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SEROLOGIC PRETESTING OF LABORATORY ANIMALS FOR STUDIES WITH HAEMOPHILUS PERTUSSIS

by

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A THESIS

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INTRODUCTION

The investigations leading to the present report were begun in the early part of 1939 with the purpose of studying immune responses to Hemophilus pertussis by means of complement fixation. After only a few weeks of preliminary work, it was realized that "standardized" conditions could not easily be maintained from time to time, lending added significance to the statement made about two years later by Flosdorf et al, (24) with regard to complement fixation in pertussis: that "------- such a test has not been developed.": and by Lapin (52) in 1943, that the contradiction among the authors with regard to serologic testing "----merely serves to emphasize the necessity for standardization of procedures in agglutination and complement fixation." Even more discouraging is the fact that these statements - to the writer's knowledge - are still true, some 40 years after Bordet and Gengou (5) announced discovery of the causative agent of whooping cough.

Judging from the reports in the literature, and from personal experience, it was apparent to the writer that efforts directed toward the standardization of a complement fixation procedure had been blocked by an interfering principle completely hidden among the intricacies of the system; and it was certain that no progress could possibly be expected before this principle could be better understood and controlled. In following the only paths of thought opened by the data already accumulated, and the attitudes of apparently well informed investigators, a further

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scouring of the literature seemed practically superfluous since it lengthened the list of conflicting reports, without offering any definite clues for the solution of the problems involved. In the already great volume of publications, especially on laboratory studies with H. pertussis, there was:

1. A marked diversity in results obtained by various investigators employing the same or similar procedures for the study of test material (sera and antigens).
2. A lack of correlation among the results of several test procedures employed in individual investigations.
3. A lack of uniformity in the use of one procedure by individual investigators over a period of time.

It is evident from the above that the effect of an unknown principle was involved not only in the serology of pertussis, but also in phases other than those of a purely serological nature. It seemed to the writer, therefore, that the matter of primary importance to the serology of pertussis in particular, and to the pertussis problem in general, was not the publication of additional data on immune responses, but rather an exploration into the mechanisms or principles responsible for these incongruities. Efforts directed toward this end were finally rewarded in the accumulation of the data to be presented here; data which suggested the necessity of subjecting to rigorous scrutiny, the serum of all animals which would in any way be involved in studies with H. pertussis. Scattered throughout all the pertussis literature was evidence in support of this conclusion, hidden there by certain masking factors.

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HISTORICAL BASIS FOR THE STUDY

General divergence of opinion in the entire field.

Most of the investigators since 1906 have been in accord with Bordet and his co-workers on some of the bacteriological aspects of whooping-cough:

That H. pertussis is the etiologic agent of this disease, (5)

That the organism is present in the spray of whooping-cough patients most often in the early weeks of the disease, and rarely after the fourth or fifth week; (5), (9)

That the presence of blood in the medium is very essential for isolating and maintaining the organism in a virulent undissociated state (5), (6), (8), (10).

Other phases in the bacteriology of H. pertussis, however, have been the cause of divergent opinion. For instance, Chievitz and Meyer (11) introduced the cough plate method of diagnosis, which has found a considerable number of proponents such as Gardner (31), Gold & Bell (35), Gajzago & Gottche (28), Soare & Turai (81), Donald (16), Sauer (76), and many others, especially among health workers who use the method daily. Banks (4), and Maclean (55), have not been so well satisfied with it, at least under certain conditions. Anderson (1), and Miller (61), favored the use of nasal swabs in certain instances.

In addition to this, considerable cause for concern has been found in the preparation of the medium to be used for culture of the organism:

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while most investigators have favored the use of fresh blood medium, Shiga et al (77), Brunwiede et al (47) and Mishulow et al (63) have favored use of the "chocolate" blood medium. Most investigators have used whatever blood seemed convenient (horse, human, rabbit, sheep, ox, etc.); but Mishulow et al (63) preferred horse blood to human (64), while on the other hand, Toomey & Takacs (87) and Lu et al (92) have shown decided changes in their cultures from use of horse blood. They, therefore, preferred human or sheep blood.

In the serological and immunological aspects, the experiences have been equally diversified, but the reports are too numerous to be discussed individually. Briefly, the situation which exists can be stated as follows: While the responses of humans and other animals to B. pertussis antigens in one form or another, have been studied by complement fixation, agglutination, animal -- usually, mouse -- protection, neutralization, opsonocytophagic, and skin tests, or by the ability of the organism to cause sickness or death, there has been no unanimity of opinion among investigators as to the value either of the methods, or of the results obtained. Furthermore, while some have claimed good correlation among the results of two or more of these methods others have taken the opposite stand. A study of the chronological order in which the various reports have appeared in the literature indicates that there has been no general trend toward acceptance of one method in preference to another. There seems to be no indication that any one procedure has become so well developed with the passage of time, that it could claim wider usage than ever before, and merit higher than any other similar procedure. This is rather surprising, in view of the fact that at the very beginning and in the early years of the work, Bordet and his co-workers gave rather comprehensive accounts of the results to be expected in most phases of

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study with H. pertussis:

They studied the lethal effects of the organism for laboratory animals (5), and suggested the presence of a pertussis toxin (5), (6), which was sensitive to heat, and not antigenic(7).

They studied responses to the organism by agglutination, and complement fixation (5), (8), (10), and by skin tests (7).

They showed the presence of reacting substances in the serum of normal horses, rabbits and guinea pigs (8); and injected animals with suspensions of organisms to produce anticera for the study of the antigenic properties of different strains (6), (10).

They mentioned the effect of immune serum on the course of the infection (6) as well as the effect of media (6), (8), (10) and immune serum (6) on the organism itself.

Of the many authors who have exerted their efforts to elaborate on the work of this group, comparatively few have added anything vital to a better understanding, especially of the antigenic nature, of the pertussis organism when used in vitro. Rather, this accumulating literature has, in its many divergent opinions, presented many disturbing inconsistencies to the field. Certain authors have felt that they could on the basis of their own work, explain some of the inconsistencies which have arisen; and it would perhaps be in order to give a few examples.

In the use of an agglutination test, for instance, Arnheim(2) pointed out that false negative tests may be due to a prozone phenomenon, since agglutination is hindered in low dilutions of serum. Bordet & Sleeswyk (8) also mentioned a similar phenomenon due to anti-agglutinins. Auto-agglutination of antigens has been found a disturbing factor by Odaira (67), Kristensen(46), Lapin et al (48) and many others. Odaira (67)

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treated the antigens to reduce auto-agglutination, while Hansen (37) stressed the necessity of using only suspensions which do not auto-agglutinate. Mosdorf et al (24) claimed best results with living organisms; and Miller et al (62) required a mixture of 3 to 5 fresh strains. Furthermore, the time and temperature of incubation of the tests have seemed to be important factors determining the success of the test.

As for complement fixation, Olmstead & Luttinger (69) have enumerated a large number of factors which they have felt could have been responsible for some of the discrepancies, and added that "An explanation of some discrepancies is still to be found." Giese (32) considered some failures due to the amounts of complement and antigen used, while Cajzago & Gottche (28) claimed that a reliable antigen is difficult to obtain. Mishulow et al (66) attributed discrepancies in complement fixation results to the method of testing, and admitted that their own method was such as to result in a test of low sensitivity.

The opsonocytophogic test has been found by Glynn & Cox (34), and Singer-Brooks & Miller (79) to be complicated by several sources of error: the number of cells counted, the age and concentration of the antigen suspension and certain physical factors in handling the reagents used; the presence of normal opsonins, variations in the leucocyte content of the blood, etc.

The skin test, both in humans and in laboratory animals, was considered by Paterson et al (71) to be limited by unknown factors in the antigen, since results seemed to vary with the lot of injection material used.

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The passive mouse protection test, according to Lapin (49), is complicated by an unaccountable variation in virulence of the cultures. Mishulow et al. (66) considered the intramuscular route for administration of the test serum as an important factor in avoiding the "non-specific" results reported by Matthieson & Clark (57) in tests with normal and non-pertussis immune animal sera.

These are only a few of the explanations, offered by various investigators, for the inconsistencies in reported results, but they are sufficient in themselves to emphasize the fact that full realization of all the intricacies of the various test systems has often been lacking. This is especially true of the complement fixation test, because of its very involved nature; and as result of the writer's experience with this system, it is now possible to offer another explanation for some of the difficulties encountered during the course of previous investigations not only in complement fixation, but perhaps in other fields as well.

In partial support of the writer's statement is the fact that most investigators have, at some time during the course of their work (especially in complement fixation), considered their antigens responsible for the inconsistencies in their results. By so doing, the emphasis of their work has been well placed, but apparently not widely enough applied since certain problems have still remained unsolved.

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Inconsistencies among reports regarding H. pertussis antigens in complement fixation.

Of the many publications reporting experiences with complement fixation in pertussis studies, very few have given descriptions of methods and reagents used; and of these, none could be considered so complete as to be reproducible in every respect. This lack of reproducibility resulted in a statement made in 1914 by Williams (90), which, it appears, is still applicable today: "-----that many of these methods do not stand the test of sufficient controls." She apparently attributed this to eccentricities of the antigens, since she mentioned "---- a hypothetic ferment which seems to make the antigen unstable." and tried several methods of preparation to surmount this obstacle. She, also, gave none of the particulars, however.

The preparation of the test antigens has always been a cause for concern; and only a few of the authors have attempted to describe the reagent which they have considered satisfactory. Aside from the reagent of Meier (58), and Hollstein (91), who used, among other things, extracts of tissues from fatal cases of whooping-cough, these antigens may be classified under two main types:

1. Saline suspensions of variously treated whole organisms in varying concentrations, and obtained from growth on variously prepared media.
2. Extracts of whole organisms.

