STUDIES IN THE CHEMISTRY OF HEMOGLOBIN.

III. THE EQUILIBRIUM BETWEEN OXYGEN AND HEMOGLOBIN AND ITS RELATION TO CHANGING HYDROGEN ION ACTIVITY.

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INTRODUCTION.

Knowledge concerning the combination between oxygen and hemoglobin, which had been obtained prior to 1912, was summarized by Barcroft in his book, "The Respiratory Function of the Blood" (1). To this understanding Hoppe-Seyler and his contemporaries, and later, Barcroft, Haldane, and Hill, contributed notably. Since this book appeared it has been found that the picture presented was too simple and neglected certain other interdependent variables such as hydrogen ion activity, salt concentration, and distribution of water and electrolytes between plasma and cells.

Beginning with the appearance of the paper by Christiansen, Douglas, and Haldane (2), and followed by the papers of Henderson, of Parsons, of Van Slyke and his collaborators—to mention only a few of the investigators involved—data have accumulated rapidly. As a result, we now have in all cases at least a qualitative picture of the equilibria involved. In certain cases, notably that of the acid-base equilibrium, our knowledge is relatively satisfactory, both theoretically and quantitatively; in others, either theory has outstripped precise data, or quantitative data lack a theoretical explanation. None the less empirical relationships

* Lc Conte Memorial Fellow, University of California, 1924-1925, the period in which the experiments herein described were performed. R,c-Research Fellow in Medicine, National Research Council, 1927.
have been demonstrated in many instances. But despite the fact that the equilibria involving hemoglobin, hydrogen ion activity, and oxygen tension have received particular attention, there is as yet no adequate theoretical explanation, nor do satisfactory data correlating these equilibria over a wide range of hydrogen ion activity and percentage saturation of hemoglobin with oxygen exist.

The experiments described in this paper were, therefore, undertaken in order to make such data available, and, if possible, to provide a theoretical interpretation for them. To this end we have constructed isohydrionic oxygen dissociation curves for the pH range 4.91 to 8.88, derived from experimental values for percentage saturation of hemoglobin at varying oxygen tensions, obtained in heavily buffered solutions of crystallized horse hemoglobin.

Methods.

A description of the methods employed may conveniently be divided into four portions: the preparation of pure hemoglobin; the elaboration of solutions of known hydrogen ion activity from this material; equilibration of the solutions with suitable gas mixtures; and, finally, the analysis of the equilibrated solutions for their oxygen content.

Preparation of Hemoglobin.

Hemoglobin was prepared by a modification of a method previously described by one of us (3). This method depends on the separation of red blood cells from diluted plasma by means of the Sharples centrifuge, and repeated crystallization of oxyhemoglobin from a cold, concentrated solution, after complete oxygenation and adjustment of the pH to a point slightly more acid than 6.8.

3 liters of horse red blood cells¹ were diluted with 12 liters of 1.5 per cent sodium chloride solution which had previously been cooled to 2°. The resulting volume of 15 liters was then run

¹ Obtained from the Massachusetts Antitoxin Laboratory through the courtesy of its director, Dr. Benjamin White.
through a modified bowl\(^2\) of the Sharples laboratory centrifuge rotating at a speed of about 30,000 r.p.m. The bowl, from which the vanes at the lower end had been removed, nearly eliminated hemolysis during centrifugation. Consequently, we could wash the corpuscles once by this procedure. Inasmuch as we used large volumes of salt solution, most of the plasma proteins and salts were separated from the cellular suspension at this stage of the preparation.

In order to prevent possible immediate crystallization the concentrated cells were allowed to run into 100 cc. of distilled water for each liter of cells originally present. Occasionally crystallization did occur even after the addition of distilled water. In this event the suspension was vigorously stirred and \(1 N\) KOH added slowly until the crystals dissolved. Concentrated alkali had to be added with extreme care in order to avoid denaturation and the formation of methemoglobin.

In order to separate hemoglobin from cellular débris the partially laked suspension of erythrocytes was again passed through the Sharples centrifuge. This time the ordinary bowl was used, rotating at 35,000 to 40,000 r.p.m. in order to lake the corpuscles mechanically. Very few cell bodies were then observed microscopically.

By this procedure an initial volume of 3 liters of erythrocyte suspension had been concentrated to about 2 liters of 25 to 28 per cent hemoglobin solution. From this solution oxyhemoglobin could readily be crystallized as rhomboid plates. The factors favoring crystallization are concentration of solution, hydrogen ion activity in the neighborhood of the isoelectric point, low temperature, and complete oxygenation. In order to attain the latter end, the alkaline solutions were stirred by a motor-driven Luther

