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AGGLUTINATION STUDIES

Thesis for Degree of M. S.

Ferne F. Loomis

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THESIS

agglutination

Bacteriology

AGGLUTINATION STUDIES

Agglutination Studies

Thesis

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THESIS

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INTRODUCTION

The Agglutination test is now generally recognized as having great significance in laboratory diagnosis. Only comparatively recently has active research been conducted along this line, and when all the findings are completed the results seem astonishingly meagre as compared with the significance of the problems involved.

In general, further work on the agglutination test is justified by the fact that the subject is not complete, and that the test is widely used in the identification of diseases in man and animal. Any improvement that can be made may be of considerable value in the system now used, and further may make the test adaptable to other diseases.

In taking for the subject of this thesis "Agglutination Studies" we recognized the broadness of the field, and the necessity for concentrating on a few definite phases of the subject. Accordingly the work has been divided into the following sections:

1. Cross agglutinations of the four organisms studied, namely: Bacillus sanguinarium, Bacterium pullorum, and Bacillus typhosus, selected because of their close relationship, and Pseudomonas pyocyanea, chosen as a representative of another distinct group, totally different from the other three organisms.

2. Salt and acid agglutinations of the three organisms.

3. The effect of incubation on the hydrogen-ion concentration of the tubes.

4. The effect of the preservative phenol on the agglutination test for Bacterium pullorum. This was instigated by the report of Rebraesier (69) that pseudo-agglutinations with this organism might be avoided by the omission of phenol from the tests.

5. The effect of organic substances on the agglutination.

6. The application of the above findings in increasing the sensitivity of the antigen.

7. Routine use of the antigens prepared as above.

A. In testing cloudy sera.

B. In the standardization of B. pullorum antigen.

In this laboratory the problem of cloudy reactions has proven a distinct annoyance in the routine tests for Bacillary White diarrhea in chickens, since the standard agglutination test does not function with cloudy sera. In order to avoid cloudy sera it is necessary to starve the birds for 48 hours before bleeding. This definitely interferes with production, and is greatly objected to by the owners. Hence the introduction of a method for testing cloudy sera may be considered a distinct addition to laboratory technique, both from the standpoint of the laboratory worker, and for the convenience of chicken breeders.

The standardization of antigens is of great importance, especially in testing for B. pullorum. Hallmann (1) in conducting research over a period of two years found the variability of different strains of this organism and of the same strain at different times, to be very great. This necessarily has resulted in inaccurate tests, depending upon the particular strain used for the antigen suspension. This difficulty has been overcome, in part, by making up large quantities of anitgens from a known strain and preserving for use in the tests. However, a method of standardizing antigens against a known preserved serum, in such a way that the results would be constant and dependable, would be a decided improvement upon the present method.

REVIEW OF LITERATURE

Kolmer (2) defines agglutinins as "antibodies that possess the power of causing bacteria, red blood corpuscles, and some protozoa, suspended in a fluid to adhere and form clumps.

The first observations of the agglutination reaction were made by Metchnikoff, Charrin and Rogers (2) who in 1889 noted unusual developments of Pseudomonas pyocyaneus when grown in immune serum.. They did not recognize the importance of the reaction and went no further with the experiment.

Seven years later Gruber and Durham (2) and Bordet(2) while investigating the Pfeiffer phenomenon discovered the agglutination reaction as such and believed it to be a

particular function of immune serum. In addition, Gruber observed that absolute specificity did not hold, since agglutinins would react with antigens of allied species of bacteria.

In 1896 Pfaundler discovered the thread reaction or Pfaudler's phenomenon, in which bacteria developed long thread-like growths when grown in immune serum. At this time the reaction was considered an important diagnostic test, but was later supplanted by the agglutination test.

During the same year, Widal and Grunbaum (2)* favored the theory that agglutinins are dell receptors having a haptophore group which combines with the agglutinogen of the bacterial protoplasm and a zymophore group which is directly responsible for agglutination. Due to their complexity he designated them as receptors of the second order.

Grunbaum (2) made practical application of the agglutination test in diagnosing typhoid fever. They discovered that agglutinins for B. typhosis developed in the serum of typhoid patients in the early stages of the disease, and applied this knowledge as a diagnostic test known as the Grober-Widal reaction.

From the first there have been various theories as to the mechanism of agglutination.

Gruber (2) believed that the agglutin in acted upon the bacterial membrane increasing its viscosity which in turn caused clumping of the bacteria.

Paltauf's (2) idea was that the agglutinogen united

*Ehrlich.

with the agglutinin and was precipitated on the surface of the bacteria.

Bordet's(2) view was that of molecular physics and consisted of two phases: first, the preparation of the cells by the agglutinin which alters the molecular attraction between them and the fluid in which they are suspended, and second, produced by the addition of salt which increases the surface tension of the combined bacterium-agglutinin particles so that they form clumps, thus decreasing the surface tension.

In 1901 Joos (3) in summarizing a series of experiments with Bacillus typhosus concluded that salt was necessary for agglutination, and that in the absence of salt the serum became inactivated through the action of bacilli, without decreasing the vitality of the latter.

In 1902 Nicolle and Trenel (4) arrived at the conclusion that all free cells possessed the power to agglutinate, and sensitiveness to agglutination, particularly bacterial cells. They considered motility and the presence of the bacterial membrane to be essential factors for pronounced agglutination. At this time also, Defalle (5) stated his theory that agglutinins were formed in the organism through the action of the bacterial membranes. Hence the degree of agglutination would depend upon the development of the membranes.

Smith (4) advanced the idea that agglutination resulted from the action of salts on the precipitate present on the bacteria.

One year later, Joos (7) made the statement that the role of salt in agglutination was that of actively assisting the combination of the agglutinable and agglutinating substances. He was successful in substituting other salts for sodium chloride.

In 1903 Smith and Reagh (8) distinguished two classes of agglutinins, one acting upon flagella and the other upon the body of the bacteria and found that the flagellar agglutinins might be demonstrated with a much lower degree of immunity than those which act upon the body of the bacilli.

Upon continued research (9) these men also found certain agglutination relationships between related bacteria.

During the same year Wassermann (10) applied the knowledge of agglutinins and precipitins to vaccination and immunity.

A little later Nicolle (11) urged a uniform technique for agglutination tests. He emphasized the importance of the flagella in producing the reaction.

In a later paper Smith and Reagh (12) stated the theory that the presence of immune agglutinins produced an affinity between the motile and non-motile races, making it possible to distinguish between the flagella and body agglutinins of the motile bacilli.

In 1904 Beyer and Reagh (13) found that the application of heat would further distinguish between flagellar and body agglutinins.

Rossi (14) believed that the flagella were primarily responsible for the phenomena of agglutination, possessing great sensitivity toward the agglutinating substances of specific sera.

In 1907 Michaelis and Rona (15) found that when an indicator is used in determining the pH of a solution, the color is affected by the presence of neutral salts and the results may be wrongly interpreted due to an apparent decrease in acidity.

In 1908 Loeb (16) made a study of agglutination concluding that this phenomenon resulted from a softening of the surface of the organism and the subsequent cohesion of the particles, as differentiated from the precipitation of colloids.

Shortly after, Michaelis (17) published a contrary statement claiming that the agglutinating agent acts principally through its ability to increase the surface tension of the agglutinable material, the change in consistency being a secondary consideration.

In 1908 Bechold (18) demonstrated that the positive ion of the added salt is responsible for agglutination, and believed the variations in the effect produced by different cations to be due to four things: first, the extent of dissociation of the salt; second, the valence of the ion; third, the motility of the ion; fourth, the electro-affinity of the ion.

