CCXXXIII. ON THE DIFFUSION CONSTANTS OF THE AMINO-ACIDS

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IN connexion with the ultracentrifugal studies of the action of enzymes on proteins a systematic investigation was started on the diffusion constants of the amino-acids. When ovalbumin was digested with papain, Annetts [1936] and Svedberg & Eriksson-Quensel [1934] found that amino-acids were split off during the process of digestion and in the ultracentrifuge these amino-acids showed up as non-centrifugable material. In order to get an idea of the size of these dissociation products Annetts made a number of diffusion experiments on them and compared the average diffusion velocities so found with those of well-defined polypeptides and amino-acids. Since the proteins are constituted of only a small number of amino-acids a knowledge of their constants might be of great value in their identification.

The method used for measuring the diffusion constants was that of Lamm [1928; 1929; 1937; *v. also* Lamm & Polson, 1936]. In this method a uniform transparent scale is photographed through the diffusion cell. The gradient in refractive index at the diffusion boundary produces a distorted image of the scale. The scale line displacement is proportional to the concentration gradient when the refractive index is a linear function of the concentration.

The arrangement of apparatus is shown in Fig. 1.

Fig. 1. Arrangement of apparatus for measuring diffusion constants.

A is a source of illumination (quartz mercury arc lamp), *B* a filter which transmits light of a wave-length not absorbed by the solution examined, *C* the scale, *D* the diffusion cell in a water thermostat *E*, and *F* the camera. The camera objective has a focal length of 100 cm. and is placed in such a position that the scale image has a magnification of about 1.

The thermostat heating element was a chrome nickel wire 70 cm. long and 0.03 cm. in diameter. It was heated by a 50-cycle current of 1.5 amp. The water in the thermostat was well stirred and its temperature was kept constant to within 0.005° by a Vertex contact thermometer.

The diffusion cell used for the water solutions in this investigation was that of Lamm [1937]. The cell is sketched in Fig. 2. The experiments were performed as follows. The bottom half of the vertical opening was filled with the solution and the slide driven across with the help of the screw arrangement. The solvent

was then placed in the top half. After 1 hr. temperature equilibration in the water thermostat the slide was drawn out very carefully. A very sharp boundary was formed in each case.

Fig. 2.

Fig. 2a.

Fig. 2. Diffusion cell for measuring diffusion constants in water.

Fig. 2a. Diffusion cylinder for measuring diffusion constants in organic solvents.

The diffusion cell employed for measuring the diffusion constants of substances in alcohol solutions is sketched in Fig. 2a. *A* is a cylindrical vessel with two inlet tubes *b* and *g*. This cylindrical vessel is attached to a well-ground cylindrical optically homogeneous quartz tube *B*. *B* is connected to a cylindrical tube *C* through a capillary tap and tube. *c* is a well-ground steel ball with the same diameter as the outer diameter of the quartz tube, i.e. 1.2 cm. This steel ball moves in a guiding steel framework as shown in the figure. Attached to the steel ball *c* is a thin silk thread. With a clockwork *a* the steel ball can be raised slowly. The rate varies with the diameter of the wheel.

In performing an experiment the following procedure was followed. The cylinder *C* was filled with mercury which was allowed to run into the cylinder *B* through the stopcock. The rubber stopper *e* was covered with mercury as shown in Fig. 2a. With a thin pipette 2 ml. of the solution to be investigated were run in through the inlet *b*, on to the mercury in *B*. By allowing more mercury to run into the cylinder *B* the surface of the liquid was raised to the top of *B*. The steel ball was then dropped on to the solution. 10 ml. pure solvent were run into *A* through the inlet *b*.

After half an hour's temperature equilibration the clockwork was set in motion and the steel ball was thereby raised at the rate of 2 mm. in 5 min. A sharp boundary between the solvent and the solution was thus formed. When the ball was well removed from the mouth of the cylinder the mercury was allowed to run out slowly through the narrow capillary *d*. The boundary formed was thus lowered in the tube *B* to a suitable position for photographing. The clockwork was stopped and the ball lowered into its position.