\(^2\) The bowl, at first used, was devised by W. B. Wescott and patented by the American Protein Products Company, Boston, to whom we are indebted for its use. The essential features are, (1) the elimination of vanes in the bottom of the usual bowl, and (2) the insertion of four vanes half way up the bowl. By means of its device great peripheral velocity is imparted rather gradually to the cell suspension, and the impact of rapidly moving vanes on red cells, which have no peripheral velocity on entering the bowl, is avoided. Hemolysis is largely avoided by this device. We have lately found that merely removing the vanes gives satisfactory results and is much simpler as the bowl does not have to be balanced.
stirrer in the cold room at 2°; the speed of stirring was regulated by a rheostat, so that changes in the viscosity of the liquid were counteracted. The Luther stirrer consists of an open ended T-tube fused to a solid glass rod. About 2 inches above the cross-piece there is another opening into the tube. This device provides effective agitation and complete oxygenation. During stirring the reaction was adjusted to about pH 6.6, either by the gradual addition of \( \frac{2}{3} \text{m} \) phosphate buffer in an amount sufficient to give final concentration of 0.1 m phosphate, or by the addition, drop by drop, of 0.1 m HCl.\(^3\) Because of irreversible changes inactivating the hemoglobin, this must be done with extreme care. At intervals during the titration, drop samples were placed on microscope slides. A few seconds’ exposure to air sufficed to evaporate and concentrate the solution, inducing crystal formation. If rods appeared, the solution was still too alkaline and the titration was continued until a sample revealed rhomboid plates microscopically. After the first appearance of crystals another 5 to 10 cc. of 0.1 N HCl per 500 cc. of solution were added, inducing the rapid formation of crystals readily distinguishable macroscopically by the sheen of the suspension. Too much acid must be avoided since denaturation of hemoglobin occurs very rapidly. This is shown by a color change from red to dark brown. From 160 to 320 cc. of 0.1 N HCl generally sufficed to crystallize oxyhemoglobin in 1 liter of concentrated solution. The rather wide limits are presumably due to differences in initial hydrogen ion and hemoglobin concentrations. The steps of washing the erythrocytes, separation of hemoglobin from the cells, and crystallization could easily be completed in 2 hours.

After crystallization had begun the solution was stirred about 1 hour longer and then allowed to stand in the cold room at least another hour. Centrifugation for 15 minutes in the usual laboratory centrifuge yielded a closely packed crystalline sediment occupying a volume equal to that of the supernatant liquid. Centrifugation was performed either in chilled cups or, preferably, in a centrifuge mounted in the cold room.

\(^3\) Crystallization can also be induced by the addition of sulfuric, acetic, and phosphoric acids, and presumably by the addition of any acid strong enough to change the pH to 6.6. The shape of the crystals obtained depends on the acid used.
The crystalline hemoglobin was then purified by repeated washing and by recrystallization. Washing was done by mixing the crystals with 0.5 to an equal volume of cold distilled water. The suspension was again centrifuged for 15 minutes. This process could be repeated several times without an undue loss of material. After two washings the salt concentration was reduced to a point negligible in comparison with that of the buffer used in our experiments.

Recrystallization was invariably used when the first crop of crystals was formed in a phosphate buffer, and usually when the crystals were formed by acid titration. To the centrifuged crystals was added about half their volume of water in order to facilitate stirring. The solution was then vigorously stirred and 1 N KOH added drop by drop until solution was complete. Usually 5 cc. of alkali per 100 cc. of crystals sufficed.

Centrifugation of the solution removed any insoluble impurity. 0.1 N HCl equivalent to the KOH used in dissolving the crystals was now added, the same precautions being observed as during the first crystallization. Repeated recrystallization could be performed without undue loss of material.

In order to free preparations from electrolytes and secure crystals of a high degree of purity, a dilute isoelectric solution of hemoglobin was dialyzed under reduced pressure. We used this procedure infrequently since the high concentration of salts used in our experiments made it unnecessary.

The successful preparation of hemoglobin by the method described depends largely on bringing a concentrated solution to a proper pH and the observance of precautions already mentioned, but which, because of their importance, we think it worth while to state again: (1) The avoidance of local or general excess of acid, which leads to cleavage and possibly denaturation. (2) Rapidity of the process. Hemoglobin solutions change to methemoglobin spontaneously on standing. (3) Low temperature maintained at 2° as nearly as possible. Increasing the temperature increases the rate of transformation to methemoglobin. (4) Complete oxygenation.

If, however, these precautions are observed, one can obtain a very pure product as is suggested by ratio of protein determined...
by the Kjeldahl method to that determined by oxygen capacity and the iron ratio.4

The preparations used in our experiments were made by the hydrochloric acid titration method with the exception of Preparations 21 and 22, which were made by adding phosphate buffer.

**Preparation of Buffered Hemoglobin Solutions for Equilibration.**

Buffered hemoglobin solutions of desired pH were made by adding to a suspension of oxyhemoglobin crystals phosphate or borate solutions, together with enough alkali to change the pH of hemoglobin to a given value. The quantities were so chosen that final concentrations of phosphate were 0.167 M, those of borate M/9, and that of hemoglobin 0.001 M.6 Electrometric measurement indicated that pH of fully reduced solutions deviated from the intended values by less than 0.01 in pH.

The phosphate hemoglobin solutions were prepared by addition to a suspension of oxyhemoglobin crystals of sufficient M KH₂PO₄ and 0.884 M KOH to bring the hemoglobin to the desired pH, and to make the final concentration of phosphate 0.167 M. The amount of KOH necessary to change the hemoglobin and phosphate to the desired pH was determined by reference to titration curves for reduced hemoglobin6 and buffer. The final concentration of the hemoglobin was approximately 6.4 gm. per 100 cc. or approximately 0.001 M, on the basis of a molecular weight of 66,800, consistently assumed in this paper.

The pH finally attained depended chiefly on the buffering value of the phosphate and to a slight extent on that of hemoglobin. Whereas the concentration of the former (0.167 M) is relatively large compared with that of the latter (0.001 M, or in terms of the probable number of acid groups affected by oxygenation, 0.004 N), it is evident that the phosphate buffering effect must be correspondingly great. Moreover, slight errors in the amount of

4 Unpublished experiments of one of us. This subject requires further investigation since the nitrogen factor of Abderhalden on which our observations were based was doubtless made on the basis of dry weights of material prepared by the alcohol-ether method.

