The accuracy of bond-length estimations

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The density increments of proteins

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The density increment, symbolized \( k_d \), of a protein is defined as the difference between the density of the protein solution and that of its dialysate, divided by the protein concentration in g./ml.

The density increment of the protein has been shown to be a function of the protein concentration and of the hydrogen ion and the salts present in the dialysate.

Under the conditions investigated, the influence of protein concentration is so small that density increments of haemoglobin are constant within 0·2% over a range of protein concentration up to 48%.

A fall in pH value from 7·8 to 6·35 increases the density increment from 0·248 to 0·253.

Detailed calculations (table 9) show that the increase is mainly due to the combination of haemoglobin with anions.

Salts, if present in high concentration, may cause a considerable reduction in the density increment of the protein. Theoretical formulae, (11) and (17), have been given which relate this reduction to the specific volume of the protein and the net excess of water bound by the protein.

The apparent density of protein is determined when the density of a protein solution is equal to that of its dialysate. The apparent density of human-serum albumin dialysed against an equimolecular mixture of \( \text{KH}_2\text{PO}_4 \) and \( \text{K}_2\text{HPO}_4 \) was found to be 1·219 at 1·0° C.

1. INTRODUCTION

The difference between the density of a protein solution and the density of an ultrafiltrate or dialysate with which the protein is in diffusion equilibrium is of importance for the derivation of exact formulae for the distribution of a protein in a gravitational or centrifugal field (Adair 1935; Roche, Roche, Adair & Adair 1935). It is here suggested that this difference in densities divided by the protein concentration expressed in g./ml. should be termed the ‘density increment’, symbolized \( k_d \).

Measurements of the density increments have important practical applications. If the density increment be a constant or a known function of the protein concentration, it is possible to compute the protein concentration from measurements of density. If the protein concentration be known, it is possible to compute the density.
A number of measurements of the specific volumes of proteins have been given by Svedberg & Pedersen (1940). In general, it has been found that the specific volume of a protein is a constant, and it may be inferred that the density increases in direct proportion to the protein concentration. The experimental evidence, usually obtained in conjunction with ultracentrifugal studies of dilute protein solutions, has not been published in detail. The linear relationship between protein concentration and density has been accepted as a basis for the rapid methods for determinations of serum protein concentrations from density measurements (Phillips Van Slyke, Dole, Emerson, Hamilton & Archibald 1945; Lloyd, Cheeck, Sinclair & Webster 1945).

The present communication records a series of measurements of densities made over a range of protein concentrations from 7 to 45 g./100 ml. The determinations were made in order to study densities under the conditions used in osmotic-pressure measurements of haemoglobin and of serum proteins, in which series of protein solutions were dialyzed against repeated changes of standard buffer solutions. With a dialysate of constant composition, the concentration of inorganic ions in the protein solution is a function of the protein concentration, and it is therefore desirable to study the effects of the distribution of ions on the density of the protein.

2. Experimental methods

Dilute solutions of CO haemoglobin from ox blood were prepared and analyzed as described by Adair & Adair (1934b). Concentrated solutions were obtained by ultrafiltration followed by dialysis for at least 7 days at 0° C. Highly concentrated solutions were prepared in the ultrafilter shown in figure 1 which is arranged so that dilute protein solution is forced into the membrane from below and the concentrated protein is thus continuously stirred.

The water content and total solids of dialyzed solutions were determined by freezing samples containing approximately 150 mg. protein in weighing bottles and drying the frozen samples over P₂O₅ in a desiccator evacuated by an oil pump. The solutions were analyzed for phosphates, chlorides and total base and the weight of protein was calculated by subtracting the weight of anhydrous inorganic salts from the total solids.

Standard types of pycnometers are unsuitable for concentrated protein solutions which are exceedingly viscous, tend to form films of great stability surrounding air bubbles, and on evaporation set like adhesives and block the orifices of the pycnometer. A modified type of pycnometer is shown in figure 2. A tube graduated in cu.mm. is sealed to a bulb of 3 ml. capacity fitted with a tap made with precision to avoid trapping of air bubbles. The tap is lubricated with vaseline, excess lubricant is removed with chloroform, and the pycnometer is then cleaned with nitric acid and alcohol, washed, dried and calibrated.

Protein solutions were put into short test-tubes, the pointed tip of the pycnometer inserted and the mouth of the test-tube covered with tinfoil to minimize
evaporation. The graduated tube of the pycnometer was connected with an aspirator and the suction pressure adjusted to give a slow rate of flow. The time taken to fill the pycnometer may be as much as 1 hr. if the protein concentration exceeds 40%. After weighing, the pycnometer was put in an air thermostat and left in a horizontal position for about 1½ hr. before the volume was read on the graduated scale. The pointed tip was protected with a rubber cap during temperature equilibration.

![Figure 1. Ultrafilter for preparation of concentrated protein solutions.](image1)

![Figure 2. Pycnometer.](image2)

3. **Densities of ox CO haemoglobin in distilled water**

Gasometric analyses of a series of haemoglobin solutions subjected to prolonged dialysis against distilled water showed CO₂ contents ranging from 0.7 to 1.2 g.mol./mol. (67,000 g.) of haemoglobin. Solutions of haemoglobin in water were therefore prepared by dilution of a stock solution which had been dialyzed against glass-distilled water and concentrated by ultrafiltration at 1.0°C.
Table 1 records the densities of five aqueous haemoglobin solutions of concentrations from 7.7 to 42 %, at refrigerator and at room temperature. The last column of table 1 shows the result of a more sensitive test of a linear relationship between density and concentration, obtained by applying formula (1):

\[ k_d^0 = (\rho - \rho_w)/(c_p - \rho_w)/0.01C, \]  

where \( \rho' \) = density of protein solution, \( \rho_w \) = density of water, \( c_p \) = protein concentration expressed in g. anhydrous protein per ml., \( C \) = % concentration in g./100 ml., and \( k_d^0 \) = the density increment.

