THE VIRUS OF PSITTACOSIS

III. Serological Investigations

ALFRED S. LAZARUS* AND K. F. MEYER

George Williams Hooper Foundation, University of California,
San Francisco, California

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In previous communications (Lazarus and Meyer (1939a, 1939b)) the virus of psittacosis has been investigated with respect to its filterability and general properties. The present report deals with the serological reactions which could be elicited with the washed and concentrated suspensions of elementary bodies.

AGGLUTINATION

Review

The literature contains little reference to serological studies on the psittacosis virus, with the exception of work concerning the complement fixation reaction, which has been extensively investigated by Bedson (1933). Since the complement fixing antibodies were not considered in this study, no further reference need be made to this phase of the subject.

Bedson (1932) attempted to produce antipsittacosis sera in guinea pigs and mice, with some success. Agglutination tests performed with twice-washed mouse elementary bodies and antiserum from guinea pigs gave positive agglutinations in dilutions of 1 in 8. The reaction was shown to have been specific, but such low titers are rather unsatisfactory. In the same study, neutralizing antibodies were demonstrated in an equally low dilution. No reference was made to precipitin tests, and no

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2 Edith Claypole Memorial Research Fellow in Pathology, 1936-1938.
attempts have been described employing neutralization of the virus on the chorio-allantoic membrane of the chick.

The particular problems involved in the virus-antibody reaction must be only special examples of principles applicable to all antigen-antibody reactions. The modern trends of thought concerning these reactions are well expressed by Marrack in "The Chemistry of Antigen-Antibody Reactions" (1938). The problem of applying these general concepts to viruses has been extensively discussed by Burnet, Keogh and Lush (1937) in their comprehensive monograph.

In view of the demonstration of two antigenic groups in the vaccinia elementary body (Craigie and Wishart (1934)) and the presence of at least 3 different types of foot-and-mouth virus (Topley and Wilson (1936)), there is reason to believe that some virus particles, at least, may closely resemble the bacteria in respect to antigenic properties in general. The demonstration of a polysaccharide component in vaccinia elementary bodies has further verified these views (Craigie (1932, 1935)).

For technical reasons the study of the in vitro aggregation reactions of vaccinia elementary bodies has advanced much further than that of any other animal-pathogenic virus. The important preliminary work was accomplished by Gordon (1925), Burgess, Craigie and Tulloch (1929) and Craigie and Tulloch (1931), although Paschen (1913) had observed the elementary bodies many years previously. The method of Craigie (1932) rendered it practicable to obtain large amounts of crude material very rich in the elementary particles of the virus. In general, the principles outlined in the above studies have been followed in the investigations of psittacosis virus.

Schultz, Bullock and Lawrence (1928) claimed that the agglutinations and precipitations observed in vaccinia studies were the result of the presence of bacteria, both in the antigenic material and in the reagents used in the actual tests. This theory has been disproved by the careful investigations of Craigie and Tulloch (1931) and has been completely discredited by the use of bacteria-free elementary body suspensions by Smadel and Wall (1937). Sterility tests with psittacosis ele-
mentary body suspensions were completely negative and no account need be taken of the possible influence of bacteria.

In addition to the studies of vaccinia agglutinations, work along these lines has also been reported for varicella and zoster (Amies (1934)), for Rous tumor virus (Ledingham and Gye (1935)) and myxoma (Rivers and Ward (1937)).

Experimental technic

The purified suspensions of L.C.L. bodies previously described (Lazarus and Meyer (1939a)) were used in the principal investigations of agglutination reactions. The determination of agglutination titers of animals receiving other types of psittacosis suspensions as antigenic material will be referred to in a later section.

The agglutination reaction in guinea pig sera was first investigated. Normal guinea pigs of approximately 300 grams weight were given 0.5 ml. of washed elementary bodies by means of intraperitoneal injections. This dose was approximately $1.5 \times 10^9$ active elementary bodies. The first inoculation was accompanied by a slight elevation of temperature (to about 104°F.) and a loss of appetite for about 48 hours, but this reaction was not observed after subsequent injections. The same apparent malaise has been observed by Bedson (1933). One week after the first inoculation, the animals were given 1 ml. of a fresh suspension of washed elementary bodies (approximately $3 \times 10^9$) by the same intraperitoneal route, and subsequent inoculations were made at intervals of from 5 to 8 days.

The guinea pigs were bled by cardiac puncture, the sera allowed to separate overnight in the ice chest and removed, centrifugalized and stored without preservative. Normal serum was collected before the first inoculation.

The technic of the agglutination test was similar to that described by Craigie (1932). Pyrex test tubes with outside dimensions of 10 × 75 mm. were carefully washed and then sterilized by dry heat. As a diluent in the tests, buffered saline, prepared as already described (Lazarus and Meyer (1939a)), was used.
This saline was made with freshly prepared double distilled water, and was autoclaved just before use. For agglutination, a relatively light suspension of elementary bodies yielded the most satisfactory results, and the stock suspension diluted to contain approximately \(1 \times 10^9\) elementary bodies per ml. was found to be most satisfactory. 0.25 ml. of the different dilutions of immune sera were placed in the tubes, using sterile 1 ml. pipettes and aseptic technic. Saline and normal serum controls were used throughout. To the tubes was then added 0.25 ml. of the elementary body suspension. The same results were obtained whether the elementary bodies were freshly prepared, or were held in the ice chest long enough to cause them to lose their infectiousness for mice, as has already been discussed. All glassware used in the tests was subjected to careful washing and neutralization.

