A STUDY OF THE MECHANISM OF THE AGGLUTINATION AND ABSORPTION OF AGGLUTININ REACTION, TOGETHER WITH AN EXAMINATION OF THE EFFICACY OF THESE TESTS FOR IDENTIFYING SPECIMENS OF THE MENINGOCOCCUS ISOLATED FROM 354 CASES OF CEREBRO-SPINAL FEVER.

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At the present time owing to the difficulties arising from war conditions, and the stress of work due to the special circumstances in which the study of bacteriology is being prosecuted, we have had in some cases to modify established methods of bacteriological procedure and introduce new technique.

The difficulty of obtaining an adequate supply of pure carbohydrates for performance of sugar tests and the even greater difficulty of obtaining men trained in laboratory work in bacteriology has hastened this change in general procedure to a very considerable extent. It is important that the nature—so far as this is susceptible of examination in the present state of our knowledge—and limitation of procedures which have in the past been commonly made use of, or have recently been introduced, be carefully scrutinised.

Such scrutiny would serve the useful purpose of preventing undue enthusiasm for, and its converse, unmerited condemnation of, methods which when correctly appreciated and properly carried out afford valuable information.

In the following paper the first three sections are devoted to a consideration of the mechanism and limitations of the agglutination phenomenon, and of the absorption test. The last section (4), is occupied with an analytical survey of the actual result of application of the agglutination test—checked in a large proportion of the cases by the absorption test—to over 300 specimens of meningococcus isolated from the C.S. fluid of cases and examined at the Central Laboratory, up to the present time.

In order fully to appreciate the potential errors of the agglutination phenomenon, and its amplification, the absorption of agglutinin test, in their application to the identification of micro-organisms and the establishment of the relationship which these bear to disease processes, it is essential that the factors concerned in the mechanism of these reactions should be borne in mind.

Our knowledge of these reactions has progressed considerably of recent years, and I think that we may fairly state now that the views of Ehrlich in respect of the phenomenon under consideration are no longer tenable. The same may be said of the theories advanced by Gruber and Durham, Nicolle, Paltauf and Dineur, to explain the clumping of micro-organismal suspensions when exposed to the action of corresponding anti-sera.

The view advanced by Bordet that the process of agglutination occurs in two distinct phases, viz. (1) the union of antigen with antibody, (2) the flocculation of the united antibody antigen "couple" by the electrolytes of the fluid in which the reaction is carried out, has never been controverted, but, on the contrary, has been corroborated by all the experimental work carried out since Bordet's original discovery.

I shall, therefore, discuss the problem of agglutination from the standpoint of Bordet.

In the process of agglutination three separate systems react with one another, the antigen, the antibody and menstruum in which these are suspended; and the reaction is divisible into two phases of (1) union of antigen with antibody, (2) flocculation of the antibody-antigen complex.

I. POINTS TO BE NOTED CONCERNING THE UNION OF ANTIGEN WITH ANTIBODY.

(*a*) *Influence of the reaction of the menstruum in which the reagents are suspended.*

Two of the interacting substances—the antigen and the antibody—are, so far as is known, of the nature of complex colloids. The complexity of these colloids is further enhanced by the fact that the active bodies are probably only constituents of still more complex systems. The conditions, therefore, which determine the union, or interaction, of antibody with antigen are not so far susceptible of complete investigation. Nevertheless, there is evidence that this union occurs only under certain definable conditions. Thus in the case of organisms of the

colon-typhoid group at least it will not take place if the fluid be too acid or too alkaline, i.e. the interaction of the two "active" colloids is determined to some extent by the reaction of the menstruum and it appears to take place most readily when the suspending fluid is isolectric with the mixture of colloids.

The following simple experiments illustrate this point:

To investigate the action of acid on the process of sensitisation a culture of organisms was suspended in saline with HCl present in concentration equal to N/100 and sensitised with specific agglutinating serum—titre 1/8000—in concentration of 1/100. The mixture was allowed to stand at room temperature for 48 hours, it was then centrifuged at high speed to deposit the bacteria. The supernatant fluid was drawn off and the deposit washed with distilled water. The material was washed twice in this way and then suspended in distilled water. This suspension was added to a series of tubes containing the following reagents in such concentration that, with the addition of the bacillary suspension, the concentrations shown in the following table were obtained.

TABLE I.

B. paratyphosus β and its Homologous Serum. (*HCl N*/100.)

Electrolyte	N/20	N/100	N/1000	N/10,000	N/100,000
NaCl	–	–	–	–	–
Na_2SO_4	–	–	–	–	–
$BaCl_2$	–	–	–	–	–

Note. In the above table and in all which follow,
+ = Agglutination.
– = No agglutination.
? = Doubtful result.

The tests were done using the macroscopic method, the volume in each tube being 1 c.c. In the experiments with *B. paratyphosus* the readings were made after 2 hours at 37° C., with the meningococcus, after 24 hours at 55° C.

Control experiments with a number of strains of the same and other organisms showed that when sensitisation was carried out with sera of much lower titre—1/2000—in absence of acid the organisms agglutinated readily in all cases at a concentration of NaCl equal to N/100. The converse of the experiment with NaOH gave a similar result.

If the reaction of the menstruum then be too acid or too alkaline the union of antigen with antibody is inhibited, and no complex being formed, the system is not susceptible to the flocculating action of electrolytes.

Certain colloidal complexes appear to be more susceptible than others to this disturbing influence, and on this account great care should be taken to prevent the introduction of technical errors.

(*b*) *Influence of the electrolytes of the menstruum.*

There is some evidence too (unpublished investigations on biological lysis of red blood cells deprived of their electrolytes by prolonged washing in isotonic sugar solutions) that the formation of an antibody-antigen "couple" is also conditioned by the presence of dissolved salts in the menstruum and that the result obtained depends largely on the nature of the electrolytes in the fluids employed for suspending the reagents. In these experiments it is of course essential that all the fluids used be isotonic with the red blood cells. The "inactive" diluents employed by me in these experiments are solutions of glucose or saccharose.

The work of Pauli[1] on the relation which the concentration of electrolytes bears to the coagulation temperature of protein points in the same direction, for it will be seen from the second section of the present communication that the behaviour of sensitised organisms—i.e. antibody antigen "couple"—strongly recalls the reaction of denaturated proteins, and Pauli's investigations indicate that the process of denaturation is also to some extent conditioned by the presence of salts. It is interesting to note that Pauli's work calls attention to the fact that the reaction between salt and protein in this connection is probably of the nature of a surface condensation.

(*c*) *Influence of the presumably inactive constituents of the antigen and antibody colloids upon the process of sensitisation.*

It must be clearly understood that the active substance of (or the peculiarly active physical state of) the serum, or of the organismal colloids, that leads to the initial sensitisation in the process of agglutination, does not necessarily include all the constituents of the serum or of the organism.

The (presumably) inactive substances may be present in such quantity or in such a physical state that they protect the united antibody-antigen complex from the flocculating action of electrolytes. I conceive of certain examples of the "negative phase phenomenon" in agglutination as being due to such a mechanism. The following is an example of such a negative phase reaction with the serum of an animal (rabbit) on being immunised with meningococcus, Type III.

[1] *Zeitschr. Chem. Ind. Koll.* 1908, III. 2.

TABLE II.

Serum of Rabbit "C" in course of Immunisation.

	1/50	1/100	1/200	1/400	1/800
Coccus used for immunisation	–	–	+	+	+

It is to be noted that after being stored for some time this particular serum no longer exhibited a negative phase.

(*d*) *The quantitative relationship between antigen and antibody in the process of agglutination.*

The quantitative relationship between antigen and antibody in the process of agglutination is of considerable moment, in view of the fact that upon this directly depends the validity of the absorption of agglutinin test in its application to bacteriological research.

Mainly owing to the work of Eisenberg and Volk, this relationship is now known to obey the same laws as those governing the phenomenon of adsorption or surface condensation.

When a foreign substance is introduced into a two phase-colloid-"solution" it tends according to its physico-chemical attributes to distribute itself throughout the system in a peculiar way. Often it will be found to condense itself on the surface of the disperse phase.

For example, if gum arabic be introduced into a tube containing suspended particles of finely divided barium sulphate in water (such a suspension of barium sulphate may be regarded as a suspensoid colloid admittedly of large particle, dispersed in the water) the gum condenses on the surface of the particles of barium sulphate and a complex of barium sulphate plus gum is formed. It may be noted in this instance that the gum arabic acts as a protective colloid and inhibits the sedimentation of the barium sulphate due to gravity.

