3. Other derivatives of piperonal and of piperonylic acid have been prepared and described.

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

THE MOLECULAR WEIGHT OF THE HEMOCYANIN OF LIMULUS POLYPHEMUS

BY THE SVEDBERG AND FRANCIS F. HEYROTH[1]

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Gross differences have been reported by several workers[2] in the copper content, oxygen-combining curves, isoelectric points and various other properties of the hemocyanins obtained from different animals. This investigation was undertaken to determine whether a similar difference could be observed in the molecular weights of the hemocyanins or respiratory pigments of two species, the vineyard snail, *Helix pomatia*, and the horseshoe crab, *Limulus polyphemus*. Measurements in the ultracentrifuge by Svedberg and Chirnoaga have shown the former protein to have a molecular weight of 5,000,000 in solutions containing 0.05–0.1% of hemocyanin near the isoelectric point, 5.2. The copper contents of the hemocyanins of *Limulus* and *Helix* are 0.17 and 0.28%, respectively.[3]

Experimental

Preparation of Material.—We are indebted to Dr. A. C. Redfield for a sample of the hemocyanin of *Limulus* prepared by him at Woods Hole in 1926. He has described its preparation as follows:[3] "The fresh serum from a large number of animals was salted out by the addition of ammonium sulfate to half saturation, and in this condition preserved for some six months. The precipitated hemocyanin was separated by filtration, redissolved in 5% saturated ammonium sulfate containing 0.001 *N* ammonium hydroxide, filtered free of all insoluble residue, and reprecipitated by adding just sufficient saturated ammonium sulfate. This precipitate was separated by centrifugation. In order to secure a satisfactory separation, it was found desirable to bring the reaction to approximately *P*H 8.0 by the addition of ammonium hydroxide. The process of redissolving and salting out was then repeated two more times."

The material so prepared was dissolved in 5% saturated ammonium sulfate and further purified by dialysis before beginning this series of experiments. The dialysis was conducted in collodion bags in the ice chest against *N*/10,000 and later *N*/1000

[1] Fellow in Medicine of the National Research Council.

[2] References in Svedberg and Chirnoaga, THIS JOURNAL, **50**, 1399 (1928), and in the papers of Stedman and Stedman, *Biochem. J.*, **20**, 938, 949 (1928).

[3] Redfield, Coolidge and Shotts, *J. Biol. Chem.*, **76**, 185 (1928).

sodium hydroxide. After three weeks the alkali was replaced by distilled water, which was repeatedly changed during a month.

The suspension obtained had a specific electrical conductivity of 1.07×10^{-5} mho. The solubility of the hemocyanin of *Limulus* in electrolyte solutions is not as great as that of *Helix*, a suspension of which may be brought into solution by the addition of but traces of salts. A 2.27% stock solution of the *Limulus* hemocyanin was made from the supension by adding 3 cc. of $M/15$ $Na_2HPO_4 \cdot 2H_2O$ and 1.5 cc. of $M/15$ KH_2PO_4 per 100 cc. of final solution. The reaction of this solution, P_H 6.63 (electrometrically determined), permitted the preparation of a sufficiently concentrated solution for the pycnometric determination of the partial specific volume, V, of the protein. At the same time the reaction was judged to be not too far from the isoelectric point of the protein. This is not accurately known, Stedman and Stedman[2] merely stating that the method of maximal precipitation indicates it to be somewhat greater than 6.3. Toluene was employed as a preservative as it is readily removed from the solutions just before use by bubbling nitrogen gas through them.

The partial specific volume was determined by the pycnometric method described for the other proteins studied in this Laboratory. The concentration of the solutions was determined by transferring 1 cc. of the solution to a porcelain crucible, evaporating down to coagulation on a water-bath and then drying to constant weight at 105°. The content of anhydrous salt per cc. of the solution was subtracted from this weight. The mean value of V obtained in three determinations at two protein concentrations was 0.735 at 20°. This is, within the limits of experimental error, the same as that of the hemocyanin of *Helix*.

