

## MOLECULAR WEIGHT, MOLECULAR VOLUME, AND HYDRATION OF PROTEINS IN SOLUTION

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The gram molecular weight and volume of a dissolved substance may be calculated from the osmotic pressure of the solution. Osmotic pressure is affected only slightly by hydration and so furnishes no precise information as to the size of the hydrated molecule as it exists in a solution. The radius of the hydrated molecule in solution, and hence the gram molecular volume of the hydrated solute, may be determined from diffusion measurements. The difference between this figure and the gram molecular volume, as found by osmotic pressure, therefore represents the amount of hydration. The hydration may also be calculated from viscosity measurements. These two independent methods for the estimation of hydration give essentially the same values for the hydration of crystalline hemoglobin and crystalline trypsin.

### *Molecular Weight from Osmotic Pressure*

The gram molecular weight of a substance in solution may be defined as that quantity of dry substance which, when dissolved in 1 liter of solvent, gives rise to an osmotic pressure of 22.4 atmospheres at 0°C. If the osmotic pressure of a solution is known, therefore, its molar concentration may be calculated. Since there are  $6.06 \times 10^{23}$  molecules in a gram molecule the average weight of the individual molecules may be found if the weight concentration of the solution is also known. This figure represents the average dry weight of the individual molecules of solute for which the membrane is impermeable but furnishes no definite information as to their size. Solvation of the molecules increases their size but does not change the number of molecules and affects the osmotic pressure only by decreasing the quantity of free solvent. This decrease in the quantity of free solvent

is not noticeable experimentally except in concentrated solutions or when the solvation is large.

Calculations of the molecular weight from osmotic pressure determinations involve the following assumptions:<sup>1</sup>

1. The system is at equilibrium.
2. The membrane is permeable to the solvent but impermeable to the solute in question.
3. The osmotic pressure is proportional to the concentration (van't Hoff's law).
4. The molecules of solute are all of the same size.

In the case of collodion membranes and aqueous solutions of proteins the first three conditions are fulfilled but the fourth may or may not be true. The protein molecules themselves may vary in size and in addition they may be combined with small ions or molecules which are thus prevented from free diffusion through the membrane, as in the Donnan equilibrium. In this case the osmotic pressure is due to both the protein molecules and the excess concentration of inorganic ions and the value calculated for the molecular weight represents the average of these various molecular species present. The complication due to Donnan equilibrium may be avoided experimentally by measurements made at the isoelectric point of the protein. The effect of neutral salts also furnishes a test for the presence of such Donnan pressures.

#### *Radius of Molecules from Diffusion Measurements*

The radius of the molecule determines the rate of diffusion in accordance with Einstein's equation (1)

$$D = \frac{R T}{N} \frac{1}{6\pi r \eta}$$

$R$  = gas constant

$T$  = absolute temperature

$N$  = Avogadro's number  $6.06 \times 10^{23}$

$\eta$  = viscosity of solvent

$r$  = radius of molecule

molecular volume =  $4/3 \pi r^3 N$

<sup>1</sup>For a discussion of the osmotic pressure of hemoglobin solutions see Adair, G. S., *Proc. Roy. Soc. London, Series A*, 1928, **120**, 573.

Thus, if the diffusion coefficient of the solute is known the radius of the molecules and hence the gram molecular volume may be calculated. This value for the radius represents the radius of the particle which actually moves in the solution and therefore includes any solvent carried with the molecule. The following assumptions are involved in Einstein's equation:

1. The diffusing particles are few and large compared to the molecules of the solvent.
2. They are spherical.
3. They are electrically neutral.
4. They are impelled by a force equal to the osmotic pressure as given by van't Hoff's law against a resistance as given by Stokes' law.

As in the case of osmotic pressure the effect of ionization is the most important source of error with protein solutions. The presence of charged molecules may again be tested for by determining the effect of neutral salts and of the pH. If the molecules are not of the same size a constant value for the diffusion coefficient will not be obtained but the value will decrease as the experiment proceeds since the smaller particles will diffuse out faster. It is important, therefore, to continue the experiment until a large proportion of the solvent has diffused out; or better, to repeat the measurement on the first part of the diffusate, in order to be sure that the diffusion coefficient is actually the same for all of the solute. Otherwise entirely erroneous values may be obtained. The determination may be made conveniently and accurately as described by Northrop and Anson (2).

