STUDIES IN THE PHYSICAL CHEMISTRY OF THE PROTEINS.

VI. THE ACTIVITY COEFFICIENTS OF THE IONS IN CERTAIN OXYHEMOGLOBIN SOLUTIONS.

BY EDWIN J. COHN AND ADELA M. PRENTISS.

(From the Department of Physical Chemistry in the Laboratories of Physiology, Harvard Medical School, Boston.)

(Received for publication, July 20, 1926.)

1.

INTRODUCTION.

Proteins differ from most other chemical individuals by virtue of the large dimensions of their molecules, and the large number of their reactive groups. These characteristics not only distinguish proteins from other classes of substances, but also distinguish proteins from each other. Thus the proteins of lower molecular weight are, on the whole, more readily crystallizable than larger proteins, more soluble, less viscous, and less readily precipitated from solution by neutral salts (10). Each of these properties, however, depends not only upon the size, but also upon the charged condition of the molecule. The manner in which the size and the charged condition of molecules influence their characteristics need not be identical in the case of the large polyvalent proteins and the small ions with which inorganic chemistry is generally concerned. Comparable methods of study may, however, be pursued, and may ultimately reveal the fundamental relations between size and charge upon which behavior depends.

The Viscosity of Oxyhemoglobin.—Proteins appear to be more viscous the larger their molecular weights (10). Thus oxyhemoglobin is less viscous than the serum globulins, gelatin, or casein, but more viscous than egg albumin. Oxyhemoglobin is less viscous than serum albumin, however, although the latter has a lower molecular weight.
This, and other exceptions to the generalizations that have been made, indicate that the size of proteins, though a dominant property, is not the only one to determine their behavior.

Arrhenius (4) has shown that the relation between the viscosity of a solution and its concentration can be expressed by his logarithmic formula:

$$\log \frac{\eta}{\eta_0} = \theta c$$

in which $\eta$ is the viscosity of the solution, $\eta_0$ the viscosity of the solvent, $\theta$ a proportionality constant characterizing the viscosity of the solute, and $c$ the concentration of the solute, expressed in his later papers as gm. solute per 100 gm. solvent. In order to apply his equation to certain protein solutions that had been investigated, Arrhenius assumed that their large molecules were hydrated, and that concentration in such solutions was expressed with sufficient accuracy if account were taken of the water held by the hydrated molecules, by means of an hydration factor, $n$, representing the number of gm. of solvent associated with each gm. of solute, and therefore withdrawn from the free solvent (4, 19).

The constant $\theta$, calculated by Arrhenius from the extensive observations of Chick and her collaborators, increases steadily with the molecular weight of the protein. The value of the hydration factor calculated by Arrhenius was as great as 1.0 for all the proteins studied with the exception of egg albumin. In this case $\theta$ and $n$ were as high as 0.0184 and 0.7 respectively.

Not all of the proteins studied by Chick were at their isoelectric points. Casein is too insoluble near its isoelectric point, and its viscosity was measured in alkaline solution. Most of the other proteins were also studied as alkali salts. In 1921 Loeb studied the viscosity of isoelectric egg albumin (24). His measurements, calculated by means of the Arrhenius equation, are in Table I. Two conclusions must be drawn from these measurements. The first, that the value of the viscosity constant, $\theta$, becomes smaller near the isoelectric point. The second, that $n$, the “hydration factor,” becomes negligibly small in the case of isoelectric egg albumin. Both of these observations were explicitly made by Loeb.
Loeb recognized that the viscosity of proteins was influenced both by their size and by their charge. Working before the molecular weights of the proteins were known, he noted the difference in behavior between a small and a large protein and wrote: “it is possible to account for the viscosities of protein solutions on the basis of Einstein’s law when the relative volume occupied by the protein in solution is small, and on the basis of Arrhenius’s formula when the volume exceeds the limits within which Einstein’s formula holds. According to our view the former is true when the protein exists in the solution exclusively or almost exclusively in the form of isolated mole-

cules or ions or particles too small to occlude water and this seems to be the case for solutions of crystalline egg albumin” (24), page 80).

Although the viscosity constant $\theta$ is also smaller in the case of isoelectric serum albumin, pseudoglobulin, and euglobulin, the so-called hydration factor never becomes negligible in the case of these proteins, indeed never becomes lower than 1.0.

The viscosity of oxyhemoglobin has recently been studied near its isoelectric point, and the results are given in Table II. They demonstrate that the Arrhenius equation adequately defines the change in viscosity with concentration of this protein, and that the factor $n$
is very small, if not negligible, for isoelectric oxyhemoglobin as for the isoelectric egg albumin studied by Loeb.