**Table 1. Densities and density increments of solutions of haemoglobin in distilled water (density \( \rho_w \))**

<table>
<thead>
<tr>
<th>temperature ( t ) (°C)</th>
<th>concentration ( C )</th>
<th>density ( \rho )</th>
<th>density increment ( k_d^0 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7</td>
<td>42.108</td>
<td>1.1068</td>
<td>0.254</td>
</tr>
<tr>
<td>1.7</td>
<td>33.682</td>
<td>1.0856</td>
<td>0.254</td>
</tr>
<tr>
<td>1.8</td>
<td>24.998</td>
<td>1.0635</td>
<td>0.254</td>
</tr>
<tr>
<td>1.8</td>
<td>15.479</td>
<td>1.0394</td>
<td>0.255</td>
</tr>
<tr>
<td>1.9</td>
<td>7.712</td>
<td>1.0195</td>
<td>0.253</td>
</tr>
<tr>
<td>16.0</td>
<td>41.982</td>
<td>1.1035</td>
<td>0.249</td>
</tr>
<tr>
<td>14.8</td>
<td>33.602</td>
<td>1.0831</td>
<td>0.250</td>
</tr>
<tr>
<td>15.0</td>
<td>24.939</td>
<td>1.0614</td>
<td>0.250</td>
</tr>
<tr>
<td>17.6</td>
<td>15.448</td>
<td>1.0373</td>
<td>0.250</td>
</tr>
<tr>
<td>19.1</td>
<td>7.697</td>
<td>1.0176</td>
<td>0.249</td>
</tr>
</tbody>
</table>

Since all values for the density increment at 1.8 °C are equal within the limits of experimental error, it may be concluded that there is a linear relationship between density and protein concentration over the range from 8 to 42 %, More dilute solutions were investigated by Svedberg & Fåhreus (1926), who found that the specific volume was constant.

The apparent specific volume, symbolized \( v_p \), is defined by formula (2):

\[ v_p = (v - v_w x_w)/x_p, \]  

where \( v \) = specific volume of solution, \( v_w \) = specific volume of pure water, \( x_w \) = weight fraction of water, and \( x_p \) = weight fraction of protein.

According to this definition, the mass of water displaced by 1 g. protein is equal to \( v_p \rho_w \), and the density of a solution which contains \( c_p \) g./ml. is equal to \( \rho_w + c_p - c_p v_p \rho_w \). Comparison with formula (1) shows that the apparent specific volume is correlated with the density increment by formula (3):

\[ k_d^0 = (1 - v_p \rho_w). \]  

Since \( \rho_w \) is constant, the statement that \( v_p \) is constant implies that \( k_d^0 \) is also constant.
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The experiments recorded in table 1 do not exclude the possibility of small changes in the specific volumes, as such changes might be masked by the experimental errors which may be as much as 0.002. A more sensitive method, the dilution of haemoglobin solutions in a dilatometer, was therefore tested. Preliminary measurements showed that when a 40% solution of haemoglobin was diluted with an equal volume of water, the apparent specific volume of the protein diminished by 0.00026 ± 0.00003 ml. Smaller contractions, difficult to measure accurately with the apparatus available, were obtained when the initial concentration was 20% or less.

If the changes in specific volume are known, changes in density increment can be calculated by formula (3). The contraction observed, 0.00026, would increase the density increment only by approximately 0.1%.

4. Ox CO haemoglobin dialyzed against sodium phosphate buffer pH 7.4

Figure 3 records measurements of density at 0°C of CO haemoglobin dialyzed against a buffer mixture containing 0.03 g.mol. Na₃HPO₄ and 0.01 g.mol. NaH₂PO₄, pH 7.4 and ionic strength 0.1. It will be seen that the observed points lie on a straight line calculated by the formula \( \rho = 1.051 + 0.24872C; \) 1.051 is the density of the buffer, 0.24872 is the mean density increment. Over the range of concentrations from 7 to 43.49%, the maximum difference between observed and calculated densities was 0.0004, and the mean difference was 0.0002.

\[ \text{Figure 3. Densities of solutions of ox CO haemoglobin dialyzed against 0.03 M-Na₃HPO₄ + 0.01 M-NaH₂PO₄.} \]

\[ \rho = \text{density at 0°C; } C = \text{g. protein/100 ml. solution.} \]
Table 3 gives the values for the density increments defined by formula (4) for different protein concentrations:

\[ k_d = (\rho - \rho_0)/c = (\rho - \rho_0)/0.01C, \quad (4) \]

where \( k_d \) = density increment for the protein in the presence of salts, \( \rho = \) density of protein solution, and \( \rho_0 = \) density of dialysate, or 'outer fluid'.

In this work, all values of \( \rho \) and \( c \) were measured at atmospheric pressure. In theoretical applications of formula (4) to systems in osmotic equilibrium, it is understood that corrections are applied to give densities and concentrations at the pressures necessary for osmotic equilibrium.