#### *Calculation of Hydration from Osmotic Pressure and Diffusion Measurements*

If the osmotic pressure and the diffusion coefficient of a solution are known, then the degree of hydration of the molecules of the solute can be determined as follows:

- Let  $M$  be the gram molecular weight of the dissolved substance as determined from osmotic pressure measurements,  
 $r$  the average radius of the molecules as determined from diffusion measurements,  
 $S$  the specific volume of the dry substance,

then the gram molecular volume of hydrated molecules equals  $4/3 \pi r^3 N$  and the gram molecular volume of non-hydrated molecules equals  $S M$ .

Volume of water of hydration (if water is used as solvent) equals

$$\frac{4}{3} \pi r^3 N - S M$$

and

$$\frac{4/3 \pi r^3 N - S M}{M}$$

equals volume of water of hydration per gram of dry solute, or

$$\frac{4/3 \pi r^3 N - S M}{N}$$

equals volume of water of hydration per molecule solute.

#### *Determination of Hydration from Viscosity Measurements*

An independent method for the determination of the amount of hydration of substance in solution is the measurement of viscosity (3). This method applies to the case of molecules or particles large as compared with the size of the molecules of the solvent and consists in determining the relative viscosity of the solution as compared with the viscosity of the solvent. The volume of the solute may be calculated by aid of the empirical formula

$$\eta = \frac{1 + 0.5 \phi}{(1 - \phi)^4}$$

where  $\eta$  equals the relative viscosity of solution and  $\phi$  equals the volume of solute expressed as the fraction of the total volume of the solution. The formula was found to hold well for a large number of solutions or dispersions of molecules of relatively large size.

The two methods of determining the degree of hydration were used here in the case of such substances as hemoglobin and crystalline trypsin, and the results show that there is quite a close agreement between the two methods.

The results are summarized in the following table:

	Molecular weight	Average radius of hydrated molecule	Water of hydration per gm. dry wt.	
			Osmotic pressure diffusion method	Viscosity method
			<i>cm.</i> <sup>3</sup>	<i>cm.</i> <sup>3</sup>
Hemoglobin.....	67,000	$2.73^* \times 10^{-7}$	0 to 0.14	0.13†
Isoelectric gelatin.....	61,500	$(5.4 \times 10^{-7})$	(5.8)	5.9
Crystalline trypsin.....	35,000	$2.6 \times 10^{-7}$	0.54	0.49

*Technique.*—Osmotic pressure, Northrop and Kunitz (4), Adair (5); diffusion, Northrop and Anson (2).

\* Svedberg, T. (Colloid chemistry, American Chemical Society Monographs, New York, The Chemical Catalog Co., 2nd edition, 1928, 165) obtains a value of  $0.0342 \text{ cm.}^2/\text{day}$  corresponding to a molecular radius of  $3.35 \times 10^{-7} \text{ cm.}$  Svedberg's measurements were made while the molecules were moving under the influence of centrifugal force and the difference in the value may be due to the fact that the molecules are not spherical. In this case they might be oriented in a gravitational field and would, therefore, move through the liquid at a rate different from that determined by diffusion alone.

† Adair and Robinson (*J. Physiol.*, 1931, **72**, 28) obtained a value of 0.2 ml. water per gm. hemoglobin from measurements of the water absorbed by the dry protein from ammonia sulfate solutions.

#### EXPERIMENTAL

##### *Hemoglobin*

*Osmotic Pressure.*—Measurements of Adair (5).

*Diffusion.*—Northrop and Anson (2).

*Viscosity.*—Measurements were made of the viscosity at  $5^\circ\text{C.}$  of various concentrations of CO-hemoglobin in  $\text{M}/20$  phosphate buffer pH 6.8 using an Ostwald viscosimeter; specific volume of dry hemoglobin equals 0.75.

