THE RESPIRATORY PROTEINS OF THE BLOOD.

IV. THE BUFFER ACTION OF HEMOCYANIN IN THE BLOOD OF LIMULUS POLYPHEMUS.

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Although the buffer action of the blood of man has received most thoroughgoing analysis (Van Slyke, 1921) the similar phenomena in the body fluids of invertebrates have been described only in such a way as to define the magnitude of the problem without at all adequately contributing to its solution. Quaglariello (1916) showed that relatively large amounts of acid or alkali must be added to the blood of certain invertebrates in order to produce a given change in its reaction. Thus in the cephalopods, Eledone and Octopus, some 200 mg. of sodium hydroxide were required to bring the reaction of a liter of blood from its normal value of pH 7.36 or 7.54 to pH 9.0. In the case of the horseshoe crab, Limulus polyphemus, we find that 400 mg. of sodium hydroxide are required to produce this effect. This is 1000 times the amount required to produce a comparable effect in distilled water. To what components of the blood is this large buffer effect due?

Parsons and Parsons (1923-24) amplified the observations of Quaglariello by working out the carbon dioxide dissociation curves of a number of marine invertebrates. They point out that those bloods which combine with carbon dioxide in quantity are rich in hemocyanin. On the other hand Nitzescu and Cosma (1927) conclude from a study of the carbon dioxide dissociation curve of the snail, Helix pomatia, that in this species protein substances (hemocyanin) do not play the principal rôle in the acid-base equilibrium. Redfield, Coolidge, and Hurd (1926)
point out that the carbon dioxide-combining power of different species is not strictly comparable to their hemocyanin content as judged by the capacity for uniting with oxygen. This may be due entirely to specific differences in the properties of hemocyanins of different organisms or it may be attributable to varying quantities of other buffer substances in these bloods. We have attempted to evaluate these alternatives in the case of the blood of *Limulus polyphemus*. This blood is well adapted to the inquiry because it is one of those in which the buffering action is largest—relative to oxygen capacity—and in which one might expect to find other buffer substances taking an important part.

The substances known to be of significance as buffers in mammalian blood are the carbonates, phosphates, and proteins. The very interesting and important buffering action, described by Collip (1920–21, 1921) due to calcium carbonate of the shells of certain pelecypods and crustacea need not concern us here, since we have defined our problem in terms of phenomena observable in the blood in vitro. *Limulus* blood may contain as much as 10 or 20 volumes per cent of carbon dioxide, equivalent to 0.005 or 0.010 mols of bicarbonate per liter. While this bicarbonate is conceivably of importance in buffering other acids, because of the ease with which the highly diffusible carbonic acid may be eliminated through the gills, from another point of view the bicarbonate exists in the blood because carbonic acid has been buffered, rather than as one of the primary buffer substances of the blood. This is shown by the fact that when the carbon dioxide pressure is reduced to zero the blood no longer contains more than a trace of carbonate, the base normally bound as bicarbonate having reverted entirely to the other buffers of the system.

The importance of phosphates as buffers in *Limulus* blood may be approximated from analyses of the ash which have been made by Genth (1852), Gotch and Laws (1884), and McGuigan (1907). McGuigan, whose values are typical, found the blood to contain 2.676 per cent ash, of which 0.34 per cent was estimated to be P₂O₅. If all this phosphorus were present in the blood as salts of phosphoric acid, their concentration would be about $1.3 \times 10^{-3}$ molar. In changing from a reaction of pH 7.3 to 9.0 less than half of the phosphate would be converted from the primary to the secondary phosphate. Consequently
the phosphate could not combine with more than about $0.5 \times 10^{-3}$ mols of base per liter. We have found, however, that *Limulus* serum will combine with about 400 mg. of NaOH, or $10 \times 10^{-3}$ mols per liter, in undergoing this change in reaction. The phosphates, therefore, cannot account for more than 5 per cent of the total buffer action in the blood of *Limulus*. That inorganic constituents should not be of importance in this regard is not surprising when it is considered how little this animal regulates the salt content of its serum (Macallum, 1910).