The amount of foreign substance which a disperse phase can adsorb depends, *caeteris paribus*, upon the extent of the surface which it presents for the foreign substance to condense upon.

The law which governs this process is of wide application in physical chemistry and is applicable to the adsorption of hydrogen and other gases by carbon at different pressures and the adsorption of dissolved substances from various strengths of their aqueous solutions by the same reagent. The law holds good for solutions of any substance in a variety of solvents.

The amount of gas adsorbed, or the quantity of solute removed from a solution by carbon, does not increase either in proportion to the

pressure under which the reaction is carried out, in the former instance, or to the concentration of the dissolved substance in solution in the latter. The amount of gas, or material adsorbed from solution, increases much more slowly in proportion than the increase of pressure exerted, or the heightening of concentration of solute in the fluid.

In the agglutination reaction we introduce serum—a foreign substance—into a two phase system consisting of bacteria dispersed in saline. Such suspensions of bacteria have properties akin to those of non-rigid or emulsoid colloid solutions.

In many respects they recall solutions of fresh proteins which are flocculated like these by high concentrations of ammonium or magnesium sulphate. Like the proteins too, certain suspensions are flocculated by one concentration of those reagents while similar suspensions of other micro-organisms require higher concentrations of these salts to bring this about.

The foreign substance—serum—distributes itself between the saline and the bacteria and if a sufficiently large bacterial surface *of the requisite character* be available, the whole of the serum will be condensed thereon. If the surface be not sufficiently extended, a certain amount of serum will remain unattached and will be demonstrable in the fluid after removal of the antibody-antigen complex by centrifuging.

The following table from Eisenberg and Volk[1] calls attention to the adherence of this reaction to the law of adsorption as the figures of column II are in remarkable agreement with those in column III, the former of which indicate observed results while the latter give the results as calculated on the general formula of adsorption.

The figures of column I represent the amount of agglutinating serum, estimated in arbitrary units, exposed to adsorption by a given (arbitrary

TABLE III.

Column I	Column II	Column III	Column IV
Units of Agglutinin exposed to Adsorption	Units *observed* after Adsorption	Units which ought to be demonstrable after Adsorption *calculated* on "The Adsorption Formula"	Units adsorbed (*observed*) expressed a percentage of that exposed to Adsorption
2	0	0·02	100 %
20	0	0·7	100 %
40	0	2·1	100 %
200	20	19·7	90 %
400	60	52·9	85 %
2000	500	478	75 %
10000	3500	3890	65 %
20000	9000	9160	55 %

[1] *Zeitschr. f. Hyg.* 1902, XL. 155.

but constant) quantity of emulsion of the homologous organism. Those of column II indicate the potency of the demonstrable remaining agglutinin observed in the fluid after the serum and organisms have interacted. The potency of the agglutinin is expressed in terms of the same unitage as in the figures of column I. Column III indicates the potency of agglutinin which should be present after the reaction if the union of antigen and antibody were strictly amenable to the law of adsorption. Column IV indicates the quantity of agglutinin adsorbed, expressed as percentages of the amount exposed to the adsorbing action of the organismal suspension.

The above results are so striking that they scarcely call for comment, but the importance of the findings cannot be exaggerated. It is obvious that the absorption of agglutinin test is limited in its application, and that extreme care must be exercised in its technique if reliable results are to be obtained.

Attention is here called to the qualification noted in the previous paragraph, viz. that the condensing surface must be of the requisite character, that is that the suspension must be homologous with the serum to be adsorbed.

It is this which introduces that factor in the phenomena of immunology—specificity—concerning which we remain in complete ignorance.

Until the conditions which determine the specificity of serological tests are known, all the so-called immunity reactions must remain fundamentally empirical, so that at present their value as an aid to the further investigation of bacteriological problems can only be gauged by the results obtained.

I might also call attention here to the fact that in addition to the specific adsorption of an antibody to its homologous antigen, there may sometimes be some non-specific condensation of antibody upon a heterologous antigen or other disperse phase suspended in the menstruum to which the serum is added. It follows that in carrying out the absorption of agglutinin test one must bear in mind the possibility of thus introducing avoidable error; the test must be performed under such conditions that

(*a*) the serum is sufficiently dilute,

(*b*) that the emulsion of organisms is sufficiently dense to bring about complete absorption of specific antibodies, but

(*c*) that the emulsion is not too dense lest it introduce error owing to non-specific condensation of the serum.

The import of these points will again be noted in considering the

practical application of the absorption of agglutinin test, for it will be seen from the later sections of the present communication that it is necessary to elaborate a special technique for every variety of organism or rather for every group of organisms that one proposes to examine.

(*e*) *Analogies between the union of antibody with antigen and certain experiments of colloidal chemistry.*

Gengou[1] in an illuminating article on the subject of molecular adhesion calls attention to certain important points. He shows, for example, that the apparently different and opposed phenomena of dissociation and agglutination may be due to one and the same mechanism. Those colloids which are in themselves stable bring about dissociation of material suspended in fluids, while unstable colloids added to the same suspension may bring about its flocculation. This point will be further considered in the second part of this article, which deals with the purely physical factor of agglutination—i.e. precipitation.

One point of great import having its counterpart in the phenomenon of agglutination is, that when a colloid is adsorbed to a suspension of, for example, barium sulphate, there results a barium sulphate colloid complex. If the fluid containing such a complex be centrifuged, it is found that the deposit obtained consists of the whole complex and not of either constituent alone.

The supernatant fluid is inert and will no longer bring about either dispersion or flocculation, as the case may be, of further quantities of barium sulphate suspension that may subsequently be added to it, provided that the quantity of adsorbable colloid added in the first instance is not too large. The law governing adsorption in general is applicable to these complexes.

An especially interesting point may here be noted, namely, that the complex of a colloid adsorbed to a suspension may have a very different effect upon that suspension than does the colloid alone.

Of this phenomenon Gengou also cites an excellent example: gum arabic added to suspensions of barium sulphate markedly stabilises these and renders them relatively insusceptible to gravity. On the contrary, the "$BaSO_4$-gum complex," obtained by centrifuging such a mixture, brings about very rapid flocculation of further suspensions of barium sulphate in water subsequently added to it. In the first experiment, sedimentation of the barium is inhibited owing to the particles of that substance being protected one from another by a

[1] *Arch. Intern. Phys.* 1908, VII. fasc. 1 and 2.

coating of gum and, as the gum shows no tendency to flocculate, the suspension is stable. In the second experiment, on the contrary, each droplet of gum with its nucleus of barium sulphate forms a particle on the surface of which the barium sulphate which is further added will be adsorbed and condensed.

This second quota of barium sulphate is however unprotected and is therefore liable to flocculate, which it will do all the more rapidly because the particles of the system are much larger and heavier than before, and do not now show the mutual repulsion for one another exhibited by the droplet of gum.

I call attention to these points as they serve to illustrate how apparently paradoxical may be the interaction of relatively simple colloidal systems with one another, and to indicate the paramount necessity for special care being observed when carrying out tests in which the interacting systems appear to be almost infinitely complex as is the case in the immunity reactions.

(*f*) *Influence of the physical state of the reacting systems upon the union of antibody with antigen.*

In Part II of the present communication, attention is called to the fact that sensitised organisms—serum-organism "complex"—behave in certain cases as does denaturated protein. It is remarkable that in respect of agglutination, if either of the interacting colloids be denaturated by heat prior to their being mixed together, agglutination may not take place. Indeed, serum exposed to relatively high temperature may even develop inhibitory properties, thus Priestley[1] finds that if agglutinating sera be heated to certain temperatures, which vary according to the serum under investigation,—temperatures between 60° C. and 70° C. for 30 minutes—they may no longer produce agglutination of emulsions of the homologous organism in any dilution; the same serum heated to 75° C. for a similar time may become not only inactive but even inhibiting, and will interfere with the agglutinating power of the (same) unheated serum.

If it be exposed to a still higher temperature it not only fails to produce agglutination, but, further, it loses this inhibitory property.