**Table 2. Density increments at 0°C of haemoglobin solutions equilibrated with 0.03m-Na₂HPO₄ + 0.01m-NaH₂PO₄**

<table>
<thead>
<tr>
<th>Concentration (g./100 ml.)</th>
<th>Density increment</th>
<th>Concentration (g./100 ml.)</th>
<th>Density increment</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.67</td>
<td>0.2478</td>
<td>35.47</td>
<td>0.2483</td>
</tr>
<tr>
<td>12.64</td>
<td>0.2495</td>
<td>37.21</td>
<td>0.2491</td>
</tr>
<tr>
<td>14.65</td>
<td>0.2485</td>
<td>39.76</td>
<td>0.2477</td>
</tr>
<tr>
<td>21.22</td>
<td>0.2507</td>
<td>40.45</td>
<td>0.2498</td>
</tr>
<tr>
<td>25.74</td>
<td>0.2482</td>
<td>41.22</td>
<td>0.2494</td>
</tr>
<tr>
<td>30.61</td>
<td>0.2484</td>
<td>43.49</td>
<td>0.2493</td>
</tr>
<tr>
<td>31.96</td>
<td>0.2495</td>
<td>45.67</td>
<td>0.2468</td>
</tr>
<tr>
<td>33.01</td>
<td>0.2482</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3. Specific volumes at 1.0°C of haemoglobin dialyzed against 0.03m-Na₂HPO₄ and 0.01m-NaH₂PO₄ at 0°C**

\[
\begin{align*}
C & : 0.0000 & 34.8000 & 46.7000 \\
k_d & : 0.2492 & 0.2487 & 0.2486 \\
v_p \text{ (apparent)} & : 0.7476 & 0.7481 & 0.7482 \\
v_p \text{ (partial)} & : 0.7476 & 0.7484 & 0.7486 \\
v_{sv} \text{ (partial)} & : 0.9947 & 0.9945 & 0.9944 \\
\end{align*}
\]

The mean value for the density increments recorded in table 2 is 0.2487. The maximum deviation from the mean was 0.00195, and the mean deviation was 0.00073 or 0.3% of the value of \( k_d \). The maximum range of variation is greater than the range shown in table 1 because each measurement in table 2 is influenced by the accidental errors in analysis of the protein solution. The mean value of 0.2487, based on the analyses of fifteen solutions, has greater weight than the mean value of 0.254 in table 1, which depends on the analysis of one solution.

Independent evidence concerning the degree of constancy of the density increments of haemoglobin in the presence of salts can be obtained by measuring the distribution of salts across the membrane and the changes in the specific volume of the protein which take place when concentrated protein solutions are diluted with
buffer in a dilatometer. A theoretical formula (15), showing the effects of both factors on the density increments, is given in § 7 (b).

If a protein solution be in dialysis equilibrium with a sodium phosphate solution, according to Donnan's theory of membrane equilibrium the concentration of disodium phosphate inside the membrane should be determined by a cubic equation, which must be inconsistent with a simple linear relationship between protein concentration and density. Theoretical calculations show that the unequal distribution of ions should increase the density increment as the protein concentration increases, but that the magnitude of the increase is very small, approximately 0.02% of the mean density increment for haemoglobin solutions at pH 7.4, over a range of concentrations up to 40%.

The hydration of proteins and the association of anions with haemoglobin and the serum proteins (Adair 1928; Adair & Adair 1934a, b, 1936) are factors which must affect the protein density and which are not included in Donnan's theory. The theoretical calculations were therefore supplemented by chemical analyses. The results recorded in figure 4 show that there are linear relationships between the concentrations of sodium ions and phosphates and the concentration of protein up to 40%. The measurements of salt distribution therefore agree with the hypothesis.

![Graph](image)

**Figure 4.** Molarities of sodium ion and total phosphate in solutions of ox CO haemoglobin, dialyzed against 0.03 M-Na$_2$HPO$_4$ + 0.01 M-NaH$_2$PO$_4$ at 0° C. C = g. protein/100 ml. solution.
that there is a straight line relationship between density and protein concentration and that the density increment is constant within the limits of experimental errors.

The influence of the specific volume of the protein on the density increment was investigated by mixing in a dilatometer at $0^\circ$C sodium phosphate buffer pH 7.4 with concentrated haemoglobin solutions which had been dialyzed against the same buffer. Preliminary experiments showed that there is a diminution of approximately 0.0005 ml. in the specific volume of haemoglobin diluted from 46.7 to 8.7%. Estimates of specific volumes and density increments at different protein concentrations, based on dilatometer measurements, are recorded in table 3, which also includes preliminary values for the partial specific volume of the protein $\bar{\nu}_p$ and the partial specific volume of solvent $\bar{\nu}_{av}$. The values recorded are not corrected for the small effects caused by the unequal distribution of salts.

5. RELATIONSHIP BETWEEN DENSITY INCREMENTS AND PROTEIN CONCENTRATION FOR HAEMOGLOBIN SOLUTIONS IN EQUILIBRIUM WITH MIXTURES OF SODIUM PHOSPHATES AND SODIUM CHLORIDE

Table 4 records measurements of density increments of ox CO haemoglobin equilibrated with sodium phosphate buffer of ionic strength 0.1 and pH values 6.38, 6.95 and 7.74. The results, together with those given in table 2, show that in this range of pH the density increment is independent of the protein concentration within 0.002 unit but is a function of the pH. The increase of the mean density increment caused by a diminution in the pH value is caused by the excess of phosphates inside the membrane. Analyses of haemoglobin, base and phosphate concentrations and calculations of the specific volume of haemoglobin at different pH values are given in table 9.

