ON THE MECHANISM OF THE SERUM SENSITIZATION OF ACID-FAST BACTERIA.

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(Received for publication, April 15, 1927.)

The peculiar surface properties of acid-fast microorganisms make them readily susceptible of direct study by the interfacial tension method, a procedure not heretofore applied to analysis of the interactions of bacteria and sera. The acid-fast bacteria are thus especially favorable material for study of the mechanism of serum sensitization.

I. Directly Observable Alterations in the Bacterial Surface Produced by Sensitization.

The surfaces of acid-fast bacteria grown on glycerol agar slants, and suspended in 0.8 per cent sodium chloride solution, as has already been pointed out, are usually vigorously wetted by a neutral oil, tricaprylin. When these bacteria are suspended in fresh sera and subsequently washed in salt solution and studied in a saline-tricaprylin interface, they are no longer easily wetted by the oil. They are on the contrary in equilibrium[1] in the oil-saline interface and pass into the oil only at the expense of mechanical work.

Strongly sensitized clumps of acid-fast bacteria may be carried ahead of the advancing oil phase, or, if the clump is adherent to the slide, it retards locally the advance of the oil and may even draw out the interface to form a "peninsula" or vacuole.[2]

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[1] Mudd, S., and Mudd, E. B. H., *J. Exp. Med.*, 1924, xl, 633, 647; 1926, xliii, 127.

[2] Similar phenomena have been described and figured for sensitized red blood cells (*J. Exp. Med.*, 1926, xliii, 127). However, the passage into the oil of clumps of sensitized acid-fast bacteria differs from that of sensitized erythrocytes in that no surface processes or "tails" are ever to be seen with the bacterial clumps.

Serum sensitization thus transforms the predominantly oil-miscible[3] surface of the acid-fast bacteria into a surface more readily wet by saline solution than by the oil. All stages of this transformation may be seen. A strong immune serum causes in low concentration just perceptibly decreased oil miscibility of its homologous microorganism. With increasing concentration of the sensitizing serum the resulting bacterial surface change usually increases to a maximum in the highest serum concentrations. Normal sera or immune sera with unrelated heterologous organisms cause detectable oil immiscibility in the higher concentrations only.

The *cohesion* of films of sensitized and unsensitized typhoid bacilli has been measured by Northrop and De Kruif; they found that, other factors being constant, the cohesion increased with the concentration of sensitizing immune serum, at least until maximum sensitization was reached.[4] This relation of sensitization to cohesion is very strikingly shown in the present experiments with acid-fast bacteria.

Suppose an even bacterial suspension virtually free of clumps to have been made, and equal portions mixed with serial dilutions of homologous immune serum. All tubes are centrifuged, the supernatant liquid decanted, and the sediment resuspended in salt solution. The sensitized bacteria resuspend in clumps whose size and coherence, as estimated from their resistance to dispersion by agitation, increase with the concentration of serum used for sensitization.[5,6] If now all tubes are centrifuged again and the sediment studied in the tricaprylin-saline interface the same relation of sensitization to cohesion is again brought out. The loose aggregates of unsensitized bacteria on contact with the advancing oil are explosively dispersed and flung into the oil. Weakly sensitized clumps are dispersed less completely, the dispersion occupies perceptible time, and the component bacteria are carried away from the clumps for greater distances along the the oil-saline interface before passing into the oil. Very strongly sensitized clumps may resist dispersion by the interfacial stresses altogether. In general, for any one bacterial suspension the degree of coherence of clumps produced by the sensitization and the degree of oil immiscibility produced by sensitization run roughly parallel.

The characteristic increase in resistance to wetting by oil produced by the serum sensitization of red blood cells and acid-fast bacteria has been called in an

[3] See Foot-note 11 in preceding paper.

[4] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 664.

[5] Mudd, S., *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 569.

[6] Mudd, S., *J. Immunol.*, 1927, xiii, 113.

earlier paper[2] "a positive stabilization reaction." A more convenient term is "positive interface reaction."

To summarize, serum sensitization of the acid-fast bacteria causes two definite and directly observable changes in the bacterial surface:

1. A change from a surface readily wet by oil to a surface more readily wet by aqueous salt solution than by oil. This change is directly observed by microscopic examination of the bacteria in a saline-oil interface; thus detected, the surface alteration is said to constitute a "positive interface reaction."

2. An increased cohesiveness of the sensitized bacteria. This may be detected either by centrifuging the bacteria and then shaking up the sediment (resuspension reaction) or by microscopic observation of the clumps in the saline-oil interface.

II. Specificity of the Interface Reaction and Its Relation to Agglutinin Binding and Complement Fixation.

The procedure in obtaining the comparative titers given in this paper has been as follows:

The microorganisms were suspended in 0.8 per cent sodium chloride solution, with grinding when necessary, and the clumps thrown down by centrifugation. Portions of the relatively even supernatant suspension were mixed with serial dilutions of serum and put in the ice box overnight. The sera were unheated except where otherwise specified. The macroscopic *agglutination* readings were made in the morning. All tubes were centrifuged; the sediment was then either resuspended in the same serum dilutions, or else the serum was decanted and the sediment resuspended in a few drops of 0.8 per cent salt solution. The unsensitized bacteria resuspended evenly, the sensitized in clumps. This procedure has been termed the *resuspension* reaction. Its details and uses have been given in full elsewhere;[6] it is a simple and practical way of determining the binding of agglutinin by difficultly agglutinable strains. The bacteria were then washed once or more in salt solution and studied in the saline-tricaprylin *interface*. (Triolein was used as test oil in a few experiments.) Suspensions of the same strains and the same sera were used simultaneously by Dr. J. Furth in the *complement fixation* reaction.[7,8] The titer given is in each case unless otherwise indicated the highest dilution of serum which produced a perceptible effect.