It is not the purpose of this paper to discuss the significance of the hydration term in the Arrhenius viscosity equation. This quantity may be a measure of the hydration of the protein molecule, or of certain electrical forces at its surface which lead to an effective size

TABLE II.
Viscosity of Oxyhemoglobin in the Neighborhood of Its Isoelectric Point.
Temperature = 25°C.

<table>
<thead>
<tr>
<th>Preparation and experiment No.</th>
<th>Solvent</th>
<th>Concentration, gm. per liter.</th>
<th>log ( \eta / \eta_0 )</th>
<th>( 10^{(\log \eta / \eta_0)} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>xxiii, 7</td>
<td>H_2O</td>
<td>16.33</td>
<td>0.0216</td>
<td>0.0130</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18.88</td>
<td>0.0285</td>
<td>0.0148</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20.76</td>
<td>0.0289</td>
<td>0.0136</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32.66</td>
<td>0.0516</td>
<td>0.0153</td>
</tr>
<tr>
<td></td>
<td></td>
<td>37.76</td>
<td>0.0587</td>
<td>0.0150</td>
</tr>
<tr>
<td></td>
<td></td>
<td>41.52</td>
<td>0.0550</td>
<td>0.0127</td>
</tr>
<tr>
<td>xxiv, 15</td>
<td>Phosphate 1/6 of pH 6.8.</td>
<td>12.44</td>
<td>0.0155</td>
<td>0.0123</td>
</tr>
<tr>
<td></td>
<td></td>
<td>31.10</td>
<td>0.0483</td>
<td>0.0150</td>
</tr>
<tr>
<td></td>
<td></td>
<td>77.80</td>
<td>0.1339</td>
<td>0.0159</td>
</tr>
<tr>
<td>xxviii, 18</td>
<td></td>
<td>16.74</td>
<td>0.0282</td>
<td>0.0166</td>
</tr>
<tr>
<td></td>
<td></td>
<td>23.44</td>
<td>0.0350</td>
<td>0.0146</td>
</tr>
<tr>
<td></td>
<td></td>
<td>32.82</td>
<td>0.0536</td>
<td>0.0158</td>
</tr>
<tr>
<td></td>
<td></td>
<td>45.95</td>
<td>0.0717</td>
<td>0.0149</td>
</tr>
<tr>
<td></td>
<td></td>
<td>53.61</td>
<td>0.0933</td>
<td>0.0165</td>
</tr>
<tr>
<td></td>
<td></td>
<td>64.33</td>
<td>0.1114</td>
<td>0.0162</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td>0.0148</td>
</tr>
</tbody>
</table>

greater than the volume calculated from its molecular weight and its specific gravity. The increased viscosity may, however, depend in some other way upon the electrically charged condition of the protein molecule. In any case the forces represented by \( n \) are far smaller, at their respective isoelectric points, for egg albumin than for serum albumin, and for oxyhemoglobin than for the serum globulins.

*The Dissociation of Oxyhemoglobin.*—These observations regarding
viscosity suggest that next in importance to size, in determin-
ing the behavior of proteins, is the charged condition of their
molecules. There have been two approaches to the study of pro-
teins as electrolytes. In the one the attempt has been made to
define protein-containing systems in terms of laws which have
been developed for smaller ions. Whether the measurements upon
the systems were upon their conductivity, their migration velocity, or
their combining capacity, the same observation has been made;
that proteins obeyed the laws of classical chemistry, and behaved as
though they possessed but a small number of reactive groups. The
conductivity measurements upon protein solutions, the large number
of electromotive force measurements upon their acid-or base-com-
bining capacities, and the solubility measurements upon their base-
combining capacity, have been quantitatively defined by the mass
law on the assumption that the valence of the proteins was of a
low order. On this basis it has often been possible to estimate the
affinity or dissociation constants with which the groups of the pro-
tein appeared to be reacting.

