CCCVIII. OSMOTIC EQUILIBRIA OF HAEMOCYANIN IN A GRAVITATIONAL FIELD.

By JEAN ROCHE, ANDRÉE ROCHE, GILBERT SMITHSON ADAIR
AND MURIEL ELAINE ADAIR.

From the Biochemical Laboratory, University of Marseilles and the Physiological Laboratory, Cambridge.

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RECENT work on the sedimentation of haemocyanin in the ultracentrifuge has been reviewed by Svedberg [1933], who has given the value 5,100,000 for the molecular weight of the haemocyanin of the snail. A brief note on the possibility of demonstrating the existence of very large molecules by osmotic methods has been published by Adair *et al.* [1934].

The present communication records a method for the preparation of the haemocyanins, which eliminates certain impurities, and data of the osmotic pressures of blood (or haemolymph), once crystallised and thrice crystallised haemocyanins, using material obtained from the snail (*Helix pomatia*), the crab (*Carcinus moenas*) and the octopus (*Octopus vulgaris*). In addition, a method is described for studying the distribution of proteins in a gravitational field. This method differs from those used by previous workers for large colloidal particles in a gravitational field or for the proteins in centrifugal fields, in that two phases are equilibrated in a gravitational field by means of a semipermeable membrane. The thermodynamical treatment of such equilibria has been given by Adair [1935].

1. Preparation of haemocyanin.

The comparison of a number of preparations of haemocyanin is of interest, for it is possible that the average size of the particle might depend on the procedure adopted for separating the protein from impurities. In this work, the following preparations have been examined.

(a) Blood, dialysed against a mixture of salts calculated from the analyses of snail's blood [Arvanitaki and Cardot, 1932], or against the *M*/15 phosphate mixtures used for the preparations 2 and 3.

(b) Once crystallised haemocyanin. The haemocyanins of the snail, the crab and the octopus have been prepared by prolonged dialysis of the bloods at 0° against distilled water. It has been known since the publication of Dhéré's researches [Dhéré, 1908; Dhéré and Burdel, 1914] that on dialysis of the blood of *Helix pomatia*, crystalline haemocyanin is deposited after a period varying from a few weeks to two months. Although the haemocyanins are globulins, precipitating at the isoelectric point, many authors have stated that with the blood of certain animals, including *Limulus*, no precipitation or crystallisation is obtained on dialysis. In this work it has been observed that even with animals such as *Limulus*, the protein is deposited in an amorphous or a crystalline state, according to the rapidity with which diffusible ions are eliminated, although in certain cases it may be necessary to continue dialysis for at least two months.

The vessels used for dialysis were made of sparingly soluble glass. In some experiments, the time required for the formation of a precipitate was reduced by

the addition of a trace of potassium hydrogen phosphate or of an isoelectric acetate buffer solution to the water used for dialysis.

Each preparation of octopus haemocyanin was made from the blood obtained by the vascular puncture of about 5 animals. In the cases of *Helix* and *Carcinus* several hundred animals were required. The blood of *Helix* was obtained by cardiac puncture; it may be noted that the majority of the preparations used were made in the winter months. The blood of *Carcinus* was obtained by puncture of the articulations.

In all cases, the blood was dialysed against distilled water, changed twice daily, for about 8 days. As a general rule, a whitish deposit of protein was obtained after about a week's dialysis. It was found that this deposit contains only traces of copper, and it is therefore not haemocyanin. In preparations from the blood of crustaceans, this precipitate contains the orange carotenoid astacin (tetronerythrin), which is sometimes present in quantity.

When the first deposit of protein and other impurities was obtained, the supernatant material was transferred to a new membrane and dialysis was continued. An additional precipitation of impurities sometimes took place, in which case the material was again transferred to another membrane. The solution was not considered to contain pure haemocyanin unless it remained absolutely limpid for at least 8 days. At the end of 3 to 10 weeks, the pigment deposited in the crystalline state in the case of *Helix*, and in crystals mixed with amorphous matter in the case of *Octopus* and of *Carcinus*. In a few experiments instead of waiting for spontaneous crystallisation, the protein was brought to the isoelectric point by the addition of $N/40$ HCl, drop by drop, with continuous stirring. If dialysis were then continued for some weeks, it was possible to obtain abundant deposits of haemocyanin.

