

ON THE MECHANISM OF OPSONIN AND BACTERIOTROPIN ACTION

VI. AGGLUTINATION AND TROPIN ACTION BY PRECIPITIN SERA. CHARACTERIZATION OF THE SENSITIZED SURFACE

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In the preceding communication we have advanced a theory of tropin action and offered in its support evidence that we feel to be convincing. The effectiveness of immune sera in agglutinating, altering the surface properties and promoting the phagocytosis of antigens has been found to be dependent upon the presence of substances in the globulin fractions of the serum which combine with and form a deposit on the surface of the antigen. Phagocytosis, the characteristic behavior of the sensitized antigens in resuspension, interface and cataphoresis tests, and agglutination follow essentially as consequences of the properties of the substances deposited on the antigen surface.

In the present communication we shall describe building up artificial surfaces and obtaining agglutination, characteristic surface properties and phagocytosis in accordance with prediction from the theory. In this phase we are following and extending the work of F. S. Jones (1, 2). We find with Jones that precipitin sera will cause the agglutination and phagocytosis of collodion particles previously treated with homologous precipitinogen. The active substances are found both in the euglobulin and pseudoglobulin fractions of the antisera. Precipitation, agglutination, altered cataphoretic properties and phagocytosis are brought about in approximately corresponding degree. And finally the properties of maximally sensitized precipitinogen-treated collodion particles, of specific immune precipitate, and of maximally sensitized acid-fast bacteria are, within the limits of our methods, identical.

Thus we have powerful evidence in support of a rational conception of these diverse serological phenomena as all special consequences of one underlying phenomenon. The combination of antigen and antibody is determined by specific chemical affinities. The effects following this combination, at least with the antigens we have thus far studied, namely precipitation, agglutination, changes in surface properties and phagocytosis are consequences primarily of the properties of the antibody-protein combined with and deposited upon the antigen surface. The actual phenomena exhibited as a consequence of the deposit of the antibody-protein on the antigen surface of course depend also upon accessory factors such as electrolytes, temperature, the presence of leucocytes and other environing conditions.

Experimental Methods

Immune Sera.—Rabbits were injected five times usually at 5-day intervals with solutions of the antigen. These were crystalline egg albumin,* edestin,* casein (Hammarsten), human serum and horse serum. The egg albumin and human serum and their antisera were most satisfactory.

Preparation of Precipitinogen-Treated Collodion.—Loeb (3) showed that protein could be adsorbed on the surface of collodion particles. Brilliant application of this fact to serology was made by F. S. Jones (1, 2).** We have followed essentially the technique of Jones. Spherical collodion particles of about 1 to 2 μ in diameter were furnished us through the kindness of Dr. M. Kunitz. One volume of a dense suspension of these particles was allowed to stand usually for several hours in contact with one volume of human or horse serum or of a saturated solution of the protein. The collodion-precipitinogen mixture, after sufficient contact, was centrifugalized, the supernatant fluid decanted and the sediment resuspended in excess of saline. The suspension was again centrifugalized, the supernatant fluid decanted and the sediment resuspended in 0.85 per cent saline.

Tests.—The precipitinogen-treated, washed collodion particles were set up with serial dilutions of the precipitin sera or of the globulin or albumin fractions of these sera. Agglutination was read at short intervals and often again after keeping the tubes overnight in the ice box. Resuspension was often omitted, but was otherwise done according to the usual technique (5). The interface reaction could not be done since the collodion particles themselves are not wetted by oil.

* For the egg albumin we are indebted to Dr. J. Freund, and for the edestin to Professor D. Wright Wilson.

** Specific agglutination of protein-treated collodion particles was demonstrated independently by Freund (4).

Cataphoresis was carried out as previously described(5).* In the earlier experiments the test particles were suspended in 0.85 per cent sodium chloride solution. In later work the particles were suspended in M/50 acetate buffer of pH 5.2. When isoelectric point determinations were made the particles were suspended in two or more buffers whose reactions were graduated in intervals of 0.4 pH. Buffers were found such that the test particles moved toward the anode in one, toward the cathode in the next in series. The isoelectric point was then estimated by interpolation.

The phagocytosis tests, staining and counting were as previously described (5). The use of essentially the same methods of detecting phagocytosis of collodion particles as had been used with acid-fast bacteria was made possible by the earlier observation of Freund (6) that collodion particles are acid-fast by the ordinary Ziehl-Neelsen technique.