The same is true to some extent of bacteria. Thus Eisenberg and Volk[2] find that bacteria heated above 65° C. may no longer agglutinate in presence of immune serum. This seems to be true of most organisms; it has been my experience with representatives of the colon-typhoid

[1] *Journ. Hyg.* 1917, xv. 500. [2] *Zeitschr. f. Hyg.* 1902, xl.

group of bacilli, meningococci and *B. tetani* when heated to 75°–80° C. for 30 minutes.

I conceive of this alteration in agglutinability as depending upon an alteration in the physical state of the reagents. Its import cannot be overrated for it implies that there are different optimum conditions for the demonstration of each type of reaction between antibody and antigen, so that here again the essentially empirical nature of the agglutination test is emphasised until at least these optimum conditions have been determined in respect of the particular complex that is under investigation.

What precisely happens on "overheating" an antibody or an antigen is difficult to say and in the first instance—overheating of the serum—the problem appears incapable of solution by simple experiment. Michaelis, Eisenberg and Volk, and Bail, have, however, demonstrated that it is the process of flocculation, and not that of the union of antigen with antibody which is thus inhibited.

In the latter case—"overheating" of the antigen—it seems that the modification of the antigen is such that the complex which it forms with the serum is not susceptible to flocculation by electrolytes. That the antibodies of the serum unite with the organisms is shown by the fact that they are removed by absorption with emulsions of "overheated" bacteria.

The following experiment illustrates this phenomenon in the case of the meningococcus.

An emulsion of meningococcus (a Type III coccus was used) standardised to contain four thousand million cocci per c.c., was divided into two portions, A and B.

A was exposed to a temperature of 65° C. for 30 minutes and B to a temperature of 80° C. for the same time.

Each was then used for saturating a specimen of Type III serum following the standard technique.

The following day agglutination and saturation experiments were set up with A and B, the following results being obtained:

TABLE IV.

	Unsaturated serum				Saturated serum. Coccus added was heated to 65° C. for 30 m.			
	1/100	1/200	1/300	1/400	1/100	1/200	1/300	1/400
Coccus heated for 30 m. to 65° C	+	+	+	+	–	–	–	–
Coccus heated for 30 m. to 80° C.	+	–	–	–	–	–	–	–

Note. The temperature to which the organisms must be exposed, and the time during which they are exposed thereto, varies considerably. Some organisms are more susceptible than are others to the influence of heat. This probably depends on the reaction of, and the electrolytes present in, the menstruum. Pauli's work on the coagulation of the proteins would lead one to expect such variation.

The complexity of the whole mechanism is further enhanced by the fact that agglutinogen so-called is not a definable substance. All organisms contain—in terms of the nomenclature used by Ehrlich and his followers—more than one agglutinin-producing antigen, as is shown by Joos[1]. This author shows that in the case of *B. typhosus* one of these or a group of these is thermostable and another, or others, thermolabile. Even more important than the finding of Joos are those of Scheller[2] that heated bacteria may absorb agglutinins from the sera with more avidity than do emulsions of the same organisms when they have not been heated.

The influence, then, which previous heating exercises upon organisms that are to be agglutinated is not unimportant. Heating to certain temperatures may enhance both the power which a given suspension of organisms has of combining with antibodies, and the flocculability of the complexes which the organisms form with these, while heating to 80° C. although it does not necessarily inhibit the formation of a "comples" does inhibit flocculation.

The conditions under which such heating, before exposure of the suspension to the agglutinating serum is carried out, are important. A saline suspension heated to 65° C. is agglutinable in the case of most organisms, but a similar suspension heated to 65° C. in saline containing 0·5 per cent. pure phenol may not flocculate on exposure to the same serum.

The assumption that there is a multiplicity of agglutinogenic antigens in one organism as conceived by Joos, renders the whole subject of agglutination unnecessarily complex. If it be true, it is not improbable that the optimum conditions for the flocculation of one particular antigen-antibody complex will differ from the optimum conditions for the flocculation of another, although the two antibodies are each constituents of the same serum and the two antigens constituents of the same organism.

In another connection the (assumed) "multiplicity" of so-called agglutinin-producing antigens is of importance, for the more specific response on the part of an animal to inoculation with emulsions of certain organisms occurs more readily than the less specific or group response. There is really another explanation of this which is dealt with in Part II.

This is particularly true of meningococcus and is almost equally

[1] *Centralbl. f. Bakteriol.* 1903, xxxiii.

[2] *Centralbl. f. Bakteriol.*, Orig., 1904, xxxvi. pp. 427, 694; 1905, xxxviii. p. 100.

true of *B. tetani*. For this reason in working with these organisms I invariably employ only the serum of animals whose course of immunisation has not been prolonged beyond ten days.

A similar fact, and one of considerable importance, particularly in the study of the pathogenic anaerobes, is, that if an animal be immunised with mixed cultures of certain organisms, the antibodies for one of the organisms comprised in the mixture may be demonstrable in the blood serum of the animal some days before those corresponding to the others are manifest.

It has so far been my experience—although this is admittedly limited—that the response of the animal to the more pathogenic constituent of the mixture occurs earlier and is more easily demonstrable than is the response to the less pathogenic constituent.

II. THE SECOND PHASE OF AGGLUTINATION.

The second phase of the agglutination test, that of demonstrating the formation of a complex by its flocculation, is relatively simple compared with the first phase. Experiments conducted with a view to its elucidation in the case of organisms of the colon-typhoid group show the mechanism of flocculation of sensitised bacteria in this group to be similar to that concerned in the aggregation of suspensions to certain denaturated proteins.

It must be remembered in this connection that different proteins react differently in the presence of various reagents, as is shown by Chick and Martin[1]. These authors demonstrate that the aggregation of particles of denaturated egg-white is conditioned by the presence of electrolytes and by the range of hydrogen ion concentration over which the reaction is extended.

The same is true of the aggregation of particles of denaturated serum-protein but the figures applicable to egg-white are not applicable to serum-protein, etc.

In the case of serum-protein, the flocculation of the particles occurs when electrolytes are present in low concentration, and if these be raised to certain concentrations which bring about aggregation of egg-white, no aggregation of serum-protein occurs.

In the same way it is highly probable that certain antibody-antigen complexes are much more susceptible to the flocculating, or to the dispersing, power of electrolytes than are other similar complexes—

[1] *Journ. Physiol.* 1912–1913, XLV. 261 and 295.

that is "similar" only in that they consist of organisms which have adsorbed their homologous antibodies.

Considering, then, the second phase of the reaction, one may ask the question, Why is it that a suspension of organisms remains more or less stable until the organisms are sensitised? when the organisms are sensitised, What is the mechanism which determines their clumping together in masses sufficiently large to render them susceptible to gravity?

As Liefman has shown[1], unsensitised organisms are flocculated only by the process of salting out by means of highly concentrated solutions of magnesium sulphate and ammonium sulphate and therein resemble natural proteins.

Their resemblance to fresh proteins is all the more marked in that some species of organisms are precipitated by certain concentrations of these reagents, while others require stronger solutions to bring this about.

Sensitised organisms—serum-organism complex—on the contrary, behave like denaturated proteins and, like rigid colloids, are susceptible to flocculation by low concentrations of salts.

The unsensitised organisms in a suspension are repelled by and themselves repel, those adjacent to them by virtue of their carrying a surface electric charge according to the Lippman-Helmholtz hypothesis. The conditions which bring about the neutralisation of this charge will determine the flocculation of the suspension.

The charge carried by protein particles in suspension depends in kind and in degree upon

(1) the reaction of the menstruum in which they are suspended,

(2) the electrolytes present.

In enquiring into the second phase of agglutination therefore the relation which these two factors bear to one another must be considered and the relation which each, or both, bear to the degree of sensitisation to which the organisms have been subjected. Furthermore the influence which other physical conditions may have upon the process must not be lost sight of.

(*a*) *Influence of the reaction of the suspending fluid, in which the interacting bodies are dispersed, upon the process of flocculation, and influence of the valency of the electrolytes upon the process in presence of acid and alkali.*

Considering these questions *seriatim*, it is found that the reaction of the suspending fluid exerts a marked influence on the phenomenon under

[1] *Centralbl. f. Bakteriol.*, Referate, 1913, LXXV. 14.

consideration. This would naturally be expected in view of the fact that the surface charge carried by particles of protein—organisms may be regarded as large protein particles—in suspension depends for its sign upon the acidity or alkalinity of the fluid. When the menstruum is acid the particles carry a positive charge and when alkaline, a negative charge.