6 Assuming a molecular weight of 66,800. This value accords closely with the values obtained by Svedberg (4) and Adair (5).

6 Unpublished data by Cohn and Ferry.
alkali or acid added to reduced hemoglobin produce errors in the pH for the reduced solution of less than 0.01 in pH. The change in pH attendant upon completely oxygenating the reduced solutions is more serious in its magnitude even in the case of phosphate-buffered solutions and increases with increasing pH. It can be shown that this shift varies from approximately 0.02 in pH at pH 6.55 to about 0.06 in pH at pH 7.95. At pH 7.38 the shift is equal to about 0.04 in pH. It is to be noted that the greater changes in pH occur just where the phosphate buffer system becomes inefficient. The numerical values given for the change in pH are, of course, maximal, inasmuch as the change is proportionately less with incomplete oxygenation.

At points more alkaline than pH 7.95 we made use of boric acid-borate buffers. Such buffers are somewhat less satisfactory, largely because considerable uncertainty exists concerning the precise mechanism of their action. Because of this it is difficult to predict variations in the apparent ionization constant of boric acid in solutions of different ionic strengths. We found that increase in the borate concentration of the solution above 1/9 caused appreciable variation in pH even with a constant buffer ratio. Consequently we used 1/9 borate solutions, although heavier buffering would otherwise have been desirable.

The final hemoglobin borate-buffered solutions were 7 per cent, or slightly more than 0.001 M in hemoglobin and 1/9 in borate. The borate solutions were prepared in accordance with the method of Clark (6). To a solution of 0.667 M boric acid and 0.667 M NaCl we added enough 0.667 N NaOH to adjust the hydrogen ion activity of the final buffered hemoglobin solution to the desired point. In the case of these more alkaline solutions, the error due to the addition of incorrect amounts of alkali to the hemoglobin is more serious than is the pH shift following oxygenation. We have, however, made calculations which indicate that the error in the former case probably does not exceed 0.02 in pH. The change following oxygenation is limited because of the fact that at points more alkaline than pH 8.00 most of the hemoglobin, whether oxygenated or reduced, probably exists as salt. Consequently the change in the value of the ionization constants has little effect on the pH. At pH 8.88 we have calculated that the shift is about 0.01 in pH.
We have further controlled the accuracy of the pH estimations by determining the hydrogen ion activities of the buffered solutions of Preparations 28 and 29 electrometrically. The measurements were made on solutions which had previously been reduced by repeated evacuation and equilibration with oxygen-free hydrogen. No deviation exceeding 0.01 in pH was detected between observed and calculated values. This indicated that except for changing pH due to oxygenation, our oxygen dissociation curves are within the limits of experimental error, indeed isohydrionic.

Methods of Equilibration and Sampling.

Samples of buffered hemoglobin were equilibrated with analyzed gas mixtures and sampled by a method similar to that described by Austin et al. (7) as their first saturation method.

Suitable mixtures of oxygen and nitrogen were made each day and stored in a carboy over water acidulated with sulfuric acid. They were then analyzed with a Henderson-Haldane apparatus.

In order to equilibrate the buffered hemoglobin solutions with the gas mixtures, 10 to 15 cc. of solution were placed in a blood sampling tube of the usual design, and connected with a tonometer by means of rubber tubing. Following evacuation the tonometer was filled with gas, slightly in excess of atmospheric pressure, and the hemoglobin solution introduced by appropriate manipulation of stop-cocks and the leveling bulb. Subsequently the tonometer was rotated in a water bath at 25° ±0.02°. After a few seconds, the gas was brought to atmospheric pressure by quickly opening and closing the tonometer stop-cock without removing the tonometer from the water bath. After 5 minutes rotation, the tonometer was removed from the water bath and connected with the sampling tube into which the hemoglobin solution was next drawn. Again the tonometer was evacuated and filled with gas mixture and, as before, the hemoglobin was reintroduced into the tonometer, which was then rotated in the water bath. This procedure was repeated in all four times, except at low oxygen pressures, when five periods of rotation were employed.
As a result of a set of analyses performed after each period of rotation, we feel confident that equilibrium was actually reached at the end of this process. In order to be certain that the gas mixture in the tonometer was in fact identical with that in the carboy, we washed all connections with the mixture before connection was established either with the tonometer or the sampling tube.

After the completion of equilibration, the solution was returned to the sampling tube, and the tonometer removed. A pipette with stop-cock similar to those described by Van Slyke was substituted for the tonometer, and both pipette and connecting tube washed out with the gas mixture. The pipette was then filled and its contents rapidly transferred to a Van Slyke blood gas apparatus.

Following the removal of a sample for analysis, the remainder of the hemoglobin solution was returned to the tonometer and saturated with air at room temperature. This was done by washing out the tonometer frequently with moist air and shaking vigorously for at least 3 minutes. We believe that this constitutes an important step in the procedure, since large errors in the estimation of percentage saturation due to the formation of inactive hemoglobin during the process of equilibration, and resulting errors in the estimation of active hemoglobin, were thereby avoided. These errors are particularly significant on the acid side of the isoelectric point. A further advantage of this procedure is that errors due to changes in the concentration of hemoglobin, resulting from evaporation during manipulation, are avoided by this procedure.

**Analysis for Oxygen Content.**

The determination of the oxygen content of each sample was made with the Van Slyke constant volume blood gas apparatus, with carefully evacuated reagents. We believe that these analyses were accurate to about 0.05 volume per cent and that the combined errors of gas analysis, equilibration, sampling, and blood gas analysis rarely exceeded ±0.1 volume per cent, or, in general with the solutions here used, about 1 per cent saturation.