The contents of the membranes were centrifuged when a sufficient amount of haemocyanin was obtained. The purified protein was then dissolved in $M/15$ Sørensen phosphate buffer, usually at p_H 7.5; concentrated solutions, up to 10% of protein, could be prepared in this manner.

(c) Thrice crystallised haemocyanin. The once crystallised material was washed with water on the centrifuge, dissolved by the addition of sodium chloride, as described in detail by Dhéré and by Burdel [1922], reprecipitated by dialysis, rewashed and redissolved and finally reprecipitated by dialysis and dissolved in phosphate buffer.

2. *Experimental methods for measuring the distribution of proteins in a gravitational field.*

Perrin [1908] devised methods for the determination of the distribution of microscopic or ultramicroscopic particles over short distances in a gravitational field. Burton [1922] and Porter and Hedges [1922] have made observations over longer distances (0.5 to 90 cm.) and concluded that there are forces acting between the particles which prevent the application of the simple formulae given by Perrin. Svedberg [1928] has criticised the methods of Burton and of Porter and Hedges, drawing attention to the difficulty of obtaining equilibria in long tubes. It may be noted that earlier work with his ultracentrifuge, using long tubes, was unsuccessful, and in the present form the distances are of the order of 5 mm. In the case of proteins, the theoretical formulae given by Adair [1935] indicate that distances of 2 cm. or more are required to produce measurable differences of concentration in a gravitational field.

Preliminary experiments with globulin (unpublished) indicated that the difficulties inherent in the use of long tubes could be, in some respects, eliminated

by the use of a system of the type shown in Fig. 1, where a cylindrical collodion membrane is filled with protein solution and placed in a tube of dialysate. In this apparatus, equilibration may take place by diffusion and sedimentation along the axis of the tube, as in the apparatus used by previous investigators, but in addition, the approximation to the equilibrium distribution may be facilitated by the movement of solvent across the membrane.

A solution of uniform composition with a density greater than that of the solvent is not in osmotic equilibrium, because the difference in densities of columns of protein solution and dialysate should lead to a difference in osmotic pressure and therefore solvent should flow out in the lower part of the membrane, causing an increase in concentration, to correspond with the higher pressure. At the same time, solvent should flow in and reduce the concentration of protein in the upper part of the column.

It is probable that, if a cell with two walls of glass or quartz were used, the most accurate method of determining the concentration gradient would be by the use of an interferometer, whereby the concentration of the protein at different depths could be estimated and the process of equilibration followed by taking readings on successive days. In the absence of an optical device of sufficient accuracy, a preliminary investigation has been made by leaving the protein solutions contained in cylindrical collodion membranes to equilibrate for a given time and then withdrawing samples from different depths for analysis, as described below. The measurements of the concentration gradient were combined with measurements of osmotic pressures which were made by a modification of the procedure described by Adair [1925].

The collodion membranes, from 5 to 20 cm. in length, were prepared by pouring 3 coats of collodion over rotating glass moulds of approximately 1.13 cm. diameter, giving membranes of capacity 1 ml. per cm. of length. The membranes were mounted on 3 cm. lengths of pressure tubing, slightly distended by a short length of glass tube shown in Fig. 1. The permeability of the membranes was adjusted so that at a pressure of 450 mm. mercury, approximately 0.01 ml. water per cm. of length flows out in one minute. The membranes were filled with haemocyanin solution dissolved in *M*/15 Sørensen phosphate buffer mixtures and attached to glass tubes of external diameter 6 mm. and internal diameter 1.4 to 3 mm. graduated in cm. and mm. The membranes were then fixed in tubes of buffer solution as shown in the figure. The temperature was regulated at 0° by placing the tubes in thermos flasks containing ice and water, which were kept in a refrigerator.