In criticizing Bechold's work, Buxton and Shaffer (19) showed that the dissociation of the salt is negligible giving as an example sulphates, which although always less dissociated than chlorides produce the same results. These men agreed with Weltham's theory that the coagulating powers of the ions increases geometrically with the increase in valence. They also maintained that there was little evidence in favor of the motility theory. They showed the definite coagulating powers of heavy metals, especially for normal bacteria; also that the resulting metallic hydroxide and the hydrogen ions worked in opposite directions. They observed the phenomenon of "irregular series" in which a given salt will cause coagulation in the highest concentrations, this followed by a region of no action, with a lower concentration, and at still lower concentration is again brought about, ceasing at the minimum concentration.

This is different from the anterior zone phenomenon, in which there is a certain range producing coagulation, while above and below there is no coagulation, the upper zone of coagulation being called the "anterior zone".

In comparing the value of different suspensions Buxton and Teague (20) later found a marked difference in coagulation of (1) unorganized suspensions (2) bacteria (3) immune bacteria. NaCl and $CaCl_2$ coagulate immune bacteria, but not normal bacteria. Anterior zones appear with normal bacteria and immune bacteria but not with unorganized

suspension. Coagulation begins with a lower concentration of salt with immune than with normal bacteria. $FeCl_3$ and $AlCl_3$ produce irregular series with unorganized suspensions; $FeCl_3$ with immune bacteria, and not at all with normal bacteria.

In investigating the electric charges of the above suspensions these men (21) found that each carries a negative charge in pure water but that in salt solutions the sign is reversed between the zones of no coagulation. They did not consider this neutralization of the charge responsible for coagulation.

Their conclusion was that "bacteria show no irregular series but always anterior zones unorganized suspensions the reverse". (22).

In 1910 Cornetto (23) arrived at the conclusion that NaCl acts upon the agglutinins to prevent both the receptor groups from combining, leaving the functioning group free.

While Kraus maintained that if a serum precipitated the filtrate of a given bacterial culture it would also agglutinate the homologous bacteria. Gachtgens (24) found that the precipitins appear in the serum before agglutinins and concluded that these work independently. He showed that the precipitation reaction is more dependable with the layer method than when the serum and tested solutions are mixed.

In 1913, Michaelis (25) in a series of experiments proved that proteins may be identified by means of acid

agglutination tests since the optimum concentration of hydrogen ions precipitation proteins is constant and characteristic for each protein. He showed also that bacteria may be identified in the same manner, even the most closely related species having a different hydrogen ion optimum.

This test is even more delicate than agglutination with specific antisera, since in the latter method the different strains may hinder the test, while with the acid agglutination test, the hydrogen ion concentration is alone responsible and the kind of acid used does not interfere with the test.

The practical application of Michaelis' test has proven successful. Bacillus typhosus agglutinates at a hydrogen ion concentration of 4 to 8 x 10^{-5}; Bacillus para typhosus at 16 to 132 x 10^{-5}. Bacillus Coli is not agglutinated by acids.

Later in the same year Beniask (25) corrorborated Michaelis' experiments. His conclusions were as follows: "Neither the acid, as such, nor the anion, is responsible for the agglutination, but only the hydrogen ions. This acid agglutination is produced only by hydrogen ion concentration within certain definite limits, characteristic for each species of bacteria. There is also an optimun concentration constant for each species". This method has been found of particular importance in differentiating the members of the colon group of bacteria.

Still later Poppe (27) partly substantiated Michaelis' work, but was unable to differentiate the members of a

group, and at about the same time Pauli and Klecker (28) made a study of the relation of proteins to inorganic colloids and the salts of heavy metals. They divided the colloids into two classes and called the first group suspensoids, while the second were named lyocolloids. The latter show greater viscosity and stability than the suspensoids, which are very sensitive toward electrolytes and become less stable with age. Copper sulphate is an example of the suspensoids and ferric hydroxide of the lyocolloids. In the precipitation of proteins an excess of the suspensoids does not interfere with precipitation, while the addition of electrolytes hinders the reaction. Lyocolloids, on the contrary, if used in excess may completely inhibit precipitation. The addition of salts has a favorable effect. Hydroxyl ion assists in the precipitation with positive lyocolloids, hydrogen ions with the negative ones. The precipitation of excessive amounts of protein, by either the suspensoids or lyocolloids, is hindered by electrolytes, only a small fraction of the protein being precipitated.

These men also noted peculiarities in the behavior of salts of heavy metals when used with solutions of electrolyte-free proteins. When very small amounts of the salts are used precipitation results, more concentrated solutions dissolve the precipitate and still larger amounts of salts again cause precipitation. The following salts followed this rule, ferric chloride, copper sulphate, Mercuric chloride and zinc sulphate. The explanation offered is that in the dilute solutions,

hydrolysis of the salts permits th formation of insoluble compounds with the proteins. These compounds form the precipitate. When certain concentrations of the salt is reached the hydroxy-protein compound is replaced by an ionized salt-protein compound and no precipitate is visible. A concentrated salt solution depresses the ionization, which in turn forces a precipitation of the undissociated compound.

Two years later, Michaelis and Pechstein (29) were able to determine the iso-electric point of casein.

A little later, Michaelis and Adler (30) reported that "The acid agglutination optimum for Bacillus typhosus agglutinated with hydrochloric acid is at the same hydrogen ion concentration as with acetic or lactic acids."

In 1912 Dean (31) published a paper on agglutination drawing the following conclusions: An agglutinating serum contains two essential factors, these being first, the specific antibody, and second, a non-specific substance which may be serum globulin. The interaction of antigen with antibody causes an aggregation of the molecules of the globulin, resulting in turbidity. If the antiserum is greatly diluted it may still contain an adequate amount of the specific antibody , while the globulin, or non-specific substance, becomes insufficient. This may be remedied by the addition of globulin from normal serum.

Stuber (32) advanced the theory that agglutination is caused by the fatty substance within the bacteria. He considered these substances to be true agglutinogens,

which by stimulation of the sympathetic nervous system lead to the formation of agglutinins. The specificity of these fatty substances is only relative.

In 1917 Garrow (33) made a comparison of the microscopic and macroscopic methods of agglutination. He found the macroscopic method to be thirty times as sensitive as the microscopic method, due to the different conditions under which the tests are conducted namely: The time limit for the microscopic test is much less than for the macroscopic; the microscopic test is conducted at room temperature and the macroscopic may be incubated in a water bath at a higher temperature. In the latter method convection currents aid in the union of antigen and agglutinin.

In 1918 Topley (34) made a definite study of the effect of convection currents on the rapidity of agglutination, and found a marked difference especially with the antigens which showed relatively poor agglutination. He also noted more rapid flocculation in tubes in which the fluid contents were only partly submerged in the water bath.

In 1918 Sierkowskie (35) demonstrated the possibility of obtaining an increase in the agglutination titer with Bacillus typhosus and the vibrio of cholera, by a simultaneous increase in the amount of serum and suspension of three to ten times, in such a way that the degree of the dilution of serum and other constituents remained constant. He obtained a further increase in titer by adding small amounts of five to ten per cent acetic acid, the action

being depressed by a stronger concentration. He also found that the titer might be increased, especially with B. typhosus, by cultivation on media which is weakly acid or weakly alkaline, or by warming the bacterial suspension to 56 degrees, but not over 64 degrees.

During the same year, Bauer (36) experimented with fatty acids and soaps, concluding that oleric, stearic, and palmitic acids have the greatest effect on agglutination, while soaps and isohydric mineral acids have weaker action. The higher fatty acids were emulsified with sodium cholate in non-agglutinable concentrations before use in the tests.

In 1918 Nicolle, Jouan and Debains (37) were able to produce agglutination by treating gonococcus serums, which otherwise failed to agglutinate, with normal HCl, heating in boiling water, cooling them, and neutralizing with NaOH. Several races of pneumococci were likewise treated and gave similar tests. The above results were due, according to Porges, to hydrolysis of the mucous capsule.

Radsma (38) produced agglutination of red blood corpuscles, suspended in a large amount of isotonic solution of dextrose, by the addition of 0.04 % sodium chloride, and believed that some hydrophil colloid played a part in maintaining the stability of a suspension of corpuscles.