The following tables illustrate experiments designed with a view to showing the influence of acid and alkali upon the process of agglutination of sensitised bacteria.

METHOD. A 24 hours' agar culture of *B. paratyphosus* β is washed off in saline and exposed at 22° C. or 37° C. for 24 hours to the action of 1/1000 anti-paratyphosus β serum of titre 1/8000.

The supernatant fluid is pipetted off and the deposit washed by centrifuging at least twice in distilled water. The deposit is then suspended in distilled water, filtered to remove any gross particles that may remain and exposed to the action of NaOH N/100 in varying concentration in presence of a number of electrolytes, each of which was, when all the reagents were mixed, equimolecular with 0·9 per cent. NaCl. The following table shows the readings of this experiment after 2 hours at 37° C.

TABLE V.

Bacillus paratyphosus and Homologous Serum.

Complex—constant. Electrolyte—constant. Hydroxide Ion—variant.
2 hours at 37° C.

Electrolyte	NO NaOH Control	1 c.c.	0·9 c.c	0·8 c.c.	0·7 c.c.	0·6 c.c.	0·5 c.c.	0·4 c.c.	0·3 c.c.	0·2 c.c.	0·1 c.c.
NaCl	+	−	−	−	−	−	−	−	−	−	+
K_2SO_4	+	−	−	−	−	−	−	−	−	−	+
KI	+	−	−	−	−	−	−	−	−	−	−
$NaHCO_3$	+	+	+	+	+	+	+	+	+	+	+
Na_2HPO_4	+	+	+	+	+	+	+	+	+	+	+

This experiment shows that the hydroxide ion interferes with the process of flocculation and that a relatively small concentration of that ion has a marked inhibitory effect. The results with $NaHCO_3$ and $NaHPO_4$ show that if replaceable hydrogen be present in the electrolyte this inhibitory effect is negatived.

The converse of this experiment is shown in the following table. The organisms were sensitised with 1/100 serum, then washed twice as before and exposed to the action of varying proportions of N/50 HCl in presence of the electrolytes named.

TABLE VI.

Bacillus paratyphosus and Homologous Serum.

Complex—constant. Electrolyte—constant. Hydrogen Ion—variant.
2 hours at 37° C.

Electrolyte N/20	1 c.c.	0·9 c.c.	0·8 c.c.	0·7 c.c.	0·6 c.c.	0·5 c.c.	0·4 c.c.	0·3 c.c.	0·2 c.c.	0·1 c.c.
NaCl	–	–	–	–	–	–	–	–	–	–
Na_2SO_4	–	–	–	–	–	–	–	–	+	+
NaCit (Neutral)	+	+	+	+	+	+	+	+	+	+
$BaCl_2$	–	–	–	–	–	–	–	–	–	–

Note. The citrate result is "falsified" in respect of the influence of valency owing to the replaceable Na of the salt.

It may be noted that the above results indicate that the inhibitory effect of hydrion is more marked in the presence of monovalent anions than in the presence of divalent anions. The reason for this will be considered later.

I have purposely omitted from this section of the work a detailed consideration of the influence of polyvalent kations or anions upon the process of flocculation, as these are somewhat difficult to investigate. The polyvalent kations have much the same influence upon unsensitised organisms as they do upon sensitised organisms, owing probably to the formation of metal hydroxides in the system in the form of precipitable gels. The polyvalent anions also present difficulties owing to their dissociation; thus, using citrates, if a neutral solution of the salt be employed the following difficulty arises, the effect of acid cannot be readily estimated owing to the presence of available hydroxide which will neutralise the acid, while the effect of alkali is also masked owing to the presence of replaceable hydrogen groups.

The mechanism whereby electrolytes bring about the precipitation of sensitised organisms is explicable on the basis that they neutralise the surface charge carried by the particles. It follows therefore that the precipitating ion in the case of acid suspensions of protein is the anion, while in that of alkaline suspensions, it is the kation. It is interesting to note that Burton[1] found that the addition of aluminium ion to hydrosols of gold or silver in certain concentrations, leads to the precipitation of the metal from its sol; lower concentrations of the Al ion did not have this effect, and the particles of the sol in the case of the metals under consideration (which bear a negative charge) continued to move in the same direction in an electric field as before *but at a diminished rate.*

[1] *Univ. Toronto Stud. Phys. Lab.* 36.

If concentrations of Al ion, greater than that required to bring about the precipitation of the sol, were employed, the particles remained dispersed, but their direction of movement in the electric field was reversed, showing that they had now become positively charged.

In the case of the sopper sols, which carry a positive charge, exactly the opposite was noted: polyvalent anions in this case lead to precipitation, and ultimately if added in sufficient concentration to dispersion, accompanied by a reversal of surface charge as evidenced by reversal of direction of movement in the electric field.

The relative precipitating value of uni-, di- and tri-valent kations in respect of negatively charged colloids and of uni-, di- and tri-valent anions in respect of those bearing a positive charge may be expressed according to Linder and Picton[1] by the formula: $1 : x : x^2$.

We have evidence that, in the case of sensitised bacilli of the colon typhoid group at least, the precipitating value of salts obeys in general the same rules in respect of valency, although the actual figures applicable to the precipitation of colloidal As_2S_3 (the reagent used by Linder and Picton) by electrolytes are not applicable to the flocculation of serum-organism complexes. These facts show that in acid suspension or in alkaline suspension, sensitised bacteria behave as do denaturated protein particles, and the results obtained agree in principle with the findings of Hardy[2] and of Chick and Martin[3].

I here recall the fact that these authors show that the precipitation of denaturated egg-white is determined by somewhat different physical conditions from those that bring about precipitation of denaturated serum-protein. It follows therefore that the optimum conditions for the flocculation of each type of antigen-antibody complex would have to be defined before the most specific agglutination results could be obtained. Here again the essentially empirical character of the agglutination test, as ordinarily performed, is manifest.

(*b*) *The relation which exists between the degree of sensitisation and the precipitating value of the electrolytes present in the menstruum.*

This point is of considerable importance especially if a proper appreciation of the absorption of agglutinin test is to be obtained. The following examples indicate that this relationship is not a simple one, and they serve to call attention to its important bearing upon the question under consideration.

[1] *Journ. Chem. Soc.* 1895, LXVI. and LXVII.
[2] *Journ. Physiol.* XXIV. 170 and *Proc. Roy. Soc.* 1900, LXVI. 101.
[3] *Journ. Physiol.* XLV. 261.

If emulsions of *B. Aertrycke* and *B. paratyphosus* β be exposed to a serum specific to either, it not infrequently happens that both organisms are equally well agglutinated. One may describe such a finding thus: that both *B. Aertrycke* and *B. paratyphosus* β form with e.g. anti-paratyphosus β serum, complexes which are flocculable by 0·9 per cent. NaCl.

It does not necessarily follow, however, that these two complexes are identical, and if one can show that the complexes differ from one another, the validity of the absorption test, in its application to the identification of micro-organisms, is much enhanced. In effect the proposition is: Does *B. paratyphosus* β form exactly similar (that is, similar from the standpoint of flocculation) complexes with its homologous serum as does *B. Aertrycke* with the same, and therefore heterologous?

A priori, one might hazard a guess that this query is to be answered in the negative; for, were it answered otherwise, the absorption of agglutinin test would be inexplicable. By varying both the dilution of a serum and the concentration of electrolytes in which an agglutination is carried out, it can be shown that a whole series of complexes, varying in their susceptibility to flocculation, may be formed when an organism is exposed to the action of its homologous serum. The following table illustrates an experiment of this kind in which both serum and electrolyte are variants while the organismal suspension is a constant.

TABLE VII.

Electrolyte = NaCl					
N/20	N/40	N/100	N/200	N/400	Dilutions of Serum
+	+	+	+	+	1/100
+	+	+	+	+	1/500
+	+	+	+	?	1/1000
+	+	+	?	–	1/2000
+	+	?	–	–	1/4000
–	–	–	–	–	1/8000
Electrolyte = $BaCl_2$					
N/400	N/500	N/666	N/1000	N/2000	
+	+	+	+	+	1/100
+	+	+	+	?	1/500
+	+	+	–	–	1/1000
+	+	–	–	–	1/2000
+	–	–	–	–	1/4000
–	–	–	–	–	1/8000

This experiment shows that an organism can form, along with its own antibody, a variety of complexes differing *inter se* in their suscepti-

bility to flocculation. These complexes cannot be regarded as different in kind, but only in degree. Is it not highly probable then that the complex formed by union of an organism with an heterologous antibody would differ markedly from that obtained by interaction with homologous antibody?