The level of the solution in the manometer was read daily for periods from 7 to 30 days, and in most cases the level was reset at a higher or lower point to see if the same pressure was reattained. After equilibration, the levels of the protein solution and of the dialysate, marked h and y_0 respectively in Fig. 1, were

Fig. 1. Apparatus for measurement of osmotic pressure and concentration of protein at different depths, h = height of protein solution in manometer; y = level of dialysate. Rigid collodion membrane enclosing haemocyanin solution indicated by broken line.

read with an accuracy of 0.02 cm. on the glass scale. The distance between y_0 and y_1 , the end of the rubber tube, was estimated with an accuracy of 0.05 cm. with a ruler. The collodion tube was then lifted out of the dialysate, detached from the manometer and secured by a clamp at the point where the rubber tube is made rigid by the short glass tube. A sample of protein solution down to the level y_2 (a few mm. below the edge of the rubber) was then removed and used for the measurement of the capillarity correction. The rigidity of collodion membranes made by the method referred to above is sufficiently great for them to be lifted out of the dialysate without appreciable distortion, and it was therefore possible to estimate the depth of the solution at y_2 (defined as the distance between y_0 , the surface of the dialysate, and y_2) as the sum of the distance between y_0 and y_1 , measured with the membrane in the dialysate and the distance between y_1 and y_2 , measured with the membrane exposed to the air.

A capillary pipette, secured in a clamp, was then dipped into the solution and a sample down to the depth y_3 , about 4 cm. below y_2 , was removed very slowly by suction, controlled by a rubber tube and a glass tap. It is impossible to avoid all disturbance of the column, and uniformity of all details of procedure is desirable, so that as far as possible, the errors due to admixture with relatively light solution initially above y_2 and with the relatively heavy solution initially below y_3 cancel out. The sample from y_2 to y_3 was transferred to a refractometer beaker and mixed by means of the capillary pipette. Similar samples from y_3 to y_4 and from y_4 to y_5 were obtained by the same method. The refractive indices of the dialysate and of all the samples were determined by means of the Zeiss dipping refractometer. In a few experiments, the nitrogen content of individual samples was determined by the method of Kjeldahl. As a general rule, the samples from the refractometer beakers were afterwards pooled and the mixture used for measurements of nitrogen, density and refractive index.

3. Relationship between refractions, concentrations and densities of haemocyanin solutions.

In this work, the calculations of the protein concentration from nitrogen determinations were based on the values for the nitrogen content of the dry protein given by Roche [1934] and quoted in Table I. The refractive indices of protein solutions equilibrated with phosphate buffers have been discussed theoretically by Adair and Robinson [1930, 1]. Their measurements showed that under these conditions, the refractive indices of solutions of serum albumin and globulin are directly proportional to the protein concentration C , calculated from nitrogen determinations. In the present work, it was found that the refractive index of the solutions of haemocyanin was directly proportional to the concentration up to 10%, and it follows that the concentration can be calculated by the simple formula No. 1.

$$C = (R' - R'') \left(\frac{1}{\alpha'} \right) \quad \text{.....(1),}$$

C = g. dry protein per 100 ml. solution; R' = refractive index of protein solution; R'' = refractive index of dialysate; α' = an empirical constant, equal to the refraction differences ($R' - R''$) for a 1% solution.

It may be noted that Redfield's [1934] value of 0.00198 for the specific refraction increment of *Limulus* haemocyanin dissolved in water exceeds the values for α' given in Table I. The difference is not due to accidental errors; in the case of *Helix*, for example, where in a series of 9 determinations the maximum and minimum values are 0.00186 and 0.00175 respectively, the probable error of the

mean (0.00179) is less than 0.00001. One factor which may cause differences between α' and the specific refraction increment for aqueous solutions is the ionisation of the proteins and the unequal distribution of salts across the membrane.