The tubes with their contents were placed in an ordinary serological rack and the mouths of the tubes were protected by a piece of sponge rubber sterilized by autoclaving. This in turn was covered with a block of wood secured in place by large rubber bands. This procedure prevented evaporation during the prolonged incubation period and also prevented the possibility of the virus particles being allowed to dry and be carried into the atmosphere by air currents. Cotton stoppers did not prevent evaporation and resulted in lint in the tubes, while small individual rubber stoppers were too awkward and difficult to manipulate. The covered racks were then incubated at a temperature of 46 to 48°C for 20 to 36 hours.

**Results**

When agglutination reactions were carefully conducted in the manner described, convincing and consistent results were obtained. The results were easily read with the naked eye, although a magnifying lens was often of service in determining the exact end-point of the titration. Care had to be taken not to shake the tubes too violently, because agglutinated elementary bodies were almost completely dispersed by excessive agitation, and further sedimentation required long periods of time. The
same phenomenon has been observed with vaccinia elementary bodies (Parker and Rivers (1935)). The chick membrane elementary bodies when agglutinated by guinea pig sera clumped in a granular form, and complete sedimentation was observed

![Antibody Response of Guinea-Pigs to Suspensions of Washed Concentrated L.C.L. Bodies](image1)

**Fig. 1. Result of 9 Intraperitoneal Injections**

only in the lowest dilutions. When sera from rabbits was used, large loose flakes were formed and the supernatant fluid was often completely clear. This point will be discussed in later sections.

The progress of agglutinin production being of considerable interest, an attempt was made to follow the rise and fall of agglu-
tinins in immunized guinea pigs. Figures 1 and 2 represent two typical curves produced by long continued injections of washed elementary bodies. All doses, represented by arrows, consisted of approximately $3 \times 10^9$ freshly isolated and washed elementary bodies, except the initial inoculation, which contained only half as many L.C.L. bodies. There were no symptoms resulting from these massive doses of highly infectious material, except for the slight rise in temperature previously mentioned as occurring after the first inoculation.

It will be immediately observed that although these sera were far superior to those obtained by Bedson (1933), no rise in titer over 1 in 320 could be induced, in spite of long continued inoculation with active material (fig. 2). In fact, other animals showed the same production of agglutinins following only 2 or 3 injections. The maintenance of a titer of 1 in 80 after discontinuance of injections is also noteworthy.

The failure to raise the titer above 1 in 320 is closely analogous to the results obtained using bacteria as antigens. It is well known that when the titer of any particular bacterial antibody has been forced up to a certain level, which of course varies widely with the nature of the antigen and the responsiveness of the animal injected, it becomes impossible to induce any further rise in the concentration of antibody in the circulating blood. This fact was pointed out as early as 1901 (Goldberg (1901)), and its application to the antigenicity of L.C.L. bodies lends further credence to the rapidly growing conviction that the elementary bodies are no more than highly parasitic organisms requiring an intracellular habitat for survival and reproduction, and lacking the necessary enzyme systems for independent existence.

Effect of incubation time and temperature

In the thorough investigations of Craigie (Craigie and Tulloch (1931) Craigie (1932)), the necessity for relatively long periods of incubation to elicit maximum agglutination of vaccinia elementary bodies was discussed. Similar results have been observed in studies of psittacosis material. It appears logical that
the incubation period must be longer than that required for bacterial agglutination, not because of any peculiarity inherent in the elementary bodies themselves, but because the particles and the clumps they form are much smaller than those resulting from bacterial agglutinations, and the time required to produce a visible reaction is therefore longer. This view is strengthened by the observation that psittacosis agglutination tests gave the same results after 60 hours whether the 48° incubation period lasted through the first 36 hours or only through the first 12 hours. Obviously, the higher temperature gave the usual result in the first few hours, and the visible reaction came about at any
temperature after the proper time interval had elapsed. Table 1 shows the degree of agglutination apparent at various times during the incubation period of a typical test.

In the experiment tabulated in table 1, the rack was removed from the incubator at the end of 36 hours and held at room temperature during the remainder of the 60 hours. It will be noted that the final titer did not increase after 36 hours, but that the intensity of the agglutination showed some improvement. In a parallel experiment, the rack was removed at the end of 18 hours incubation and the final results were identical with those in table 1 at the end of 60 hours. This verifies the fact that once union between antigen and antibody has occurred, it is only a matter

| TABLE 1 |
| Effect of time and temperature on agglutination |

<table>
<thead>
<tr>
<th>DILUTION OF ANTISERUM</th>
<th>TIME OF INCUBATION IN HOURS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16 at 45°</td>
</tr>
<tr>
<td>1:20</td>
<td>+</td>
</tr>
<tr>
<td>1:40</td>
<td>+</td>
</tr>
<tr>
<td>1:80</td>
<td>?</td>
</tr>
<tr>
<td>1:160</td>
<td>-</td>
</tr>
<tr>
<td>1:320</td>
<td>-</td>
</tr>
<tr>
<td>Saline</td>
<td>-</td>
</tr>
<tr>
<td>Normal serum 1:20</td>
<td>-</td>
</tr>
</tbody>
</table>

+ to ++++ = degrees of agglutination visible macroscopically, ? = questionable agglutination, = = negative.
of sufficient time elapsing before the results are visible, and the temperature of incubation after such a union is of little consequence. With a view to standardizing the technic, all incubations referred to in this report were done at 46 to 48°C. for 36 hours, followed by 24 hours at room temperature.

*Heat-labile and heat-stable agglutinogens*

Craigie and Wishart (1934) have reported the presence of at least two agglutinogens in a strain of vaccinia elementary bodies. The more labile L agglutinogen had its agglutinability and its ability to absorb agglutinin impaired or destroyed by exposure to a temperature as low as 56°C., while the S agglutinogen was stable at temperatures up to 95°C. Bedson (1933) showed that steamed formalinized psittacosis virus lost none of its immunizing powers.

