THE HEAT OF AN ANTIBODY-ANTIGEN REACTION

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Serological reactions are now generally regarded as essentially chemical in nature. Therefore, if this view is correct, the heat of combination of antibody and antigen should be of a magnitude which appears reasonable in the light of our knowledge of classical thermodynamics.

The first extended study of serological reactions from the physicochemical standpoint was that of Arrhenius (1), who, with Madsen, obtained values for the temperature coefficient of the reaction between tetanolysin and its antibody which enabled them to calculate a heat evolution of 5480 calories per mole of antigen. The first attempt to measure the heat of such reactions directly was that of Bayne-Jones (2) who used a differential microcalorimeter of the type designed by A. V. Hill. A heat evolution of 0.0645 calorie per Lf unit was observed when diphtheria toxin reacted with antitoxin.

Recent analyses of highly purified toxin indicate that 1 Lf unit contains about 0.00046 mg. of nitrogen. Since the molecular weight of diphtheria toxin is about 70,000 (8), and it contains about 16 per cent of nitrogen, we may calculate that Bayne-Jones' result corresponds to the impossibly large value of $1.53 \times 10^4$ kilocalories per mole of toxin. Concentrated preparations of toxin, containing as much as 10,000 Lf units per cc., have recently been obtained (7), and were such solutions mixed with the appropriate amount of antitoxin, with comparable volumes, one would

1 The $L_f$ unit is the amount of toxin giving most rapid flocculation with 1 standard unit of antitoxin.
predict from Bayne-Jones' result a rise in temperature of over
100°. Smith and Marrack (9) found no detectable rise in tempera-
ture (with a thermometer graduated in tenths of a degree) with a
toxin containing 500 Lf units per cc. Thus the heat of reaction
found by Bayne-Jones seems to be very much in error. Further
reason for so thinking lies in the fact that he observed a slow
liberation of heat over a period of about 1 hour, contrasting with
the extremely rapid rate with which serological reactions are now
known to proceed during the first (combination) stage. A possible
explanation is that the slow liberation of heat might have been
due to the increased heat of stirring of the increasingly viscous
reacting mixture. No increase of viscosity would have occurred
in the control Dewar flask containing no toxin. The observed
temperature difference attributed to the toxin-antitoxin reaction
might thus have had a purely mechanical origin. The tempera-
ture began to fall very slowly after 72 minutes and this could
perhaps have been due to separation of the precipitate attended
by a decrease in viscosity. In our own experiments we found a
very large change in viscosity.

The most recent attempt to estimate the heat of a serological
reaction was that of Follensby and Hooker (5), also with diphtheria
toxin and antitoxin. Using a relatively crude method of titration
(subject to about 20 per cent error) they found no influence of
temperature on the equilibrium of the toxin-antitoxin reaction,
and estimated that the heat of reaction was not over 1000 calories
per mole of toxin.

The present communication reports a new direct measurement, at
31°, of the heat of a serological reaction, that between the purified
hemocyanin of the sea snail, _Busycon canaliculatum_, and its anti-
body produced by a horse injected with this antigen. The
hemocyanins were regularly found to be powerful antigens by
Hooker and Boyd (6).

_Preparation of Materials_—The hemocyanin had been purified
by repeated precipitation near its isoelectric point. The antigen
solution contained 0.9 per cent sodium chloride, added to make it
isotonic with the serum. The pH of these reagents, and of sam-
ple of normal (non-immune) serum from three different horses,
was brought to the same value, as measured by the glass electrode,
by addition of the required small amounts of 0.2 N sodium hydrox-
ideal conditions for a calorimetry experiment. The preliminary experiments were done without addition of preservative to the reactants; in later experiments the addition of merthiolate (Lilly) to a concentration of 0.02 per cent was used to prevent bacterial growth, and resulting alterations of pH.

