STUDIES IN THE SEROLOGY OF SYPHILIS

I. The Mechanism of the Flocculation Reactions

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In alcoholic solution, the normal tissue lipoids used as "antigen" in the serum-diagnosis of syphilis are probably molecularly dispersed. Upon dilution with water, however, there is a marked aggregation of the lipoid molecules. The originally transparent solution becomes opalescent, gives a Tyndall phenomenon, and by darkfield examination, innumerable highly refractile lipoid particles in active Brownian motion can be seen. Their average size, the number visible, and therefore the degree of opalescence, depend upon the method of admixture of the two liquids (Sachs and Rondoni, 1909).

If, to a diluted antigen in which all these microscopically visible particles are discrete, one adds syphilitic serum, there is, as Jacobsthal (1911) observed, a rapid clumping of the apparently unchanged lipoid micellae into coherent aggregates. Depending upon the concentration of the reagents, the temperature, time of incubation, etc., these aggregates may or may not exceed the limits of colloidal stability to form the optically visible "precipitate" first noted by Michaelis (1907). As he predicted, this precipitation phenomenon has found wide-spread diagnostic application. The Sachs-Georgi and Kahn precipitation tests, the Vernes flocculation reaction, the Murata test, the Sigma reaction, the Meinicke-Trübungs, Meinicke-Klärungs, and Müller-Ballungs reactions, and the Hinton agglutination reaction are all basically the same. The lipoid antigen, modified by the addition of tolu balsam, cholesterol, glycerol, NaCl, etc., forms a more or less stable "solution" (suspension) in normal serum; but in syphilitic serum there is a visible aggregation.

The causes of this aggregation and the optimal conditions for its production are the subjects of the present paper.

I. Surface Properties of the Lipoid Antigen

Colloids in general are divided more or less arbitrarily into two heterogeneous groups, between which there is no sharp distinction, but which, at the extremes, are quite characteristic (Svedberg, 1924): hydrophobic, with no affinity to water, and therefore readily flocculated by electrolytes (gold sol; Fe_2O_3 sol), and hydrophilic (proteins; glycogen).

The lipoid antigen in alcoholic solution is probably molecularly dispersed. Diluted with water, however, the molecules aggregate to form a colloidal suspension, the properties of which are intermediate between those of the hydrophobic and hydrophilic classes. The colloidally dispersed lipoid is amphoteric, flocculating at its isoelectric point, pH 1.9 (Fig. 3), but relatively stable at more alkaline reactions. Thus, at pH 6.0 (Figs. 1 and 2) it requires about 1 N concentration of univalent cations to cause aggregation of the dilute sol (0.04 per cent), and, as usually found with negatively charged particles, about 1/40 as much of bivalent cations ($BaCl_2$). The critical potential, the minimum compatible with stability, is 2 to 5 millivolts, much lower than that of most hydrophobic colloids, and strongly suggesting some other factor making for stability, analogous to hydrophilic colloids.

Protocol 1

The procedure followed in the preparation of a concentrated antigen was in essential details that used by Kahn (1925). 250 gm. of dehydrated powdered beef heart were shaken for 10 minutes with 1000 cc. ether, the filtrate discarded and the process repeated twice again with the residue. The dry powder remaining was weighed, and extracted for 3 days with 95 per cent ethyl alcohol (5 cc. per gram). Cholesterin is added to the pale yellow filtrate to any desired concentration.

5 cc. of antigen containing 1.5 per cent of beef heart lipoid and 0.2 per cent of added cholesterin were shaken with 5 cc. of NaCl N/7, and the suspension centrifuged. The sediment is shaken up in 10 cc. H_2O, forming a suspension containing approximately 0.7 per cent lipoid, varying quantities of which are dropped into electrolytes of various concentration. The pH is kept within an approximate range by phosphate buffers (final concentration M/300), the exact value being determined potentiometrically. The total volume is brought up to 4 cc. with H_2O, the tubes shaken, and flocculation read after 24 hours at room temperature. The data are summarized in Figs. 1 and 2.

One other point should be mentioned here, which will be referred to again in another connection. The concentration of electrolytes necessary to produce flocculation (coagulation value) is not a fixed quantity, an intrinsic property of the given colloid, but varies markedly with the concentration of the sol. As seen in Table I and Fig. 2, the more particles per unit volume, the less stable is the suspension. A smaller concentration of electrolyte suffices to produce aggregation; and the
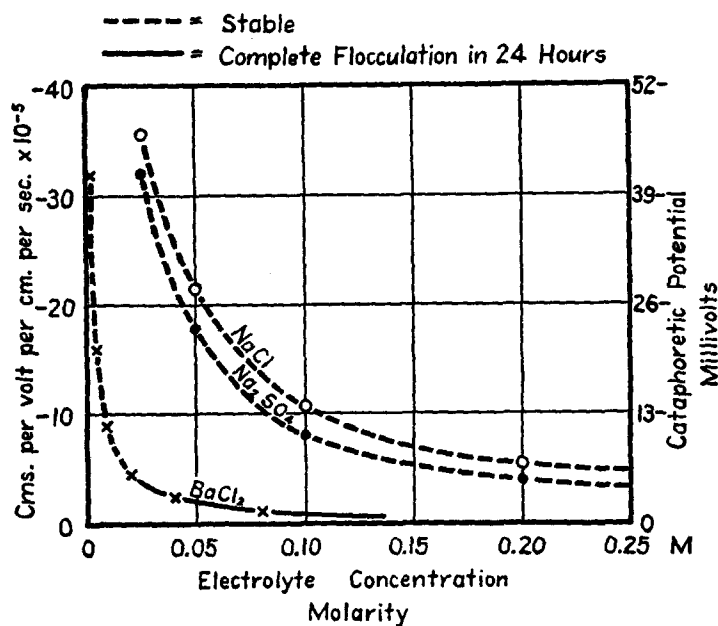

FIG. 1. Effect of electrolytes upon electrokinetic potential and stability of lipoid sol (0.04 per cent) at pH 6.0.

surface charge necessary to keep the particles dispersed and thus ensure stability becomes progressively higher. The theoretical considerations involved are discussed by Freundlich (1922).