Also in 1918, by means of cross agglutination experiments with anti-typhoid, anti-paratyphosus A, anti-paratyphosus B, and anti cholera sera of high titre,

Caprone (39) demonstrated that homologous species are agglutinated to the same degree either in saline suspension or a growing broth culture, but that heterologous series show agglutination in higher dilutions in the broth cultures, especially before the period of heavy growth.

In 1.19 Buchanan (40) made the statement "Bacterial agglutination is a colloidal phenomenon best studied in the light of colloidal and physical chemistry".

During this same year Bergel (41) studying the mechanism of hemagglutination and hemolysis decided that the lipoid of the red cells is the true antigen possessing the haptophase groups and that the receptor with which it combines is a non-specific lipase, a constituent of the mono-nuclear white cells. His explanation is that a specific immune body is formed which remaines attached to the cell for a time and is then set free. He considered this immune body to be a zymogen, which, activated by the complement already present, attaches itself to the lipoid part of the homologous red cells.

At about the same time, Mansfield (42) presented a physiological explanation of agglutination. He claimed that bacteria are maintained in a permanent suspension through the action of a protective colloid, and that agglutination is produced by the action of a digestive ferment which acts upon the colloidal substance. He offered as proof, the fact that there is a direct proportion between the concentration of the agglutinin

and the speed of the reaction, as is also true of other ferments. The temperature curve conforms to the same rule. He considered the presence of sodium chloride important to the reaction.

During the years 1921 and 1922, numerous findings were made. In 1921 Lanelli (43) offered the theory that the passage of an electric current through agglutinating sera would result in the division of the agglutinins into two parts, each of which had no agglutinating powers. The agglutinability was restored when the two portions were again combined.

Also in 1921 Barratt (44) offered an equation for estimating the action between agglutinin and antigen, $y\,t = m$, where y = the concentration of the agglutinin, t = the agglutination period, and m = a constant.

Later in the same year, Hall and Tinsley (45) made the statement, "Changes in agglutinability of organisms are associated with variations in culture media".

In 1921 Gates (46) was able to produce agglutination mechanically by the use of the centrifuge, the results coinciding with those obtained with the standard test. The advantage is the elimination of the inconstant time factor.

At about the same time Schachenmeier (47) was able to demonstrate the use of purified bacterial fats as agglutinogens, with only relative specificity.

Soon after, Fabry (48) was able to show that by attenuating microorganisms (B. typhosis) by growing them

on media to which was added increasing amounts of phenol, the agglutinating powers became greater as the strength of the organism lessened.

During the same year, Ichii (49) recorded his experiments with the colon-typhoid group of bacilli. He found that acid culture media favored agglutination while alkaline media retards. In using formalin he found the most favorable concentration to be 0.01 %, 0.05 % to 0.2% showed a retarding action on agglutination. Traces of sodium chloride were sufficient for agglutination, while the addition of 0.1 % phenol, or 0.01 % mercuric chloride had no effect. He considered 37° the optimum temperature.

Soon after Hersch (50) in substituting immune serum for a fraction of the saline used in regular tests, found a resulting increase in aldalinity which he believed to be due to the differences in the dissociation constants of the reacting substances and their products.

Gunn (51) was able to produce agglutination of cholesteral suspensions with ricin. He considered this to be a non-specific action due to the precipitation of one colloid by another of the opposite sign.

Von Szent Gyorgi (52) classified pathogenic microorganisms into tow groups. In the first group he placed those organisms which are concerned with local disease processes, and produce no immunity, as Bacillus pestes, Bacillus mallei, and Bacillus anthrasis. These organisms have a tendency to flocculate when grown in fluid

medium, and give a weak agglutination reaction. The second group comproses those organisms causing acute diseases resulting in immunity, as Bacillus typhosus and vibrio cholerae. These remain in suspension when cultivated in fluid media and give a strong agglutination reaction.

In 1922 Northrup and DeKruif (53) in studying the agglutination by electrolytes found that electrolytes in low concentration affect the potential primarily while higher concentrations decrease the attractive force between the organisms.

In a similar report (54) the same men, experimenting with agglutination in the presence of proteins normal serum and immune serum, found that when proteins or serum were added to bacterial suspensions, the zone of acid agglutination broadened, and the iso electric point shifted to that of the added substance. Proteins used were egg albumen and globulen.

At the same time, Eggerth and Bellows (55) obtained practically the same results using egg albumin, gelatin, protalbumen, hemoglobin, edestin, and heteralbuminose, adding them to suspension of B. coli. They found that at reactions more acid than the iso electric point the bacteria had a positive charge and failed to flocculate. This lessened stability was also shown in suspensions of sellusose, nitrate, cellulose acetate and oil emulsions when similarly treated with proteins.

Northrup and DeKruif (56) upon further study with Bacillus typhosus with regard to the concentrations of

various salts required to produce agglutination, found it convenient to divide electrolytes into two classes. The first calss comprising those in which the agglutinating concentration is not influenced by the concentration of the suspension, and which do not reversethe sign of the charge. Class two includes those whose agglutinating concentration is increased with an increased turbidity of the suspension. These with the exception of zinc sulphate reverse the charge. To quote from the paper, "These results are contrary to the idea that the combination is caused by a difference in the sign of the charge carried by the immune body and the organism. They agree with the assumption that the immune body forms a film on the surface of the organism so that the effect of the charge is the result of this film.

Wolf (57) advanced the revolutionary theory that antigens are fatty in nature.

DeKruif and Northrup (58) found that by repeated washing with distilled water, they were able to completely remove the immune body from sensitized bacteria. Contrary to their original belief this removal was as complete at pH_7 as at pH_3.

Hohn (59) in 1923 studied the effect of culture media upon the agglutinability of B. typhosus. He found that when grown on clear agar the bacilli loose the power to agglutinate much more readily than when the medium has not been cleared or when mannitol, galactose

or dextrose are added to the cleared agar. 0.1 % galactose is especially valuable for use with cleared media.

In 1922 Shionoya (60) investigated the effect of ions on agglutination, and found that as the valency of the cation increased, the limiting concentration at which agglutination would occur became lessened, the relation being such that the curve of the valencies and limiting concentrations equalled a straight line.

During the same year, Yamaguchi (61) reported that the increased viscosity resulting from heating emulsions of Bacillus typhosus lessened the agglutinability. These could be restored to almost the original viscosity and agglutinability by the addition of acids. He also (62) treated emulsions of the same bacillus with a 1.0% solution of KOH. When neutralized, these suspensions showed a lessened titre with serum immunized with live cultures but no change in agglutinability was noted when serums from animals immunized with killed cultures of the organism were used.

Also in 1923 Hine (63) found that the agglutinin-antigen complex became dissociated upon standing at room temperature. When normal serum was substituted for immune serum only partial dissociation occured. The organisms used were staphlococci.

In the same year Krumwiede, Cooper and Provost (64) stated that " the absorptionof specific agglutinins must be used to determine agglutinative identity.

Evans and Small (65) found that serum dilutions of Bacillus influenzae were made in beef infusion broth at pH 7.6 instead of normal salt solution the results were more satisfactory especially with the less sensitive antigens.

In 1924 Ozata (66) discovered that warm alkali with a concentration of .01 normal might be used in the separation of antigen and agglutinin, 42° being the favored temperature. He found a 10 % sucorose solution more favorable than a solution of NaCl.

Landsteiner (67) and Sander Scheer, also in 1924 by means of partial absorption methods were successful in differentiating the blood of different species, even when the precipitins were much alike. They inclined to the belief that the essential difference between agglutinins and precipitins is a chemical one which determines the specificity of antigens.

Jacobitz (68) differentiated between artificial acid agglutination used by Michaelis, and biological acid agglutination induced by the acid formed by the bacteria during their growth in sugar solutions. When the former method is used the bacteria die quickly while in biological acid agglutination they may live for a long while.