**Table 4. Influence of haemoglobin concentration and pH on density increments at $0^\circ$C. Sodium phosphate buffers of ionic strength 0.10**

<table>
<thead>
<tr>
<th>pH 6.38</th>
<th>pH 6.95</th>
<th>pH 7.74</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C$</td>
<td>$k_d$</td>
<td>$C$</td>
</tr>
<tr>
<td>27.1</td>
<td>0.255</td>
<td>26.7</td>
</tr>
<tr>
<td>34.1</td>
<td>0.253</td>
<td>37.3</td>
</tr>
<tr>
<td>43.3</td>
<td>0.253</td>
<td>43.7</td>
</tr>
</tbody>
</table>

The experiments recorded in table 5 show the influence of the two variables, salt concentration and protein concentration, on the density increment. Samples of ox CO haemoglobin were equilibrated with four buffer mixtures of 0.015 M-Na$_2$HPO$_4$ and 0.005 M-NaH$_2$PO$_4$, to which were added 0, 0.1, 0.5 and 1.0 M-NaCl. The fifth column of table 5 gives the total weight of salts in g./100 g. water, and shows that with the two more dilute buffer mixtures there is a net excess of salt inside the membrane, and the ratio salt/water increases with increasing protein concentration. If the salt concentration exceeds 3%, this ratio diminishes, and it follows that there
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is a net excess of water inside the membrane. The figures given in the seventh column indicate that the density increments are constant within 0.002 unit over the range of protein concentrations investigated, both when there is a net excess of salts and when there is a net excess of water inside the membrane.

**Table 5. Influence of salt concentration and protein concentration on density increments at 0° C**

\[
\begin{array}{cccccc}
\text{[NaCl]} & C & H_2O & \text{salts} & \text{salts/H}_2\text{O} & \text{density} & k_d \\
0.0^* & 0.00^* & 99.98^* & 0.27^* & 0.27^* & 1.0025^* & — \\
0.0 & 15.97 & 88.02 & 0.28 & 0.32 & 1.0427 & 0.253 \\
0.0 & 33.84 & 74.60 & 0.27 & 0.36 & 1.0871 & 0.251 \\
0.0 & 43.30 & 67.52 & 0.29 & 0.43 & 1.1110 & 0.250 \\
0.1^* & 0.00^* & 99.84^* & 0.86^* & 0.86^* & 1.0070^* & — \\
0.1 & 9.76 & 92.54 & (0.81) & — & 1.0311 & 0.247 \\
0.1 & 25.82 & 80.55 & 0.74 & 0.92 & 1.0711 & 0.248 \\
0.1 & 28.08 & 78.83 & (0.73) & — & 1.0764 & 0.247 \\
0.1 & 36.65 & 72.47 & 0.69 & 0.95 & 1.0981 & 0.248 \\
0.5^* & 0.00^* & 99.20^* & 3.20^* & 3.23^* & 1.0240^* & — \\
0.5 & 13.08 & 89.48 & (2.87) & — & 1.0543 & 0.232 \\
0.5 & 21.94 & 82.90 & 2.64 & 3.19 & 1.0742 & 0.229 \\
0.5 & 34.62 & 73.46 & 2.28 & 3.10 & 1.1036 & 0.230 \\
1.0^* & 0.00^* & 98.39^* & 6.12^* & 6.22^* & 1.0451^* & — \\
1.0 & 22.96 & 81.37 & 4.94 & 6.07 & 1.0926 & 0.207 \\
1.0 & 33.87 & 73.33 & 4.36 & 5.95 & 1.1157 & 0.208 \\
\end{array}
\]

Figures with asterisks refer to buffer solution. Figures in brackets obtained by interpolation. Figures for salts computed from the concentrations of the ions Na, Cl, HPO₄ and H₂PO₄.

6. Density increments of serum proteins dialyzed against m/15-phosphate buffer, pH 6.91

Solutions of proteins prepared from human serum were dialyzed at 0° C against a Sorensen m/15-phosphate buffer containing m/30-disodium phosphate + m/30-potassium dihydrogen phosphate, pH 6.81 at 18° C and 6.91 at 0° C.

The results obtained with three different fractions are summarized in table 6. The fraction euglobulin represents material precipitated by adding one volume of saturated ammonium sulphate to two volumes of serum. The fraction designated pseudo-globulin represents material precipitated by ammonium sulphate over the range of saturation from 33 to 50%. Both these fractions were reprecipitated twice. The fraction designated albumin represents protein which remains in solution when saturation with ammonium sulphate is raised to 60%. One experiment with crystalline albumin is recorded. Popják & McCarthy (1946) have made measurements on whole serum.

The second column of table 6 gives the concentrations C of total colloid (protein and lipoids) in g./100 ml. solution, obtained by deducting the weight of phosphate,
sodium and potassium from the total dry weight. The densities of the protein solutions were directly proportional to the protein concentration. The density increments were constant within the limits of experimental error, as shown by the figures in the third column of the table.

**Table 6. Density increments at 0° C of human serum proteins dialyzed against m/30-Na₂HPO₄ + m/30-KH₂PO₄, pH = 6·9 at 0° C**