With the advance in knowledge concerning the size of proteins and
the number of their reactive groups it has become apparent that,
however adequate these definitions of behavior, they might need
reinterpretation. D’Agostino and Quagliariello studied the combin-
ing capacity of serum albumin over a restricted range (3) and at-
ttempted to estimate the dissociation constants of the groups in the pro-
tein from the slope of the titration curve. We now know that not
two, but at least twenty, groups in the protein molecule were combin-
ing base over the range that they studied. The titration curve de-
scribing the combination of this protein with acid may be calculated
on the basis of a single dissociation constant, although seventy groups
in the albumin molecule are involved ((10), page 380). In a recent
study of the combination of reduced and carboxyhemoglobin with
base, Hastings and his collaborators have concluded that “the quanti-
tative increase in the base bound by hemoglobin upon combination
with CO is consistent . . . . with the hypothesis that the dissoci-
ation constant of one acid group is increased” ((18), page 334). This
calculation was made on the basis of a molecular weight of 16,700.
Since the molecular weight of hemoglobin is probably four times this
value four groups must be involved in this reaction, which nonetheless appears to be of the first order. Certain of these phenomena may depend upon the distance between the reactive groups at the surface of vast protein molecules (2, 5, 23, 31).

The isoelectric point (21, 26) has been defined in terms of acid and basic dissociation constants. The extension of this notion to the slopes of titration curves has been used as an indication either of the number of groups that are reactive in the neighborhood of the isoelectric point, or of their strength (9, 35). A distinction must, however, be made between the affinity constants which define the behavior of proteins, and the number of groups that are known to be reactive. One acid and one basic group which are largely dissociated may increase the slope of a titration curve more than a larger number of acid and basic groups of such strength that there is no change in their ionization over a wide pH range.

The titration curves of serum and egg albumin are steeper at their isoelectric points than are those of other known proteins. The observations that even insoluble proteins are readily soluble when they are largely ionized has led to the notion that readily soluble proteins are largely ionized. The solubility of albumins in the neighborhood of their isoelectric points has thus been associated with their being highly dissociated both as acids and as bases. The narrow range of hydrogen ion activities which changes the direction of migration of these proteins in an electric field may be considered additional evidence in favor of this view.

Of the proteins that have been studied thus far, excepting only the albumins, oxyhemoglobin has the steepest slope to its titration curve in the neighborhood of its isoelectric point. Its titration curve, from pH 6 to 7.5, is most readily interpreted by assuming the presence of

1 The hemoglobin molecule contains four atoms of iron. The analytical evidence of Hufner, Jaquet, and Osborne suggested that the molecular weight of dog hemoglobin was either 50,000 or 66,800 (11). Ultrafiltration experiments have demonstrated that dog and horse hemoglobin were of the same size, and were larger than serum albumin, 45,600. They have not rendered it possible to decide between 50,000 and 66,800. Adair's (1) consideration of the osmotic pressure of oxyhemoglobin has led him to suggest the larger value, and Svedberg (33, 34) has recently demonstrated, by his centrifugation method, that 66,800 is the molecular weight of this protein.
both positive and negative charges upon the oxyhemoglobin molecules throughout this range. The view deduced from the behavior of albumins suggests that the relatively great solubility of oxyhemoglobin in the neighborhood of its isoelectric point depends upon its dissociation. Like the albumins its migration changes in direction over a very narrow range of hydrogen ion activities (15, 27, 28) and in this respect, as in certain others, oxyhemoglobin is related more closely to the albumins than to most of the globulins.

The Solubility of Oxyhemoglobin.—Oxyhemoglobin has certain properties which give it a position intermediate between the albumins and the globulins. Its molecular weight is larger than that of egg and serum albumin, but smaller than that of pseudoglobulin, of euglobulin, or of gelatin. It is fairly soluble in water in the neighborhood of its isoelectric point, but much less soluble than the albumins. Its solubility in the absence of salts is much greater than that of these animal globulins, or of such vegetable globulins as edestin, but like them its solubility is increased by neutral salts. Neutral salts increase the solubility of oxyhemoglobin to a much smaller extent, however, than that of any other globulin that has thus far been studied.

The effect of neutral salts in increasing the solubility of slightly soluble substances is not restricted to the globulins, however, nor to the proteins. The solubility of most slightly soluble salts is increased by the presence of other salts. Many investigations of this phenomenon have been carried on in different laboratories. The inorganic salts that have been investigated have usually been of low valence types (22) but Brønsted and his collaborators (6, 7, 8), have studied certain compounds of higher valence. 6 years ago S. P. L. Sørensen and I attempted to extend to the serum globulins the observations that Brønsted had made upon the cobaltamines. This attempt failed to yield conclusive results for two reasons. In the first place serum globulin is usually a mixture of euglobulin and pseudoglobulin, and therefore solubility, as Mellanby (25) and Sørensen (32) have shown, is rarely independent of the amount of saturating body. 2 We have now chosen for study oxyhemoglobin, a smaller

2 In two preparations studied by us ((11), Table X) solubility appeared to be independent of the saturating body.
molecule, which is readily crystallizable, and can be so purified that a single chemical individual can be studied. In the investigation to be reported, solubility in a given solvent was always independent of the amount of the saturating body, and remained constant as long as any saturating body was present. In this respect, therefore, it obeyed the laws of classical chemistry.

