Dilution and acidification	Normal Serum 3369, heated 56° for 30 min.	Heated 87° for 20 min.	5.05

The mean of the first seven values in Table II is plotted in Figs. 1 and 2 as the horizontal line at pH 5.2. These were all euglobulin samples freshly precipitated from immune sera and subjected to no heating or drying, except in three instances the heating involved in inactivation of the immune serum. Eleven additional euglobulin samples have been precipitated by dilution and acidification or salted out[25] of anti-protein precipitin rabbit sera. The range of isoelectric points for these eleven samples was pH 4.9 to pH 5.2, with a mean value of 5.1. Eleven isoelectric point estimations have also been made

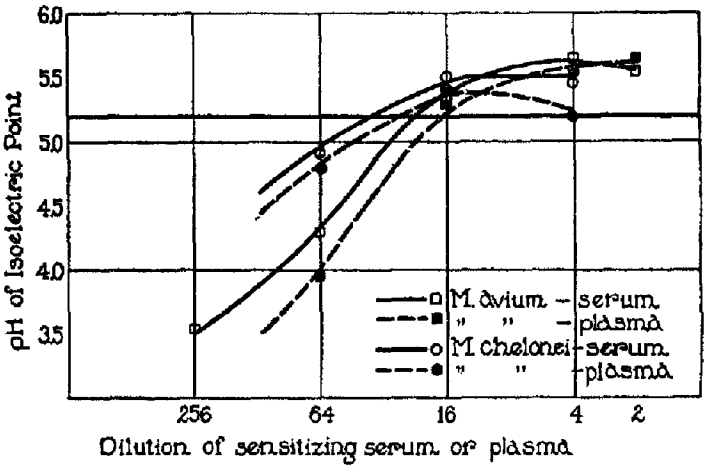

FIG. 2. Progressive change of the isoelectric points of acid-fast bacteria with sensitization. Exposure to homologous immune serum or plasma progressively shifts the isoelectric points until they pass to the alkaline side of that of serum globulin, indicated by the horizontal line at pH 5.2.

upon pseudoglobulin fractions salted out of rabbit sera, dried and kept for varying lengths of time. The isoelectric points found for these pseudoglobulins ranged from pH 4.4 to pH 5.3 with a mean of 4.9; however drying and aging may have shifted the isoelectric point of some of these pseudoglobulin samples somewhat toward the acid side* as heating seems to have done for one or more of the euglobulin samples in Table II.

* We are informed by Dr. L. D. Felton that drying regularly shifts the point of minimum solubility of horse globulin toward the acid side.

The isoelectric points of sensitized bacteria in two other experiments are graphed in Fig. 2. The features to be emphasized are again (1) that the isoelectric point of the sensitized antigen shifts toward the alkaline side with increasing concentration of sensitizing serum or plasma, and (2) that in the highest concentrations of serum the isoelectric points reached are slightly but distinctly above the values for

TABLE III

Comparison of Isoelectric Points with Buffers Containing Monovalent and Trivalent Anions

Experiment No.	Test object	Dilution of sensitizing serum	Isoelectric point with acetate or phosphate buffer pH	Isoelectric point with citrate buffer pH
5	*M. chelonei*	1:2	5.7	5.05
	" "	1:4	5.7	—
	" "	1:16	5.1	—
	" "	1:∞	2.8 or lower	
	Euglobulin from sensitizing serum		5.0	4.6
6	*M. avium*	1:2	5.05	4.45
	" "	1:4	5.0	—
	" "	1:16	4.8	—
	" "	1:64	3.8	—
	" "	1:∞	not reversed in 0.1 N HCl	
	Euglobulin from another antiserum		5.1	4.45
12	*M. chelonei*	1:2	5.55	5.0
	" "	1:4	5.35	—
	" "	1:16	4.8	—
	Euglobulin from normal serum		5.05	4.4

the isoelectric points of the precipitated or salted-out euglobulin and pseudoglobulin.

It seemed that the explanation of the isoelectric point of strongly sensitized bacteria being on the alkaline side of serum globulin might conceivably lie in deposition on the bacteria from the lower serum dilutions of some residual fibrinogen. It was to test this possibility that bacteria were sensitized with plasma as well as with serum.

Plasma, however, gave no higher, indeed not as high values as serum, thus lending no support to this hypothesis.

