

*The Osmotic Pressure of Hæmoglobin in the Absence of Salts.*

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*Introduction.*

In the general theory of hæmoglobin solutions the osmotic pressure of the pure protein does not occupy an important place, because it is simply one of many possible combinations of hæmoglobin, water, hydrogen ions, and salts. There is, however, the definite advantage in studying pure hæmoglobin solutions that, if the protein be in equilibrium with distilled water, there is no need to consider secondary complications in the distribution of salts, due to the presence of the membrane.

If a very acid protein were used, it would be necessary to consider the pressure due to hydrogen ions, but the iso-electric point of hæmoglobin is so near neutrality that the hydrogen-ion pressure is immeasurably small.

The osmotic pressure of purified hæmoglobin solutions aroused the interest of early investigators, because it offered a means of determining the molecular weight. The equivalent weight calculated from iron analyses was about 16,700, and the molecular weight must be  $N$  times as great, where  $N$  is the number of iron atoms in the molecule. According to van't Hoff's formula, the osmotic pressure should be proportional to the concentration, and if results are expressed in the form of millimetres of mercury (reduced to 0° C.) per 1 per cent. of protein, the calculated pressure is  $10\cdot21/N$ .

It is usually stated that Hüfner proved that the molecular weight of hæmoglobin was the same as the equivalent, 16,700. His results are stated below, together with the data obtained by other workers on the osmotic pressure of pure hæmoglobin.

Hüfner and Gänsser, 9·3–12·1 mm.; Reid, 3·5–4·4 mm.; Roaf, 5·3 mm.; Wilson, 7–11 mm. The accepted explanation of these variable results, mainly due to Prof. A. V. Hill, is that hæmoglobin forms in pure water a uni-molecular solution, and that the lower pressures obtained by Reid and others were due to an aggregation of the molecules caused by traces of salts.

There is, however, an alternative view. Protein preparations almost always contain traces of combined acids or bases, and these diffuse away very slowly when dialysed against distilled water. It is here suggested that these impurities caused irregular osmotic effects, formerly attributed to changes in aggregation.

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These two theories differ most sharply in relation to the observations of Hüfner and Gänsser. The aggregation theory rests on the assumption that theirs was the purest preparation, whilst according to the "ionic impurity" theory they obtained the largest pressure, because their solution contained the greatest concentration of impurities. It is true that Hüfner used crystallized hæmoglobin to make up his solution, but Sørensen has shown since that the crystallization of a protein does not give the pure substance, but its salt. Hüfner's material, therefore, was almost certainly impure.

It has been shown that even in the absence of proteins salts may cause large temporary pressures, lasting for 11 days, with the relatively impermeable parchment membranes (*ref.* Donnan and Harris), and for two or three days with more permeable membranes of the type used by Reid (*ref.* Reid, 1904). Hüfner and Gänsser used parchment membranes, and measured the maximum osmometer reading which occurred during the first day or two. According to my experience, the maximum pressure varied from 5 to 20 mm., according to the type of membrane used, and it bore no relation to the final osmotic pressure.

The work of Hüfner and Gänsser received popular recognition, but, in my opinion, it is not to be compared with the work of the earliest investigator, Reid,\* who used a stirred osmometer with membranes more permeable by salts. Even with the improved membranes and methods described before (Adair<sup>1</sup>), it was difficult to obtain exact equilibria with distilled water, and with the parchment system of Hüfner and Gänsser it is practically certain that the osmometer readings were not osmotic equilibria.

*Osmotic Pressure and Conductivity.*

The measurement of the electrical conductivity offers a method of deciding between the alternative theories, because it will show if Reid's results or Hüfner's correspond to the purest solution. The conductivity method is of great value in this problem, because the impurities are not exactly known; their concentrations may be too small for chemical analysis, and in any case ash analyses could not be used because of the risk of losing volatile impurities, such as ammonia and carbon dioxide.