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[7] Furth, J., *J. Immunol.*, 1926, xii, 273.

[8] Mudd, S., and Furth, J., *J. Immunol.*, 1927, xiii, 369.

The binding of antibody as detected by the interface reaction has been found to be serologically specific and to indicate antigenic relationships among acid-fast bacteria which correspond to those indicated by agglutinin binding, and, except for the anomaly mentioned in the next paragraph, to those indicated by complement

TABLE I.

Comparative Titers with a Saprophyte, an Avian and a Bovine Tubercle Bacillus, and Their Corresponding Antisera.

		Antiserum against "milk" bacillus	Antiserum against Arloing bacillus	Antiserum against Bovine III
Suspension of "milk" bacillus	Agglutination	1:640++*	1:40+*	1:40+*
	Resuspension	1:640 flocculi*	1:2.5	1:5+ (others?)
	Interface reaction	1:80	1:1.25+, 1:10−	1:2.5
	Complement fixation	1:1000		
Suspension of Arloing bacillus	Agglutination	1:80	1:640++*	1:80
	Resuspension	1:5	1:640 flocculi*	1:160
	Interface reaction	1:2.5−	1:640	1:80
	Complement fixation		1:500	1:100
Suspension of Bovine III	Agglutination	1:80+*	1:40	1:320
	Resuspension		1:20	1:160
	Interface reaction	1:5	1:5− or ∓	1:80
	Complement fixation	1:100	1:100	1:500

Moderate cross-reactions between *M. avium* (homogeneous culture of Arloing[7]) and *M. tuberculosis* (Bovine III strain). "Milk" bacillus practically unrelated serologically. All antisera from rabbits.

* Titer not reached.

fixation. The specificity of the interface reaction is illustrated by Tables I and II.

The anomaly just referred to is that certain sera homologous with such difficultly agglutinable strains as virulent mammalian tubercle bacilli may give higher interface and agglutination titers with heterol-

ogous than with homologous microorganisms. Examples of such anomalous titers are given in Tables III and IV.

This anomaly has occurred when the heterologous was closely related to and much more easily agglutinable than the homologous strain. The complement fixation titers in such instances have been normal, *i.e.* higher with the homologous than with the heterologous strain. The crossed combinations, *i.e.* antiserum for easily agglutinable strain with homologous easily agglutinable and heterologous difficultly agglutinable strain, have likewise been normal for interface and agglu-

TABLE II.

Comparative Titers with Three Species of "Tubercle" Bacilli of Cold Blooded Animals and Their Corresponding Antisera.

		M. marinum antiserum	*M. chelonei* antiserum	*M. ranæ* antiserum
M. marinum suspension	Agglutination	1:80	All negative	1:20
	Resuspension	1:320	All negative	1:2½
	Interface reaction	1:160	1:5	1:10
	Complement fixation	1:1000	1:20 negative	1:20 negative
M. chelonei suspension	Agglutination	1:40	1:1280*	1:80
	Resuspension	All negative	1:320	1:10
	Interface reaction	1:2½∓	1:80	1:5
	Complement fixation	1:50 negative	1:1000	1:100
M. ranæ suspension	Agglutination	1:5	1:10	1:2560*
	Resuspension	1:2½	1:10	1:2560*
	Interface reaction	1:2½	1:5	1:640
	Complement fixation	1:50 negative	1:50	1:2000*

Little or no serological relationship found between the three species. All antisera from rabbits.

* Titer not reached.

tination reaction as well as for complement fixation. Similar anomalies in agglutination experiments have been noted by Griffith[9] and by Wilson.[10] It may be necessary to use "absorption tests" in order to differentiate such strains with certainty.

The agglutination, resuspension, interface, and complement fixation titers have all been obtained for some forty-three combinations

[9] Griffith, A. S., *Tubercle*, 1924–25, vi, 417.

[10] Wilson, G. S., *J. Path. and Bact.*, 1925, xxviii, 1, 69.

TABLE III.

Comparative Titers with Virulent Bovine[8] *(816) and Avirulent Bovine (III) Tubercle Bacilli, "Leprosy Bacillus" of Kedrowsky,*[7] *and Corresponding Antisera.*

		Bovine 816 antiserum	Bovine III antiserum	"Leprosy bacillus" of Kedrowsky antiserum
Bovine 816 suspension	Agglutination	1:5		
	Resuspension	1:80		
	Interface reaction	1:10		
	Complement fixation	1:1000		
Bovine III suspension	Agglutination	1:400	1:400	1:800
	Resuspension	1:100	1:200	1:400
	Interface reaction	1:200	1:100	1:200
	Complement fixation	1:500	1:500	1:100±
"Leprosy bacillus" of Kedrowsky suspension	Agglutination	1:200+*	1:200	1:800+++*
	Resuspension	1:25	1:50	1:800+*
	Interface reaction	1:12½	1:12½ negative	1:800
	Complement fixation			1:1000*

Higher interface and agglutination titers with easily agglutinable strains Bovine III and "leprosy bacillus" in Bovine 816 antiserum than with difficultly agglutinable Bovine 816 in Bovine 816 antiserum. Cross-reactions between Bovine III and "leprosy bacillus" show ordinary specificity. All antisera from rabbits.

