solvent, nature of catalyst, and especially by hydrogen-ion concentration. Pseudocodeine can thus be reduced in excellent yield to a new non-phenolic dihydropseudocodeine.

2. The degradation of dihydropseudocodeine to dihydro-ϵ-methylmorphimethine, and conversion of the latter to tetrahydro-ϵ-methylmorphimethine is described. The preparation of tetrahydro-ϵ-methyl-morphimethine through extension of the special hydrogenation method to ϵ-methylmorphimethine has been accomplished.

3. The degradation product from tetrahydropseudocodeine is correctly described and its relationship to hexahydro-ϵ-methylmorphimethine established.

UNIVERSITY, VIRGINIA

[CONTRIBUTION FROM THE LABORATORY OF PHYSICAL CHEMISTRY OF THE UNIVERSITY OF UPSALA]

THE MOLECULAR WEIGHT OF THE HEMOCYANIN OF OCTOPUS VULGARIS

BY THE SVEDBERG AND INGA-BRITTA ERIKSSON

RECEIVED JULY 8, 1932 PUBLISHED DECEMBER 13, 1932

In three previous papers[1,2,3] reports have been given of the determination of the molecular weights and the sedimentation constants of hemocyanin from *Helix pomatia* and *Limulus polyphemus* by means of the ultracentrifugal methods developed in this Laboratory. Both of these hemocyanins (called *h*-hemocyanin and *l*-hemocyanin) differ markedly from all other proteins by having molecular weights of the order of millions. It seemed desirable to extend this study to other members of the hemocyanin group. The chemical and physico-chemical properties of the hemocyanin of *Octopus vulgaris* (*o*-hemocyanin) are well known, the protein having been the object of a great number of investigations.[4] The blood of octopus is comparatively easy to obtain in sufficient quantities and the protein is very stable. It was therefore chosen as a proper object for a detailed ultracentrifugal investigation.

Material.—The *o*-hemocyanin was prepared by one of us (T. S.) during a stay at the Stazione Zoologica of Naples, Italy.[5] To 50 cc. of octopus blood drawn in the manner described by Henze[6] was added 50 cc. of

[1] Svedberg and Chirnoaga, THIS JOURNAL, **50**, 1399 (1928).

[2] Svedberg and Heyroth, *ibid.*, **51**, 539 (1929).

[3] Svedberg and Heyroth, *ibid.*, **51**, 550 (1929).

[4] *E. g.*, Dhéré, Thèse, Fribourg, 1909; Dhéré, *J. physiol. path. gén.*, 1915–1922; Quagliariello, in Wintersteins "Handb. d. vergl. Physiolog.," Jena, 1922; Schmitz, *Z. physiol. Chem.*, **194**, 232 (1931); **196**, 71 (1931).

[5] For the interest and help shown him on this occasion by the director of the Station, Prof. R. Dohrn, and by Prof. L. Califano, sincere thanks are expressed.

[6] Henze, *Z. physiol. Chem.*, **33**, 370 (1901).

saturated ammonium sulfate solution and some toluene as a preservative. After standing for a week in the refrigerator at 0°, the precipitate was centrifuged off and bottled up with some toluene. Examined in the microscope it proved to be crystalline. It was brought to Upsala in this state. Here it was washed two times on the centrifuge with one-half saturated ammonium sulfate solution. A part of the precipitate was dissolved in water and then dialyzed against distilled water for a week. The hemocyanin which had completely precipitated in the collodion bag was divided into two parts; one was dissolved in phosphate buffer 0.005 *M* in Na_2HPO_4 and 0.005 *M* in KH_2PO_4 (P_H 6.8), the other in 0.07 *M* sodium acetate. Another part of the ammonium sulfate precipitate (crystals) was dissolved in 0.01 *M* Na_2HPO_4 and dialyzed against the same solvent for a week (P_H approx. 8.0). A further portion of 50 cc. of octopus blood was precipitated with ammonium sulfate and a trace of acetic acid according to the method given by Henze.[6] This material, however, deteriorated with time and therefore was not used in the present work. For the sake of comparison with the crystallized *o*-hemocyanin, a quantity of octopus blood (75 cc.) to which some toluene had been added was also taken to Upsala for investigation.