Precipitin tests were set up by adding 0.2 cc. portions of the precipitin serum or serum fraction to 1 cc. each of the serial dilutions of the precipitinogen solution.

Fractionation of the Precipitin Sera.—Fractionation was carried out as previously described (7).

Agglutinin and Tropin Action by Precipitin Sera; Further Verification of the Theory of Tropin Action

Precipitation tests with the dissolved proteins, and agglutination, cataphoresis and phagocytosis tests with the proteins adsorbed on collodion particles were performed as routine with all of the antiserum fractions and often also with the whole antisera. In the majority of experiments two antigens and their antisera were included and both homologous and heterologous combinations were tested. The data were finally analyzed with reference to certain questions designed, like those in the preceding paper, to test deductions from our theory of tropin action. In addition to verifying the theory the data have yielded strong support of the unitarian hypothesis of antibody action (8).

* A Zeiss water-immersion lens of 0.75 n.a. and 1.9 mm. working distance has been found to be in certain respects superior to the objective previously described. The water-immersion feature is a nuisance; however, for studying particles which settle out quickly, this may be more than compensated for by the advantages of more critical focus and the greater magnification afforded by the water-immersion lens.

The questions and the results of analyses of the data are given below. Details have for the most part been omitted.

I. When precipitinogen-treated collodion particles are sensitized with immune serum or one of its fractions, what is the order of relative effectiveness in (1) phagocytosis, (2) agglutination, (3) cataphoresis, (4) precipitation?

Whole serum was found generally to be more effective in bringing about all of the reactions than either of its globulin fractions; both globulin fractions were strikingly more effective in all of the reactions than the albumin fraction. In precipitation and phagocytosis the euglobulin and pseudoglobulin fractions were about equally effective; in agglutination pseudoglobulin was oftener more effective than euglobulin, in cataphoresis euglobulin was oftener more effective than pseudoglobulin. With the exception of this slight discrepancy in the relation of the euglobulin and pseudoglobulin fractions, therefore, the sera and fractions were effective in the same order in all of the reactions.

It has been supposed that the euglobulin fraction contains all the precipitin (9); certainly in our work we have found it fairly equally distributed between the euglobulin and pseudoglobulin fractions. Possibly it is not irrelevant to the perennial controversy over the distribution of antibodies in the globulin fractions to point out that two of the chief students of globulin chemistry, Chick (10) and Sørensen (11), believe that a clean-cut separation of euglobulin and pseudoglobulin is impracticable. According to Sørensen, "neither fractionation nor dialysis nor a combination of the two methods results in a complete separation of the two globulins." He believes that the euglobulin and pseudoglobulin are associated as a labile compound E_pP_q .

II. When precipitinogen-treated collodion particles are sensitized with immune serum or one of its fractions, are the effects in promoting phagocytosis approximately proportional to the effects on 1) agglutination, 2) cataphoresis, and 3) precipitation?

The correspondence between tropin, agglutinating, cataphoretic and precipitating effects with whole serum and with both globulin fractions was excellent in practically all experiments. There was not good correspondence between the several tests when the serum albumin fraction was used. The results are thus precisely similar to those obtained with acid-fast bacteria (7).