Preliminary measurements of the visible and ultraviolet light absorption showed the hemocyanin of *Limulus* to possess in general the same type of absorption curve as that obtained from *Helix*. In the experiments here reported the light absorption in the short-waved ultraviolet region only was employed. Chlorine and bromine filters were used as previously described[4] for the isolation of this region of the radiations from a quartz mercury vapor lamp.

Determination of the Molecular Weight

A. Sedimentation Velocity Method.—In this method the molecular weight is given by the equation $M = RTs/D(1 - V\rho)$ in which s is the specific sedimentation velocity or rate of movement of the protein under unit centrifugal field expressed in cm. per second, D is the diffusion constant expressed in cm.^{2} per day, ρ the density of the solution, R the gas constant and T the absolute temperature. The ultracentrifuge designed for low and medium centrifugal fields was employed in all of the runs. A speed of approximately 8400 r.p.m. (4100 times the force of gravity) was used and each run was continued for seven and a half hours, as this hemocyanin did not move down as rapidly as did that of *Helix*. Since an increase in the concentration of *Helix* hemocyanin solutions has been shown to produce a great depression of the values of the diffusion constant, while affecting but slightly the specific sedimentation velocity, a series of runs with *Limulus* hemocyanin at concentrations varying from 0.03 to 0.98% was deemed necessary. Three different quartz cells were used to secure the necessary thickness of layer of the variously concentrated

[4] Svedberg and Nichols, THIS JOURNAL, **48**, 3081 (1926).

solutions necessary in order to secure photographs of suitable contrast by means of exposures of from fifteen to forty-five seconds. Thus, the thickness of the layer of solution used was 2 mm. for 0.65 and 0.98% solutions, 8 mm. for the range of 0.09 to 0.12%, and 14 mm. for the more dilute solutions of 0.03 to 0.06%. Imperial process plates were employed. Dilutions of the stock solution into phosphate buffer of *P*H 6.63 were made immediately before each run. The results of each of the runs are given in Table I, in which Col. 1 gives the concentration of the protein, Col. 2 the specific sedimentation velocity and Col. 3 the diffusion constant. In almost all of the runs fifteen values of each were calculated at successive half-hour intervals. In the case of the diffusion constants the first three values in each series have been omitted and then only the next ten following values have been employed in the calculation of the mean value for each run. The first three values are inaccurate as, since the rate of centrifugation was not great under the centrifugal field employed, no region of pure buffer had yet appeared. The last few values are also in most cases in error because there is then no longer present a region of the solution within which there is no change of concentration with distance from the center of rotation.

TABLE I

SEDIMENTATION VELOCITY MEASUREMENTS

Concn., g. per 100 cc.	s, cm./sec. $\times 10^{12}$	D. cm.²/day	"Concentration" of non-centrifugible matter
0.98	3.59	0.0139	3 "%"
.65	3.65	.0189	5.5
.13[a]	3.48	.0190	17.5
.12	3.32	.0178	8
.09	3.74	.0227	17
.06	3.52	.0141	19
.06	3.55	.0162	18
.06	3.28	.0146	21
.03	3.74	.0221	103?
.03	3.79	.0181	49
Mean	3.57	.0177	
Mean using only the runs free from a drift in D.		.0147	

[a] This solution was made from the precipitate obtained by dialyzing a 0.03% solution. See section on Reversibility of the Disintegration.

The values of s are fairly constant throughout the range studied and show no definite trend with change of concentration. The diffusion values are, however, very irregular and lead to great variations in the molecular weights calculated in each of the runs. Thus the molecular weights vary from 1.31×10^6 to 2.05×10^6. Such variations point to the possibility that more than one variety of light-absorbing substance may be present in the solutions employed. That such is the case becomes apparent from a consideration of the drift with time which occurs in the

successive diffusion constants obtained during the course of several of the runs. Table II gives the values of the diffusion constants obtained at successive half-hour intervals, and these are seen to increase throughout several, but not all, of the runs. As has been previously explained, this excessive blurring of the protein solution–solvent boundary may result from the gradual separation arising from the sedimentation at different rates of two or more proteins of not very greatly different frictional resistances.