Oxygen was absorbed in the pipette by means of an alkaline solution of sodium hydrosulphite and anthraquinone sulfonate made up as described by Fieser (8). The stop-cock of the burette was
Because of the necessity for making measurements of the oxygen content and capacity simultaneously, it was impossible, with the two burettes available, to make these measurements in duplicate. We believe that our analytical results are accurate to about 0.05 volume per cent, or in the case of solutions of about 0.001 M hemoglobin, to rather better than 1 per cent saturation.

Results.

We have collected the experimental data in Table I. Columns 1 to 4 refer to the hydrogen ion activity and ionic strength, the number of preparation, the number of experiment, and oxygen tension, respectively. In Column 5 appears the figure for the total oxygen contained in the sample expressed as volumes per cent. This value is in fact the sum of two quantities, free and chemically bound oxygen. The former quantity may itself, possibly, consist of two parts, an amount of oxygen in physical solution, and a fraction considered as "absorbed," as suggested by Conant (9). At the present time there is no positive evidence for the existence of the last mentioned fraction. We have, therefore, disregarded this possibility.

In order to estimate the solubility of oxygen in the salt solutions used, we have employed the theory of Debye and Hückel. According to this theory, gases, as non-electrolytes should be subject only to "salting out" effects. Therefore, the first term of the Debye-Hückel equation drops out, leaving an equation of the form $- \log \frac{S}{S_0} = K_s \mu$ in which $K_s$ is a proportionality constant characteristic of the salt used, $S$ and $S_0$ the solubilities in the solvent and pure water respectively, and $\mu$ the ionic strength. This has been discussed by Scatchard (10). From a consideration of published data for the solubility of oxygen in salt solutions (11) it appears that this law holds. Unfortunately there exist no data for phosphate solutions. It would, of course, be desirable to have data bearing directly on our problem. Unfortunately these do not exist and it is extremely difficult to secure such data since relatively large changes in the electrolyte content of solutions alter the solubility coefficient to an extent which is not large compared to the experimental error.

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The results are expressed in volumes per cent. $pO_2$ in mm.

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TABLE I—Continued.
used the value for sodium chloride, \( K_s = -0.14 \). This is justified since variations in \( K_s \) are not very great from salt to salt and also since the data for gas solubilities in electrolyte solutions are themselves so unsatisfactory that the value chosen accords within the experimental error with that obtained from the data for various salt solutions.

In addition to the salting out effect produced by the phosphates, we have also considered the effect of hemoglobin itself in lowering gas solubility. The solubility may be assumed to be proportional to the volume of phosphate solution present per cc. of buffered hemoglobin solution. This proportionality factor should be equal to the density of the buffered hemoglobin solution less the weight of hemoglobin in 1 cc. divided by the density of the phosphate solution. No data exist for the densities of such buffered solutions, nor did it appear of sufficient importance to secure the data ourselves. We have, therefore, estimated this factor as being approximately equal to 0.94. This estimate is probably accurate to about \( \pm 2 \) per cent.

---

TABLE I—Concluded.

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<th>( O_2 ) capacity.</th>
<th>( O_2 ) combined, 100 per cent saturated.</th>
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* Electrometric measurement not made. \( p_{aH} \) interpolated from E.M.F. curve for phosphate-Hb system.
In Table II are given values for the solubility correction per mm. of oxygen pressure in terms of the ordinary solubility coefficient $\alpha$, together with figures indicating their derivation. In order to convert these values to volumes per cent one must multiply by 100.

In Column 6 of Table I appears the value for oxygen capacity; that is, the total amount of oxygen per cc. of solution after saturation with air. In Columns 7 and 8 appear the values for the combined oxygen after equilibration with the indicated oxygen tension and of another aliquot equilibrated with air respectively. Column 9 is self-explanatory.

The data are graphically represented in Figs. 1 and 2.

The data here presented are, we believe, in general accurate to about 1 per cent saturation, but in the case of the more acid solutions, changes in the hemoglobin occurred so rapidly that such data possess only qualitative significance.

\[
\log \frac{S}{S_0} = -0.14
\]
Fig. 1.

Fig. 2.
DISCUSSION.

The classical interpretation of the equilibrium between oxygen and hemoglobin has been based on the hypothesis that they combine according to an equation of the form $\text{Hb}_n + n\text{O}_2 \rightleftharpoons \text{Hb}_n\text{O}_{2n}$. In this case a simple equilibrium constant may be written

$$K = \frac{a\text{Hb}_n\text{O}_{2n}}{a\text{Hb}_n \cdot a\text{O}_2^n}$$

This should hold provided that the proper values are used for the activities. If we replace activities by stoichiometric concentrations, we must write

$$K' = \frac{[\text{Hb}_n\text{O}_{2n}]}{[\text{Hb}_n][\text{O}_2]^n}$$

(1)

where $K' = K \frac{\gamma'}{\gamma_1}$ and $\gamma_1$ and $\gamma'$ are activity coefficients referring to oxyhemoglobin and reduced hemoglobin, respectively.

The simple theory has previously been proved faulty. Further proof is given by the curves in Fig. 3, in which log $R = \log \frac{\text{Hb}}{\text{HbO}_2}$ has been plotted against log $p\text{O}_2$. In order to conform to the theory, these curves should represent a family of straight lines—a consideration already discussed fully by one of us (12) and by Brown and Hill (13). Only at pH 7.38 does this hypothesis nearly fit, and here the value for $n$ is approximately 2.6, a value closely comparable to the value, 2.4, found at a similar pH in human blood. The fact that the assumption of integral values of $n$ in Equation 1 does not accord with the facts, is shown in Fig. 4 in which oxygen dissociation curves constructed according to this hypothesis are compared with the data obtained at pH 6.77, and with a curve drawn according to a theory subsequently discussed.

