STUDIES WITH H. PERTUSSIS

V. Agglutinogenic Relationships of the Phases

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In 1931, Leslie and Gardner concluded, on the basis of a study of 43 strains of *Hemophilus pertussis*, that the organism as isolated from clinical whooping cough is of a single type. They further concluded, however, that the organisms on laboratory-culture media can undergo a semi-reversible change through four distinct phases, each demonstrable serologically as well as on the basis of virulence and toxicity. Their latter observations were in conflict with the conception of Bordet and Sleeswyk (1910) that only a single variant results from subculture of stock strains on a blood-free medium. The work of Leslie and Gardner (1931), did, however, reconcile the work of Bordet and Sleeswyk (1910) with the conflicting view of Krumwiede, Mishulow and Oldenbusch (1923) that there were two permanent types which bore no relation to culture medium. Apparently the two permanent types were two avirulent phases, Type A being a Phase IV and Type B a Phase III organism. However, Toomey, Ranta, Robey and McClelland (1935) and Toomey (1937) reported their inability to confirm the work of Leslie and Gardner (1931) with respect to distinct or definite degraded phases of existence and further disputed the work on a theoretical basis. We cannot agree with the premises on which the dispute is based. Toomey *et al.* (1935 and 1937) have stated that if agglutination titer is the basis of Phases, there are theoretically on a mathematical basis 25,000 Phases possible with a serum of 1:25,000 titer, these being merely modifications between the virulent and avirulent or "resting" state. This criterion of

titer was not that which Leslie and Gardner (1931) used, but rather the *qualitative* differences in specificity which they observed.

More recently, Eldering and Kendrick (1937, 1938) and Bradford and Slavin (1937) have isolated from cases of whooping cough a "Para" form of the organism, called atypical by the latter authors. This "Para" form apparently cannot exactly be considered as another type of *H. pertussis*, as perhaps is suggested by the terminology of Bradford and Slavin (1937), because its growth requirements and characteristics upon fresh isolation are so different. Further, the biochemical reactions are slightly different, *H. pertussis* being essentially inert on the basis of the usual criteria, whereas the "Para" form produces catalase and also a brown pigmentation in the plain agar or broth culture medium in which it is grown. On this basis there would appear to be a species difference. Because of this, and also because of the serological relationship of the freshly isolated strains to Phase I, *H. pertussis*, Eldering and Kendrick (1937, 1938) have felt that these "Para" organisms bridge the gap between *H. pertussis* and *Brucella bronchiseptica* and that *pertussis* and *bronchiseptica* should be cogeneric.

In making a serological comparison with *H. pertussis* it would appear to be pertinent to include the degraded phases. We have done this in a study to establish the agglutinogenic relationship of the degraded phases of *H. pertussis* to the "Para" form and to *Brucella bronchiseptica*. In the course of the work we have been able essentially to confirm the work of Leslie and Gardner (1931) and to add a few new findings in this connection.

EXPERIMENTAL

Source of strains

Twenty-five strains of organisms have been used, including 14 *H. pertussis* in Phase I. Four of these latter were isolated in this laboratory from cough plates, the others were recent isolations kindly sent to us by Drs. Kendrick, Sauer, and Toomey. Mr. Roos of Sharp and Dohme sent us several strains used in production work as well as a number of old stock strains known

not to be in Phase I. Dr. Kendrick also furnished us with strains of *B. para-pertussis* and *B. bronchiseptica.* Dr. Bradford sent us two of his "atypical" strains. Drs. Toomey and Sauer also sent us several strains of old stock cultures. We wrote to Drs. Leslie and Gardner for strains but unfortunately they no longer had either cultures or sera available in 1938.

All the strains were found upon receiving them either to be Phase I or not, as specified. The "para" strains and "atypical" strains were identical with respect to biochemical and growth characteristics. Immediately subcultures of all strains as received were prepared and Cryochem-preserved (Flosdorf and Kimball, 1940a). This proved to be fortunate because in one case a strain identified after receiving it as a Phase III (no. 538) was found upon continued subculture on plain glycerol agar for about nine months to have reverted to a full Phase I. This unexpected change was observed in the acquired ability of the strain to absorb Phase I agglutinins. It was confirmed by the ability of its own anti-serum, produced about the time the altered absorption characteristics were first noted, to agglutinate Phase I organisms, although antiserum prepared immediately after the strain was received did not. Upon opening a Cryochem container of the original organism it was found to have characteristics identical with those which it had when received. Consequently, we now rely upon periodic opening of desiccated cultures of the degraded strains as well as of Phase I strains.