The data given in Table I show that the hydration of hemoglobin under the conditions of the experiment decreases with the dilution and is about 0.1 ml. per gm. of hemoglobin at concentrations below 2 per cent. The diffusion experiments of Northrop and Anson were done under the same conditions of hemoglobin in the range of 1–2.5 per cent. The experiments of Northrop and Anson show that at  $5^\circ\text{C.}$  the diffusion coefficient for 2.5 per cent hemoglobin in  $\text{N}/20$  phosphate buffer pH 6.8 is between 0.0434 and  $0.0401 \text{ cm.}^2/\text{day}$ . The calculated radius of the molecules is between  $2.65 \times 10^{-7}$  and  $2.86 \times 10^{-7}$ . Hence  $4/3\pi r^3 N$  equals between 47,300  $\text{cm.}^3$  and 59,500  $\text{cm.}^3$ . From osmotic pressure measurements (Adair)  $S M = 67,000 \times 0.75 = 50,000$ .

Volume of water of hydration per mole = between 0 and 9,500  $\text{cm.}^3$   
 “ “ “ “ “ “ gram = “ 0 “ 0.14  $\text{cm.}^3$

Thus it is seen that in the case of hemoglobin the amount of water of hydration per gram of protein, as obtained by viscosity measurements,

is so small as to be within the experimental error of the diffusion measurements.

### *Crystalline Trypsin*

*Osmotic Pressure Measurements.*—Northrop and Kunitz (6).

*Diffusion Measurements.*—Scherp (7).

*Viscosity.*—Viscosity measurements of solutions of crystalline trypsin were made under conditions similar to those employed in the determination of the molecular weight of crystalline trypsin by means

TABLE I  
*Viscosity Measurements of CO-Hemoglobin, pH 6.8 at 5°C.*

Concentration of protein	Relative density at 5°C.	Relative viscosity*	Calculated volume of solute in cm. <sup>3</sup> /100 cm. <sup>3</sup> solution	Specific volume per gm. protein	Volume of water of hydration per gm. hemoglobin
<i>gm./100 ml. solution</i>				<i>cm.<sup>3</sup></i>	<i>cm.<sup>3</sup></i>
2.10	1.006	1.084	1.85	0.88	0.13
4.20	1.012	1.175	3.65	0.87	0.12
6.30	1.018	1.290	5.60	0.89	0.14
8.36	1.024	1.445	7.90	0.95	0.20
10.45	1.030	1.610	10.15	0.97	0.22

\* These values are much lower than those reported by Lewis and Loughlin (*Biochem. J.*, London, 1932, **26**, 480) and give rise to correspondingly lower values for the hydration. This difference is not due to the salt present since repetition of the measurements with salt-free hemoglobin solution gave practically the same figures for the viscosity of the solution as found for hemoglobin solution in *M*/2 phosphate buffer.

of osmotic pressure measurements, as well as in the determination of the diffusion coefficient of crystalline trypsin as carried out by Dr. Scherp in this laboratory.

The procedure was as follows. A solution of crystalline trypsin in *M*/10 acetate buffer pH 4.0 was made salt-free by dialysis in the cold room against *N*/10,000 hydrochloric acid. The dialyzed trypsin was then diluted with equal volume of saturated magnesium sulfate in *M*/10 acetate buffer pH 4.0 and dialyzed against a definite volume of trypsin-free 0.5 saturated magnesium sulfate in *M*/10 acetate buffer pH 4.0 until equilibrium was established as indicated by the reading of a manometer tube inserted in the collodion bag containing the

trypsin solution. The outside solution was found to be free of any trypsin. A series of dilutions was then made of the trypsin solution by means of the outside magnesium sulfate solution and viscosity measurements were made at 5°C. The results are shown in Table II. The specific volume of dry trypsin was taken as 0.75 ml./gm. which was found to be common for proteins of the albumin type. The average value of the water of hydration of crystalline trypsin when dissolved in 0.5 saturated magnesium sulfate pH 4.0 was thus found by the viscosity measurements to be 0.5 ml. per gm. dry protein.