<table>
<thead>
<tr>
<th>Protein</th>
<th>C</th>
<th>kₐ</th>
<th>vₙs</th>
<th>vₚ</th>
</tr>
</thead>
<tbody>
<tr>
<td>euglobulin</td>
<td>5·76</td>
<td>0·271</td>
<td>0·35</td>
<td>0·726</td>
</tr>
<tr>
<td>euglobulin</td>
<td>7·84</td>
<td>0·269</td>
<td>0·55</td>
<td>0·727</td>
</tr>
<tr>
<td>euglobulin</td>
<td>10·58</td>
<td>0·271</td>
<td>0·43</td>
<td>0·725</td>
</tr>
<tr>
<td>euglobulin</td>
<td>12·70</td>
<td>0·272</td>
<td>0·54</td>
<td>0·724</td>
</tr>
<tr>
<td>euglobulin</td>
<td>13·31</td>
<td>0·273</td>
<td>0·50</td>
<td>0·723</td>
</tr>
<tr>
<td>pseudo-globulin</td>
<td>3·95</td>
<td>0·256</td>
<td>(0·91)</td>
<td>0·737</td>
</tr>
<tr>
<td>pseudo-globulin</td>
<td>10·68</td>
<td>0·257</td>
<td>0·89</td>
<td>0·736</td>
</tr>
<tr>
<td>pseudo-globulin</td>
<td>12·90</td>
<td>0·259</td>
<td>0·93</td>
<td>0·734</td>
</tr>
<tr>
<td>albumin</td>
<td>7·32</td>
<td>0·256</td>
<td>(0·54)</td>
<td>0·740</td>
</tr>
<tr>
<td>albumin</td>
<td>10·19</td>
<td>0·258</td>
<td>0·45</td>
<td>0·738</td>
</tr>
<tr>
<td>albumin</td>
<td>15·01</td>
<td>0·258</td>
<td>0·57</td>
<td>0·738</td>
</tr>
<tr>
<td>albumin</td>
<td>20·70</td>
<td>0·257</td>
<td>0·60</td>
<td>0·739</td>
</tr>
<tr>
<td>albumin</td>
<td>23·89</td>
<td>(0·258)</td>
<td>0·53</td>
<td>0·738</td>
</tr>
<tr>
<td>crystalline albumin</td>
<td>6·40</td>
<td>0·266</td>
<td>(0·60)</td>
<td>0·729</td>
</tr>
</tbody>
</table>

Interpolated figures in brackets. vₙs and vₚ are defined by formula (17).

These observations suggest, first, that density increments are constant for mixtures as well as for pure proteins, and secondly, that the density increments are at least approximately constant even if the pH value is much more alkaline than the isoelectric point, which may be below 4·6 for serum albumin at 0° C.

7. Relationships between density increments, specific volumes and membrane equilibria of salts

(a) Ideal systems. The relationship between the density increment and the specific volume of a protein in equilibrium with a dialysate is given by the simple formula (5) in an ideal system where the molalities of the diffusable substances, or the weights per g. water, are the same on both sides of the membrane. Formula (6) applies if the concentrations of the diffusable substances be equal:

\[ k_d = (1 - v_p \rho_0), \]

\[ k_d = (1 - v_p \rho_o), \]

where \( v_p \) = apparent specific volume of protein.

(b) Density increment of isoelectric proteins. In non-ideal solutions, the molalities of the diffusable substances differ in a protein solution and in its dialysate, and, as a general rule, the mean specific volumes of the diffusable substances differ. In the
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region of the isoelectric points of the proteins investigated, the differences in specific volumes are small. Formulae (7) to (19) refer to the density increments of systems in which the mean specific volumes of the diffusible substances are the same on both sides of the dialyzing membrane.

The relationship between the concentrations of the diffusible substances and the density increments is stated in formula (7):

\[ k_d = (1 - v_p \rho_w) + (1/c_p) (c_{sp} - c_{so}) (1 - v_s \rho_w), \]  

where \( c_{sp} = \) g. diffusible substances/ml. protein solution, \( c_{so} = \) g. diffusible substances/ml. dialysate, and \( v_s = \) apparent specific volume of diffusible substances calculated from the density of the dialysate \( \rho_0 \) by formula (8):

\[ \rho_0 = \rho_w + c_{so}(1 - v_s \rho_w). \]

The relationship between the density increment and the distribution ratio \( r = c_{sp}/c_{so} \) is stated in formula (9):

\[ k_d = (1 - v_p \rho_w) - (1/c_p) (1 - r) (\rho_0 - \rho_w). \]

If water or diffusible substances be combined with or attracted by the protein, the density increments may be represented by formulae (10), (11) and (12):

\[ k_d = (1 - v_p \rho_0) + b_{tw}(1 - v_w \rho_0) + b_s(1 - v_s \rho_0), \]  

\[ k_d = (1 - v_p \rho_0) + b_w(1 - v_w \rho_0), \]  

\[ k_d = (1 - v_p \rho_0) + b_s(1 - v_s \rho_0), \]

where \( b_{tw} \) and \( b_s \) denote the total weights of water and of diffusible substances associated with 1 g. protein, and \( b_w \) and \( b_s \) denote the net excess of water or of diffusible substances found by chemical analyses, and defined by formulae (13) and (14). Formula (10) is less useful than formulae (11) and (12) because of the lack of accurate methods for measuring either \( b_{tw} \) or \( b_s \):

\[ b_w = (1/c_p) (c_{wp} - c_{wp0}[c_{sp}/c_{so}]), \]  

\[ b_s = (1/c_p) (c_{sp} - c_{so}[c_{wp}/c_{wp0}]), \]  

\[ b_s = -b_w(c_{so}/c_{wp0}) = +b_{ts} - b_{tw}(c_{so}/c_{wp0}). \]

If \( b_s \) and \( v_p \) can be represented by empirical formulae, \( b_s = b_s^* + k_b c_p \) and \( v_p = v_p^* + k_v c_p \), where \( b_s^*, k_b, v_p^* \) and \( k_v \) are constants, the density increment is then given by formula (15):

\[ k_d = k_d^* - k_v \rho_0 c_p + k_b(1 - v_s \rho_0) c_p, \]  

\[ k_d^* = (1 - v_p^* \rho_0) + b_s^*(1 - v_s \rho_0). \]

In practice, changes in \( b_s \) are usually less than the experimental errors in determinations of small differences of salt concentration.
G. S. Adair and M. E. Adair

Formula (17), a modification of formula (9), has useful practical applications, because the function \( v_{ns} \) is almost independent of the protein concentration:

\[
k_d = (1 - v_p \rho_w) - v_{ns}(\rho_0 - \rho_w),
\]

\[
v_{ns} = \frac{1}{c_p}(1 - r).
\]

\( v_{ns} \) can be determined in systems in which the conditions for analysis are favourable, namely, when the protein concentrations are high and \( 1 - r \) is therefore sufficiently large for precise measurements. Formula (17) can then be used to calculate \( 1 - r \) when the concentrations are too low for accurate analyses.

