ON THE MECHANISM OF OPSONIN AND BACTERIOTROPIN ACTION

V. Experimental Test of a Theory of Tropin Action

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In previous papers we have reported that sera which have promoted the phagocytosis of acid-fast bacteria have regularly produced certain alterations in the bacterial surfaces (1, 2, 3) in corresponding degree. Conversely sera which produced these changes in the bacterial surfaces promoted phagocytosis correspondingly, although certain exceptions to the the latter rule were found in using sera which had been altered by heating or aging. Both surface and tropin effects were clearly due to the deposit of certain substances from the immune sera on the surfaces of the homologous bacterial antigen; heating or aging in some cases impaired the phagocytosis-promoting action of these substances without similarly impairing their capacity to combine with the bacteria. The correspondence between the effectiveness of fresh rabbit immune sera in producing surface changes and in promoting phagocytosis was found to hold with sera of various strengths, during the course of immunization, and during the course of tuberculous infection and reinfection.

It then became of interest to determine the distribution of the substances responsible for the surface and tropin effects in the several fractions of immune sera. This subject is treated in the present paper.

Methods

Fractionation of Sera

Fresh serum, without preservative, is diluted with an equal volume of 0.85 per cent NaCl solution. To one volume of serum, diluted, one volume of 66 per cent

saturated ammonium sulfate solution is added. The mixture is shaken thoroughly and allowed to stand at room temperature for 15 minutes. It is then filtered in the ice box using a rather thick, soft filter paper. To the clear filtrate enough ammonium sulfate is added to bring the concentration of ammonium sulfate to 50 per cent. This mixture is shaken thoroughly and allowed to stand 15 minutes at room temperature and filtered in the ice box. To the clear filtrate again enough ammonium sulfate is added to obtain a saturated solution; this is shaken thoroughly and after standing 15 minutes at room temperature, filtered in the ice box. The first precipitation separates the "euglobulin" fraction; the second the "pseudoglobulin" fraction, and in the third the albumin is brought down. After the filtration of each fraction the dried filter paper is temporarily stored in large petri dishes in the ice box. The precipitates are dissolved and washed off from the filter paper, using liberal amounts of distilled water. The solutions are then dialyzed for 4 days in the ice box, using parchment thimbles, and large quantities of water which are frequently changed. When dialysis is completed the fractions are evaporated to the original volume, with the aid of an electric fan, and enough sodium chloride is added to make 0.85 per cent solutions. If the solutions are not completely clear, centrifugation may be resorted to. If it is desired to separate the hydrophobic pseudoglobulins from the hydrophilic portion, after dialysis of the pseudoglobulin fraction, the contents of the dialyzing bag are centrifugalized at once. The sediment of hydrophobic pseudoglobulin can thus be separated by decantation.

Testing of Surface and Phagocytosis-Promoting Effects

The methods used in testing the effects of the sera and serum fractions were those already described in an earlier paper (1).

An even suspension of each antigen* was made and adjusted to a standard turbidity. Serial dilutions of each serum and serum fraction to be used were made. 1 cc. of antigen suspension was mixed with each dilution of serum or serum fraction. All tubes stood at room temperature usually for 30 to 90 minutes and were then put in the ice box over night. In the morning *agglutination* or sedimentation was read without shaking up the tubes. All tubes were centrifuged until clear; the supernatants were decanted, two drops of 0.85 per cent sodium chloride solution were added to each tube and all tubes were shaken up in the *resuspension* test. Excess of saline solution was added to each tube and all were again centrifuged. The supernatant fluids were again decanted, and the sensitized sediments brought to as even a suspension as possible in their original volume of saline. These suspensions of washed sensitized antigen were routinely used for *cataphoresis* and *interface* reactions, and for the tests of phagocytosis of washed antigen.

* The term "antigen" is for convenience applied in this paper to the whole bacteria; it is of course understood that the bacterial cell usually contains more than one antigen.

Phagocytosis of washed antigen was tested by mixing 0.2 cc. of polymorphonuclear leucocyte suspension with 0.2 cc. of each washed, sensitized antigen suspension in a vial and rotating all the vials for 15 minutes on a Robertson agitator. Phagocytosis in the presence of serum or serum-fraction dilution was conducted by rotating in each vial a mixture of 0.2 cc. of leucocytes suspension, 0.1 cc. of serum dilution and 0.1 cc. of stock antigen suspension (of twice the concentration of the washed suspension mentioned above). The making of smears, staining and counting in the phagocytosis tests were as already described.

