LXXVIII. THE DETERMINATION OF DIFFUSION CONSTANTS OF PROTEINS BY A REFRACTOMETRIC METHOD.

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IN the calculation of the molecular weights of proteins from sedimentation data it is essential that their diffusion constants should be known accurately. At first diffusion constants were measured from the spreading at the sedimenting boundary in the ultracentrifuge [Svedberg, 1925, 1, 2] and the concentration changes were determined from measurements of the light absorption of the solutions. But in the ultracentrifuge it is impossible to control the temperature to a degree necessary for accurate diffusion measurements. A further objection to this method of measuring diffusion constants is that the time of diffusion in the centrifuge cell is far too short for an extended series of measurements. Therefore Tiselius and Gross [1934] applied the light absorption method to the measurement of diffusion at a boundary in a tube at rest. Small fluctuations in the results persisted due to variation of the photographic blackening caused by irregularities in the photographic emulsion or by traces of impurities in the solutions.

In the work to be described in this paper the diffusion apparatus was similar to that used by Tiselius and Gross but the concentration gradients were measured by a refractometric method developed by one of us for use in the ultracentrifuge [Lamm, 1928; 1929].

In this method a uniform transparent scale is photographed through the diffusion cell. The refractive index gradient at the diffusion boundary produces a distorted image of the scale in which scale line displacement is proportional to the concentration gradient when the refractive index is a linear function of the concentration.

The arrangement of the apparatus is shown in Fig. 1.

Fig. 1. Diffusion apparatus. *C* represents the scale and *D* the diffusion tube.

A is a quartz mercury arc lamp, *B* a filter which transmits light of a wavelength 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 one.

The thermostat heating element was a chrome nickel wire 70 cm. long and 0.03 cm. in diameter. It was heated by a 60-cycle current of 1.5 amperes. The water in the thermostat was well stirred and its temperature was kept constant by a Vertex contact thermometer to within 0.005°.

Details of the diffusion tube [Svedberg, 1925, 1, 2] are shown in Fig. 2. The protein solution was placed in the limb *d* and the buffer or solvent in limb *a*. The limb *a* has an inner diameter of 1 cm. The tube was placed in the thermostat and, when thermal equilibrium has been reached, the stopcock *c* was opened and the protein was forced through the capillary tube *b* by slowly increasing the air pressure on the solution in limb *d*. When the boundary between the two solutions had reached a suitable position in limb *a* the stopcock was closed. Photographs of the diffusion process were taken at suitable intervals.

Fig. 2.

Fig. 3.

Fig. 2. Diffusion tube. The capillary *b* fits into the tube *d*. The diffusion process takes place in the limb *a*.

Fig. 3. Diffusion gradient curve for ovalbumin. The circles represent the ideal distribution. The abscissa axis is the direction of the diffusion process.

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 rate of change of refractive index with height was plotted against the height.

The equation for this curve has been developed by Wiener [1893] and has the form:

$$\frac{dn}{dx} = \frac{n_1 - n_0}{2\sqrt{\pi Dt}} \cdot e^{-x^2/4Dt} \quad \dots\dots(1),$$

where n_1 is the refractive index of the solution,

n_0 is the refractive index of the solvent,

D is the diffusion constant,

t is the time since diffusion started,

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

Several equations were derived from the above equation from which the diffusion constants were calculated. We give a few equations employed:

$$D = \frac{\mu^2}{2t} \cdot \left(\frac{l-b}{l} \right)^2 \quad \text{.....(2).}$$

μ equals half the distance between the two inflection points. The position of the inflection 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.

$$D = \frac{A^2}{4\pi t H_{\max}^2} \left(\frac{l-b}{l} \right)^2 \quad \text{.....(3),}$$

where A is the area enclosed by the curve and $H_{\max}$ the maximum ordinate of the curve.

$$D = \frac{x_1^2 - x_2^2}{4t \ln \frac{H_2}{H_1}} \left(\frac{l-b}{l} \right)^2 \quad \text{.....(4),}$$

x_1 is the abscissa at ordinate H_1 ,

x_2 is the abscissa at ordinate H_2 .