We have attempted to cause strains to change phase by varying the amount of blood in the medium. So far we have not carried a single strain through all the phases, but we can say that it is evident that there is a wide variation among strains in their tendency to undergo change in either direction through the various phases.

Confirmation of Leslie and Gardner

By cross agglutination, and by agglutinin-absorption, using the technique of 3 successive absorptions with whole organisms as published previously (Flosdorf and Kimball, 1940a), we have made full confirmation of the existence of Phases I, III and IV

of Leslie and Gardner (1931), each having a separate and distinct specificity. We have yet to find a Phase II, but Leslie and Gardner (1931) have pointed out that it occurs but rarely. Except for the one Phase III strain noted above (no. 538), the cultural or growth requirements follow those described by Leslie and Gardner (1931). We have also tried Hornibrook's semi-synthetic medium (1939), some of which was kindly furnished us by Dr. Hornibrook, and found that it supports a light growth of Phase I organisms. Strains in Phases III and IV grow much more luxuriantly, strains characterized as per page 465.

The degree of cross-agglutination obtained was not found to be identical with that reported by Leslie and Gardner (1931). This, however, was found to vary as a function of the intensity of immunization as suggested by these workers, similar to the observation noted by Eldering and Kendrick (1938) with respect to the broadness of specificity of para-pertussis. Consequently, this finding is not at variance with that of Leslie and Gardner (1931).

In figure 1 is given a graphical representation of the average degree of cross reaction of the several phases together with the ease of homologous and heterologous absorption. In this figure results of all combinations of homologous and heterologous absorptions of the three phases studied are shown and as such it represents an extension of the findings by absorption as reported by Leslie and Gardner (1931). There was a difference noted, however, in the characteristics of absorption by our Phase III strains. For example, from Phase IV antiserum one Phase III strain (588) completely removes the Phase IV agglutinins, whereas another Phase III strain (538) does so but partially. Similarly, absorption of a Phase I antiserum (484) with Phase IV organisms results in partial removal of agglutinins against strain 538, but not at all those against strain 588. Leslie and Gardner's experiments parallel to these (1931) give results that are similar to those we obtained with the 538 strain.

However, absorption of a Phase III antiserum by Phase IV organisms was found by Leslie and Gardner (1931) not to remove Phase III agglutinins. We find, on the other hand, upon ab-

sorption of our 538 serum with Phase IV organisms, partial removal of agglutinins against 538 but no removal of 588 agglutinins. The latter is consistent with our other results just noted with the 588 strain of Phase III organisms. Likewise, absorption of 588 serum with Phase IV organisms does not result in removal of agglutinins against either 538 or 588. In this respect, in contrast with the previously mentioned results

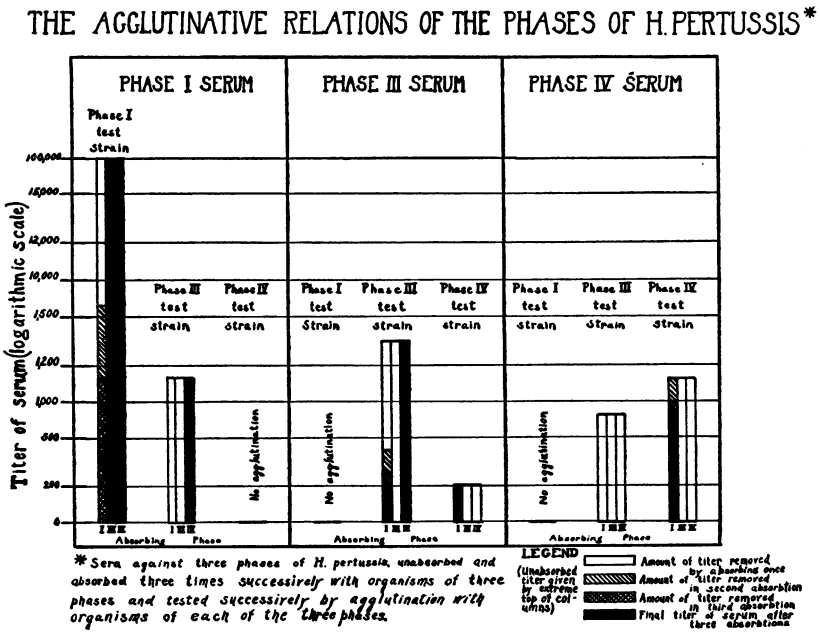

FIG. 1

of Leslie and Gardner (1931), their results with Phase III antiserum are more like ours with 588 rather than 538.