The blood of different individuals of *Limulus* differs greatly in its oxygen- and carbon dioxide-combining power, depending on how long and under what conditions the animal has been kept in captivity. This has enabled us to compare the buffering power of bloods naturally differing in protein content. Using the usual technique (Redfield, Coolidge, and Hurd, 1926) we have determined the carbon dioxide bound by four specimens of serum at different carbon dioxide pressures. These data are illustrated by the points in Fig. 1. Through these points have been drawn curves spaced relative to one another as they should be if the carbon dioxide-combining power is proportional to the nitrogen content of the blood. This has been done in accordance with the principle that at any hydrogen ion concentration the quantity of carbon dioxide combined will be proportional to the quantity of buffer present. Consequently we have drawn a standard curve through the points yielded by the serum containing 8.95 mg. of nitrogen per cc. Through this we have drawn a number of lines which converge at the origin. These lines are each the locus of a single hydrogen ion concentration. Each theoretical curve was then constructed so as to transect each of these lines of equal hydrogen ion concentration at ordinates bearing the same proportions to the ordinate of the standard curve, as the nitrogen content of the specimen bears to the nitrogen content of the standard curve. It may be seen from Fig. 1 that theoretical curves drawn in this way very adequately describe the position of experimental points obtained in bloods having a 4-fold variation in nitrogen content. This result indicates that the buffering of *Limulus* blood must be attributed largely to its nitrogenous compounds. What nitrogenous substances does it contain?
Non-protein nitrogenous substances occur in almost negligible quantities in this blood, there being only 0.12 gm. of nitrogen non-coagulable by heat in 1000 cc. of serum according to our analyses. Alsberg (1914) observed as much as 0.45 gm. per 1000 cc. in certain specimens.

Alsberg (1914) inquired into the protein content of *Limulus* serum and concluded that in addition to hemocyanin there was another protein present derived from the blood corpuscles in the process of clotting. In quantity he considered it insignificant compared to the total protein content. Redfield, Coolidge, and Shotts (1928) point out that purified hemocyanin contains more copper per gm. of protein nitrogen than does serum. The
difference is such (90 × 10⁻⁴ mg. of Cu per mg. of N in the case of serum as compared with 99 × 10⁻⁴ mg. of Cu per mg. of N in the case of hemocyanin) as to indicate that not more than 10 per cent of the protein is other than hemocyanin. Whether this other protein is present in the native blood one cannot say: undoubtedly it was present in the sera with which we have experimented.

The foregoing considerations suggest strongly that hemocyanin is the principal buffer substance of Limulus blood. If so, it should be possible to imitate the buffer action of blood by similar solutions of purified hemocyanin. We have consequently prepared titration curves of Limulus serum, and of purified hemocyanin dissolved in water and in solutions of NaCl and mixtures of NaCl and MgCl₂. From such titration curves the buffer value at any hydrogen potential may be deduced and used as a basis for comparing the properties of the different solutions.

Titration Curve of Limulus Serum.—This was obtained from the blood of a single animal freed of clot by shaking and filtering. The specimen was preserved with toluene and kept on ice during the several days required for the measurements. Measurements of hydrogen potential were made with the hydrogen electrode upon samples prepared by adding 10 cc. of water containing varying quantities of HCl and NaOH to 10 cc. of serum. These samples were allowed to stand overnight in the cold and were then placed in a tonometer which was evacuated in order to free the samples of oxygen and carbon dioxide. Hydrogen was then introduced into the tonometer and the sample saturated with this gas by rotating the tonometer for 10 minutes. The sample was next passed into the Clark electrode vessel which was rocked for 30 minutes before the potential was measured. Hydrogen potentials were estimated, the potential of the 0.1 N calomel electrode being taken as 0.3356 at 25°.

The quantity of hemocyanin present in the stock specimen of serum was estimated from its copper content. Analyses of three samples by the method described by Redfield, Coolidge, and Shotts (1928) yielded 0.0878, 0.0884, and 0.0888 mg. of Cu per cc. of solution. Taking copper to compose 0.173 per cent of Limulus hemocyanin, we estimate that the serum contained 0.0511 gm. of hemocyanin per cc. The samples on
which measurement was made were diluted with an equal volume of water containing acid or alkali and consequently contained approximately 2.5 per cent hemocyanin.