The electrical conductivity of the salts of hæmoglobin is high compared with that of the pure protein, as shown in Table I. As dialysis was continued, the conductivity measurements were enormously decreased and the osmotic

\* The work of Reid was discredited by Prof. Roaf on the ground that alcohol was used, but my results, obtained without the use of alcohol, agree approximately with those of Reid.

pressure tended to reach a constant steady value. The flat region of the curve is shown in fig. 1.

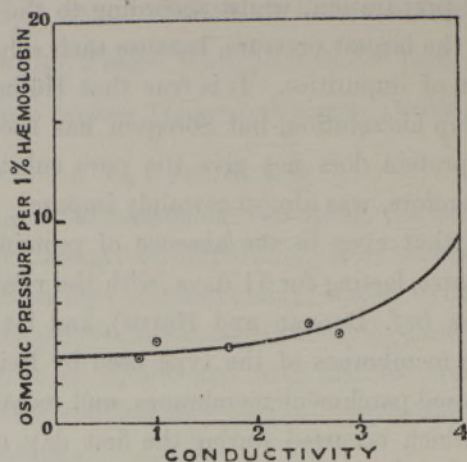


FIG. 1.

The conductivity measurements show that the degree of purity attained in these experiments was greater than that recorded by previous workers, and the character of the curve suggests that further purification would have no further effect on the osmotic pressure. The purest preparation obtained gave a pressure of 3.2 mm., which is in accordance with Reid's figures. Since the high pressures corresponding to Hüfner's value, 10.2, are correlated with high conductivities, it is probable that his results were due to undialysed acid or base.

Table I.

Osmotic pressure of purified hæmoglobin (from sheep's blood).

No. 23, sodium salt of hæmoglobin.

No. 11, 14, 13, 10, 9, dialysed hæmoglobin (8.6 grams per 100 c.c. water).

$\pi$  = osmotic pressure per 1 per cent. protein, reduced to 0° C. (Experimental temp. 23° C.)

$pH''$  = negative log of hydrion concentration in outer liquid.

Conductivity.	$pH''$	$\pi$	No.
0.00092.....	11.0	21.6	23
0.00025.....	6.8	4.8	11
0.00028.....	7.0	4.4	14
0.00010.....	6.9	4.1	13
0.00017.....	6.8	3.8	10
0.00008.....	6.6	3.3	9

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At the iso-electric point of a protein, the amount of electrolyte held is a minimum, therefore the measurement of the hydrogen-ion concentration of the dialysate (initially pure water) is a useful method of estimating the degree of purification.

The results are given in Table II. It will be observed that the minimum osmotic pressure is found at the iso-electric point, as in Loeb's work, and moreover, the pressure at this point is very much smaller than 10·2. Hüfner's pressure corresponds to pH 5·5, and it is quite certain that acid would be bound at such hydrogen-ion concentrations. Ionizing salts of hæmoglobin would be formed which would give a much larger osmotic effect than the "partial protein pressure."

Table II.

Osmotic pressures of hæmoglobin at different hydrogen-ion concentrations.

pH.	$\pi$	Grams Hb per 100 c.c. solution.	pH.	$\pi$	Grams Hb. per 100 c.c. solution.
5·0	21·5	0·74	6·8	4·5	4·00
5·4	13·4	1·20	6·8†	6·8	0·97
6·5	3·2	1·20	7·2	5·0	1·20
6·7*	2·4	3·70	9·6	15·6	3·67
6·8	2·4	6·20	10·2	21·4	1·20
6·8	3·5	6·20			

\* Iso-electric point—minimum osmotic pressure.

† Denaturation observed.

*Relation between Osmotic Pressure and Hæmoglobin Concentration.*

This relation offers a method of testing the aggregation theory, because polymerization ought to increase with increasing concentration. Measurements were made of the osmotic pressures of different concentrations of hæmoglobin, with distilled water for outer liquid, having a hydrogen-ion concentration about  $10^{-6.7}$ .

According to the aggregation theory, the pressure-concentration ratio should diminish in strong solutions, but the observed points (fig. 2) show an increase. The data contradict the aggregation theory.