In figure 1 we have used our results with the 588 strain of Phase III organisms.

An unrepresentative phase of H. pertussis

An interesting finding was a variant strain (589) that is representative of neither Phase III nor IV. It is agglutinated by one (580) but not by all (e.g. 484) Phase I antisera. This dif-

ference is a function of the particular strain of Phase I organism used for immunization and not of the intensity of immunization. The Phase I sera which do not agglutinate the unclassified organism do agglutinate representative strains of Phase III to a high titer, in one case to 1:6400. Furthermore, the 580 organism in all other respects is exactly typical of Phase I, except in cross reaction with *B. bronchiseptica* and *"Bacillus para-pertussis"* as will be discussed later. Strain 580 is not agglutinated by any Phase III or Phase IV antiserum that we have prepared.

Antiserum against the unclassified organism, 589, does not agglutinate any strain of Phase I organism, so the organism is definitely not in Phase I. The unclassified organism is agglutinated by both III and IV antisera so it is not in Phase II. However, Phase III antisera agglutinate the unclassified organism to a titer which is lower than that for Phase III organisms and more nearly that for Phase IV strains. Absorbing the antiserum of the unclassified organism with organisms in Phase IV, however, does not remove the homologous agglutinins but it does remove the Phase IV cross agglutination. This would indicate the unknown organism to be in Phase III and not homologous with Phase IV. Phase IV antiserum absorbed with the unclassified organism has its agglutinins against the absorbing strain as well as against III and IV removed, but this is not a distinguishing characteristic, inasmuch as both Phases III and IV strains absorb in this manner.

However, since the strain is one which is not agglutinated by all Phase I antisera, not being agglutinated in the least by some, even with Phase I sera that behave representatively with other Phase III strains, the unclassified strain cannot be considered as Phase III. Similarly, there is a difference in Phase I strains, indicated by these results, because even though both 580 and 484 are alike and are typically Phase I in their agglutinative characteristics with respect to other phases of *H. pertussis* than strain 589, this difference with respect to this unclassified strain does exist. Furthermore, these same two phase I strains differ, as will be brought out later, in their cross reactivity with

B. para-pertussis and *B. bronchiseptica.* Accordingly, it must be concluded that variation in the pertussis group is even more complex than found by Leslie and Gardner (1931), both with respect to multiplicity of Phases and of cross reactivity with other species as well as with respect to tendency to undergo such variation. Tentatively, we are referring to this unrepresentative avirulent strain as in Phase X, realizing that it is close to

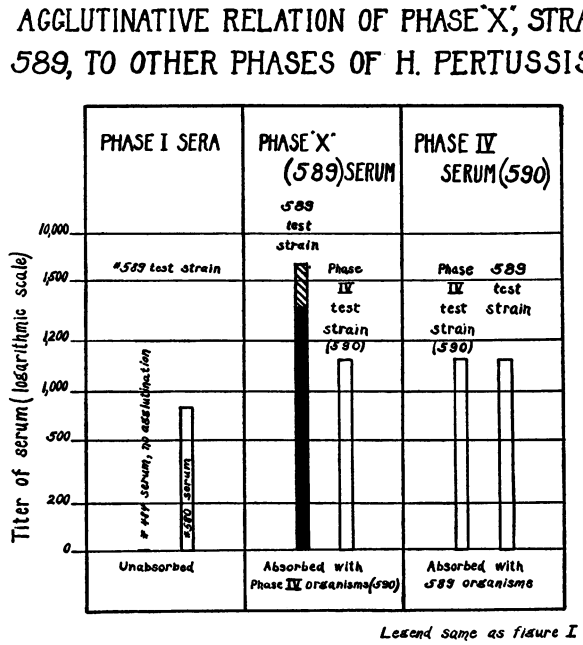

FIG. 2

Phase III. A single strain does not justify shifting of the present Phase IV or any other rearrangement of phases to accommodate it, hence the designation as Phase "unclassified" ("X"). Both 580 and 484 are classified as Phase I regardless of the minor variations in heterologous agglutinative reactivity of their antisera just noted.