Table I. *Nitrogen content, refraction increments and density coefficients of haemocyanins equilibrated at 0° with M/15 phosphate buffer, p_H 7.5.*

Species	<i>Helix</i>	<i>Octopus</i>	<i>Carcinus</i>
Nitrogen percentage of dry protein [Roche, 1934]	15.22	16.09	16.83
α' (Formula 1)	0.00179	0.00184	0.00187
$1/\alpha'$	559.0	543.0	535.0
k (Formula 2)	0.27	0.27	0.27

The measurements of densities recorded in Fig. 2 show that there is a straight line relationship between density and the concentration C , as stated in the empirical formula No. 2.

$$D' = D'' + kC/100 \quad \dots\dots(2).$$

The percentage error in this series of measurements of densities was greater than in the measurements of refractive indices, and the constant k could not be estimated to more than 2 significant figures. The values for different species are given in Table I.

Fig. 2. Densities at 0° of solutions of haemocyanin equilibrated with M/15 phosphate buffer, p_H 7.5, density 1.009. Circles, *Helix*; squares, *Octopus*; crosses, *Carcinus*.

4. Measurements of the distribution of haemocyanin in a gravitational field.

The concentrations of samples of haemocyanin from different depths are recorded in Table II. The calculations of concentrations and ratios have been made and recorded to at least one decimal place more than the accuracy of the measurements of refractive index and other data. The values of the ratio C_2/C_1 in the sixth column represent the concentration of the second sample divided by the concentration of the first sample. The values for C_2/C_1 for different experiments are not strictly comparable, because the mean depths are not identical. In the seventh column the observed values have been used to calculate the value of the ratio C_2/C_1 on the assumptions that the difference in depth between the first and second samples is 1 cm. and that the increase in C_2 is proportional to the depth over the range investigated.

Table II. *Increase of concentration of haemocyanin with depth in a gravitational field.*

Letters H, O and C denote *Helix*, *Octopus* and *Carcinus* respectively. Dialysate *M*/15 phosphate buffer, p_H 7.5, except for H 2 and H 7, which were at p_H 6.8, and H 8, which was blood of *Helix* with dialysate 0.1435 *M* NaCl, 0.0087 *M* KCl, 0.0159 *M* CaCl₂, 0.0428 *M* MgCl₂ [Arvanitaki and Cardot, 1932]. C 30 *Carcinus* blood.

Species and exp. no.	Mean depth. First sample	Mean depth. Second sample	Concentration. First sample	Concentration. Second sample	Ratio C_2/C_1	Ratio for 1 cm.
H 3	7.39	10.52	6.142	6.246	1.0170	1.0051
H 4	8.05	11.88	4.335	4.407	1.0166	1.0043
H 5	8.65	11.88	3.435	3.496	1.0178	1.0055
H 7	8.35	11.43	3.513	3.715	1.0576	1.0187
H 2	7.68	10.38	2.624	2.733	1.0417	1.0154
H 8	8.93	12.88	2.922	3.019	1.0333	1.0084
H 8	12.88	17.58	3.019	3.161	1.0470	1.0100
O 20	8.67	11.99	8.985	9.152	1.0186	1.0056
C 11	8.19	11.87	3.192	3.233	1.0128	1.0035
C 12	8.35	11.71	3.269	3.328	1.0181	1.0054
C 14	6.95	10.31	8.134	8.351	1.0267	1.0080
C 30	6.15	10.20	4.129	4.160	1.0075	1.0019
C 30	10.20	14.90	4.160	4.222	1.0015	1.0032

In the series of experiments on *Helix* haemocyanin, marked H, which were equilibrated for periods from 10 to 16 days, the ratio varied from 1.004 to 1.019. In the experiments on *Octopus* and on *Carcinus* haemocyanins, marked O and C respectively, carried out later, the times of equilibration were increased to about 25 days. The ratios show a range of variation from 1.002 to 1.008. It may be noted that the chief sources of error, including imperfect equilibration and accidental disturbances in taking the sample, tend to give low values for the ratio.