![Graph showing titration curve of Limulus serum.](image)

**Fig. 2.** Titration curve of *Limulus* serum. The quantity of NaOH added per 10^6 gm. of hemocyanin present in the serum is indicated along the ordinate. Negative values on the ordinate indicate the addition of equivalent quantities of HCl to the serum.

The titration curve of reduced *Limulus* serum so obtained is shown in Fig. 2. The values of hydrogen potential plotted are the best representative values of a series of two to six different
measurements of the same sample. The temperature at which
the measurements were made varied between 23-25°.

_Titration Curves of Solutions of Hemocyanin._—These were
obtained from specimens made from Preparation XIIA of Red-
field, Coolidge, and Shotts (1928). This preparation had been
purified by repeated salting out with ammonium sulfate, followed
by dialysis until free of sulfate. It contained 0.10 gm. of hemo-
cyanin per cc. as estimated from the dry weight; of this weight
0.167 per cent was copper. By diluting 2 cc. of hemocyanin
with 8 cc. of water, containing various amounts of HCl or
NaOH, specimens were obtained containing 2 per cent hemo-
cyanin and of varying hydrogen potential. These samples were
saturated with hydrogen and their hydrogen potential measured
in exactly the same way as were the samples of serum. The
temperature of the electrode vessels was 21-23°.

The preparation employed contained $26 \times 10^{-5}$ mols of NaOH
per gm. of hemocyanin as determined by the quantity of dilute
HCl required to produce the maximum precipitation of the
hemocyanin in the region of the isoelectric point. In order to
determine the quantity of NaOH present in the samples meas-
ured this quantity was added to the amount of NaOH mixed
with the sample in preparing the final dilution. When HCl
was added to the sample the quantity added per gm. of hemo-
cyanin was subtracted from $26 \times 10^{-5}$ in order to give the
amount of NaOH left unneutralized in these preparations. The
quantity of NaOH present per gm. of protein thus obtained is
plotted as the ordinates of titration Curve A in Fig. 3.

The measurements have not been corrected for the amount
of base remaining uncombined because this is not a significantly
large quantity below pH 11. Beyond this reaction the hemo-
cyanin undergoes some change, as indicated by the appearance
of a purple color similar to the biuret reaction, which would
appear to invalidate any conclusions which might be drawn
concerning the maximum base-binding power of hemocyanin.

The titration curve of pure hemocyanin is clearly quite differ-
ent from that of serum. Whereas the former rises in a rather
uniform slope from the point of neutrality to pH 10.5, after
which it is inflected upward with great rapidity, the latter rises
more steeply between pH 6.5 and 7.5; it is then flattened out
Fig. 3. Titration curve of *Limulus* hemocyanin; Curve A in the absence of salt; Curve B in the presence of 0.5 M NaCl.
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between pH 8.0 and 9.0, after which it again rises steeply in the region about pH 10.0.

*Titration Curve of Hemocyanin in the Presence of Neutral Salts.*—From the fact that the addition of salt greatly alters the equilibrium between hemocyanin and oxygen, and that it also modifies the color of this substance, it was natural to suspect that the addition of neutral salts to the hemocyanin solution would modify the titration curve also. Sørensen, Linderstrøm-Lang, and Lund (1927) have recently given a theoretical interpretation of such phenomena as observed by them in solutions of egg albumin, and Simms (1929) has studied similar effects in gelatin preparations.

The titration Curve B in Fig. 3 was obtained from a 2 per cent solution of hemocyanin containing NaCl in the concentration of 0.5 mol per liter. The hemocyanin was the same preparation used in the previous series of measurements, and the measurements for the two curves were actually made in alternation.

The addition of NaCl brought the titration curve of purified hemocyanin much closer in shape to that of the native serum. At hydrogen potentials below pH 9.0 the curves are much alike. The abrupt rise on the alkaline end of the curve occurs sooner than in the salt-free hemocyanin but not as soon as in

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**TABLE I.**

Composition and Ionic Strength of Limulus Serum.