The advantage of using the above described diffusion tube is that the solvent or solution never comes in contact with grease from the taps or slides as in the other methods. A further advantage is that the volume of solution necessary for a determination is reduced to 2 ml.

This diffusion cell resembles the cell designed by Lamm [1928; 1937] in that the boundary between the solvent and the solution is formed on top of the

tube *B* and then lowered to a suitable position for photographing in the tube. Lamm [1928] however formed the boundary by allowing the solvent to run on to the solution through a tapering end of the cylinder. He then lowered the boundary in the cell by a type of water manometer arrangement. He also constructed an apparatus in which he formed the boundary by means of a thin coarse glass filter on top of the tube *B* which he then lowered to a suitable position for observation. The objection to using a glass filter plate is that the solution and the solvent can be mixed up in the pores of the filter during the time of levelling, and furthermore it is uncertain whether all the substance is washed away from the filter during levelling.

Method of calculation

The scale displacements obtained from the micro-comparator readings of the distorted scale and a standard reference scale were plotted against the comparator readings of the distorted scale lines (Fig. 3), i.e. the gradient of refractive index was plotted against the height.

Fig. 3. Diffusion gradient curve of a monodisperse substance.

Fig. 3 a. Diffusion gradient curve for a polydisperse substance.

The photographic line displacement (*Z*) of any line from its position on the undeviated scale [Lamm, 1928; 1929] is given by

$$Z = Gab \frac{dn}{dx}, \quad \text{.....(1)}$$

where *G* is the photographic enlargement factor,

a the thickness of the liquid column in the diffusion cell,

b the optical distance from the scale to the centre of the tube,

Z the photographic line displacement,

and dn/dx the gradient of refractive index at the displaced line position.

By integration of equation (1) it follows that

$$A = \int_{-\infty}^{+\infty} \left(Gab \frac{dn}{dx} \right) dx = Gab (n_1 - n_0). \quad \text{.....(2)}$$

The equation obtained by plotting the scale line displacement against the scale lines has been developed by Wiener [1893] and has the form

$$\frac{dn}{dx} = \frac{n_1 - n_0}{2\sqrt{\pi Dt}} \cdot e^{-\frac{x^2}{4Dt}}, \quad \text{.....(3)}$$

where *D* is the diffusion constant,

t the time since diffusion started,

and *x* the distance of a point in the cell from the original boundary.

The other symbols have their previous significance.

Several equations were derived from the above equation from which the diffusion constants can be calculated [v. Lamm & Polson, 1936]. For solutions of one component the following equation gave the most reliable values:

$$D = \frac{\mu^2}{2t} \left(\frac{l-b}{l} \right)^2 \quad \dots\dots(4)$$

μ = half the distance between the two inflexion points.

The position of the inflexion points was obtained by dividing the maximum height of the curve by $\sqrt{e}$.

l represents the optical distance from the scale to the camera objective. b is the optical distance from the scale to the centre of the tube and is frequently called the scale distance. The other symbols have their previous significance. A more complete account of the methods of calculation is found in an earlier publication [Lamm & Polson, 1936]. The diffusion constants of the amino-acids investigated here were in all cases corrected to the basis of diffusion in pure water at 20° by means of the relation:

$$D \text{ corr.} = D\eta_s/\eta_{20} \cdot \frac{T_{20}}{T_x}, \quad \dots\dots(5)$$

where η_s is the viscosity of the solvent at absolute temperature T_x , and η_{20} the viscosity of distilled water at 293° absolute.

Since the diffusion constants of the amino-acids are relatively large in comparison with the diffusion constants of the proteins great care was taken in observing the starting time of diffusion as accurately as possible. The time required for drawing out the slide of the diffusion cell was determined and the diffusion constants were calculated from the time when the slide was half-way across the cell.

Since the basic and acid dissociations of the amino and carboxyl groups are very small the effect of the electric charge upon the diffusion velocity can safely be neglected.

