QUANTITATIVE STUDIES OF BRUCELLA PRECIPITIN SYSTEMS

I. PRECIPITATION OF HOMOLOGOUS ANTISERA BY BRUCELLA ENDOANTIGENS*

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Quantitative relationships in the precipitation of antibodies by their specific antigens have been studied in the past few years by several groups of workers (1-15). Heidelberger and coworkers (8-15) have developed a quantitative theory of the precipitin reaction and have applied it to the study of various antigen-antibody systems. They have shown that "if the combination of antigen or hapten were considered to take place in a series of bimolecular competing reactions between multivalent antigen and antibody, simple equations expressing in several instances the entire course of the precipitin reaction could be derived from the law of mass action."

These equations were found to be of the type

\[ \text{antibody N precipitated} = 2RS - \frac{R^2}{A}S^* \] ........................ (1)

in which R is the ratio of antibody nitrogen to antigen nitrogen at a reference point in the equivalence zone, S is the amount of antigen or hapten nitrogen added, and A is the amount of antibody nitrogen precipitated at the reference point (10).

Dividing equation (1) through by S, the equation

\[ \frac{\text{antibody N}}{S} \text{ in the precipitate} = 2R - \frac{R^2}{A}S \] ........................ (2)

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is derived. This is the equation of a straight line obtained by plotting \( \frac{\text{antibody N}}{S} \) in the precipitate against the amounts of antigen N added. This equation permits the evaluation of the constants \( 2R \) and \( \frac{R^2}{A} \) for any given serum.

An empirical relation (11, 12) yielded in some instances an even closer approximation to a straight line than that obtained by (2). The equation of this line is

\[
\text{antibody N precipitated} = 3R'' - 2 \sqrt{\frac{(R'')H(S)}{A}} \tag{3}
\]

Since these relationships were found to apply to each of the several systems studied by Heidelberger and associates, it seemed of interest to extend these studies to antigens from the brucella group of organisms. This was made possible by the preparation of purified antigens, which have been designated endoantigens from the brucella group of organisms (16). These substances are comparable to the "somatic O antigens" which Boivin and Mesrobeanu (17) believe present in all Gram-negative organisms. The endoantigens contain polysaccharide, lipide, and possibly amino acids, are very toxic for normal guinea pigs and precipitate brucella antisera to extreme dilutions. The endoantigens from the three species of the brucella are not identical.

Among the antigen-antibody systems studied by Heidelberger and associates, there were two systems, crystalline egg albumin-egg albumin antibody and crystalline horse serum albumin and its homologous antibody, in which the antigens were colorless nitrogen-containing compounds. Since the brucella endoantigens come within the scope of this class of compounds it was decided to employ their procedure for this type of reaction (12) in the study of brucella antigen-antibody systems.

**EXPERIMENTAL**

*Antisera.*—The antisera were prepared by injecting separate goats intravenously with suspensions in saline of one-tenth of an agar slant of a virulent strain of each of three species of brucella. The strains used were from the stock collections maintained at this laboratory.

*Endoantigens.*—Endoantigens for *Br. abortus*, *Br. suis*, and *Br. melitensis*, subsequently referred to as BcA, BcS, and BcM respectively, used in this experiment were prepared as described in a previous publication (16). The endoantigens were prepared for the precipitation tests as follows: A dilution of 1:500 by dry weight in distilled water was prepared. This was clarified by heating
in flowing steam at pH 8 for 10 minutes. The pH was then adjusted to 7 and the solutions were centrifuged and filtered. The opalescent solutions were then diluted so that 1 ml. of each contained 1 mg. dry weight of endoantigen. Sufficient NaCl was added to make a concentration of 0.85 per cent. The nitrogen content of the endoantigens was 8.56 per cent, 10.19 per cent, and 3.69 per cent for the abortus, suis, and melitensis preparations respectively.

Effect of Temperature and Dilutions on Antibody Precipitated by Endoantigens of Brucella from Their Homologous Antisera.—Since Heidelberger and Kendall noted that the time and temperature of incubation influenced the end-results of the precipitin systems studied and that some specific precipitates are more soluble than others, it seemed advisable to determine the effect of temperature and dilution in the three precipitin systems to be considered. The results of this determination are recorded in Table I.