By taking H_2 as the maximum ordinate of the curve, x_2 disappears and equation (4) reduces to

$$D = \frac{x_1^2}{4t \ln \frac{H_{\max}}{H_1}} \left(\frac{l-b}{l} \right)^2 \quad \text{.....(4a).}$$

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

$$Z = Gab \frac{dn}{dx} \quad \text{.....(5),}$$

where G is the photographic enlargement factor,

a is the inner diameter of the diffusion tube,

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

Z is the photographic line displacement,

$\frac{dn}{dx}$ is the gradient of refractive index at the displaced line position.

By integration of the equation we get the relation:

$$A = \int_{-\infty}^{+\infty} \left(Gab \frac{dn}{dx} \right) dx = Gab (n_1 - n_0) \quad \text{.....(6).}$$

All the symbols have their previous significance. From this equation the refractive index increment $\frac{dn}{dc}$ of the protein solution may be calculated.

In addition to the above methods, calculation of diffusion constants was accomplished by a method which is used for obtaining the dispersion in statistical work. It depends on the assumption of treating the curves obtained from diffusion as ideal dispersions. By plotting the normal dispersion curve on that experimentally found conclusions can be drawn about the uniformity of the dispersion. The method of drawing the normal curve is that used by Pearson [1894].

The method is shortly illustrated as follows:

The base line of the curve (Fig. 3) is divided into equal units, for convenience the unit of 1 mm. was taken. We thus divide the base line into 20 to 30 units. Let the ordinates at 1, 2, 3, 4, 5, ... units be $y_1, y_2, y_3, y_4, y_5, \dots$. Then the n th

moment of the area about the vertical through the point of zero deviation is given by

$$1^n y_1 + 2^n y_2 + 3^n y_3 + 4^n y_4 \quad \dots\dots(7).$$

Now

$$\mu_n' = \frac{1^n y_1 + 2^n y_2 + 3^n y_3 + 4^n y_4}{A} \quad \dots\dots(8),$$

where A equals the area of the probability curve as before. We thus calculate μ_1' and μ_2' .

The distance of the centroid from the point of zero deviation is the ratio of its first moment to its area A and equals μ_1' . Having found the position of the centroid of the curve we calculate the second moment of the probability curve about the centroidal vertical. From μ_1' and μ_2' we calculate the standard deviation. This is given by the following relation¹

$$\sigma = \sqrt{\mu_2' - \mu_1'^2} \quad \dots\dots(9).$$

In terms of the diffusion constant the standard deviation σ equals $\sqrt{2Dt}$.

From tables compiled by Pearson [1894] the normal distribution curve can be plotted and the deviation of the experimental curve from the normal curve studied.

Preparation of the proteins.

Ovalbumin was prepared from hen's eggs according to Sørensen and Høyrup [1918], human CO-haemoglobin according to Ettish and Groscurth [1933]. Horse serum albumin and globulin were prepared by McFarlane according to the method described by him [1935].

Erythrocrucorin was obtained from the blood of *Planorbis corneus*.

Lactoglobulin was supplied by Dr K. O. Pedersen. The sample was prepared by Palmer [1934].

Gliadin was prepared and fractionated according to the method of Haugaard and Johnson [1931].

Before diffusion the respective protein solutions were dialysed against the buffers in order to make the electrolyte concentrations in both buffer and solution the same. Enough electrolyte was added to depress all charge effects [Tiselius, 1930].

Measurements.

Table IA gives the values obtained from an experiment with an electrolyte-free solution of ovalbumin.

In column I is given the time since the diffusion was started, column II the area enclosed by the diffusion curve and the base line, column III the maximum height of the curve, column V the diffusion constant calculated from formula (3) and column VI the diffusion constant calculated from formula (2). The symbols have their previous significance.

Table IB-H shows the results of experiments using different ovalbumin concentrations at p_H 4.6. For a buffer was used 0.02 M sodium acetate, 0.02 M acetic acid and 0.2 M NaCl.

The diffusion constants were in all cases corrected to the basis of diffusion in pure water at 20° by means of the relation

$$D \text{ corr.} = D \cdot \eta_s / \eta_w \quad \dots\dots(10),$$

where η_s is the viscosity of the solvent at 20°,

η_w is the viscosity of the distilled water at 20°.

¹ Sheppard [1897-98] calculated the correction which should be considered in calculating the moments of frequency curves; μ_2 in equation (9) becomes $\mu_2 - \frac{1}{12}$ when applying his correction, which is small in our present case.

Table II gives the results of an analysis of the curve obtained from the exposure after 24 hours in Table I E.

The curve was analysed by means of formula (4).