The amino-acids form association products in solution due to the presence of the carboxyl and amino groups. This effect explained their anomalous depression of the freezing-points [Pfeiffer, 1927] and anomalous refractive indices at high concentrations [Frankel, 1930]. From this anomalous behaviour of the depression of the freezing-point Loughlin, quoted by Lewis [1931], calculated the degree of association of glycine. The association factor increased from 4 to 13 % in the interval of concentration 3.0–16.35 %.

It was thus found necessary to measure the diffusion constants at at least three different concentrations and to extrapolate to infinite dilution. However small the variations of the diffusion constants in low concentrations seem to be the effect should always be considered since the degree of association is different for different amino-acids.

Experimental

In the following tables are given the results of diffusion experiments on the amino-acids at different concentrations in pure water at 20°. The influence of salts on the diffusion constants was also investigated in a few cases.

The influence of salts on the diffusion constant is simply to increase the viscosity of the solvent whereby the diffusion velocity is lowered.

An experiment was performed in order to find out whether there exists any difference in the diffusion constants of the optical isomerides of proline. Since both isomerides were not available a few determinations were made on *l*-proline.

Table I

Diffusion of glycine in water at 20°

Conc. g./100 ml.	Time in hr.	$D \times 10^7$ cm. ² /sec.
0.33	1	94.0
	1.5	94.4
	Average	94.2
0.66	1.25	92.8
	2.0	93.1
	Average	93.0
1.00	1	92.6
	2.33	92.0
	Average	92.3

Diffusion of 0.5% glycine in different concentrations of NaCl

Conc. NaCl	Time in hr.	$D \times 10^7$ cm. ² /sec.
2.0 M	1.66	78.1
	2.25	76.0
	Average	77.05
Corrected according to eq. (5)		92.0
1.0 M	1.25	84.4
	1.75	84.4
	Average	84.5
Corrected according to eq. (5)		92.0
0.5 M	1.50	88.8
	2.50	87.4
	Average	88.1
Corrected according to eq. (5)		91.8

The diffusion constant when extrapolated to zero concentration gives a value of 95.0×10^{-7} cm.²/sec.

Table II

Diffusion of dl-α-alanine in water at 20°

Conc. amino- acid g./100 ml.	Time in hr.	$D \times 10^7$ cm. ² /sec.
0.25	0.33	81.5
	1.5	80.4
	2.25	82.4
	Average	81.4
0.5	0.7	80.8
	1.25	80.0
	2.0	79.1
	2.5	81.0
	Average	80.2
1.0	0.33	80.0
	1.5	79.0
	2.25	77.3
	Average	78.8

Diffusion of 0.5% dl-α-alanine in NaCl

Conc. NaCl	Time in hr.	$D \times 10^7$ cm. ² /sec.
0.5	1.5	79.3
	2.5	78.4
	Average	78.8
Corrected according to eq. (5)		82.1

When extrapolated to infinite dilution the diffusion constant becomes 82.6×10^{-7} cm.²/sec.

Table III. *Diffusion of dl-valine in water at 20°*

Conc. valine g./100 ml. solution	Time in hr.	$D \times 10^7$ cm. ² /sec.
0.25	1	73.7
	1.75	70.7
	2.75	72.0
	Average	72.1
0.5	1	70.9
	1.5	70.6
	Average	70.7
1.0	1.33	67.3
	2.0	68.6
	3.0	68.8
	Average	68.2

When extrapolated to infinite dilution the diffusion constant becomes 73.5×10^{-7} cm.²/sec.

Table IV. *Diffusion of dl-leucine in water at 20°*

Conc. leucine g./100 ml. solution	Time in hr.	$D \times 10^7$ cm. ² /sec.
0.25	0.75	62.9
	2.00	63.5
	Average	63.2
0.5	0.75	62.5
	1.75	59.0
	2.35	61.6
	Average	61.0
1.0	2.0	61.4
	3.0	61.0
	Average	61.2
0.5% dl-leucine in 0.5 M NaCl		
	0.75	61.2
	1.75	60.0
	3.00	58.0
	Average	59.7

Corrected according to eq. (5) this becomes 62.2×10^{-7} cm.²/sec.