In view of the interesting findings in regard to the agglutinins produced by unaltered vaccinia elementary bodies, it was considered of value to determine whether or not the elementary bodies of psittacosis showed any such antigenic complexity.

A preliminary experiment showed that a suspension of freshly prepared L.C.L. bodies, after being heated for 1 hour at a temperature of from 70 to 75°C., was still agglutinated by a known antiserum, but not by a normal guinea pig serum. This observation called for further investigation.

A suspension of freshly washed and concentrated elementary bodies was heated at 70 to 75°C. for 1 hour and then cooled. 0.5 ml. of this suspension was added to the same amount of a 1 in 10 dilution of antiserum having a titer of 1 in 160. This antiserum was produced in guinea pigs by the inoculation of freshly prepared virus. The mixture was incubated in the usual manner and showed a very good +++ agglutination at the end of 60 hours. The tube was then centrifuged at 3,000 R.P.M. in the ordinary horizontal centrifuge for 15 minutes, and the clear supernatant fluid removed. The sediment, when stained by the Castaneda method, showed clumps of elementary bodies obviously agglutinated by the antiserum. Saline and normal
The virus of psittacosis

serum controls were entirely negative when treated by the same procedure.

The absorbed serum was then set up with both freshly prepared elementary bodies, and with elementary bodies which had been heated at 70 to 75°C. for 1 hour. The mixtures were incubated in the usual manner. At the end of 60 hours, the heated elementary bodies showed no signs of agglutination, while the unheated material gave a definite ++ agglutination. Saline and normal serum controls were again negative, and agglutination was once more verified by stained smears. These findings were verified by further experiments of the same type, but due to the difficulty of obtaining large amounts of elementary bodies in the necessary state of purification, it was not possible to determine more accurately the temperature range at which the heat-labile component was destroyed.

On the basis of the above results, one may conclude that the elementary body suspensions, as prepared for purposes of immunization, contained at least two components, one destroyed at 70 to 75°C. for 1 hour and the other resisting the same treatment. These agglutinogens produced their specific agglutinins in the sera of inoculated guinea pigs, and these agglutinins were demonstrable by the usual absorption methods. It thus appears that the psittacosis elementary bodies are complex antigenically.

Cross-relationship with vaccinia agglutinogens

In view of the fact that both psittacosis and vaccinia elementary bodies possessed an antigenic complexity, it was considered of interest to determine whether or not one of these antigenic components might be common to both viruses.

A suspension of vaccinia elementary bodies was prepared in the same manner as the L.C.L. body suspensions were made. The vaccinia virus was obtained from a strain of egg membrane virus furnished in 1935 through the courtesy of Dr. E. W. Goodpasture of Vanderbilt University, and carried since that time by routine chorio-allantoic membrane passage. This virus has
gradually lost its invasive qualities for the skin of the normal rabbit, and now produces few or no lesions after over 350 egg-membrane passages. The elementary body suspensions prepared from this virus had the same appearance as the psittacosis suspensions already described.

Antivaccinia serum was prepared by hyperimmunization of normal rabbits with calf vaccinia virus. The shaved rabbits were given 0.4 cc. each of a 1 in 100, 1 in 1,000, 1 in 3,000 and 1 in 10,000 dilution of the virus by the usual scarification technic. This was followed in 19 days by the inoculation of 1 ml. of a 1 in 10 dilution of the same virus intravenously and the latter dose was repeated after another 17 day interval. The serum was collected and stored without preservative by cardiac bleeding 6 days after the last inoculation.

### TABLE 2

Cross agglutinations with psittacosis and vaccinia elementary bodies and antisera

<table>
<thead>
<tr>
<th>ANTISERUM</th>
<th>HEATED PSIITACOSIS ELEMENTARY BODIES</th>
<th>UNHEATED PSIITACOSIS ELEMENTARY BODIES</th>
<th>HEATED VACCINIA ELEMENTARY BODIES</th>
<th>UNHEATED VACCINIA ELEMENTARY BODIES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccinia.....</td>
<td>Negative 1:20</td>
<td>Negative 1:20</td>
<td>Positive 1:10</td>
<td>Positive 1:160</td>
</tr>
<tr>
<td>Psittacosis...</td>
<td>Positive 1:160</td>
<td>Positive 1:320</td>
<td>Negative 1:10</td>
<td>Positive 1:40</td>
</tr>
</tbody>
</table>

Preliminary experiments showed that the psittacosis antiserum was able to agglutinate vaccinia elementary bodies in a titer of 1 in 40 by the usual technic. The vaccinia antiserum prepared in rabbits gave a doubtful agglutination of psittacosis elementary bodies in a dilution of 1 in 10. These observations led to further experiments with both heated and unheated elementary bodies of both types. These suspensions were set up with both vaccinia and psittacosis antisera. Table 2 summarizes in composite form the principal results obtained by such investigations.

The data in table 2 represent the highest significant dilution of the materials used. All saline and normal serum controls were negative in the dilutions used.

It is rather difficult to draw clear conclusions from such findings. The antipsittacosis serum was able to agglutinate unheated vaccinia elementary bodies, but not heated material.
It must be emphasized that the elementary bodies of both viruses were obtained from chick material, but that the vaccinia antiserum was prepared in rabbits receiving calf material only. The results observed were not due to chick antibodies in the guinea pig sera, as will be proved in a later section. Apparently the cultivation of the viruses on the chorio-allantoic membrane had resulted in the presence of some common factor, which made it possible for the vaccinia elementary bodies to be agglutinated in low titer by antipsittacosis serum. The only possible conclusion to be drawn from these puzzling observations is that under the conditions of the experiments performed, some common antigenic factor was present in egg membrane viruses of psittacosis and vaccinia, perhaps a component acquired by the intracellular habitat. Due to the difficulty of obtaining large amounts of material, it was not possible to investigate this phenomenon as thoroughly as might be desired.