<table>
<thead>
<tr>
<th></th>
<th>Ionic concentration</th>
<th>Concentration ( \times ) valence$^\mathbf{2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Limulus serum.</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na</td>
<td>8.885</td>
<td>0.386</td>
</tr>
<tr>
<td>K</td>
<td>0.4589</td>
<td>0.0127</td>
</tr>
<tr>
<td>Ca</td>
<td>0.3613</td>
<td>0.00903</td>
</tr>
<tr>
<td>Mg</td>
<td>0.9955</td>
<td>0.0361</td>
</tr>
<tr>
<td>Cl$^{-}$</td>
<td>16.608</td>
<td>0.1640</td>
</tr>
<tr>
<td>SO$_4^{2-}$</td>
<td>1.1185</td>
<td>0.0464</td>
</tr>
<tr>
<td>Hemocyanin</td>
<td>50.00</td>
<td>1.1132</td>
</tr>
<tr>
<td>Ionic strength</td>
<td></td>
<td>0.5566</td>
</tr>
</tbody>
</table>

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the serum. The reason for the steep rise in the titration curve of serum is obvious when it is recalled that *Limulus* serum contains a considerable quantity of magnesium, the hydroxide of which becomes insoluble at about pH 10. The titration curve of sea water, which contains much the same quantity of
magnesium as does Limulus serum, has a similar steep upward inflection just beyond pH 10 (Haas, 1916).

We have attempted to imitate the titration curve of Limulus serum with a solution in which the concentration of hemocyanin and magnesium is the same as that in serum and having a total ionic strength equal to that of serum secured by the addition of sodium chloride. Table I gives the composition of Limulus serum based on Macallum's (1910) analyses for inorganic salts and our estimate of the hemocyanin content of the specimen of which we have determined the titration curve. The ionic strength of the known components, with the exclusion of the hemocyanin is 0.5566.

We prepared a mixture containing 0.44 mols of NaCl, 0.04 mols of MgCl₂, and 50 gm. of hemocyanin per liter. This solution has an ionic strength of 0.56 and a hemocyanin and magnesium content very similar to native serum. Samples of this solution were diluted with equal volumes of water containing NaOH or HCl and the hydrogen potential measured. The temperature at which the measurements were made varied between 21–23°. These data corrected for the combined base in the hemocyanin, are presented in Fig. 4.

Comparison of the titration curves of serum and of this mixture of NaCl, MgCl₂, and hemocyanin shows that the curves are not only very similar in shape but that they have identical slopes at all reactions between pH 6 and 10. Beyond this point there is a slight discrepancy due to the failure of the magnesium in the artificial mixture to duplicate entirely the conditions existing in serum.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>pH</th>
<th>Excess of base. Mols per 10⁵ gm. hemocyanin.</th>
</tr>
</thead>
<tbody>
<tr>
<td>14</td>
<td>7.2</td>
<td>22</td>
</tr>
<tr>
<td>16</td>
<td>7.4</td>
<td>28</td>
</tr>
<tr>
<td>29</td>
<td>7.9</td>
<td>36</td>
</tr>
<tr>
<td>33</td>
<td>7.0</td>
<td>18</td>
</tr>
<tr>
<td>35</td>
<td>8.0</td>
<td>37</td>
</tr>
</tbody>
</table>
Since the slope of the titration curves of the artificial mixture and the natural serum is the same at all physiological reactions, one may conclude that within the limits of experimental error the buffering of *Limulus* serum within this range may be accounted for by its hemocyanin.

Aside from the discrepancy at extreme alkalinity the difference in the curves in Figs. 2 and 4 consists solely in the fact that the ordinates of the curve for the artificial mixture are $18 \times 10^{-5}$ mols per gm. greater than those of the natural serum at corresponding hydrogen potentials. This difference clearly represents the quantity of base, in excess of acid, present in the natural blood. Consequently Fig. 4 may be used to determine approximately the quantity of base present in any sample of *Limulus* blood of which the hydrogen potential is known. Such determinations on a number of samples are given in Table II.