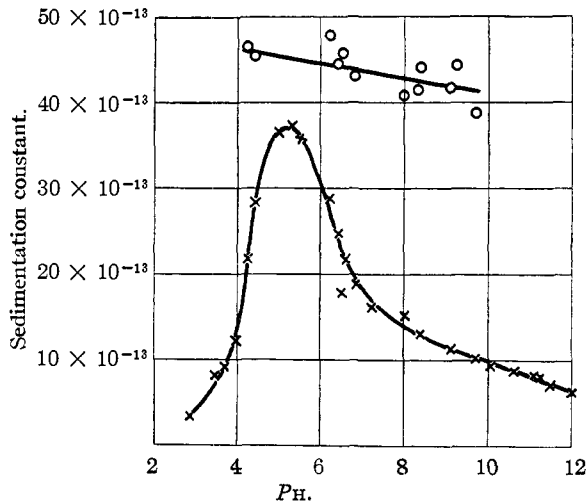

Fig. 1.

The partial specific volume was measured pycnometrically at 20° in the concentration range 1.3–3.2% and the P_H range 6.8–8.4. No systematic drift with concentration or P_H was observed. From five determinations the mean value 0.740 resulted. This is almost identical with the values 0.738 and 0.735 previously found for *h*-hemocyanin and *l*-hemocyanin,[1,2] respectively.

Sedimentation Constant and P_H Stability Region.—The velocity of sedimentation of the crystallized *o*-hemocyanin was measured in the P_H range 2.9 to 12.0. In order to secure as accurate P_H values as possible most of the determinations were carried out in the following way. Samples

TABLE I

CRYSTALLIZED HEMOCYANIN FROM OCTOPUS BLOOD, SEDIMENTATION VELOCITY MEASUREMENTS

Concn. of protein 0.4–0.8%; centr. force about 100,000 times gravity; thickness of col. of soln., 1.2 cm.; source of light, mercury lamp; light filter, nickel oxide glass; aperture of lens, F:50; plates, Imperial process; time of exp., 15 sec.; methol hydroquinone developer, 1 min.