5. The estimation of the osmotic pressure of haemocyanin.

In the majority of protein solutions, the osmotic pressure can be calculated with sufficient accuracy by the formula (3).

$$p_0 = \frac{10D'}{13.595} (h - x) \quad \dots\dots(3),$$

p_0 = osmotic pressure in mm. mercury at 0°, measured in a membrane permeable by electrolytes but impermeable by the protein; D' = density of the solution; 13.595 = density of mercury; h = observed height of column of solution expressed in cm. solution; x = rise due to capillarity.

It will be noted that p_0 represents the osmotic pressure in the protein solution at the point y_0 in Fig. 1, where the depth of the dialysate is equal to 0. At a point y cm. below y_0 the protein solution is subjected to a higher pressure on account of its greater density, and the total osmotic pressure p at the depth y is given by formula (4), which is exact.

$$p = p_0 + \frac{10}{13.595} \int_0^y (D' - D'') dy \quad \dots\dots(4).$$

In the majority of protein solutions, the integral is small in comparison with p_0 , but in the case of haemocyanin, the integral is of greater importance, as shown by the data in Table III, obtained for a specimen of snail's blood (Exp. 8). The densities at different depths recorded in the table were calculated by applying formula (2) to the measurements of concentration computed from refractive

Table III. *Osmotic pressures, Exp. H 8, calculated by formulae (4) and (5).*

Depth	Concentration	$(D' - D'')$	p (formula 4)	p (formula 5)
0	2.682	0.0072	0.358	0.358
8.93	2.922	0.0079	0.408	0.405
12.88	3.019	0.0082	0.431	0.426
17.58	3.161	0.0085	0.460	0.451

indices. The value for zero depth was estimated by extrapolation. It will be observed that the density difference $(D' - D'')$ given in column 3 does not increase very rapidly with the depth, and it follows that the pressure calculated by formula (5) must be approximately equal to the exact value given by formula (4).

$$p = p_0 + \frac{10}{13.595} (D' - D'') y \quad \dots\dots(5).$$

In formula (5), y is equal to the mean depth of the sample taken for analysis and D' is the density of the sample. The range of error involved is small, as shown by the figures in the fifth column of Table III, where p has been calculated without making any allowance for the increase in $(D' - D'')$ below the point where $y = 0$. It will be seen that the pressure p may be about 28% greater than p_0 calculated by formula (3), in the case of the haemocyanin of the snail. Similar calculations of the proportion of the total pressure due to the term $(D' - D'') y$ can be made from the experimental data recorded in Tables II and VII.

6. Relationship between the osmotic pressure and the protein concentration.

In a previous paper [Roche *et al.*, 1932] two different methods of stating the composition of protein solutions were described, namely, the "corrected percentage concentration", symbolised C_v , expressed in g. dry protein per 100 ml. solvent, and the concentration, symbolised C , expressed in g. dry protein per 100 ml. protein solution. The theoretical treatment of the distribution of haemocyanin in a gravitational field may be simplified as described in the following section 7 by the use of the term C rather than C_v , because the densities of the solutions are directly proportional to C as stated in formula (2) and the correlation of such measurements with determinations of osmotic pressure is facilitated by the use of C rather than of C_v .

The curves in Fig. 3 show the relationship between the osmotic pressure and the concentration C when the haemocyanins prepared from the blood of the snail, the octopus and the crab are equilibrated with $M/15$ phosphate buffers of p_H 7.4 and 7.5 at 0° . It may be noted that the pressures are extremely small, especially in the more dilute solutions. In spite of the difficulties inherent in the measurements of low pressures, and of the additional term in the calculations of pressures defined by formula (5), the points lie close to the smooth curves shown in the figure. The points shown in this figure do not give a complete record of the data for *Helix*. One additional series of measurements made at p_H 6.8 is summarised in Table IV. The difference between the osmotic pressures at p_H 6.8 and 7.5 seems to be less than 0.1 mm.