In some cases, the authors have specified the technics which they have considered essential to the preparation of a satisfactory reagent. Yet,

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there has been no tendency among other investigators to adopt one method in preference to any other. Following is an illustration of this.

As result of their investigations, Gundel & Schluter (36) advocated use of a lecithinized alcohol extract of H. pertussis as being more specific, while Olah & Munsagi (61) using a suspension of whole organisms, claimed there is no advantage in such an extract. Lapin (52) on the other hand, reversed the conclusion of Olah & Munsagi (61) by supporting use of the lecithinized alcohol extract antigen of Weichsel & Douglas (89) claiming, as did also Gundel et al (36) that a simple suspension of H. pertussis is unsuitable because it gives frequent nonspecific and anticomplementary reactions. In partial disagreement with both sides, the work of Olmstead & Lovitsky (70) indicated that there is no method which can definitely assure one of a satisfactory antigen, since "There is such great individual variation in antigens of the same strain made by the same method, that comparison of methods is difficult." This statement, though corroborating that of Williams (90) certainly does not clear the confusion; and the point to be derived from this example is that regardless of the individual experiences and preferences, reported in the literature, the main difficulties encountered in the complement fixation system have been in the behavior of the test antigens. Further analysis of these reports indicated the difficulties to be in some way connected with the anticomplementary activities of these antigens.

Though most of the reports in the literature fail to make any description whatsoever of the antigens used, those of Bordet & Gengou (5), and Eaton (72) claim their antigens were not anticomplementary. Evans & Maitland (17), and Giese (32) compensated for "slight" anticomplementary properties by the addition of an extra small amount of complement, while Olmstead

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& Luttinger (69), Long & Bliss (54), Mishulow et al (65) and others adjusted the system by employing 1/3 to 1/4 of the anticomplementary dose of antigen in the tests. Olmstead & Povitsky (70) later presented the novel idea of comparing the anti-complementary to the complement fixing potencies of an antigen to determine a numerical "Index of Fixation" for the suitability of an antigen. This latter method has apparently not been widely adopted, however; and, judging from the works of Manicadite (56), Miller (60), Long & Bliss (54) and others, the procedure generally employed has been to prepare the antigens by whatever method seemed suitable or convenient to the investigator, and to titrate them daily. In other words, these investigators have apparently accepted the anticomplementary property as an inherent, unavoidable characteristic of H. pertussis antigens, and have used this characteristic as the point of reference from which the suitability of an antigen could be judged. In view of the experiences in preliminary work, the writer has felt, however, that such a basis for the selection and titration of an antigen is not well founded.

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PRELIMINARY WORK ON COMPLEMENT FIXATION WITH H. PERTUSSIS

During the course of considerable preliminary study, the complement fixation system was put to test on more than 1500 sera from humans of varying age and pertussis history. An analysis of some of the results obtained was included in reports by Kendrick (43), (44) on one of the Grand Rapids immunization studies, (which, incidentally, furnished the writer with some of the material required in his complement fixation work). Not indicated in Kendrick's reports, is the fact that the complement fixation test on sera of immunized subjects was positive as long as 18 to 22 months (longest period tested) after immunization, in contrast to the reports of Singer-Brooks et al (79), Gajzago et al (29), (30), Lapin et al (48), Lapin (50), (52), (53), who claimed that the complement fixation test is generally negative by the third to the eighth month. Also not indicated are the results of tests which were not directly related to Kendrick's work, and of others which had to be discarded because of sporadic inconsistencies in the behavior of the complement fixation system. Evidence of these inconsistencies lie in the fact that oftentimes there was an increase in positivity of the serum with increase in serum dilution; or the results of repeated tests on individual sera could not be duplicated.

After repeated experience with this type of behavior, an analysis of the results revealed close correlation between the occurrences of the inconsistencies and the anticomplementary activity of the antigens. Not only were suspensions of variously treated organisms or their extracts at times markedly anticomplementary; the anticomplementary property of any given lot of antigen seemed to fluctuate from time to time. Some of the antigens made available through the kindness of Drs. Kendrick and Eldering at the

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Western Michigan Division of the Michi an Department of Health Laboratories in Grand Rapids, had been in the refrigerator for as long as 14 years; and it seemed to the writer that if fluctuations in anticomplementary activity were due to changes in the antigen itself, these suspensions certainly, had had ample time to become stablized. Yet, their anti-complementary activity followed the same undulations as freshly prepared material, with no general trend in any particular direction. In so doing, this activity failed to fit any logical pattern; and therefore, could not in itself be considered as a cause of the disturbances in the antigens. It seemed rather to be an effect, the cause of which must necessarily lie elsewhere in the complement fixation system. In that case, the only hope for the future of this problem would consist in determining the cause, rather than attempting to minimize its effect, as had been the general inclination. To that end, it seemed necessary first of all to establish a stabilized starting point and since the complement activity of fresh guinea pig serum was known to depreciate considerably in a few days, it seemed logical to begin with this reagent.

A report on a study of preserved complement based on the work of Ginsburg & Kalinin (26a) has already appeared in the literature (26); and in this study it was found that guinea pig serum, to which 80 mg. of sodium chloride and 40 mg. of boric acid crystals had been added for each ml. of volume, retained a satisfactory complement activity for as long as three to four months. The use of guinea pig serum stabilized in this manner was adopted for the work with H. pertussis. With such a complement on hand, it was now possible to gain a fuller and a clearer conception of the so called "anticomplementary" activity of the antigens; and the results of further tests brought out the following facts:

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1. The anticomplementary activity of antigens varied widely with different lots of complement.
2. Different cultures of H. pertussis differed in anticomplementary acitivity to some extent, but retained the same relationship to each other when tested with different lots of complement.
3. The antigens became more and more anticomplementary with increase in age of the complement.
4. It was not possible to satisfy the anticomplementary activity of antigens by mixing them with an amount of complement equivalent to this activity (in a method similar to that described a few years later by Laran (84) for "Wasserman" tests on anticomplementary sera).
5. In addition to (4) above, it was found that the anticomplementary activity of antigens was enhanced by an increase in the amount of complement serum used(within demonstrable limits).
6. The anticomplementary activity of antigens for constant amounts of complement remained fairly constant over very wide range of antigen dilutions, in contrast to the activity of anticomplementary sera, which decreases very rapidly with increase in dilution.

These results were very suggestive of antigen-antibody reaction, and it was considered possible that the apparent anticomplementary properties and the instability of antigens were due rather to the presence of antibodies in the guinea pig complement serum.

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ANTIBODY CONCEPT OF ANTICOMPLEMENTARY ACTIVITY

The factors which militated against obtaining definite direct proof for this antibody concept of anticomplementary activity of antigens were twofold:

1. In the early part of the work, the initial lack of experience in the field necessitated reliance upon the results and opinions of previous investigators. Of all the reports that had come to the writer's attention, only that of Miller (60) mentioned complications in the complement fixation system involving use of guinea pig complement containing antibodies (for Brucella bronchiseptica). All the investigators including Miller had apparently assumed the anticomplementary activity of pertussis antigens to be an unfortunate, unpredictable characteristic, entirely separate from other activities; and the only methods suggested to cope with it, when it existed, were either to increase the amount of complement, or to decrease the amount of antigen. No one, till the present time, has so much as attempted to present the basis for such an attitude toward the anticomplementary activity of antigens. This was naturally rather misleading to the writer as a less informed worker.

2. On the other hand, when it had been decided by the writer to disregard the impressions left by previous authors, and to develope the problem independently, it seemed necessary first of all to pretest the guinea pigs. This presented serious technical difficulties:

In the works of Bordet and Sleeswyk (8), Krumwiede et al, (47)

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Toomey & Takacs (86), Dienst (15), Evans & Maitland (18) and Evans (19) could be seen favorable possibilities of employing an agglutination test; but the agglutination phenomenon was unfortunately found to be definitely inadequate, as will be shown later.

From the works of Sprunt et al (82), (83), Culotta et al (12), and Evans & Maitland (18) came the suggestion of culturing material from the air passages for the presence of Br. bronchiseptica which Miller (60) has considered responsible for such antibodies. However, besides other objections (technical and theoretical), there is no evidence that such a culture could give any definite direct information regarding the presence or absence of antibodies in the animal's serum.

To test the guinea pigs by complement fixation seemed the most plausable alternative; but in this case, there was need for an "antibody-free" complement with which to test the guinea pigs. Such a complement, of course, is not readily attainable without first pretesting the animals which are to be used as the source of such a complement. Complement fixation procedures for pretesting guinea pigs, without use of a previously standardized complement, have been studied and described for the "Wasserman" procedures by Holmer (45), Harris(38), Austin (3), Giordano & Carlson (33), Diamond (14), and Wadsworth (88). These methods, however, have the following objections:

They employ a system of "auto-fixation"; and since the complement activity of individual guinea pigs vary greatly, the individual sera are not tested under identical - or standardized - conditions. Furthermore, there is no evidence supporting the assumption that the reacting substance (antibody, reagin) in any given guinea pig serum is necessarily capable of fixing its homologous complement

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in the presence of the test antigen. Also, the amount of complement present in the guinea pig serum, at the dilutions used, may be so high as to make a positive (fixation) reaction impossible, thereby leading one to a false sense of confidence in the sera considered satisfactory.

In view of these inadequacies, other methods of pretesting were sought, methods devoid as much as possible, of assumed factors, and rigidly controlled in every respect. A report on the results of tests on a small group of guinea pigs, employing an earlier pretesting method has already been made by the writer (27). This work served as impetus to more concentrated efforts which resulted in:

Development of reproducible, standardized procedures both for pretesting the sera of guinea pigs to be used for the complement supply, and for detecting H. pertussis antibodies in routine complement fixation tests.