Solvent	Total molar	P_H history of soln.	$s_{20} \times 10^{13}$ A	B
HAc	0.2	6.8→8.4→ 2.9	..	3.5
HAc, NaAc	.2	6.8→8.4→ 3.5	..	8.3
HAc, NaAc	.176	6.8→ 3.7	..	9.3
HAc, NaAc	.2	6.8→8.4→ 4.0	..	12.5
HAc, NaAc (1% in NaCl)	.02	6.8→ 4.3	46.7	21.6
HAc, NaAc (1% in NaCl)	.02	6.8→ 4.4	45.4	28.5
HAc, NaAc	.2	6.8→ 4.7	Soly. too low	for detn.
HAc, NaAc	.2	6.8→ 5.0	..	40.4
HAc, NaAc	.2	6.8→8.4→ 5.0	..	32.8
Na_2HPO_4, KH_2PO_4	.2	6.8→8.4→ 5.3	..	37.4
Na_2HPO_4, KH_2PO_4	.2	8.1→ 5.7	..	35.9
Na_2HPO_4, KH_2PO_4	.2	6.8→ 6.2	..	33.4
Na_2HPO_4, KH_2PO_4	.2	8.1→ 6.2	..	24.8
Na_2HPO_4, KH_2PO_4	.2	6.8→ 6.2	47.8	28.6
Na_2HPO_4, KH_2PO_4	.2	6.8→8.4→ 6.4	44.7	24.7
Na_2HPO_4, KH_2PO_4	.2	6.8→ 6.5	45.1	17.8
Na_2HPO_4, KH_2PO_4	.2	8.1→ 6.6	..	21.6
Na_2HPO_4, KH_2PO_4	.2	6.8	43.1	17.2
Na_2HPO_4, KH_2PO_4	.2	6.8	..	17.9
Na_2HPO_4, KH_2PO_4	.2	6.8	..	20.5
Na_2HPO_4, KH_2PO_4	.2	8.1→ 7.3	..	17.8
Na_2HPO_4, KH_2PO_4	.2	6.8→8.4→ 7.3	..	15.8
Na_2HPO_4, KH_2PO_4	.2	6.8→ 7.3	..	15.6
Na_2HPO_4, KH_2PO_4	.2	6.8→ 8.0	42.3	14.8
Na_2HPO_4, KH_2PO_4	.2	6.8→ 8.0	39.4	15.8
Na_2HPO_4	.2	6.8→ 8.3	41.2	..
Na_2HPO_4	.2	6.8→ 8.4	43.1	13.2
Na_2HPO_4	.2	8.4	45.2	..
Na_2HPO_4, NaOH	.2	6.8→8.4→ 9.1	41.9	11.5
Na_2HPO_4, NaOH	.2	6.8→ 9.2	44.4	..
Na_2HPO_4, NaOH	.2	6.8→8.4→ 9.7	37.8	10.2
Na_2HPO_4, NaOH	.2	6.8→ 9.7	40.3	..
Na_2HPO_4, NaOH	.2	6.8→8.4→10.0	..	9.5
Na_2HPO_4, NaOH	.2	6.8→10.6	..	8.9
Na_2HPO_4, NaOH	.2	6.8→8.4→11.1	..	8.4
Na_2HPO_4, NaOH	.2	6.8→8.4→11.2	..	8.1
Na_2HPO_4, NaOH	.2	6.8→8.4→11.5	..	7.2
Na_2HPO_4, NaOH	.2	6.8→8.4→12.0	..	6.3

of the hemocyanin solution about 0.5% in protein were dialyzed (in the cold) against the proper buffer solutions and the P_H measured in the buffer after equilibrium with the protein had been reached. In Table I and Fig. 1 the measurements are summarized.[7]

Another series of determinations of sedimentation velocity was carried out on octopus blood in the P_H range 2.8 to 11.1. The sedimentation constants are summarized in Table II and Fig. 2.

TABLE II

OCTOPUS BLOOD, SEDIMENTATION VELOCITY MEASUREMENTS IN BUFFER SOLUTIONS

Experimental conditions as in Table I

Solvent	Total molar	P_H of soln.	$s_{20} \times 10^{13}$ A	B
HAc	0.2	2.8	..	5.3
HAc, NaAc	.2	3.5	..	9.9
HAc, NaAc	.2	4.0	..	12.9
HAc, NaAc	.2	4.2	..	14.3
HAc, NaAc	.2	4.5	..	21.5
HAc, NaAc	.2	5.0	..	30.7
HAc, NaAc	.2	5.2	50.2	26.8
Na_2HPO_4, KH_2PO_4	.2	5.3	47.6	31.7
Na_2HPO_4, KH_2PO_4	.2	5.5	48.0	30.4
Na_2HPO_4, KH_2PO_4	.2	5.7	..	33.1
Na_2HPO_4, KH_2PO_4	.2	5.7	47.6	21.6
Na_2HPO_4, KH_2PO_4	.2	6.2	44.2	..
Na_2HPO_4, KH_2PO_4	.2	6.2	47.5	..
Na_2HPO_4, KH_2PO_4	.2	6.2	44.9	..
Na_2HPO_4, KH_2PO_4	.2	6.8	43.6	..
Na_2HPO_4, KH_2PO_4	.2	7.1	42.0	17.5
Na_2HPO_4, KH_2PO_4	.2	7.2	40.7	15.8
Na_2HPO_4, KH_2PO_4	.2	7.4	40.5	20.3
Na_2HPO_4, KH_2PO_4	.2	8.0	44.1	..
Na_2HPO_4	.2	8.4	44.9	..
Na_2HPO_4, NaOH	.2	8.7	45.1	9.3
Na_2HPO_4, NaOH	.2	9.1	45.3	..
Na_2HPO_4, NaOH	.2	10.2	..	9.9
Na_2HPO_4, NaOH	.2	11.1	..	9.2

A third series of measurements of the sedimentation velocity was made on untreated octopus blood at different protein concentrations, using 1% sodium chloride as diluting agent. The determinations are given in Table III and in Fig. 3. The mean of the six last determinations (1.0–0.5%) is also represented by a point in the diagram Fig. 2 (at P_H 7.9).