II. The Effect of Normal Serum upon the Lipoid Antigen

Serum proteins in solution are, of course, highly hydrophilic. When adsorbed at the surface of a heterogeneous phase, however, they may act in either of two diametrically opposed senses, depending upon the

nature of the absorbent. They may retain their hydrophilic properties and form a protective film around a hydrophobic particle, preventing its flocculation by electrolytes; or they may become denatured when adsorbed, lose their hydrophilic properties, and become instead water-insoluble, hydrophobic; they then sensitize the adsorbing particle, making it even more susceptible to flocculation by electrolytes.

The nature of this denaturation is not clearly known; full discussions are to be found elsewhere (Freundlich, 1922, 1924). The point of interest is that serum globulin, when adsorbed by a colloidally dispersed heterogeneous phase, may form either a protective or sensitizing

TABLE I

Effect of Increasing Concentration of Lipoid Sol (Antigen) upon Its Stability and Coagulation Value (Fig. 1)

NaCl, normality	0.1	0.2	0.4	0.8	1.6
Concentration of lipoid in sol:					
0.002 per cent	0	0	0	0	2
0.008 per cent	0	0	0	1	2
0.032 per cent	0	0	0	3	4
0.128 per cent	1	1	3	4	4
0.512 per cent	3	3	4	4	4

Figures in body of table indicate agglutination after 24 hours.

4—complete, with clear fluid.

3, 2, 1—degrees of aggregation.

0—no optically visible aggregates.

film, depending upon the nature of the adsorbent, the determining factors being unknown.

When the lipoid antigen is added to normal serum, one can demonstrate a remarkably avid adsorption of the serum protein by the particles of the colloidally dispersed lipoid.

Protocol 2

5 cc. of antigen were shaken with 5 cc. of NaCl N/7, the suspension centrifuged to remove the alcohol, and resuspended to 10 cc. in H_2O. Series of tubes were set up similar to those outlined in Tables II and III, differing only in the concentration of normal, *i.e.*, Wassermann and Kahn negative serum. Cataphoretic velocities were measured in the simple Michaelis (1926) chamber, care being taken to make

readings one-fifth of the distance from the top and bottom of the chamber to avoid endosmotic currents, and in both directions to counteract drifts due to gravity. The values given for the velocity in the tables and in the figures were obtained by measuring the time required for the particle to pass between two points on an ocular-micrometer scale. The absolute values of this velocity of the electrokinetic potential it implies (see note to Tables II and III) have only a qualitative significance, the important factor being the isoelectric point, the hydrogen ion concentration at which the charge on the particle changes sign (equal ionization as acid and base), and at which there is therefore no movement in an electrical field.
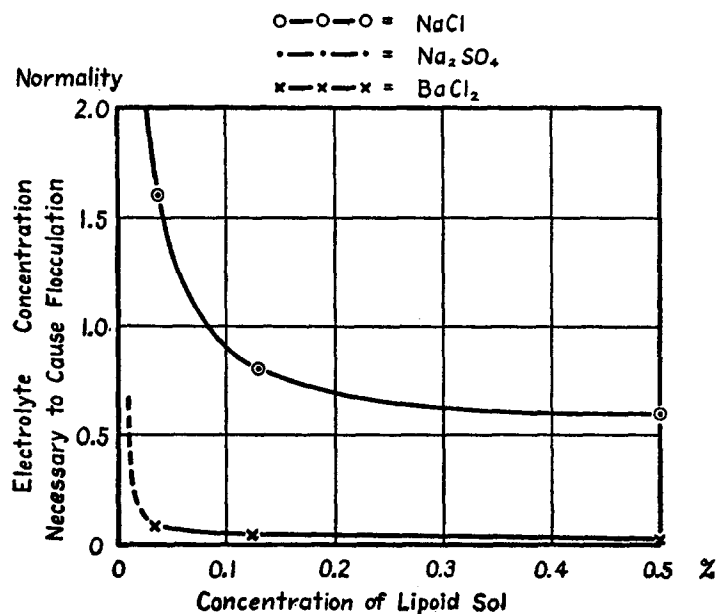

FIG. 2. Effect of concentration of lipoid upon its stability (pH 7.4).

Hydrogen ion concentrations were determined with the quinhydrone electrode, the error being ±0.05 pH.

The solid lines in all the figures indicate zones of flocculation, uniformly corresponding to zones of minimal potential; + signs indicate a particle positively charged towards the water, migrating to the cathode in an electrical field, while — signs imply a negative electrokinetic potential.

A. Isoelectric Point.—As little as 1:4000 serum (*i.e.*, 1:50,000 protein) suffices to alter the surface properties of the lipoid particles significantly. The zone of optimal flocculation, coinciding with the