Light could be thrown on this problem by setting up an experiment in which the suspension of organisms and the serum remain constant, the only variant being the concentration of electrolytes. The following experiment illustrates this.

METHOD. Equal volumes of standard suspensions of *B. Aertrycke* and *B. paratyphosus* β were each sensitised in presence of anti-Aertrycke and anti-paratyphosus β sera. The agglutinated bacilli were washed twice in distilled water, shaken and filtered through paper to produce an homogeneous suspension which was then "dispersed" in distilled water. Thereafter each of the emulsions so obtained was exposed to the influence of varying concentrations of NaCl and of $BaCl_2$. The following results were obtained.

TABLE VIII.

1 hour at 37° C.

Serum used	Organism	Concentrations of Electrolyte, NaCl					
		N/20	N/40	N/80	N/100	N/200	N/400
Anti-Aertrycke serum ...	B. Aertrycke	+	+	+	+	–	–
Titre = 1/2000	B. paratyph. β	?	–	–	–	–	–
Anti-paratyphosus β serum	B. Aertrycke	+	+	+	–	–	–
Titre = 1/8000	B. paratyph. β	+	+	+	+	+	+

The results obtained with $BaCl_2$ are not shown in the above table. They fully corroborate those obtained with NaCl.

Note. When a simple agglutination was set up in saline with the above organisms in presence of homologous and heterologous sera, it was found that the anti-Aertrycke serum agglutinated *B. Aertrycke* and *B. paratyphosus* β equally well and *vice versa.*

The result indicates, I think, that serum and electrolyte being constant, the physical properties of what might be termed a "co-complex" differ from those of what might be called a "specific complex," the latter being more susceptible to the flocculating action of electrolytes. This result also bears a striking resemblance to Pauli's investigations noted in Section I of the influence which salts have upon the heat coagulation of protein, for it appears that the salts in Pauli's experiments may have played a double rôle

(1) by influencing the process of denaturation and

(2) by determining the occurrence of actual flocculation.

The relation which the valency of the precipitating ion of the electrolyte bears to the titre of the serum here calls for attention. If a number of electrolytes equimolecular with NaCl be used in place of that salt, it is found that none of them increase the titre of the agglutinating serum as is shown by the following table illustrating such an experiment:

TABLE IX.

Electrolyte	Dilutions of serum 1/100	1/500	1/1000	1/2000	1/4000	1/8000	1/10,000
NaCl	+	+	+	+	+	+	−
NaF	+	+	+	+	+	+	−
Na_2SO_4	+	+	+	+	+	+	−
K_2SO_4	+	+	+	+	+	+	−
Na_2CO_3	−	−	−	−	−	−	−
Na_2HPO_4	+	+	+	+	+	+	−
$BaCl_2$	+	+	+	+	+	+	−
NaCit (Neutral)	+	+	+	+	+	+	−
Aq. Dest.	+	−	−	−	−	−	−

Note. The positive result with 1/100 dilution of serum is probably due to the salts which the serum contains.

On comparing this result with that indicated in Table VII one sees that as one approaches that dilution of serum which might be called its "threshold value" the electrolyte has to be added in greatly increasing concentration, in order to bring about aggregation of the bacteria.

Thus in Table VII it is seen that $BaCl_2$ brings about flocculation in concentration—N/400 in presence of 1/4000 serum—while an increase in concentration of the same salt to N/6·5 does not show marked increase in the titre, which is apparently the same as that with NaCl 0·9 per cent.

(*c*) *Effect of other physical factors upon the second phase of agglutination.*

It might be expected that the surface tension—intrinsic pressure—and viscosity—internal friction—of the menstruum, would influence the process of flocculation because of the important rôle which these play in determining the disposition of particles in suspension. It has been found, however, that, within fairly wide limits, provided the electrostatic conditions of the particles in suspension do not show alterations concomitant with variation of these, they in themselves have but little effect in enhancing or inhibiting the aggregation of sensitised bacteria.

One point which is so obvious that it may easily be forgotten, is drawn attention to by Gengou: that if the particles deprived of their

surface charge are to come together to form flocculi, they must not be too far dispersed from one another in the suspension, for, under such circumstances their coalescence will be delayed and the initial lag in this phase of the reaction may be lengthened beyond the time arbitrarily allowed in the laboratory for the demonstration of agglutination.

Admittedly this must occur but infrequently. Nevertheless, attention is called to it because it substantiates the plea that to be of value for comparison the agglutination test must be carried out under standard conditions. One of the most easily standardised factors in the process is the bacterial content of the emulsion employed.

The temperature to which the reagents are exposed before or during the test is also of moment, for variation of this condition may well affect both phase I and phase II of the reaction.

I have already discussed how it may influence the union of antigen and antibody. Its influence on the process of flocculation is even more striking, for it may affect this in two distinct ways:

(1) by producing a continuous movement of the interacting bodies in the suspension,

(2) by encouraging or inhibiting, owing to its altering the physical state of certain complexes, their precipitation by electrolytes.

Considering these factors in more detail the following points appear:

(1) It is known that gentle shaking of an antibody-antigen complex suspended in saline will hasten in a remarkable manner the process of flocculation. This hastening of the process is probably due to the fact that shaking brings the particles of the suspension closer together and makes one particle roll over the other. As these particles are no longer mutually repellent, they tend to coalesce and having once coalesced remain together unless violently dispersed by rough handling.

If the reaction be carried out at 55° C. I have observed that the convection currents in the tubes are more active than if the test be made at 37° C. These convection currents have exactly the same effect as gentle shaking of the tubes and are not too active at this temperature to break up the flocculi. The higher temperature therefore is, *caeteris paribus*, a more satisfactory one at which to perform the test than is body heat; provided always of course that the possibility of introducing an experimental error due to this elevation of temperature is borne in mind and such error eliminated before incubation at the higher temperature is adopted as a routine procedure.

(2) The mechanism whereby the higher temperature enhances flocculation depends on the fact that certain proteins are more easily

flocculated after exposure to an increase in temperature varying according to the particular protein under examination: they are in fact altered somehow in respect of their physical properties.

Gengou, in the article already quoted, gives a striking example of this. If unheated serum be mixed with a suspension of $BaSO_4$, the serum stabilises the suspension; but if the serum be previously heated to 65° C. it induces rapid flocculation of suspensions of the same substance. In the former instance, the serum, adhering to the $BaSO_4$, and itself showing no tendency to flocculate, protects the $BaSO_4$, so increasing the stability of the suspension. In the latter instance, on the contrary, the serum does tend to flocculate of its own accord and adhering to the $BaSO_4$, which is a fairly heavy substance, remaining in suspension in water only for a limited time, and only if it be sufficiently finely divided, the union of the already flocculable protein to the heavy barium suspension will result in aggregation and sedimentation taking place with great rapidity.

One would naturally expect then that as proteins are rendered more susceptible to flocculation by elevation of temperature, that the formation of certain complexes, while not demonstrable at 37° C., might well be demonstrable at, for example, 55° C. This is particularly likely to be the case for the process of sensitisation, i.e. the formation of a complex, as we have seen, is akin to the process of denaturation.

There is an obvious criticism to the employment of the higher temperature for agglutination—Does the reaction remain specific under the new conditions brought about by the change from 37° C. to, e.g. 55° C.? The answer to such criticism is to be sought by experiment and in experience.

If a sufficient number of organisms be investigated under the new conditions, if a sufficiency of controls be included in the experiments—always especially bearing in mind the employment as a control of a normal serum of the same species of animal as that used for preparing the agglutinating serum—if the results obtained are corroborated by the absorption of agglutinin test, the new conditions may be found to be valid in that they yield specific results.

From this again, it can be seen that the test is empirical, for until we know the optimum conditions for the demonstration of agglutination in respect of these factors, the value of the agglutination and the absorption of agglutinin tests, for the identification of micro-organisms, can only be established on results obtained.

III. PRACTICAL CONSIDERATIONS AND DISCUSSION.

The points raised in Sections I and II of this communication indicate that the whole process of agglutination depends upon a number of finely balanced physico-chemical reactions. The formation of a flocculable "complex," and its sedimentation, are both governed by physical forces, and it is apparent that the fine balancing of these forces may in some instances be of paramount import if consistent results are to be obtained.