In four experiments on the whole blood of *Helix*, at p_H 7.5, containing about 3.09% haemocyanin, the values of the ratio $p/C = \pi$ were 0.15, 0.30, 0.21 and 0.22 respectively. These values are of the same order as those obtained for crystallised haemocyanin, and it may be inferred that the process of purification does not cause great changes in the structure of the molecules. Further evidence on this point was obtained in an experiment on crab's blood at p_H 7.4, where the pressure of 1.47 mm. at a concentration $C = 4.06$ is in agreement with the curve for purified haemocyanin, shown in Fig. 3. The results recorded in Table V

Fig. 3. Osmotic pressure at 0° of haemocyanin equilibrated with $M/15$ phosphate buffer p_H 7.5. Circles, *Carcinus*; squares, *Octopus*; crosses *Helix*; broken lines, theoretical osmotic pressures of proteins of molecular weights 710,000 and 1,800,000, calculated according to van't Hoff's law.

Table IV. Osmotic pressure of *Helix haemocyanin* at p_H 6.8.

Exp. no.	<i>C</i>	<i>p</i>	$\pi (=p/C)$
1	3.41	0.35	0.10
7	3.62	0.32	0.08
58	3.49	0.31	0.09
59	3.16	0.41	0.13

Table V. Osmotic pressure and membrane potentials of thrice crystallised haemocyanin at p_H 7.4.

E = potential in m.v. at 0°; p_i = calculated pressure of diffusible ions.

Exp. no.	Animal	<i>C</i>	<i>E</i>	p_i (calc.)	<i>p</i> (observed)
1 (3)	<i>Octopus</i>	3.55	-0.44	1.03	1.02
2 (3)	<i>Octopus</i>	2.84	-0.36	0.69	0.54
3 (3)	<i>Helix</i>	1.96	-0.23	0.28	0.30
4 (3)	<i>Helix</i>	0.90	-0.19	—	0.06

show that material subjected to three crystallisations gives pressures similar to those obtained with material once crystallised.

The relationship between the osmotic pressure p , expressed in mm. mercury at 0°, and the molecular weight is formulated below:

$$p = RT\phi c_p = RT\phi \frac{10C}{M} = 170,330\phi \left(\frac{C}{M}\right) \quad \dots\dots(6),$$

where T = absolute temperature; R = gas constant. At 0° , RT = product of pressure and volume of 1 g. mol. of a perfect gas = 760 mm. $\times$ 22.414 litres = 17,033; c_p = g. mol. protein per litre protein solution; M = molecular weight, or the mean molecular weight if the protein be polydisperse; ϕ = a coefficient, equal to the ratio p/RTc_p .

The term ϕ represents the sum of the osmotic effects due to three factors: (1) the volume occupied by the protein hydrate, (2) the "partial pressure" symbolised p_i , due to the excess of diffusible ions inside the membrane, referred to in Donnan's theory and (3) attractive and repulsive forces between the protein particles. Previous investigations on haemoglobin [Adair, 1928] and serum proteins [Adair and Robinson, 1930, 2] have shown that ϕ approaches unity at low protein concentrations, so that accurate values of molecular weights can be determined by extrapolation. In the case of haemocyanin, the osmotic pressures of the dilute solutions are so small that the application of methods of extrapolation must be postponed.

Table VI includes a series of values of the ratio M/ϕ which is equal to $10RTC/p$ calculated from the curves shown in Fig. 3. It will be seen that this ratio diminishes with increasing concentrations, in accordance with observations on haemoglobin and on serum proteins referred to above, which shows that ϕ is greater than unity even in the case of 3% solutions, and it may be inferred that the molecular weights of the haemocyanins are greater than the values for such solutions entered in the second column of Table VI.