cataphoretic isoelectric point, shifts from pH 1.9±, that of the lipoid antigen particles, towards a more alkaline reaction, the degree of shift depending upon the concentration of serum protein. It is significant that the maximum change is to pH 4.9, midway between the iso-electric points of serum albumen and serum globulin; the particle is then completely covered with protein, and has the same surface properties. With less serum, however, the amount of protein ad-sorbed does not suffice to cover the cell; the charge and the isoelec-
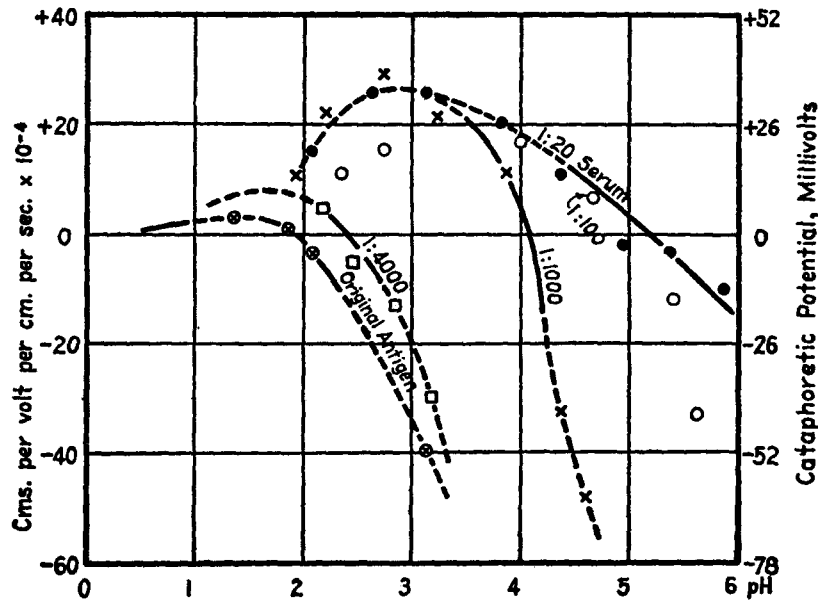

FIG. 3. Effect of normal serum upon cataphoresis of the antigen particles.

tric point, determined as they are by a mosaic of protein and lipoid, therefore have some intermediate value, the exact value of which de-pends upon the proportions of the two types of surface. This is more clearly shown in Fig. 4.

In addition to affecting the isoelectric point, this adsorbed normal serum protein changes the surface charge (and cataphoretic velocity) of the antigen at all reactions, corresponding to a change from a surface of lecithin to one of serum protein, the degree of shift again depending upon the extent of the adsorbed film.

TABLE II

Effect of pH upon Surface Properties of the Lipoid Antigen in Absence of Serum
(Fig. 3, Curve ⊗—⊗)

HCl, N/1, cc	3.8	1.0	0.25	0.062	0.05			
" N/100, cc						1.6	0.4	0.1	0.025
H₂O, cc	0	2.8	3.55	3.75	3.75	2.2	3.4	3.7	3.8
Antigen suspension, cc	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
pH	<1	<1	1.35	1.86	2.0	2.51	3.14	3.8	4.56
Cataphoretic velocity cm. per (volt per cm.) per sec. × 10⁻⁵	<1	+1	+3.6	+1.4	−3	−16	−40	>−80	>−80
Surface charge* millivolts	<+1	+1.3	+4.7	+1.8	−4	−20	−52	>−100	>−100
Agglutination in 2 hours	±	0	+	++++	+++	0	0	0	0

TABLE III

Effect of pH upon Surface Properties of the Lipoid Antigen in 1:1000 Normal Serum
(Fig. 3, x — x Curve)

HCl, N 1/10, cc	1.28	0.64								
HCl, N 1/100, cc			3.2	0.8	0.4	0.2	0.1			
HCl N 1/1000, cc								0.8	0.4	0.2
H₂O	3.2	3.86	1.3	3.7	4.1	4.3	4.4	3.7	4.1	4.3
Antigen suspension + 1:100 serum	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
pH	1.64	1.93	2.21	2.88	3.23	3.88	4.38	4.6	5.14	5.4
Cataphoretic velocity cm. per volt per cm. sec. × 10⁻⁵	+2.5	+10	+22.4	+29.1	+21.3	+10.3	−32	−48	>−80	>−80
Electrokinetic potential,* millivolts	+3	+13	+27	+38	+28	+11.4	−39	−62.4	>−100	>−100
Macroscopic agglutination in 2 hours	0	0	0	0	0	±	+++	±	0	0

+ = Positive charge; migration to cathode.
− = Negative charge; migration to anode.

* Electrokinetic potential (electrostatic units) $= \dfrac{4\pi\eta}{KX}\,u$

$$= \frac{4\pi \times \text{coefficient of viscosity}}{\text{dielectric constant} \times \text{potential gradient (E.S.U.)}} \times \text{velocity}$$

Potential (volts) $= 300\left[\dfrac{12.57 \times 0.0093\ (23°\mathrm{C.})}{80}\right] \times$ velocity per volt per cm. per sec. × 300

$= 13 \times$ velocity per volt per cm. per sec.

Protocol 3

To each of a series of tubes were added 0.2 cc. of phosphate buffer (m/15) at pH 7.4, varying quantities of serum, and H₂O to 4 cc. A similar control series was prepared, containing varying quantities of NaCl 0.85 per cent instead of serum as in the first series. A washed suspension of antigen was added to each tube and cataphoretic velocity determined. The experimental data are summarized in Fig. 5.
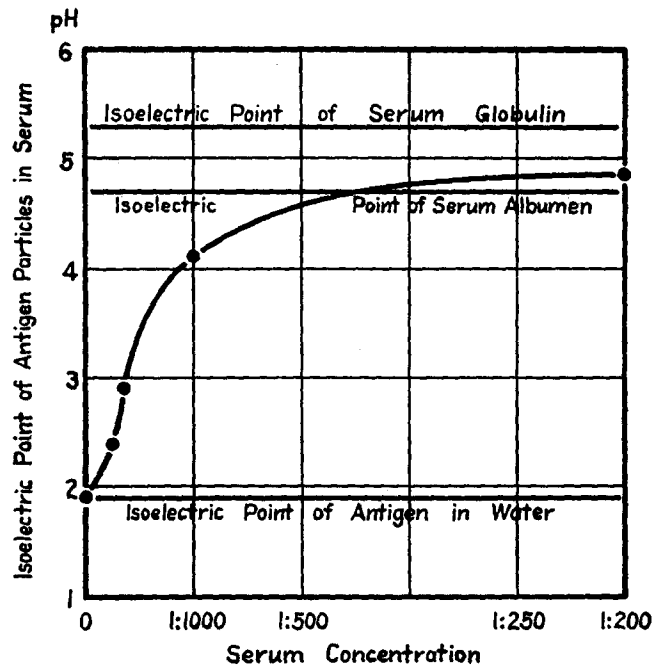

FIG. 4. Isoelectric point of antigen particles in serum is determined by adsorbed serum protein.