In 1926 Rebrassier (69) in studying the effect of phenol in the pseudo-agglutination found that by omitting phenol from the antigens, this trouble could be practically eliminated.

TECHNIC EMPLOYED

The sera were obtained from four rabbits which had been previously injected with suspensions of killed cultures of Bacillus sanguinarium, Bacillus pullorum, Bacillus typhosus, and Pseudomonas pyocyanea respectively. The organisms were cultivated for twnety-four hours on liver agar slants, the growth washed off with physiological salt solution, the suspension diluted to correspond with tube 1 of McFarland's nephelometer and heated in a water bath at 60° C. for one half hour before use. These suspensions were then kept at 10°C.and injected intraperitoneally in one half c.c. amounts at intervals of three days, for a period of four weeks. At this time the rabbits were bled from the ear and the sera tested against the respective antigens. Since each serum gave a positive test at a concentration of 1:640, the titre was considered to be sufficiently high for study. The rabbits were bled from the heart and the sera obtained were preserved at 10° C. for future use.

CROSS AGGLUTINATION STUDIES

As a preliminary to the agglutination studies, cross agglutinations of the four organisms selected for study were made. These cross agglutinations were conducted with the usual antigen prepared by washing the growth from liver agar cultures, incubated at 37° for

TABLE I CROSS AGGLUTINATIONS

Serum dilutions	1- 20	1- 40	1 - 80	1 - 160	1 - 320	1 - 640
	Pseudomonas pyoryanea serum					
Antigens						
Ps.pyoryanea	++++	++++	++++	+++	++	+
B. sanguinarium	-	-	-	-	-	-
B. pullorum	-	-	-	-	-	-
B..typhosus	++	+	±	-	-	-
	Bacillus sanguinarium serum					
B. Sanguinarium	++++	++++	++++	++++	+++	++
Ps.pyoryanea	++	+	-	-	-	-
B. pullorum	++++	++++	++	±	-	-
B. typhosus	++++	++++	++++	++++	++	+
	Bacterium pullorum serum					
B. pullorum	++++	++++	++++	+++	++	+
Ps. pyoryanea	-	-	-	-	-	-
B. typhosus	++++	++++	++++	++	+	-
B. sanguinarium	++++	++++	++	+	±	-
	Bacillus typhosus serum					
B. typhosus	++++	++++	++++	++++	+++	++
Ps. Pyoryanea	++	+	-	-	-	-
B. pullorum	++++	++++	+++	+	-	-
BS sanguinarium	++++	++++	++++	+	±	-

48 hours, with physiological salt solution to which 0.5 % phenol had been added as a preservative. The antigen was diluted to a turbidity of 1 according to McFarland's nephelometer.

The tests were set up using serum-antigen dilutions of 1;20, 1:40, 1:80, 1:160, 1:320, and 1:640, physiological salt solution being used in making the dilutions. The results obtained (Table I) were as follows:

Pseudomonas pyocyanea serum tested with its homologous antigen gave a titre of 1:640, while with B. typhosus antigen a + test was obtained at 1:40, with a ± at 1:80. With B. sanguinarium and B. pullorum antigens negative results were obtained in all dilutions.

With B. sanguinarium serum, the homologous antigen gave a titre of 1:640, B. typhosus a + at 1:640, B. pullorum a ++ at 1:80, with a ± at 1:160; while B. pyocyanea antigen gave a + at 1:40, with a ± reaction at 1:80.

It will be noted that B. typhosus antigen gave the highest titre, as would be expected, while Ps. pyocyanea, an unrelated organism, had the lowest titre.

B. pullorum serum with its homologous antigen gave a distinct positive at 1:640 with B. typhosus antigen a + at 1:320, B. sanguinarium + at 1:;60 with a ± at 1:320, while Ps. pyocyanea gave only a ± at 1:20.

These results are all comparable, indicating a close relationship between B. typhosus, B. sanguinarium and B. pullorum, and the totally different nature of Ps. pyocyanea.

STUDIES WITH SALT AND ACID AGGLUTINATION

Having determined the titres of the various sera, both on the homologous and heterologous antigens, it was possible to study the effect of altering the test from the standard procedure.

The first modification to be studied was a comparison of the effect produced by the various salts as compared to sodium chloride .85% ordinarily used with the standard test, incubating at 37°, 25°, and 10° C. and also according to the rapid method.

In conducting the standard tests for the four organisms under consideration, the set-up in each case was as follows: 0.05 c.c. of serum, 0.2 c.c. of antigen suspension having a turbidity of 1, and 0.85 c.c. of 0.85 % salt solution in each tube. Dilute hydrochloric acid was added to obtain the desired hydrogen-ion concentration. The serum-antigen combination of each of the four organisms was tested at pH values 3.0, 4.0, 4.2, 4.4, 4.6, 4.8, 5.0, 5.2, and 5.4 with each of the eight salts used, namely copper acetate, sodium sulphate, barium chloride, ammonium chloride, magnesium sulphate, potassium chloride, calcium carbonate, and sodium chloride. Duplicate sets were incubated at 37°, 25° and 10° respectively, andalso with the rapid method.

Of the salts used, calcium carbonate and magnesium sulphate were the least effective. The former, when used with B. sanguinarium serum and antigen, with the incubation

at 37°, effected only a + reaction at pH 3 and 4, while the latter produced the same results, with an additional ± reaction at pH 4.2.

When used with B. pullorum serum and antigen $CaCO_3$ permitted a ++ reaction at hydrogen pH 3.0, + at pH 4.0, and ± at pH 4.2, while $MgSO_4$ produced only a + at pH 3.0 and 4.0 with a ± at 4.2. With B. typhosus serums and antigen $CaCO_3$ allowed a ++++ at pH_3, ++ at pH_4 and ± at $pH_{4.2}$ while $MgSO_4$ caused a ++ at pH_3 with ± at pH_4 and 4.2

With Ps. pyrocyanea serum and antigen $CaCO_3$ gave a + + at pH 3.0 and ± at pH 4.0 and 4.2; $MgSO_4$ ++ at pH 3.0 + at pH 4.0 and ± at pH 4.2.

Ammonium chloride was slightly more effective, since when used with B. sanguinarium a +++ reaction was produced at pH 3.0, ++ at pH 4.0 and ± at pH 4.2/

With B. typhosus a +++ reaction was given at pH 3.0 + at pH 4.0 and ± at pH 4.2, while with Ps. pyrocyanea serum and antigen a ++++ reaction occured at pH 3.0, ++ at pH 4.0, + at pH 4.2 and ± at pH 4.4.

Barium chloride and potassium chloride were next in order of efficiency. With B. sanguinarium the effect of these salts was identical. Both gave a ++++ reaction at pH 3.0 and 4.0, ++ at pH 4.2 and ± at pH 4.4.

With B. pullorum the degree of agglutination produced by these two salts was again the same. The reaction produced at pH 3.0 and pH 4.0 was in each case ++++ while a +++ was indicated at pH 4.2, ++ at 4.4, and ± at 4.6.

The maximum results were obtained by the use of copper acetate, Sodium sulphate and Sodium chloride. The results of these salts being much the same.

With B. sanguinarium all three of the last mentioned salts gave a ++++ reaction at pH 3 and 4, Na_2SO_4 gave a +++ at pH 4.2, while copper acetate and NaCl gave a ++. Copper acetate and Na_2SO_4 produced a + at pH 4.4 while ± was indicated in the NaCl test.

Copper acetate and Na_2SO_4 gave a ± reaction at pH 4.6.

With B. pullorum serum and antigen the results at pH 3 and 4 were the same, all three salts permitting a ++++ reaction. At pH 4.2, Na_2SO_4 and NaCl gave a ++++, while Copper acetate gave a +++ reaction. At pH 4.4, NaCl gave a +++ test while Copper acetate and Na_2SO_4 gave ++. At pH 4.6 the test was + for each salt. With B. typhosus each of the three salts gave +++ at pH 3 and 4. Copper acetate and NaCl gave +++ at pH 4.2, while Na_2SO_4 gave ++. At pH 4.4 NaCl produced a ++ reaction while copper acetate gave a +, and Na_2SO_4 ±/ NaCl and Copper acetate gave ± at 4.6.