The values given in Tables I, II and III and in Figs. 1 and 2 show that solutions of *o*-hemocyanin contain two components. The sedimentation constant of one of them, A, is practically independent of P_H within the range 4.3–9.7 and has a mean value of 43.2×10^{-13}. The sedimentation

[7] In cases where more than one determination was carried out at the same P_H the mean value has been used for the diagram.

constant of the other component, B, varies strongly with P_H. The ratio of the two components changes with P_H in a very peculiar way.[8] There is

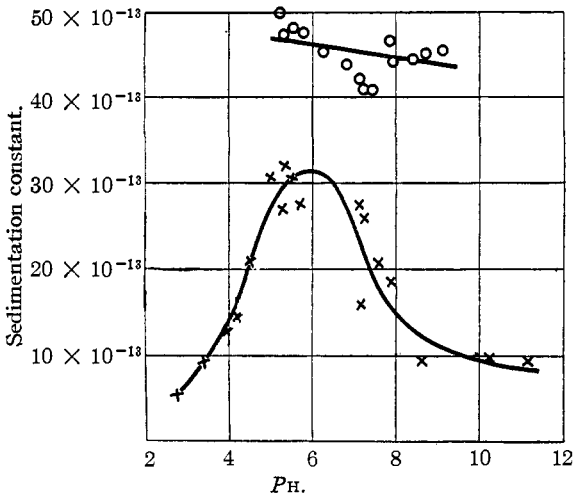

Fig. 2.

one acid region, P_H 4.3–4.5, and one alkaline region, P_H 7.9–9.7, where the component A predominates and a middle region, P_H 5.0–7.2, where com-

TABLE III

OCTOPUS BLOOD, SEDIMENTATION VELOCITY MEASUREMENTS AT DIFFERENT DILUTIONS

Experimental conditions as in Tables I and II

State of the blood	P_H of soln.	Concn. of protein	$s_{20} \times 10^{13}$ A	B
Oxygenated, undiluted	...	10.0	11.7	..
Oxygenated, diluted 1% NaCl	...	7.0	18.4	..
Oxygenated, diluted 1% NaCl	...	4.0	30.4	..
Oxygenated, diluted 1% NaCl	...	2.0	40.7	..
Oxygenated, diluted 1% NaCl	...	1.5	44.2	..
Oxygenated, diluted 1% NaCl	...	1.0	48.1	..
Oxygenated, diluted 1% NaCl	7.9	0.7	47.3	18.6
Oxygenated, diluted 1% NaCl	7.9	0.5	45.8	..
Reduced by bact., dil. 1% NaCl	7.9	0.7	44.5	..
Reduced by evac. and H_2, dil. 1% NaCl	7.9	0.7	49.7	..
Reduced by evac. and N_2, dil. 1% NaCl	7.9	0.7	49.5	..

[8] Percentage values were calculated from the microphotometric records on the assumption that the light absorptions of the two components are identical at the wave length used (366 mμ). A comparison of two solutions of the same protein concentration, one of them containing practically only component A and the other practically only component B, showed that the light absorption of B is somewhat higher than that of A. There seems to be some other factor except the P_H which also influences the ratio between the components, occasionally giving rise to irregular results. We have not been able to locate this factor and therefore refrain from giving numerical values of the ratio A:B.