* Titer not reached.

TABLE IV.

Comparative Titers with Avian (Egret) and Human[8] *(H 37) Tubercle Bacilli and Corresponding Antisera.*

		Egret (avian) antiserum	H 37 antiserum
Egret (avian) suspension	Agglutination	1:400+++*	1:12.5+
	Resuspension	1:400 granular*	1:50
	Interface reaction	1:400+	1:12.5±
	Complement fixation	1:1000	1:20–50
H 37 human suspension	Agglutination	1:12.5±	1:12.5−
	Resuspension	Inconclusive	1:12.5
	Interface reaction	1:12.5−	1:12.5−
	Complement fixation	1:100	1:500

In H 37 antiserum agglutination and interface titers higher for egret strain than for H 37. Complement fixation shows ordinary specificity. Antisera from rabbits. Test oil triolein.

* Titer not reached.

of serum and bacterial suspension. The interface reaction proves to be the least sensitive, *i.e.* to have the lowest average titer. The ratios of the average titers of the several reactions to that of the interface reaction are:

Agglutination titer:interface titer::3.5:1
Resuspension titer:interface titer::2.4:1
Complement fixation titer:interface titer::13.6:1

The resuspension titer:interface titer ratio has been less inconstant than the others. Moreover in those cases already cited (for instance Tables III and IV) in which the agglutination and resuspension titers were anomalous and the complement fixation titers normal, the interface titers paralleled the agglutinin binding and not the complement fixation titers. Since, therefore, agglutination as estimated by the improved or resuspension method, cohesion as estimated by the resuspension and interface methods, and the bacterial surface change (decreased oil miscibility) detected by the interface reaction all show general parallelism, it seems safe to conclude that the bacterial surface change, like the other effects, is at least in considerable part due to the binding of agglutinins by the bacterial surface.

Similar decrease in surface oil-miscibility and increase in cohesion has been found as a result of serum sensitization of mammalian red blood cells, but in this case the surface change was correlated with the binding of hemolytic sensitizer and was relatively independent of agglutinin binding.[2]

The importance of the physicochemical nature of the bacterial surface itself in agglutination is particularly well shown with the acid-fast bacteria. It has already been pointed out that agglutination as ordinarily detected may be entirely absent under circumstances in which the resuspension and interface reaction show abundant agglutinin to have been bound by the bacterial surface.[6,11] Conversely, with readily agglutinable strains agglutination may be complete in tubes in which interfacial observation shows that only a part of the bacterial surface has been altered by deposit of antibody. The latter point is illustrated in Tables V and VIII.

[11] It has repeatedly been shown also that inagglutinable bacteria may adsorb agglutinins (Buchanan, R. E., *J. Bact.*, 1919, iv, 82).

TABLE V.

"Milk" Bacillus Suspension with Homologous Rabbit Antiserum.

	Final serum concentration						
	5 per cent	2.5 per cent	1.2 per cent	0.62 per cent	0.31 per cent	0.16 per cent	Control
Agglutination	Complete	Complete	Complete	Complete to +++	+++	++	Tr.
Resuspension	All bacteria are in one or more great masses				Even suspension with some gross flocculi	Even suspension with some gross flocculi	Even suspension
Interface reaction	++++	++	+	−	−		−

TABLE VI.

Resuspension and Interface Reactions with Sera of Normal Goats and Suspension of M. tuberculosis (Strain Bovine III).

Serum from	Final serum concentration															
	80 per cent		40 per cent		20 per cent		10 per cent		5 per cent		2.5 per cent		1.25 per cent		0.62 per cent	
	Resuspension*	Interface*	Resuspension	Interface	Resuspension	Interface	Resuspension	Interface	Resuspension	Interface	Resuspension	Interface	Resuspension	Interface	Resuspension	Interface
Goat 25, unheated	Coarse flocculi	++++	Coarse flocculi	+++	Fairly coarse flocculi	+++	Fine flocculi	++ or +		+ weak	Even suspension	+ weak	Even suspension	±	Even suspension	−
Goat 25, inactivated	Even suspension	+	Fine flocculi	±		−	Almost even suspension		Almost even suspension	−						
Angora goat unheated	Even suspension		Coarse flocculi	+++	Finer flocculi	++	Even suspension	+	Even suspension	−	Even suspension		Even suspension	−	Even suspension	
Angora goat inactivated	Even suspension	+ very weak	Almost even suspension	−			Even suspension									

* Bacteria washed after about 3 hours contact with serum; bacteria in other tubes in serum dilutions overnight.

TABLE VII.