The earlier attempt to study the nature of globulin action was premature in a second respect. Brønsted's equation, defining the solvent action of neutral salts, applied to dilute solutions. An extension to concentrated solutions, of equations defining the change in solubility of difficultly soluble substances was advanced by Debye in 1923 (12, 13). This equation has been found adequate to describe the effect of neutral salts upon the solubility of oxyhemoglobin in the neighborhood of its isoelectric point.

II.

THEORETICAL.

"When, at a given temperature, a solid salt is in equilibrium with a solution, the activity of that salt in the solution is fixed. It cannot be changed by any change in the nature of the solution, such as would be produced by the addition of other electrolytes. . . . whatever happens (isothermally) to the solution, the activity coefficient of the salt in question must remain inversely proportional to the mean molality of its ions" (22), page 369), and therefore to its solubility. Since the activity must remain constant in a saturated solution, change in its solubility yields the activity coefficient. If we define the activity as the solubility in the absence of salt* $S_0$ and call the solubility in any other solution $S$, then the activity coefficient, $\gamma$, is equal to the ratio:

$$\gamma = \frac{S_0}{S}$$

(1)

It has long been known that the activity coefficients of salts differed with their valence type. Many equations defining this relation have

*A distinction should in reality be made between $S_0$, the solubility in pure water, and $S_{oo}$ the solubility at infinite dilution (7). The quantity measured in this investigation was $S_{oo}$, but the distinction may, for present purposes, be ignored.
been advanced. But only the last of these, due to Debye, need be considered here. Debye has shown that in dilute solution the negative logarithm of the activity coefficient of an ion is proportional to the square of its valence, and to the square root of a quantity representing the electrical environment of the ion. This quantity is just twice the "ionic strength" of Lewis, $\mu$, expressed, however, as mols per liter, instead of mols per 1000 gm. water. The proportionality constant includes the absolute temperature, the Boltzmann constant, and the dielectric constant of the solution. At ordinary temperatures these reduce to the value 0.5. The Debye equation for the activity coefficient of an ion, of valence $Z$, in dilute solution may be written (14):

$$- \log \gamma = 0.5 Z^2 \sqrt{\mu}$$

or for a salt of ions $Z_1$ and $Z_2$:

$$- \log \gamma = 0.5 Z_1 Z_2 \sqrt{\mu}$$

In solutions more concentrated than $\mu = 0.01$ the distance between the ions is no longer so great in comparison with their dimensions as to warrant considering their charges as occupying a point in space. The above equations are therefore extended to concentrated solutions by including a term for the mean effective diameter of the ions in solution, $b$, expressed as cm., and a term expressing the effective thickness of the ionic atmosphere. This reciprocal distance, $\kappa$, has been estimated by Debye as equal to $0.33 \times 10^4 \sqrt{\mu}$. The expanded equation thus becomes for an ion:

$$- \log \gamma = \frac{0.5 Z^2 \sqrt{\mu}}{1 + \kappa b}$$

or for a salt:

$$- \log \gamma = \frac{0.5 Z_1 Z_2 \sqrt{\mu}}{1 + \kappa b}$$

These equations may be employed in the study of the change in solubility of a slightly soluble substance by combining equation (1) with (4) or (5):

$$\log S/S_0 = \frac{0.5 Z^2 \sqrt{\mu}}{1 + \kappa b} = \frac{0.5 Z_1 Z_2 \sqrt{\mu}}{1 + \kappa b}$$
In the studies with which we are concerned the molecular concentration of the solvent salt was great in comparison with that of the solute. Phosphate solutions were chosen as the solvents for the oxyhemoglobin, since they would permit the measurement of solubility at any desired pH and any value of the ionic strength, and at the same time effectively buffer the oxyhemoglobin solutions.