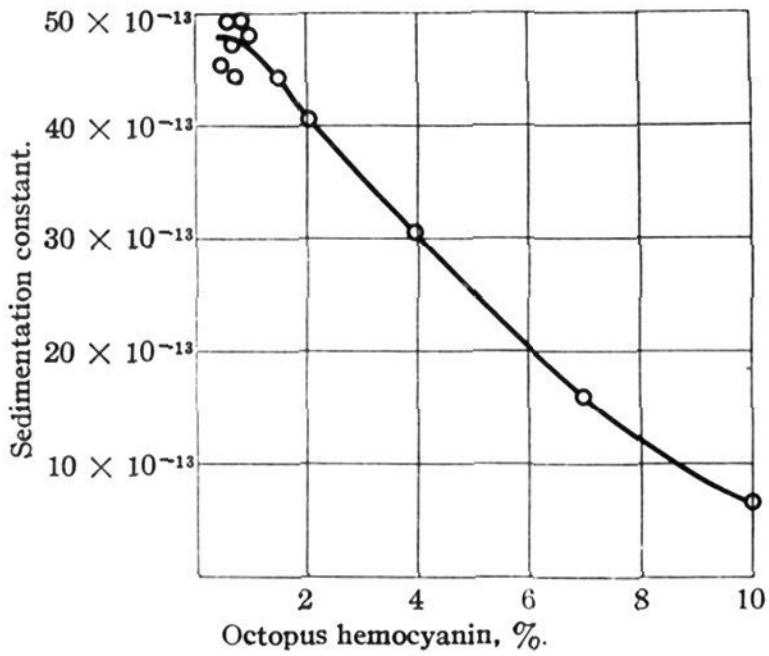

Fig. 3.

ponent A is fairly abundant. In the two regions P_H 4.5–5.0 and 7.2–7.9 the concentration of A is practically zero. The two components A and B are not two different proteins but represent two different kinds of aggregation in equilibrium. Only component A which has a sedimentation constant (and accordingly a molecular weight) practically independent of P_H over a certain P_H range should be termed a distinct molecular species like *h*-hemocyanin and *l*-hemocyanin. The change from A to B and from B to A is reversible. This fact is borne out by the following determinations. A solution at P_H 6.8 containing only about 5% of the high molecular component was brought to P_H 8.4 and showed there a content of 73%. When the P_H was lowered to 7.3, the high molecular component disappeared completely. To illustrate the striking change in constitution of the *o*-hemocyanin brought about by a shift in the P_H, the sedimentation pictures of runs at 6.5, 7.3 and 8.0 are given in Fig. 4. At P_H 6.5 (Fig. 4, A)

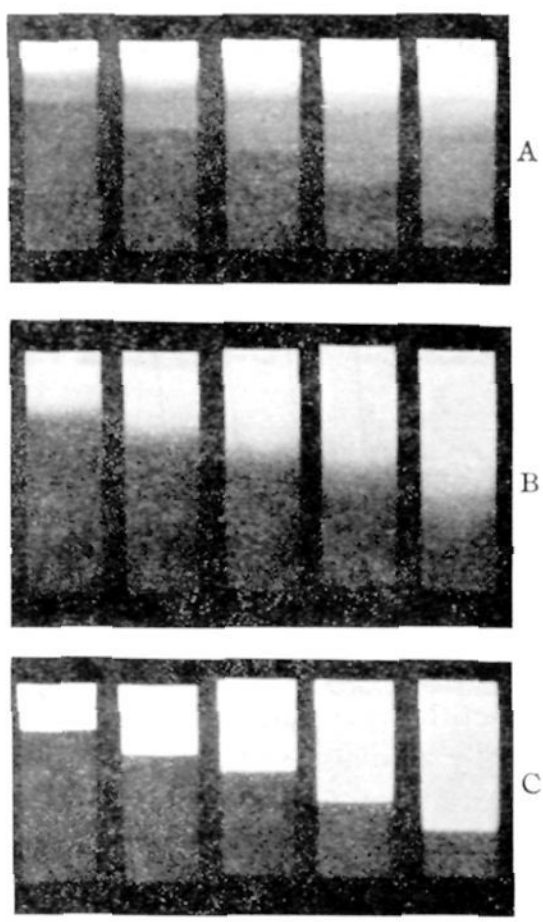

Fig. 4.