With Ps. pyocyanea ++++ was indicated in each case at pH 3 and 4, the NaCl test showed ++++ at pH 4.2, while copper acetate and Na_2SO_4 gave +++ reaction. At pH 4.4 NaCl produced a ++ while the tests with Na_2SO_4 and copper acetate indicated a + reaction (Table II)

The tests incubated at 25° averaged one point less than those given for the 37° incubation.

Those placed at 10° for 24 hours showed at the most only doubtful reactions, but when held for 48 hours the results compared favorably with those obtained by incubating at 37° fo 24 hours.

TABLE II Effect of salts on Agglutination.

B. Sanguinarium

pH	Cu. acetate	Na_2SO_4	$BaCl_2$	NH_4CL	$MgSO_4$	KCl	$CaCO_3$	NaCl
3.0	++++	++++	++++	+++	+	++++	+	++++
4.0	++++	++++	++++	++	+	++++	+	++++
4.2	++	+++	++	±	±	++		++
4.4	+	+	±			±		±
4.6	±	±						
4.8								

B. pullorum

pH	Cu. acetate	Na_2SO_4	$BaCl_2$	NH_4CL	$MgSO_4$	KCl	$CaCO_3$	NaCl
3.0	++++	++++	++++	+ ++	+	++++	++	++++
4.0	++++	++++	++++	++	+	++++	+	++++
4.2	+++	++++	+++	+ ±	±	+++	±	++++
4.4	++	++	++			++		+++
4.6	+	+	±			±		+
4.8								

TABLE II (Continued) Effect of salts on Agglutination

B. typhosus

pH	Cu acetate	Na_2SO_4	$BaCl_2$	NH_4Cl	$MgSO_4$	KCl	$CaCO_3$	NaCL
3.0	++++	++++	++++	+++	++	++++	+++	++++
4.0	++++	++++	+++	+	±	+++	++	++++
4.2	+++	++	+	±	±	+	±	+++
4.4	+	±						++
4.6	±							±
4.8								

Ps. pyocyanea

3.0	++++	++++	++++	++++	++	++++	+++	++++
4.0	++++	++++	++++	++++	+	++++	±	++++
4.2	+++	+++	++	++	±	++	±	++++
4.4	+	+	±	±		±		++
4.6								
4.8								

With the rapid method, results corresponding with those given for incubation at 37° were obtained. Here the buffer solutions were substituted for the colorimetric method of obtaining the desired pH, since by so doing the time required for the set up was materially lessened. The same serum antigen dilution was used as in the above tests, the tubes shaken for two minutes, and incubated for 15 minutes at 37°.

The results would seem to indicate that physiological sodium chloride solution is as efficient as other salts in promoting agglutination of the four organisms studied.

In view of the fact that these results checked with those previously obtained by other investigators, the evident conclusion seemed to be that physiological sodium chloride as ordinarily used in the routine agglutination tests is as efficient as other salts, at least those tested did not seem to average higher in results produced than those given by .85 % NaCl.

Change of Hydrogen-ion Concentration During Incubation.

The next point in question was to what extent incubation at 37° would affect the hydrogen-ion concentration of the agglutinating mixture.

Accordingly set-ups were arranged for the four organisms, using 0.05 c.c. of serum, 0.25 c.c. antigen (turbidity 1), 0.5 c.c. buffer solution, 0.5 c.c. physiological salt solution.

The tests were arranged in duplicate for each organism, at the end of thirty minutes, and 24 hours incubation indicator was added to each tube, and the contents compared with the pH standards to determine the extent in change of the hydrogen ion concentration. The results were as follows: With the half hour incubation, the tests averaged one point increase in alkalinity, while with the 24 hours incubation, the average change in pH value was 1.5 toward the alkaline side.

While this has no particular significance, the conclusion may be made that the pH of the incubating tubes tend to move toward the alkaline side.

The Effect of Omitting Phenol from B. Pullorum Antigen

At this time an attempt was made to substantiate Rebrassiers (69) claim that the so-called pseudo-reactions in the agglutination of B. pullorum might be

avoided by the omission of phenol.

Routine tests were run in the usual manner, but omitting phenol from the antigen used. Tests were arranged in duplicate, with incubation at 37°, and 25°C. for 24 hours, and 10° for 48 hours with the following results:

Almost with out exception, the tubes incubated at 37° for 24 hours showed sufficient growth to interfere with reading the tests.

In those incubated at 25°C. the growth was less apparent, but was evident to an undesirable degree.

Incubation for 48 hours in the ice box eliminated the growth factor but the length of time necessary for agglutination is undesirable for routine work.

The antigen prepared without phenol was also tested according to the rapid method, using 0.05, 0.025, 0.0125 amounts of sera, 0.2 c.c. of antigen with a turbidity of 1, and 0.5 c.c. of physiological salt solution being added to each tube. The tests were shaken for two minutes at 37° and compared with tests similarly conducted but in which phenolized antigen was used.

This method was repeated several times with varying results. At times the tests from which phenol was omitted seemed clearer than those in which phenol was used. At other times no difference was apparent in the results produced by the two methods.

The evident conclusion is that phenolized antigen is more dependable for routine work.

THE EFFECT OF ORGANIC COMPOUNDS ON AGGLUTINATION

The next step was an attempt to influence the degree of agglutination by the addition of organic compounds.

Accordingly various organic compounds, namely, blood albumen, edestin, lecethin, and hemoglobin were prepared in suspensions of 1:400, 1:2000, 1:10,000, 1:50,000 and 1:250,000; giving as final dilutions in the tests 1:800, 1:4000, 1:20,000, 1:100,000, and 1:500,000.

These varying suspensions were added in 0.5 c.c. amounts to tubes adjusted with buffer solutions to hydrogen-ion concentration of pH 4.4, 4.8, 5.0, 5.4, 5.6, 5.8, 6.0, 6.2 and 6.4 using B. pullorum antigen with a turbidity of 1 and positive serum with a dilution of 1:40. Incubation was at 37° fo 24 hours. The following results were noted

Lecethin with a dilution of 1:800 produced a ++ reaction at pH 6.4, 6.2, and 6.0, + at 5.8, ± at 5.6 and 5.4, negative at pH 5.0, 4.8 and 4.4; and with a 1:4000 dilution, negative at 6.4, + at 6.2, ± at 6.0 and negative for the rest of the series. Higher dilutions of lecethin produced no effect.

The value of edestin proved slightly less than that of lecethin. The tests were not clear, and while some agglutination was apparent in the same tubes as those noted with the use of lecethin, the tests were not satisfactory.

The same was true of blood albumen. The tests were not satisfactory even when a dilution of 1:800 was used. For this reason the use of these substances was not continued further.

When hemoglobin was used in the same manner with incubation at 37° fo 30 minutes, 0.5 c.c. of a 1:800 suspension produced a ++++ reaction at pH 4.4, 4.8, 5.0, 5.4, 5.6, 5.8, 6.0, and 6.2, with a +++ reaction at pH 6.4. The 1:4000 suspension produced a negative reaction at 6.4, ++ at 6.2, + at 6.0, and ± at 5.8, 5.6, 5.4, 5.0, 4.8, and 4.4. A 1:20,000 suspension gave a negative test at 6.4, ++ at 6.2, + at 6.0 and 5.8, and a negative at 5.6, 5.4, 5.0, 4.8 and 4.4. A suspension of 1:100,000 gave a negative test at pH 6.4, + at 6.2, ± at 6.0, and negative for the rest of the series. After being held at 37° for 24 hours the tube containing hemoglobin dilution 1:4000 and buffer at pH 6.2 increased to a +++ reaction. The other tubes were not altered. (Table III)

This was a distinct increase over normal agglutination of B. pullorum serum and antigen without the addition of hemoglobin, since the previous tests had given only a ± reaction at pH 4.8 and above.