In figure 2 is summarized the relation of strain 589 to other known phase strains.

Relationship of B. para-pertussis and B. bronchiseptica to H. pertussis in the various phases

Antiserum against *B. para-pertussis* agglutinates Phase I *H. pertussis* organisms to a degree which varies with the intensity of immunization, as found by Eldering and Kendrick (1938), although in general we found the cross-agglutination to be higher than they reported. Similarly, Phases III, IV and X are agglutinated. This cross-agglutination seems to vary in degree not only with the intensity of immunization but also with the strain of *B. para-pertussis*; in one case the degraded phases were found to be agglutinated as strongly as Phase I strains were, although in another case the degraded strains were not agglutinated at all, whereas Phase I strains were.

Phase I *H. pertussis* antiserum, however, did not in all cases agglutinate *B. para-pertussis*. It is interesting that the 580 antiserum did not agglutinate any strains of *B. para-pertussis*, this serum being the one that was found to agglutinate the Phase X strain (589).

With respect to *B. bronchiseptica*, one strain of organisms, the older[1] strain (644), is not agglutinated by antisera against degraded *H. pertussis* or *B. para-pertussis*. Its antiserum does not agglutinate any pertussis or para-pertussis organism. With the more recently isolated[1] strain (645), however, cross-agglutination occurs in both directions with Phase IV, *H. pertussis*. Strain 645 is agglutinated by serum from rabbits intensively immunized with Phase III pertussis but Phase III organisms are not agglutinated by antiserum against 645. In addition, *B. para-pertussis* antiserum agglutinates 645 even though *B. para-pertussis* organisms are not agglutinated by 645 antiserum. The findings with *B. para-pertussis* and the anti-sera to the strains of *B. bronchiseptica* are not in complete agreement with previous findings of Eldering and Kendrick (1938), but because of the apparent differences in strains and the difficulty in reading agglutination of an organism so salt-sensitive as *B. para-pertussis*, little significance can be attached to this discrepancy.

[1] Strain as received from Dr. Pearl Kendrick.

More interesting, however, is the fact that the younger strain of *B. bronchiseptica* (645) is agglutinated by an antiserum against some strains of Phase I *H. pertussis* but not by all (e.g. 484) even if of very high titer from intensive immunization. The older strain of *B. bronchiseptica* is not agglutinated by any of the Phase I antisera used. One strain of Phase I *H. pertussis* (580) whose serum agglutinates *B. bronchiseptica* is the same which was found to agglutinate the Phase X strain (589) of *H. pertussis* but not *B. para-pertussis* as discussed earlier.

Relationship among the various phases of H. pertussis

In considering the serological findings by cross-agglutination and by agglutinin-absorption, as set out in figure 1 and in the figures of Leslie and Gardner (1931), certain facts may be noted and interpreted:

Phase I, Phase III and Phase IV organisms generally are in each case agglutinated to highest titer in antiserum versus the homologous phase.

Phase I. Phase I organisms are not agglutinated by antiserum versus Phases III or IV nor does absorption of antiserum versus Phase I with Phase III or IV organisms appreciably diminish Phase I agglutinins. From these data the conclusion may be drawn that the Phase I agglutinogen is completely lost in the transformation to Phases III and IV.

Antiserum versus Phase I agglutinates Phase III organisms; occasionally the heterologous titer equals the homologous, particularly after intensive immunization; moreover absorption of serum versus Phase III by Phase I organisms materially reduces Phase III agglutinins. Phase I organisms therefore must contain some Phase III agglutinogen, presumably in part upon their effective surfaces.

Antiserum versus Phase I does not agglutinate Phase IV organisms, nor does absorption of serum versus Phase IV by Phase I organisms greatly reduce Phase IV agglutinins. Phase I organisms therefore contain little if any Phase IV agglutinogen.

Phase III. Phase III organisms are agglutinated by antiserum versus Phase IV, and antiserum versus Phase III agglu-

tinates Phase IV organisms, although to low titer. Absorption of Phase IV antiserum with Phase III organisms removes Phase IV agglutinins partially or completely. The surfaces of phase III organisms would therefore appear to contain Phase IV agglutinogen. As indicated above, there is no indication of the presence of Phase I agglutinogen in Phase III.