We are, then, compelled to assume either (1) that the reduced-oxyhemoglobin activity coefficient ratio varies with oxygenation, or (2) that the correction for dissolved oxygen should itself be corrected on account of the presence of "adsorbed" oxygen, or (3) that the theory does not accord with the facts. As far as the
first alternative is concerned, one might, on account of the fact—clearly shown by L. J. Henderson (14)—that oxyhemoglobin is a stronger acid than reduced hemoglobin, expect variation in the activity coefficient ratio in unbuffered hemoglobin solutions. But in the buffered solutions used by us, we are unable to describe a mechanism which would vary the ratio in such a manner as to give a single isohydrionic curve, to say nothing of the family of
curves in Fig. 3. The second alternative has as yet no experi-
mental support* and seems unlikely. We are, consequently,
compelled to adopt the third alternative.

Henderson (15) has developed an empirical equation of the
form \( y = a - bx - cx^2 \), in which \( y = \log pO_2, x = \log R, b \) and
c are constants, while \( a \) is a constant which is a linear function of
\( \log pCO_2 \) having the value of \( \log pO_2 \) at 50 per cent saturation.
We have been unable to fit all our curves by an equation of this
type. Further, it presents no apparent theoretical justification.

We have also developed a simple empirical expression which
may be written:

\[
K = \frac{[Hb][pO_2]}{[HbO_2]} = 0.5 + \frac{[H^+]}{0.013 (7.4 \times 10^{-8} + [H^+])} (\log \% HbO_2 - 2.016) \tag{2}
\]
in which the symbols have the same meaning as in Equation 1.

* J. B. Conant—personal communication—has not been able to secure
any experimental evidence substantiating this view.
In this equation the equilibrium constant is a linear function of the log of the mol fraction of oxyhemoglobin. This expression accords with the data at most of the pH values used in our experiments. This is shown in Fig. 5, in which we have plotted $K$ against log per cent HbO$_2$. Only at pH 6.55 does the deviation from the data become significant. This expression moreover demon-
Chemistry of Hemoglobin. III

...ates the observed fact that oxygenation increases the affinity of hemoglobin for oxygen, since the value of $K$ decreases as oxygenation at constant pH progresses. Further, it indicates the fact that increasing pH increases the affinity of hemoglobin for oxygen by a decreasing value of $K$. We have thus far been unable to attach any theoretical significance to this expression. It may, nevertheless, serve as a useful tool in further investigation.

The experimental data at any pH may be described in terms of equations in which hemoglobin appears as a mixture of forms each of which reacts according to Equation 1, where $n$ is an integer. This form of expression was suggested by Redfield (16) who found that the oxygen dissociation curve of the hemocyanin occurring in Limulus polyphemus could be satisfactorily described in these terms. One may assume a mixture of equal parts of hemoglobin such that $n = 2$ and $n = 4$, or a mixture such that there is present one-fifth in the form in which $n = 1$, and four-fifths in which $n = 4$. Accordingly equations may be written

\[
\text{Per cent saturation} = \frac{50K_1 [O_2]^2}{1 + K_1 [O_2]^2} + \frac{50K_4 [O_2]^4}{1 + K_4 [O_2]^4}
\] or

\[
\text{Per cent saturation} = \frac{20K_1 [O_2]}{1 + K_1 [O_2]} + \frac{80K_4 [O_2]^4}{1 + K_4 [O_2]^4}
\]

in which $K_1$, $K_2$, and $K_4$ are the equilibrium constants of Equation 1 when $n$ is 1, 2, and 4, respectively. All the curves may be fitted on either assumption. Further, these constants vary systematically with pH. In this form of expression only two constants are needed to fit a single isohydrionic curve. But at present the

Although we have not presented curves drawn according to this hypothesis, they may be constructed by means of the following constants when $pO_2$ is used as a measure of $O_2$ concentration:

\[
\begin{array}{cccccccc}
\text{pH} & 6.55 & 6.77 & 7.00 & 7.38 & 7.65 & 7.95 & 8.40 & 8.88 \\
n = 2 & 15.8 & 13.8 & 11.5 & 7.9 & 5.8 & 4.3 & 3.2 & 2.5 \\
n = 4 & 22.6 & 19.4 & 15.5 & 9.5 & 6.0 & 3.5 & 1.7 & 0.89 \\
n = 1 & \sqrt[4]{K_1} & 15.8 & 13.8 & 11.5 & 7.9 & 5.8 & 4.3 & 3.2 & 2.5 \\
n = 4 & \sqrt[4]{K_4} & 21.8 & 18.8 & 15.2 & 9.7 & 6.5 & 4.2 & 2.5 & 1.9 \\
\end{array}
\]

The values given above vary with pH if $pK$ is assumed to be 7.0.
molecular weight of this protein, 66,800, does not accord with this assumption, at least in its explicit form.

As another alternative we may use the theory of intermediate compound formation suggested by Adair (17). This theory follows rather naturally from the observed molecular weight of hemoglobin (66,800), which together with the established iron-oxygen ratio (18), implies that the 4 iron atoms in the hemoglobin molecule are each through the radicals they represent capable of combining with 1 oxygen molecule.