Table I. *Diffusion of ovalbumin.*

A, electrolyte-free; B-H, buffered. Bracketed average figures represent D_μ corrected according to formula (10).

Conc. of protein %	Time in sec.	Area cm. ²	Max. height	$(1 - b/l)^2$	$D_A \times 10^7$ cm. ² /sec.	$D_\mu \times 10^7$ cm. ² /sec.
A. 0.88	36 000	—	—	0.871	—	8.06
	50 400	—	—	0.871	—	8.53
	86 400	183.6	15.8	0.871	7.71	8.10
	118 800	189.1	12.9	0.871	8.27	7.64
	172 800	182.7	10.35	0.871	8.00	7.72
Average						8.01
B. 1.4	86 400	195.4	16.3	0.911	7.67	7.50
	115 200	195.2	14.3	0.911	7.50	7.50
	129 600	194.0	13.4	0.911	7.55	7.34
Average						7.44 (7.64)
C. 0.91	21 600	189.8	29.8	0.871	8.30	7.58
	29 700	186.1	25.9	0.871	7.49	7.41
	43 200	187.2	21.6	0.871	7.89	7.50
Average						7.50 (7.71)
D. 0.88	28 800	182.9	25.2	0.871	7.06	7.72
	43 200	180.0	20.8	0.871	7.58	7.35
	86 400	188.7	14.95	0.871	8.17	7.66
	115 200	180.0	12.9	0.871	7.50	7.46
	172 800	182.5	10.4	0.871	7.46	7.55
Average						7.55 (7.76)
E. 0.83	79 200	170.0	14.35	0.871	7.75	7.61
	86 400	170.0	14.0	0.871	7.43	7.43
Average						7.52 (7.73)
F. 0.7	28 800	168.2	23.3	0.871	7.66	7.57
	86 400	162.9	13.6	0.871	7.28	7.42
	115 200	161.6	11.7	0.871	7.30	7.52
Average						7.50 (7.71)
G. 0.5	36 000	104.4	12.3	0.871	9.54	8.42
	71 100	105.2	9.2	0.871	8.73	8.16
	110 000	100.0	7.2	0.871	9.04	8.03
Average						8.20 (8.40)
H. 0.15	14 400	48.0	7.8	0.8035	10.75	9.87
	43 200	52.0	5.0	0.8035	10.20	10.30
	86 400	52.9	3.7	0.8035	9.34	9.01
	93 600	52.6	3.6	0.8035	9.34	9.64
Average						9.71 (9.98)

Table II.

D_μ corrected according to formula (10) = 7.75×10^{-7} cm.²/sec.

Time in sec.	H_1	$H_{\max}$ cm.	x cm.	$(1 - b/l)^2$	$D_\mu \times 10^{-7}$ cm. ² /sec.
86 400	1.0	14	0.892	0.871	7.60
	4.0	14	0.615	0.871	7.60
	6.0	14	0.504	0.871	7.55
	8.5	14	0.385	0.871	7.45
Average					7.55

From the foregoing tables it can be seen that D_μ is constant between the concentration limits 0.7 and 1.4 % having a value of 7.71×10^{-7} cm.²/sec. Below a concentration of 0.7 % the diffusion constant increases on dilution.¹

In Fig. 3 the normal distribution curve is compared with the diffusion curve obtained from the exposure after 24 hours (Table I E). No deviations within small experimental errors are noticeable. Thus the diffusion process follows closely the ideal distribution law.

Human carbon monoxide-haemoglobin. Table III A gives the results of diffusion constants measurement on electrolyte-free CO-haemoglobin when diffused into distilled water.

Tables III B-E and IV give the results of experiments with different concentrations of CO-haemoglobin. For buffer was used 0.0085 *M* Na₂HPO₄, 0.014 *M* KH₂PO₄ and 0.1 *M* NaCl. The p_H was 6.5.

Table III.

A, electrolyte-free; B-H, buffered. Bracketed average figures represent D_μ corrected according to formula (10).