B. Flocculation.—Despite the fact that, as just shown, serum protein is strongly adsorbed by the lipoid antigen, there is no significant change in its stability, as determined by its tendency to flocculate. True, the isoelectric point, and therefore the range of optimum flocculation, shift towards that of the adsorbed protein: but away from this reaction the suspension is even more stable than before the addition of serum. The adsorbed protein remains hydrophilic, and acts as a film of pro-
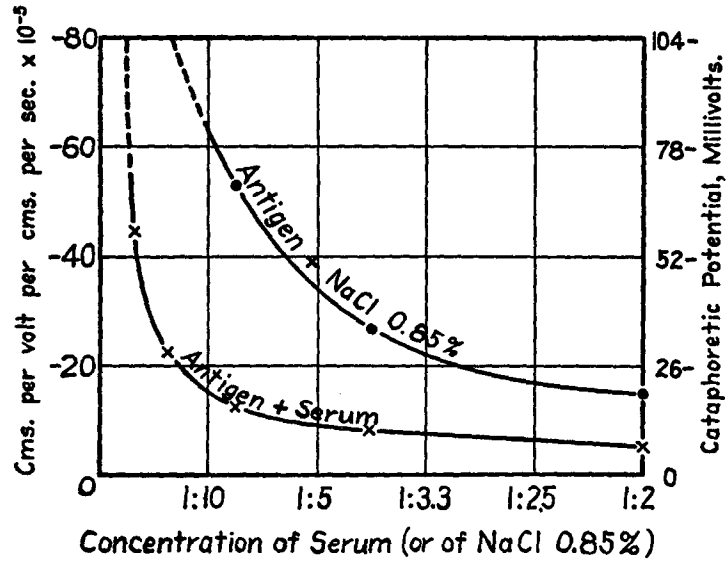
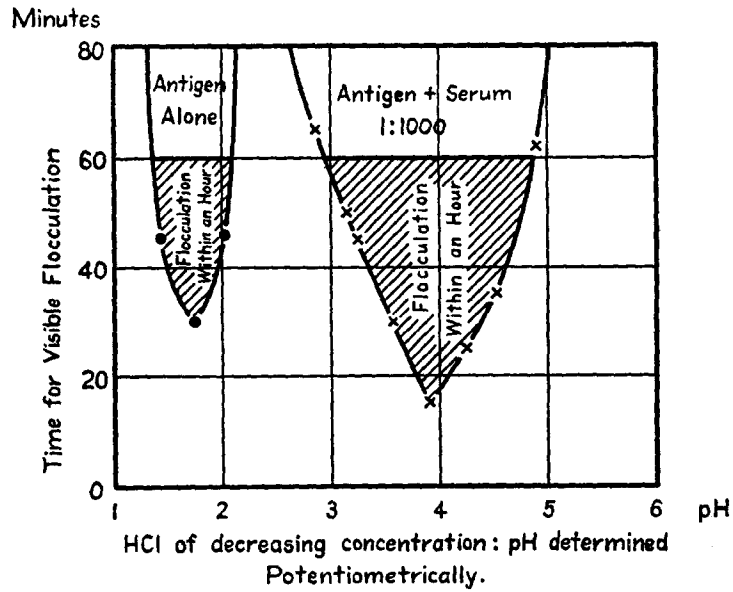FIG. 5. Effect of normal serum upon surface properties of antigen at pH 7.4.

FIG. 6. Effect of normal serum upon flocculating properties of antigen.

tective colloid, preventing even the normal flocculation of antigen at its own isoelectric point (Fig. 6): while at serum reaction (pH 7.4) just as much electrolyte is required to cause flocculation in the presence of serum as in its complete absence.

Possibly, at high electrolyte concentration ($e.g., > M/2$) the adsorption of normal serum protein is prevented, allowing the flocculation of the uncovered lipoid particle by electrolytes. At any rate, the critical potential and the coagulation value, and therefore stability, are not affected by normal serum.

To summarize, normal serum protein is strongly adsorbed by the lipoid antigen,* forming a protective film of hydrophilic protein around the constituent particles, and adding to their stability away from their isoelectric point.

III. Properties of the Lipoid in Syphilitic Serum

When the antigen is placed in syphilitic serum, there is, of course, the same non-specific adsorption of normal serum protein. In addition, however, it combines with a specific component, so-called "reagin," with a striking change in its surface properties.

A. Composition of the Lipoid-Reagin Precipitate.—
Like true antibodies, this "reagin" is always associated with the globulin fraction of serum protein (Kapsenberg, 1924) (Sahlmann, 1922) (Gloor and Klinger, 1920). Such evidence as there is to the contrary (Felke, 1921; Skrop, 1923) has been vigorously discredited by Stern (1923). *A priori,* then, the precipitate should consist of the antigen plus the reagin-globulin with which it has combined. On this point, however, there is conflicting evidence.

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* This adsorption of normal serum protein at all reactions is in itself the strongest evidence against the theory (Epstein and Paul, 1922) that syphilitic reagin is a positively charged colloid, which combines with the negatively charged lipoid particle, with mutual discharge and precipitation. There is no experimental evidence for this theory (Bauer and Nyiri, 1921), (Stern, 1923, 1924); moreover, it fails to explain why flocculation is not obtained when syphilitic serum is added to any negatively charged suspension. The fact that normal protein is adsorbed by lipoid even when both have the same charge (both negative at serum reaction) shows that one can not predicate opposite charges as the cause for the combination of lipoid with reagin, and their subsequent flocculation.