Certain minor departures from the routine outlined were made from time to time, but these were unimportant. For instance resuspension and cataphoresis were occasionally tested with the particles suspended both in the original serum or serum fraction dilutions and also after washing.

Increased cohesiveness of the antigen surface due to sensitization is indicated by positive resuspension and interface reactions. Decreased surface electric potential difference is indicated by decreased velocity of migration in cataphoresis. Altered wetting properties of the antigen surface are indicated by the interface reaction. The degree of phagocytosis-promoting or tropin effect is indicated by comparison of the percentage phagocytosis of sensitized and control bacterial suspensions.

Correlation between Bacterial Surface and Tropin Effects Produced by Immune Sera and Their Fractions

The immune sera and their fractions have proved to be effective in the same order in altering the surface and in causing agglutination and phagocytosis of their homologous bacteria. The order of effectiveness has been uniformly: whole serum > euglobulin > pseudoglobulin > albumin. In only one of eleven experiments was any deviation from this order detected; in this experiment euglobulin was more effective than pseudoglobulin in some reactions, less effective in others.

As heretofore the correspondence between the surface changes and the agglutination and phagocytosis brought about by whole immune sera has been found in this work to be exceedingly close. The correspondence between surface, agglutinating and bacteriotropic effects has been exceedingly good also when the euglobulin or pseudoglobulin fractions of immune sera have been used. Of course the parallelism between surface and tropin effects was not perfect in all cases; for

instance the phagocytosis titers tended to run a little higher than those of the surface reactions when Arloing, Prague or chelonei strains were used as antigens, a little lower when Bovine III was antigen; also minor quantitative discrepancies occurred from time to time. Such occasional slight discrepancies are, however, inevitable in an extensive

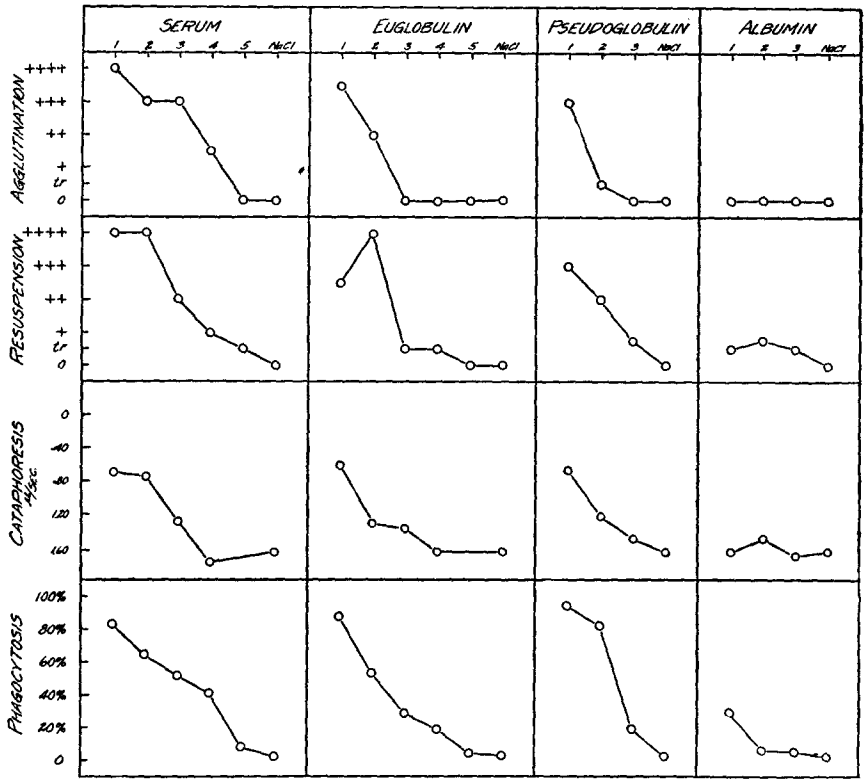

FIG. 1. Parallelism between surface changes and phagocytosis in presence of immune serum and its globulin fractions. Abscissae are successive dilutions of antiserum or serum fraction in powers of four. (Thus 3 is a dilution of $1:4^3$ or 1:64.) *M. avium* (Arloing strain) and homologous antiserum.

experiment of this character in which the tests cannot be perfectly precise and necessarily include in each case variables not common to the other tests. With these slight reservations in mind the impressive fact has been the close correspondence between surface, agglutinating and tropin effects when whole serum or its euglobulin or pseudoglobulin fractions have been used.