Phase IV. Phase IV organisms are agglutinated to low titer by antiserum versus Phase III and antiserum versus Phase IV agglutinates Phase III organisms. Both these observations might be explained, however, by the presence of Phase IV agglutinogen in Phase III. Absorption of antiserum versus Phase I with Phase IV organisms resulted in partial removal of agglutinins against Phase III, strain 538, but in no removal of agglutinins against Phase III, strain 588. These results and the other results of absorption experiments recorded in this paper indicate that there is an additional "common" antigen in certain Phase III strains (e.g. no. 538), which is present also in small amounts in Phase I and Phase IV organisms.

Unless this assumption of the common antigen is made, one would need to assume the presence of the single Phase III antigen in Phase IV organisms. Therefore, since an antigen from Phase IV exists in Phase III strains, we would then have Phase III and Phase IV strains qualitatively alike, differing only in quantitative relationship of the two antigens. This, however, would not explain satisfactorily the cardinal difference in these two phases, i.e., the Phase III is agglutinated by serum versus Phase I, whereas IV is not. Likewise, without this assumption the difference between strains 538 and 588 is difficult to interpret. The assumption of the several antigens might be arranged symbolically in simplest terms as in table 1, "c" being "common".

Using the symbols of table 1, "c" would be present also, in small amount, or more deeply seated, in both Phase I and IV organisms. The small amount or location of "c" would explain the failure of Phase I antiserum to agglutinate Phase IV organisms even though the latter will absorb partially the no. 538 agglutinins from the former. This latter condition would be parallel to the absorption of Phase III agglutinins by Phase I

organisms from Phases III and IV antisera even though these sera do not agglutinate Phase I organisms, the amount of the major Phase III agglutinogen, which by these symbols would be "b", in Phase I organisms being small or deeply seated. Unless such an assumption of the additional common antigen, which may be common to I, III and IV organisms, is made, we are forced to leave the question open as to whether or not Phase IV organisms contain any Phase III agglutinogen. In order to explain Phase X and other cross reactions, such a simple symbolic arrangement would require further elaboration.

In order to obtain further evidence with regard to certain of the above interpretations which were based on experiments with intact organisms, the whole sonic extract, SX–I, of Phase I organisms (Flosdorf, Kimball and Chambers, 1939 and Flosdorf

TABLE 1

Suggested simplest agglutinogenic structure of H. pertussis

PHASE	STRAIN	ANTIGENS
I		a, (b, c, small)
III	538	b (major), c
III	588	b (major), d
IV		c, d (major)

and Kimball, 1940b) has been used. Antisera against SX–I agglutinates Phase III organisms to almost the same titer (1:1600) as Phase I (1:3200), but Phase IV is agglutinated to very low titer (1:40). Upon absorption of either Phase I or Phase III antiserum with SX–I, however, the agglutinins for Phase III cannot be removed. There was not the slightest reduction in Phase III agglutinin titer in Phase III antisera, even by absorbing the serum with an amount of SX–I which was 72 times the quantity which would be required on the basis of serum-titer (Flosdorf and Kimball, 1940c).

Accordingly, it appears that although some Phase III agglutinogen exists in Phase I organisms, sufficient in amount to elicit agglutinins by injecting either the whole organisms or SX–I, there is insufficient to absorb the Phase III agglutinins when the

antigen is in solution. Of course, the fact that Phase III agglutinins are elicited by either the whole Phase I organisms or SX–I offers no basis for estimation of the relative amount of agglutinogen present in the whole organisms. Likewise, the fact that the whole Phase I organisms absorb Phase III agglutinins is irrelevant, quantitatively, because other factors may be concerned in such reactions with particulate matter. On the other hand, the lack of absorption of Phase III agglutinins by SX–I with such a favorable ratio of antigen solution to serum seems to be a reasonable basis for assuming that very little Phase III agglutinogen exists in the solution. The excellent Phase III agglutinin response by SX–I upon injection is merely an indication that the Phase III agglutinogen is of high antigenic activity.

The low Phase IV antigenic activity of SX–I confirms the observation based on study with whole organisms that little Phase IV agglutinogen exists within the Phase I organisms.

Accordingly, it would appear that Phase I organisms are not merely Phase III or IV organisms with a Phase I coating on the surface, but antigenically are different in their entirety except for small amounts of other antigens. In general the results obtained with SX–I are consistent with the interpretations suggested above in connection with the relationships among the phases. All these findings indicate an extreme complexity of the species *H. pertussis*.