Jacobsthal (1911) considered the aggregates to the lipoidal, as did Niederhoff (1921), and Epstein and Paul (1921, 1922). Meinicke (1919), on the other hand, misled by his two-phase reaction, considered the precipitate to be entirely proteid, disregarding the fact that microscopically one can see the aggregation of the lipoid particles. Following Georgi (1919), who showed that the precipitate is not completely soluble in alcohol and ether, the exact analyses of Klostermann and Weisbach (1921) indicate that about 20 per cent of the precipitate can not be redissolved in alcohol and ether, and that of the insoluble residue at least one half (10 per cent of the total) is serum globulin; while Scheer (1921) considers the globulin to constitute 36 to 60 per cent of the total precipitate.

The following qualitative experiment shows clearly that the precipitate does consist chiefly (80 to 90 per cent) of the antigen lipoid, plus some specific component of syphilitic serum with which it has combined, a substance known to be associated with the globulin fraction of serum, giving the routine tests for protein and which can therefore be called reagin-globulin.

Protocol 4

Preparation of Large Quantities of Antigen-Reagin Precipitate.—5 cc. of the alcoholic extract containing 0.6 per cent cholesterin were mixed with 5 cc. of NaCl N/7 and the white suspension thus obtained shaken for a few minutes with 100 cc. of strongly positive syphilitic serum. After $\frac{1}{2}$ hour at 56°C. and 24 hours at ice box temperature the suspension was diluted several times with saline in order to facilitate sedimentation, and centrifuged at 2500 r.p.m. for 30 minutes. The sediment was washed twice in 250 cc. NaCl N/7, once in H_2O and finally centrifuged in a graduated tube until its volume remained constant.

Qualitative Analysis of the Precipitate.—A known volume of sediment was extracted with 15 cc. of alcohol, then with 15 cc. of ether, and finally again with 15 cc. of alcohol. The residual precipitate was centrifuged in a hematocrit tube until its volume remained constant. As seen in Table IV, from 10 to 20 per cent by volume of the antigen-reagin precipitate is insoluble in alcohol and ether.

The combined alcohol and ether extractions, representing 80 to 90 per cent of the total precipitate, when evaporated to dryness and redissolved in alcohol, form as efficient an antigen as the original cholesterinized extract; the antigenic lipoids are carried down unchanged in the precipitate. The 10 to 20 per cent residue is insoluble in water and saline; but since it contains N, and give the routine tests for protein (biuret, Millon, xanthoproteic), it must consist in part, if not wholly, of denatured serum protein.

A quantitative gravimetric analysis of the precipitate has no real

significance. The proportion of alcohol-soluble lipoids to serum glo-
bulin will depend upon the reagin-titre of the serum used and the
relative amounts of serum and antigen; moreover, such large per-
centages of protein as found by Scheer represent non-specific normal
protein, incompletely removed by washing.

Protocol 5

8 cc. of saline were shaken with 8 cc. of cholesterinized antigen, the resulting
suspension centrifuged, resuspended to 15 cc. and added with shaking to 100 cc.
of strongly positive syphilitic serum. After 24 hours in the ice box the mixture
was diluted with 300 cc. NaCl, $N/7$ (pH 7.4) and centrifuged. The sediment,

TABLE IV

Composition of Antigen-Reagin Precipitate

Antigen-reagin precipitate	Residue after extraction with alcohol and ether	Alcohol-ether soluble (volumetric)
cc.	*cc.*	*per cent*
0.2	0.038	81
0.5	0.05	90
2.0	0.28	86

taken up in 50 cc. NaCl, is Suspension 1, containing at most 1:50 parts of serum.
After 2 hours at room temperature, the suspension was again centrifuged, and
again made up to 50 cc. (Suspension 2, <1:2500 serum). The final suspension
obtained after 4 such washings represents at least $1:10^7$ serum dilution, verified
by the N content of the supernatant fluid.

The isoelectric point of each suspension was determined catophoretically and
by the zone of optimal flocculation. The experimental data are summarized in
Fig. 7, Table V corresponding to Curve 4 of the figure.

B. Effect of Washing.—

Normal serum alters the surface properties of the lipoid particles by
virtue of the protein adsorbed. The following experiment shows that
in the lipoid-reagin precipitate also, the protein detected chemically
is present as a film around the lipoid particles, but more or less irre-
versibly adsorbed, and thus similar to antibody protein bound by a
specific antigen.

Clearly, the protein taken up by the antigen from syphilitic serum
is present as an incomplete film around the individual lipoid particles,
not removed by washing. The isoelectric range of Suspension 4,

washed so thoroughly that it contains $<10^{-7}$ parts of free serum, is pH
$3.4\pm$, the same as antigen suspended in 1:2000 serum, instead of the
normal value of pH 1.9. Once the normal serum has been removed
(1 to 2 washings), repeated further washing makes no more change in
the surface properties than is indicated by the shaded zone of Fig. 7.
Assuming an approximately equal ionization of the lipoid and protein
surfaces per unit area, the shift in isoelectric point to pH 3.4 implies
a lipoid particle roughly one-half covered with protein.

TABLE V

*Effect of pH upon Surface Properties of the Lipoid-Reagin Precipitate, Washed until
Supernatant Is Practically N Free (Fig. 7, Curve □—□)*

HCl, N/10, cc..................	1	0.25					
" N/100, cc................			1.25	0.62	0.31	0.16	0.08
H₂O, cc.......................	2.8	3.55	2.55	3.2	3.5	3.65	3.70
Precipitate suspension, cc.......	0.2	0.2	0.2	0.2	0.2	0.2	0.2
pH............................	1.64	2.2	2.52	2.81	3.13	3.56	4.22
Cataphoretic velocity cm. per volt per cm. per sec. $\times 10^{-5}$...	+1.3	+10	16	+12	+10.8	−13.3	−40
Electrokinetic potential,* milli-volts......................	+1.7	+13	+20.8	+15.6	+14	−17.3	−52.4
Agglutination in 2 hours........	+	0	0	0	+++	++++	0

— = Negative charge; migration to anode.

+ = Positive charge; migration to cathode.