We know nothing of the spatial arrangements of these groups. But no matter what their spatial relations may be, we may assume that each group combines, when fully saturated, with 1 oxygen molecule. We have, therefore, to deal with the series of compounds Hb, HbO₂, HbO₄, HbO₆, HbO₈, in which Hb represents the presence of 4 atoms of iron, and may write:

\[
\begin{align*}
\text{Hb} + \text{O}_2 & = \text{HbO}_2, \quad K_1 = \frac{\alpha_{\text{HbO}_2}}{\alpha_{\text{Hb}} \cdot \alpha_{\text{O}_2}} \\
\text{HbO}_2 + \text{O}_2 & = \text{HbO}_4, \quad K_2 = \frac{\alpha_{\text{HbO}_4}}{\alpha_{\text{HbO}_2} \cdot \alpha_{\text{O}_2}} \\
\text{HbO}_4 + \text{O}_2 & = \text{HbO}_6, \quad K_3 = \frac{\alpha_{\text{HbO}_6}}{\alpha_{\text{HbO}_4} \cdot \alpha_{\text{O}_2}} \\
\text{HbO}_6 + \text{O}_2 & = \text{HbO}_8, \quad K_4 = \frac{\alpha_{\text{HbO}_8}}{\alpha_{\text{HbO}_6} \cdot \alpha_{\text{O}_2}}
\end{align*}
\]

The symbols used here have the usual significance: \( \alpha_{\text{Hb}} \), \( \alpha_{\text{HbO}_2} \), \( \alpha_{\text{HbO}_4} \), \( \alpha_{\text{HbO}_6} \), \( \alpha_{\text{O}_2} \), refer to the activities of reduced, monooxy-, dioxy-, trioxygeny-, and tetraoxyhemoglobin and oxygen respectively. These are the substances postulated by the theory of intermediate compound formation. Substituting the product of an activity coefficient and the stoichiometric concentration for each of the terms involving hemoglobin and \([\text{O}_2]\) for the pressure of oxygen, we can write:

\[
\begin{align*}
[\text{Hb}_4\text{O}_2] & = K_1' \gamma' [\text{Hb}_4] [\text{O}_2] \\
[\text{Hb}_4\text{O}_4] & = K_1'K_4' \gamma' [\text{Hb}_4] [\text{O}_2]^3 \\
[\text{Hb}_4\text{O}_6] & = K_1'K_4'K_6' \gamma' [\text{Hb}_4] [\text{O}_2]^5 \\
[\text{Hb}_4\text{O}_8] & = K_1'K_4'K_6'K_8' \gamma' [\text{Hb}_4] [\text{O}_2]^7
\end{align*}
\]
where $K_1' = \frac{K_1}{\gamma_1}$, $K_2' = \frac{K_2}{\gamma_2}$, $K_3' = \frac{K_3}{\gamma_3}$, $K_4' = \frac{K_4}{\gamma_4}$, and $\gamma_1'$, $\gamma_2'$, $\gamma_3'$, and $\gamma_4'$, the activity coefficients for $[Hb_4]$, $[Hb_4O_2]$, $[Hb_4O_4]$, $[Hb_4O_6]$, and $[Hb_4O_8]$ respectively.

The concentration of combined oxygen may now be written:

$$Hb_4\gamma' (K_1'[O_2] + 2 K_1'K_2'[O_2]^2 + 3 K_1'K_2'K_3'[O_2]^3 + 4 K_1'K_3'K_4'[O_2]^4)$$

and the total concentration of protein

$$Hb_4\gamma' (1 + K_1'[O_2] + K_1'K_2'[O_2]^2 + K_1'K_2'K_3'[O_2]^3 + K_1'K_2'K_3'K_4'[O_2]^4)$$

Now since 4 oxygen atoms combine with hemoglobin when oxygenation is complete, we may write:

$$\text{Per cent saturation} = \frac{\frac{1}{4} \cdot \text{concentration of combined } O_2}{\text{total } Hb \text{ concentration}} \times 100$$

$$= \frac{100 \gamma'[Hb_4](K_1'[O_2] + 2 K_1'K_2'[O_2]^2 + 3 K_1'K_2'K_3'[O_2]^3 + 4 K_1'K_3'K_4'[O_2]^4)}{4 \gamma' [Hb_4](1 + K_1'[O_2] + K_1'K_2'[O_2]^2 + K_1'K_2'K_3'[O_2]^3 + K_1'K_2'K_3'K_4'[O_2]^4)} \quad (3)$$

If all the oxygen-binding groups have the same affinity for oxygen, the saturation of any one group has no chemical effect on any of the others—as might be the case if they were widely separated in space—and the order of saturation is governed by probability, then, as Adair has shown,

$$K_3' = \frac{3}{8} K_2', K_5' = \frac{1}{5} K_1', K_6' = \frac{1}{16} K_1'$$

and the expression reduces to

$$\text{Per cent saturation} = y = \frac{0.25 K_1'}{1 + 0.25 K_1'}$$

a transformation of Equation 1. This is known not to hold. Adams (19) and later Simms (20) have developed a similar treatment for the ionization of polyvalent organic acids.