the sedimentation boundaries of the two components moving with different velocities are clearly seen, while at P_H 7.3 (Fig. 4, B) only one boundary corresponding to the movement of the low molecular component is visible. At P_H 8.0 the high molecular component predominates and the picture shows—in reproduction—only that boundary (Fig. 4, C). Owing to the slower diffusion and probably also to a higher degree of homogeneity the boundary of the high molecular component is much sharper than that of the low molecular component. The centrifugal field was about the same in the three runs, *viz.*, a little over 100,000 times gravity, but the time between the exposures was ten minutes in Expt. B against only five minutes in Expts. A and C so as to allow the low molecular component to sediment sufficiently. The curves giving the relation between distance from meniscus and relative concentration for the three runs are reproduced in Fig. 5, A, B, C. A small amount of the low molecular component is present in the third run, as borne out by the microphotometric curves in Fig. 5, C although not visible in the reproduction Fig. 4, C.

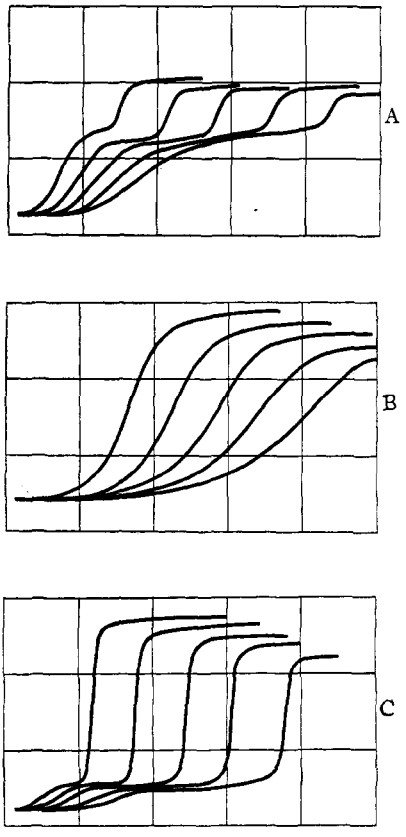

Fig. 5.

In order to find out whether the ratio of the two components depends on the total protein concentration runs were made with solutions of crystallized *o*-hemocyanin containing 1, 2 and 3% at P_H 6.8 where component B predominates. One would expect the formation of component A which has the higher sedimentation constant (and the higher molecular weight) to be favored by an increase in the total protein concentration. No such effect, however, was noticed the concentration of component A being practically zero also in these concentrated solutions.

An attempt was also made to study the influence of high dilution upon the ratio of the two components by using chlorine and bromine filters when taking the sedimentation pictures. Owing to the presence of some low-molecular substance of high light-absorbing power in the short ultraviolet, however, this experiment failed.

The rapid fall of the sedimentation velocity with increasing protein concentration is demonstrated by Fig. 3. Determinations of the change of viscosity with concentration showed that the phenomenon is not merely a viscosity effect but is largely due to an interaction between the protein molecules similar to the effect already observed in solutions of *h*-hemocyanin.[1]

As shown by the diagrams in Figs. 1 and 2 the sedimentation velocity values found for diluted octopus blood agree well with those found for dilute solutions of crystallized *o*-hemocyanin. The maximum of the curve for component B is shifted slightly in the alkaline direction and is not quite so high as for the crystallized product. The mean value of the sedimentation constant of all the determinations on diluted blood (1.0–0.4%) for component A is 45.8×10^{-13} against 43.2×10^{-13} for the same component in the solutions of the crystallized product, which is probably within the limits of experimental error. The *o*-hemocyanin therefore exists in the blood with the same molecular weight as in the purified product. From the data of Table III it follows that the sedimentation and, accordingly, the molecular weight is the same in the oxygenated and the reduced state.