Titrations of Human and Rabbit Sera against Human and Bovine Tubercle Bacilli, Virulent Strains P 15 B and Bovine C.

		Final serum concentration								
		80 per cent	40 per cent	20 per cent	10 per cent	5 per cent	2.5 per cent	1.25 per cent	0.62 per cent	Control
Human serum, Patient N. Br., with human bacteria	Agglutination	−	−	−	Slight tr.	Tr. to +	−	−	−	−
	Resuspension	Medium flocculi	Fine flocculi	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	
	Interface	+ weak	−					−	−	
Human serum, Patient N. Bl., with human bacteria	Agglutination	−	−	−	−	Tr. to +	−	−	−	−
	Resuspension	Fine flocculi	Fine flocculi	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	
	Interface	+	+ weak	+ weak	−					
Normal rabbit serum I with human bacteria	Agglutination	Slight tr.	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	Slight tr.	
	Resuspension	Medium flocculi	Medium to fine flocculi	Very fine flocculi to even suspension	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	
	Interface	++	++ to +	+	+	+ weak	−	−		
Normal rabbit serum II with human bacteria	Agglutination	−	Tr.	Tr. to +	+	+	− to tr.	− to tr.	−	
	Resuspension	Medium to fine flocculi	Fine flocculi	Very fine flocculi	Some fine flocculi	Some fine flocculi	Some fine flocculi	Even suspension	Even suspension	
	Interface	++	+	+	+ weak	+ weak	+	−	−	
Human serum, Patient N. Br., with Bovine C bacteria	Agglutination	Tr.	Tr.	Tr.	Tr. to +	+	− to tr.	− to tr.	−	
	Resuspension	Very fine flocculi	Fine flocculi	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension
	Interface	−	−				−		−	

Human serum, Patient N. Bl., with Bovine C bacteria	Agglutination	−	−	Tr.	−	Tr. to +	Tr.	Slight tr.	−
	Resuspension	Even suspension	Even suspension	Even suspension	Even suspension	Stringy bodies	Stringy bodies	Even suspension	Even suspension
	Interface	+	+	−					
Normal rabbit serum I with Bovine C bacteria	Agglutination	Tr.	+	+	Tr. to +	Tr. to +	−	Tr. to +	−
	Resuspension	Fine flocculi	Fine flocculi	Fine flocculi	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension
	Interface	++	++ to +	+	−				
Normal rabbit serum II with Bovine C bacteria	Agglutination	−	Tr. to +	Tr. to +	Tr.	+	+	Tr.	−
	Resuspension	Fine flocculi	Fine flocculi	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension	Even suspension
	Interface	+	+ weak	−					

Patient N. Br. pregnant and with four plus Wassermann at time blood was taken for tests tabulated. Chest negative.
Patient N. Bl. with negative Wassermann at time blood was taken and for at least a year previously. Chest negative.
Rabbits normal. Test organisms a virulent bovine tubercle bacillus, Bovine C, and a virulent human tubercle bacillus, P 15 B.

III. Effects of Sensitization with Normal Sera.

The bacterial surfaces are modified by treatment with fresh normal sera in a manner quantitatively less but qualitatively indistinguishable by the methods used, at least, from the effects of immune sera. Sensitization with normal sera decreases the oil-miscibility and increases the cohesion of the acid-fast bacteria, but to a less degree and with lower titer than homologous immune sera.

Heating sheep, goat, rabbit, and human sera for 30 minutes at 56°C. usually decreased but did not abolish their effect on the bacterial surface. Similar inactivation of guinea pig sera left them without detectable effect on the bacterial surface. The test organisms used with normal sera have been *M. tuberculosis:* Strains H 37 (human), P 15 B (human), Bovine III, and Bovine C; "butter" bacillus; and *M. chelonei* (turtle). A titration is given in Table VI.

The sera of nine patients from the syphilis clinic have been tested against Bovine III.

These patients with the exception of two cases of arrested pulmonary disease were without clinical tuberculosis. The Wassermann reaction on the same serum specimen was four plus in two cases, was and had been negative in one case, and was negative or weakly positive but had formerly been four plus in the other six cases. The final serum concentration used for sensitization was in each case 80 per cent, and the test oil in the interface reaction was triolein. The sensitized bacteria, as in all other cases, were washed before study in the interface.

All these sera caused the characteristic bacterial surface change before, and all with one exception after heating for 30 minutes to 56°C. Another human serum, that of a girl of 12, negative for tuberculosis and with negative Wassermann, gave a two plus interface reaction after heating.

Titrations of two human and two normal rabbit sera against a human and a bovine tubercle bacillus are given in Table VII. The test oil in this case was tricaprylin.

IV. The Agglutination Prezone.

Since the important work of Northrop and De Kruif,[12] it has been recognized that the main features of bacterial agglutination can be

[12] Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 639, 655.

formulated in terms of the repellent forces of the electrical charges on the bacteria, which tend to keep them apart, and the cohesive force which tends to hold the bacteria together once they have collided. Substances which reduce the charge below a certain critical level ordinarily cause agglutination unless the cohesive force is also reduced.