Control experiments

In order to evaluate accurately the above results, it was necessary to rule out the possibility that one or both of the viruses investigated had become contaminated with the other. The ease with which vaccinia virus can spread through animal quarters is well known, and since both viruses were being constantly incubated and transferred in close proximity, it was considered possible that through carelessness or accident, some mixture might have occurred. Accordingly, elementary body suspensions of both types were inoculated intraperitoneally into mice, the psittacosis in a dilution of 1 in 10 and the vaccinia undiluted. The mice receiving the psittacosis material died with positive findings in 2 and 4 days. The mice receiving the undiluted vaccinia suspension were anatomically negative for psittacosis when sacrificed 70 days after inoculation. These results were verified by another experiment of the same type, and it was therefore known that the vaccinia virus suspensions contained no psittacosis virus as a contaminant.

To rule out the possibility of the psittacosis virus having been contaminated with vaccinia virus, intradermal inoculations were
made with suspensions of both types in separate normal white rabbits. Although neither suspension gave a definite vaccinia lesion, due to the peculiarities of the vaccinia virus strain used, further inoculation with calf virus verified the fact that the psittacosis material contained no vaccinia virus, since the animal receiving the psittacosis virus gave a positive vaccinia reaction upon inoculation with the calf virus, while the other rabbit showed a definite vaccinoid reaction, indicating partial immunization by the previous inoculation. These results were likewise verified by further experiments of the same type.

Further evidence of the purity of the strains was supplied by the picture given in the chorio-allantoic membrane. The psittacosis virus, as already described (Lazarus and Meyer (1939a)), gave an easily recognizable lesion without discrete foci, with death of the embryo between the third and fourth days. On the other hand, the vaccinia virus gave occasional discrete lesions typical of this virus, and the membrane showed none of the edema characteristic of the psittacosis infections. The eggs infected with vaccinia virus consistently showed death of the embryo between the second and third day. Thus, through animal experiments and pathological pictures, it appears certain that no mixture of the two viruses was responsible for the results described above.

Although the tests for protein in the wash water of the psittacosis suspensions were negative after the second washing following trypic digestion, it is a known fact that biological tests for the presence of protein are much more delicate than any known chemical test. Accordingly, it was considered necessary to rule out the possibility of the presence of anti-chick-protein antibodies in the sera used to agglutinate psittacosis elementary bodies of chick origin.

Normal chick protein was prepared by grinding chorio-allantoic membranes removed from uninoculated eggs. The ground material was suspended in buffered saline, extracted overnight in the ice chest, centrifuged and the supernatant fluid removed. This material gave a very heavy precipitate when tested with sulfosalycilic acid. Guinea pigs and rabbits were inoculated
with this material by intraperitoneal and intravenous injections. The material was antigenically active, as was shown by the induction of anaphylactic shock in one guinea pig. The sera from these animals were not able to agglutinate the washed elementary body suspensions of psittacosis virus. Tests were made after 1 injection and after 5 injections, but no positive agglutination resulted when the sera were set up with L.C.L. body suspensions. It therefore appears certain that the results observed when psittacosis elementary bodies were agglutinated by antiserum were not due to the presence of antibodies against chick protein. These anti-chick sera were also used as control material for the precipitin tests described below.

**Agglutination of Proteus vulgaris**

Lillie (1930) noted the resemblance of the L.C.L. bodies to the rickettsia group and proposed the name "*Rickettsia psittaci*" for the psittacosis inclusion bodies. Meyer (1935) pointed out that there is no evidence at hand that psittacosis is an insect-borne disease and that the name proposed by Lillie was therefore inappropriate.

In view of the microscopic resemblance of L.C.L. bodies to the rickettsia group, it was considered of interest to determine whether the antipsittacosis serum was able to agglutinate proteus strains, a phenomenon well known in connection with rickettsia infections. Accordingly, suspensions of various proteus strains were prepared and washed by the usual technic. These suspensions were set up against a strongly positive psittacosis antiserum and were incubated in the usual manner for bacterial agglutinations.

The results observed were of little positive significance. *P. vulgaris* OX 19 and OX 2 were not agglutinated by the sera used. *P. vulgaris* OX K and OX Muar were agglutinated in dilutions of 1 in 20, a titer of very doubtful significance.

The same strains were set up against an antivaccinia rabbit serum. All agglutinations were negative by the standard technic.

It appears, in the light of the above results, that neither the
psittacosis nor vaccinia elementary bodies bear any close serological relationship to the rickettsia group.

_Agglutinins in other sera_

The results reported above were obtained by the use of sera from guinea pigs immunized with suspensions of washed and concentrated elementary bodies. It was considered of value to verify these results by appropriate tests of sera from other sources. The author is indebted to Miss Bernice U. Eddie of the Hooper Foundation for her coöperation in supplying the materials used in the studies below.

The agglutination tests described below were performed by the standard method described above, and appropriate saline and normal serum controls were included in each experiment. For the sake of brevity, the results are recorded in table form.

_Discussion_

The results shown in table 3 bring to light some interesting data. That the agglutination reaction is not due to the presence of chick protein in the antigenic material is proved by the positive agglutinations observed in guinea pig 1453, monkey 709, monkey "leather collar," and human AM. None of the foregoing had received chick protein in any form. Normal human, monkey, rabbit and guinea pig sera were consistently negative in all the above tests.