In contrast to this were the results when the albumin fractions of antisera were used. The presence of serum albumin in the phagocytic mixture usually induced a moderate degree of phagocytosis. This effect was moderate at a dilution of albumin of 1 to 4, was weaker or

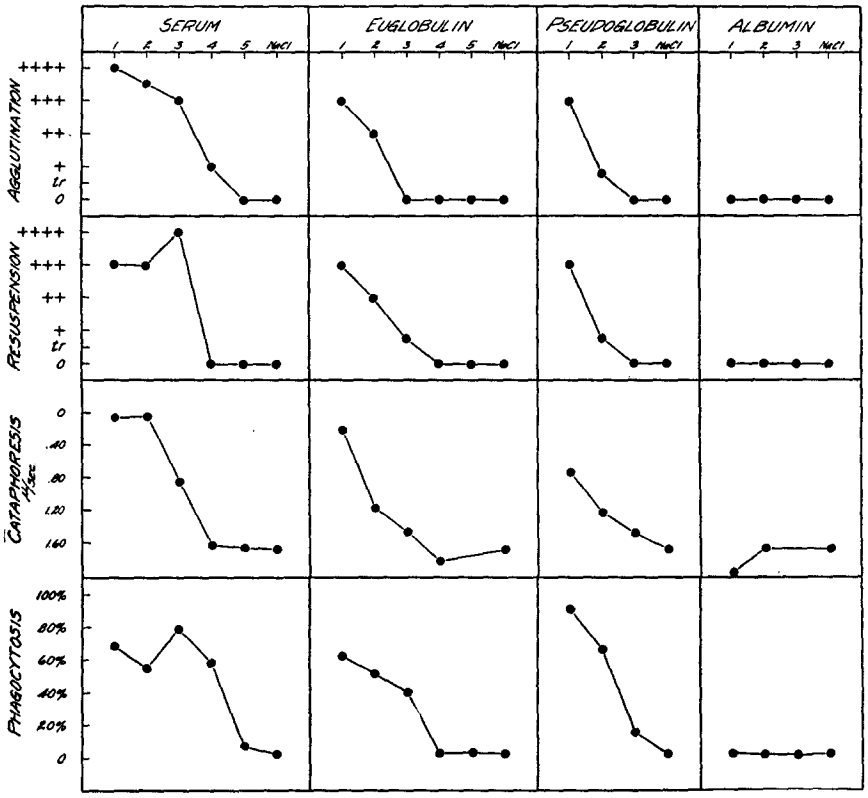

FIG. 2. Parallelism between surface changes and phagocytosis after sensitization with immune serum and its globulin fractions. The same antigen and antiserum as in Fig. 1. The antigen was sensitized and washed, then exposed to phagocytosis.

absent at dilutions of 1 to 16 and 1 to 64, and did not extend to higher dilutions. Serum albumin on the contrary caused only very slight and irregular surface or agglutinating effects and these were essentially out of correspondence with the phagocytosis-promoting effect.

TABLE I

The Effect of Washing upon the Phagocytosis of Bacteria Sensitized with the Globulin and Albumin Fractions of an Immune Serum

	Serum dilutions				NaCl contro
	1:4	1:16	1:64	1:256	
Series A: *M. chelonei* in anti-chelonei serum-euglobulin					
Agglutination	++++ to +++	++++ to +++	++++ to +++	++++ to +++	0
Resuspension	+++	+++	−	++	0
Interface	+++	+++ to ++	−	+	0
Cataphoresis, μ/sec	0	0	−	0.19	1.80
Phagocytosis, washed, %	90	97	−	92	4
Phagocytosis, unwashed, %	97	91	97	97	3
Series B: *M. chelonei* in anti-chelonei serum-pseudoglobulin					
Agglutination	++++ to +++	++++ to +++	++ to +	0	0
Resuspension	++	+	+	0	0
Interface	++ to +	+ to tr.	±	−	−
Cataphoresis, μ/sec	0	0.61	1.11	1.53	1.98
Phagocytosis, washed, %	100	92	83	8	6
Phagocytosis, unwashed, %	98	87	91	16	3
Series C: *M. chelonei* in anti-chelonei serum-albumin					
Agglutination	0	0	0	−	−
Resuspension	tr.	tr.	0	−	−
Interface	0	−	−	−	−
Cataphoresis, μ/sec	1.61	1.65	1.41	−	−
Phagocytosis, washed, %	8	4	6	−	−
Phagocytosis, unwashed, %	54	7	3	−	−