The precipitation of brucella goat antisera by homologous endoantigens is as complete when carried out at 37°C. for 2 hours followed by overnight at 4°C. as when the precipitation is carried on entirely at the lower temperature for 48 hours. 24 hours at 4°C. does not suffice for complete precipitation. The specific precipitates formed are so little soluble in saline solution that this factor may be ignored in the present studies in which a volume of 2 ml. was maintained throughout.

In Tables II, III, and IV are recorded the data obtained from the addition of increasing amounts of the three endoantigens to 1 ml. of their respective homologous antisera. The total volume in each case

### TABLE I

Total Nitrogen Precipitated from 1 ml. Serum by the Amount of Endoantigen Indicated in 1 ml. Saline, unless Otherwise Indicated

<table>
<thead>
<tr>
<th>Serum + 0.1 mg. homologous endoantigen</th>
<th>37°C. 2 hrs.</th>
<th>4°C. overnight</th>
<th>4°C. 24 hrs.</th>
<th>4°C. 48 hrs.</th>
<th>37°C. 2 hrs.</th>
<th>4°C. overnight</th>
<th>Total volume 10 ml.</th>
<th>Difference per ml. between columns 2 and 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Br. melitensis</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
</tr>
<tr>
<td>Br. suis</td>
<td>0.024</td>
<td>0.018</td>
<td>0.023</td>
<td>0.011</td>
<td>0.0016</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Br. abortus</td>
<td>0.054</td>
<td>0.047</td>
<td>0.052</td>
<td>0.042</td>
<td>0.0015</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.076</td>
<td>0.071</td>
<td>0.075</td>
<td>0.055</td>
<td>0.0026</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
## TABLE II

### Abortus Endoantigen + Abortus Antiserum

<table>
<thead>
<tr>
<th>BcA added</th>
<th>BcAN added</th>
<th>BcAN pptd.</th>
<th>Total N pptd.</th>
<th>Antibody N pptd.</th>
<th>Ratio AN/BcAN</th>
<th>Antibody pptd.</th>
<th>Ratio A/BcA</th>
<th>Antibody pptd.</th>
<th>Tests on supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>0.0042</td>
<td>0.0448</td>
<td>0.0400</td>
<td>9.66</td>
<td>0.253</td>
<td>5.00</td>
<td>0.164</td>
<td>0.182</td>
<td>Antibody excess</td>
</tr>
<tr>
<td>0.10</td>
<td>0.0085</td>
<td>0.0767</td>
<td>0.0683</td>
<td>8.03</td>
<td>0.426</td>
<td>4.25</td>
<td>0.318</td>
<td>0.337</td>
<td></td>
</tr>
<tr>
<td>0.20</td>
<td>0.0171</td>
<td>0.1120</td>
<td>0.0949</td>
<td>5.54</td>
<td>0.593</td>
<td>2.96</td>
<td>0.592</td>
<td>0.600</td>
<td></td>
</tr>
<tr>
<td>0.50</td>
<td>0.0428</td>
<td>0.2176</td>
<td>0.1748</td>
<td>4.08</td>
<td>1.092</td>
<td>2.18</td>
<td>1.150</td>
<td>1.125</td>
<td>Excess BcA</td>
</tr>
<tr>
<td>0.80</td>
<td>0.0684</td>
<td>0.2622</td>
<td>0.2033</td>
<td>3.41</td>
<td>1.270</td>
<td>1.82</td>
<td>1.300</td>
<td>1.309</td>
<td></td>
</tr>
<tr>
<td>1.00</td>
<td>0.0856</td>
<td>0.2912</td>
<td>0.2121</td>
<td>2.68</td>
<td>1.325</td>
<td>1.43</td>
<td>1.273</td>
<td>1.379</td>
<td></td>
</tr>
<tr>
<td>1.10</td>
<td>0.0941</td>
<td>0.2780</td>
<td>0.1965</td>
<td>2.41</td>
<td>1.228</td>
<td>1.29</td>
<td>1.273</td>
<td>1.379</td>
<td></td>
</tr>
<tr>
<td>1.20</td>
<td>0.1027</td>
<td>0.2684</td>
<td>0.1840</td>
<td>2.18</td>
<td>1.150</td>
<td>1.16</td>
<td>1.273</td>
<td>1.379</td>
<td></td>
</tr>
<tr>
<td>1.50</td>
<td>0.1284</td>
<td>0.2352</td>
<td>0.1500</td>
<td>1.76</td>
<td>0.937</td>
<td>0.941</td>
<td>1.273</td>
<td>1.379</td>
<td></td>
</tr>
<tr>
<td>2.00</td>
<td>0.1712</td>
<td>0.2016</td>
<td>0.0964</td>
<td>0.91</td>
<td>0.602</td>
<td>0.49</td>
<td>1.273</td>
<td>1.379</td>
<td></td>
</tr>
</tbody>
</table>