It may likewise be observed that the agglutination titer in monkeys was never as satisfactory as the titer in guinea pigs and rabbits receiving the same type of material. This emphasizes the known fact that monkeys are not the most satisfactory animals to use for studies of psittacosis virus. It is likewise noteworthy that the mouse virus suspensions were not antigenically adequate, since it was observed early in these studies that it was almost impossible to free the virus from mouse-organ cells. In the process of preparing the antigenic material used above, much virus was undoubtedly discarded with the sediment of centrifugalized mouse material, while the same applied to a greatly lessened extent with guinea-pig and chick virus. This
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once again emphasizes the fact that the virulence and hence the antigenicity of the material used runs parallel with the number of elementary bodies present, and lends further support to the belief that the L.C.L. bodies are actually the virus particles.

The type of agglutination observed in all tests conducted varied with the type of serum used. Rabbit serum consistently

<table>
<thead>
<tr>
<th>TABLE 3</th>
<th>Agglutination tests performed with miscellaneous sera</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOURCE</td>
<td>IDENTIFICATION</td>
</tr>
<tr>
<td>Rabbit</td>
<td>4</td>
</tr>
<tr>
<td>Rabbit</td>
<td>8</td>
</tr>
<tr>
<td>Rabbit</td>
<td>92</td>
</tr>
<tr>
<td>Rabbit</td>
<td>94</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>1,074</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>1,453</td>
</tr>
<tr>
<td>Monkey</td>
<td>47</td>
</tr>
<tr>
<td>Monkey</td>
<td>71</td>
</tr>
<tr>
<td>Monkey</td>
<td>709</td>
</tr>
<tr>
<td>Monkey</td>
<td>1,837</td>
</tr>
<tr>
<td>Monkey</td>
<td>L.C.*</td>
</tr>
<tr>
<td>Human</td>
<td>BE</td>
</tr>
<tr>
<td>Human</td>
<td>AL</td>
</tr>
<tr>
<td>Human</td>
<td>AM</td>
</tr>
</tbody>
</table>

* L.C. = Leather collar.

gave large flaky clumps, which tended to settle completely, leaving the supernatant fluid crystal clear in all but the highest dilutions. On the other hand, human, monkey and guinea pig sera gave a granular agglutination with clumps which were easily disrupted. They settled very slowly and left the supernatant fluid clear only rarely, and then in tubes having a concen-
tration of serum far greater than the end-point of the titration. These results were observed consistently.

The results with human sera in table 3 emphasize the fact that the complement-fixation reaction is more delicate than the agglutination test. Human sera BE and AL both gave positive complement fixation reactions but no agglutinins were demonstrable in the dilutions noted. Comparison with guinea pig results shows that the agglutination test would probably be positive too late to be of any value in clinical diagnosis, whereas the complement fixation test was positive for human serum AM shortly after the onset of the disease. It is regretted that earlier specimens of this serum were not available for agglutination tests.

SUMMARY

1. Agglutinins for psittacosis elementary bodies have been produced in guinea pigs by the inoculation of washed L.C.L. body suspensions.
2. The technic of the agglutination test and the influence of incubation time and temperature have been studied.
3. The presence of heat-labile and heat-stable agglutinogens in psittacosis elementary bodies has been demonstrated.
4. Control experiments have proved that the agglutination reaction was not due to the presence of foreign protein or of virus contamination.
5. Cross-agglutination reactions with vaccinia virus and vaccinia antiserum have given some indication of a common antigenic factor with psittacosis virus.
6. Agglutinins have been demonstrated in the sera of human, monkeys and guinea pigs which were immunized with virus from sources involving no contact with chick protein.
7. No significant agglutinin titer for *P. vulgaris* strains was demonstrable in antipsittacosis serum.

MERRILL "MASS FACTOR" IN AGGLUTINATION

Review

Merrill (1936) has discussed the conditions under which aggregation reactions may be expected to occur. This worker
pointed out that an analysis of the requirements of reactions *in vitro* indicates that we need not yet assume a special mechanism for the immunological reactions of viruses *in vitro*. Merrill points out that the lack of observable reactions is quite probably due to the fact that insufficient antigenic mass is present.

As Merrill emphasizes, the most important consideration in *in vitro* reactions is how much actual antigen, having regard both to the number of particles and the size of the particles, is present in the virus preparations available. A rough approximation to the available experimental results is given by the assumption that 0.001 mgm. of antigen per ml., irrespective of the particle size or molecular weight, is the minimal amount capable of producing a visible aggregation with an active immune serum. Over the whole range of antigenic particles, from relatively simple molecules through virus particles to bacteria and red blood cells, there is a consistent deviation from this approximation, the smaller antigens (molecules) reacting with somewhat smaller numbers per ml., while the larger particles (bacteria and red blood cells) must be present in considerably larger numbers. Merrill's table is reproduced in figure 3.

It will be noted in figure 3 that the theoretical threshold serological reaction curve is based on 2 points, the mass of both antigens being 0.001 mgm. per ml. The diazo dye precipitation reaction was experimentally determined, and that point linked with the point represented by 0.001 mgm. per ml. of red blood cells by the dotted line. This line then represents the theoretical threshold of positive serological reactions, assuming that a mass of 0.001 mgm. per ml. is required. Actually, as previously discussed, there is a consistent deviation from this approximation, and the solid line in figure 3 represents the threshold curve as connecting two experimentally determined points, those for the pneumococcus specific soluble substance and for red blood cells. Along this line may then be placed the theoretical points for the various materials mentioned. It will be noted that the experimental point for paratyphoid bacilli coincides closely with the theoretical, while the two points for vaccinia virus are well within the limits of experimental error.