* See note at bottom of Table III.

Antigen therefore combines irreversibly with a specific protein of
syphilitic serum. It is significant that Otto and Winkler (1922) ar-
rived at the same conclusion by an entirely different line of investi-
gation. They found that the washed lipoid-reagin precipitate sen-
sitized 80 per cent of thirty-two guinea pigs to the subsequent injection
of human serum, proving the presence of serum protein in the precipi-
tate; while of the controls, nineteen guinea pigs injected with the lipoids
washed after immersion in negative serum, only 20 per cent showed
slight sensitivity to human serum, subsequently injected, and none
died of anaphylactic shock.*

* Similarly, agglutinated bacteria (Braun, 1909) or red cells (Altmann, 1912)
sensitize an animal to the subsequent injection of serum of the same species as the
antiserum.

There is therefore a striking analogy to the so-called specific immune reactions. Bacteria, red cells, and lipoid antigen all adsorb normal, as well as antibody protein: but while the normal serum protein, remaining hydrophilic, is readily removed by washing, the antibody globulin is firmly bound, and has been shown (chemically, immunologically, and by surface properties) to form a more or less irreversible denatured film around the antigen (Eagle, 1929, 2, 3; 1930).
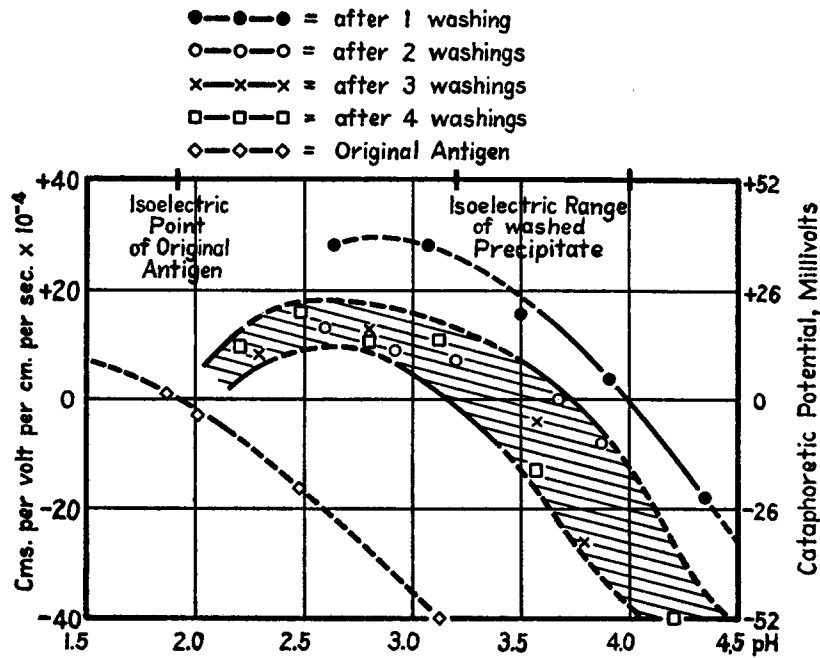

Fig. 7. Effect of washing upon the surface properties of the lipoid-reagin precipitate.

It has already been shown (Eagle, 1930) that the flocculation of bacteria or of red cells by the antiserum is due to this film of antibody protein, denatured by its combination with the antigen, and therefore sensitizing the antigen-antibody complex to flocculation by electrolytes. The obvious implication is that the flocculation of the lipoid syphilitic serum is an exactly similar process, an hypothesis which is confirmed in the following sections.

C. *Flocculating Properties of the Lipoid-Reagin Complex.*—

Normal serum protein affected only the isoelectric point and the cataphoretic potential of the antigen, without changing its critical potential. The adsorbed film retained its hydrophilic properties, and the suspension therefore remained stable up to about 1 M NaCl or 0.02 M BaCl$_2$, the exact coagulation value depending upon the concentration of lipoid.
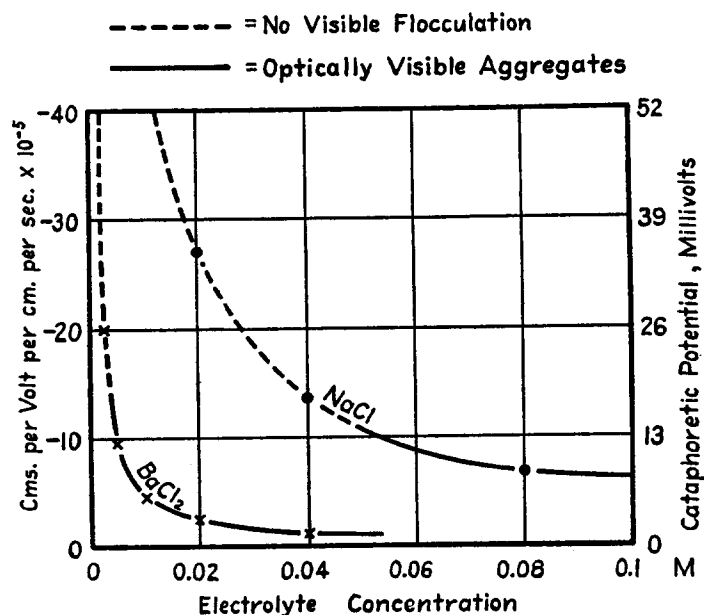

FIG. 8. Coagulation value of lipoid-reagin precipitate.

In marked contrast, the lipoid-reagin complex is flocculated at any hydrogen ion concentration by traces of electrolyte. The reagin with which the antigen particles combine not only changes their isoelectric point and lowers their cataphoretic potential exactly as does normal serum protein, but in addition, raises their critical potential from 1 to 5 millivolts to 10 to 15 millivolts (Figs. 8 and 9), an increase in cohesive tendency of approximately fivefold.