The smooth curve drawn in the figure is not without interest. It is calculated from the formula

$$p = 2.55 \text{ cHb} \left( \frac{1}{1 - 0.0168 \text{ cHb}} \right) \quad (1)$$

$p$  = osmotic pressure.

$\text{cHb}$  = grams hæmoglobin per 100 c.c. of solvent.

Most of the observed points are higher than the theoretical curve, because it was impossible to remove all the bound electrolytes in every experiment.

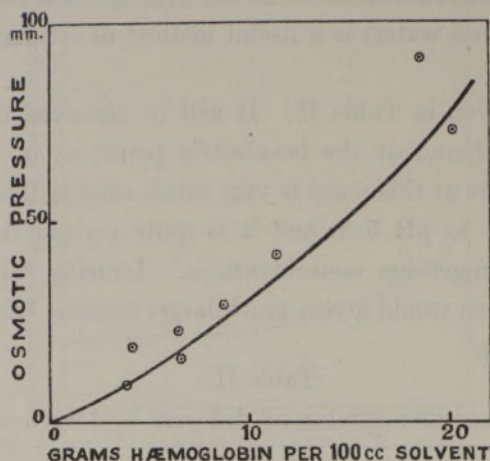


FIG. 2.

The empirical constants 1.68 and 2.55 were worked out for the pressure-concentration curve when the solvent was a mixture of chlorides and phosphates isotonic with blood. A similar curve was obtained with N/10 sodium as solvent. (Adair.<sup>2</sup>) This result is of theoretical importance, for it shows that the osmotic pressure curve is the same in the distilled water experiments as it was in the salt solutions, not only in the general form of the curve but even in the numerical values of the constants. This agreement is an additional argument against the theory that aggregation is caused by salts.

The significance of the formula will be described later in relation to the data which were used to establish it, but there are a few points which may be stated now, in order to explain the meaning of the empirical constants. In dilute solutions, the term in brackets can be neglected, and the pressure per 1 per cent. of hæmoglobin ( $p/cHb$ ) = 2.55. This figure is about one-fourth of the pressure obtained by Hüfner, so it is probable that the molecular weight is four times the equivalent.

The constant 0.0168 can be explained by re-writing the formula

$$p (v - 1.68) = 255, \quad (2)$$

$v = 100/cHb$  = cubic centimetres of solvent per gram of hæmoglobin.

The term 1.68 corresponds to the "b" in van der Waal's equation, written in a simplified form below

$$p (V - b) = R.T. \quad (3)$$

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Sørensen and others have suggested that the pressure of a protein should be proportional to the concentration, if the latter is expressed in grams per 100 c.c. of solvent (not solution), but this theory is not borne out by the experimental data for hæmoglobin.

*Discussion of the Evidence for the Molecular Weight of Hæmoglobin.*

The osmometer work described here is definite evidence against the theory that pure hæmoglobin has the molecular weight 16,700. Whether the conductivity or the hydrogen-ion concentration is used as a criterion of purity, the molecular weight is three or four times the equivalent. It is almost impossible to obtain exact results in the absence of salts, therefore the absolute value will be discussed in a later communication.

The view that the molecular weight was larger than the equivalent was advanced by Waymouth Reid, the first worker on the subject. The conclusions of Hüfner and Gänsser have already been discussed. When ionizing salts of hæmoglobin are present, the observed pressures are higher than the partial pressure due to hæmoglobin. This effect probably explains the results observed recently by Wilson (1925). Pressures are recorded which are in rough agreement with Hüfner's results, but the statement is made that: "The osmometer flask was kept loosely stoppered so that sufficient carbon dioxide entered to make the outer fluid somewhat more acid than  $pH\ 7.0$ ."