Equation (1): mg. antibody pptd. = 3.4 (BcA) - 2.20 (BcA)^2; A = 1.313 mg. antibody

Equation (3): endoantigen in ppt. = 4.3 - 2.90 √BcA; BcA = 0.965; A = 1.407 mg.

* Determination of excess antigen not run in duplicate.

## TABLE III

### Suis Endoantigen + Suis Antiserum

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td>mg.</td>
<td></td>
</tr>
<tr>
<td>0.05</td>
<td>0.0051</td>
<td>0.0443</td>
<td>0.0392</td>
<td>7.68</td>
<td>0.243</td>
<td>4.90</td>
<td>0.239</td>
<td>0.249</td>
<td>Antibody excess</td>
</tr>
<tr>
<td>0.10</td>
<td>0.0102</td>
<td>0.0793</td>
<td>0.0691</td>
<td>6.77</td>
<td>0.432</td>
<td>4.32</td>
<td>0.431</td>
<td>0.427</td>
<td></td>
</tr>
<tr>
<td>0.15</td>
<td>0.0153</td>
<td>0.1050</td>
<td>0.0897</td>
<td>5.86</td>
<td>0.560</td>
<td>3.73</td>
<td>0.572</td>
<td>0.559</td>
<td></td>
</tr>
<tr>
<td>0.20</td>
<td>0.0203</td>
<td>0.1225</td>
<td>0.1022</td>
<td>5.03</td>
<td>0.638</td>
<td>3.19</td>
<td>0.671</td>
<td>0.652</td>
<td></td>
</tr>
<tr>
<td>0.30</td>
<td>0.0305</td>
<td>0.1465</td>
<td>0.1160</td>
<td>3.80</td>
<td>0.725</td>
<td>2.41</td>
<td>0.720</td>
<td>0.745</td>
<td>Neither antibody nor BcS</td>
</tr>
</tbody>
</table>

Equation (1): mg. antibody pptd. = 5.27 (BcS) - 9.57 (BcS)^2; A = 0.723 mg. antibody

Equation (3): endoantigen in ppt. = 6.73 - 7.75 √BcS; BcS = 0.335; A = 0.751 mg.
was 2 ml. The precipitations were carried out at 37°C for 2 hours followed by 24 hours at 4°C. The precipitates were centrifuged, carefully drained, and washed twice with 1 ml of ice cold saline with careful rinsing of the tubes and agitation. A third washing seemed to have no influence on the precipitates. Nitrogen determinations were run by a modification of the micro Kjeldahl method. The supernatants were tested for the presence of excess antigen and antibody by adding to aliquots a corresponding fraction of antibody and antigen, respectively. In the regions of excess antigen and in the inhibition

<table>
<thead>
<tr>
<th>Table IV</th>
<th>Melitensis Endoantigen + Melitensis Antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BcM added</td>
</tr>
<tr>
<td>0.05</td>
<td>0.0018</td>
</tr>
<tr>
<td>0.10</td>
<td>0.0037</td>
</tr>
<tr>
<td>0.15</td>
<td>0.0055</td>
</tr>
<tr>
<td>0.20</td>
<td>0.0073</td>
</tr>
<tr>
<td>0.30</td>
<td>0.0110</td>
</tr>
<tr>
<td>0.50</td>
<td>0.0184</td>
</tr>
<tr>
<td>0.70</td>
<td>0.0258</td>
</tr>
<tr>
<td>1.00</td>
<td>0.0369</td>
</tr>
<tr>
<td>1.20</td>
<td>0.0442</td>
</tr>
<tr>
<td>1.50</td>
<td>0.0553</td>
</tr>
<tr>
<td>2.00</td>
<td>0.0738</td>
</tr>
<tr>
<td>3.00</td>
<td>0.1106</td>
</tr>
<tr>
<td>4.00</td>
<td>0.1476</td>
</tr>
<tr>
<td>5.00</td>
<td>0.1844</td>
</tr>
</tbody>
</table>

