NOTE ON THE PROTEINS OF THE BLOOD OF *LIMULUS POLYPHEMUS* L.

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The blood of *Limulus polyphemus* L. has been shown to contain the protein haemocyanin and white corpuscles. The blood clots with great rapidity. Loeb\(^1\) found that clotting is not preventable by the usual chemical methods but only by methods which prevent agglutination of the corpuscles. Further, Loeb has shown that the clot is formed by the agglutination of the corpuscles, indicating the absence of fibrinogen in the blood. By a study of the composition of the clot Alsberg and Clark\(^2\) showed that the clot contains no fibrin, but is composed mainly of a protein, the analysis of which showed C : S : N ratios more like those of glutins than fibrins. The nitrogen content of the clot protein was found to be 15.6 per cent. Haemocyanin, which remains in the serum, contains 16.8 per cent of nitrogen. This serum has a specific gravity of 1.040 as found by Gotch and Laws.\(^3\) They first studied the haemocyanin of Limulus and concluded that heating the serum does not completely coagulate the haemocyanin, because the liquid still remains blue after boiling. Howell\(^4\) states that in order to produce complete coagulation it is necessary to heat the

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Proteins of Blood of Limulus

serum a long time at 80°. Halliburton⁵ gives the coagulation
temperature at 65–66°. Alsberg and Clark⁶ prepared the haemo-
cyanin of Limulus by fractional precipitation with ammonium
sulphate and removal by dialysis of the ammonium sulphate
from the purified product. The substance purified in this way
could not be crystallized. It differs from the haemocyanin of
Octopus investigated by Henze,⁷ both in its percentage composi-
tion and in its physical characteristics. Limulus haemocyanin is
easily precipitated, practically quantitatively, by weak acids,
but by this method of precipitation more or less of the copper is
dissociated. It is, also, precipitated, practically quantitatively,
by dilute solutions of zinc sulphate. This method was first em-
ployed by Kobert⁸ to precipitate the haemocyanin of Eledone
moschata. Fredericq⁹ has shown that haemocyanin is the only
protein in the plasma of Octopus. Henze confirmed the results
of Fredericq and found, further, that haemocyanin constituted
9 per cent of the blood. From the observations of Fredericq,¹⁰
who found that the protein content of the blood of the river
crayfish, Astacus flaviatilis, varies according to the state of nutri-
tion of the animal, it would seem that the protein content of
the blood of Crustacea is quite variable. Slight variability is well
known to occur in higher animals including man, as shown by
Voit,¹¹ Lewinski¹² and Benedict.¹³

In view of the scanty data existing concerning the distribution
of nitrogenous substances in the blood of the lower animals, fur-
ther information on this subject would seem to be desirable.

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⁵ Halliburton: On the Blood of Decapod crustacea, Journ. of Physiol.,
vi, pp. 300–335, 1885.
⁶ Alsberg, C. L. and E. D. Clark: The Haemocyanin of Limulus poly-
⁸ Kobert, R.: Ueber Haemocyanin nebst einigen Notizen über Haemery-
thrin, Pfüger’s Archiv, xcviii, p. 411, 1903.
⁹ Fredericq, L.: Influence du milieu ambiant sur la composition du sang
des animaux aquatiques, Arch. de Zool., 2 Série, iii, 1885.
¹⁰ Fredericq, L.: Note sur le sang de l’ecrivisse, Libre jubil. dédié à Charles
Van Bambeke, Bruxelle, 1899.
¹² Lewinski, J.: Pfüger’s Archiv, c, p. 611, 1903.
¹³ Benedict, F. G.: Publication No. 77, Carnegie Institution of Wash-
ington.
Some information concerning the blood of *Limulus polyphemus* is given in the following work. At the same time, since the observations on the coagulation of haemocyanin differ, the behavior of this substance to heat was studied.