The diffusion constant extrapolated to infinite dilution becomes 63.3×10^{-7} cm.²/sec.

Table V. *Diffusion of dl-proline in water at 20°*

Conc. proline g./100 ml.	Time in hr.	$D \times 10^7$ cm. ² /sec.
0.25	1.16	80.0
	1.75	78.8
	2.33	79.6
	Average	79.4
0.5	1.5	79.3
	2.0	78.2
	2.5	79.3
	Average	78.9
1.0	2.5	78.0
	3.16	77.6
	Average	77.8

The diffusion constant at infinite dilution = 80.1×10^{-7} cm.²/sec.

Table V (*cont.*)*Diffusion of l-proline in water at 20°*

Conc. <i>l</i> -proline g./100 ml.	Time in hr.	$D \times 10^7$ cm. ² /sec.
0.5	1.5	80.4
	2.0	78.2
	2.5	81.3
	Average	80.0
0.5	1.10	80.1
	1.75	78.8
	2.50	79.3
	Average	79.4

As can be seen from the foregoing tables the difference between the diffusion constants of *dl*-proline and *l*-proline lies within the experimental error, i.e. 1–2%.

Table VI. *Diffusion of tryptophan in water at 20°*

Conc. tryptophan g./100 ml.	Time in hr.	$D \times 10^7$ cm. ² /sec.
0.25	1.6	60.5
	2.5	61.5
	3.5	59.2
	Average	60.4
1.0	2.5	58.3
	3.25	58.7
	Average	58.5

The diffusion constant extrapolated to infinite dilution becomes 61.4×10^{-7} cm.²/sec.

Table VII. *Diffusion of arginine in water at 20°*

Conc. arginine g./100 ml.	Time in hr.	$D \times 10^7$ cm. ² /sec.
0.25	0.75	56.9
	1.75	56.9
	2.5	58.0
	Average	57.2
1.0	2.03	58.4
	3.00	57.7
	Average	58.0

The diffusion constant at infinite dilution was taken as the mean of the values at the two concentrations, i.e. 57.6×10^{-7} cm.²/sec., since it does not seem to increase on diminishing concentration.

The diffusion constant of the polypeptide leucylglycylglycine was also measured. The results at two different concentrations are given below.

Table VIII. *The diffusion of leucylglycylglycine*

Conc. g./100 ml.	Time in hr.	$D \times 10^7$ cm. ² /sec.
0.25	1.55	47.6
	3.25	46.7
	4.50	45.0
	Average	46.4
1.0	1.45	45.9
		46.4
		47.2
	Average	46.5

The diffusion at infinite dilution thus equals 46.4×10^{-7} cm.²/sec. This value compares well with the value obtained by Annetts [1936].

Of great interest from a technical point of view is a comparison between the diffusion constants reported in the present investigation with the results of Mehl [1937] obtained with the porous disk method of Northrop & Anson [1929]. The diffusion constants were calculated with reference to the diffusion constant of KCl as standard substance for which a diffusion constant of 149.3×10^{-7} cm.²/sec. was assumed.

In the following table the results obtained by the author are compared with those of Mehl. The results of Mehl were reduced to 20°.

Table IX

Amino-acid	Annetts	Polson	Mehl	Mehl/Polson
Glycine	90.8	95.0	85.13	0.896
Alanine	—	82.6	72.62	0.879
Valine	—	73.5	63.42	0.863
Leucine	—	63.3	—	—
Proline	—	80.1	72.36	0.903
Tryptophan	57.3	61.4	—	—
Arginine	—	57.6	—	—
				Average 0.8825

From Table IX it is seen that there is not good agreement between the values obtained by Mehl and the results here reported. If the values of Mehl were divided by a constant, 0.8825, the writer's results were obtained. There exists therefore no doubt but that Northrop's method can be applied with safety to low-molecular substances. High-molecular weight substances give more doubtful results since the glass filter discs often become clogged during the manipulations, thus making the results less reliable [Hecht *et al.* 1937].