If there be a net excess of water inside the membrane, \( v_{ns} = (v_p + b_w v_w) \) and \( v_{ns} \) may be termed the ‘non-solvent volume’ (Weber & Nachmannsohn 1929).

If \( v_{ns} \) be known, the correction for the weight of salts in gravimetric analyses for protein content may be computed by the formula \( c_{sp} = c_{w0}(1 - v_{ns} c_p) \).

If there be a straight-line relationship between \( k_d \) and \( \rho_0 \), an independent measurement of \( v_{ns} \) can be made by determining \( k_d \) at two different values of \( \rho_0 \):

\[
v_{ns} = \frac{[(k_d)_1 - (k_d)_2]/(\rho_0)_2 - (\rho_0)_1}{(\rho_0)_2 - (\rho_0)_1}.
\]

The range of application of formula (19) was tested by measuring the density increments of human-serum albumin dialyzed against the concentrated phosphate buffers specified in table 7. The densities of the three concentrated buffers are symbolized \( (\rho_0)_2 \), and the density increments of albumin in equilibrium with these buffers are symbolized \( (k_d)_2 \). \( (k_d)_1 \) is the density increment 0·2576, determined for albumin dialyzed against a more dilute buffer, \( m/30-KH_2PO_4 + m/30-Na_2HPO_4 \), of density \( (\rho_0)_1 = 1·0081 \).

**Table 7. Density Increments, Non-Solvent Volumes and Net Hydration of Human Serum Albumin Dialyzed Against Potassium Phosphate Buffers at 1·0° C**

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Density ( (\rho_0)_2 )</th>
<th>Density ( (\rho_0)_1 )</th>
<th>Density Increment ( (k_d)_2 )</th>
<th>Density Increment ( (k_d)_1 )</th>
<th>Non-Solvent Volume ( v_{ns} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄ ( m )</td>
<td>0·5290</td>
<td>0·7970</td>
<td>0·2680</td>
<td>0·1401</td>
<td>1·800</td>
</tr>
<tr>
<td>K₂HPO₄ ( m )</td>
<td>0·5290</td>
<td>0·7970</td>
<td>0·2680</td>
<td>0·1401</td>
<td>1·800</td>
</tr>
<tr>
<td>Density</td>
<td>1·265</td>
<td>1·1870</td>
<td>1·2422</td>
<td>1·2200</td>
<td></td>
</tr>
<tr>
<td>( (\rho_0)_2 - (\rho_0)_1 )</td>
<td>0·1184</td>
<td>0·1789</td>
<td>0·2341</td>
<td>0·4700</td>
<td></td>
</tr>
<tr>
<td>( (k_d)_2 - (k_d)_1 )</td>
<td>+0·1175</td>
<td>+0·0386</td>
<td>0·0288</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Solvent Volume ( v_{ns} )</td>
<td>1·1800</td>
<td>1·2100</td>
<td>1·2200</td>
<td>0·4800</td>
<td></td>
</tr>
<tr>
<td>( b_w )</td>
<td>0·4400</td>
<td>0·4700</td>
<td>0·4800</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The values of \( v_{ns} \) calculated from density increments by formula (19) were constant within 0·04 ml. over a range of salt concentrations from 1·0 to 2·0 \( m \) and agreed with values calculated from the salt distribution by formula (18) and recorded in table 8.

The apparent density of dissolved protein is defined as the density at which \( k_d = 0 \), that is, when the density of the protein solution is equal to that of its dialysate. By interpolating between positive and negative values for \( k_d \) in table 8, the apparent density of albumin was found to be 1·219 at pH 6·6. The apparent density is a func-
tion of the pH and of the nature of the salts present. The net hydration for albumin is 0.47 ± 0.04 g/g. protein. In a former investigation (Adair & Adair 1936) the density of horse-serum albumin crystals was found to be 1.237, and the net hydration 0.344 at pH 5. At pH 3.2, the density was 1.278 and the hydration was 0.185.

**Table 8. Estimation of non-solvent volumes \( v_{na} \) from phosphate determinations. Ref. Table 7**

<table>
<thead>
<tr>
<th>Protein g./100 ml</th>
<th>( H_4PO_4 ) dialysate</th>
<th>( H_3PO_4 ) protein solution</th>
<th>( r )</th>
<th>( v_{na} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>15.2</td>
<td>1.058</td>
<td>0.865</td>
<td>0.818</td>
<td>1.19</td>
</tr>
<tr>
<td>8.0</td>
<td>2.105</td>
<td>1.908</td>
<td>0.906</td>
<td>1.17</td>
</tr>
</tbody>
</table>

(c) Density increments of proteins combined with acids and bases. Experimental determinations of density increments of solutions of ionized proteins are simpler than theoretical calculations. Theoretical formulae are valuable for the estimation of the specific volumes of proteins, especially where it is necessary to prepare the protein solution by dialysis against buffers. This procedure must be used when the protein is unstable in the absence of electrolytes.