A realization of the importance of undetected, "nonspecific" antibodies in laboratory animals other than guinea pigs, and their possible effect on some of the work that has appeared in the literature.

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METHODS

Equipment and supplies

Besides the usual test tubes, racks and other equipment necessary for performance of serological tests--the Kahn test, for instance-, it was found convenient and more accurate, ever since the very beginning of this work, to employ bent-tipped pipettes like the one described and illustrated in 1943 by Kappaport & Kappaport (73). These can easily be made by heating the tip of the appropriate pipette (0.02 ml., 1.0 ml., and 2.0 ml) sufficiently to allow bending at no more than 5 mm. from the end. The angle of the bent tip should be approximately at 45° with the axis of the pipette; and for the right-handed operator, directed to the right when the pipette is held in position for use.

Reagents and titrations

Distilled water kept in the refrigerator.

Saline: 0.9% NaCl in distilled water.

Antigen: A saline suspension of a smooth ("Phase I") culture of H. pertussis grown on Bordet-Gengou medium. It is once-washed; killed with heat, and employed in a concentration of 20 billion/ml. or less depending on antigenic activity as determined by titration.

Antigen titration: In determining antigenic activity, suspensions of 2, 4, 6, 8, etc., billion/ml are prepared; 0.1 ml. of each is added to 0.04 ml. of a positive serum in serial dilution in Kahn test tubes; 2.0 units of "standard" complement, and 0.1 ml. of saline are added to each mixture, and the tubes are placed in the refrigerator for five hours. Then, they are placed at room temperature for 15 minutes; 0.2 ml. of

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sensitized cells is added; the tubes are incubated in a water bath at 37° C. for 15 minutes and inspected. The titer selected is the lowest concentration capable of maximum fixation, plus one billion; in other words, if an 8 billion/ml. suspension and above is capable of maximum fixation, the test titer is a 9 billion/ml. suspension.

It is not necessary to use a "polyvalent" mixture if the strain on hand has all the characteristics of a smooth ("Phase I") culture. Also, it seems best to store the stock of antigen at 4-8° C in any convenient concentration (100 billion/ml. or more); the required dilution can be made as needed.

Complement: The guinea pigs to be used for complement, preferably should be between 3 weeks and 3 months of age (150-500 grams). They are bled to the desired extent, and by the method considered most convenient. The individual specimens are placed in separate containers, left at room temperature for an hour, and are then transferred to the refrigerator at 4-8° C.

As soon as possible (within 12-15 hours) after the guinea pigs are bled, the sera must be individually tested by the appropriate complement selection procedure to be described later, the satisfactory sera pooled and the complement of the pooled serum stabilized by addition of 80 mg. sodium chloride and 40 mg. boric acid crystals for each ml. volume (26). Every precaution is taken throughout every step to avoid unnecessary rise in the temperature of the sera that are to serve as complement. In preparing the complement for use, the stabilized serum is diluted 1:10 with distilled water, and further dilutions are made with saline.

Complement titration: A series of 25 tubes is arranged (in a Kahn rack) containing increasing amounts of saline in increments of 0.02 ml.; that is, 0.02, 0.04, 0.06, 0.08, etc. proceding from tube 1 to tube 25.

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To each tube is added 0.2 ml. of a 1:20 diluted stabilized complement (prepared by mixing 0.3 ml. of the complement serum with 2.7 ml. of distilled water and 3.0 ml. of saline). After thorough mixing, 0.1 ml. of the resulting dilution in each of the 25 tubes, is transferred to a corresponding tube in a second series containing 0.2 ml. of saline. To each of the latter is added 0.2 ml. of sensitized cells, after which the rack of tubes is shaken, and placed in a 37° C. water bath for 15 minutes.

The complement titer is indicated by the highest numbered tube in which there is complete hemolysis. The titers represented in the tubes (1-25) are 1:11, 1:12, 1:13, etc. to 1:35; and at the titer dilution 0.1 ml. contains 2.0 complement units.

For convenience in performing the tests, three complement dilutions are required.

Dilution I: 0.1 ml. containing 2 units
(Complement at its titer)

Dilution II: 0.1 ml. containing 1.5 units.
(3 parts of Dilution I, plus 1 part of saline)

Dilution III: 0.1 ml. containing 1.0 unit.
(1 part of Dilution I, plus 1 part of saline)

Amboceptor: Antisheep red cell rabbit serum, titrated for the highest saline dilution capable of maximum sensitization of washed sheep red cells.

Sheep cells: a 5% saline suspension prepared from a packed volume of three times washed sheep blood.

Sensitized cells: The required volume of the amboceptor at its titer is poured into an equal volume of the washed sheep cell suspension. The mixture is agitated gently for a few seconds, and allowed to stand

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15 minutes with occasional agitation before use.

Test serum: The clear serum heated at 56° C. for 30 minutes.

Procedure for selection of the "preliminary" complement.

In the absence of a satisfactory ("standard") complement, it is necessary first of all, to obtain a "preliminary" complement, by means of which the sera for the "standard" reagent can be selected.

As soon as the serum begins to separate from the clotted guinea pig blood specimens obtained as already described (see Reagents), an approximately 0.5 ml. sample of each is transferred to other tubes, and cleared by minimal centrifugation. Exactly 0.1 ml. of each clear serum is transferred to a corresponding tube in series, and placed immediately in the refrigerator at 4-8° C.: (these are to be diluted with 3.5 ml. saline (1:36) immediately before use). The remainder of each sample is heated at 56° C. for 30 minutes.

For each guinea pig serum to be tested, a series of 5 to 7 Kahn tubes is arranged in Kahn-test racks, and the reagents are delivered as closely to the bottom as possible (bent-tipped pipette), in the order given in Table I.

TABLE I

Procedure for selection of the "preliminary" complement

		Tube						
		1	2	3	4	5	6	7
Heated guinea pig serum	(ml.)	0	0	0	0	0.04	0.04	0.04
Antigen suspension 15 billion/ml.	(ml.)	0	0	0.1	0.1	0.1	0.1	0.0
Homologous guinea pig serum (1:36)	(ml.)	0.05	0.1	0.05	0.1	0.05	0.1	0.1
Saline	(ml.)	0.25	0.2	0.15	0.1	0.15	0.1	0.2

Note: Tubes 3 and 5 may be omitted if desired.

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The tubes are thoroughly shaken and placed in the refrigerator at 4-8° C. for 5 hours, after which they are placed at room temperature for 15 minutes. To each tube is added 0.2 ml. of sensitized cells; the tubes are shaken and placed in a 37° C. water bath for 15 minutes. The tests are inspected; and the sera to be accepted for the complement pool are indicated by the tests displaying complete hemolysis in tubes 2 and 7, and no more than one+ fixation in either tube 4 or 6. Tube 1 gives indication of specimens that are unusually low in complement activity by displaying partial hemolysis. Tubes 3 and 5 give additional information concerning the "antibody" activity of the serum, but need not be taken into consideration in the final analysis of the tests.

The sera considered satisfactory are pooled, and, if necessary, cleared by minimal centrifugation. The pool is immediately stabilized by addition of the salts, and stored in the refrigerator. This "preliminary" complement can be considered serviceable for no more than two weeks.

Procedure for selection of the "standard" complement.

A "standard" complement cannot be obtained without first having on hand a complement serum previously found satisfactory. A previously selected "standard" complement is preferred, but in its absence, a "preliminary" complement must be obtained and used in the procedure for the selection of the "standard" complement.

Small samples of guinea pig sera, obtained as already described, are heated at 56° C. for 30 minutes; and tests are set up on each as indicated in Table II, delivering the reagents as closely as possible to the bottom of the tubes, and in the order given.

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TABLE II

Procedure for selection of the "standard" complement.

	Tube 1	Tube 2	Tube 3
Heated guinea pig serum (ml.)	0.04	0.04	0.04
Antigen: 15 billion/ml. (ml.)	0.1	0.1	0.0
Complement (units)	1.5	1.0	1.0
Saline (ml.)	0.1	0.1	0.2

Note: Included with these tests is the set of controls to be described later.

The tubes are thoroughly shaken and placed in the refrigerator at 4-8° C. for 5 hours, after which they are placed at room temperature for 15 minutes. To each tube is added 0.2 ml. of sensitized cells, and the tubes are shaken and placed in the 37° C. water bath for 15 minutes. The tests are inspected, and the sera selected for the "standard" complement pool are ordinarily those indicated by the tests in which all three tubes display complete hemolysis. Occasionally, sera with complete hemolysis in tubes 1 and 3, and a ± or a one + fixation in tube 2 may be included; but in this case it is a good policy to plan on replacing such a complement serum with a more satisfactory one as soon as possible within one month.

The sera considered satisfactory are pooled and, if necessary, cleared by minimal centrifugation. The pool is immediately stabilized by addition of the salts, and stored in the refrigerator. With an "antibody-free" complement on hand, one is now prepared to perform the routine complement fixation test.

Technic of the "routine" complement fixation test.

Results of greatest reliability cannot be expected unless a "standard"

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complement, selected as already indicated, is available for the tests.

The tests are set up as indicated in Table III, delivering all reagents as closely as possible to the bottoms of the tubes, and in the order given.

TABLE III

The "routine" complement fixation test

		Tube 1	Tube 2	Tube 3
Test serum	(ml.)	0.04	0.04	0.04
Antigen	(ml.)	0.1	0.1	0.0
Complement	(units)	2.0	1.5	1.5
Saline	(ml.)	0.1	0.1	0.2

Note: Included with the tests is the system of controls to be described below.

The tubes are thoroughly shaken and placed in the refrigerator for 5 hours, after which they are placed at room temperature for 15 minutes. To each tube is added 0.2 ml. of sensitized cells; the tubes are shaken and placed in a 37° C. water bath for 15 minutes.