It is therefore more than a coincidence that such dissimilar particles as bacteria, red cells, protein micellae, and beef heart lecithin should

have exactly the same isoelectric point, cataphoretic potential, and in particular, the same critical potential, after sensitization with the homologous antiserum (antibacterial, hemolytic, precipitating and syphilitic respectively). In all these antigen-antibody complexes, the surface of the originally dissimilar antigen particles has been covered with an identical film of specific globulin, in some way altered by its combination so as to lose its affinity to the aqueous phase.
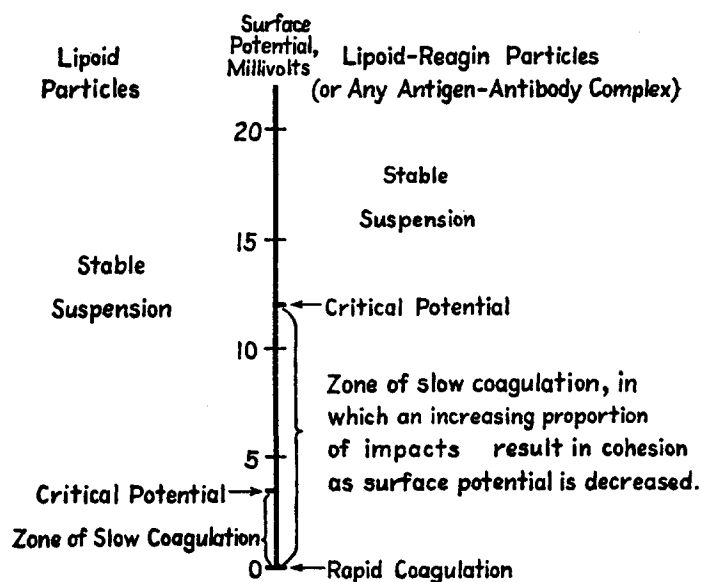
Lipoid
Particles

Surface
Potential,
Millivolts

Lipoid-Reagin Particles
(or Any Antigen-Antibody Complex)

20

Stable

Suspension

15

Stable

Suspension

Critical Potential

10

Zone of slow coagulation, in
which an increasing proportion
of impacts   result in cohesion
as surface potential is decreased.

5

Critical Potential

Zone of Slow Coagulation

0   Rapid Coagulation

FIG. 9. Contrast between surface properties of antigen particles before and after combination with reagin.

At serum reaction, it ionizes as Na^+ globulinate$^-$, negatively charged because of the greater mobility of the inorganic ion. In the absence of electrolytes, this mutually repellent surface charge suffices to prevent cohesion of the particles as they approach during Brownian movement: the suspension is stable. Upon the addition of electrolytes the charge is depressed (Fig. 8). So soon as it falls below the critical value for denatured globulin (10 to 15 millivolts), the globulin-coated particles can approach within their radius of attraction, and aggregates form which sediment. The discharging ion is the one opposite in

charge to the particle, the cation; and salts with bivalent cations, which are 20 to 40 times as effective in discharging, are correspondingly more active in causing, flocculation. Thus (Fig. 8), the coagulation value for NaCl (or Na_2SO_4) is 1/20 to 1/15 M, for BaCl M/400 to M/200; quantities to be contrasted with the corresponding values for the original antigen suspension, before it acquired its sensitizing cohesive globulin film.

At first sight, it seems paradoxical that normal serum protein, when adsorbed, forms a protective hydrophilic film, while the reagin-protein serves to decrease the stability of the lipoid with which it has combined, by forming a sensitizing film of denatured hydrophobic protein. There are, however, many examples of serum protein serving in both capacities. Aside from the specific reactions already cited there is the action of protein-containing spinal fluid upon a colloidal solution of mastic or gold: in large quantities, the fluid may protect the sol against flocculation (hydrophilic film of protein), in smaller quantities, sensitize; there is the prozone in specific agglutination; the sensitizing action of globulin on Fe_2O_3 sols, and its protective action on dyes, etc. Moreover, the forces making for the loose reversible adsorption of normal serum protein are quite different from the specific affinity between the lipoid and the reagin globulin, as shown by the irreversibility of the latter combination, a difference further illustrated in the following section.

D. Complement-Fixing Properties of the Lipoid-Reagin Precipitate.—

The loss of its affinity for water is not the only change produced in a specific antibody globulin when it combines with antigen (bacteria, red cells, or dissolved protein). In addition to becoming denatured, and thus causing the flocculation of the antigen with which it has combined, it develops a marked avidity for complement (the hemolytic substance present in fresh serum), adsorbing it irreversibly (complement fixation). When the antigen-antibody complex is heated to 100° for a few seconds, this remarkable property is completely destroyed, presumably because of the heat-coagulation of the active film of denatured antibody (Eagle, 1929, 2).

If the mechanism of the precipitation of lipoid antigen by syphilitic serum is, as outlined in the preceding sections, exactly analogous to these specific antigen-antibody reactions, the precipitate formed should

possess the same complement-fixing properties as agglutinated red cells, bacteria, or a protein-antiprotein precipitate.

Antigen alone does not fix complement; nor does an adsorbed film of normal serum protein endow it with this property. As already shown by Wassermann (1921), and Ravenel and Dulaney (1925), however, the precipitate obtained with syphilitic serum fixes complement powerfully (Table VI, Row 1).

Protocol 6

x cc. of the washed lipoid-reagin precipitate were incubated with 0.4 cc. of 1:10 complement for ½ hour at 37°, and residual complement determined by a method already described (Eagle, 1929, 1). An individual experiment is given in detail.

	0.4	0.2	0.1	0.05	0.025	0.0125	0.006
Precipitate suspension, cc.	0.4	0.2	0.1	0.05	0.025	0.0125	0.006
Time for hemolysis of sensitized cells added after ½ hour, in seconds	>1800	>1800	>1800	360	135	105	90
Per cent complement fixed	>90	>90	>90	70	40	15	<10

0.4 cc. of complement causes hemolysis in 90 seconds
0.2 " "   "   "   "   " 150 "
0.1 " "   "   "   "   " 480 "
0.05 " "   "   "   "   " 900 "

A series of experiments are summarized in Table VI, omitting similar technical details.