The combination of a protein with an acid or base may cause an expansion in volume (Weber & Nachmansohn 1929; Linderström-Lang & Jacobsen 1941) and an unequal distribution of ions across the membrane. It follows that the mean specific volumes of the diffusible substances may be unequal in the protein solution and in the dialysate, and a correction term \( q \), defined by formula (20), must be added to the simpler formula (7) given in § 7 (b):

\[
q = (v_{sp} - v_{sp}) (c_{sp} \rho_{w} / c_{p}),
\]

\[
k_{d} = (1 - v_{p} \rho_{w} ) + (1/c_{p}) (c_{sp} - c_{sp}) (1 - v_{w} \rho_{w}) + q,
\]

where \( v_{p} \) = apparent specific volume of the protein, \( v_{sp} \) = mean apparent specific volume of diffusible substances in the dialysate (formula (8)), and \( v_{sp} \) = apparent specific volume of diffusible substances present in the protein solution.

For a direct measurement of \( v_{sp} \) it would be necessary to determine the weight of water, diffusible salts, acids and bases in the protein solution after dialysis equilibrium had been reached, to prepare an aqueous solution containing identical amounts of water and diffusible substances, and to measure the density of this solution. The direct method may be difficult.

If \( v_{sp} \) be known, the apparent specific volume of the protein may be calculated by formula (22):

\[
V = w_{w} v_{w} + w_{p} v_{p} + w_{sp} v_{sp},
\]

where \( V \) = volume of protein solution containing \( w_{p} \) g. protein, \( w_{w} \) g. water and \( w_{sp} \) g. diffusible substances.

An alternative method has been used to calculate the specific volume of haemoglobin after equilibration at 1.0°C with sodium phosphate buffers of ionic strength...
0·1, described in table 9. The terms \([\text{Na}]_b\), \([\text{H}_3\text{PO}_4]_{pl}\) and \([\text{HPO}_4]_{pl}\) represent the molarities of the diffusible ions in the protein solutions. The determined values for these ions, given in table 9, were used to calculate the theoretical molarities \([\text{Na}_2\text{HPO}_4]_l\), \([\text{NaH}_2\text{PO}_4]_l\) and \([\text{H}_3\text{PO}_4]_l\) in a solution free from protein.

\[
[\text{H}_3\text{PO}_4]_l = [\text{HPO}_4]_{pl} + [\text{H}_2\text{PO}_4]_{pl} - [\text{Na}]_b \quad \text{for acid solutions},
\]

\[
[\text{NaH}_2\text{PO}_4]_l = [\text{Na}]_b \quad \text{for acid solutions}.
\]

\[
[\text{Na}_2\text{HPO}_4]_l = [\text{Na}_b] - [\text{H}_2\text{PO}_4]_{pl} - [\text{HPO}_4]_{pl} \quad \text{for alkaline solutions},
\]

\[
[\text{NaH}_2\text{PO}_4]_l = [\text{H}_2\text{PO}_4]_{pl} + [\text{HPO}_4]_{pl} - [\text{Na}_2\text{HPO}_4]_l \quad \text{for alkaline solutions}.
\]

**Table 9. Specific volume at 1° C of ox CO haemoglobin dialyzed against sodium phosphate buffers of ionic strength 0·10 at 1° C**

<table>
<thead>
<tr>
<th>pH of dialysate</th>
<th>6·35</th>
<th>6·93</th>
<th>7·39</th>
<th>7·82</th>
</tr>
</thead>
<tbody>
<tr>
<td>density of dialysate</td>
<td>1·0072</td>
<td>1·0061</td>
<td>1·0052</td>
<td>1·0051</td>
</tr>
<tr>
<td>[Na]_b of dialysate</td>
<td>0·0854</td>
<td>0·0746</td>
<td>0·0701</td>
<td>0·0675</td>
</tr>
<tr>
<td>[HPO_4]_b of dialysate</td>
<td>0·0129</td>
<td>0·0238</td>
<td>0·0294</td>
<td>0·0308</td>
</tr>
<tr>
<td>[H_2PO_4]_b of dialysate</td>
<td>0·0593</td>
<td>0·0262</td>
<td>0·0105</td>
<td>0·0053</td>
</tr>
<tr>
<td>[Na]_b protein solution</td>
<td>0·0447</td>
<td>0·0442</td>
<td>0·0454</td>
<td>0·0488</td>
</tr>
<tr>
<td>[HPO_4]_b protein solution</td>
<td>0·0181</td>
<td>0·0281</td>
<td>0·0271</td>
<td>0·0214</td>
</tr>
<tr>
<td>[H_2PO_4]_b protein solution</td>
<td>0·0748</td>
<td>0·0316</td>
<td>0·0108</td>
<td>0·0044</td>
</tr>
<tr>
<td>H_2O (g./ml. protein solution)</td>
<td>0·6738</td>
<td>0·6702</td>
<td>0·6741</td>
<td>0·6723</td>
</tr>
<tr>
<td>protein (g./ml. protein solution)</td>
<td>0·4331</td>
<td>0·4385</td>
<td>0·4339</td>
<td>0·4245</td>
</tr>
<tr>
<td>density (g./ml. protein solution)</td>
<td>1·1169</td>
<td>1·1154</td>
<td>1·1137</td>
<td>1·1105</td>
</tr>
<tr>
<td>(k_d)</td>
<td>0·2533</td>
<td>0·2494</td>
<td>0·2487</td>
<td>0·2480</td>
</tr>
<tr>
<td>(v_{as}) (formula (18))</td>
<td>-0·2800</td>
<td>-0·1000</td>
<td>+0·3100</td>
<td>+0·6700</td>
</tr>
<tr>
<td>(v_{p}) (formula (17))</td>
<td>0·7489</td>
<td>0·7512</td>
<td>0·7489</td>
<td>0·7487</td>
</tr>
<tr>
<td>(v_{p}) (formula (25))</td>
<td>0·7459</td>
<td>0·7485</td>
<td>0·7474</td>
<td>0·7487</td>
</tr>
<tr>
<td>(q)</td>
<td>-0·0032</td>
<td>-0·0028</td>
<td>-0·0015</td>
<td>-0·0000</td>
</tr>
<tr>
<td>(b_1)</td>
<td>0·0092</td>
<td>0·0055</td>
<td>0·0024</td>
<td>0·0005</td>
</tr>
<tr>
<td>H combined g.mol./67,000 g. haemoglobin</td>
<td>10·30</td>
<td>6·80</td>
<td>3·10</td>
<td>-0·40</td>
</tr>
</tbody>
</table>