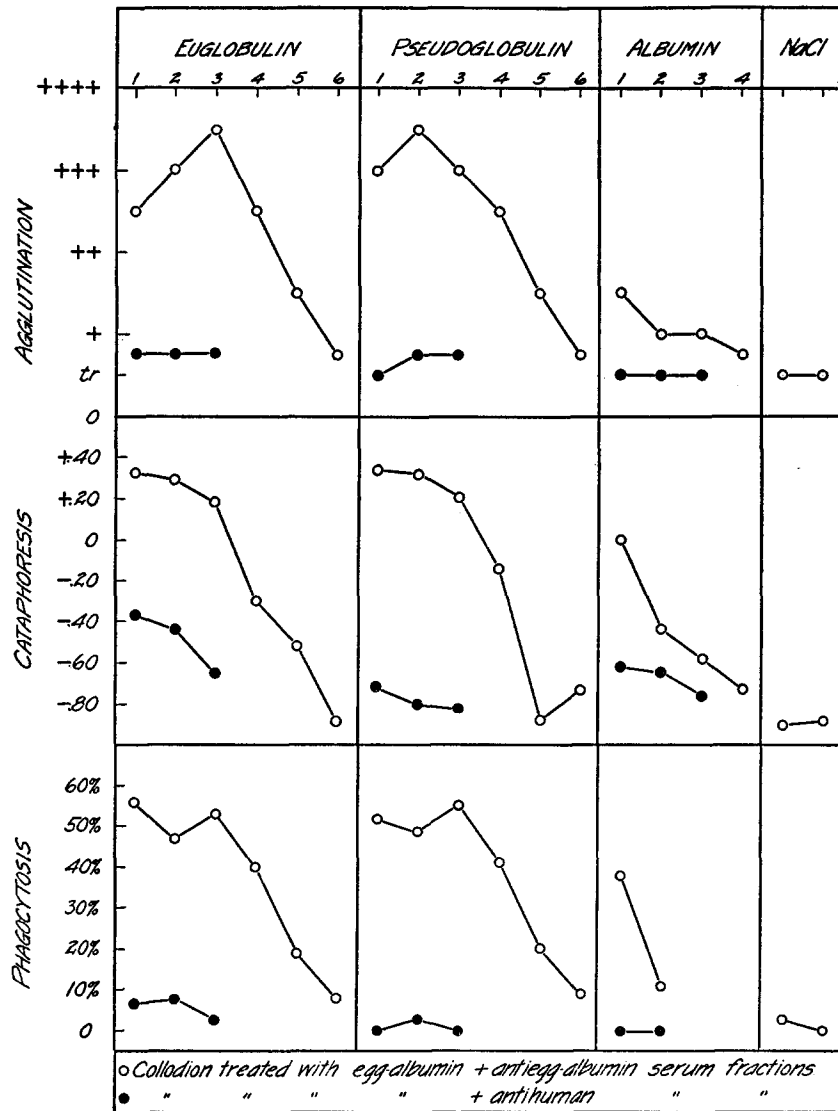


FIG. 1. Parallelism between agglutination, cataphoresis and phagocytosis of precipitinogen-treated collodion particles after sensitization with globulin fractions of precipitin sera. Abscissae are successive dilutions of antiserum or antiserum fraction in powers of four. (Thus 4 is a dilution of 1:4⁴ or 1:256.) All particles were washed after sensitization. Cataphoresis was conducted in m/50 acetate buffer of pH 5.2; graphed as μ /sec. per volt/cm. Note specificity of the reactions.