In carrying out the agglutination test and the absorption of agglutinin reaction, it is essential that those factors which are susceptible of standardisation be standardised; especially in view of the complexity of certain of the reagents employed.

(1) While serum cannot be standardised satisfactorily, as its activity can only be estimated in terms of purely arbitrary units, I think that it is advisable to employ only the serum of animals whose course of immunisation has been of short duration.

(2) To use only a serum which is found to react consistently with several representative strains of each serological type of organism which it is proposed to investigate, and

(3) To elaborate a standard routine technique and scrupulously to adhere thereto throughout each series of experiments which are to be compared one with another. The standards adopted for the examination of one group of micro-organisms may differ considerably from those adopted for the study of another group, e.g. typhoid technique differs somewhat from that used for tetanus.

The standardisation of technique involves:

(*a*) The employment of sera of approximately equal titre in all experiments dealing with allied organisms. The titre selected for one species would not necessarily be that selected for another, but in respect of all members of one group the titration of the serum to a definite point should be rigidly adhered to.

(*b*) The use of standardised suspensions of organisms of approximately definite bacterial content and always prepared in exactly the same way, both for the agglutination test and for the absorption of agglutinin test.

The need for the exercise of such care in the preparation of the suspensions is emphasised by the findings of Scheller[1] who shows that higher agglutination titres are obtained with *B. typhosus* previously heated to 65° C. than are obtained with the same organism unheated.

[1] *Centralbl. f. Bakteriol.* Orig., 1904, xxxvi. pp. 427, 694 and 1905, xxxviii. p. 100.

Further, these heated bacilli are more avid of antibodies than are the unheated organisms.

(*c*) Saline or other electrolyte employed should be made only with distilled water and, as it is easily prepared, should be made up freshly each day.

(*d*) The nature of the medium on which the cultures are made, especially the standardisation of the reaction, should be noted, and care should be taken to avoid injuring the medium. It is also advisable to get rid of condensation water before proceeding to wash off the growth. The reason for these precautions is that if the medium be alkaline there is danger that a too alkaline suspension be obtained and so inhibit the reaction. If medium, especially agar, be present in the suspension, it is conceivable that it may act as a protective colloid and so may inhibit both the union of antigen with antibody and the process of flocculation.

(*e*) I have found that, when performing the absorption of agglutinin test in the examination of meningococci, it is advisable to use freshly prepared sera as these give clearer cut results than do sera which have been in store for some months, even under the best conditions. The reason for the discrepance between freshly made sera and those that have been stored, I am not at present in a position to explain.

(*f*) If carbolised suspensions are used the strength of the phenol should not be greater than 0·5 per cent. It should only be added after the emulsion is heated—if heated emulsions are employed—and only the purest phenol can be added to suspensions if comparable results are to be obtained.

The results quoted in Sections I and II of this communication show why we are compelled to standardise the reagents and the procedure of the reactions under consideration owing to the errors which are liable to be introduced, were such not done.

If the standards just laid down be adhered to, the results obtained over long periods are found to be comparable with one another and, notwithstanding the apparent complexity of the tests, they are relatively easily carried out and give consistent results after a certain amount of experience has been obtained. Indeed when the necessary experience has been obtained, they are carried out more easily and quickly than are fermentation tests, for these call for extreme care in the isolation of cultures and in the assessment of the results given by growth in the carbohydrate media. Moreover the reactions with these may be much delayed. When a positive reaction is obtained in the case of a fermentation test one must be assured that the result is due to the organism under

examination and not to a contamination. Any one who has been engaged on the study of certain groups of bacteria and notable on a study of the anaerobes, cannot but be deeply impressed with the possibility of error due to this cause in case of inexperienced workers. On the other hand, when a negative result is obtained in the case of a carbohydrate test, one must be satisfied that not only did the inocula live in the sugar medium, but that multiplication took place. These points can only be verified by subculture and re-isolation which occupies valuable time, and the results obtained are worthless, unless the investigator be highly skilled.

It has been my experience that, when my agglutination tests have given unsatisfactory and discrepant results, some detail of my technique has been at fault and a repetition of the test with rigid attention to detail always resulted in consistent findings being obtained.

It will be noted that the question of specificity has not so far been discussed in the present communication, for reasons indicated in the introductory paragraph. I wish to point out, however, that the theory of specificity of serum reactions, which, consciously or unconsciously, dominates nearly all the writings on the subject, is the purely philosophical hypothesis of Ehrlich. This savant visualised organisms as consisting of a number of definite "antigens" certain of which are common to each member of a group of organisms and others specific to individual members (serological types) of the group, in exactly the same way that he visualised each type of antibody as having a peculiar chemical structure. Thus an "agglutinin" was diagrammatised as having a combining chemical group and a flocculating chemical group, and an "amboceptor" as having one group which combined with "antigen" and another with complement. Whenever a new phenomenon was demonstrated in serology, it was only necessary to add another side-chain, or construct a new diagram, and the new reaction was explained.

According to this view, each of the antigens of one organism is assumed to be able to call forth, when that organism is inoculated into an animal, a response specific to itself and the antibodies for each are assumed to be chemically different one from another.

Such an assumption is not by any means necessarily valid, and indeed, if we assume without question its validity, it follows that the experiment described in Section II, subsection (*c*) (Table No. VII) of this article, would indicate that the complex formed by *B. paratyphosus β* with 1/100 dilution of its homologous serum in presence of NaCl

N/400 might differ not merely in degree but in kind from the complex resulting from the union of the same quantity of the same emulsion with 1/4000 dilution of the same serum in presence of the same electrolyte, or with N/20 NaCl in presence of the lower dilution of the serum.

I conceive, therefore, of group reactions being due possibly, not to closely allied organisms having a definite group antigen common to all the members of the group, but to each member of a group of allied types or related species consisting wholly of specific antigens which however form complexes with heterologous sera more or less easily flocculated in presence of certain concentrations of certain salts, depending upon how close or how remote the relationship may be between the serum and the organism forming the complex.

To conjure up a new antigen common to all those members of a group of organisms which happen to react with one serum, and to extend the process indefinitely as has been done in order to explain why sera are not strictly specific, lays the whole subject of agglutination and other serological tests under suspicion. The same objection may justifiably be made to such reasoning concerning the multiplicity of antigens in one organism as Bordet—rightly I think—advanced in this criticism of the whole side-chain theory of immunity. Bordet says: "Everyone agrees naturally that the numerous antibodies which the study of immunity has brought to our knowledge and which are active on such different elements as bacteria, cells, toxins and the like, should not be considered as identical, inasmuch as they may be distinguished as regards specificity, or, in other words, since they unite with different antigens. But, in addition to this incontestable difference of specificity, Ehrlich has imagined another one which is more far-reaching and which deals with the molecular structure of the antibody. Indeed he classes these antibodies in accordance with their molecular structure into three genera: antitoxins, with a single combining group, agglutinins and precipitins with a single combining group and an additional functional group which brings about agglutination or precipitation; and finally sensitisers which have two combining groups in their molecules, uniting on the one hand with the cell that is affected and on the other side with the alexin (complement) and hence the name of amboceptor.

"In every instance, according to this classification, the phenomena observed are attributed to special properties in the antibody and never to those in the antigen. As a matter of fact, these phenomena should be related not as regards antigen or antibody considered separately, but as regards the complexes which result from their union, and it is

evident that the special properties of the antigen must affect markedly, and perhaps to a preponderating degree, the qualities of such complexes."

It is apparent from this quotation that Bordet's attention is focussed especially upon the antibody. But when the above was written the evidence was not clear that the same criticism could be applied to Ehrlich's conception of antigens. Indeed Bordet apparently agrees to some extent with Ehrlich's view, or at least he does not specifically refute it, that there may be a number of *definite* and therefore chemically different agglutinogenic antigens in one variety of micro-organism. In view, however, of the more recent findings in the study of the physico-chemical aspect of the subject of serology, it is quite logical to apply the same reasoning to the antigen as Bordet applies to the antibody. Further, in view of the results indicated in Section II, subsection (*b*), of this communication the application of the criticism is even wider than that indicated by Bordet in respect of antibodies, since it can be applied to the discussion of the mechanism of agglutination in the presence of heterologous serum in contrast to that in presence of homologous serum.