DISCUSSION

It seems clear that variation in *H. pertussis* is even more complex than was found by Leslie and Gardner (1931). The serological complexities within this one species emphasize the danger of comparing with finality other species of organisms with it, unless extreme precautions are taken, first, to consider thoroughly the precise relation of a given strain of *H. pertussis* to other strains of both homologous and heterologous phases of *H. pertussis*; second, to insure that the strain is not undergoing change while being studied; and, third, to consider the possibility of variation within the other species of organism with which comparison is being made.

Even though variation in *H. pertussis* may be more complex than postulated by Leslie and Gardner (1931), we cannot agree with Toomey *et al.* (1935, 1937) that there are theoretically 25,000 phases on the mathematical basis of agglutination titer. Our experimental results do not conform with the conclusions of the latter workers but definitely do confirm Leslie and Gardner (1931) with respect to the existence of separate and qualitatively distinct phases, even though we have been able to extend somewhat the findings of the latter workers. It would appear on the basis of our work that unless one uses some acceptable method of stabilization of strains, such as periodical subculturing from cryochem-containers of even the degraded strains, it is difficult to be certain that the strains are not changing during the course of the investigation. Indeed, unless the strains are stabilized there is little value in spending the necessary time and effort required for full characterization. Prior to our realization of this fact, we accidentally produced one multiphasic serum when a Phase III organism returned to Phase I while on plain glycerol agar in the interval between the first and second series of immunization, in production of a high-titer serum. This was extremely confusing until the matter was finally clarified as discussed in this communication.

The findings of this investigation do suggest that for use in production of vaccines it would be desirable to characterize all strains rather fully. Then, only those Phase I strains which meet the accepted criteria (Sauer, 1939), and in addition show broad agglutinative reactivity, particularly with *B. para-pertussis*, should be used, maintaining them in stabilized desiccated form. This would be preferable to using a mixture of organisms which individually show narrow specificity to only Phase I *H. pertussis* in one case and to *B. para-pertussis* in the other, for reasons pointed out by Sauer (1939). That is, Sauer (1939) states that merely to include a small amount of *B. para-pertussis* in a *H. pertussis* vaccine could be expected to provide but very little protection against atypical strains. We believe, however, using an organism which is fully active in all its Phase I characteristics but whose antibodies are more universal in their affinities, would

provide the solution to the problem. This, of course, assumes that the more universal antigenic structure of the antigenically broader Phase I organism would have additional antigenic groupings without loss of homologous Phase I characteristics. In choice of such master strains, therefore, in order to be certain that nothing has been lost against the major organism, Phase I antigenic response and protective value in animals against typical Phase I *H. pertussis* should, of course, be included in the full characterization of the strains to be chosen for stabilization for vaccines.

The authors wish to express their appreciation to Dr. Stuart Mudd for his helpful suggestions.

Note: In table 1 of paper II of this series (Flosdorf and Kimball, 1940a) a Phase III organism by typographical error was erroneously mentioned as Phase IV. The data given in that table itself indicates that the degraded organism could not be Phase IV.

SUMMARY

1. The existence of separate and qualitatively distinct phases of *Hemophilus pertussis* as postulated by Leslie and Gardner (1931) has been confirmed, more recent workers notwithstanding. However, evidence is presented that variation is even more complex in minor relationships than found by the former workers.

2. Strains may undergo change in either direction through the various phases during the period of their study, even reverting to Phase I while on a simple medium. Hence precautions must be taken to avoid this. Drying from the frozen state in multiple containers and periodic opening of these will avoid this spontaneous phase variation.

3. Strains may be found which although representative of a given phase in most respects, may differ in some. Accordingly, full characterization of strains in any phase is required in studying them before making comparison with other species.

4. Data pertaining to the cross agglutinative characteristics of the various phases of *Hemophilus pertussis* with *Bacillus parapertussis* and *Brucella bronchiseptica* are presented.

5. Evidence is presented which suggests that Phase I organisms are not simply Phase III organisms with a Phase I surface coating, but are antigenically different in their entirety. Little Phase III and virtually no Phase IV agglutinogen is contained within Phase I organisms.

6. Master strains of broad specificity maintained in stabilized desiccated form are suggested for use in production of vaccines for whooping cough.

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