	Conc. of protein %	Time in sec.	Area cm. ²	Height cm.	(1 - b/l) ²	$D_A \times 10^7$ cm. ² /sec.	$D_\mu \times 10^7$ cm. ² /sec.
A.	0.8	39 600	122.0	13.8	0.9137	9.48	7.56
		86 400	113.0	9.0	0.9137	8.49	8.72
		97 200	110.0	8.5	0.9137	8.01	8.28
		108 000	121.0	8.3	0.9137	9.09	9.74
		129 600	113.9	7.2	0.9137	9.79	9.51
B.	3.8	86 400	536.0	47.6	0.9137	6.84	6.84
		97 200	541.8	44.5	0.9137	7.00	6.66
		158 400	524.4	35.6	0.9137	6.40	6.66
		180 000	539.4	33.6	0.9137	6.66	6.76
		Average					6.73 (6.83)
C.	2.8	57 600	391.0	42.2	0.9137	6.94	6.86
		86 400	395.0	34.4	0.9137	7.00	6.71
		100 800	399.0	32.7	0.9137	6.43	6.89
		144 000	400.0	27.4	0.9137	6.90	6.70
		172 800	396.4	25.0	0.9137	6.77	6.71
		Average					6.77 (6.90)
D.	0.82	86 800	112.5	9.9	0.9137	7.02	6.87
		100 800	118.0	9.4	0.9137	7.30	6.74
		115 200	120.0	9.1	0.9137	7.00	6.81
		172 800	114.0	7.2	0.9137	6.73	6.66
		Average					6.77 (6.90)
E.	0.4	68 400	143.5	13.6	0.8433	6.98	7.29
		86 400	144.2	12.0	0.8433	7.16	7.25
		144 000	143.0	9.5	0.8433	6.76	7.00
		154 800	140.0	8.8	0.8433	7.00	7.25
		Average					7.19 (7.34)

¹ Very little is known about this phenomenon. If the increase of the diffusion constant on dilution can be ascribed to dissociation of the protein molecules we ought to observe a fall of the sedimentation constant on dilution. This effect has been observed in the case of human CO-haemoglobin by Dr K. O. Pedersen of this laboratory [unpubl.]. Together with the authors' results (see results on CO-haemoglobin) we must conclude that CO-haemoglobin dissociates on dilution. That ovalbumin and serum albumin dissociate on dilution cannot yet be stated since the centrifugal evidence is still lacking.

Table IV.

Concentration 0.2%. D_μ corrected according to formula (10) = 7.54×10^{-7} cm.²/sec.

Time in sec.	$D_\mu \times 10^7$ cm. ² /sec.
32 400	7.65
86 400	7.31
97 200	7.38
129 600	7.21
Average	7.39

Table V. *Diffusion of 2% CO-haemoglobin against 1% CO-haemoglobin.* D_μ corrected according to formula (10) = 6.98×10^{-7} cm.²/sec.

Time in sec.	$D_\mu \times 10^7$ cm. ² /sec.
61 200	6.90
72 000	6.87
86 400	6.88
129 600	6.78
Average	6.85

Table VI. *Analysis of curve 158 400 sec. (Table III B).* D_μ corrected according to formula (10) = 6.74×10^{-7} cm.²/sec.

Time in sec.	$H_{\max}$	H_1	x cm.	$(1 - b/l)^2$	$D_\mu \times 10^7$ cm. ² /sec.
158 400	17.8	2.8	0.928	0.9137	6.61
158 400	17.8	4.8	0.780	0.9137	6.69
158 400	17.8	6.8	0.672	0.9137	6.76
158 400	17.8	9.8	0.528	0.9137	6.73
158 400	17.8	11.8	0.432	0.9137	6.56
158 400	17.8	13.8	0.332	0.9137	6.31
Average					6.61

From the foregoing tables it can be seen that the diffusion constant of CO-haemoglobin remains constant between the limits 3.8 and 0.8%. Below a concentration of 0.8% an increase in the diffusion constant is observed. This effect has previously been observed by Tiselius and Gross [1934] in the case of horse CO-haemoglobin.

The diffusion constants calculated from equation (3) vary very much; those calculated from equation (2) remain fairly constant.

A strong increase in the diffusion constant is observed in electrolyte-free solution and the values do not remain constant but increase with time from 7.56×10^{-7} cm.²/sec. to 9.5×10^{-7} cm.²/sec.

The diffusion constant of CO-haemoglobin has been determined by various writers. Tiselius and Gross, using the light-absorption method of Svedberg, determined a value of 6.3×10^{-7} cm.²/sec. at 20°. Northrup and Anson [1929] using the porous disc method obtained a value of 7.75×10^{-7} cm.²/sec. at 20°.