Upon inspection of the tests, the tube readings are recorded with the symbols, -, ±, +, 2, 3, 4, designating the degrees from zero to complete fixation. Intermediate reactions can be designated with a prime (') mark: a reaction between 2 and 3 would be written 2': between 3 and 4, 3', etc. The final report is estimated according to Table IV.

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TABLE IV

Scheme for final report

Report		Reactions in tube 1	2	3
Negative	from	-	-	-
	to	±	+	-
Doubtful	from	±	2	-
	to	2	3	-
Positive	from	2	4	-
	to	4	4	-

The controls of the complement fixation system

These controls are included daily to give indication of possible changes in the activity of the complement as well as the "anticomplementary" activity of the particular antigen suspension. They consist of two series of seven tubes each, containing the reagents indicated in Table V, and carried through the same manipulations as the tests which they control.

TABLE V

Controls of the complement fixation system.

Complement control series	Tubes 1	2	3	4	5	6	7
Complement (Dil. III)* (ml.)	0.05	0.06	0.07	0.08	0.09	0.10	0.11
Saline (ml.)	0.25	0.24	0.23	0.22	0.21	0.20	0.19
Antigen control series							
Complement (Dil. III)* (ml.)	0.05	0.06	0.07	0.08	0.09	0.10	0.11
Antigen (ml.)	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Saline (ml.)	0.15	0.14	0.13	0.12	0.11	0.10	0.09

* See description of complement under Reagents.

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Tubes 1 to 7 of both series carry corresponding doses of complement; and while there should be a smooth curve of increasing hemolysis from left to right, with complete hemolysis in tubes 6 and 7, the hemolysis in corresponding tubes should be identical. However, a slight difference is allowable; and the system can still be considered as operating satisfactorily provided the degree of hemolysis in any one tube of the "antigen control" is no less than that in the second previous tube of the "complement control."

Other uses for the system of controls: This system of controls is an extremely important part of the complement fixation technic, and it is used experimentally in two ways:

1. With a satisfactory complement on hand, it is used to determine the tolerence of the complement fixation system for a particular lot of antigen. By "tolerence" is meant: the largest amount of antigen which, under the conditions of these controls, causes no significant destruction of very small amounts of complement. As an example of its use in this respect see Table XIII.

2. With a satisfactory antigen on hand the system is used (as in Table XIIc) to determine the suitability of a given lot of complement.

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EXPERIMENTAL FINDINGS

Application of the complement selection procedures.

Over a period of about 2½ years, tests were made on a total of 1,021 guinea pigs and the results are summarized in Tables VI and VII. The series in the respective tables are divided into groups giving the date of bleeding, the number of guinea pigs bled and an analysis of the fixation results in each group. Unless otherwise stated, the tube numbers given refer to the tube of the respective procedure outlined in Table I or Table II.

On the 629 guinea pigs tested by the "preliminary" procedure for complement selection there were 508 for which the results (Table VI) could be divided into two series: (1) based on reactions in tube 4; and (2) based on reactions in tube 6. Also included in a separate part of Table VI are results of tube 6 (only) on the additional 121 guinea pigs. For sake of convenience, the (+) columns indicate the number of guinea pig sera which under the conditions of the test gave a 4 + fixation; the (±) columns, those with less than a 4 + fixation; and the (-) column, those with no fixation.

In this series of 508 guinea pigs, 76.0% of the sera reacted with pertussis antigen in tube 4, while in tube 6 the percentage of reacting sera was 88.4, indicating the system in tube 6 to be more sensitive than that in tube 4, considering the series as a whole.

There were 422 animals tested by the "standard" procedure for complement selection and the results given in Table VII were classified on the following basis: the column (S +) (strong positive), indicates the number of guinea pig sera with which there was a 4 + fixation in both tubes 1 and 2; column (W +) (weak positive), those with which there was at least a 3+ in tube 2

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TABLE VI

Results of tests on 629 guinea pigs in the "preliminary" procedure for complement selection.

Group No.	Date	No. of guinea pigs	Results in tube* 4			6		
			+	±	−	+	±	−
1	1-6-45	37	22	5	10	28	7	2
2	1-16-45	18	5	6	7	16	•	2
3	1-23-45	52	33	9	10	46	3	3
4	2-6-45	36	15	19	2	32	4	•
5	3-6-45	24	18	6	•	23	1	•
6	3-30-45	34	28	6	•	33	1	•
7	4-30-45	20	20	•	•	20	•	•
8	5-2-45	20	20	•	•	20	•	•
9	6-4-45	32	12	18	2	32	•	•
10	6-18-45	16	15	1	•	15	1	•
11	9-17-45	16	10	5	1	8	5	3
12	9-3-45	33	29	4	•	26	7	•
13	1-14-46	40	7	21	12	8	23	9
14	4-29-46	34	7	16	11	29	5	•
15	8-19-46	23	•	•	23	2	4	17
16	10-28-46	23	•	1	22	10	5	8
17	11-12-46	20	15	3	2	18	1	1
18	12-23-46	30	11	7	12	5	8	17
Total		508	247	139	122	364	85	59
%			76.0%		24.0	8[illegible].4%		11.6%
19	7-9-45	28				12	7	9
20	7-23-45	34				16	9	9
21	8-23-45	6				2	2	2
22	2-4-46	30				22	7	1
23	2-11-46	23				11	11	1
Total		121				63	36	22
%						82%		18%

* The tube number refers to the tube described in Table I.

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TABLE VII

Results of tests on 422 guinea pigs in the "standard" procedure for complement selection.

Group No.	Date	No. of Guinea pigs	Results			
			3+	2+	±	-
1	11-14-44	17	6	3	3	5
2	11-21-44	14	6	•	•	8
3	11-28-44	15	5	•	1	9
4	12-5-44	18	15	2	•	1
5	12-19-44	14	5	3	3	3
6	1-2-45	44	35	6	1	2
7	10-1-45	26	9	5	4	8
8	10-22-45	29	7	14	7	1
9	11-19-45	30	7	1	4	18
10	12-16-45	22	8	10	3	1
11	9-4-46	21	2	3	2	14
12	9-30-46	23	12	7	1	3
13	12-23-46	30	10	18	•	2
14	2-3-47	29	5	13	5	6
15	4-1-47	27	6	16	2	3
16	4-8-47	40	11	15	5	9
17	6-16-47	23	7	9	4	3
Total		422	156	125	45	96
%			77.3%			22.7%

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and less than a 3+ in tube 1, column (±) those with less than a 3+ but more than a ± in tube 2, column (-) those with no fixation.

In this series of 422 guinea pigs, 77.3% reacted with pertussis antigens; leaving 22.7% of the animal sera which could be considered satisfactory for use as complement. In other words the results given in both Tables VI and VII show that, in this laboratory at least, a very high percentage of the normal healthy guinea pigs cannot be used to supply complement for the tests with H. pertussis; nor is it possible to predict from time to time how many satisfactory sera will be available. In fact, there was a period of about nine months between 1/2/45 and 10/1/45 (groups 6 and 7, Table VII) when it was not possible to obtain a "standard" complement because it was also impossible to obtain a satisfactory "preliminary" complement (see groups 4 to 12 in Table VI) with which to test the guinea pigs. The proportion of reacting sera seemed to increase from the early part of February 1945, reached a peak in the latter part of April or early in May, and then decreased again, thereby allowing selection of a serviceable complement serum in early October.

Relative sensitivity of the "preliminary" and "standard" procedures.

Among the groups of results given in Tables VI and VII, groups 18 and 13 respectively are the results obtained when the 30 guinea pigs bled on 12/23/46 were tested by both of the complement selection procedures already described with the exception of some slight modifications indicated in Tables VIII and IX respectively in which the essential features are given. The tubes are labeled with letters in order to avoid confusion with tube numbers given in other tables.

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TABLE VIII

Features of the "preliminary" procedure used in tests on 30 guinea pigs bled 12-23-46.

		Tube*		
		A	B	C
Heated guinea pig serum	(ml.)	0	0.04	0.04
Antigen	(ml.)	0.1	0.1	0.0
Homologous guinea pig serum (dil. 1 + 35)	(ml.)	0.1	0.1	0.1
Saline	(ml.)	0.1	0.1	0.2

* Note: Tubes A, B, and C, correspond to tubes 4, 6, and 7 respectively of Table I.

The "preliminary" complement selected by this procedure, was pooled, stabilized within 15 hours of bleeding, and designated for the record as P-23. It was then titrated and found to have 2.0 units per 0.1 ml. in a 1:25 dilution (or 500 units per ml.). On the next day, portions of the same guinea pig sera were tested by means of a modification of the "standard" procedure using P-23 for complement (Table IX).

TABLE IX

Features of the "standard" procedure used in tests on 30 guinea pigs bled 12-23-46

		Tube*				
		D	E	F	G	H
Heated guinea pig serum	(ml.)	0.01	0.02	0.04	0.04	0.04
Antigen suspension 11.8 billion /ml.	(ml.)	0.1	0.1	0.1	0.1	0.0
Complement, (P-23)	(units)	1.5	1.5	1.5	1.0	1.0
Saline	(ml.)	0.1	0.1	0.1	0.1	0.2

*Note: Tubes F, G, and H correspond to tubes 1, 2, and 3 respectively of Table II.

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The results of these two tests on the individual guinea pig sera are given in Table X, in which tube A, (identical with tube 4 of Table I, represents the results of a technic similar to the pretesting technic of Kolmer and others previously mentioned. Though the reactions in column A are in general of a higher level than those in column B (tube 6 of Table I), the test as a whole is definitely less sensitive than the "standard". (F and G being identical with 1 and 2 respectively of Table II).