Equation (1): \( \text{mg. antibody pptd.} = 1.325 \times (\text{BcM}) - 0.714 \times (\text{BcM})^2; A = 0.612 \text{ mg} \)

Equation (3): \( \text{endoantigen in ppt.} = 1.58 - 0.968 \sqrt{\text{BcM}}; \text{BcM} = 1.184; A = 0.624 \text{ mg} \)

* Determination of excess antigen not run in duplicate.

† Calculated by both methods.
zones the excess antigen was determined by reference of the total N precipitated from the aliquot of the supernatant, to graphs of total nitrogen precipitated plotted against endoantigen nitrogen precipitated in the region of antibody excess. In some instances, where indicated, better results were obtained by calculating excess antigen by the method given by Heidelberger and Kendall in their study of the egg albumin system (12). All determinations were made in duplicate unless otherwise indicated.

In Fig. 1 are presented the graphs produced from the data in Table IV. The graphs for the two other endoantigens are extremely similar and are not presented.

DISCUSSION

From Tables II, III, and IV and from Fig. 1, it is evident that these three precipitin systems proceed in general, in the same manner as those studied by Heidelberger et al. Thus, by plotting the experimental combining ratios of antibody N and endoantigen N against milligrams of endoantigen N precipitated and against the square root
of endoantigen N precipitated, it is possible in each case to derive
equations which describe the behavior of the system.

Data obtained in the study of cross precipitations (21), and further
data which have not been included in this paper, have shown some
suggestion that the nitrogen content of the endoantigen may not
always be intimately connected with its ability to precipitate anti-
serum. For this reason Fig. 1 and equations (1) and (3) for each of
the endoantigens have been prepared from substance-combining
ratios, rather than N-combining ratios. Although the endoantigens
upon careful examination (16) have seemed to be pure substances,
this suggestion of occasional lack of correlation necessitates considera-
tion of the present data as provisional, to be corrected by a purity
factor if that is found necessary upon further examination of the
endoantigens.

If the data for Brucella abortus endoantigen be graphed it will be
noticed that the initial ratios are much too high to strike the graphs of
the linear equations. Yet, if the data be graphed according to equa-
tions for systems showing an antibody-antigen combining ratio
greater than 2R, agreement with the experimental ratios is entirely
lacking. Similar data encountered by Heidelberger et al. were believed
to show that, whereas the reaction behaved generally according to
equation (2), there was a small amount of antibody present capable of
reacting with the antigen in ratios greater than 2R. It will be noticed
that the values of R and A in equation (1) are located at the beginning
of the equivalence zone in the equation developed for the Br. suis
system, but that these two values are located well within the equiva-
lence zone in the equations developed for Br. abortus and Br. melitensis.

The data obtained in the inhibition zones gave similar values
whether treated by the simple or by the more accurate complex calcu-
lation developed by Heidelberger and associates. Since these authors
have demonstrated conclusively (11, 12, 15) that the entire amount of
dissolved antigen-antibody complex in the supernatant in this zone is
precipitable by additional antibody, their method was adopted in this
work as perfectly valid.

Since brucella sera are usually calibrated by the determination of
agglutinin content, and since agglutinins and precipitins have been
shown to be identical (20), the data would imply that the endoantigens
may be used for the more accurate calibration of brucella antisera. For this it would be necessary, as suggested by Heidelberger and Kendall (8), to determine the combining ratios of endoantigen and antibody in a given serum at two, or preferably three points in the region of antibody excess. From these ratios, plotted against the square root of the endoantigen precipitated, an equation of type (3) could be derived, which would permit the calculation of the maximum specific nitrogen of the serum. Conversely, calibrated antisera could be used to detect small quantities of endoantigen.

SUMMARY

It has been shown that the precipitation by the endoantigens of the three species of brucella of their homologous antibodies may be described by equations developed from the law of mass action.

The endoantigens may be used for the accurate calibration of brucella antisera.

The nitrogen-containing constituent of the endoantigens does not always seem to be intimately connected with the ability to precipitate the specific antibodies.

BIBLIOGRAPHY