The peculiar behavior of the *o*-hemocyanin with regard to its dependence on P_H is without any parallel in the other proteins so far investigated by means of the ultracentrifugal technique. The phenomenon seems at first sight very difficult to explain but may perhaps be correlated with some interesting facts found by Roche[9,10] when studying the titration curves of the hemocyanins. The hemocyanin from helix[10] gave a curve with one inflection point at a P_H corresponding to that of the isoelectric point 5.2. The hemocyanin from octopus,[9] however, gave a titration curve with three inflection points, one at P_H 4.8, the isoelectric point, one at 6.3 and one at 7.5. The first and third inflection points are characterized by great change in P_H when the amount of acid or alkali present is but slightly changed. The affinity of the *o*-hemocyanin to an acid or a base is low at these points. Now these two minima of reactivity found by Roche correspond very nearly with the two regions where the concentration of component A is practically zero. It does not seem unlikely that the power of forming the huge molecules of this component is lost when the protein has become electrochemically saturated.

Molecular Weight and Molecular Symmetry.—Four sedimentation equilibrium runs, one on crystallized *o*-hemocyanin and three on diluted octopus blood, were carried out at a P_H of 8.4 where the solution consists almost entirely of component A. In Table IV the results are given.

From the mean of all the sedimentation constant measurements in dilute solutions, 44.7×10^{-13}, and the mean molecular weight, 2,050,000, we get for the molar frictional constant $f = M(1 - V\rho)/s$ the value 11.9 $\times$

[9] Roche, *Arch. de physique biol.*, **7**, 207 (1930).

[10] Roche, *Compt. rend. de sc. de la soc. de biol. de Lyon*, **107**, 1145 (1931).

TABLE IV

OCTOPUS HEMOCYANIN, SEDIMENTATION EQUILIBRIUM MEASUREMENTS

Solvent, 0.15–0.20 M Na_2HPO_4; P_H of soln., 8.4; concn. of protein, 0.5% at start

Nature of soln.	Mean speed	Mol. wt.
Cryst. o-hemocyanin	1600	1,700,000
Diluted octopus blood	1500	2,000,000
Diluted octopus blood	1530	2,200,000
Diluted octopus blood	1530	2,300,000
		Mean 2,050,000

10^{16}. A spherical molecule of the same specific volume would have a frictional constant $f_s = 6\pi\eta Nr$ equal to 9.6×10^{16}, where η is the viscosity, N the Avogadro constant and r the radius which is expressed by $(3MV/4\pi N)^{1/3}$. The o-hemocyanin molecule therefore deviates markedly from the spherical shape (dissymmetry number $f/f_s = 1.24$).

The expenses connected with this investigation have been defrayed by grants from the Nobel Fund of Chemistry, from the Rockefeller Foundation and from the Foundation "Therese och Johan Anderssons Minne."

Summary

1. The sedimentation constant, the P_H stability range and the molecular weight of octopus hemocyanin (called o-hemocyanin) have been determined by means of the ultracentrifugal methods.

2. Within the range P_H 4.3–9.7 solutions of crystallized o-hemocyanin contain two components, one of high molecular weight, A, and one of lower molecular weight, B. The sedimentation constant of A is almost independent of P_H and has a mean value of 43.2×10^{-13}, that of B varies strongly with P_H, showing a maximum at about P_H 5.2. Component A predominates in the regions P_H 4.3–4.5 and 7.9–9.7; it is less abundant in the region P_H 5.0–7.2 and has a very low concentration in the regions P_H 4.5–5.0 and 7.2–7.9. The change from one component to the other is reversible. On the acid side of P_H 4.3 and on the alkaline side of P_H 9.7 only component B exists.

3. A similar series of determinations on octopus blood gave analogous results. The mean value 45.8×10^{-13} was obtained for the sedimentation constant of component A.

4. Only component A, the sedimentation constant of which is independent of P_H, should be termed a molecular species like *Helix* hemocyanin and *Limulus* hemocyanin. From sedimentation equilibrium measurements a molecular weight of about 2,000,000 was derived.

5. The molecule of o-hemocyanin (component A) is not spherical in shape. The dissymmetry number is 1.24.

UPSALA, SWEDEN