The prezone in specific bacterial agglutination has not fitted well into this formulation. Studies by cataphoresis have indicated that the negative bacterial charge is reduced to a minimum in concentrated agglutinating sera.[13] Fig. 2 of Shibley's paper of 1924[13] is particularly instructive in this connection. This shows the bacterial surface charge falling with increasing serum concentration until the critical level is reached and agglutination occurs; further concentration of

TABLE VIII.

Egret Avian Suspension with Antiegret Rabbit Serum.

	Final serum concentration						
	8 per cent	4 per cent	2 per cent	1 per cent	0.5 per cent	0.25 per cent	NaCl
Agglutination	+	+	++	+++	++++	+++	−
Interface reaction	++++	++++	+++ or ++	+	+	+	−

Agglutination shows prezone, interface reaction does not.

serum reduces the charge along the same curve but agglutination ceases.

That the prezone is not due to lack of cohesiveness of the bacterial surface in concentrated sera is shown by our own results. Tables VII and IX of this paper and Tables IV and VI of an earlier communication[6] show that spinning the sensitized cells in the centrifuge abolishes the agglutination prezone. Similarly interfacial observation of the sensitized washed bacteria shows that the characteristic change in surface properties increases to a maximum after treatment

[13] Wells, H. G., The chemical aspects of immunity, New York, 1925, 137; Northrop, J. H., and De Kruif, P. H., *J. Gen. Physiol.*, 1921–22, iv, 655; Shibley, G. S., *J. Exp. Med.*, 1924, xl, 457; Freund, J., *Am. Rev. Tuberc.*, 1925, xii, 124; Falk, I. S., and Jacobson, M. A., *J. Infect. Dis.*, 1926, xxxviii, 182; Shibley, G. S., *J. Exp. Med.*, 1926, xliv, 674.

TABLE IX.

Bovine III Suspension with Homologous Goat Antiserum.

	Final serum concentration							
	8 per cent	4 per cent	2 per cent	1 per cent	0.5 per cent	0.25 per cent	0.12 per cent	0.6 per cent
Agglutination.....	+	+	++	+++	++++	+++	++	+
Resuspension.....	Very coarse flocculi	Very coarse flocculi	Flocculi	Flocculi	Flocculi	Even suspension	Even suspension	Even suspension
Interface reaction....	++++	++++	++++	++++	++ or +		+ weak	+ weak

Agglutination shows prezone; resuspension and interface reactions do not.

with concentrated sera and is without prezone (Tables VII to IX of this paper, and Table IV of a previous paper[6]).

Since the agglutination prezone then is not due to a failure of concentrated sera to reduce the bacterial surface charge or to increase the cohesiveness of the bacteria and may be abolished by centrifugation, we suggest that the prezone in such cases is due to interference with the collisions of the bacteria which are of course prerequisite to their clumping. Whether the interference with the colliding of the bacteria is chiefly due to increased viscosity of the menstruum, or to an accumulation of excess colloidal material in the bacteria-serum interfaces is uncertain; possibly both effects play a part.

It seems that the observations cited in the preceding paragraph are incompatible with the conception of these prezones as due to combination of the bacteria with Ehrlich's "agglutinoids."[14]

Krumwiede, Cooper, and Provost[15] have reported the production of prezones or the exaggeration of existing prezones by addition of material containing products of bacterial autolysis or media constituents. These workers also attribute their prezones to interference with agglutination by excess colloidal material.

V. Partial Reversibility of the Bacterial Surface Change.

Since the pioneer observations of Hahn and Trommsdorff[16] and Landsteiner[17] a number of investigators have demonstrated the possibility of recovering antibodies by partial dissociation of antigen-antibody combinations.[18] In the acid-fast group Aronson[19] has reported partial recovery of complement-fixing antibodies from combination with *M. tuberculosis*, or with alcoholic or saline extracts of tubercle bacilli. It seemed probable therefore that subjecting the sensitized bacteria to conditions that have been described as favoring dissociation might cause alteration of the bacterial surface in the

[14] Park, W. H., and Williams, A. W., Pathogenic microörganisms, Philadelphia and New York, 8th edition, 1924, 192.

[15] Krumwiede, C., Cooper, G., and Provost, D. J., *J. Immunol.*, 1925, x, 93.

[16] Hahn, M., and Trommsdorff, R., *Münch. med. Woch.*, 1900, xlvii, 413.

[17] Landsteiner, K., *Münch. med. Woch.*, 1902, xlix, 1905; Landsteiner, K., and Jagić, N., *Münch. med. Woch.*, 1903, l, 764.

[18] Huntoon, F. M., *J. Immunol.*, 1921, vi, 117.

[19] Aronson, J. D., *Nat. Tuberc. Assn. Tr.*, 1922, xviii, 517.

direction of the normal. This expectation has been verified by experiment, and further evidence has thus been furnished that the changes observed to follow sensitization are consequent on combination of antibodies with materials in the bacterial surfaces.

Serial dilutions of serum were mixed with bacterial suspension and allowed to stand in the ice box overnight. The sensitized bacteria were then washed, resuspended in saline, and studied in the interface. Into certain tubes of the series 0.1 per cent Na_2CO_3,[19] was distributed and into the other, usually alternate, tubes 0.8 per cent NaCl. Since we have not found evidence of appreciable dissociation by washing in NaCl, these saline tubes served as controls for the alkali-treated bacilli.[20] All tubes were returned to the ice box for five days or more. The tubes were then centrifuged, decanted, the sediment washed in NaCl, and studied in the interface.