The activity coefficients of phosphate solutions have been determined, and will be reported elsewhere. The solubility of oxyhemoglobin has been determined in phosphate solutions of ionic strength varying from 0.04 to 1.00. The solvent action of these phosphate solutions has been studied at pH 6.4, 6.6, and 6.8. Phosphate solutions of the desired reactions were obtained by employing the following formula:

\[ \text{pH} + \log \left( \frac{(\text{KH}_2\text{PO}_4)}{(\text{K}_2\text{HPO}_4)} \right) = \text{pK} - \log \gamma_1/\gamma_2 \]  

(7)

The use of the Debye equation in the characterization of phosphate systems has led to a new value for the second dissociation constant of phosphoric acid:

\[ \text{pK} = 7.16 \]  

(8)

and to the following definition of the difference in the activity coefficients of the univalent and bivalent phosphate ions:

\[ \log \frac{\gamma_1}{\gamma_2} = \frac{1.5 \sqrt{\mu}}{1 + 1.5 \sqrt{\mu}} - K_s \mu \]  

(9)

where the salting out term, \( K_s \), has the following values for the ratio:

\[
\begin{align*}
\left( \frac{\text{KH}_2\text{PO}_4}{\text{K}_2\text{HPO}_4} \right) & = 9 \quad 5 \quad 3 \quad 2 \quad 1 \quad 1/2 \quad 1/8 \\
K_s & = -0.25 \quad -0.18 \quad -0.11 \quad -0.05 \quad 0.03 \quad 0.07 \quad 0.12 
\end{align*}
\]

In most of the systems studied the salting out term may be neglected. The mean effective diameter, \( b \), multiplied by the reciprocal distance, \( \kappa \), has been found to be \( 1.5 \sqrt{\mu} \). In the phosphate solutions used as solvents for the oxyhemoglobin this value can, as a first approximation, be substituted in equation (6) which thus becomes:

\[ \log \frac{S}{S_0} = \frac{0.5 \ Z^2 \ \sqrt{\mu}}{1 + 1.5 \sqrt{\mu}} = \frac{0.5 \ Z_1 Z_2 \ \sqrt{\mu}}{1 + 1.5 \sqrt{\mu}} \]  

(10)
But two quantities remain undetermined in this equation, $S_0$ and the apparent valence type of the protein.

III.

EXPERIMENTAL.

The oxyhemoglobin used in these investigations has been prepared by a method worked out in this laboratory that will be described by Ferry and Green (16). In principal it depends upon the very property with which we are concerned, namely that oxyhemoglobin, which is present in the red blood corpuscle to over 30 per cent, is soluble in the neighborhood of its isoelectric point, in a concentrated phosphate buffer at 0°C., only to the extent of 4 per cent. The oxyhemoglobin used was always crystallized twice, and was then washed repeatedly with the phosphate solvent to be employed. Since oxyhemoglobin crystals contain a large amount of water we have often found it necessary to wash the crystals for at least 12 hours, five or ten times, before solubility became constant. Thereafter the solubility remained constant.

The oxyhemoglobin crystals were placed in 250 cc. centrifuge cups, covered with the phosphate solvent, and continuously agitated by means of a mechanical stirrer. The centrifuge cups were immersed in an air-stirred ice water bath at 0°C. and the entire apparatus remained in a cold room throughout the experiment. The stirring continuously oxygenated the hemoglobin. When equilibrium had been reached the undissolved crystals were separated from the solution by centrifugation, and the supernatant liquid filtered. The measurement of solubility, as well as the preparation of oxyhemoglobin, was carried out in the cold.

The solubility of the oxyhemoglobin was determined by analyses of the nitrogen in aliquot parts of the filtrate. Triplicate nitrogen analyses were generally made. The results have been calculated as gm. oxyhemoglobin per 100 cc. solution, on the assumption that oxyhemoglobin contains 17.7 per cent nitrogen. The first experiments were conducted approximately at the isoelectric point of the oxyhemoglobin, as measured by Michaelis (27, 28) and Ferry (15). These investigators give 6.78 as the isoelectric point determined by cataphoresis.
The phosphate solvents of different ionic strength were therefore so constituted as to have approximately this pH. The results are recorded in Table III. In this first experiment one solvent was