Tiselius and Gross's method of diffusing one concentration against another was followed in one experiment. The results are given in Table V. A 2% CO-haemoglobin solution was diffused against a 1% solution. No difference within experimental error was observed between this value and those in Tables III B-D.

An analysis of curve 158 400 sec. (Table III B) by means of equation (4a) gives the results tabulated in Table VI. We observe only a slight drift in the values.

A diffusion curve was compared with the normal curve (Fig. 4) but no deviation could be seen.

Fig. 4.

Fig. 4. Comparison between the diffusion gradient curve and the normal curve for CO-haemoglobin.

Fig. 5.

Fig. 5. Diffusion curve and ideal distribution points for serum albumin.

Serum albumin. Table VII A-C gives the results of experiments with serum albumin in different concentrations.

The buffer used was 0.019 *M* acetic acid, 0.181 *M* sodium acetate and 0.2 *M* NaCl.

In Table VIII we give the results of an analysis of curve 133 200 from Table VII B.

Table VII.

Bracketed average figures represent D_μ corrected according to formula (10).

	Conc. of protein %	Time in sec.	Area cm. ²	Height cm.	$(1 - b/l)^2$	$D_A \times 10^7$ cm. ² /sec.	$D_\mu \times 10^7$ cm. ² /sec.
A.	2.0	86 400	568.0	52.4	0.8347	5.67	5.88
		129 600	567.0	42.8	0.8347	5.77	5.80
		136 800	560.0	40.5	0.8347	5.80	5.90
		151 200	557.0	38.8	0.8347	5.80	5.89
		Average					5.87 (6.38)
B.	0.93	86 400	262.0	23.5	0.854	6.11	5.97
		133 200	264.0	19.4	0.854	5.90	5.94
		151 200	261.3	15.1	0.854	5.85	5.98
		162 000	263.0	17.7	0.854	5.80	5.92
		Average					5.95 (6.46)
C.	0.5	79 200	145.0	13.1	0.8347	6.50	6.22
		100 800	146.0	11.8	0.8347	6.48	6.23
		138 800	145.0	10.5	0.8347	5.85	5.80
		154 800	143.0	9.6	0.8347	6.11	5.95
		Average					6.05 (6.57)

Table VIII.

 D_μ corrected according to formula (10) = 6.46×10^{-7} cm.²/sec.

Time in sec.	H_1 cm.	$H_{\max}$	x cm.	$(1 - b/l)^2$	$D_\mu \times 10^7$ cm. ² /sec.
133 200	2	19.4	0.928	0.8347	6.07
133 200	4	19.4	0.772	0.8347	6.04
133 200	7	19.4	0.612	0.8347	5.90
133 200	10	19.4	0.492	0.8347	5.86
133 200	13	19.4	0.382	0.8347	5.85
133 200	16	19.4	0.368	0.8347	5.99
Average					5.95

We observe that the diffusion constant does not vary very much between 2 and 0.93 %. At 0.5 % there is a considerable increase in the diffusion constant. The experimental curve from exposure 133 200 (Table VII B) shows a marked departure from the normal curve, see Fig. 5. It has been found that the diffusion of serum albumin followed the ideal distribution law very closely when material prepared by a different method was employed. The investigation is not completed and the results will be published in a later paper.

Serum globulin. This protein proved to be not uniform; it was composed of a main component of sedimentation constant 7.1×10^{-13} cm.²/sec. and two other components of higher sedimentation constants together with small molecules.

The protein was diffused into a buffer composed of 0.019 *M* acetic acid, 0.181 *M* sodium acetate and 0.2 *M* NaCl; p_H 5.6.

No reliable diffusion constants could be obtained as will be seen from Table IX. In Table IX a curve obtained was analysed by means of formula (4a).

Table IX.

Time in sec.	H_1 cm.	$H_{\max}$	x cm.	$(1 - b/l)^2$	$D_\mu \times 10^7$ cm. ² /sec
108 000	3	31	0.652	0.8543	3.61
108 000	4	31	0.588	0.8543	3.75
108 000	5	31	0.532	0.8543	3.76
108 000	10	31	0.460	0.8543	3.70
108 000	16	31	0.348	0.8543	3.62
108 000	22	31	0.240	0.8543	3.02
108 000	25	31	0.192	0.8543	2.95

From the above figures it is evident that no diffusion constant can be given. The large drift in the apparent diffusion constant is caused by the different mobilities of the differently sized particles.