Table VI. Values of the ratio M/ϕ at different concentrations of haemocyanin.

Animal	$C=3.0$	$C=6.0$	$C=9.0$
<i>Helix</i>	1,800,000	1,400,000	—
<i>Octopus</i>	710,000	630,000	570,000
<i>Carcinus</i>	550,000	440,000	330,000

The values obtained by the osmotic method are comparable with ultracentrifugal measurements. The sedimentation velocity of the haemocyanin of *Carcinus*, recorded by Svedberg [1933] corresponds to a molecular weight of $620,000 \pm 40,000$, a figure rather larger than $M/\phi = 550,000$ estimated by the osmotic method, but it is obvious from the data in Table VI that M/ϕ tends to increase with dilution, and a value of $M = 620,000$ is consistent with the osmotic measurements. In the case of the haemocyanin of *Octopus*, the measurements of sedimentation velocity made by Svedberg and Eriksson [1932] show that the molecular weight depends upon the p_H value. In certain ranges the molecular weight is about 2,000,000, but at $p_H 7.5$ the mean molecular weight may be about one-fifth of this value ($400,000 \pm 150,000$), a figure which is below the value $M/\phi = 710,000$ obtained by the osmotic method. It is not unlikely that the difference may be accounted for by the fact that the centrifugal measurements are made on dilute (0.5%) solutions at 20° , whereas the osmotic measurements were made on more concentrated solutions at 0° .

In the case of the haemocyanin of *Helix*, which has been studied in detail by ultracentrifugal methods, the molecular weight is 5,100,000, a value more than three times as great as M/ϕ estimated by osmotic methods. One possible explanation of the difference is the pressure due to the excess of diffusible ions inside the membrane, symbolised p_i . Calculations of p_i have been made as described by Adair and Robinson [1930, 2] and recorded in Table V. It appears that the effect of the term p_i must be balanced by interionic attractions, because the observed pressures for concentrated solutions are lower than the

(extrapolated) values for p_i . Osmotic abnormalities of this type were observed by Hammarsten [1924] and have been discussed thermodynamically by Linderstrøm-Lang [1926]. In the circumstances, it is by no means certain that the discrepancy can be accounted for by the term p_i .

In the case of a substance of molecular weight 5,100,000 the presence of relatively small amounts of less polymerised particles may cause marked alterations in the mean molecular weight, and it is not unlikely that the relatively low value of 1,800,000 for M/ϕ is partly due to the polydisperse state of the solution. In experiments where chloroform was added as a preservative, M/ϕ was reduced to 700,000, an observation which suggests that the haemocyanin of *Helix* tends to form degradation products.

7. *Theoretical calculation of the distribution of a protein in a gravitational field from measurements of osmotic pressure.*

In the system shown in Fig. 1, where the density of the protein solution D' is greater than that of the dialysate, symbolised D'' , an increase in the depth must be correlated with an increase in the osmotic pressure, which is defined as the difference between the hydrostatic pressure in the colloidal solution and the dialysate.

$$dP = (D' - D'') dy \quad \dots\dots(7),$$

where dP = increase in osmotic pressure, expressed in cm. water of density 1.00.

In the gravitational field, the hydrostatic pressure and composition of the dialysate may change slightly with depth, but the effects of these changes must be small, and the terms P , D' and D'' in formula (7) should agree with measurements made using solutions with different protein concentrations equilibrated with a dialysate of constant pressure and composition.

The term $(D' - D'')$ is then equal to $kC/100$, as stated in formula (2); $k = 0.27$, in solutions of haemocyanin equilibrated with the standard phosphate buffer mixtures. The pressure p , in mm. mercury, is given by formula (6):

$$p = RT\phi (10C/M),$$

where $RT = 17,033$ at 0° . If the pressure P be expressed in cm. water, RT must be given in the same units, and it follows that $P = 23,158\phi (10C/M)$.