In the experiment at pH 6·35 (table 9) \([Na]_b\) is 0·0477 m and total phosphate is 0·0930 m. It is evident that in this case the aqueous dispersion medium free from protein must contain free phosphoric acid: \([H_3PO_4]_l = 0·0930 - 0·0448 = 0·0482\).

A part of the difference between \(\rho\), the density of the protein solution, and \(\rho_w\), the density of water, is due to the diffusible substances in the dispersion medium, and this part, symbolized \(\Delta sp\), can be calculated by formula (23):

\[
\Delta sp = 0·144[Na_2HPO_4]_l + 0·092[NaH_2PO_4]_l + 0·059[H_3PO_4]_l. \quad (23)
\]

The numbers represent the molar density increments determined by measuring the densities of solutions of each salt present. If \(\rho_s\) denotes the density of a salt solution of molar concentration \([S]\), the molar density increment \(K_s\) is defined as \((\rho_s - \rho_w)/[S]\).

The molar density increments vary with the salt concentration. The numbers in formula (23) represent values for solutions with an ionic strength of 0·1.
The density increments of proteins

The difference between the density of the dialysate and the density of water, symbolized \( \Delta_{s0} \), may be calculated by formula (24):

\[
\Delta_{s0} = \rho_0 - \rho_w = 0.144[\text{Na}_2\text{HPO}_4]_0 + 0.092[\text{NaH}_2\text{PO}_4]_0.
\]  

(24)

The values of \( \Delta_{sp} \) and \( \Delta_{s0} \) were used to calculate the specific volume of the protein by formula (25):

\[
k_d = (1 - v_p \rho_w) - (1/c_p) (\Delta_{s0} - \Delta_{sp}).
\]  

(25)

The specific volumes, \( v_p \), in table 9 calculated by formula (25) are from 0.000 to 0.003 ml. lower than the approximate values given in table 9 which were calculated by the simpler formula (17), derived for isoelectric protein.

Table 9 gives four values for the correction term \( q \), defined by formula (20), which were calculated by formula (26):

\[
q = v_{ns}(\rho_0 - \rho_w) - (1/c_p) (\Delta_{s0} - \Delta_{sp}).
\]  

(26)

It will be observed that \( q \) becomes more negative as the number of molecules of hydrogen ions combined with the protein is increased.

A negative value of \(-0.0021\) for the correction term \( q \) was obtained for haemoglobin equilibrated with a more concentrated salt solution,

\[
1.0\text{M-}\text{NaCl} + 0.015\text{M-}\text{Na}_2\text{HPO}_4 + 0.005\text{M-}\text{NaH}_2\text{PO}_4,
\]

referred to in table 5.

A positive value of \(+0.0037\) for \( q \) was obtained for human-serum albumin equilibrated with \( \text{m/15-phosphate buffer pH 6.9} \) referred to in table 6, when albumin existed in the form of an alkaline salt with ten equivalents of alkali combined with 67,000 g. albumin. The positive correction for albumin is due to the fact that the dispersion medium must contain a high proportion of disodium phosphate, a salt with a low specific volume. It follows that \( v_{sp} \) in formula (20) is smaller than \( v_{s0} \).

8. The specific volume of haemoglobin

Haemoglobin is stable in solutions free from salts, and it is therefore possible to make direct measurements to test the accuracy of the calculations of specific volumes by formulae (17) and (25) given in table 9.

Direct measurements on aqueous solutions showed that the apparent specific volume is 0.7459 at 1.7° C; the values for specific volumes of haemoglobin in phosphate buffers, calculated by formula (25), range from 0.7459 to 0.7487 (table 9). These figures agree with direct measurements within the limits of accuracy required for molecular-weight determinations. The difference, 0.0028 ml. or 0.37 %, between the values observed for aqueous solutions and those calculated for haemoglobin in the presence of salts may be partly due to experimental errors, but it is possible that sodium phosphates at \( \text{pH 7.4} \) may increase the apparent specific volume of haemoglobin.
Direct measurements of the effects of hydrogen ions on the specific volume of horse haemoglobin at 30°C have been published by Svedberg & Nichols (1927), who record values of 0.7545, 0.755 and 0.7575 for the pH values 6.0, 6.5 and 7.56. Their measurements agree with the data in table 9 in showing that the specific volume is constant within 0.003 ml. over a considerable range of pH values.

The specific volume of ox haemoglobin in water was determined as 0.751 at 15°C. Svedberg & Pedersen (1940) give the value 0.749 for horse haemoglobin at 20°C.

REFERENCES

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