Of ten series of sensitized bacteria prepared as described, six showed alteration of surface properties toward the normal as a result of treatment with Na_2CO_3. Results in the other four series were doubtful. Five of the positive instances are given in Table X.

Results of tests by resuspension were similar to those with the interface reaction. The sensitized bacteria digested with sodium carbonate and subsequently washed and resuspended in sodium chloride, were regularly in finer clumps than those in the alternate tubes digested with sodium chloride, as illustrated in Table XI. This result it is true is of less significance than the very convincing dissociation of antibody detected by the interface reaction, because the alkali might conceivably have dispersed the bacterial clumps simply through increasing the negative electric charge of the material on the bacterial surface. However, the only mechanism that, to our mind at least, satisfactorily explains both the observed interface and resuspension results after sodium carbonate treatment is dissociation of antibody from the bacterial surface.

VI. What Chemical Groups Are Responsible for the Bacterial Surface Change?

The evidence already cited indicates clearly, we believe, that fat-soluble material in the bacterial surface is coated over by the sensitiz-

[20] Indeed the impression has been gained that bacteria either normal or sensitized kept 24 hours or longer in NaCl tend to become slightly less oil-miscible.

ing serum antibodies or possibly by serum substances secondarily bound by these antibodies.[21] This shows that the actual chemical

TABLE X.

Dissociation of Antibodies by Sodium Carbonate with Return of Bacterial Surface toward Normal.

Tubes	Sensitized bacteria before treatment	Sensitized bacteria after treating with Na_2CO_3	Sensitized bacteria after treating with NaCl
A 1	++++	±	
A 2			+++
A 3	++	−	
A 4			+
B 1	++	+	
B 2	++ to +		++
C 1	++	+	
C 2	+	−	
D 1	++++	++	
D 2			+++
E 1	++	+	
E 2			++
E 3	++ to +	∓	
E 4			+

+ to ++++ indicate degrees of positive interface reaction. − indicates a normal (unsensitized) bacterial surface.

Series A (Bovine C), *M. tuberculosis* sensitized with homologous rabbit antiserum; serum concentration in Tube A 1, 20 per cent, in A 2, 10 per cent, etc.

Series B (Bovine III), *M. tuberculosis* sensitized with homologous goat antiserum; serum concentration in Tube B 1, 10 per cent, in B 2, 5 per cent.

Series C, *M. ranæ* sensitized with normal horse serum; serum concentration in Tube C 1, 80 per cent, in Tube C 2, 40 per cent.

Series D, *M. tuberculosis* (Bovine C) sensitized with homologous rabbit antiserum; serum concentration in Tube D 1, 20 per cent, in D 2, 10 per cent.

Series E, *M. tuberculosis* (Bovine C) sensitized with homologous rabbit antiserum; serum concentration in Tube E 1, 5 per cent, in E 2, 2.5 per cent, etc.

[21] It is evident from the parallel complement fixation studies described in Section II above that complement is bound by the sensitized bacteria. To what extent this complement material contributes to the observed bacterial surface change is uncertain. Even from inactivated sera there is evidence to indicate

group[22] which combines with the antibody responsible for the interface reaction is at least very closely associated with fat-soluble material in the bacterial surface. It is not clear from these data, however, whether the binding group is a part of the lipin molecule, is conjugated with it, or is merely in very close physical association with the lipin.

Kurt Meyer[23] showed that alcohol extracts of the tubercle bacillus make active "antigens" for the complement fixation reaction. Wadsworth, Maltaner, and Maltaner[24] prepared extracts of tubercle bacilli with various fat solvents

TABLE XI.

Dissociation of Antibody by Treatment with Sodium Carbonate. Resuspension Reactions.

	Final serum concentration			
	80 per cent	40 per cent	20 per cent	10 per cent
Sensitized bacteria before treatment	Medium flocculi	Medium flocculi	Medium flocculi	Medium flocculi
After treatment with Na_2CO_3	Almost even suspension		Even suspension with some medium flocculi	
After treatment with NaCl		Medium flocculi		Medium flocculi

M. tuberculosis (Bovine III) sensitized with serum of goat infected with Bovine C.

and found the acetone-insoluble lipoids to be potent complement fixation "antigens." Pinner has lately reported alcohol-soluble, acetone-insoluble sub-

that mid-piece (globulin) may be bound (Dean, H. R., *Proc. Roy. Soc. London, Series B*, 1911–12, lxxxiv, 416; Leschly, W., *Z. Immunitätsforsch., Orig.*, 1916, xxv, 219).

[22] We do not know how many antigens or antibodies are concerned in the sensitization process. The singular or plural nouns are used in this connection without implication.

[23] Meyer, K., *Z. Immunitätsforsch., Orig.*, 1912, xiv, 359; xv, 245.

[24] Wadsworth, A., Maltaner, F., and Maltaner, E., *J. Immunol.*, 1925, x, 241.

stances from tubercle bacilli to be active "antigens" in complement fixation and biologically active antigens when injected into rabbits.[25]

Freund, by agglutination and cataphoresis experiments, has demonstrated the possibility of sensitizing by serum the particles obtained by alcohol extraction of the tubercle bacillus.[26] This strongly suggests that a binding group is at least in some sort of chemical combination with the lipin.