Regarded superficially this concept would appear to invalidate the application of the absorption of agglutinin test to the identification of micro-organisms. For, if a complex be formed, the flocculation of that complex should result in removal of antibody from the fluid, as stress has been laid upon the fact that neither organism nor serum is flocculated in the process of agglutination, but that the floccules consist of both serum and organism in some form of physico-chemical combination.

Far from invalidating the test, however, this argument could be used further to establish its value. It is known that some complexes are especially easily flocculated under certain circumstances and that in such cases only a very low concentration of antibody requires to be united with the organism in order to determine the formation of a complex flocculable in presence of e.g. 0·9 per cent. NaCl. The results obtained, performed with suspensions made in saline, indicate therefore that the degree of adsorption of antibody in the case of heterologous mixtures is much less than in that of homologous mixtures and indeed, in most cases, the adsorption which occurs with heterologous mixtures is almost negligible. This really means that phase I of the agglutination test is always specific, while phase II shows specific results only if the physical conditions of the experiment are such as to allow of the specificity of phase I being demonstrated.

IV. RESULTS OBTAINED IN APPLYING THE AGGLUTINATION AND THE ABSORPTION OF AGGLUTININ TEST TO THE INVESTIGATION OF GRAM-NEGATIVE COCCI WHICH PRODUCE PRIMARY MENINGITIS IN MAN.

In view of the fact that the validity of applying the agglutination and absorption of agglutinin tests to the demarcation of the meningococcus group of organisms, and to the differentiation of these *inter se*, may be regarded as debatable. I think it would be of some interest to give a summary of the results obtained in the examination by the methods in question of a consecutive series of this organism obtained from the cerebro-spinal canal.

We may divide those results into those which are quite specific and show that the organism under examination when tested by means of the agglutination method, using the four type sera of Gordon, react with one and with only one of these.

(*a*) *Results which are absolutely unequivocal in respect of the four type sera.*

Provided that the sera issued by the Central C.S.F. Laboratory for the investigation of epidemic meningitis among troops, were absolutely specific, and if these sera represent all the organisms which might be designated meningococci, every specimen of coccus isolated from the cerebro-spinal fluid in cases of the disease should, when tested with these sera, give a reaction corresponding to one of those symbolised as *a*, *a*1, *a*2, etc. in the following table. No specimen should be met with giving a reaction corresponding to that symbolised as *x*.

It is, however, too much to expect of this, as of any biological method of investigation, that the experimental error will be completely eliminated. It would indeed be surprising if the gram-negative diplococci which produce primary meningitis in man did not show relationship one to another when tested by biological methods.

Of the 356 cocci investigated by the agglutination test in the Central Laboratory, 234 gave absolutely specific results as follows:

(1) 54 different specimens gave a positive reaction to a dilution of 1/400 with type I serum only.

(2) 13 reacted with type I serum to a dilution of 1/200.

(3) 1 reacted with this serum to a dilution of 1/100.

(4) 80 gave unequivocal results to a dilution of 1/400 with type II serum and reacted with no other serum.

(5) 39 behaved similarly to a dilution of 1/200 of the same serum.

TABLE X.

"Formula" of Reaction	Dilutions of Type Agglutinating Sera											
	Type I			Type II			Type III			Type IV		
	1/100	1/200	1/400	1/100	1/200	1/400	1/100	1/200	1/400	1/100	1/200	1/400
a	+	+	+	−	−	−	−	−	−	−	−	−
*a*1	+	+	−	−	−	−	−	−	−	−	−	−
*a*2	+	−	−	−	−	−	−	−	−	−	−	−
b	−	−	−	+	+	+	−	−	−	−	−	−
*b*1	−	−	−	+	+	−	−	−	−	−	−	−
*b*2	−	−	−	+	−	−	−	−	−	−	−	−
c	−	−	−	−	−	−	+	+	+	−	−	−
*c*1	−	−	−	−	−	−	+	+	−	−	−	−
*c*2	−	−	−	−	−	−	+	−	−	−	−	−
d	−	−	−	−	−	−	−	−	−	+	+	+
*d*1	−	−	−	−	−	−	−	−	−	+	+	−
*d*2	−	−	−	−	−	−	−	−	−	+	−	−
X	−	−	−	−	−	−	−	−	−	−	−	−

The cultures reacting according to formulae *a*2, *b*2 and *c*2 have been classed as not agglutinating, as, without verifying such a finding by means of the Absorption Test, and thereby definitely placing such cocci, it would be inadvisable to regard them as conforming to any of the four serological types.

(6) 1 coccus reacted in presence of 1/100 dilution of type II serum.

(7) 20 cocci reacted specifically to type III serum to a dilution of 1/400.

(8) 3 reacted similarly, but to a dilution of 1/200.

(9) 16 cocci reacted specifically to type IV serum up to a dilution of 1/400.

(10) 7 gave a specific reaction with this serum to a dilution of 1/200.

That is, 234 of the 348 cocci gave unequivocal type results.

In making this total of the number of cocci which react specifically, I have excluded two strains, No. 3 and No. 6, owing to their poor agglutination.

(11) 8 strains of the organism failed to react in any way with any of the four type sera. Two of these were forwarded to the laboratory not as cultures but as suspensions.

Commenting on these results, it is remarkable, when it is borne in mind that we are dealing with what are admittedly closely-allied organisms, that so high a proportion is susceptible to classification by the test employed.

(*b*) *Results in which the reactions are not absolutely specific but show a group relationship.*

(1) Examples of what may be called the 1–3 group type and the 2–4 group type.

Most authorities on the meningococcus agree that there are two main types of organism included in the meningococcus group. By employing the absorption of agglutinin test, it has been shown that type I of the French authorities includes a small sub-group which, tested by this method, fails to absorb its antibodies from type I serum.

This small group is designated type III at the Central Laboratory and it will be seen from the results in the previous paragraph that a number of strains of this organism are differentiated by agglutination alone from the other types, including type I, by means of a specific type III serum.

In view of the relationship, we should expect that a number of organisms which react with type I serum would also react to a greater or less degree with type III serum.

In all, 63 specimens of the organisms under consideration react with both type I and type III serum. The following table indicates the degree of reaction obtained with these cocci.

TABLE XI.

"Formula" of Reaction	Dilutions of Type Agglutinating Sera											
	Type I			Type II			Type III			Type IV		
	1/100	1/200	1/400	1/100	1/200	1/400	1/100	1/200	1/400	1/100	1/200	1/400
A	+	+	+	−	−	−	+	−	−	−	−	−
B	+	+	+	−	−	−	+	+	−	−	−	−
C	+	+	−	−	−	−	+	−	−	−	−	−
D	+	+	−	−	−	−	+	+	+	−	−	−
E	+	−	−	−	−	−	+	+	+	−	−	−
F	+	−	−	−	−	−	+	+	−	−	−	−
G	+	+	+	−	−	−	+	+	+	−	−	−

(1) 31 cocci react in a manner corresponding to the formula designated A in the above diagram.

(2) 19 according to formula B.

(3) Three according to formula C.

These may be regarded in all probability as type I cocci.

(4) Three react according to formula D.

(5) Two to formula E.

(6) One to formula F.

(7) Four to formula G.

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These organisms were all proved on subsequent examination—even those reacting to formula G—by the absorption of agglutinin test to be identical with either type I or type III cocci.

As in the case of the 1–3 group a similar affinity is noted between groups 2 and 4. Type IV is not recognised by the French authors and like type III in its relation to group I is regarded by some as merely a variant of II. Using the absorption of agglutinin test, type IV can be clearly differentiated from type II. The following table illustrates the reactions obtained with specimens of cocci which show agglutination in presence of type II and type IV serum.

TABLE XII.

"Formula" of Reaction	Dilutions of Type Agglutinating Sera											
	Type I			Type II			Type III			Type IV		
	1/100	1/200	1/400	1/100	1/200	1/400	1/100	1/200	1/400	1/100	1/200	1/400
A	–	–	–	+	+	+	–	–	–	+	–	–
B	–	–	–	+	+	+	–	–	–	+	+	–
C	–	–	–	+	+	–	–	–	–	+	–	–
D	–	–	–	+	–	–	–	–	–	+	+	+
E	–	–	–	+	+	–	–	–	–	+	+	–
F	–	–	–	+	+	+	–	–	–	+	+	+

(1) 12 cocci react according to formula B.