In a paper entitled "The Nature of Oxyhæmoglobin, with a Note on its Molecular Weight," Barcroft and Hill record experiments on the heat of reaction of oxygen and hæmoglobin and the oxygen bound at different pressures. The experiments were interpreted with the aid of the equation quoted below, on the assumption that  $n$  represents the average number of iron atoms in the molecule. The authors have since pointed out that  $n$  may have other meanings (*ref.* Brown and Hill),

$$y = Kx^n / 1 + Kx^n. \quad (4)$$

$y$  = oxygen bound at pressure  $x$ , divided by oxygen bound at full saturation (oxygen capacity),  $x$  = oxygen pressure,  $K$  = constant.

Barcroft and Hill obtained the following results with their dialysed preparation:—

- (1) The oxygen dissociation curve was fitted by formula (4) when the value of  $n$  was taken to be about 1.0. The pressure of oxygen at half saturation was 8 mm.

- (2) The heat of reaction calculated by applying van't Hoff's formula was 28,000 calories. The observed heat of reaction was about 30,000 calories.

From these it was concluded that  $n$  was unity, and that therefore the molecular weight of hæmoglobin was 16,700.

This conclusion is opposed to the results of measurements of osmotic pressure, given earlier, and the observed heat of reaction has not been obtained by any later worker. The explanation of this discrepancy has been given elsewhere (*ref.* Adair<sup>3</sup>).

The exponent  $n$  was found to be equal to unity, owing to the balancing of two opposing factors—the change in acidity on oxidation which tends to give an  $n$  smaller than unity, and the fundamental form of the dissociation curve which tends to give an  $n$  larger than unity. Barcroft and Hill worked at a hydroxyl-ion concentration where the uncorrected value of  $n$  was in the region of unity.

The oxygen dissociation curves of dilute solutions of pure hæmoglobin have not been studied, but Hartridge and Roughton have developed the theory that  $n$  is unity in dilute solutions, containing salts. Unfortunately, the range of oxygen pressures which could be studied was hardly sufficient to give a decisive test. These authors, and also Adair (1925), have suggested that  $n$  need not be the same as the number of iron atoms in the molecule.

It is impossible to frame a complete theory\* of the reaction between oxygen and hæmoglobin, but it may be useful to prove the proposition that  $n$  may be smaller than the number of iron atoms in the molecule. The proof given below was advanced in a Fellowship Dissertation (1921), but was not published as it involved many assumptions which could not be tested. Prof. Milne has pointed out to me that similar assumptions have been made in applying the theory of detailed balancing to the ionization of atoms at high temperatures, discussed by Einstein, Klein and Rosseland and others (*ref.* Milne, E. A., 'Phil. Mag.', vol. 47, p. 209).

Let Hgb be a molecule, which can combine with four oxygen molecules.

Let

$u_0$  = concentration of Hgb.

$u_1$  =       "       "       HgbO<sub>2</sub>.

$u_2$  =       "       "       Hgb(O<sub>2</sub>)<sub>2</sub>

$u_3$  =       "       "       Hgb(O<sub>2</sub>)<sub>3</sub>

$u_4$  =       "       "       Hgb(O<sub>2</sub>)<sub>4</sub>

$x$  =       "       "       oxygen.

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Let  $\kappa, \kappa_2, \kappa_3, \kappa_4$  and  $\lambda_1, \lambda_2, \lambda_3, \lambda_4$  be reaction velocity co-efficients defined by equations 5, 6, 7 and 8.

$$\delta u_1/\delta t = 4 \cdot u_0 \kappa_1 x - 1 \cdot u_1 \lambda_1 + 2 \cdot u_2 \lambda_2 - 3 \cdot u_1 \kappa_2 x, \quad (5)$$

$$\delta u_2/\delta t = 3 \cdot u_1 \kappa_2 x - 2 \cdot u_2 \lambda_2 + 3 \cdot u_3 \lambda_3 - 2 \cdot u_2 \kappa_3 x, \quad (6)$$

$$\delta u_3/\delta t = 2 \cdot u_2 \kappa_3 x - 3 \cdot u_3 \lambda_3 + 4 \cdot u_4 \lambda_4 - 1 \cdot u_3 \kappa_4 x, \quad (7)$$

$$\delta u_4/\delta t = 1 \cdot u_3 \kappa_4 x - 4 \cdot u_4 \lambda_4. \quad (8)$$