Generally, the test performed as in tube A, results in a lower grade reaction than the test performed as in tube B (compare results of tube 4 with those of tube 6 in Table VI); but in the case of these 30 guinea pigs the results were reversed. An explanation for this cannot easily be given without a discussion of other experimental data which are beyond the scope and purpose of the present report. Suffice it now to note that both tubes are necessary in order to avoid including in the complement pool, those sera which react in one tube and not in the other. Though 18 specimens gave reactions in tube A, and 14 specimens in tube B, one is not justified in selecting the preliminary complement on the basis of tube A only. There were specimens (No.'s 4, 6, 10, and 25) which reacted in tube B and not in tube A; and which gave a fairly strong reaction in the "standard" procedure.

On the basis of tube A there were only 12 of the 30 guinea pig sera that could be considered satisfactory for complement, while on the basis of tubes A and B the number was narrowed down to 9. By means of the "standard" procedure, however, the number was further reduced to 2; all

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TABLE X

Comparison of results of the "preliminary" and "standard" procedures in tests on 30 guinea pigs.

Serum No.	Procedure: Preliminary — Tube A	Tube B	Tube C	* a	Standard — Tube D	Tube E	Tube F	Tube G	Tube H	* b
1	4	4	-		4	4	4	4	-	
2	3	3	-		3	4	4	4	-	
3	-	-	-	x	-	-	-	-	-	x
4	-	2	-		±	+	4	4	-	
5	+	-	-		-	-	±	3	-	
6	-	±	-	x	±	±	3	4	-	
7	-	-	-	x	-	-	3	4	-	
8	3	-	-		±	+	3	4	-	
9	2	-	-		2	2'	3'	4	-	
10	-	2	-		3	not done		4	+	
11	3	4	-		3	4	4	4	-	
12	4	4	-		4	4	4	4	±	
13	4	4	-		2'	3	4	4	-	
14	4	4	-		4	4	4	4	-	
15	-	-	-	x	2	2'	3'	4	±	
16	4	±	-		2'	3	3'	4	-	
17	4	+	-		2	2'	3	4	-	
18	4	+'	-		4	4	4	4	-	
19	3	-	-		3'	3	2'	4	-	
20	-	-	-	x	±	±	2	4	-	
21	-	-	-	x	±	±	2'	3'	-	
22	4	-	-		3'	3	2'	4	-	
23	4	3	-		4	4	4	4	-	
24	3	-	-		3	2'	+	4	-	
25	-	±	-		3	3'	3'	4	-	
26	-	-	-	x	-	-	-	-	-	x
27	-	-	-	x	-	+	2	4	-	
28	-	-	-	x	-	-	±	3	3	
29	4	-	-		3'	3	2'	3'	-	
30	4	-	-		-	±	2	4	-	

* In column (a) are indicated the guinea pig sera selected for the pool of the "preliminary" complement F-23; and in column (b) those that would have been considered satisfactory for the "standard" reagent.

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of the other 28 guinea pig sera reacted with H. pertussis under the very strict conditions required by this test, thereby indicating its greater sensitivity.

Furthermore, there is another point, involving columns 3 and H, which must be mentioned because apparently it has not come to the attention of previous investigators. These two columns are similar in that they are each an anticomplementary control of the guinea pig serum itself (there is no antigen present). Under the conditions of the "preliminary" procedure, 0.04 ml. of the 30 heated guinea pig sera was in no case anticomplementary in the presence of their homologous complement, whereas under the exacting conditions of the "standard" procedure, four specimens were found to be somewhat anticomplementary for the pool, P-23. In this respect, then, it is not safe to assume that any guinea pig serum which has good complement activity can be considered a satisfactory reagent. Obviously, though, the anticomplementary factors in any given serum may have no effect on their homologous complement, they may be effective in reducing the activity of heterologous complement. Certainly, such sera should not be included in the complement pool.

From an analysis of Table X, it follows, that there are two reasons for using only complement selected by the "standard" procedure, for routine tests in pertussis:

1. Interfering antibodies have been eliminated from the complement, or reduced to a noninterfering minimum.
2. Anticomplementary factors of the guinea pig serum itself have also been detected and eliminated from the complement.

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Effect of selected complement on the complement fixation system.

Due to the practice of severe pretesting of guinea pigs for complement selection it has been possible to increase the sensitivity, efficiency and dependability of the routine complement fixation test by a large margin. In Table XI is given a comparison of the salient differences in the complement fixation system before and after instituting the practice of complement selection.

TABLE XI

Salient differences in the complement fixation system resulting from complement selection

	Before	After
	Practice of complement selection	
1. Antigen: Titration method	Anticomplementary dose	Complement fixing potency
Concentration tolerated by the system	0.04- 2.0 billion/ml.	.25 billion/ml. or more
Frequency of titration	daily	once for each lot
2. Fixation period	18 hours at 4-8° C.	5 hours at 4-8°C.
3. Serviceability of stabilized complement	about 2 weeks	as long as 3 months

As result of employing a selected complement, it was no longer necessary to titrate antigens every day. The routine complement fixation test could be performed within one day using the 5 hour refrigerator fixation period; and the complement remained serviceable as long as 3 months, or practically as long as it still retained a satisfactory complement titer. Especially noteworthy (in Table XI) is the marked increase in the amount of antigen that could be employed in the complement fixation system, as

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result of complement selection, without danger of complications. While the complement fixation system would never tolerate more than a 2.0 billion/ml. suspension when an unselected complement had been used, it would tolerate a suspension as high as 25.0 billion/ml. or more with a selected complement; and it must be kept in mind that the complement is selected chiefly for its lack of interfering antibodies. A good example of the effect complement selection has on the antigen concentration tolerated by the system, is brought out by the following experiment in which a single lot of antigen was tested in varying amounts for its "anti-complementary" activity on varying amounts of four different lots of complement; the latter were chosen because they varied in antibody content.

The "anticomplementary" effect of a typical antigen on different lots of complement.

The four lots of complement chosen for this experiment, are characterized in Table XIIa., which gives the complement lot number, date of bleeding, the number of guinea pigs bled, the number of sera in each lot and the resulting volume. Also given are the respective titers and ages of the lots at the time of the experiment.

TABLE XIIa

Description of four experimental lots of complement.

Complement No.	Date of Bleeding	No. of Animals	No. of sera in pool	Volume	Titer*	Age* of Serum
P-26	4-3-47	40	10	28.5 ml.	1:14	3.5 weeks
P-25	4-1-47	27	4	19.0 ml.	1:15	4.5 weeks
P-23	12-23-46	30	9	23.0 ml.	1:10	18.5 weeks
S-50	4-1-47	27	6	25.0 ml.	1:18	4.5 weeks

* Note: at time of the experiment.

In Table XIIb are given the reactions obtained when the sera included

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in each pool were tested by the "standard" complement selection procedure; and the pools are given from left to right in order of increasing antibody content as judged purely by inspection.

TABLE XIIb

Reactions of "standard" complement selection procedure on the guinea pig sera composing the four experimental pools.

P-26			P-25			P-23			S-50		
Tube											
1	2	3	1	2	3	1	2	3	1	2	3
–	–	–	–	–	–	–	–	–	+	+	–
–	–	–	2'	4	–	3	4	–	–	–	–
–	–	–	–	+	–	3	4	–	4	4	–*
–	–	–	4	4	–*	4	4	–	4	4	–
–	–	–				2	4	–	3	4	–
–	–	–				2'	3'	–	–	±	–
–	–	–				–	–	–			
–	–'	–				2	4	–			
–	–'	–				±	3	3			
–	–	–									

* In agglutination tests these were the only two sera that agglutinated a suspension of H. pertussis antigen.

With the four complement sera characterized as in Tables XIIa and XIIb, the experiment was set up as indicated in Table XIIc. The antigen employed in this experiment was the very same lot that had been used to test the animals composing the four experimental pools; and one unit of it was equal to 0.1 ml. of an 11.8 billion per ml. suspension. The values of 0.25, 0.5, 1.0, 2.0 and 4.0 units specified in Table XIIc corresponded to concentrations (in organisms per ml.) of 2.9, 5.9, 11.8, 23.6, and 47.2 billion respectively.

In this experiment, the reagents were brought together in the usual total volume of 0.3 ml., and allowed the usual incubation periods before and after addition of the sensitized cells. The results given in the table are in terms of percentage hemolysis, (-) indicating complete and (0), no hemolysis.

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TABLE XIIc

The "anticomplementary" effect of one lot of antigen on four different lots of complement.

Complement No.	Units of Antigen	Units of Complement 0.5	0.7	0.9	1.0	1.1
P-26	0.0	70	95	99	-	-
	0.25	70	95	99	-	-
	0.5	70	95	99	-	-
	1.0	70	90	99	-	-
	2.0	40	60	95	-	-
	4.0	0	0	50	80	92
P-25	0.0	80	92	99	-	-
	0.25	80	92	99	-	-
	0.5	60	90	99	99	-
	1.0	60	75	95	-	-
	2.0	0	0	0	0	50
	4.0	0	0	0	0	0
P-23	0.0	80	90	99	-	-
	0.25	60	70	97	-	-
	0.5	40	60	80	95	99
	1.0	0	0	50	70	80
	2.0	0	0	0	0	50
	4.0	0	0	0	0	0
S-50	0.0	70	95	99	-	-
	0.25	0	50	60	70	75
	0.5	0	0	20	50	60
	1.0	0	0	0	0	0
	2.0	0	0	0	0	0
	4.0	0	0	0	0	0

Note: (-) equals 100%, and (0) equals no hemolysis.

The first series in each of the four groups of reactions in Table XIIc gives the percentage hemolysis due to the specified amounts of the respective complement serum alone; and serves as a control on the other five series of the group. A comparison of all four control series, shows the curves of hemolysis to be identical even though the complement sera had to be used at different titers.