### Table III.

**Solubility of Oxyhemoglobin in Phosphate Solutions.**

<table>
<thead>
<tr>
<th>Preparation and experiment No.</th>
<th>No. of washings with solvent</th>
<th>Date of saturation of solvent</th>
<th>Ionic strength of phosphate solvent.</th>
<th>Concentration of phosphate: (KH₂PO₄) + (K₂HPO₄).</th>
<th>Mol fraction of phosphate as K₂HPO₄.</th>
<th>Solubility of oxyhemoglobin: gm. per 100 cc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>x₁, 7</td>
<td>7</td>
<td>Mar. 5</td>
<td>0.040</td>
<td>0.027</td>
<td>0.222</td>
<td>2.51</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>&quot;</td>
<td>2.83</td>
<td>0.35</td>
<td>0.266</td>
<td>3.73</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>&quot;</td>
<td>2.08</td>
<td>0.25</td>
<td>0.320</td>
<td>3.40</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>May 1</td>
<td>2.63</td>
<td>0.35</td>
<td>0.372</td>
<td>3.50</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>&quot;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>&quot;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>2.51</strong></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>x₃₁, 14</td>
<td>7</td>
<td>Feb. 19</td>
<td>0.125</td>
<td>0.079</td>
<td>0.266</td>
<td>4.02</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>&quot;</td>
<td>2.47</td>
<td>0.136</td>
<td>0.320</td>
<td>4.01</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>&quot;</td>
<td>2.61</td>
<td>0.136</td>
<td>0.372</td>
<td>4.18</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Apr. 6</td>
<td>2.78</td>
<td>0.136</td>
<td>0.419</td>
<td>5.16</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>&quot;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>&quot;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td><strong>2.62</strong></td>
</tr>
</tbody>
</table>

pH of solvent = 6.4.

pH of solvent = 6.6.
TABLE III—Concluded.

<table>
<thead>
<tr>
<th>Preparation and experiment No.</th>
<th>No. of washings with solvent</th>
<th>Date of saturation of solvent</th>
<th>Ionic strength of phosphate solvent.</th>
<th>Square root of ionic strength.</th>
<th>Concentration of phosphate: ((\text{KH}_2\text{PO}_4) + (\text{K}_2\text{HPO}_4)).</th>
<th>Mol fraction of phosphate as (\text{K}_2\text{HPO}_4).</th>
<th>Solubility of oxyhemoglobin: gm. per 100 cc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>xxxvii, 4</td>
<td></td>
<td></td>
<td>0.040 0.1225 0.250 0.640 1.00 0.20</td>
<td>0.35 0.5 0.8 1.0</td>
<td>0.0213 0.062 0.121 0.293</td>
<td>0.428 0.500 0.537 0.594</td>
<td>3.36 3.39 4.94 4.15 3.84 3.47 3.30 3.45 4.73 6.62 6.62 6.66</td>
</tr>
<tr>
<td>Average....................</td>
<td></td>
<td></td>
<td>3.32 3.81 4.85 6.66</td>
<td></td>
<td></td>
<td></td>
<td>3.32 3.81 4.85 6.66</td>
</tr>
</tbody>
</table>

employed which has not been recorded. A phosphate solution of ionic strength 0.01 yielded as high a solubility as a solvent four times the concentration. This was interpreted as being due to insufficient buffering of the oxyhemoglobin. The phosphate solution of ionic strength 0.04 was retained in subsequent experiments, partly to illustrate this phenomenon, although it probably did not completely buffer the oxyhemoglobin solutions.

The second pH at which the solubility of oxyhemoglobin was studied was 6.6. It was found that solubility at the same ionic strength was uniformly lower at pH 6.6 than 6.8. Accordingly another series of measurements were made at pH 6.4. These demonstrated that the minimum of solubility occurred in the neighborhood of pH 6.6. Moreover, the irregular behavior of the most dilute solvents largely disappeared in this more acid series. Finally other experiments were performed with certain of the same phosphate solvents, but with
other oxyhemoglobin preparations, in order to determine the reproducibility of the material, and of the method. All of these are recorded in Table III, and the average solubilities calculated.

The logarithms of the average solubilities are recorded in Table IV, and are graphically represented in Fig. 1. It will be noted that, with the exception of the measurements on the most dilute phosphate solvents, the points fall upon parallel curves. Apparently the influence of the pH was not upon that component of solubility which is effected by the salt, but upon $S_0$. The distance between these paral-

![Fig. 1.](image)

Fig. 1.