Adair (17) has been able rather successfully to express his own data, obtained at 37° on human hemoglobin, in terms of the equation:

$$y = \frac{0.25 K_1 [O_2] + 0.25 (K [O_2])^2 + 0.25 (K [O_2])^3 + (K [O_2])^4}{1 + (K [O_2]) + 0.5 (K [O_2])^2 + 0.333 (K [O_2])^3 + (K [O_2])^4}. \quad (4)$$
a form which is the result of substituting the following values in
Equation 3: \( K = K_1', K_2' = 0.5 \ K_1', K_3' = 0.667 \ K_1', K_4' = 3 \ K_1' \). These values do not, however, satisfy our data obtained
on horse hemoglobin at 25°. But if we substitute the values
\( K_2' = 0.32 \ K_1', K_3' = 0.8 \ K_1', K_4' = 10.5 \ K_1' \) in Equation 3, we
obtain Equation 5.\(^{10}\)

\[
\psi = \frac{0.25 K_1' [O_2] + 0.50 \times 0.32 (K_1' [O_2])^2 + 0.75 \times 0.256 (K_1' [O_2])^3 + 2.7 (K_1' [O_2])^4}{1 + K_1' [O_2] + 0.32 (K_1' [O_2])^2 + 0.256 (K_1' [O_2])^3 + 2.7 (K_1' [O_2])^4}
\]  

(5)

a form in which it is possible satisfactorily to describe our data. We have, moreover, constructed the curves in Figs. 1 and 3
according to this equation. It has thus been possible, with four
constants bearing a definite ratio to each other at all pH values
from 6.55 to 8.88, to describe the equilibrium between oxygen and
hemoglobin at any pH within this range.

The agreement between the theoretical curves and the data is
rather gratifying, especially when the deviations are critically
considered. Turning to Fig. 1, we note that agreement between
data and theory is very good up to pH 7.95, a point at which we
have previously noted the inefficiency of phosphate buffers. Furthermore, the discrepancy is serious only above 90 per cent
saturation, and quantitatively about what one would expect for a
shift of 0.05 in pH. That this may be the correct explanation is
suggested by the rather better agreement at pH 8.40 and 8.88,
where borate buffers were used. Turning now to Fig. 3, we again
note excellent agreement up to pH 7.95. In the case of the curve
for pH 7.95, both the magnitude and the direction of the deviation
are what one would expect on the ground of inefficient phosphate
buffering, with consequent shift in pH. In the case of the curves
for pH 8.40 and 8.88, agreement between theory and data is
reasonably good until the value for log \( R \) assumes a negative value
greater than -1.2. This is the point where small errors in per
cent saturation result in large errors in log \( R \).

None the less, it is to be remembered that we have used four
constants and that—as our empirical equations have shown—any
equation that will describe one curve will describe the rest.

We wish also to call attention to the fact that we have made no
correction for possible variation of the activity coefficients of the

\(^{10}\) It can be shown that positive roots of this equation are uniquely
determined.
various ionic species of hemoglobin, of which there must be at least five present. We know no means of estimating this factor at present and the correction is in part implicitly assumed in the various $K'$ factors. But we are assuming that the thermodynamic environment is constant for the entire family of curves, an assumption that is certainly incorrect since $\mu$ varies from 0.288 to 0.464.

Again, these results may be applicable only to horse hemoglobin prepared according to the method described. It is not proper to extrapolate these results to whole blood without further study.

But in spite of our reservations, if one makes the single assumption of the existence of intermediates, the molecular weight of hemoglobin (66,800), together with the iron-oxygen ratio, necessitates the general equations developed above. It must be admitted, however, that there is at present no direct experimental evidence in favor of this assumption.

We can now turn to the effect of changes in hydrogen ion activity on the equilibrium studied. Even from Figs. 1 and 2 it is apparent that the affinity of hemoglobin solutions for oxygen passes through a minimum somewhere between pH 6 and 7. This fact was first suggested by the work of Rona and Ylppö (21). The phenomenon is more clearly shown in Fig. 6, in which we have plotted the oxygen pressure sufficient to produce 50 per cent saturation against pH.

Fig. 6 is also useful in showing that the constant $a$ in Henderson's equation is not a linear function of pH, but must be explained on some other basis. Inspection of this figure also strengthens the conviction, previously expressed by Adolph and Ferry (22), that oxygen binding is in some way dependent on the ionization, and therefore, on the acid or basic dissociation constants of hemoglobin. The curve in this figure is strongly reminiscent of a titration curve, and suggests that, while acid dissociation constants may be chiefly effective on the alkaline side of the point of minimum oxygen binding, basic constants may predominate at more acid reactions. We are unable to analyze this curve further.

Let us next consider the first equation of the series upon which the theory of intermediates is based, $K_1' = \frac{[\text{Hb}_4 \text{O}_5]}{\gamma' [\text{Hb}_4] [\text{O}_5]}$. The constants for the equilibrium between oxygen and the unionized free acid and its ionized salt are probably different, so that we may write, after Henderson:
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\[ K_a = \frac{[H_2Hb_O_3]}{[H_2Hb] \cdot [O_2]} \]

where \( K_a' = K_a \gamma_{H_2Hb} \gamma_{H_2Hb_O_3} \)

\[ K_s = \frac{[B_2Hb_O_3]}{[B_2Hb] \cdot [O_2]} \]

where \( K_s' = K_s \gamma_{B_2Hb} \gamma_{B_2Hb_O_3} \)

\[ K_R = \frac{[B_2Hb] \cdot [H^+]^2}{[H_2Hb]} \]

where \( K_R' = \frac{K_R}{\gamma_{B_2Hb}} \)

if we assume that over the pH range studied the acid group affected by oxygenation behaves as though it were a dibasic acid \( H_2Hb \) with salt \( B_2Hb \). We should then write:

\[ K_1' = \frac{[\text{total monoxyhemoglobin}]}{[\text{total reduced hemoglobin}]} = \frac{(K_s' [H_2Hb] + K_s' [B_2Hb]) [O_2]}{([B_2Hb] + [H_2Hb]) [O_2]} \]