For the observations on the distribution of nitrogen in the blood and in the serum of Limulus, blood from animals which had been kept from one to twelve weeks in a float in the harbor at Woods Hole was drawn directly into a tared bottle and weighed. In Table I the order of experiments indicates decreasing length of time after capture. In each of the first four experiments of Table I blood was taken from a different animal. In the fifth experiment a measured volume of blood was taken from a number of animals. Its weight was estimated by calculating the weight of

**TABLE I.**

_Distribution of Nitrogen in Limulus Blood._

<table>
<thead>
<tr>
<th>NO.</th>
<th>BLOOD</th>
<th>N IN CLOT</th>
<th>PROTEIN IN CLOT</th>
<th>N IN HAEMOCYANIN</th>
<th>HAEMOCYANIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18.26</td>
<td>0.0045</td>
<td>0.0388</td>
<td>0.158</td>
<td>0.0483</td>
</tr>
<tr>
<td>2</td>
<td>26.004</td>
<td>0.0104</td>
<td>0.0672</td>
<td>0.258</td>
<td>0.298</td>
</tr>
<tr>
<td>3</td>
<td>28.393</td>
<td>0.0135</td>
<td>0.0865</td>
<td>0.304</td>
<td>1.63</td>
</tr>
<tr>
<td>4</td>
<td>53.424</td>
<td>0.0494</td>
<td>0.316</td>
<td>0.590</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>393.5*</td>
<td>0.376</td>
<td>2.403</td>
<td>0.610</td>
<td></td>
</tr>
</tbody>
</table>

*This determination was made on 390 cc. of composite blood of a number of animals.*

the measured expressed serum, assuming that 1.040 was its specific gravity. Obviously the figure obtained is not strictly comparable with the others given in the table, because the influence of the clot protein is not considered. During the clotting, as shown in the table, the blood yielded 390 cc. of serum and 2.4 grams of clot protein. In all experiments the weighed portions of blood were kept at about 10°C. in an ice box for twenty-four hours. The clot, after draining carefully from supernatant liquid, was washed first with 5 per cent sodium chloride solution, then with distilled water. Nitrogen in the insoluble residue was then determined by the Kjeldahl method. In determination 1 of Table I the haemocyanin was completely precipitated in the filtrate from the clot by 10–15 cc. of 5 per cent zinc sulphate solution. The
precipitated haemocyanin, separated by filtration, was washed with 5 per cent zinc sulphate solution. Nitrogen in the residue was determined. After saturating the filtrate from the haemocyanin with zinc sulphate, an insignificant precipitate was formed, which contained only traces of nitrogen. The calculations of clot protein and of haemocyanin in Table I and of haemocyanin in Table II are based on the nitrogen content for the respective proteins as given above.

It was found that the filtration of solutions containing zinc sulphate was too slow to allow suitable washing of residues. Therefore, in all subsequent experiments recorded in Table II the haemocyanin was precipitated by dilute acetic acid. The precipitate was removed by filtration through paper, washed with water faintly acidulated with acetic acid and its nitrogen content determined. The filtrate was nearly neutralized with sodium carbonate and brought to boiling. The coagulum was removed by filtration on paper, washed and its nitrogen content determined. Nitrogen was also determined by the Kjeldahl method in the filtrate.

One fact clearly brought out by a study of these tables is that in the blood of *Limulus polyphemus* very little protein other than the clot protein and the haemocyanin is present. For, after the removal of both of these proteins saturation with zinc sulphate indicates the presence of a minute amount of nitrogen in non-coagulable form. Its properties were not studied. Since the clot of Limulus blood is formed by the agglutination of the cells,