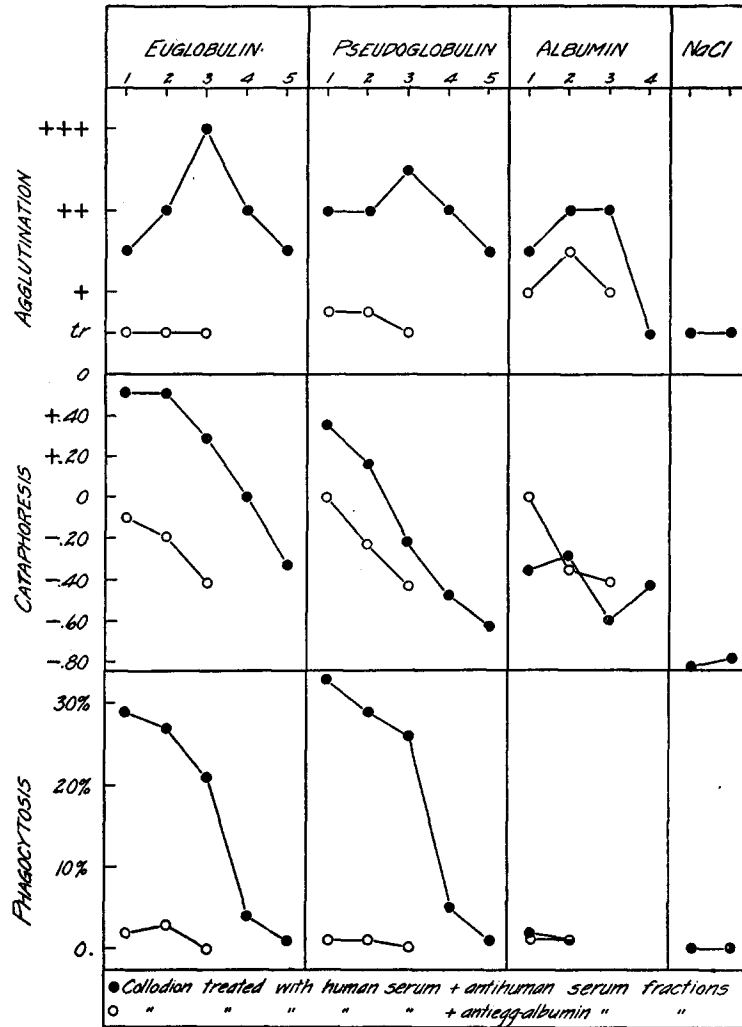


FIG. 2. General correspondence between agglutination, cataphoresis and phagocytosis of precipitinogen-treated collodion particles after sensitization with globulin fractions of precipitin sera. Antisera the same as in Fig. 1. All particles washed after sensitization. Cataphoresis as in Fig. 1. The agglutination zones are not paralleled in the other reactions.

III. When precipitinogen-treated collodion particles are sensitized with an immune serum fraction is there more interaction with homologous than with heterologous antigen, *i.e.*, are the reactions specific?

The reactions were clearly specific with whole serum, with euglobulin and with pseudoglobulin. This was not the case with serum albumin.

Agglutination by the albumin fraction was greater with homologous than with heterologous antigen in four cases, equal with homologous and heterologous antigens in five. The effect of albumin on cataphoresis was greater with homologous antigen in six cases, greater with heterologous antigen in three cases. The phagocytosis-promoting effect of albumin was greater with homologous antigen in two cases, with heterologous antigen in two cases, and equal with homologous and heterologous antigens in one case; phagocytosis was absent in four cases in which the particles were washed after treatment with serum albumin. It is possible that a trace of specific antibody may have been carried over into the albumin fractions in some experiments and may account for the slightly greater average effect of the albumin fraction with homologous antigen in the agglutination and cataphoresis tests.

IV. What is the effect of washing precipitinogen-treated collodion particles after sensitization with whole homologous antiserum or with one of the several fractions?

The phagocytosis of precipitinogen-treated collodion particles sensitized by whole homologous immune serum or a globulin fraction and then washed proceeded virtually unimpaired. As with the acid-fast bacteria the deposit on the particle surface produced by immune serum or its globulin fractions was a sufficient cause for phagocytosis even in the absence of dissolved tropin.

The phagocytosis found in the presence of the albumin fraction, on the contrary, was much reduced or abolished by washing, as was also the case with acid-fast bacteria. Lack of specificity, lack of correspondence with the other reactions and reduction by washing, therefore, distinguish this phagocytosis-promoting effect of albumin from true tropin action.

Characterization of the Sensitized Surface

The isoelectric point of a protein or other amphoteric substance is a property dependent upon and to a considerable degree characteristic of its chemical structure. The term was originally used in an empiri-

cal sense by Hardy (12) as the reaction at which no migration of the test substance occurs in an electric field. The isoelectric point for the case of a pure amphoteric substance later received mathematical definition in terms of the acid and basic dissociation constants of the substance (13). We shall, however, use the term "isoelectric point" in the original sense of Hardy.