(2) Three according to formula C.

(3) Two react to formula C.

(4) Three react to formula D.

(5) One reacts to formula E.

(6) Seven react to formula F.

In all then 28 specimens give II–IV group reactions, 17 of these are presumably type II cocci, three are presumably type IV and eight are equivocal, reacting equally well with type II and type IV sera.

The total number of organisms giving I–III and II–IV group reactions is therefore 91.

(2) There remain, therefore, 23 cocci which cannot be placed either as specifically type cocci or as cocci showing the common group relationship of I–III or II–IV.

The following table illustrates the reactions of these cocci with the four type sera.

TABLE XIII.

Register No. of Culture	Dilutions of Type Agglutinating Sera											
	Type I			Type II			Type III			Type IV		
	1/100	1/200	1/400	1/100	1/200	1/400	1/100	1/200	1/400	1/100	1/200	1/400
29	+	+	+	+	−	−	−	−	−	−	−	−
42	+	+	+	+	−	−	−	−	−	−	−	−
72	+	+	+	+	−	−	+	−	−	+	−	−
70	+	+	+	+	−	−	+	−	−	+	+	−
174	+	+	+	+	−	−	+	+	−	+	−	−
84	+	+	+	+	−	−	+	−	−	−	−	−
77	+	+	+	+	+	−	+	+	−	−	−	−
396	+	+	+	+	−	−	+	+	−	−	−	−
82	+	+	−	+	−	−	−	−	−	−	−	−
31	+	+	+	+	+	+	−	−	−	−	−	−
170	−	−	−	+	+	+	+	−	−	−	−	−
214	+	−	−	+	+	+	−	−	−	−	−	−
379	+	+	−	+	+	+	−	−	−	−	−	−
315	−	−	−	+	+	+	+	−	−	+	−	−
168	−	−	−	+	+	−	+	−	−	−	−	−
335	−	−	−	−	−	−	+	+	+	+	+	−
181	−	−	−	−	−	−	+	+	−	+	−	−
179	−	−	−	+	+	−	+	+	−	−	−	−
196	−	−	−	+	+	+	+	+	+	+	−	−
Ex. I	+	+	−	−	−	−	−	−	−	+	+	+
Ex. II	+	+	−	−	−	−	−	−	−	+	+	+
75	+	−	−	+	−	−	−	−	−	+	+	−
398	−	−	−	+	+	+	+	−	−	+	+	+

Of these cocci, therefore,

(1) Nine react in such a manner that they may be regarded as probably type I cocci.

(2) Five as probably type II.

(3) Two as probably type III.

(4) Three as probably type IV.

(5) Four are equivocal in their reactions.

These cocci have also been submitted to the absorption test and the majority of them qualify definitely as belonging to types I, II, III, or IV.

Summarising the agglutination results, then, 346 of the 356 cocci react with one or more of the type sera of Gordon. Ten specimens did not agglutinate. Included in the ten which fail to react are two which were received as suspensions and two which were ruled out owing to their reacting in presence of 1/100 dilution and no higher dilution of any serum.

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Revising the results, one may say that only eight out of 354 failed to give an agglutination—an experimental error of 2·2 per cent.

In order further to control the findings obtained by the agglutination test as applied to the investigation of spinal strains of the organism, it has now been the practice of the Central Laboratory for some time past, to verify these by means of the absorption of agglutinin reaction. The details of the technique of this reaction I shall here not discuss as this is fully described in another paper[1].

In this series of observations, 107 cocci were completely investigated, being tested each with all of the type sera. Of these 107 cocci, 101 qualified definitely as belonging to one or other of the types defined by Gordon; six could not be so placed, but of these three had died before the investigation was completed.

This enquiry brought to light an interesting point in respect of type II. Absorption showed that the sera produced by certain cocci agglutinated all the representatives of the group, but was not absorbed by contact with all of these. Other strains gave sera which were less potent in that they did not agglutinate so many strains to the full dilution of 1/400. They did, however, agglutinate both the organism used for immunising the animal and a number of other strains provisionally classed as type II to the full titre, and they were absorbed by the majority of the members of the group.

Type II appears to include a complex sub-group and shows considerable variation among the cocci comprised therein. A type II serum can be produced which is suitable for the agglutination test in that all the members of the group are agglutinated by it, but such a serum is absorbed only by a limited number of specimens of type II cocci.

The investigation, by the absorption test, of cocci provisionally classed as type II is therefore a difficult procedure and more than one serum must usually be absorbed before the organism can be definitely placed.

Ten strains provisionally classed as type II cocci and available for examination at one time, were used for absorbing three different type II sera. The results obtained in the experiment are summarised in Table IV which follows.

One of the cocci absorbed all three sera, it would be suitable for the preparation of an agglutinating serum; seven absorbed two of the sera, and two absorbed only one.

[1] The differentiation by means of the absorption of agglutinins test of the types of meningococci obtained from the cerebro-spinal fluid of cases during the current outbreak of cerebro-spinal fever. *Journ. Roy. Army Med. Corps*, July, 1917.

TABLE XIV.

Coccus		H. serum	Mi. serum	D. serum
(xxvi)	Ou.	–	+	+
(xxxi)	Wy.	–	+	+
(v)	D.	–	–	+
(xvi)	F.	+	–	–
(iv)	Ja.	–	+	+
(xxix)	New.	+	+	+
(i)	A.	+	+	P.
F^1	Har.	+	+	P.
(xxviii)	Eng.	–	+	+
(xxxii)	Clen.	–	–	+

+ = Complete or marked saturation.
– = No saturation.
P. = Result equivocal.

By employing them for immunising animals, a coccus which produces a serum similar to that designated H in the above table, one obtains a serum suitable for the agglutination of type II cocci but unsuitable for the absorption test.

In testing cultures, especially those of naso-pharyngeal origin, the least specific (i.e. most difficult to absorb) serum should be used in order that we may be assured that cultures are not being discarded because of an ultra-specificity of the serum employed.

For similar reasons when we are doing absorption with cocci provisionally classed as type II the reaction should always be performed in duplicate, (*a*) with the most "specific" and (*b*) with the least "specific" type II sera available.

CONCLUSION.

The results reviewed in this section indicate that the organisms responsible for the current outbreak of cerebro-spinal fever amongst the military forces, are comprised, with remarkably few exceptions, in the four types of meningococcus defined by Gordon.

It might conceivably be contended, however, that the agglutination test does not serve to distinguish the meningococcus from other gram-negative cocci that are not infrequently found in the naso-pharynx, and which are so like that organism both in morphological characters and cultural reactions as to be indistinguishable from it thereby.

While engaged in studying the spinal strains of the organism by means of the absorption of agglutinin test, I took the opportunity of examining a number of specimens of gram-negative cocci, obtained from

the naso-pharynx, which had been regarded as meningococci because of their agglutination reactions. In respect of agglutination and absorption of agglutinin these cocci behaved as did the strains of spinal origin.

When examining the naso-pharynx of a large number of men not infrequently there are obtained cultures of organisms closely resembling the meningococcus, which fail to react under standard conditions with any of the type sera. Are these meningococci or are they not?

The investigation of a consecutive series of 40 such cocci has been completed and it was found that none of them absorbed the agglutinin from any of the four type sera. In examining these, the standard technique was used and each coccus was tested against five sera—type I, type III, type IV and two specimens of type II—to eliminate possible error due to the complexity of type II.

Some of the pharyngeal cocci which fail to absorb the type sera may, when inoculated into animals, produce agglutinins which in high concentrations agglutinate certain strains of meningococci. This is especially liable to occur, if the process of immunisation be prolonged. It might, therefore, be suggested that such organisms could in course of time evolve into meningococci.

Up to the present, however, they have not been, in our experience, encountered invading the meninges with sufficient frequency during the current epidemic to justify their being regarded as pathogenic. It seems, I think, inadvisable—in the present state of our knowledge at least—to accept a hypothesis, based on group agglutination, that the evolution of pathogenic from non-pathogenic organisms, if it occur at all occurs sufficiently rapidly to invalidate the employment of serological methods in the identification of micro-organisms of known pathogenic significance.

Surely the most ardent evolutionary hypothesist would not insist that the old world apes must needs be endowed with the intellect and other attributes of man because the serum of those animals gives a positive reaction with anti-human precipitin?