Substituting for \( H_2Hb \) its equivalent, \( B_2Hb \cdot [H^+]^2 \), we may write:

\[ K_1' = \frac{K_s' [B_2Hb] [H^+]^2}{K_R' + K_s' [B_2Hb]} \]

Simplifying

\[ K_1' = \frac{K_s' [H^+]^2 + K_s' K_R'}{K_R' + [H^+]^2} \] (6)

We can now solve for \( K_R' \) and write:

\[ K_R' = \frac{[H^+]^2 (K_s' - K_s)}{[K_s' - K_1']} \] (7)

If we now let \( K_s' = 0.045 \) and \( K_s' = 0.345 \) and \( K_R' = 0.158 \times \)

\[ ^{11} \text{Redfield and Mason (23) have shown that upon the addition of HCl, Limulus hemocyanin is transformed from a colored to a colorless form, which does not combine with oxygen, according to a similar equation. It is also to be noted that the change in solubility of carboxyhemoglobin in concentrated salt solutions over the range pH 6.6 to 7.4 may be described by assuming that it dissociates as a divalent acid (24).} \]
we may draw the curve shown in Fig. 7. The values for
$K_1'$ obtained from our data are:

<table>
<thead>
<tr>
<th>pH</th>
<th>8.88</th>
<th>8.40</th>
<th>7.95</th>
<th>7.65</th>
<th>7.38</th>
<th>7.00</th>
<th>6.77</th>
<th>6.55</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_1'$</td>
<td>0.35</td>
<td>0.32</td>
<td>0.21</td>
<td>0.13</td>
<td>0.09</td>
<td>0.06</td>
<td>0.05</td>
<td>0.042</td>
</tr>
</tbody>
</table>

\[12\] The value for pK$_B'$ = 7.80 differs from that found by Hastings (25) for reduced hemoglobin. Hastings' value of pK$_B'$ = 8.18 obtained in solutions of $\mu = 0.30$, is, however, based on titration of all the acid groups. We have good reasons for believing that only a small number of these groups is involved in the oxygenation process. This has already been shown by Redfield and Mason (23) for hemocyanin.
These points are shown as rectangles on Fig. 7. The size of the rectangles indicates graphically the probable order of their accuracy. It is worth while noting again that the error is greatest at pH 7.95.

This procedure gives a theoretical basis for the variation of $K_i$ with pH and together with Equation 5 will explain the equilibrium between oxygen and solutions of purified horse hemoglobin over the range pH 6.55 to 8.88. The fact that only a few of the many acidic hydrogen atoms in hemoglobin are intimately connected with the process of oxygenation supports the idea that hemoglobin may be a complex iron salt somewhat analogous to the complex cyanides (26).

**CONCLUSIONS.**

1. An improved method for the preparation of hemoglobin is described. This depends on repeated crystallization of oxyhemoglobin from a cold concentrated solution after complete oxygenation and adjustment of the pH to a point slightly more acid than pH 6.8.

2. Buffered solutions of hemoglobin of given pH were made by the addition of water, phosphate or borate buffers, together with enough alkali to change the pH of reduced hemoglobin to the same value. These solutions were equilibrated with known gas mixtures and analyzed by the methods of Van Slyke.

3. Oxygen dissociation curves were constructed from the data so obtained and show the familiar S-shape and the decrease of percentage saturation with increasing acidity up to pH 6.55. At higher hydrogen ion activities the percentage saturation again rises. The affinity of hemoglobin solutions for oxygen passes through a minimum between pH 6 and 7, apparently at 6.55.

4. It is impossible to describe these data in terms of the simple mass law equation. More variables than have been commonly used must be employed.

5. The equilibrium between oxygen and hemoglobin solutions may be described by means of a simple empirical equation of the form

$$\frac{[\text{Hb}] [pO_2]}{[\text{HbO}_2]} = 0.5 \frac{[H^+]}{0.013 (7.4 \times 10^{-3} + [H^+])} (\log \% \text{HbO}_2 - 2.015)$$
6. These experimental findings may also be expressed in terms of equations in which it is assumed that hemoglobin behaves as though it were a mixture of substances, each obeying the general form of the mass law equation where \( n \) is an integer.

7. These data are also described in terms of the theory of intermediate compound formation by the equation

\[
\text{Per cent saturation} = \frac{0.25 K' \left[ O_2 \right] + 0.5 \times 0.32 (K' \left[ O_2 \right])^2 + 0.75 \times 0.256 (K' \left[ O_2 \right])^3 + 2.7 (K' \left[ O_2 \right])^4}{1 + K' \left[ O_2 \right] + 0.32 (K' \left[ O_2 \right])^2 + 0.256 (K' \left[ O_2 \right])^3 + 2.7 (K' \left[ O_2 \right])^4}
\]

8. If the theory of intermediate compound formation be assumed, the change in position of oxygen dissociation curves with changing pH, over the range 6.55 to 8.88, may be explained on the assumption that the salt and acid forms of hemoglobin have different affinities for oxygen. A theoretical expression of the form

\[
K' = \frac{K'_{R} K'_{S} (\text{[H}^+\text{]}^2 + K'_{R})}{\text{[H}^+\text{]} + K'_{R}}
\]

will explain the phenomenon.

9. Owing to the large number of assumptions involved, any theory that may at present be advanced must await further investigation for its verification.

BIBLIOGRAPHY.

16. Redfield, A. C., personal communication.
CORRECTION.

On page 228, Vol. lxxx, No. 1, November, 1928, line 23, read 103° for 130°.