Series D: *M. tuberculosis* in anti-chelonei serum-euglobulin

Agglutination	+++ to ++	++	++	++ to +	tr.
Resuspension	++ to +	+	+	–	0
Interface	++++ to +++	++ to +	+	tr.	0
Cataphoresis, μ/sec	0	0	0	0.59	1.58
Phagocytosis, washed, %	27	30	11	10	3
Phagocytosis, unwashed, %	56	45	48	45	1

Series E: *M. tuberculosis* in anti-chelonei serum-pseudoglobulin

Agglutination	++	++ to +	+ to tr.	tr.	tr.
Resuspension	tr.	++ to +	++ to +	+	+ to tr.
Interface	+	tr.	–	–	–
Cataphoresis, μ/sec	0	0.32	0.58	1.00	–
Phagocytosis, washed, %	38	15	8	–	2
Phagocytosis, unwashed, %	61	49	25	14	0

Series F: *M. tuberculosis* in anti-chelonei serum-albumin

Agglutination	tr.	tr.	tr.	–	–
Resuspension	++ to +	tr.	0	–	–
Interface	0	–	–	–	–
Cataphoresis, μ/sec	0.85	1.14	1.11	–	–
Phagocytosis, washed, %	9	4	2	–	–
Phagocytosis, unwashed, %	28	11	10	–	–

The Effect of Washing the Sensitized Bacteria

Whether phagocytosis was conducted in the presence of the whole immune sera, or whether the bacteria were sensitized and washed and then exposed to the leucocytes, made very little difference. In an occasional experiment the percentage phagocytosis was slightly less after washing, but in the majority it was not affected. The same was true for the euglobulin and pseudoglobulin fractions. Illustrative experiments are given in Figs. 1 and 2 and Table I.

In contrast to the whole serum and globulin fractions, the phagocytosis-promoting effect of the serum albumin was in all cases much reduced or was abolished by washing. This constitutes a second point of differentiation between the phagocytosis-promoting effect of albumin and the true tropin effects of the globulin fractions. A third difference, that of specificity, is brought out in the paper following this.

The Effect of Heating the Sera before Fractionation

Each of three batches of immune serum was divided into two portions, of which one was heated at 56°C. for 30 minutes. The heated and unheated portions of each serum were fractionated and tested with the homologous bacterial strain.

The surface and tropin effects of an anti-avian (Arloing) and of an antichelonei serum were only very slightly if at all weakened by the heating except that, in the case of the antichelonei serum, a definite agglutination and phagocytic prozone appeared in the heated portion; there was no prozone in resuspension, interface or cataphoresis reactions. Such prozones in the other reactions have occasionally been noted in our work with immune sera heated to 56°C. for 30 minutes. They are evidently similar to the more pronounced agglutination prozones produced by Shibley (4) by heating antisera to higher temperatures. The phagocytosis-promoting properties of immune sera may then be impaired by heating, as well as by aging (2), even in instances in which the surface reactions indicate no impairment of the capacity of the serum to combine with antigen.

The surface and tropin effects of the euglobulin fractions of the immune sera were only very slightly if at all weakened by the heating. The surface and tropin effects of the pseudoglobulin fractions of the antisera were not appreciably weakened by the heating.