Using the values for pressures and densities given above, formula (4) can be restated in terms of the concentration C .

$$\frac{10RT}{M} d(\phi C) = \frac{kC}{100} dy \quad \dots\dots(8).$$

The integration of this formula, allowing for the variation of ϕ with concentration, has been described by Adair [1935]. In the special cases where C_l , the concentration of the lower sample at the depth y_l , is only slightly greater than C_u , the concentration of the upper sample at the depth y_u , the change in ϕ is small, and the ratio C_l/C_u may be calculated by the approximate formulae (9) and (10):

$$\int \frac{dC}{C} = \ln \frac{C_l}{C_u} = \frac{M\phi^{-1}k(y_l - y_u)}{1000RT} \quad \dots\dots(9),$$

$$\frac{C_l}{C_u} = \exp. \left(\frac{M\phi^{-1}(y_l - y_u)}{85.8 \cdot 10^{-6}} \right) = \left[1 + \frac{M\phi^{-1}(y_l - y_u)}{85.8 \cdot 10^{-6}} \right] \quad \dots\dots(10).$$

If the solutions be as dilute as those used by Svedberg, 0.1 to 0.5%, the term ϕ should approach unity, and the ratio C_l/C_u can be calculated from the molecular weight. If M be equal to 5,100,000, as in the case of *Helix*, the ratio should be approximately 1.059 for an increase in depth of 1 cm. The ratio

diminishes to 1.006 for a molecular weight of 500,000. The observed ratios given in the last column of Table VII are lower than these calculated values, but the experiments were made at much higher protein concentrations, at which ϕ is greater than unity, as shown by the measurements of osmotic pressure recorded in Table VII, column 3.

Table VII. *Comparison of observed increase of concentration of haemocyanin with depth and values calculated from osmotic pressure measurements.*

Exp. no.	C	p	M/ϕ	Ratio calculated	Ratio observed
H 3	6.35	0.85	1,260,000	1.0146	1.0051
H 4	4.48	0.49	1,550,000	1.0180	1.0043
H 5	3.55	0.22	2,700,000	1.0314	1.0055
H 7	3.63	0.32	1,950,000	1.0227	1.0187
H 8	3.09	0.44	1,100,000	1.0128	1.0084
O 20	8.88	2.56	590,000	1.0068	1.0056
C 11	3.20	1.06	510,000	1.0059	1.0035
C 12	3.33	1.25	450,000	1.0052	1.0054
C 14	8.17	3.71	370,000	1.0043	1.0080
C 30	4.06	1.46	470,000	1.0055	1.0019

The values of M/ϕ calculated from measurements of osmotic pressure are given in column 4. The ratios in column 5 are calculated from these measurements by formula (10). Considering the technical difficulties of measuring small pressures and small differences in concentrations, the agreement between the observed and the calculated values is within the limits of experimental error, in the case of the observations on *Octopus* and *Carcinus*. In a number of the experiments on *Helix*, the observed ratio is much lower than that calculated. It must be remembered that the observed ratios in Table VII represent the distribution of the protein at the time the osmometer was taken down, and the chief object of the comparison of the observed and calculated results was not to test the validity of formula (10) but to use the formula as a test for the existence of a state of equilibrium. In the circumstances it may be inferred that although a number of the osmometers containing the haemocyanin of *Helix* failed to reach a state of equilibrium, the osmometers containing *Octopus* and *Carcinus* haemocyanin, which were allowed to equilibrate for a longer period (up to 25 days) gave an approximation to the equilibrium distribution within the limits of experimental error.

SUMMARY.

A method is described for the preparation and recrystallisation of haemocyanin.

Measurements are recorded of the osmotic pressures of haemocyanins prepared from the blood of *Helix pomatia*, *Carcinus moenas* and *Octopus vulgaris*.

The mean molecular weights of the haemocyanins, calculated from the osmotic data, are compared with those calculated by Svedberg from ultracentrifugal measurements.

A preliminary study has been made of the distribution of haemocyanin in a gravitational field.

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