While it may be concluded that hemocyanin is the only significant buffer in *Limulus* serum, it is equally clear that its value in this regard is dependent to an important degree upon the electrolytic environment which the salts of serum provide. The influence of the other components of the solution upon the buffering of hemocyanin is clearly brought out by estimating the molecular buffer values of the solutions at various hydrogen potentials. The molecular buffer value is defined by Van Slyke (1922) as

$$\beta_M = \frac{dB}{CdpH}$$

where B is the molecular concentration of base added and C is the molecular concentration of the buffer substance. Since we are dealing with measurements made on reduced solutions of hemocyanin, we designate their molecular value by the symbol $\beta_R$. The molecular concentration of hemocyanin is estimated on the assumption that its molecular weight is 73,400 (Redfield, Coolidge, and Shotts, 1928). While this value is not securely established, it represents the weight of *Limulus* hemocyanin containing 2 atoms of copper and which Redfield, Coolidge, and Montgomery (1928) have shown to be equivalent to 1 molecule of oxygen. In consequence our values of $\beta_R$ arc strictly compar-
able with those of Van Slyke and his collaborators (1922) who use the equivalent oxygen-combining capacity of hemoglobin in estimating the buffer values of this substance. The value of $\beta_n$ has been estimated by multiplying the "slopes" of the titration curves in Fig. 3, which give buffer values in mols of base.

Fig. 5. Molecular buffer values, $\beta_n$, of Limulus hemocyanin solutions: Curve A in the absence of salt; Curve B in the presence of 0.5 M NaCl.
per $10^6$ gm. of hemocyanin, by 73,400 to obtain the molecular buffer value as defined. The results of this estimation are shown in Fig. 5 which brings out the following relations.

1. The molecular buffer value of reduced *Limulus* hemocyanin in the absence of salts varies very little over a range of pH from 6.0 to 10.

2. The presence of 0.5 mol of NaCl, presumably through altering the dissociation constants of certain of the acidic groups of the hemocyanin molecule, causes a considerable increase in the buffer value below pH 8 at the expense of the buffer value between pH 8 and 9. As a result the effectiveness of hemocyanin as a buffer in the physiological range is increased up to some 40 per cent.

3. Compared on the basis of equivalent oxygen capacity the buffer value of reduced hemocyanin is about 4 times as great as that of hemoglobin. This difference is to be attributed to the inferiority of hemocyanin as an oxygen carrier; rather than to any superiority of this protein as a binder of base, for when the buffer value is estimated in terms of base bound per gm. of protein: $\beta_n$ for hemocyanin is about 15 mols per gm. of protein per pH unit and $\beta_n$ for hemoglobin is 15.9. Weight for weight there is little difference in the buffer values of these two proteins.

**Change in Buffering Due to Oxygenation.**—The foregoing estimates of buffering power in *Limulus* blood have been made on the basis of experimental measurements on solutions containing hemocyanin in the reduced condition. It is well recognized that in the blood of mammals a very considerable part of the effective buffering of the blood is due to changes in the strength of its acidic groups brought about simultaneously with the reduction of the hemoglobin. Kerridge (1926) has shown that a similar phenomenon occurs in the blood of the crab, *Maiasquinaldo*, and Redfield, Coolidge, and Hurd (1926) have demonstrated the same relation in the blood of the squid, *Loligopealei*. The latter authors were unable to demonstrate any difference in the carbon dioxide content of oxygenated and reduced blood of *Limulus polyphemus* under comparable carbon dioxide pressures. Consequently the influence of oxygenation upon the buffering of *Limulus* blood must be very small.
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SUMMARY.

1. Phosphates and proteins other than hemocyanin are present in quantity sufficient to account for only a small part of the buffering of Limulus blood.
2. The titration curve of pure hemocyanin does not closely reproduce that of serum.
3. The addition of NaCl to hemocyanin alters the titration curve so that it approaches more closely to that of serum.
4. A solution containing hemocyanin and magnesium chloride in amounts comparable to serum, and having the total ionic strength of serum made up by the addition of NaCl, has a titration curve very similar to that of serum.
5. It is concluded that the properties of hemocyanin as influenced by its electrolytic environment will account for the buffer phenomena of Limulus blood.
6. Samples of natural Limulus serum are found to contain from 18 to \(37 \times 10^{-8}\) mols of base, in excess of acid per gm. of hemocyanin.

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