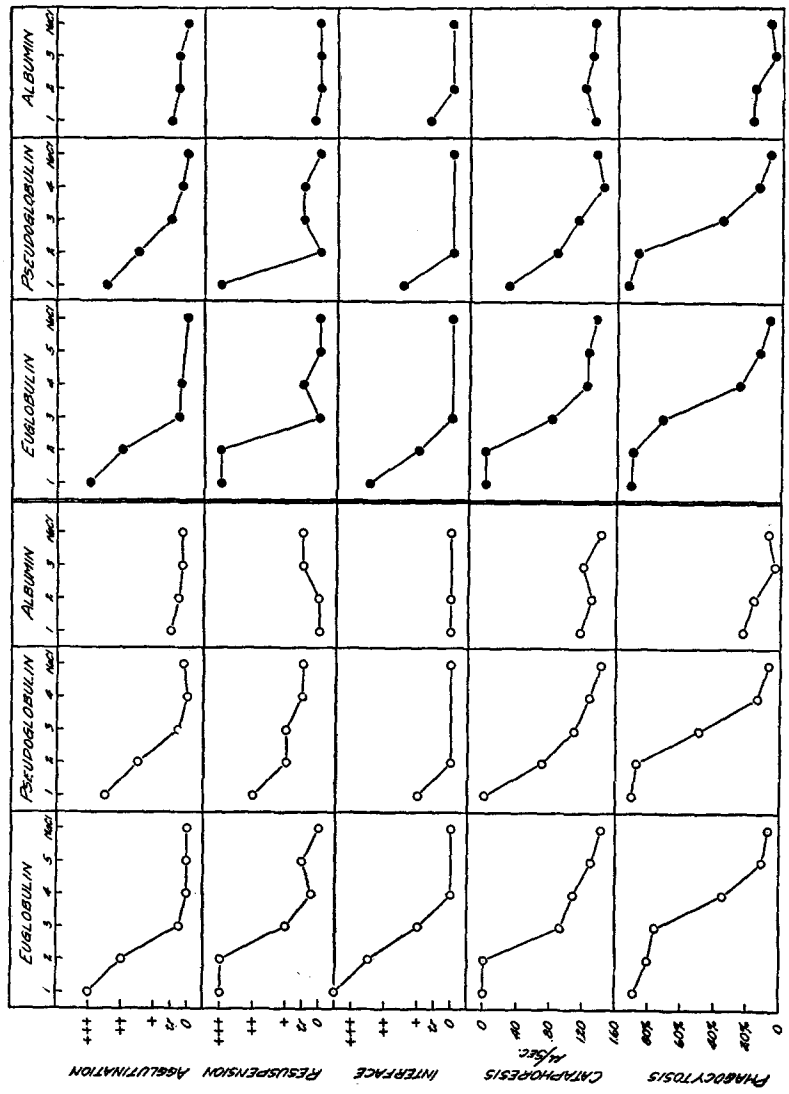

FIG. 3. Parallelism between surface changes and phagocytosis after sensitization with globulin fractions of immune serum. *M. avium* (Arloing strain) sensitized with serum fractions and then washed. Open circles: fractions from Anti-Arloing serum heated for 30 minutes at 56°C. before fractionation. Black circles: fractions from same Anti-Arloing serum unheated.

DISCUSSION

It is clear, then, that the specifically active substances in our immune sera are distributed between the globulin fractions. The small phagocytosis-promoting effect of serum albumin differs in several respects from the tropin action of whole sera and their globulin fractions. The data lead very directly to the conclusion, therefore, that the interaction of antigen and fresh immune serum results in the deposit on the antigen surface of a substance or substances contained in the globulin fractions of the antiserum. The surface reactions, agglutination and increased phagocytosis are quantitatively correlated with this surface deposition, and without a doubt are consequences of the properties of the substances deposited on the antigen surface and of the special environing conditions of the several tests.

The question very naturally arises as to whether this conception of antibody action is in harmony with previous work; this is the case.

It was demonstrated by early investigations that antigens in sensitization acquire a deposit of material which at least has properties in common with the serum of the species from which the antiserum has come.

Ehrlich and Morgenroth (5) demonstrated in 1901 that immune sera produced by a given species, A, when injected into another species, B, led to the development of anti-immune sera which could neutralize the antibodies in immune sera in species A. Bordet (6) showed similarly that normal serum of species A injected into another species produced anti-sera which would neutralize the antibodies in sera of species A. Bordet further showed that such anti-immune sera could even interact with antibodies already united with their antigens and could alter the state of their union with antigen. Thus ox cells sensitized with rabbit immune serum and washed, were "cured" by treatment with anti-sensitizer produced by injection of normal rabbit serum into guinea pigs; addition of complement did not cause hemolysis of the sensitized ox cells. The interaction of sensitizer and anti-sensitizer was shown to be a true combination. Finally it was shown that normal rabbit serum could partially displace rabbit sensitizer from its union with anti-sensitizer.

Bacteria sensitized with immune horse-serum and injected into guinea pigs by Braun (7) made the guinea pigs anaphylactic to horse serum. Bacteria sensitized with immune horse-serum were stated to absorb the anaphylactic antibodies from the serum of guinea pigs and the precipitating antibodies from the sera of rabbits injected with horse serum. Altmann (8), by immunizing animals with sensitized red blood cells, thoroughly washed, obtained sera which gave precipitation and

complement fixation with serum of the species from which the sensitizer had come.

More recent studies by physical-chemical methods have closely associated the sensitizing substance or substances with the globulin of the immune serum.