Even more interesting are the results in the second part of the table. A film of normal serum protein does not interfere with the specific reaction between the antigen particles and subsequently added syphilitic serum (reagin): the normal protein is only loosely bound and does not obscure the specific reacting groups of the lipoid. The lipoid-reagin precipitate, however, incubated with very strongly positive syphilitic serum, does not give any further reaction; the first incubation has covered the reacting groups of the lipoid with closely adherent reagin-globulin, so firmly bound as not to allow combination with more reagin (Row 3). Heating at 100° for a few seconds coagulates and destroys this complement-fixing film of protein without affecting the underlying lipoid. Their reacting groups once again free, the antigen particles give powerful fixation with the same syphilitic serum which was previously ineffective (Row 6).

The reagin film therefore differs from adsorbed normal protein not

only in its water-insolubility, and tendency to flocculate; but also in its ability to fix (adsorb) complement. Moreover, unlike normal protein, it attaches so firmly to the specific groups of the lipoid as to prevent any

TABLE VI

Complement-Fixing Properties of the Lipoid-Reagin Precipitate

Precipitate suspension, cc..........	0.1	0.05	0.025	0.0125	0.006	0.003	0.0015	
Per cent complement fixed by x cc. of lipoid-reagin precipitate suspension..........	>90	60	35	20	10	<10	<10	
Per cent complement fixed by x cc. suspension + 0.4 cc. of 1:40 antigen.......	>90	60	35	20	10	<10	<10	No free reagin
Per cent complement fixed by x cc. suspension, + 0.1 cc. very strongly positive serum..........	>90	70	40	25	10	<10	<10	No free antigen (at most, minimal quantities)
Per cent complement fixed by x cc. of suspension, heated to 100°C. for 1 minute..	0	0	0	0	0	0	0	Adsorbing reagin film destroyed by heat
Per cent complement fixed by x cc. heated suspension, + 0.4 cc. antigen 1:40.....	0	0	0	0	0	0	0	No free reagin
Per cent complement fixed by x cc. heated suspension, + 0.1 cc. very strongly positive syphilitic serum	>90	>90	>90	>90	>90	75	40	**Free antigen**

further combination with additional reagin. For lack of a better term, we may call this group of properties, induced in all antibodies by their combination with antigen, denaturation, remembering it to be quite distinct from the coagulation of a protein by heat.

IV. SUMMARY AND DISCUSSION

A. The lipoid antigen used in the serum diagnosis of syphilis, when colloidally dispersed in water, forms a relatively stable amphoteric suspension with predominantly hydrophilic properties. Although the colloidal particles flocculate at their isoelectric point (pH 1.9), in more alkaline reaction the negative surface potential prevents their cohesion and must be depressed to 1 to 5 millivolts before visible flocculation is obtained, indicating a very slight affinity between the colloidal particles. The amount of electrolyte necessary to depress this surface charge below its critical value decreases somewhat with increasing concentration of the sol, but is uniformly large: in a suspension containing 0.04 per cent lipoid, 1 M univalent and 1/40 M bivalent cation are the coagulation values.

B. In normal serum, hydrophilic protein is adsorbed, forming a protective film around the individual lipoid particles, with a corresponding change in the cataphoretic potential and the isoelectric point towards those of serum protein, the degree of shift depending upon the extent of the adsorbed film. The critical potential, however, is not affected, and the lipoid remains as stable away from its isoelectric point as in the absence of serum. The water-soluble film of unchanged protein is readily removed by washing, and does not prevent the subsequent combination of the underlying lipoid with the specific component of syphilitic serum.

C. When the lipoid antigen is added to syphilitic serum, in addition to this loose adsorption of normal protein it combines more or less irreversibly with a specifically altered fraction of the serum globulin (reagin), demonstrable in the washed precipitate both chemically and by sensitization experiments. Like adsorbed normal serum, it depresses the surface potential and causes a shift in the isoelectric point; but there the similarity ends. The reagin-globulin is rendered water-insoluble by its firm combination with the lipoid, exactly as any antibody is denatured upon combination with its specific antigen (bacteria, red cells, or dissolved protein). The hydrophobic films of reagin have five times as great an affinity for each other as the original lipoid surfaces; accordingly, the critical potential is raised from its original value of 1 to 5 millivolts to 10 to 15 millivolts, that of particles of de-

natured globulin or of any antigen-antibody complex, and relatively small quantities of electrolytes (at serum pH, cations) suffice to depress the stabilizing potential below this critical level, with resultant aggregation and flocculation. In brief, a specific globulin combines with the colloidal particles of the antigen, conferring upon them the unstable properties of a suspension of denatured protein.

Like the antibody film on bacteria, or red cells, and unlike normal adsorbed protein, the reagin globulin on the lipoid particle can adsorb ("fix") complement. When this protein film is destroyed by heat-coagulation, the complement-fixing property is lost; concomitantly, the specific groups of the lipoid having been freed from the closely adherent reagin, the antigen becomes again active, able to react with more syphilitic serum.

These changes in the properties of reagin globulin upon its combination with the lipoid antigen (denaturation) are in every sense analogous to those effected in any antibody by its specific antigen, and are probably due to the same, as yet unknown, factors. It has been suggested for bacterial and red cell "agglutinins" and protein "precipitins," that the groups of the antibody determining its specificity are also those which endow it with its hydrophilic properties; when these combine with antigen, residual free hydrophobic groups determine the surface properties of the complex. The same tentative hypothesis may be offered for the denaturation of reagin globulin by the lipoid antigen.

The complete analogy between the flocculation reactions for syphilis and the so-called specific reactions (bacterial and red cell agglutination; protein precipitation) suggests that like agglutinins, precipitins, etc., reagin globulin represents an antibody response to products of infection.

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