The previous cross agglutinations tests with B. pullorum antigen, and Ps. pyocyanea serum had given a negative test in all serum-antigen dilutions.

The effect of the addition of hemoglobin suspension 1:800 was to increase the sensitivity of the reaction

Table III The effect of various concentrations of hemoglobin in the agglutination of B. pullorum serum and antigen. Incubation at 37° for 30 minutes.

PH	4.4	4.8	5.0	5.4	5.6	5.8	6.0	6.2	6.4
Concentration of Hemoglobin									
0	-	-	-	-	-	-	-	-	-
1:500,000	-	-	-	-	-	-	-	-	-
1:100,000	-	-	-	-	-	-	±	+	-
1:20,000	-	-	-	-	-	±	±	++	-
1:4,000	±	±	±	±	±	±	+	++	-
1:800	++++	++++	+++	++	++++	++++	++++	++++	++++

so that with serum dilution 1:40 upon thirty minutes incubation at 37° ± reactions were obtained at pH 4.4, 4.8, 5.0, 5.4, 5.6, and 5.8; + at pH 6.0; +++ at pH 6.2 and negative at pH 6.4. The 1:4,000 suspension gave negative tests at pH 4.4 and 4.8, ± at pH 5.0, 5.4, 5.6, and 5.8; + at 6.0; ++ at 6.2 and negative at 6.4 (Table IV). When held at 37° for 24 hours the agglutination in the tubes containing the 1:800 suspension increased to + at pH 4.4, 4.8, 5.0 and 5.4; ++++ at 5.6, 5.8, 6.0, 6.2 and 6.4. The tubes in which the 1:4,000 suspension was used increased to ± at pH 4.4, 4.8 and 5.0; ++++ at pH 6.2 and 6.4. The tubes containing a 1:20,000 suspension which had given only negative tests with 30 minutes incubation, now gave ± at pH 6.0 and + at 6.2. The 1:100,000 suspension produced only a ± reaction at pH 6.2.

These results seemed very significant. In the first place, the addition of a suspension of hemoglobin apparently increased the sensitivity of the antigen to such an extent that ++++ reactions might be produced when normally negative tests would be given.

The next step was the application of these findings to tests with cloudy sera, first using 0.05 c.c. of serum (X), 0.2 c.c. of B. pullorum antigen, 0.25 c.c. amounts of hemoglobin suspensions 1:20,000, 1:100,000 and 1:500,000 respectively. The tests were adjusted to pH values 6.2, 6.4, 6.6 and 6.8 with buffer solutions in 0.5 c.c. amounts. With 30 minutes incubation

Table IV The effect of various hemoglobin suspensions upon cross agglutination of Ps. pyocyanea serum, and B. pullorum antigens. Incubation at 37° for 30 minutes.

pH	4.4	4.8	5.0	5.4	5.6	5.8	6.0	6.2	6.4
Concentration of hemoglobin									
0	-	-	-	-	-	-	-	-	-
1:500,000	-	-	-	-	-	-	-	-	-
1:100,000	-	-	-	-	-	-	-	-	-
1:20,000	-	-	-	-	-	-	+ -	+ -	-
1:4,000	-	-	+ -	+ -	+ -	+ -	+	++	-
1:800	+ -	+ -	+ -	+ -	+ -	+ -	+	+++	-

at 37°, the 1:20,000 suspension produced a ± reaction at pH 6.6 and + at 6.8 the remainder of the tests being negative. When the above tests were incubated for 24 hours at room temperature, all tubes containing the hemoglobin suspension 1:20,000 showed a ++++ reaction, the tubes containing the higher dilutions remaining negative.

With a second cloudy serum (Y) run according to the above system, the hemoglobin suspension 1:20,000 produced a ± reaction at pH 6.2, + at 6.4 and 6.6 and ++ at 6.8, the higher suspensions having no effect with a 30 minute incubation. The 24 hour incubation produced the same results as those given for serum (X).

These sera were obtained from the routine laboratory and had given cloudy reactions with a dilution of 1:40 on the previous day. Doubtless the disappearance o of the cloudy effect was due to the ice box storage during the intervening 24 hours. Observation to this effect has been made by Mr. Hallmann (1). On the other hand the combination of the constituents in the tubes may have affected the precipitation to some extent. At this time no further studies were made to determine the disappearance of the cloudiness.

The test with serum (X) was then repeated, using the same amounts of serum, antigen, and hemoglobin suspensions, but decreasing the amount of buffer solutions to 0.25 c.c. and adding 0.25 c.c. of physiolog-

TABLE V The final standardisation of antigens prepared from 10 different strains of B. pullorum.

Antigens	13	36	7	25	40	31	29	11		Con-trol
Concentration of cholesterol										
0	++++								-	
1:409,600		++++								
1:204,800			++++							
1:102,400						++++	++++			++++
1:51,200									++++	
1:25,600				++++	++++			++++		

ical salt solution to each tube.

With a 30 minute incubation at 37° tubes containing hemoglobin suspension 1:20,000 showed a ± reaction at pH 6.0 and 6.2, + at pH 6.6 and 6.8 (Table VIII)

Upon standing at room temperature for 24 hours a + reaction was evidenced a pH 6.0; ++ at 6.2 and ++++ at 6.6 and 6.8. (Table IX)

Lessining the amount of buffer solutions decreased the tests as might be expected.

A series of tests were then run, using cloudy chicken serum of unknown reaction, in 0.05 c.c., 0.025 c.c. and 0.0125 c.c. amounts, 0.2 c.c. of B. pullorum antigen having a turbidity of 1, 0.25 c.c. of hemoglobin suspension 1:20,000 and 0.5 c.c. of buffer solution of pH 5.8 and 6.8. The rapid method was used, that is, shaking the tests for 2 minutes and incubating for 15 minutes at 37°. Those tubes containing 0.05 c.c. amounts of cloudy serum gave a ++++ reaction; 0.025 c.c. of serum produced ++ and 0.0125 c.c. a + reaction at both pH 5.8 and 6.8.

Cholesterol was not obtainable at the time the previous studies with organic compounds were made. At this point it was possible to compare its value with that of hemoglobin, which thus far had given the most satisfactory results of the various organic compounds used in the tests.

A dilution of 1:25,600 was used arbitrarily due to the results obtained with corresponding dilutions of

When a suspension of cholesterol 1:25,600 was substituted for the hemoglobin in the above tests, all tubes gave ++++ reactions.

These tests seemed to indicate that both hemoglobin and cholesterol, but especially cholesterol, might be used to increase the sensitivity of the antigen.

However, since the sera thus far tested were not known to be positive, the next point seemed to be would these organic compounds produce a similar reaction with any serum, whether positive or negative.

Accordingly, a series of tests were run by the rapid method, using cloudy sera (c),(d) and (e) in 0.05, 0.025 and 0.0125 amounts, 0.2 c.c. of a suspension of B. pullorum antigen (turbidity 1), 0.25 c.c. of buffer solutions of pH 5.2, 5.6 and 5.8 respectively for each serum used , 0.25 c.c. of hemoglobin suspension 1:500,000 being added to each tube.

Normal horse serum was selected as a control, since being from a different animal, agglutinins for B. pullorum would be absent. Therefore a satisfactory check would be furnished for the tests.

The set-up was duplicated, substituting cholesterol suspension 1:609,200 for the hemoglobin and both sets incubated in a water bath at 56°for 15 minutes.

All tubes containing chicken serum gave ++++ reactions, while the tests with horse serum were negative. This might indicate that the chicken sera tested were all positive,

or that the organic substance used would bring down a precipitate with any chicken serum.