Fig. 6. Diffusion curve and ideal distribution points for serum globulin.

The diffusion curve from which the above values were obtained was compared with the normal distribution curve and a very large departure was obtained, see Fig. 6. Such a departure is characteristic for a non-uniform dispersion.

Gliadin. Gliadin was chosen to illustrate the method employed by the writers to use the method of diffusion as a test for the uniformity of a protein.

The crude gliadin obtained by extracting gluten with dilute alcohol (60% by volume) was diffused into the same concentration of alcohol.

0.1 *M* NaCl was added to depress the charge effects. The diffusion constant calculated from the standard deviation (here called the standard deviation diffusion constant) of the frequency curve gave a value of 6.04×10^{-7} when corrected for the viscosity of the solution. The diffusion curve compared with the normal curve is given in Fig. 7. There is a very large departure from the normal curve.

Fig. 7. Diffusion curve and ideal distribution points for crude gliadin.

Fig. 8. Diffusion curve and ideal distribution points for 0° fraction of gliadin.

The crude gliadin solution in alcohol was cooled to 0°, and kept at that temperature for 4 days. A precipitate occurred which was purified by means of Haugaard and Johnson's method. This fraction in a concentration of 0.616% gave a normal diffusion constant of 4.96×10^{-7} cm.²/sec. This fraction proved to be very non-uniform according to the comparison of the diffusion curve with the normal curve (Fig. 8).

Fig. 9. Diffusion curve and ideal distribution points for -10° fraction of gliadin.

The solution obtained after the extraction of the 0° fraction was cooled to -10° and kept at this temperature for 7 days. Another fraction of gliadin

precipitated here. A 0.62% solution of this fraction after purification gave a normal diffusion constant of 5.85×10^{-7} cm.²/sec. at 20°. A diffusion curve compared with the normal curve still shows a departure (Fig. 9).

The clear solution remaining after the extraction of the 0° and -10° fractions ought to be the most uniform according to Krejci and Svedberg's [1935] ultracentrifugal study of gliadin. This fraction after purification was diffused and gave a normal diffusion constant of 6.72×10^{-7} cm.²/sec. The diffusion curve fits the normal distribution curve very well. No departure from the normal curve could be seen (Fig. 10).

Fig. 10. Diffusion curve and ideal distribution points for the remaining fraction of gliadin. The ideal form of the curve shows the uniform character of this fraction.

According to a suggestion made by Krejci and Svedberg the gliadin remaining in solution after the extraction of the -10° fraction consists of molecules of molecular weights 34,500 and 17,000 the two molecules having the same sedimentation constant. A departure of the experimental curve from the normal curve ought to be seen if this were the case. No such evidence could be obtained from the diffusion curves.

Erythrocrucorin. The erythrocrucorin was diffused into a solvent containing 0.05 *M* Na₂HPO₄, 0.05 *M* KH₂PO₄ and 0.1 *M* NaCl. The *p*_H of the solvent was 6.67.

Table X gives the results on diffusing a 0.44% solution in the above-mentioned solvent.

Table X.

D_{μ} corrected according to formula (10) = 1.962×10^7 cm.²/sec.

Time in hours	$D_{\mu} \times 10^{-7}$ cm. ² /sec.
48	1.865
72	1.885
96	1.875
144	1.850
Average	1.869

D_A is omitted in favour of D_{μ} which remained much more constant as can be seen in all the previous cases.

Lactoglobulin. The lactoglobulin was diffused into solvents of different hydrogen ion concentrations. The following solutions were used:

*p*_H 5.0: 0.012 *M* acetic acid, 0.038 *M* sodium acetate and 0.2 *M* NaCl.

*p*_H 5.4: 0.001 *M* Na₂HPO₄, 0.019 *M* KH₂PO₄ and 0.2 *M* NaCl.

*p*_H 9.5: 0.02 *M* Na₂CO₃, 0.02 *M* Na₂B₄O₇ and 0.2 *M* NaCl.

A diffusion curve was compared with the normal curve. No departure was obtained.

Table XI.

Bracketed average figures represent D_μ corrected according to formula (10).

	Conc. of protein (%)	p_H	Time in hours	$D_\mu \times 10^7$ cm. ² /sec.
A.	1	5.0	11.25	6.97
			17.25	6.95
			35.00	6.95
			Average	6.953 (7.18)
B.	1	5.4	17.00	6.91
			24.00	6.86
			30.00	7.00
			Average	6.925 (7.10)
C.	1	9.5	20.00	6.46
			27.00	6.50
			Average	6.48 (6.78)

DISCUSSION.