Furth and Aronson[27] have shown 95 per cent ethyl alcohol extracts of a considerable number of acid-fast bacteria to contain serologically specific "antigens" for complement fixation. The writer has made some simple chemical tests with the extracts used by Furth and Aronson with the hope of gaining further light on the relation of the binding groups to the lipin.[28]

Amino acid tests with the material in these alcohol extracts were unsatisfactory. Millon's reaction was negative with suspensions of the residues from evaporation of the extracts. The possibility of traces of protein being present was not, however, satisfactorily excluded.

Molisch reactions with watery suspensions of the residues of these crude alcohol extracts were in all cases strongly positive. On hydrolysis positive orcinol-HCl tests for pentose were obtained.

The specific carbohydrate haptene of Mueller[29] and of Laidlaw and Dudley[30] likewise yields a pentose. The pentose in the alcohol extracts might conceivably be of nucleic acid origin. Nevertheless, finding such a carbohydrate and lipin in association in these serologically active and specific extracts suggests the possibility of harmonizing conflicting views in the literature on "lipoidal antigens." Possibly the *"lipoidal antigens"* of Meyer, Wadsworth, Maltaner, and Maltaner, Pinner, Freund, Dienes, and others and the surface lipoid material involved in the interface reaction *may be conjugated lipins owing their serological specificity to a carbohydrate haptene.* Such an

[25] Pinner, M., *Am. Rev. Tuberc.*, 1925, xii, 142.

[26] Freund, J., *Am. Rev. Tuberc.*, 1925, xii, 124 (Tables 8 to 12).

[27] Furth, J., and Aronson, J. D., *J. Immunol.*, 1927, xiii, 265.

[28] Thanks are due these workers for the material and to Professor D. W. Wilson for aid in studying it.

[29] Mueller, J. H., *Proc. Soc. Exp. Biol and Med.*, 1924–25, xxii, 209; *J. Exp. Med.*, 1926, xliii, 9.

[30] Laidlaw, P. P., and Dudley, H. W., *Brit. J. Exp. Path.*, 1925, vi, 197.

hypothesis at least is free from the *a priori* objections to lipoid antigens on the basis of their chemical simplicity.[31]

Dienes and his coworkers prepared an active alcohol extract of the tubercle bacillus. Partial separation from the lipoids increased the potency of the active part which they regarded as possibly identical with the carbohydrate haptene of Mueller and Laidlaw and Dudley.[32] Later this active non-lipoid group was interpreted as a haptene distinct from that of Mueller and Laidlaw and Dudley.[33]

It is of course well established that the tubercle bacillus contains protein antigenic material,[34] but with this we are not concerned in this study.

The Sensitizing Material.—It is common knowledge that the antibodies are intimately associated with the serum globulins and accompany the globulins through various chemical manipulations. Although considerable progress has recently been made by Felton[35] in defining the relations of antibodies to the globulin fractions, it remains uncertain still whether the antibodies are special sorts of globulins or are separate chemical substances attached to the globulins. However this may be, evidence has most recently been advanced by Shibley[36] to indicate that the binding of agglutinin by various bacteria gives them a surface coating of globulin. Shibley's figures indicate that globulin particles and bacteria which had fixed agglutinin from concentrated homologous antisera behaved identically in an electric field. That the globulin condensed on the bacterial surface is denatured is indicated both by analogy with the properties of other proteins condensed on surfaces and by the precipitation reactions of the sensitized bacteria themselves.

Through the kindness of Dr. L. D. Felton we have been able to compare the interfacial behavior of antipneumococcus globulins with the interfacial behavior of sensitized bacteria.

[31] Heidelberger, M., *Physiol. Rev.*, 1927, vii, 107.

[32] Dienes, L., and Schoenheit, E. W., *Proc. Soc. Exp. Biol. and Med.*, 1925–26, xxiii, 106.

[33] Dienes, L., and Freund, J., *J. Immunol.*, 1926, xii, 137.

[34] Wells, H. G., De Witt, L. M., and Long, E. R., Chemistry of tuberculosis, Baltimore, 1923, 73.

[35] Felton, L. D., *J. Infect. Dis.*, 1925, xxxvii, 199, 309.

[36] Shibley, G. S., *J. Exp. Med.*, 1926, xliv, 667.

A sample each of Type I, II, and III water-insoluble antipneumococcus horse serum protein which protects, agglutinates, and gives the rest of the immunological reactions,[37] was sent us by Dr. Felton. This material had been precipitated four times at the isoelectric point of the protein containing the protective substance and repeatedly washed with water.[37] As received the protein was suspended in distilled water in small aggregates of particles. The particles were about the size of bacteria and the aggregates in the dark field closely resembled small clumps of agglutinated microorganisms.