Within each group there was an increase in inhibition of complement with increase in amount of antigen added. This inhibition was most marked for complement S-50, less for P-23, P-25 and least for P-26. There was no parallelism between the inhibitive effect of the antigen and the respective

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complement titer, for as indicated in Table XIIa, S-50 had the highest titer, and P-23 the lowest. On the other hand, since the antigen was least inhibitive for P-26 (complement serum known to contain a very low level of antibody), and more inhibitive for the other complement sera (known to contain appreciable levels of antibody), it seems very certain that the amount of antigen tolerated by the system is inversely dependent upon the level of the antibody content of the complement serum. This is further illustrated by the summary of the results of this experiment given in Table XIId in which is shown the antigen concentration tolerated by each lot of complement.

TABLE XIId

Variation in antigen concentration tolerated by various lots of complement.

Complement	Order of antibody content	Maximum concentration of antigen tolerated
P-26	least	23.6 billion/ml.
P-25		11.8 billion/ml.
P-23		2.9 billion/ml.
S-50	most	much less than 2.9 billion/ml.

Most significant is the very extreme difference between the amount of antigen tolerated by P-26, a selected "pertussis" complement, and that tolerated by S-50, a selected "Wasserman" complement; 0.25 units (2.9 billion/ml.) of H. pertussis antigen were more inhibitive for S-50 than were 4 units (47.2 billion/ml.) of the very same antigen for P-26 ---- in other words ---- more than a 16-fold difference. Such activities definitely cannot be attributed to changes in the antigen.

Effect of antigen concentration on sensitivity of the complement fixation test.

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With a selected complement on hand, therefore, it is possible to employ larger amounts of antigen; and the effect of larger amounts of antigen on the sensitivity of the complement fixation test is illustrated in Table XIII.

TABLE XIII

Effect of antigen concentration on sensitivity of the complement fixation test.

Test system									
Complement Units*	ml. of serum	Antigen (billion/ml.)--0.1 ml. used							
		16.5	14.1	11.8	8.5	4.7	1.2	0.6	0
1.5	0.04	4	4	4	4	4	3	2	-
1.5	0.02	3	3	3	3	3	2	±	-
1.5	0.01	+	+	+	+	±	±	-	-
1.5	0.005	±	-	-	-	-	-	-	-
Control system									
0.5	0	60	60	70	70	80	80	80	70
0.8	0	95	95	99	99	99	99	99	97
1.0	0	-	-	-	-	-	-	-	-
1.5	0	-	-	-	-	-	-	-	-

Note: The results in the test system are given in the usual terms of plus's.

The results in the control system are given in terms of % hemolysis: (-) equals 100%

*: Complement P-26, obtained one month previous was used here, at a titer of 1:14.

The tests set up as indicated in Table XIII were carried through the usual incubation periods, etc., and as result of this experiment it is seen that the sensitivity increases with an increase in amount of antigen to include a concentration of 8.5 billion/ml. Beyond this concentration there was no further increase in sensitivity of the test. Meanwhile, the effect of the antigen on the complement alone (see control system) remained practically constant for all concentrations. In other words, though the system would tolerate concentrations of antigen as high as 16.5

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billion/ml., it was not necessary to use much more than an 8.5 billion/ml. suspension for maximum sensitivity. Concentrations less than 8.5 billion/ml., however, resulted in a test of lower sensitivity.

Sensitivity of complement fixation compared to agglutination in tests on animal sera.

In studying the phenomenon of agglutination, several manipulations were tried and it was found that for guinea pig and other animal sera, heated or unheated, a 10 billion/ml. suspension of H. pertussis produced a more permanent reaction than a 20 billion/ml. suspension. A period of shaking followed by refrigerator incubation was also found necessary for maximum reactivity of antigen with serum, although the readings after refrigeration were often difficult to interpret. Therefore, the technic employed was as follows: 0.1 ml. of the antigen suspension was added to 0.04 ml. (or less) of serum in Kahn test tubes; the tubes were shaken at the rate of 100-150 oscillations per minute over a wide arc for five minutes; the tests were read immediately, again after 24 hours at 4-8°C., and, in some intances, once more after 72 and/or 96 hours at 4-8°C.

Some of the data accumulated with regard to agglutination tests on guinea pig sera have already been indicated in Table XIIb in which it was shown that only two, of all the guinea pig sera represented there, were capable of agglutinating a suspension of H. pertussis. This in itself is evidence enough against reliance upon such a test for complement selection; but it scarsely begins to show the true status of the situation.

In a series of 172 guinea pig sera tested both by agglutination and complement fixation, there were only 6 which agglutinated a suspension of H. pertussis antigen at one stage or another of the technic used, while 144 reacted in the "standard" procedure for complement selection. Of

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considerable interest is the fact that the contents of the tubes of the complement fixation tests, could be read for agglutination at the end of the 5 hour fixation period. The sensitivity of agglutination under these conditions was found to be as high as it was in separately performed tests employing the 5 minutes shaking period; but slightly less than the full technic described above. In these and many other tests to be reported below, at no time was there evidence of agglutination, without evidence of complement fixation; all sera that agglutinated suspensions of H. pertussis also fixed complement.

Since a high percentage of "normal" guinea pigs had been found to have antibodies capable of reacting with pertussis antigens, especially in the rigidly controlled complement fixation test described for complement selection, it seemed almost mandatory that sera of other species of laboratory animals be tested.

Tests on laboratory animals other than guinea pigs.

Unless otherwise stated, the results of complement fixation given are those of the "routine" test (see Table III). This test is less sensitive than the "standard" complement selection procedure (Table II) used in tests on guinea pig sera, because the respective tubes of the routine test contain 0.5 units of complement more than those of the latter.

Mice: These animals were bled under anaesthesia by cardiac puncture, and the sera tested individually to avoid any possibility of gaining the false impressions that sometimes result from testing pooled sera. Of 62 apparently normal mice tested, all were negative both by the routine complement fixation test and by agglutination. Tests were also made on sera of 235 mice immunized with H. pertussis. The results given in Table XIV show that of 93 immunized mice tested by complement fixation alone, only

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4 were negative; of the other 137, only 13 were negative by complement fixation while 7% were negative by agglutination.

Sheep: Sera of 13 supposedly "normal" sheep were tested by agglutination, and by both the routine complement fixation test and the "standard" complement selection procedure. The results given in Table XV, indicate that while about half (7) of the sheep sera were negative in agglutination tests, only 3 were negative in the routine complement fixation test, and none under the very strict conditions of the "standard" complement selection test.

Rabbits: The results of tests on the sera of 19 "normal" rabbits are given in Table XVIa in which it is shown that while 12 to 15 sera were negative by agglutination, only 7 were negative in the routine complement fixation test.

Of 37 rabbits immunized with H. influenzae (Table XVIb) all sera were found to react with H. pertussis in the routine complement fixation test, while only about 1/3 of them gave definite reactions in agglutination tests.

Horse: Only one "normal" horse serum, a pool of two horse blood specimens was available for testing. The result in the routine complement fixation test was strong enough for a "doubtful" report; and in the "standard" complement selection procedure, for a "positive" report. This serum also agglutinated a suspension of H. pertussis.

As result of the tests on all the animals it is evident that not only do guinea pig sera have antibodies capable of reacting with H. pertussis antigen, but the sera of other laboratory animals do as well. In order to detect such antibodies, however, the agglutination test is totally inadequate. Evidence of this has been shown in the above tables of results.

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TABLE XIV

Results of complement fixation and agglutination tests on mice immunized against B. pertussis.

No. of mice	Complement fixation test				Agglutination 5 hours at 4-8°C. **		
	+	±	-	ac*	+	-	NT
98	88	2	4	4	•	•	98
137	108	12	13	4	65	72	•
235	196	14	17	8	65	72	98

*(ac): anticomplementary

**: The tubes of the complement fixation test read for agglutination at the end of the 5 hour fixation period.

NT: Tests not examined for agglutination.

TABLE XV

Results of complement fixation and agglutination tests on sera of 13 "normal" sheep.

Report	Complement fixation		Agglutination ***		
	Routine test*	Complement selection test**	5 hours 4-8°C.	5 min. shaking + 24 hours 4-8°C.	
+	5	9	5	6	6
±	4	3	2	•	•
-	3	•	6	7	7
ac	1	1			

* The results given are based on the stipulations in Table IV for reports on the routine complement fixation test.

** The results given are classified as indicated for the tabulation of Table VII.

*** The results given for the 5 hours at 4-8°C. represent the agglutination readings in the tubes of the complement fixation test at the end of the fixation period.

ac: Anticomplementary.

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TABLE XVIa

Results of complement fixation and agglutination tests on sera of 19 "normal" rabbits.

Report	Complement fixation test	Agglutination		
		5 hours 4-8°C.	5 min. shaking +	24 hours 4-8°C.
+	8	4	4	5
±	4	•	•	2
-	7*	15	15	12

* Three of these sera reacted to a slight extent in the more sensitive tube of the test.

TABLE XVIb

Results of complement fixation and agglutination tests on sera of 37 rabbits immunized with H. influenzae.

Report	Complement fixation test	Agglutination		
		5 hours 4-8°C.	5 min. shaking +	96 hours 4-8°C.
+	36*	5	6	4
±	1	8	6	25**
-	•	24	23	8

* Of these 36 tests, all but 11 gave a 4 + in both tubes. The weaker reacting sera, however, gave at least a 3 + under the less sensitive conditions in tube 1 of the complement fixation test.