TABLE II  
*Viscosity at 5°C. of Various Concentrations of Crystalline Trypsin in 0.5 Saturated Magnesium Sulfate and M/10 Acetate Buffer pH 4.0*

Concentration of trypsin	Time of outflow	Relative viscosity	Calculated volume of hydrated trypsin in cm. <sup>3</sup> /100 cm. <sup>3</sup> solution	Volume of hydrated trypsin per gm. dry trypsin	Water of hydration per gm. dry trypsin
<i>gm./100 ml.</i>	<i>sec.</i>			<i>cm.<sup>3</sup></i>	<i>cm.<sup>3</sup></i>
0	203.4	1.000	0		
0.8	212.6	1.045	1.00	1.25	0.50
1.6	221.5	1.089	2.00	1.25	0.50
2.4	231.0	1.135	2.90	1.21	0.44
3.2	241.4	1.187	3.90	1.22	0.47
4.0	257.0	1.265	5.15	1.29	0.54
Average.....					0.49

The radius of hydrated trypsin molecules under the same conditions, as determined by Scherp from diffusion measurements, was found to be  $2.6 \times 10^{-7}$  cm. The volume of one mole of hydrated trypsin is therefore

$$(2.6 \times 10^{-7})^3 \times 4/3 \pi \times 6.06 \times 10^{23} = 44,700 \text{ cm.}^3$$

The molecular weight of the trypsin in solution under the same conditions was found by osmotic pressure measurements to be about 35,000 gm. The molecular volume of the non-hydrated trypsin equals 26,000 cm.<sup>3</sup> Hence, water of hydration per mole of trypsin equals 19,000 cm.<sup>3</sup> Water of hydration per gram dry trypsin equals 19,000/35,000 equals 0.54 cm.<sup>3</sup>/gm. Thus, the value for hydration

of trypsin, as determined by diffusion experiments in connection with osmotic pressure measurements checks quite closely with the value obtained by viscosity measurements. This agreement serves as a check for the viscosity formula and justified the application of Einstein's diffusion formula to protein solutions.

#### *Gelatin*

The hydration of gelatin, as calculated from osmotic pressure and from viscosity measurements, has been described in a previous paper (8). The value of the hydration so obtained was 6 cm.<sup>3</sup> water per gram dry gelatin in 3 to 5 per cent solutions.

Diffusion measurements were made with gelatin solutions in order to see whether the hydration, as determined by this method in connection with the osmotic pressure measurements, agrees with that calculated from viscosity. If a 5 per cent solution of gelatin pH 4.7 in M/1000 acetate buffer was allowed to diffuse, a constant value for the diffusion coefficient of 0.05 cm.<sup>2</sup>/day was obtained. However, if the first diffusate was replaced in the cell and the experiment repeated, a much larger value for the diffusion coefficient was found. Gelatin solutions, therefore, as was to be expected, are not homogeneous but the relative size of the particles or their relative amount, do not differ sufficiently to cause a noticeable drift in the diffusion coefficient as determined from any one experiment. Trial calculations show that a mixture containing 30 per cent of particles of radius 2 and 70 per cent of particles of radius 1 will diffuse in such a way as to give a value for the diffusion coefficient, as calculated from the total amount diffused, which does not vary over 10 per cent until more than 75 per cent of the total original quantity has diffused out. Such a mixture, however, would give an entirely different value for the diffusion coefficient if the measurements were repeated on the diffusate. This is the result obtained with the gelatin. The results are complicated in addition by the fact that some hydrolysis of the gelatin occurs during the experiment.

Since the value of the diffusion coefficient has no physical significance unless the diffusing particles are of nearly uniform size, the results with gelatin are of doubtful significance.

## SUMMARY

1. The gram molecular weight of a substance may be calculated from the osmotic pressure of its solution.

2. The radius of the hydrated molecule and, hence, the gram molecular volume of the hydrated solute may be determined from diffusion measurements. The hydration of the molecules may, therefore, be calculated from osmotic pressure and diffusion measurements.

3. Hydration may also be determined by viscosity measurements. Hydration of crystalline hemoglobin, crystalline trypsin, and gelatin have been determined by these methods and found to be as follows:

	Molecular weight	Average radius of hydrated molecule	Water of hydration per gm. dry wt.	
			Osmotic pressure diffusion method	Viscosity method
			<i>cm.<sup>3</sup></i>	<i>cm.<sup>3</sup></i>
Hemoglobin.....	67,000	$2.73 \times 10^{-7}$	0 to 0.14	0.13
Isoelectric gelatin.....	61,500	$(5.4 \times 10^{-7})$	(5.8)	5.9
Crystalline trypsin.....	35,000	$2.6 \times 10^{-7}$	0.54	0.49

The results with gelatin calculated from the diffusion measurements are uncertain since gelatin solutions are not homogeneous.

## REFERENCES

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