Isoelectric points found for sensitized bacteria and for globulin in three other experiments are given in Table III. In two of these values for the strongly sensitized bacteria are found to be well on the alkaline side of the globulin; in Experiment 6 the isoelectric points of the most strongly sensitized bacteria and of the globulin coincided.

The shift of the isoelectric points toward the acid side by the use of a buffer containing polyvalent anions is shown in Table III. The buffers used in this experiment were about one-twentieth molar with respect to citrate; the isoelectric points both of the sensitized bacteria and of the euglobulin are seen to be about one-half pH unit more acid than in the corresponding acetate or phosphate mixtures.

DISCUSSION

Unsensitized acid-fast bacteria have very low isoelectric points and are wetted by oils in a manner indicating a high lipin content for their surfaces. As these bacteria interact with increasing concentrations of immune sera their surface properties progressively change. The strongly sensitized bacterial surfaces have wetting properties like those of protein,[20] are cohesive,* and are isoelectric at reactions slightly more alkaline than those of precipitated serum globulin; concomitantly with the changes in surface properties the bacteria are agglutinated* and prepared for phagocytosis.

For comparison consider the effect of sensitizing an essentially different type of antigen. Loeb[22] found that collodion particles could be coated with protein, and then behaved in an electric field like particles of the pure protein. This device has been adapted to serological uses by F. S. Jones,[23] and independently by Freund.[24] We have deposited crystalline egg albumin, edestin and human serum proteins on collodion particles. These particles were then agglutinated and prepared for phagocytosis by the sera of rabbits immunized with the corresponding proteins.[25] As such protein treated collodion

* In the formulation of Northrop,[21] with which this work is essentially in harmony, the critical potential of the antigen is increased by deposit of a surface film of agglutinin.

particles were sensitized with increasing concentrations of homologous immune serum their isoelectric points progressively shifted to the alkaline side until the same values were attained as in the case of sensitized acid-fast bacteria.

In general, as far as present evidence goes, though this is still incomplete, the surface properties of diverse antigens have been found to converge with progressive sensitization toward that condition described for strongly sensitized acid-fast bacteria. There is no doubt, at least in our minds, that the properties of the maximally sensitized antigens are or approximate those of the sensitizing substance or substances of the immune serum *after deposition upon the antigen surface.** In brief the sensitizing protein forms a surface deposit on the antigen with which it specifically combines. The combination depends upon the specific chemical affinities of antigen with antibody. The surface properties, agglutination and phagocytosis of the maximally sensitized antigen depend chiefly upon the serum substances combined with and deposited on the antigen surface.

Felton[26] has succeeded in bringing the antibodies of anti-pneumococcus horse sera to a state of relative purity. He believes his best preparations to consist of about equal parts of active antibody-protein and inert serum globulin. The antibody-protein itself seems to be essentially a modified globulin or something at least in considerable part made up of globulin. Felton has reported the point of minimum solubility of his antibody-protein to be pH 6.6 to 6.8.

Is the isoelectric point of pH 5.6 to 5.8 we have found for antigens strongly sensitized with rabbit sera to be regarded as the isoelectric point of rabbit antibody-protein? Or is this value due to a mixed deposit of inert serum globulin with an antibody protein of more alkaline isoelectric point analogous to Felton's? Why should the antibody protein, which is found in the globulin fractions of serum, have a higher isoelectric point when combined with antigen than the globulin precipitated chemically from the same serum? Is there an active substance of higher isoelectric point mixed with the inert globulin or does the antibody-protein change its isoelectric point in

* There is evidence that the antibody-protein undergoes denaturization on combination with the antigen surface; see references 14 and 20, also a paper by H. Eagle which appeared when this paper was in press, (*J. Immunol.*, 1930, **18**, 169).

combining with antigen because of altered orientation or the combination of certain radicals? Answers to these questions must wait upon further investigations.

CONCLUSIONS

Sensitization with increasing concentrations of homologous immune serum shifts the isoelectric point of the antigens studied progressively to the alkaline side. Antigens maximally sensitized with rabbit sera have shown isoelectric points of pH 5.6 to 5.8. The globulins precipitated or salted out of the same immune sera have been isoelectric at pH 5.1 to 5.2.

The combination of antigen with antibody depends of course upon specific affinities; the surface properties of the sensitized antigen, agglutination and phagocytosis depend primarily upon the properties of the sensitizing serum substances combined with and deposited on the antigen surface.

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