** In these 25 tests the agglutinum was rather indefinite and easily dispersed on agitation.

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DISCUSSION

The results of tests on the sera of laboratory animals corroborate -- qualitatively, at least--the reports of other authors. Bordet et al (8), and Toomey et al (96) found "normal" horse sera to have (H. pertussis) antibodies; Bordet et al (8), Evans et al (18), and Miller (60) found such antibodies in guinea pig sera; and Chievitz et al (11), Ferry et al (21), Dienst (15), Evans (19), Evans et al (18), Huenekens (39), Kristensen (46), Long et al (54), Miller (60), Olmstead et al (70) found rabbit sera to react with H. pertussis antigens. However, since the tests employed by these investigators were not necessarily of very high sensitivity, it is possible that their results were quantitatively deficient, and they perhaps were not able to see the complete picture with all its implications. These implications, therefore, warrant a brief discussion which must necessarily follow along two paths: one directed toward the organism or organisms, if any, involved in the production of the "antibodies" in the supposedly "normal" animals, and the second toward the complications(possible and actual) resulting from the use of such experimental animals in studies with H. pertussis, regardless of the origin of the antibodies.

There are several reports in the literature in which the organism, Br. bronchiseptica, has been directly or indirectly incriminated for

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complications in studies with H. pertussis. L'Cowan (59) has found this organism in many animals, including rabbits, guinea pigs, and goats; but not in rats and mice. Smith (80) also had experience with this organism in guinea pigs and rabbits. Rhea (74) stated that Br. bronchisep-tica may be present in guinea pigs that have never shown evidence of infection with it. Singer-Brooks & Miller (79) have made reference to the "long-known" interrelationship between Br. bronchiseptica and H. pertussis, perhaps thereby implying knowledge of the work of Ferry et al (23) who found Br. bronchiseptica widely distributed among rabbits and guinea pigs, and showed (21) (22) close interrelationship between these organisms. As result of their work, it seems that Br. bronchiseptica is more likely to produce pertussis antibodies, than H. pertussis is to produce bronchisep-tica antibodies. Miller (60) has directly incriminated Br. bronchiseptica for the interfering antibodies which he found ("occasionally") present in the complement serum. This report of Miller adds considerable weight to the statement of Smith (80) written in 1913: "The importance of the guinea pig as a laboratory reagent to toxins and living bacteria is very great today---and the time has come to all attention to the necessity for rid-ding the species of those infections which tend to interfere with inves-tigations---."

Considering the pertussis problem as a whole, is it not likely that other organisms, known or yet to be known, might likewise have exerted an undetected influence on many of the investigations reported in the liter-ature? In a report by Sprunt (83) it is implied that the presence of Bacillus leoisepticum (82) besides Br. bronchiseptica in "normal" rabbits may interfere with H. pertussis experiments. Odaira (67) has mentioned Bacillus hemoglobinophilus canis as an organism related in some way to H. pertussis. Eldering & Kendrick (20) described a parapertussis organism

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of human origin, related antigenically and in other ways to H. pertussis; and Miller (61) also found this organism among human cases diagnosed as "whooping cough". Mizuka (40) isolated 12 strains of organisms from whooping cough patients which he was able to classify in five groups. None of them were related to H. influenzae, nor in any degree of certainty, to H. pertussis. On the basis of these reports, it seems certain that until all the organisms related to H. pertussis are actually known and their interrelationships exhaustively studied in rigidly controlled investigations, it is not safe to incriminate, or to hold any particular species responsible for interferences in studies with H. pertussis. Such incrimination is especially not warranted on the basis of work, reported in the literature, in which the animals used had not been rigidly pre-tested.

Regardless of the causative agent, it is obvious, from the data presented in this report, that in employing laboratory animals, their blood or serum in experimental work with H. pertussis, one may risk a complication which may have a marked effect on the final results and consequently lead to erroneous conclusions. It would be superfluous, as well as almost impossible to discuss any significant number of investigations with regard to the manner in which the factor of "normal" animal antibodies may have influenced the results because, in the first place, it has undoubtedly been possible in some cases for investigators to set certain standards, on a basis of previous experience, whereby the influence of this factor could be reduced to a non-interfering minimum even without actual knowledge of its existence. Secondly, the complete information required for such a discussion is often lacking in the literature.

On the other hand there are undoubtedly certain instances in which

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the "normal" animal antibodies have influenced the course of various investigations much in the same way as the antibodies in guinea pig sera have influenced the behavior of the complement fixation system. The degree to which this has taken place would of course differ with different stocks of animals, depending upon the antibody level of the group as well as of the individual animals. In this laboratory, for instance, young normal mice have been found to be 100% free of detectable (H. pertussis) antibodies. This may not necessarily be true for stocks available to other workers. It would, therefore, be well to mention briefly the types of manipulations through which the influence of such "normal" antibodies may unwittingly be thrown into the investigations.

1. Use of "normal" animal blood and sera for preparation of the culture media to be used for the study of cultural characteristics of the test organisms, and upon which the antigens for subsequent work are to be grown.
2. Use of "normal" animals (rabbits and guinea pigs especially) to determine the potency of antigens and their products (toxins) by means of mortality and morbidity (including skin) tests.
3. Use of "normal" animals for preparation of antisera to be used especially in animal (usually mouse) protection tests or in studies of antigenic interrelationships among strains and phases of H. pertussis, or between H. pertussis and other organisms.

Since the presence of blood is necessary for the propagation of H. pertussis, can the antibodies present in the blood used for preparation of the media, have some influence on the antigenic structure of this organism? Tooney & Tokacs (87) and Wu et al (92) have experienced marked

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deleterious effect of horse blood on H. pertussis cultures after repeated transfers on their media. That antibodies in the media do have an effect on H. pertussis has been shown by Bordet & Gengou (6) in 1907 in some work with liquid media. They found that H. pertussis does grow in the presence of its antiserum (serum heated at 56°C.) in liquid medium; but, as it grows, it becomes agglutinated and falls to the bottom of the flask. Could antibodies in the medium be a factor contributing to the phenomenon of "auto-agglutination" that has been reported by various authors from time to time? Organisms grown in the presence of antibodies could conceivably adsorb these antibodies from the medium, and agglutinate spontaneously when suspended in saline. What also can be said about the use of such antigens with adsorbed antibody in other work? The answers to these and other questions certainly cannot be found without rigidly controlled experimentation.

In employing "normal" animals to study the toxic properties of H. pertussis or its products, could not the antibodies present in the animal influence the course of the end results? The discordant conclusions in the literature with respect to this phase of study might easily have been due to differences in the level of undetected "natural" immunity of the various stocks of animals.

In the use of "normal" animals for production of specific antisera, to what extent can one expect the resulting immune sera to be specific for the product injected, if the animals already have immunologically related antibodies in their system? Might it not be possible for the product injected rather to increase the titer of the immunologically related antibodies that are already present? For instance, Evans (19), Teissier et al (39), Katsampes et al (42), Roberts et al (75),

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Silverthorne et al (78), etc. claimed that they were able to produce antibodies, (in some instances, antibacterial antibodies) upon injection of pertussis toxin, in one form or another, into experimental animals, while it seems from the work of Bordet et al (7), Lapin (51) and others that pertussis toxin has little or no antigenic properties. The fact that Flosdorf et al (25) consider pertussis toxin not very antigenic in humans is rather significant because apparently the antigenicity of pertussis toxin has been demonstrated only in laboratory animals, ("normal", healthy, and uninitiated). From this also follows the possibility that organisms which may be totally unrelated antigenically may appear to be related through the medium of undetected "normal" antibodies already present in the animals used for preparation of the "specific" antisera. This point can best be emphasized by considering the experiences of Smith (80) with infections in laboratory animals, and his statement that "An acquaintance with these infections is essential in avoiding serious misinterpretations--in the immunization of animals for agglutinins and other antibodies."

Judging from the results presented in this report, with regard to the study of guinea pig sera especially, it is evident that this statement of Smith is far more inclusive than the words imply. Certainly, in order to perform a satisfactory complement fixation test in a study of experimental material, it is necessary to have on hand a complement serum which is free of antibodies that are capable of reacting with the test antigen. Herein at least lies a plausable explanation for the results obtained, during the course of preliminary work, in studying the nature of the anti-complementary action of H. pertussis antigens.

1. The variation in anticomplementary activity with different lots of complement due, as indicated in Table XIIc, to differences

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in the antibody content of the complement sera.

2. The anticomplementary activity of antigen for constant amounts of a given complement remained constant over a wide range of antigen dilutions because constant amounts of a serum contain a constant level of antibody.

3. The increase in the anticomplementary activity of antigens with increase in the amount of complement serum used, was due to increase in the amount of antibody available in the larger amounts of serum.

4. The anticomplementary activity of antigens increased with the age of the complement serum because of the decrease in complement activity which necessitated use of larger amounts of complement serum thereby increasing the amount of antibody available to the antigen.

A complement serum, which avoids such complications as these, is evidently assured by the complement selection procedures described here.

Upon superficial analysis of some of the data given (Tables VI and VII) it would seem that the "standard" procedure has little advantage over the "preliminary" procedure in complement selection since it was shown that 22.7% of the guinea pig sera tested by the former could be considered satisfactory for use as complement, while on the basis of tube 4 of the latter there were 24% which could be considered satisfactory for the complement supply. In fact, the "standard" procedure seems to be much less sensitive than tube 6 of the "preliminary" procedure in which only 11.6% to 18.0% of the total were satisfactory; and in such a comparison, the "standard" procedure would seem to have no value.

However, is it safe to assume that the animals represented in Table

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VI and VII are comparable, just because they were obtained from the same source, bred and raised in the same colony? There are undoubtedly many unknown and unpredictable variables involved in the handling and housing of the animals which can affect their serological picture, especially if the responsible agent is a bacterium (Br. bronchiseptica), the communicability of which is not easily controlled in the breeding pens and stock cages.