Porges (9) reported that bacteria treated with a large excess of agglutinin were precipitated by salts in a range of concentration characteristic for serum globulin rather than for the unsensitized bacteria.

Coulter (10, 11) reported that strong sensitization with immune rabbit serum shifted the agglutination optimum of sheep's red blood cells from its value with normal cells of pH 4.75 to a pH of about 5.3; the latter value is close to the isoelectric point of serum globulin. The maximum combination of hemolytic sensitizer from rabbit serum with sheep red blood cells (and minimum dissociation) were stated to occur also at a pH of about 5.3.

The work of Northrop and De Kruif (12), and Northrop and Freund (13) showed that the agglutinating substances from immune sera form a deposit on the surface of the homologous bacteria.

Red blood cells (14), acid-fast bacteria (15) and certain spirochetes (16) have been shown by Mudd and Mudd to have surfaces whose wetting properties are suggestive of a high lipin content. Sensitization of each of these antigens with homologous immune serum gives it an altered surface whose wetting properties are like those of protein.

Shibley (17) found that a variety of bacteria whose surface electric potential differences vary widely, were all brought by sensitization to essentially the same potential difference. The electric behavior of strongly sensitized bacteria closely paralleled that of serum globulin.

Eagle (18) showed that the physical constants for adsorption by sensitized bacteria and red blood cells were closely similar to those for adsorption by immune precipitate. The work of Moll (19), Welsh and Chapman (20), Ottensooser (21), Wu and his associates (22), and Marrack and Smith (23) indicate that a large part of immune precipitate is protein material from the globulin fraction of the antiserum.

Heinemann and Gatewood (24) demonstrated that the bacteriotropins in antistreptococcic and antigonococcic horse sera were precipitated with the globulins.

The work cited thus shows by a number of independent methods that an antigen in specific sensitization receives a surface deposit of a substance or substances which at least have several points of resemblance to serum globulin.

The contributions of Rhumbler (25), Tait (26) and especially those

of Fenn (27) and of Ponder (28) have shown on theoretical grounds that a major requirement for phagocytosis is that the particles to be ingested shall have a surface upon which the leucocytes will spread.

We conclude, therefore, both from our own work and from that of earlier investigators, that *the contact of antigen with fresh homologous immune serum results in the deposit on the antigen surface of a substance or substances contained in the globulin fractions of the antiserum; as a consequence of this surface deposit leucocytes can spread upon and engulf the antigen.*

The conception of antibody action to which we and other students of the physical-chemistry of immune reactions have been led, represents a synthesis of certain elements from the views of the older schools associated, respectively, with the names of Ehrlich and of Bordet. Practically all investigators are agreed, so far as we know, that the combination of antigen and antibody is determined by specific chemical affinities. The beautiful demonstration that the specificity of antigen and antibody may be determined by the spacial configurations about a single carbon atom securely establishes this point of view (29, 30). On the other hand we find no evidence to support the conception of special reacting groups on the antibody responsible for bringing about the several immune reactions. The evidence, at least in the systems with which we have worked, supports the conception of precipitation, agglutination, phagocytosis, and the changes detected in the interface and cataphoresis reactions as consequences, under the several conditions of test, of the properties of the same substances deposited on the antigen surface. The combination of antigen and antibody then is specific; the consequences of that combination are non-specific expressions of certain physical-chemical attributes of the immune substances, and the phenomena exhibited are various rather because of various environing conditions than because of differences in the attributes of the immune substances. Further evidence in support of this conclusion will be presented in the paper following.

We do not wish at the present time to extend this discussion to include lytic or antitoxic reactions. Even in such cases it seems not improbable, however, that the phenomena exhibited may depend more upon the properties of the antigens than upon any special peculiarities of the specific antibodies involved.

SUMMARY

Antisera against several strains of acid-fast bacteria have been separated into their euglobulin, pseudoglobulin and albumin fractions. The globulin fractions have been found to possess the essential properties of bacteriotropic sera: thus they alter the bacterial surface properties, and, in quantitative correspondence, cause agglutination and phagocytosis; these several effects withstand washing of the sensitized bacteria; the effects are little if at all affected by inactivation of the antisera before fractionation; the combination of antibody and antigen is serologically specific.

The conclusion is drawn that the contact of antigen with fresh homologous immune serum results in the deposit on the antigen surface of a substance or substances contained in the globulin fractions of the antiserum; as a consequence of this surface deposit leucocytes can spread upon and engulf the antigen.

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