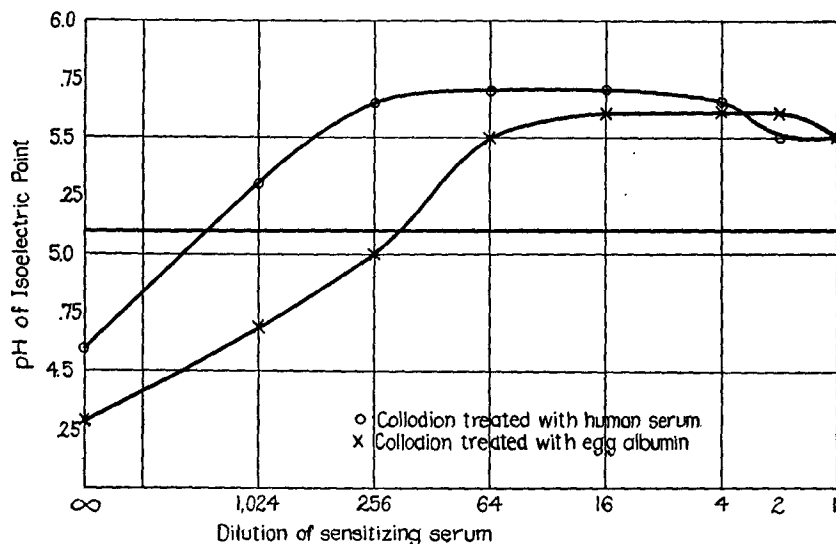


FIG. 3. Progressive change of the isoelectric points of precipitinogen-treated collodion particles with sensitization by homologous precipitin sera. The isoelectric points of the protein-treated collodion particles are about pH 4.3 and 4.6 respectively. After exposure to homologous immune serum in progressively increasing concentrations, the isoelectric points shift to plateau values of pH 5.6 and 5.7. There is a slight prozone with the highest serum concentrations. The horizontal line at pH 5.1 is the mean value for eleven determinations of the isoelectric point of serum euglobulin.

Collodion particles show a strong negative charge in the electric field. When treated with protein by the technique we have followed the particles are isoelectric at a reaction somewhat on the acid side of the isoelectric point of the protein used. This indicates that protein has been adsorbed on the surface of the collodion. The fact that the isoelectric point is on the acid side of that of the protein indicates that the charge is a resultant of that associated with the collodion and that

due to the protein. This supports the conclusion reached by Jones (2) on other grounds that the protein under such circumstances does not form a complete film on the particle.

When such protein-treated collodion particles are sensitized by strong precipitin sera homologous with the protein used, the isoelectric point is shifted to the alkaline side. The shift is progressively greater with progressing concentrations of sensitizing serum until a plateau value of

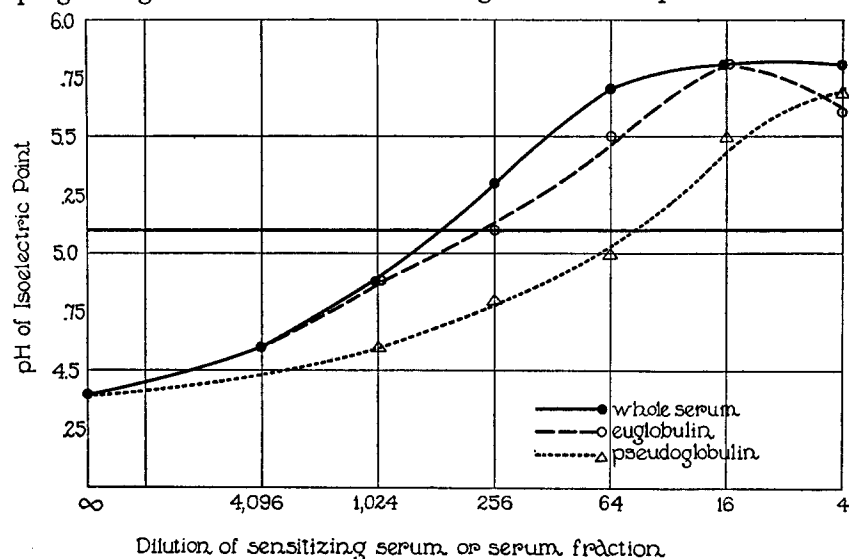


FIG. 4. Progressive change of the isoelectric points of human-serum-treated collodion particles with sensitization by homologous precipitin serum and its globulin fractions. The globulin fractions as well as the whole serum bring the isoelectric point of the antigen within the zone of pH 5.5 to 5.8. Figs. 1, 2, 4 and 5 and Table I all represent data from the same experiment.

pH 5.6 to 5.8 is reached. This effect is seen in Fig. 3. With sera of lower titer greater concentrations of sensitizing serum are required to bring the isoelectric point of the sensitized particle to the plateau value, as seen in Figs. 4 and 5. These figures show that a similar effect is produced by the euglobulin and pseudoglobulin fractions of the immune sera, but that greater concentrations are required to bring it about than with the whole serum. With still weaker immune sera the maximum value reached by the isoelectric point may be below pH 5.6.

The isoelectric points of acid-fast bacteria have been shown in another place (14) to undergo a similar change with serum sensitization. The isoelectric points of the unsensitized bacteria studied were exceedingly low, below pH 2.5; with sensitization by increasing strengths of homologous antiserum the values of the isoelectric points shifted to the alkaline side until values of pH 5.5 to 5.8 were reached.