llel curves has been estimated, and is termed $\Delta \log S_0$ in Table IV. This component of solubility should probably be ascribed to a change in ionization, and therefore to compound formation of the dissolved oxyhemoglobin. It can be treated in terms of the mass law, and will be considered at another time. It does not depend upon a change in the valence type of the oxyhemoglobin in whatever form it exists as saturating body. The subtraction of $\Delta \log S_0$ from the solubilities at pH 6.4, 6.6, and 6.8 led to results which are in such close agreement, at the same value of the ionic strength, as to suggest the identity of the saturating body in the systems studied.
The change in the solubility of oxyhemoglobin with the concentration of the phosphate at any pH, or the average of all the results corrected for the change in pH, may be employed in connection with equation (10) to estimate the apparent valence type of oxyhemoglobin. As has been noted two quantities remain undetermined in this equation, the valence type, and $S_0$. The shape of the curves in Fig. 1 suggests that oxyhemoglobin behaves as though it were either a bivalent ion, a bi-bivalent compound, or, more probably, a quadrivalent compound. In either case, equation (10) becomes:

$$\log S/\log S_0 = \frac{2}{1 + 1.5 \sqrt{\mu}}$$

The activity coefficients of oxyhemoglobin have been calculated on this assumption, and subtracted from the logarithm of the solubility in Table IV. The difference yields a constant value for $\log S_0$, and thus justifies the assumption regarding the apparent valence type of oxyhemoglobin.
Landsteiner and Heidelberger (20) have determined the solubility of very pure salt-free oxyhemoglobin. They were, as they state, concerned only with relative results, and therefore made no attempt to control the temperature. The solubilities that they observed are recorded in Table V. It will be noted that after the first experiment, they obtained results all of which are consistent with, and the lower of which are identical with, those calculated above by means of the Debye equation from the solubility in salt solutions. The solubility in water of oxyhemoglobin at pH 6.6 and 0°C. may therefore be provisionally taken as 11.2 gm. per liter. The solubility of oxyhemoglobin is

<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Solubility of oxyhemoglobin, gm. per liter</th>
<th>log So</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.0</td>
<td>0.903</td>
</tr>
<tr>
<td>2</td>
<td>15.8</td>
<td>1.199</td>
</tr>
<tr>
<td>3</td>
<td>15.1</td>
<td>1.179</td>
</tr>
<tr>
<td>4</td>
<td>10.7</td>
<td>1.029</td>
</tr>
<tr>
<td>6</td>
<td>12.0</td>
<td>1.079</td>
</tr>
<tr>
<td>6</td>
<td>11.8</td>
<td>1.072</td>
</tr>
<tr>
<td>6</td>
<td>10.7</td>
<td>1.029</td>
</tr>
<tr>
<td>6</td>
<td>10.7</td>
<td>1.029</td>
</tr>
<tr>
<td>7</td>
<td>16.6</td>
<td>1.220</td>
</tr>
<tr>
<td>Average</td>
<td>12.4</td>
<td>1.082</td>
</tr>
</tbody>
</table>

higher at more acid and at more alkaline reactions than it is at pH 6.6. The solubility at the other reactions studied may be estimated by adding to our average value of log So the values of \( \Delta \log S_0 \) deduced from solubility in salt solutions. This calculation yields a solubility at pH 6.4 and 6.8 of 12.2 and 13.1 gm. per liter respectively.

Dividing the solubility results obtained at different values of the ionic strength by these values of \( S_0 \) yields the activity coefficients of oxyhemoglobin in phosphate solutions. These are graphically represented in Fig. 2, and compared with the activity coefficients of substances of known valence type, such as the bi-bivalent compound
xantho chromate, and the tri-trivalent luteco hexacyano cobaltiate, studied by Brønsted and his collaborators. The similarity between

\[
\begin{align*}
\text{Bi-bivalent compound xantho chromate and oxyhemoglobin is striking. The} \\
\text{influence of a neutral salt upon the solubility of the bi-bivalent} \\
\text{compound xantho chromate and oxyhemoglobin is striking. The}
\end{align*}
\]
results, when potassium chloride or magnesium sulfate were employed as solvent, are somewhat higher in concentrated solution, since the mean effective diameter of the ions is somewhat lower. Under these conditions the solvent action of the potassium formate upon xanthochromate and of potassium phosphate upon oxyhemoglobin fall upon the same curve.

Activity coefficients may be calculated from these solubility measurements by means of equation (1). The activity coefficients that have been determined for oxyhemoglobin in phosphate solutions have been compared in Table VI with the ratio $S_0/S$ determined by Brønsted and Petersen (8) for xanthochromate dissolved in magnesium sulfate, and with the activity coefficients of magnesium sulfate.

### Table VI.

Comparison of the Activity Coefficients of Magnesium Sulfate, Xanthochromate, and Oxyhemoglobin.