Procedure

The calorimeter used was previously described by Conn, Kistiakowsky, and Roberts (3). In order to avoid complications due to the heat of precipitation, the reaction was carried out in two stages in the calorimeter. 840 gm. of horse antihemocyanin were put in the outer compartment of the calorimeter, and the inner can was filled with 62 cc. of "weak" hemocyanin. Advantage was taken of the fact that when horse antiprotein serum is mixed with its antigen in such proportions that antibody is in considerable excess no precipitate is formed, although combination takes place between the antibody and antigen. The first quantity of antigen added to the serum slowly produced an opalescent solution, but no precipitate, even on prolonged standing. The calorimeter was brought by electrical heating to a temperature not far from its equilibrium (with stirring) temperature. Observations on the main thermel were made over a suitable interval; whereupon the double plug in the inner can was raised, allowing the antigen to mix with the serum. After heat evolution was complete, the inner can was closed, its contents were pipetted out, and after being rinsed it was filled with 62 cc. of "strong" hemocyanin solution, and the experiment was carried out as before. The concentration of the strong hemocyanin was such as to give a precipitate promptly.

As control experiments, weak and strong hemocyanin solutions were added to normal serum in the manner described above. The concentrations of these solutions are shown in Table I. Runs were made with weak hemocyanin and normal sera from three different horses.

Two series of experiments were made, with portions of the same immune serum and different preparations of hemocyanin. Our results are presented in Table II. The molecular weight of the hemocyanin was assumed to be 6,800,000 (4). The course of the
Heat of Antibody-Antigen Reaction

Heat evolution in the first experiment of Series 2, in which weak hemocyanin and immune serum were allowed to react, is shown in Fig. 1. The curve has been corrected for heat of stirring and for heat losses to the outside. Previous experiments have shown that

<table>
<thead>
<tr>
<th>TABLE I</th>
<th>Data Pertaining to Reagents Used in Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Series No.</td>
<td>Hemocyanin</td>
</tr>
<tr>
<td>1</td>
<td>&quot;Weak&quot;</td>
</tr>
<tr>
<td>2</td>
<td>&quot;&quot;</td>
</tr>
<tr>
<td>3</td>
<td>&quot;Strong&quot;</td>
</tr>
</tbody>
</table>

The molecular weight of the antigen was assumed to be 6.8 × 10^6, that of antibody 1.6 × 10^6. The ratio by weight of antibody to antigen in the compound formed by the addition of "weak" hemocyanin was estimated from analyses of other precipitates to be about 2.0, that in the compound formed when the "strong" hemocyanin was added to be about 1.0.

FIG. 1. Evolution of heat when weak hemocyanin and immune serum were allowed to react.

Mixing of the contents of the calorimeter is complete after 3 minutes. One may therefore say that the reaction with which we are concerned here is probably a very fast one, since 80 per cent of the heat is evolved within 2 minutes after mixing.
The ratio by weight in which antibody and antigen combine in the region of antibody excess represented by our mixtures of immune serum and weak hemocyanin is not known, since no precipitate is formed. By extrapolating from the results of analyses on precipitates from mixtures in various proportions we estimated that in this particular system the maximal ratio would probably be about 2.0. From this estimated value the number of molecules of antibody (assumed to have a molecular weight of 160,000) combining with 1 of antigen, and hence the heat of reaction per mole of antibody, could be calculated. These latter are shown in the last column of Table II.

### Table II

**Heat Evolved on Mixing Hemocyanin and Normal and Immune Sera**

<table>
<thead>
<tr>
<th>Series No.</th>
<th>Heat evolved</th>
<th>$\Delta H$</th>
<th>Kilocalories × 10⁻⁴</th>
<th>Kilocalories per mole hemocyanin</th>
<th>Calculated kilocalories per mole antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>+0.350</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak hemocyanin + immune serum, (1)</td>
<td>0.000</td>
<td>+0.350</td>
<td>2.78</td>
<td>3027</td>
<td>35.6</td>
</tr>
<tr>
<td>Weak hemocyanin + 1st normal serum, (2)</td>
<td>(1) - (2)</td>
<td>+0.405</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>+0.045</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak hemocyanin + immune serum, (1)</td>
<td>+0.045</td>
<td>3.58</td>
<td>3900</td>
<td>45.9</td>
<td></td>
</tr>
<tr>
<td>Weak hemocyanin + 2nd normal serum, (2)</td>
<td>(1) - ((2) + (3))/2</td>
<td>+0.428</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weak hemocyanin + 3rd normal serum, (3)</td>
<td>(4)</td>
<td>+0.144</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strong hemocyanin + immune serum, (4)</td>
<td>+0.045</td>
<td>+0.428</td>
<td>116</td>
<td>2.7*</td>
<td></td>
</tr>
<tr>
<td>Strong hemocyanin + 4th normal serum, (5)</td>
<td>(4) - (5)</td>
<td>+0.099</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Calculated for antibody reacting with the second addition of hemocyanin.
Heat of Antibody-Antigen Reaction