The isoelectric points of specific precipitates obtained by the interaction of the dissolved proteins and their homologous precipitin sera have

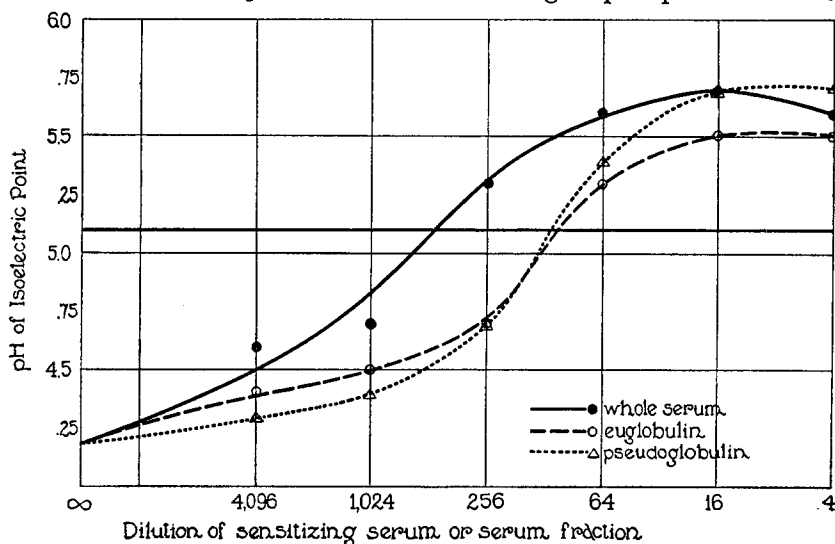


FIG. 5. Progressive change of the isoelectric points of egg-albumin-treated collodion particles with sensitization by homologous precipitin serum and its globulin fractions. The globulin fractions as well as the whole serum bring the isoelectric point of the antigen within the zone of pH 5.5 to 5.8.

also been studied. The results are given in Tables I, II and III. It is evident that the precipitates obtained in the zone of approximately optimal proportions of antigen and antibody are isoelectric in the same range of hydrogen ion concentrations as are maximally sensitized acid-fast bacteria or precipitinogen-treated collodion particles, namely pH 5.5 to 5.8.

With excess of either antigen or antibody the isoelectric points of the immune precipitates have tended to be at slightly lower pH

values.* An analogous instance in which sensitization with the highest concentrations of a high titer immune serum has resulted in a reduction of the isoelectric point to slightly below the maximum value

TABLE I
Isoelectric Points of Specific Precipitates

Immune serum	Dilution of antigen	Precipitate, amount	Isoelectric point of precipitate, unwashed, pH	Isoelectric point of precipitate, washed, pH
Antiegg albumin.....	1:10	+++ to ++	5.15	5.5
“ “	1:100	+++	5.35	5.55
“ “	1:1000	+++	5.4	5.75
“ “	1:10,000	+	—	—
“ “	1:100,000	tr.	—	—
Euglobulin from the above serum		—	5.0	5.0
Antihuman serum.....	1:10	+++ to ++	5.4	5.55
“ “	1:100	++++ to +++	5.6	5.75
“ “	1:1000	++++ to +++	5.4	5.7
“ “	1:10,000	++ to +	—	—
“ “	1:100,000	+	—	—
Euglobulin from the above serum		—	4.8	4.95

1 cc. each of progressive dilutions of egg albumin solution and of human serum were mixed with 0.2 cc. each of their respective homologous precipitin antisera. Euglobulin was obtained from the same precipitin sera by dilution and acidification. The precipitates were first concentrated by centrifugalization (unwashed); they were then suspended in distilled water, centrifugalized, the supernatant decanted and the sediment resuspended in distilled water (washed); a drop or two of the suspensions were added to several cubic centimeters of dilute acetate or phosphate buffer for the isoelectric point determinations.

is given in Fig. 3. Similar instances have occurred a sufficient number of times in as yet unpublished experiments with potent antisera so

* Incidentally these results show that the often-cited analogy between the zone phenomena in specific precipitation and that seen in the reciprocal precipitation of an electronegative and electropositive colloid is both superficial and misleading. The antigen, the precipitate, and, as far as present evidence shows, the antibody are all electronegative at the hydrogen ion concentration of blood serum.