It was thought that the effect of the high temperature might be in part responsible for the high degree of agglutination. Therefore, the previous set-up was repeated, incubating at 52° for two hours. The resulting agglutination was evident only at pH 5.8/ At this point, those tubes containing chicken serum in 0.05 c.c. amounts gave ++++ reactions, those containing 0.025 c.c. +, and those having 0.0125 c.c. gave a ± test. All tests with horse serum were negative.

Hence, several things were determined. First, cholesterol suspension 1:809,200 has an effect equal to that produced by hemoglobin 1:500,000. Second, the temperature at which the tests are incubated is a definite factor in the rate of the resulting reactions. Evidently, too, the optimum pH value is 5.8.

The effect of varying amounts of antigen and organic compounds was the next point in question.

Cloudy sera from two birds suspected of being infected with B. pullorum were run against a pool of cloudies previously tested and the same horse serum used in the previous test. The tubes were set up as follows: (1) 0.05 c.c. of the respective sera with 0.2 c.c. of a suspension of B. pullorum antigen, 0.05 c.c. of buffer solution having a pH value of 5.8 and 0.25 c.c. of hemoglobin suspension in 1:500,000; (2) 0.05 c.c. of the sera,

0.15 c.c. of antigen, 0.3 c.c. of the hemoglobin, and 0.5 c.c. of buffer solution; (3) 0.05 c.c. of the sera, 0.1 c.c.of antigen, 0.35 c.c. of hemoglobin, 0.5 c.c. of buffer solution; (4) the set up for (1) was repeated using cholesterol suspension 1:809,200 instead of hemoglobin 1:500,000; (5) was a duplicate of (2) with cholesteral substituted for hemoglobin; (6) duplicated (3) with the substitution of cholesterol 1:809,200 in place of hemoglobin 1:500,000. The results were the same in all cases the tests with chicken sera giving ++++ reactions, while those with the horse serum were negative.

Evidently, one of two conclusions must be drawn. Either all the chicken sera tested were from birds infected with B. pullorum, or the mixture was still too sensitive for accurate results.

To further dilute the serum-antigen mixture seemed the logical step. Therefore two suspected sera (B) and (L) were run with a pool of cloudies which had previously given positive tests, and horse serum, six set-ups being used for each sera. (1) 0.05 c.c. of the respective sera, 0.1 c.c. of B. pullorum antigen (turbidity 1) 0.35 c.c. of hemoglobin suspension 1:500,000, 0.5 c.c. of buffer solution having pH value 5.8., (2) 0.025 c.c. of sera, 0.1 c.c. of antigen, 0.35 c.c. of hemoglobin, and 0.5 c.c. of buffer solution., (3) 0.0125 c.c. of sera, 0.1 c.c.of antigen, 0.4 c.c. of hemoglobin, 0.5 c.c. of buffer solution., (4) was a duplicate of (1) substituting a cholesterol suspension of 1:819,200 for the hemoglobin., (5) duplicated (2) substituting cholesterol for hemoglobin;

(6) duplicated (3) with the substitution of cholesterol for hemoglobin. These tubes were incubated for 15 minutes at 56° and allowed to stand for 15 minutes at room temperature before reading. The results were as follows: In the first set of tubes, serum B gave a negative test, L ++++, the pool negative, and horse serum negative. The results for set (2) and (3) were identical with those of (1). Test (4) using cholesterol 1:809,200 gave a ++++ for serum L and for the pool. Serum B and the horse serum gave negative reactions. The results for (5) and (6) were the same as those given for (2) and (3).

The effect of increasing the serum antigen dilution was a decrease in the amount of agglutination to the extent that the pool of cloudy sera which had previously given a ++++ reaction, now became negative. Allowing the tubes to stand for 15 minutes at room temperature after being removed from the water bath resulted in the production of a decidedly clear supernatent fluid.

To re-check the results so far obtained, and to further determine the effect of temperature, serum (1) was tested in 0.05 c.c., 0.025 c.c. and 0.0125 c.c. amounts at pH values 5.0, 5.2, 5.4, 5.8, 6.0 and 6.2, using 0.1 c.c. of antigen (turbidity 1), 0.4 c.c. of organic substances as before, and 0.5 c.c. of the buffer solutions. The tests were incubated at 56° for 15 minutes with an added 15 minutes at room temperature, and in duplicate at 37° for 24 hours. All tubes indicated a ++++ reaction. However, those incubated in the

water bath at 56° gave a sharper reaction than those held at 37° for 24 hours. Likewise, the tubes in which cholesterol was used gave a clearer test than those with hemoglobin.

The same conditions were repeated, using negative chicken serum (not cloudy) with negative results in all tubes.

The question naturally arose as to whether the agglutination with cloudy sera was due to a combination with the organic compounds, not formed with clear sera. This was refuted by the microscopic test which showed a marked agglutination with serum (L) and none with the clear negative serum.

The next question to arise was that of specificity - whether other antigens would produce like results when sensitized with cholesterol. Accordingly, the previous set-up was repeated using serum (L) with B. sanguinarium, B. typhosus, and Ps. pyocyanea antigens. Positive results were obtained in all cases, indicating that the test was still too sensitive for practictical use in the diagnosis of Bacillary White Diarrhea in chickens.

The test with the various antigens were repeated, with decreasing amounts of serum (L) the latter being used in 0.0125 c.c. and 0.01 c.c. amounts, the amount of the other constituents remaining constant. ± reactions were still evident with B. typhosus and B. sanguinarium antigens. However, when the amount of serum

used was decreased to .005 c.c.. B. typhosus and B. sanguinarium antigens failed to react above pH 5.4 while B. pullorum antigen still produced ++++ agglutination at pH 5.6 and 5.8, +++ at pH 6.0, ++ at 6.2, ± at 6.4, with a negative at 6.6. Therefore the development of the test seemed to be approaching the point where reliable reactions might be expected. As a further proof of the reliability of the test, th. turbidity of the antigen was decreased to 1/2 and used in 0.2 c.c. amounts in testing four known positive sera (clear) against a known negative and a guinea pig serum, using buffer solutions of 6.4 and 6.6 in 0.4 c.c. amounts, 0.4 c.c. of cholesterol suspension 1:809,200 and 0.005 c.c. of the respective sera. Incubation was for 15 minutes at 56° and 15 minutes at room temperature.

The results were checked with the routine test, and with the microscopic test, the results in all cases being identical.

Serum R gave a ++ reaction, serum 213 ++++, serum 138 +++, and serum 187 ++, while the known negative chicken serum gave a negative reaction, and the guinea pig serum likewise failed to agglutinate.

As the cloudy sera available for the test had been limited in number, 102 chickens were bled, and the sera tested for the purpose of substantiating the reliability of the test. Of the sera thus obtained, 30 gave cloudy reactions with the routine test. Of these 30 cloudy ones (32) gave a ++ reaction with the sensitized test and likewise showed

some agglutination with the microscopic test. The other (933) gave a ++++ reaction when tested according to both methods.

The clear sera were tested by the sensitized test, the routine test, and the microscopic test, the results in all cases being identical.

When the birds C2 and 933 were starved for 48 hours and bled, the clear sera thus obtained were tested by the sensitized, routine and microscopic methods, giving the same results as those noted above.

Hence it seemed justifiable to conclude that the sensitized test as outlined may be considered a reliable means of diagnosis for the presence of B. pullorum in the sera of chickens.

The advantages of this test may be stated as follows: first, the rapidity with which it may be conducted, only 30 minutes being necessary for incubation, 15 minutes in the water bath at 56°C., and 15 minutes standing at room temperature, to allow complete precipitation. Second, only one tenth as much serum is required as in the standard test. Third, the avoidance of the loss of production caused by starving the birds to obtain clear sera.

The object of running the tests at both pH 6.2 and 6.4 is that sera vary in their reaction with the buffer solutions. Some give a clearer test at pH 6.2, while others give a slightly sharper test at pH 6.4. Hence to obtain the maximum results it seemed advisable to run all sera at both concentrations.