At equilibrium, we have—

$$\delta u_1/\delta t = \delta u_2/\delta t = \delta u_3/\delta t = \delta u_4/\delta t = 0, \quad (9)$$

Let the affinity coefficients  $k_1, k_2, k_3, k_4$  be defined by the formulæ 10, 11, 12, 13,

$$u_4/u_0 = x^4 \kappa_1 \kappa_2 \kappa_3 \kappa_4 / \lambda_1 \lambda_2 \lambda_3 \lambda_4 = \frac{1}{256} k_4^4 x^4, \quad (10)$$

$$u_3/u_0 = 4x_3 \kappa_1 \kappa_2 \kappa_3 / \lambda_1 \lambda_2 \lambda_3 = \frac{4}{64} k_3^3 x^3, \quad (11)$$

$$u_2/u_0 = 6x^2 \kappa_1 \kappa_2 / \lambda_1 \lambda_2 = \frac{6}{16} k_2^2 x^2, \quad (12)$$

$$u_1/u_0 = 4x \kappa_1 / \lambda_1 = k_1 x, \quad (13)$$

Then oxygen combined at pressure  $x$  is proportional to the sum of the term

$$\frac{1}{4}u_1 + \frac{2}{4}u_2 + \frac{3}{4}u_3 + \frac{4}{4}u_4.$$

The total hæmoglobin present would be equal to  $u_0 + u_1 + u_2 + u_3 + u_4$ .

Let  $y$  = oxygen combined divided by total hæmoglobin. Then  $y$  is given by formula 14

$$y = \frac{\frac{1}{4} k_1 x + \frac{1}{2} \frac{6}{16} k_2^2 x^2 + \frac{3}{4} \frac{4}{64} k_3^3 x^3 + \frac{4}{4} \frac{1}{256} k_4^4 x^4}{1 + k_1 x + \frac{6}{16} k_2^2 x^2 + \frac{4}{64} k_3^3 x^3 + \frac{1}{256} k_4^4 x^4}. \quad (14)$$

If the  $k$  terms are independent of the concentrations of the components, and if all the terms except  $k_4$  are small, the formula can be simplified:  $y = Kx^4/1 + Kx^4$ . Unless these conditions are fulfilled, the  $n$  calculated by Hill's method may be much smaller than the number of iron atoms in the molecule.

If the  $k$  terms are equal, the formula becomes  $y = \frac{1}{4}kx/1 + \frac{1}{4}k_1x$ .

This assumption corresponds with the theory of Hartridge and Roughton. Adair,<sup>3</sup> in collaboration with Bock and Field, suggested that  $k_4$  might be larger than the other terms.

*Summary.*

The osmotic pressure determinations for dialysed hæmoglobin made by previous workers ranged from 3.5 mm. to 12.1 mm. per 1 per cent. of protein.

Variation is to be expected, for it is theoretically impossible to prepare a hæmoglobin solution absolutely free from combined acids or bases, and there is the further risk that prolonged purification may cause changes in the protein.

300 *Osmotic Pressure of Hæmoglobin in the Absence of Salts.*

The theory formerly accepted was that the high pressures (about 10 mm.) represented pure hæmoglobin of molecular weight 16,700, the same as the equivalent determined by iron analyses. The lower pressures were attributed to aggregations of the molecules caused by salts.

Repetition of the work with iso-electric hæmoglobin gave pressures of 3.2 mm., or less, and pressures of 10 mm. or more were obtained only when conductivity and hydrogen-ion determinations showed that acid or base was bound.

The determinations of Hüfner (10 mm.) were made at an unknown hydrogen-ion concentration, and it is here suggested that most of the pressure in his experiments was due to undialysed acid or base. The molecular weight of pure hæmoglobin is about four times the equivalent.

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