<table>
<thead>
<tr>
<th>Ionic strength</th>
<th>MgSO₄</th>
<th>Xanthochromate</th>
<th>Oxyhemoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$S_0/S$ in MgSO₄ (8)</td>
<td>$S_0/S$ in KH₂PO₄ + K₂HPO₄</td>
<td>$S_0/S$ in KH₂PO₄ + K₂HPO₄</td>
</tr>
<tr>
<td>0.08</td>
<td>0.321</td>
<td>0.42</td>
<td>0.40</td>
</tr>
<tr>
<td>0.20</td>
<td>0.225</td>
<td>0.28</td>
<td>0.29</td>
</tr>
<tr>
<td>0.40</td>
<td>0.166</td>
<td>0.21</td>
<td>0.22</td>
</tr>
<tr>
<td>0.80</td>
<td>0.119</td>
<td>0.14</td>
<td>0.17</td>
</tr>
</tbody>
</table>

given by Lewis and Randall (22). The agreement between the activity coefficients of oxyhemoglobin and of xanthochromate has already been considered. It is closer than that between magnesium sulfate and xanthochromate dissolved in magnesium sulfate, although in this comparison both are bi-bivalent compounds, and the mean effective diameter of the ions in solution should be the same. In comparing two bi-univalent salts, the activity coefficients of one of which, cadmium chloride, happen to be of the same order, at certain concentrations, as those of bi-bivalent compounds, Lewis and Randall remark "we have seen other cases of slight disagreement between the activity coefficients of salts of the same valence type, but this is evidently a different kind of phenomenon. The fact is that cad-
mium chloride cannot be regarded as a strong electrolyte" ((22), page 361).

To conclude that oxyhemoglobin is bivalent or quadrivalent might be correct, but would be unjustified at the present time. Before this conclusion can be accepted it will be necessary to demonstrate that an equation deduced for ions whose dimensions are of the order $10^{-8}$ cm. holds without alteration for ions whose dimensions are of the order $10^{-6}$ cm., and to discover the nature of the saturating body. Our experiments permit neither deduction. They render it certain, however, that oxyhemoglobin behaves in this respect as though it were bivalent or quadrivalent, and that the action of neutral salts in dissolving proteins is identical to their action in dissolving other slightly soluble substances.

Salts have an even more profound influence upon the other globulins that have been studied thus far than they have on oxyhemoglobin. Presumably they are of higher valence types. It is for this reason that no analogy has been found for the solvent action of neutral salts upon globulins like edestin, whose solubility in phosphate solutions at pH 6.8 varies from 0.001, at an ionic strength of 0.25, to 60 gm. per liter at an ionic strength of 1.4, and which behaves as though it were approximately quinquevalent. The solubility of this globulin in salt solution was studied by Osborne and Harris in 1905 (30), and their results are still adequate for its characterization. In the same year Hardy (17) and Mellanby (25) studied the solubility of serum globulin in salt solutions. Since then Sørensen has shown that serum globulin is usually a mixture of pseudoglobulin and euglobulin (32). The fractions of serum globulin that have been studied, which have solubilities of the order of 0.1 gm. in a liter of water, have solubilities over 100 times as great in 0.1 molal salt solution. The valence types of such ions, and the electrical forces surrounding such vast molecules are of a kind for which inorganic chemistry has as yet found no analogy. On the other hand the relatively small solvent action of neutral salts upon hemoglobin, which renders it unique among the globulins thus far investigated, offers an opportunity of comparing the behavior of these proteins with that of slightly soluble salts of known valence.
IV.

SUMMARY.

1. The solvent action of a neutral salt upon a protein, oxyhemoglobin, has been found identical to the solvent action of a neutral salt upon a bi-valent or uni-quadrivalent compound.

2. The solubility of oxyhemoglobin in phosphate solutions of varying ionic strength has been defined by the equation: 
   \[ \log \frac{S}{S_0} = \frac{2\sqrt{\mu}}{1 + 1.5\sqrt{\mu}} \]
   in which \( \mu \) is the ionic strength, and \( S_0 \) is the solubility in the absence of salt.

3. The values of \( S_0 \) have been calculated to be 12.2, 11.2, and 13.1 grn. per liter respectively at pH 6.4, 6.6, and 6.8.

4. The relatively great solubility of oxyhemoglobin in water has been ascribed to the strong affinity constants for acid and base of certain groups in oxyhemoglobin.

5. The small change in the solubility of oxyhemoglobin effected by neutral salts suggests that but few such groups are dissociated in oxyhemoglobin in the state in which it crystallizes near its isoelectric point.

6. Certain of the other properties of oxyhemoglobin, such as its low viscosity, are considered in the light of its molecular weight and its valence type.

BIBLIOGRAPHY.