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**TABLE II.**

*Nitrogen Distribution in Limulus Serum.*

<table>
<thead>
<tr>
<th>NO.</th>
<th>SERUM</th>
<th>N IN HAEMOCYANIN</th>
<th>HAEMOCYANIN</th>
<th>N IN COAGULUM AFTER REMOVAL OF HAEMOCYANIN</th>
<th>NON-COAGULABLE N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cc.</td>
<td>gram</td>
<td>gram</td>
<td>percent of serum</td>
<td>gram</td>
</tr>
<tr>
<td>1</td>
<td>25*</td>
<td>0.1204</td>
<td>0.744</td>
<td>2.94</td>
<td>0.0011</td>
</tr>
<tr>
<td></td>
<td>25*</td>
<td>0.1210</td>
<td>0.747</td>
<td>2.96</td>
<td>0.0012</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>0.0755</td>
<td>0.466</td>
<td>1.84</td>
<td>0.0020</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>0.0696</td>
<td>0.430</td>
<td>1.74</td>
<td>0.0014</td>
</tr>
</tbody>
</table>

*These are duplicates.
as shown by Loeb,\textsuperscript{14} a considerable amount of cell protein might be expected to pass into the blood. Apparently, this cell protein is insignificant in amount, the greater part of the proteins of the blood cells of Limulus remaining in the clot. However, a minute amount of other proteins does occur, as shown by the very slight precipitate formed on saturating with zinc sulphate, or, of coagulum formed by heating the serum deprived of clot protein and haemocyanin.

There seems also to be present in the clot a minute amount of protein which can be crystallized in macroscopic rhombic plates the largest of which measure one-eighth of an inch. The yield was so small that no further study was attempted.

Another fact shown by the tables is that the amount of haemocyanin present in the blood is much less than is found in the blood of Octopus, estimated by Henze as 9 per cent.

Inspection of the tables shows, moreover, that the clot protein and the haemocyanin vary greatly in amount in different individuals. In spite of these variations it is clear that the haemocyanin is several times as abundant as the clot protein. The variations themselves are probably dependent upon the condition of nutrition of the animal. Since, as stated above, the specimens examined for the results recorded here had been kept in a float in the harbor of Woods Hole for varying periods, some of them were in a more or less advanced stage of starvation. After three months of confinement the protein content of the blood diminished from approximately 3.5 per cent to 1.50 per cent. Similar observations on the influence of starvation on the protein content of the blood of other animals are cited above.

For the determination of the coagulation temperature pure neutral solutions of haemocyanin containing a little ammonium sulphate were heated. These were obtained after precipitation of serum with the requisite amount of ammonium sulphate,\textsuperscript{15} the precipitate filtered off, redissolved and dialyzed. When heated to 48°C. opalescence appeared. This became more marked as the temperature rose but distinct floccules did not appear until 60–62°C., while the coagulation did not seem to be complete until

\textsuperscript{14} Loeb, L.: \textit{loc. cit.}

\textsuperscript{15} Alsberg, C. L. and E. D. Clark: \textit{loc. cit.}
67–68°C. was reached, which accords very well with Halliburton’s determination of 65–66°C. This is the behavior of solutions of pure haemocyanin; serum does not coagulate so readily. The observations of Howell, and of Gotch and Laws, that the haemocyanin in serum does not readily coagulate completely are in accord with the observations recorded here. The different behavior of haemocyanin in serum and in pure solution is due to the alkalinity of the serum. That alkalinity greatly affects the coagulability of protein is well known. The failure to take this alkalinity into consideration seems to have led to a certain amount of confusion in the observations of those investigating invertebrate bloods.

The small amount of protein obtained by coagulation after removal of the clot and haemocyanin, as recorded in Table II, seems to be different from the haemocyanin, as far as may be judged from the coagulation point. It begins to coagulate at 66°C., but coagulation is slight until a temperature of 72–74°C. is attained. Evidence such as this based upon the temperature of coagulation can not, of course, be regarded as conclusive.

SUMMARY.

The proteins of the blood of Limulus consist almost exclusively of the clot protein, or cell fibrin, and haemocyanin. Haemocyanin is several times as abundant as the cell fibrin. Other proteins occur only in minimal amounts. The blood also contains a small amount of nitrogen in non-coagulable form. The quantity of protein in the blood seems to vary with the condition of the animal, diminishing in starvation. There is probably, at most, less than half as much haemocyanin as in the blood of Octopus. The coagulation temperature of Limulus haemocyanin was found to be 67–68°C.

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