STANDARDIZATION OF ANTIGENS

The last division of the work was an attempt to prove that antigens might be standardized, that is that different strains of the same organism giving widely different results in the standard test might be checked with a known preserved serum, and by the addition of organic substances, be made to give a ++++ reaction.

Ten strains of B. pullorum were used in making the antigens, which varied from a straight ++++ when tested with the standard method to a negative.

Varying proportions of serum-antigen were used in the attempt to standardize these antigens. The antigens were used in various turbidities, with different amounts of saline, with and without buffer solutions, and with different dilutions of cholesterol.

The system giving the best results may be outlined as follows: Each antigen was tested for its reaction with a known ++++ serum, using 0.025 c.c. of the serum, 0.2 c.c. of antigen with a turbidity of 2, and .8 c.c. of normal saline, giving a final dilution of 1:400. Of the antigens tested # 13 alone gave a sharp ++++ reaction. The remaining antigens were then repeated, using the same serum-antigen dilution, but decreasing the amount of saline to 0.4 c.c. and adding 0.4 c.c. of cholesterol suspension 1:409,600. Antigen #5 gave a ++++ reaction, those given by the other antigens being unsatisfactory. The remaining

eight antigens were again tested, this time using 0.4 c.c. of cholesterol solution 1:204,800, the other constitutents remaining constant. At this point a ++++ reaction was given by antigen # 7. Tests on the remaining antigens were repeated, substituting cholesterol 1:102,400 for the suspension previously used. This caused ++++ reactions to appear with antigens 29, 31, and 8. Cholesterol suspension 1:51,200 was then used with the remaining antigens, resulting in a ++++ reaction with #47. It was found that by substituting 0.4 c.c. of cholesterol 1:25,600, +++ reactions could be forced with the remaining three antigens, #25, 40, and 11 (Table V)*

Since up to this time, no agglutination had ever been obtained with antigen # 11 and only very weak reactions with # 47 the above results seem to indicate more than the ever the poorest antigens may be reinforced or sensitized by the addition of cholesterol suspensions of sufficient concentration so that a reaction, equal in strength to that produced by the more effective antigens, may be secured.

Hence the conclusion may be drawn that it is possible to standardize antigens for use with a known preserved serum.

This would prevent the possibility of variations in the standard test, caused by changing from one strain to another. Likewise, antigens prepared from the same strain may be checked from time to time to secure constant results.

*Follows page 37

The value of such a standardization is apparent. As noted by Mallmann (1) in an extensive survey of B. pullorum antigens of various strains, great irregularity occurs from time to time. This irregularity is, of course reflected in the routine laboratory tests. Accurate results cannot be insured, and definite standards cannot be maintained, if antigens giving widely different results are used.

METHOD OF STANDARDIZATION OF ANTIGEN

(1) ++++ serum, having a 1-400 titre, obtained by bleeding chickens known to be infected with B. pullorum is used.

(2) Prepare antigen as usual, and dilute to a turbidity of 2.

(3) Set up 7 tubes according to the following table.

	1	2	3	4	5	6	Control
Antigen c.c.	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Serum c.c.	0.025	0.025	0.025	0.025	0.025	0.025	--
Saline c.c.	0.8	0.4	0.4	0.4	0.4	0.4	0.4
Concentration of cholesterol c.c. -							
1:409,600		0.4					
1:204,800			0.4				
1:102,400				0.4			
1:51,200					0.4		
1:25,100						0.4	0.4

(4) Shake and incubate at 56° for 15 minutes and allow to stand at room temperature for 15 minutes.

(5) Observe and record.

The tube containing the least amount of cholesterol giving a ++++ reaction has the right antigen concentration. This amount of cholesterol should be added to each tube when this particular antigen is used in the routine test.

(6) Set up at least two known negative and two known positive sera and the newly prepared antigen as

follows:

	1	2
ANTIGEN c.c.	0.2	0.2
SERUM c.c.	0.0025	0.001
Saline c.c.	0.4	0.4
Cholesterol c.c.	0.4	0.4

(7) Shake and incubate at 55° for 15 minutes in the water bath. Remove at the end of this period and allow to stand at room temperature for 15 minutes.

(8) Read the results. The negative sera should be negative in both tubes, and the positive sera should be positive in at least the first tube. In this case the correct antigen-cholesterol mixture has been determined. This combination of antigen and cholesterol is now ready for use in the routine test.

If dilutions 1:40 and 1:100 have been used according to the standard method, then dilutions of 1:400 and 1:1000 should be used in this test, as indicated by the above table. The object of the higher dilutions is to make the test applicable to general use. By increasing the sensitivity, cloudy reactions are avoided. The above procedure is tentative and should be adapted to the routine of the individual laboratory.

SUMMARY

Sodium chloride as used in the standard agglutination test produced maximum efficiency. At least none of the salts studied gave better results at the same concentration.

Incubation at temperatures below 37°C. decreases the amount of growth when phenol is omitted, but also decreases the amount of agglutination unless held for a decidedly longer period.

Hydrogen-ion concentration influences the results materially. Varying results were obtained.

The addition of cholesterol or hemoglobin causes an increased sensitivity of the antigen, resulting in higher titres for the sera used.

Cloudy reactions can be avoided by using high titres, made possible by using more unstable antigens.

Combinations have been obtained allowing the use of a dilution ten times higher than could be used with the usual antigen, still giving a negative and a positive test as usual. This makes possible the use of an antigen ten times as sensitive.

The modified test may be conducted in a much shorter time than the standard test. Fifteen minutes incubation at 56°C and 15 minutes standing at room temperature is sufficient to clear the sera.

Antigens may be standardized against a known preserved serum. Even the weakest antigens which produce no agglutin-

ation in the standard test may be brought to a ++++ reaction by the addition of a suspension of cholesterol of the correct concentration.

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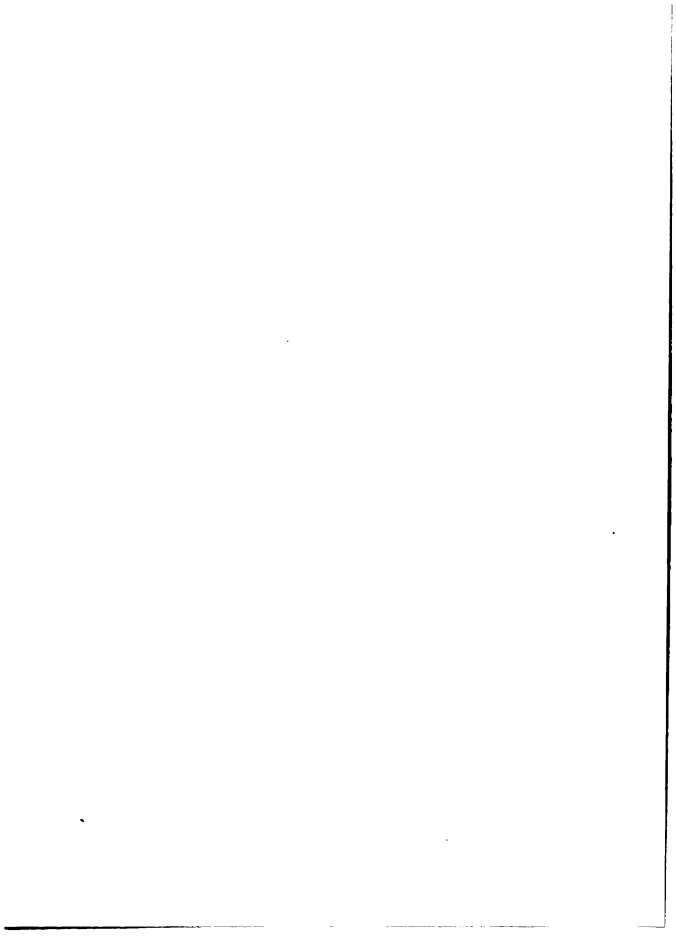

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