These globulins with immune properties were studied in the interface between distilled water and tricaprylin and between salt solution and tricaprylin. The saline suspension was prepared by cautiously adding 0.8 per cent sodium chloride solution to the protein in distilled water until the resulting mixture was only slightly cloudy. Much of the globulin was of course dissolved in so doing. The remaining aggregates did not scatter light so well as did the particles in distilled water; on contact with the interface they were quickly dispersed in the boundary plane; the resulting particles were visible for a moment spreading along the interface but were soon themselves dispersed until no longer visible. When a large aggregate was overtaken by a swiftly moving interface some of the protein dragged through into the oil with slight local retardation of the advancing interface.

In distilled water-tricaprylin interfaces, the globulin aggregates were in equilibrium. Large free floating aggregates were swept before the advancing oil with only that part of their surface which was contiguous to the boundary plane in the interface. Very small aggregates entered the interface and slid along it, but even with these the greater part of their mass was in the water rather than the oil. Small clumps adherent to the glass passed through the advancing interface, the latter being retarded locally; the aggregates were somewhat spread in passing through the boundary plane. Small clumps did not form peninsulas or vacuoles.

Globulin samples were denatured by bringing to a boil. The resulting soft coagula were broken up with glass rods and the flocculi studied in the distilled water-tricaprylin and saline-tricaprylin interfaces. The cohesion of the aggregates, their adhesion to the glass, and their resistance to passage into the oil were all plainly increased by denaturation. Even small aggregates were tightly stuck to the glass and passed through the interface without spreading but with formation of peninsulas and vacuoles.

Results with the globulins are summarized in Table XII.

It is to be noted that the changes in these globulins produced by heat denaturation were similar to those produced by heat injury of polymorphonuclear leucocytes and blood platelets.[2] This result is contrary to the expectation expressed in the earlier paper.

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[37] Felton, L. D., personal communication.

The behavior of denatured globulin particles in the saline-tricaprylin interface and that of maximally sensitized acid-fast bacteria in such interfaces have been closely similar. Indeed we could not detect differences. This evidence is in harmony with the conclusion proposed by Shibley on the basis of cataphoretic and precipitation data that bacteria maximally sensitized with agglutinating serum are coated with denatured globulin. Much more extensive test of this important generalization is desirable.

TABLE XII.

Interfacial Behavior of Globulins with Immune Properties.

	Cohesion of globulin	Adhesion to glass	Resistance to passage from aqueous to oil phase	Behavior of aggregates in interface
Native globulin in NaCl solution	Slight	Slight	Slight	Aggregates are dispersed along interface
Native globulin in distilled water	Moderate	Moderate	Moderate	Aggregates at oil-water boundary largely in water; are somewhat spread in passing through interface
Denatured globulin in NaCl or distilled water	Great	Great	Great	Aggregates at oil-water boundary almost entirely in aqueous phase. No spreading of aggregates by interface

The interfacial behavior of the denatured globulins in saline solution was indistinguishable from that of maximally sensitized acid-fast bacteria in saline solution.

SUMMARY.

Serum sensitization of the acid-fast bacteria causes two definite and directly observable changes in the bacterial surface:

1. A change from a surface readily wet by oil to a surface more readily wet by aqueous salt solution than by oil. This change is observed by microscopic examination of the bacteria in a saline-oil interface; thus detected, the surface alteration is said to constitute a "positive interface reaction."

2. An increased cohesiveness of the sensitized bacteria. This may be detected either by centrifuging the bacteria and then shaking up

the sediment (resuspension reaction), or by observation of the clumps in the saline-oil interface.

The interface reaction is serologically specific and confirms the existence of qualitative differences among acid-fast bacteria.

The interface reaction parallels the binding of agglutinins as detected by the resuspension reaction, but not agglutination as ordinarily tested for. The interface reaction is less sensitive,—*i.e.*, gives lower titers—than the resuspension reaction in about the average ratio of 1:3. The interface reaction in most instances runs approximately parallel to the complement fixation reaction; under at least one set of conditions, however, the interface reaction is correlated with the binding of agglutinin but not with the complement fixation reaction. How much of the bacterial surface must be covered with agglutinin in order to produce agglutination varies greatly with the bacterial strain used.

The bacterial surfaces are modified by treatment with fresh normal sera in a manner quantitatively less but qualitatively not observably different from the effects of immune sera.

Heating normal human, sheep, goat, or rabbit sera for 30 minutes at 56°C. has usually diminished but not abolished their effect on the bacterial surface. Similar inactivation of guinea pig sera left them without detectable effect on the bacterial surface.

The agglutination prezone is shown to be due to interference by excess colloidal material with the collisions of the bacteria prerequisite to clumping. The prezone may be abolished by centrifugation and resuspension of the sediment.

Antibodies may be partially dissociated from the sensitized bacteria by alkali, with return of the bacterial surface toward its normal, unsensitized condition.

A carbohydrate yielding on hydrolysis a positive pentose test has been detected in the specific alcohol extracts of acid-fast bacteria studied by Furth and Aronson.[27] The tentative suggestion is made that the alcohol-soluble antigens of acid-fast microorganisms may be conjugated lipins owing their specificity to carbohydrate haptenes.

Protective antipneumococcus globulins after heat denaturation have shown behavior in the saline-tricaprylin interface indistinguishable from that of maximally sensitized acid-fast bacteria. This strengthens the evidence suggesting that sensitized bacteria are coated with denatured globulin.