TABLE II
Isoelectric Points of Specific Precipitates

Antiserum	Dilution of antigen	Precipitate, amount	Isoelectric point of precipitate, pH
Whole antihuman serum.....	1:10	++	5.4
“ “ “	1:100	++++	5.7
“ “ “	1:1000	+++	5.4
“ “ “	1:10,000	++ to +	—
“ “ “	1:100,000	tr.	—
Antihuman serum euglobulin.....	1:10	++	—
“ “ “	1:100	+++	5.3
“ “ “	1:1000	+++	5.5
“ “ “	1:10,000	++	—
“ “ “	Euglobulin control	+	—
Euglobulin obtained from the above serum by dilution and acidification		—	5.0

1 cc. each of progressive dilutions of human serum were mixed with 0.2 cc. each of whole antihuman precipitin serum or of the euglobulin fraction of this precipitin serum. Euglobulin was obtained from the same precipitin serum by dilution and acidification. The precipitates were washed and isoelectric points were determined as described under Table I.

TABLE III
Isoelectric Points of Specific Precipitates

Dilution of antigen	Precipitate, amount	Isoelectric point of precipitate, pH
1:10	+++	5.4
1:100	++++	5.65
1:1000	+++	5.7
1:10,000	+	5.45
—	Euglobulin	5.25

2 cc. each of progressive dilutions of an egg albumin solution were mixed with 0.4 cc. each of anti-egg-albumin precipitin serum. Euglobulin was obtained from the same precipitin serum by dilution and acidification. The immune precipitate and the euglobulin were washed in distilled water; their isoelectric points were determined in dilute acetate buffers.

that it seems unlikely that they can be dismissed as accidental. Heidelberger and Kendall (15) have recently successfully treated the precipitin reaction between Pneumococcus III specific carbohydrate and its corresponding antibody in terms of simple mass action equations. It seems probable that in our experiments the rise of the isoelectric points through a maximum with increasing relative concentrations of antibody may correspond to the transition from the compounds AS₃, through AS₂ to AS as described by Heidelberger and Kendall. Study should be directed explicitly to this point.

TABLE IV

Acid-fast bacteria + homologous antisera or their globulin fractions, result in	{ altered surface* agglutination phagocytosis
Protein-treated collodion + homologous antisera or their globulin fractions, result in	{ altered surface* agglutination phagocytosis
Protein solutions + homologous antisera or their globulin fractions, result in	} immune precipitate*
Sheep erythrocytes + homologous antisera, result in	{ altered surface* agglutination phagocytosis

* Properties of altered surface and of immune precipitate:

1. Cohesion high.
2. Wetting properties characteristic of protein.
3. Isoelectric point at pH 5.5 to 5.8.

The wetting properties of immune precipitate when examined in an oil-water interface are also similar to those of maximally sensitized acid-fast bacteria. These properties are characteristic* for protein; for instance globulin, egg-albumin, casein or edestin particles examined in such interfaces exhibit wetting properties similar to those of the immune precipitate or the maximally sensitized bacterial surface.

The analogy between immune precipitate and the maximally sensi-

* Although characteristic, no claim is made that these wetting properties are specific for protein.

tized surface of particulate antigen is rendered virtually complete by the evidence brought forward by Cromwell and Centeno (16) to indicate that specific precipitate is phagocytized.

The chief results of the interaction of antigen and antibody in the systems we have studied are summarized in Table IV.

Earlier work on the isoelectric points of sensitized antigens is reviewed elsewhere (14). The work which has hitherto advanced farthest in characterizing the properties of the sensitized surface, that of Shibley (17), seemed to show that the surface was coated with a film of denatured serum globulin. That the sensitized surface has several points