It was considered of value to determine whether the psittacosis
particle might be placed on Merrill's diagram, since the materials were available. The methods and calculations discussed below are arrived at following the formulae of Merrill.

![Diagram of Mass Factor in Serological Reactions](http://jb.asm.org/)

**FIG. 3. THE MASS FACTOR IN SEROLOGICAL REACTIONS, AFTER MERRILL. PSITTACOSIS DATA ADDED**

**Experimental**

The calculation of the mass of the psittacosis elementary body necessitated the assumption that the particle was a perfect sphere with a specific gravity of one. The error introduced by actual deviations from these assumptions could not affect the final result to any great extent, and it probably introduced no appreciable error to assume that the mass of the particle in milligrams was equal to the volume in cubic millimeters. Since the psittacosis elementary body has been shown to have a
diameter of 200 m\(\mu\) to 300 m\(\mu\) (Lazarus, Eddie and Meyer (1937)), a figure of 250 m\(\mu\) was taken to give an approximate diameter. Applying this to the formula for the volume of a sphere, \(\frac{4}{3}\pi r^3\), the approximate mass in milligrams of each L.C.L. body was found to be \(7.7 \times 10^{-12}\). Applying this value to the curve represented by the heavy line in figure 3, it is seen that approximately \(8 \times 10^8\) virus particles per ml. would be necessary to elicit a visible reaction. This figure is of course a theoretical one based on a curve with experimentally determined values at each end.

The actual number of elementary bodies necessary to give a visible reaction was determined and checked experimentally by using dilutions of a counted L.C.L. body suspension and observing the tube in which agglutination was just visible. The counts were made in the Petroff-Hausser chamber as already described (Lazarus and Meyer (1939a)) and the standard agglutination technic was followed throughout. A 1 in 10 dilution of an elementary body suspension containing \(2.2 \times 10^9\) particles per ml. gave a definite positive agglutination test, while a 1 in 20 dilution of the same suspension gave no visible sedimentation, when set up with a known positive antiserum with the proper controls. It may, therefore, be stated that under the conditions of the test, a suspension containing \(2.2 \times 10^9\) virus particles per ml. gave a positive agglutination test, while a suspension of \(1.1 \times 10^8\) particles per ml. gave no visible clumping.

This value was then placed on the Merrill diagram and is represented by a solid dot. It will be noted that this point coincides closely with the theoretical line based on 0.001 mgm. antigen per ml. The mass of virus particles required for a positive reaction was then calculated and gave a figure of 0.0017 mgm. per ml. of psittacosis particles necessary before a positive agglutination reaction could be elicited under the conditions of the experiments. This value, it will be observed, agrees with Merrill’s statement that the larger antigenic particles must be present in greater numbers than that represented by the theoretical 0.001 mgm. of antigen required.

On the basis of the Merrill theory, positive serologic reactions
have not been obtained with viruses because there has been an insufficient number of virus particles present, and, if the theory is correct, observable reactions will be obtained when the threshold virus concentration is reached. Until it has been shown that this factor has been taken into account, one is not justified in concluding that viruses differ fundamentally in their antigenic behavior \textit{in vitro} from smaller or larger antigenic particles about which more is known.

The principles laid down by Merrill give a logical explanation for the unsatisfactory agglutination reactions with the sera of animals receiving psittacosis virus in low concentration, notably the partially purified mouse material. The theory likewise helps to explain the highly satisfactory results obtained with animals which had received large doses of washed and concentrated L.C.L. bodies. From these considerations it would seem probable that the problem of preparing immune sera against viruses in non-susceptible animals is primarily one of obtaining sufficient mass of antigenic agents. This question of obtaining sufficient mass of antigen might well be a formidable barrier in many virus diseases, but has been in part met in psittacosis.

**SUMMARY**

1. The studies of Merrill, regarding the relationship between the size of antigenic particles and the number of particles necessary to produce visible immunological reaction, have been applied to the virus of psittacosis.

2. The data obtained for psittacosis virus have conformed closely to the principles outlined by Merrill.

3. The application of Merrill's theory has shown that immunological reactions have not been obtained with some anti-psittacosis sera because insufficient concentrations of virus particles have been used as immunizing agents.

**PRECIPITATION**

\textit{Review}

No investigations of the precipitin reaction in psittacosis have as yet been published. The early work of Craige (1932) has
laid down a sound foundation for the studies of vaccinia precipitation. The important preliminary work which made the detailed investigation of the vaccinia flocculation reaction possible was done by Gordon (1925), Burgess, Craigie and Tulloch (1929) and Craigie and Tulloch (1931). The work of Craigie and Wishart and of Parker and Rivers has shown the presence of a soluble specific substance in the filtrate of vaccinia emulsions after all virus has been removed. The antibodies responsible for the precipitation reaction were the same as those causing agglutination of the washed elementary bodies. Absorption tests have shown that the antigen possesses both heat-labile and heat-stable fractions.

Precipitin reactions have been demonstrated for filtrates of myxoma virus (Rivers and Ward (1937)), using virus-free filtrates of emulsions prepared from infected skin. A specific precipitinogen was also demonstrated in virus-free serum of animals acutely ill as a result of extensive infection with myxoma virus.