Evidence of this is the marked variation in results among the various groups within each series. In Table VI, for instance, while there are several groups (5, 6, 7, 8, 10, 12) in which there were no non-reacting guinea pig sera, there are others (13, 15, 18) in which there was a good percentage of them. Likewise in Table VII there is considerable variation in the percentage of negative guinea pigs among the various groups. It would be natural to suspect variations in reaction among the groups tested by one given procedure, to be due to variation in the procedure; and due to lack of definite evidence to the contrary, such a possibility cannot safely be denied. On the other hand, the conditions of the tests were rather rigid and did not allow much chance for variation, especially since no one but the writer has had any part whatsoever in performing them, thereby, also eliminating some technical factors. Therefore, until the conditions in the breeding and stock cages can be rigidly controlled, the only way in which the two procedures can be compared is by performing both of them on the same animal sera as was done with the group of 30 guinea pigs bled 12/23/46.

From the results given in Tables VI and VII it would seem that, under these very rigid conditions of pretesting, a very low percentage of guinea pig sera would be found serviceable as complement with a consequent low

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supply of the reagent. In this laboratory, this has unfortunately been true; but it would not necessarily apply to other stocks of guinea pigs where the situation may well be different. On the other hand, it would be utter fallacy to rely upon less stringent methods of pretesting just because such methods allow one a greater supply of complement. Regardless of whether or not the pretesting method is capable of detecting them, the interfering antibodies may still be present; and what is more, their presence will still allow them the opportunity to interfere with the balance of the system. This has already been illustrated by the work with P-23, a "preliminary" complement, selected by a procedure even more stringent than is considered necessary by Kolmer (45) Harris (38) and the others for selection of the "Wasserman" complement. Some of the sera pooled for this complement still contained antibody in high enough concentration to be detected by the very rigid "standard" procedure (Table XIIb), and to cause marked effect on the amount of antigen tolerated by the system (Tables XIIc and XIId).

Furthermore, if the antibody content of the guinea pig serum is totally ignored, and the test unit of antigen is determined by the "anticomplementary dose method," one can expect marked variation in the antigen concentration tolerated by the test system from day to day,(depending on the antibody content of the guinea pig serum), as well as risk the possibility of having a system which will not tolerate the quantity of antigen necessary for maximum sensitivity. Explanation for this is simple. By resorting to the "anticomplementary titration" method proposed by other authors, the value obtained in titrations is the largest amount of antigen, which, in the presence of complement serum alone, is just barely capable of retarding complement activity to an appreciable extent. The proponents of such a method usually recommend, as a test dose, an amount equal to 1/3

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or 1/4 the anticomplementary dose. Since the evidence already presented indicates such "anticomplementary" activity to be due to antibodies in the complement serum, such a test dose is in reality 1/3 to 1/4 of the largest amount of antigen capable of reacting with these antibodies to such an extent as to bring about an appreciable fixation of the complement which is also present in the given amount of guinea pig serum. Naturally, if the complement serum contains a high concentration of antibody, the so-called "anticomplementary" activity is correspondingly high; and only small amounts of antigen can be tolerated. On the other hand, if the complement serum has a low concentration of antibody, correspondingly larger amounts of antigen can be tolerated. In this laboratory, chances for the occurence of the latter condition have been very rare for as indicated in Table XI it had never been possible, before adoption of the complement selection procedure, to employ antigens for complement fixation tests, in concentrations above 2 billion/ml. (equivalent to 400 million organisms per tube of the test).

Evidence in the literature indicates that other investigators have found it equally impossible to use the higher concentrations tolerated by a system in which a selected complement is employed. It is rather difficult to analyse many of their reports in terms of amounts comparable to those employed by the writer, because most investigations employed "1/3 to 1/4 of the anticomplementary dose" (which, of course, gives no indication of numbers of organisms); but a few examples can be given. Chievitz & Meyer (11) were able to use 400 million organisms for each tube of their test; and the same was true in the work of Jacobsen & Meyer (41) who used the same procedure. Eaton (72) was able to use 200 million, Olah & Kunsagi (68) 100 million, organisms for each tube of the tests. Other authors have specified the concentration but have not given sufficient data from

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or 1/4 the anticomplementary dose. Since the evidence already presented indicates such "anticomplementary" activity to be due to antibodies in the complement serum, such a test dose is in reality 1/3 to 1/4 of the largest amount of antigen capable of reacting with these antibodies to such an extent as to bring about an appreciable fixation of the complement which is also present in the given amount of guinea pig serum. Naturally, if the complement serum contains a high concentration of antibody, the so-called "anticomplementary" activity is correspondingly high; and only small amounts of antigen can be tolerated. On the other hand, if the complement serum has a low concentration of antibody, correspondingly larger amounts of antigen can be tolerated. In this laboratory, chances for the occurence of the latter condition have been very rare for as indicated in Table XI it had never been possible, before adoption of the complement selection procedure, to employ antigens for complement fixation tests, in concentrations above 2 billion/ml. (equivalent to 200 million organisms per tube of the test).

Evidence in the literature indicates that other investigators have found it equally impossible to use the higher concentrations tolerated by a system in which a selected complement is employed. It is rather difficult to analyze many of their reports in terms of amounts comparable to those employed by the writer, because most investigations employed "1/3 to 1/4 of the anticomplementary dose" (which, of course, gives no indication of numbers of organisms); but a few examples can be given. Chievitz & Meyer (11) were able to use 400 million organisms for each tube of their test; and the same was true in the work of Jacobsen & Meyer (41) who used the same procedure. Paton (72) was able to use 200 million, Clah & Kunsagi (60) 100 million, organisms for each tube of the tests. Other authors have specified the concentration but have not given sufficient data from

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which to calculate the number of organisms used per tube: Daughtry-Denmark (13) used a 10 billion/ml. suspension; Donald (16) a 2 billion/ml. suspension; Evans & Maitland (17) an 800 million/ml. suspension.

It has been shown (Table XIIe) that with a typical antigen such as the one employed, the concentration of at least 6.5 billion/ml. (or 650 million organisms per tube of the test) was necessary for greatest sensitivity of the system. A concentration of 4.7 billion/ml. (or 470 million per tube of the test---70 million more than the amount used by Chievitz & Meyer (11)---) resulted in a less sensitive system, and if the writer's estimation of the amounts employed by other workers is correct, it seems rather certain that very few, if any, of them were able to employ the quantity of antigen necessary for maximum sensitivity. Judging by the writer's experience, therefore, it is very likely that they, too, had been dealing with antibodies present in the complement sera; antibodies which have defied detection by the methods available at the time. If this is true, it is also evident that such antibodies could have been largely responsible for the false reactions reported in the literature: false negatives due to low tolerence of the system for sufficient quantities of antigen; and false positives due to (partial) fixation of complement by the antibodies contained in the complement serum.

During the course of the work leading to the present report, it was also found that reacting substances in guinea pig sera interfered greatly with complement fixation tests for syphilis and gonorrhea, necessitating selection of a "specific" complement for each antigen used. The importance of this cannot be overestimated, since it is very possible that failures in tests for other diseases may have been due to failure to eliminate from the complement supply, those sera which have "antibodies" capable of reacting

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with the particular antigen employed. The writer does not intend from this discussion to minimize the value of complement fixation results reported in the literature; he merely intends to point out that unless the authors are certain of an "antibody free" complement, many of the conclusions derived from their work are not necessarily well founded; and the same could be true with regard to other work involving animals that were not highly protected.

With H. pertussis especially, there is still another fact only superficially treated in this report, and deserving of further emphasis (see page 25--controls of the complement fixation system).

One may at times encounter antigens which are not very highly tolerated by a complement serum known to be satisfactory for a previous antigen. Such antigens have come to the writer's attention and in his experience have been found to behave, in the presence of a known satisfactory complement, very much as do satisfactory antigens in the presence of a known unsatisfactory complement. In other words, they do behave in a manner suggesting the presence of antibodies. Could it is be due to antibodies adsorbed on the surface of the organisms?

Since the writer does not have sufficient data to answer this question or to support further discussion of this matter, it must be left for a future report. Suffice it now to say that in order to select a satisfactory complement one must first be certain of a satisfactory antigen. In the writer's experience, such an antigen has not been difficult to obtain, from growth of a known ("Phase I") smooth H. pertussis culture on Bordet-Gengou medium.

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SUMMARY AND CONCLUSIONS

In much preliminary work leading to the present report, there were encountered marked inconsistencies in the behavior of H. pertussis antigens, which prohibited establishment of a standardized complement fixation procedure with any degree of finality. Similar difficulties have also been a common occurrence in the studies of other investigators, and this has resulted in many conflicting reports. The results of the present study, however, cannot fail to play a major role in solving some of the problems involved because of the following.

1. Results of preliminary work indicated that it was necessary to establish a procedure whereby a complement serum known to be (H. pertussis) antibody free could be obtained.

2. Such a procedure was devised, and by means of this procedure it was found that the anticomplementary activity of antigens was rather due to antibodies present in the complement serum. It was also evident that a very high percentage of the guinea pigs tested carried such antibodies.

3. Though evidence in the literature seems to point to Br. bronchiseptica as the agent chiefly responsible for these antibodies, other pretesting methods, such as culture for these organisms from the air passages, agglutination tests, etc., are entirely inadequate for selection of complement.

4. By use of a rigidly selected complement, it was possible to employ concentrations of antigen for greater than any reported in the literature. Also, concentrations far in excess of that necessary for maximum sensitivity of the test had no deleterious effect on the complement.

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5. In complement fixation tests on sera of other animals, it was found that the "normal" rabbits, sheep, and horses in this laboratory have (H. pertussis) antibodies, while the normal young mice do not. These results, however, do not necessarily apply quantitatively to animals available to other workers, and it is advisable that all stocks be pretested in order to avoid the conceivable complications which may result from use of (partially) immune animals in pertussis studies.

6. It is also recommended that the guinea pig sera to be used for complement in tests with other antigens be similarly selected.

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