TABLE V
Isoelectric Points of Euglobulin Samples

Source	Method of preparation	Isoelectric point, pH
Antiegg-albumin Rabbit 29-19	Ammonium sulfate fractionation	5.2
Anticasein Rabbits 32-97 and 33-72	“ “ “	5.1
Antiegg-albumin Rabbit 29-19	Dilution and acidification	5.0
Antihuman serum Rabbit 31-77	“ “ “	4.9
Antihuman serum Rabbit 31-77	“ “ “	5.0
Antiedestin Rabbits 32-96 and 32-98	“ “ “	5.05
Antiegg-albumin Rabbit 29-19	“ “ “	5.2
Antiedestin Rabbit 32-96	“ “ “	5.2
Antiedestin Rabbit 32-98	“ “ “	5.2
Antiegg-albumin Rabbit 29-19	“ “ “	5.1
Antihuman serum Rabbit 31-77	“ “ “	5.1
Average		5.1

of resemblance to a film of serum globulin has been brought out also by the studies of Coulter (18), of Mudd and Mudd (19) and of Eagle (20). Moreover the sensitizing substance or substances are well known to be associated with the globulin in the fractionation of the serum. However, the familiar fact of possessing specific affinities for homologous antigen constitutes one obvious point of distinction between the antibody and the ordinary serum globulin. A further distinction between the properties of the sensitized surface and the serum globulin is brought out in Tables I to V and Figs. 3 to 5.

In Table V are given the isoelectric points of eleven samples of

euglobulin obtained from the precipitin sera. These were usually precipitated by diluting a small volume of the serum with about fifteen volumes of distilled water and adding a few drops of one-tenth normal hydrochloric acid. Two samples were obtained by ammonium sulfate fractionation. The values of the isoelectric points are seen to be in all cases within a range from pH 4.9 to 5.2, with an average of 5.1. The average value is plotted as a horizontal line at pH 5.1 in Figs. 3 to 5. It is thus evident that the isoelectric points of maximally sensitized acid-fast bacteria and precipitinogen-coated collodion particles and also of immune precipitate lie well to the alkaline side of the range of values for the isoelectric points of euglobulin samples precipitated as described from the same antisera.

A greater difference in the same direction has been reported by Felton (21) between the minimum solubilities of antibody-protein and the non-specific globulin of antipneumococcus horse sera. The minimum solubilities of Felton's antibodies lie between pH 6.6 and 6.8. Several interpretations are possible for the isoelectric values of 5.5 to 5.8 we have found for the maximally sensitized surface and for the specific precipitate. Conceivably these values might represent the values of the isoelectric points of the antibodies of rabbit serum; or these values might be a resultant of the isoelectric points of a more alkaline protein analogous to Felton's with more or less non-specific serum globulin adsorbed upon it; or the uncombined antibody might be isoelectric at a higher or lower pH than 5.5 to 5.8 and obtain these values because of changes involved in the union with antigen. Answers to these questions will have to await further investigation.

Useful conceptions of the nature of antibodies have been proposed by Locke, Main and Hirsch (22) and by Manwaring (23). The denaturation of the antibody-protein when deposited upon the antigen surface has been discussed in references 17, 19 and 24.

Throughout this work we have had capable technical assistance from Mr. H. J. Henderson.

SUMMARY

As a further test of the theory of tropin action proposed in the preceding paper artificial surfaces have been prepared, and have been found to be phagocytized according to prediction from the theory.

Protein was adsorbed on collodion particles according to the technique of F. S. Jones. These particles were then agglutinated and prepared for phagocytosis by the corresponding protein precipitin sera. The precipitating, agglutinating, surface and tropin effects for each serum or serum globulin fraction have been found to be in satisfactory quantitative correspondence. All of these effects were serologically specific; all remained almost unaffected by inactivation of the immune sera for 30 minutes at 56°C. or by washing of the particles after sensitization.

The surfaces of particles maximally sensitized by homologous rabbit immune serum or one of its globulin fractions have shown certain characteristic properties, *i.e.*, they were cohesive, had wetting properties characteristic for protein, and were isoelectric at pH 5.5 to 5.8. The same set of properties were found for immune precipitate in the zone of maximal precipitation. The same properties have also been found for maximally sensitized acid-fast bacteria, and for maximally sensitized sheep erythrocytes.

These results indicate, we believe, that precipitation, agglutination, the surface changes and increased phagocytosis are all consequences of one underlying phenomenon. This phenomenon is the specific chemical combination with, and deposit on the surface of the antigen of antibody protein. The several serological reactions then follow as consequences of the properties of the sensitized surface and of the special environing conditions.

The antibody is contained in the globulin fractions of immune serum, and appears to be a globulin with physico-chemical differences from normal serum globulin.

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