Two complications were encountered in the first series of experiments. Upon mixing the hemocyanin with immune serum an increase in viscosity occurred, as shown by a very great increase in the heat of stirring, thus making accurate extrapolation to initial time difficult. Furthermore, the solutions were not protected from bacterial growth by preservative, and the experiments with strong hemocyanin in the first series of experiments were worthless.

In the second series of experiments these difficulties were largely remedied. The rate of stirring of the calorimeter was cut in half, thus reducing the heat of stirring to one-eighth its former value. This change resulted in a very much smaller increase in heat of stirring with increased viscosity, making extrapolation to initial time more accurate. The use of merthiolate in the second series of runs appeared to protect the solutions adequately; there was no evidence of bacterial attack.

DISCUSSION

The heat of reaction per mole of antigen is large, as one would expect, since it is the resultant of the heats of reaction of a number of molecules of antibody with each antigen molecule. The heat of reaction per mole of antibody is more interesting, since presumably this results from the reaction of a small number of chemical groups. Assuming a reasonable value, say \(-10,000\) calories, for the change in free energy, \(\Delta F^\circ\), in this reaction, one which goes very nearly to completion but may be reversed experimentally, one may estimate the entropy change, \(\Delta S^\circ\), from the equation

\[
\Delta S^\circ = \frac{\Delta H - \Delta F^\circ}{T} = \frac{-40000 + 10000}{304} \approx -100 \text{ entropy units}
\]

The entropy decrease in gaseous reactions involving a decrease of 1 unit in mole numbers is usually of the order of 30 entropy units for small molecules, when moles per liter are used as units of concentration. In the antibody-antigen reaction per mole of antibody the mole number decreases by 1 also.

The larger entropy decrease may of course be due entirely to the dissolved state of the molecules or to their large size. On the other hand its magnitude may perhaps be regarded as an indication that several chemical groups are involved in the combination of 1
antibody molecule. It is interesting to note that in the experiment with strong hemocyanin solution considerably less heat, both per mole of antibody and per mole of antigen, is evolved. Two factors may operate to reduce the heat evolution in this experiment. In order to form compounds of lower antibody-antigen ratio, some dissociation of antibody from the original soluble compound must take place, with a resulting heat absorption. Furthermore, it is possible that heat may be absorbed in the process of flocculation. No estimate of the magnitude of this heat change can be made.

We wish to thank the Lederle Laboratories, Inc., for their great kindness in immunizing a horse with hemocyanin and presenting one of us with the resulting antiserum, Dr. Robinson, director of the Antitoxin and Vaccine Laboratory of the Massachusetts Department of Public Health, for the gift of normal horse serum, and the Rockefeller Foundation for its support of the calorimetric work.

SUMMARY

With a sensitive calorimeter the heat evolved when an antibody (antihemocyanin from the horse) reacted with its antigen (hemocyanin of Busycon canaliculatum) was measured. In the region of antibody excess, where no precipitate was formed, a value of about 3.0 calories per gm. of antigen nitrogen was found (measured at 31°). This corresponds to about 3,300,000 calories per mole of antigen. It is believed that this value is probably accurate to about 20 per cent. By extrapolation from the results of analyses of specific precipitates, it was calculated that the above result corresponds to about 40,000 calories per mole of antibody. Possible factors affecting this value in the same and in different systems are mentioned, and the magnitude of the result is shown to be reasonable from thermodynamic considerations.

BIBLIOGRAPHY

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