Experimental technique
The technic followed in the precipitin tests with psittacosis material was similar to that outlined by Smadel and Wall (1937). These workers demonstrated a soluble precipitable substance in Seitz filtrates of vaccinia emulsions prepared from infected chorioallantoic membranes. The supernatant fluid which resulted from the first angle centrifugalization of psittacosis-infected membranes was filtered through a Seitz EK pad, which had previously been prepared by the passage of 10 cc. of broth containing 1 ml. of normal rabbit or guinea pig serum. These filtrates were shown to be non-infectious for susceptible white mice. Serial dilutions of the clear serum-colored filtrates were prepared with buffered saline solution and mixed with equal volumes of diluted immune serum. Tubes and racks similar to those employed for agglutination tests were used and the mixtures were incubated overnight at 48°C.

The results obtained using the above technic were found to vary considerably, and no completely satisfactory demonstra-
tion of precipitation occurred under the conditions of the tests, although occasional positive results pointed toward the presence of antigenically active material. A typical test result is given in table 4.

These results are typical of those obtained throughout numerous attempts to demonstrate the presence of precipitinogens in the extracts of infected tissues. It will be noted that normal guinea pig serum gave a weakly positive reaction with high concentrations of chick protein, whether from normal or infected membranes. This presence of normal precipitins made it impossible to use the more concentrated antigenic material, and the necessary dilution resulted in very weak reactions.

| TABLE 4 |
| Soluble precipitable substances in filtrates of chick membranes infected with psittacosis virus |

<table>
<thead>
<tr>
<th></th>
<th>PRITTACOSIS EXTRACT UNDILUTED</th>
<th>PRITTACOSIS EXTRACT 1:2</th>
<th>NORMAL EXTRACT 1:2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiserum 1:5</td>
<td>++</td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>Antiserum 1:10</td>
<td>+</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>Anti-chick serum 1:5</td>
<td>++</td>
<td>±</td>
<td>+</td>
</tr>
<tr>
<td>Anti-chick serum 1:10</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Normal serum 1:5</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Normal serum 1:10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Saline</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+++ , + , ± , − = degrees of precipitation.

Considerable difficulty was experienced with the so-called "anomalous flocculation" in tubes containing lower concentrations of serum. The same phenomenon has been reported by Craigie and Tulloch (1931) in their investigation of the variola-vaccinia flocculation reaction. It has been suggested by these workers that the anomalous non-specific flocculation appears to bear some resemblance to the "cloudy" reaction which sometimes interferes with the application of agglutination to the study of Salmonella pullorum. This subject is discussed in detail in the work of Valley and Casman (1930), and the reaction is apparently due to the presence of lipid-protein complexes in the serum. The presence of this phenomenon in the psittacosis
precipitin tests led to much difficulty in the proper interpretation of results. Since it was not possible to extract the infected tissue by the methods suggested by Craigie and Tulloch (1931), due to the great danger of contaminating the equipment with active virus, it appeared that the precipitin method did not lend itself successfully to a study of this particular virus.

Attempts with positive rabbit and human sera yielded equally vague results, and the occasional significant findings obtained were not sufficiently clean-cut to make them of value. Membrane emulsions were filterable with difficulty through Seitz pads, even after removal of most of the particles by centrifugation. Ultrafiltrates of such emulsions yielded results no more satisfactory than the Seitz filtrates. The presence of inhibitive substances in egg membrane filtrates has been noted by Smadel and Wall (1937), and these factors probably accounted for the variation in results. These workers also noted that filtrates of vaccinia membranes in certain passages did not contain any demonstrable serologically active substances. Apparently numerous uninvestigated factors are present in this type of material, and the low titer of precipitin sera prevents the demonstration of consistently clearcut results.

Craigie (1932) has demonstrated the antagonistic effect of complement on low titer precipitating sera. Inactivation of antipsittacosis serum in the above studies gave no noticeable improvement in results.

SUMMARY

1. The precipitinogens of filtrates of psittacosis-infected tissue have been investigated.
2. While results were not completely satisfactory, some indication of the presence of precipitins in low titer was obtained.
3. The factors contributing to the uncertain results have been investigated.

NEUTRALIZATION TESTS ON EGG MEMBRANE

Review

The use of the chorio-allantoic membrane of the developing egg for the titration of neutralizing antibodies in antiviral sera
has been advocated by Burnet and his co-workers (Burnet, Keogh and Lush (1937)). This group has devoted its efforts principally to the viruses of vaccinia, influenza, louping ill, infectious laryngotracheitis of fowls and rabbit myxomatosis. The method is of value only where well-defined lesions permit counting of pocks. The comprehensive monograph of Burnet (1936) summarizes the results obtained with these viruses.

In view of the lack of discrete lesions and because of the variability of the results in different egg membranes inoculated with the same material, it appeared unlikely that the psittacosis virus would lend itself to an accurate titration of neutralizing antibodies. This opinion was further strengthened by the fact that neutralization is difficult or impossible to demonstrate by any technic, even in sera of convalescent human cases (unpublished data). Nevertheless, an attempt was made to titrate serum-virus mixtures on the egg membrane.

**Results**

Table 5 gives the result of one such experiment. The serum virus-mixtures were held at room temperature for 1 hour before inoculation into 10 day eggs, in order that union of antigen and antibody might occur; 0.1 ml. of the serum-virus mixture was inoculated into each egg. The serum used was from a laboratory worker with a positive complement fixation reaction for psittacosis, and with some evidence of protection in neutralization tests performed in mice.

It will be immediately observed in the above experiment that observation of the time of death of the embryo is not a satisfactory method for determining the presence of neutralization of psittacosis virus, unless larger numbers of eggs and statistical methods are used. The absence of discrete lesions on the membrane makes it doubtful that this method will be of value in this application. Further experiments with immune guinea pig serum gave no indication of neutralization and the method was abandoned as unsatisfactory.