Calculation of refractive index increment $\frac{dn}{dc}$.

According to equation (6) we ought to be able to calculate the refractive index increments $\frac{dn}{dc}$ of the different proteins from the areas included by the curves with the base lines. This is verified in Table XII.

Table XII.

Ovalbumin, $\lambda=436$ m μ .						
a cm.	b cm.	G	Conc. %	Area cm. ²	Area/conc.	$dn/dc \times 10^5$
1	10.1	0.865	0.50	103.2	206.4	189.0
1	10.1	0.865	0.83	170.0	204.8	187.5
1	10.1	0.865	0.88	182.5	207.3	189.8
1	10.1	0.865	0.91	187.7	206.2	188.8
						Average 188.8
CO-haemoglobin, $\lambda=656$ m μ .						
1	7	0.825	3.80	535.4	141.0	195.4
1	7	0.825	2.80	396.4	141.5	196.0
1	7	0.825	0.82	116.0	141.1	195.5
						Average 195.6

Mr K. Andersson of this laboratory kindly placed some of his determinations of $\frac{dn}{dc}$ by the Pulfrich refractometer at our disposal; for CO-haemoglobin he obtained 194×10^{-5} for the line $\lambda=656$, and for ovalbumin for the line $\lambda=436$ he obtained a value of 187.8×10^{-5} .

Calculation of the molecular weights.

Svedberg [1925, 1, 2; 1927] developed a formula making use of the relation between sedimentation and diffusion constants from which the molecular weights can be calculated. The relationship is the following:

$$M = \frac{RTs}{D(1 - V\rho)},$$

where M is the molecular weight,
 s the sedimentation constant,
 D the diffusion constant,
 V the partial specific volume of the protein,
 ρ the density of the solvent,
 R the gas constant, and
 T the absolute temperature.

Table XIII gives the molecular weights calculated from the above formula. For the diffusion constants were taken those calculated from the standard deviation. These diffusion constants will be the most reliable.

Table XIII.

Protein	$s \times 10^{13}$ cm. ² /sec.	V	$D \times 10^7$	M	
Ovalbumin	3.55	0.749	7.76	43,800	40,500*
CO-haemoglobin	4.50	0.749	6.90	63,000	68,000*
Serum albumin	4.50	0.748	6.45	67,100	66,900†
Gliadin	2.10	0.722	6.72	27,500	26,750‡
Erythrocrucorin	33.70	0.745	1.96	1,634,000	1,539,000§
Lactoglobulin p_H 5.0	2.95	0.751	7.18	40,000	37,800*
Lactoglobulin p_H 9.5	2.76	0.751	6.78	39,600	—

* [Pedersen, unpubl.].

† [Mutzenbecher, 1933].

‡ [Krejci and Svedberg, 1935].

§ [Eriksson-Quensel, 1934].

In the last column of the above table the molecular weights as determined by Svedberg's equilibrium centrifuge method are given. We draw attention to the fact that the sedimentation and diffusion constants of lactoglobulin both fall at p_H 9.5 giving the same molecular weight as that found at p_H 5.0. The particle most probably changed its shape thereby acquiring a higher frictional constant.

SUMMARY.

1. A refractometric method has been described for measuring diffusion constants of proteins.

2. Several methods for calculating the diffusion constants from the curves are discussed.

3. The diffusion constants of several well-known proteins have been determined, *viz.* ovalbumin, human CO-haemoglobin, serum albumin, gliadin, erythrocrucorin (*Planorbis corneus*) and lactoglobulin. It has been found that the diffusion constants of ovalbumin, CO-haemoglobin and serum albumin increase very much in dilute solutions below 0.5%.

4. A method used in statistical work for obtaining the dispersion has been applied to the diffusion curves. It has been found that in monodisperse solutions the diffusion follows the ideal dispersion law very closely. In cases where we diffused polydisperse solutions we obtained departures from the normal curves.

5. From the areas included by the diffusion curves we have calculated the increments of refractive indices. The values obtained agree very closely with those determined by K. Andersson.

6. From the diffusion and sedimentation constants the molecular weights have been calculated, the values obtained agree well with those found by the equilibrium centrifuge.

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