Proline and hydroxyproline are soluble in ethyl alcohol and ethyl alcohol-water mixtures. It was of interest to know whether the proline exists in the same state in alcohol solution as in water. Two experiments were therefore performed with the diffusion cell constructed for organic solvents (Fig. 2*a*). In the first experiment 71.5 % (by weight) ethyl alcohol was used and in the second absolute alcohol.

Table X. *Diffusion of 1% proline in ethyl alcohol at 20°*

Conc. alcohol % by wt.	Time in hr.	$D \times 10^7$ cm. ² /sec.
71.5	0.75	35.9
	1.25	33.4
	2.00	32.5

Average 33.9

Corrected for the viscosity of the solvent $\eta/\eta_0 = 2.315$ (eq. (5)) D becomes 78.5.

99.5	4.08	37.0
	5.5	36.9
	6.5	36.6

Average 36.8

Corrected for the viscosity of the solvent $\eta/\eta_0 = 1.2$ D becomes 44.2.

From the results obtained in Table X it is evident that proline exists in the same state in solution in 71.5 % alcohol as in water. In absolute alcohol, however, proline most probably forms an association product since the diffusion constant is very much lower than in pure water.

In Fig. 4 a curve is drawn showing the relation between molecular weights and diffusion constants of the amino-acids. The diffusion constant of sucrose was also added in order to extend the curve. A diffusion constant of

44.2×10^{-7} cm.²/sec. it will be seen corresponds to a molecular weight of approximately 400. This suggests that the association product of proline in absolute alcohol has a molecular weight of three or four times that of proline, i.e. 345 or 460.

Fig. 4. Variation of the diffusion constants with molecular weight. The scattered points must not be regarded as experimental errors of some kind but only to a difference in the shapes of the various molecular species, nor may the drawn curve be considered as having any theoretical significance whatsoever.

If the absolute alcohol acted merely as a dehydrating agent in this case, evidence of heterogeneity should be obtained from the diffusion curve [v. Lamm & Polson, 1936; Lamm, 1937]. The normal curve drawn from the experimental diffusion gradient curve should show a departure if the substance under investigation is heterogeneous with regard to molecular weight or shape. The effect of heterogeneity on the diffusion curve is illustrated in Fig. 3a. With proline in absolute alcohol, however, no sign of heterogeneity was obtained.

SUMMARY AND CONCLUSIONS

1. The refractory method of Lamm for measuring the diffusion constants of substances is described briefly.

2. A modified form of Lamm's diffusion tube for organic solvents is introduced. Instead of forming the boundary between two liquids by means of a tapering tube or a porous glass filter, the boundary was formed by means of a steel ball and clockwork.

3. The diffusion constants of a number of water-soluble amino-acids were measured at different concentrations. The diffusion constants were dependent upon the sizes of the molecules. The highest diffusion constant was given by glycine and the smallest by arginine. The shapes of the molecules play a very fundamental role in the diffusion constants. The more stretched the molecule is the lower the diffusion constant. This is illustrated by tryptophan and arginine. Arginine which is a stretched molecule has a molecular weight of 174 and a diffusion constant of 57.6×10^{-7} cm.²/sec. Tryptophan on the other hand which is a more spherical molecule has a molecular weight of 204 and a diffusion constant of 61.4×10^{-7} cm.²/sec.

4. When proline was diffused in an alcohol-water solution of 71.5% the molecular state remained the same as in pure water. In absolute alcohol proline showed evidence of association. The molecular weight of the association product was estimated to be 345 or 460, from the knowledge of the diffusion constants of other substances.

5. The diffusion constants of the amino-acids obtained here were compared with the results obtained by Mehl with the porous disc method. Although the diffusion constants obtained by Mehl were not the same as the writer's they were at least proportional to the writer's. This suggests that Mehl used a wrong diffusion constant in the calibration of his diffusion cells.

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