The reasons for failure of the method are apparent. It has already been shown in earlier sections that a relatively massive dose is necessary to infect the egg membrane. It is likewise
The Virus of Psittacosis

A known fact that neutralizing antibodies against this virus are present in exceedingly minute amount, if at all. If the amount of virus mixed with the serum under test were reduced sufficiently to allow the neutralizing antibodies to exert any influence, the amount of virus present, even in normal controls, would be too small to infect the egg. If the amount of virus were increased so as to be sufficient for egg membrane infection, the feeble action of the neutralizing antibodies could not be demonstrated.

TABLE 5

Neutralization of psittacosis virus as tested on the chorio-allantoic membrane

<table>
<thead>
<tr>
<th></th>
<th>Dead 22 hours (trauma)</th>
<th>Hatched, 11 days</th>
<th>Hatched, 11 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 ml. immune serum</td>
<td>Dead 7 days, +</td>
<td>Dead 5 days, +</td>
<td>Dead 5 days, +</td>
</tr>
<tr>
<td>0.1 ml. of 1:100 virus</td>
<td>Dead 7 days, +</td>
<td>Dead 4 days, +</td>
<td>0</td>
</tr>
<tr>
<td>0.1 ml. of 1:1,000 virus</td>
<td>Dead 7 days, +</td>
<td>Dead 5 days, +</td>
<td>0</td>
</tr>
<tr>
<td>0.1 ml. 1:100 virus in normal serum</td>
<td>Dead 4 days, +</td>
<td>Dead 6 days, +</td>
<td>0</td>
</tr>
<tr>
<td>0.1 ml. 1:1,000 virus in normal serum</td>
<td>Dead 7 days, +</td>
<td>Dead 5 days, +</td>
<td>Dead 5 days, +</td>
</tr>
<tr>
<td>0.1 ml. 1:100 virus in immune serum</td>
<td>Dead 7 days, +</td>
<td>Dead 6 days, +</td>
<td>Dead 7 days, +</td>
</tr>
<tr>
<td>0.1 ml. 1:1,000 virus in immune serum</td>
<td>Dead 7 days, +</td>
<td>Dead 6 days, +</td>
<td>Dead 7 days, +</td>
</tr>
</tbody>
</table>

+ = positive for psittacosis, anatomically and by Castaneda stain, 0 = not done.

SUMMARY

1. Neutralizing antibodies could not be demonstrated by egg membrane inoculation, and the method was abandoned as unsatisfactory.
2. The reasons for failure of the method have been discussed.

Discussion of serological data

The foregoing sections have pointed out the close resemblance between the serological reactions of the elementary bodies of psittacosis and those of ordinary bacteria. The L.C.L. bodies have been shown to be good agglutinogens, although high titers in antiserum could not be produced; they revealed antigenic complexity; some suggestion of antigenic relationship with vaccinia elementary bodies has been obtained; they followed closely the
general principles laid down by Merrill concerning the mass of antigen necessary to produce antibodies and to show visible *in vitro* reactions; they proved poor agents for the production of precipitins; and, finally, in the reacting sera, neutralizing antibodies could not be satisfactorily demonstrated. These data all yield further evidence to support the view that the etiologic agent of this disease is a minute microorganism having much in common with ordinary bacteria, and requiring an intracellular environment for multiplication and survival. The necessity for living tissue probably indicates that this agent is not equipped with the necessary enzyme systems which would allow independent existence.

It was regretted that the investigations outlined could not be carried out in greater detail. Owing to the difficulty of securing large quantities of virus, some basic information could not be obtained and other data were relatively incomplete. The production of 5 ml. of washed elementary bodies required approximately 60 eggs and several days' operations, and it is obvious that large quantities of material in a pure state could not always be guaranteed. The seasonal drop in the fertility of eggs also caused delays in the work. To these factors must be added the extreme caution needed in manipulating material with such infectious qualities. These points may help to explain the incomplete data and the omission of certain procedures.

**GENERAL SUMMARY AND CONCLUSIONS**

1. The virus of psittacosis has been propagated on the chorioallantoic membrane of the developing egg for more than 425 consecutive bacteria-free passages without loss of infectiousness for susceptible white mice. The material obtained has been used for a wide variety of experimental studies.

2. Crude suspensions of the virus have been further purified and concentrated by means of tryptic digestion and differential centrifugation. Standardization of the purified suspensions was accomplished by turbidity and by direct count of the elementary bodies.

3. Centrifugation of the virus has resulted in concentrat-
ing the elementary bodies, but the supernatant fluid could not be entirely freed from infective particles.

4. Filtration experiments have demonstrated that the virus seldom passed through Berkefeld V and N, Chamberland L3 or Seitz EK filters. Highly successful filtration was obtained by using graded collodion membranes of the Elford type.

5. Measurements of the size of the infective particle by ultrafiltration have shown that the psittacosis virus is either the elementary body, an invisible particle not separable from the elementary body, or an invisible particle of the same size as the elementary body. The first theory seems most probable.

6. The elementary bodies have been shown to be good antigenic agents and to be antigenically complex. Satisfactory and reproducible agglutination reactions have been demonstrated. Some indication of positive precipitin tests has been obtained.

7. The mass of antigen needed for immunization and serologic reactions has been determined and the results accord well with the "mass factor" theories of Merrill.

8. Neutralization of the virus and demonstration of this reaction on the egg membrane were shown to be unsatisfactory for reasons demonstrated.

9. The virus of psittacosis, as represented by the elementary bodies, acts in all respects as a small microorganism requiring an intracellular habitat for multiplication and survival.

REFERENCES