TABLE II

"APPARENT DIFFUSION CONSTANTS" AT SUCCESSIVE THIRTY-MINUTE INTERVALS DURING EACH RUN

	0.98%	0.65%	0.12%	0.13%	0.09%
4	0.0118	0.0157	0.0161	0.0143	0.0166
5	.0129	.0177	.0140	.0190	.0199
6	.0144	.0178	.0166	.0158	.0190
7	.0142	.0170	.0184	.0199	.0192
8	.0158	.0178	.0167	.0177	.0206
9	.0176	.0207	.0164	.0189	.0210
10	.0121	.0177	.0202	.0186	.0247
11	.0132	.0211	.0195	.0224	.0221
12	.0145	.0221	.0196	.0246	.0288
13	.0127	.0213	.0201	....	.0354
Mean	.0139	.0189	.0178	.0190	.0227
	0.06%	0.06%	0.06%	0.03%	0.03%
4	0.0096	0.0110	0.0107	0.0118	0.0161
5	.0098	.0136	.0115	.0132	.0160
6	.0107	.0154	.0120	.0158	.0206
7	.0169	.0184	.0136	.0265	.0207
8	.0148	.0184	.0161	.0184	.0161
9	.0167	.0180	.0193	.0266	.0286
10	.0139	.0162	.0156	.0134	.0254
11	.0139	.0175	.0161	.0199	.0258
12	.0152	.0168	....	.0196	.0249
13	.0192	.0165	.0168	.0155	.0271
Mean	.0141	.0162	.0146	.0181	.0221

The run at the concentration of 0.98% gave no indication of the presence of molecules of varying frictional resistance, but at 0.65 and 0.12% the presence of unlike molecules was clearly indicated. This effect was even more marked in solutions of 0.09%. When, however, the dilution was extended to 0.06%, it tended to disappear. In only one of three runs at this concentration could it be detected with certainty. The presence of such a condition in the two runs at 0.03% cannot be accurately determined as the contrast obtained with reasonable exposures was too small.

That the source of this non-uniformity of the protein molecules may well be a disintegration of the molecules present in the more concentrated solutions may be concluded from measurements of the light absorption in the

region between the boundary of the protein and the meniscus of the solution. In the more concentrated solutions, 0.98 and 0.65%, there were never more than traces of light-absorbing material left in this region. The point on the concentration scale corresponding to the galvanometer reading given by the density in the photograph of this portion of the solution is in the following taken as a rough measure of the amount of non-centrifugible material present. The "concentrations" so stated do not give directly the actual concentration of the non-centrifugible material, since the value of ϵ/c may conceivably (and in fact does) change as the units are disintegrated. Such "concentration" values have been given in Col. 4 of Table I and show clearly that the amount of non-centrifugible material increases from negligible amounts, 3–5% at 0.65% and over, to 17% upon dilution to 0.09%, and even to very high values in excess of 50% in 0.03% solutions. A possible interpretation of both the time drift in the diffusion constant and the formation of non-centrifugible material is as follows.

Dilution tends to cause a disintegration of the hemocyanin molecules. In the solutions which have been but slightly diluted only a comparatively small amount of non-centrifugible material is formed. This in itself is incapable of producing the drift with time in the diffusion values. At about 0.09 to 0.65% there is present, however, a certain amount of partly disintegrated or highly hydrated material which lowers but slightly the mean specific sedimentation velocity but which is capable of moving at such a rate in the centrifugal field as to produce the observed drift in the diffusion constants. In still more dilute solutions the disintegration process proceeds further, so that there are present fewer of the molecules in the first stage of disintegration (those which are responsible for the drift) and more of the more completely disintegrated and therefore non-centrifugible material. With increasing dilution as the decomposed material becomes more and more non-centrifugible, the drift in the diffusion values tends to disappear. The material which does centrifuge down in these dilute solutions may represent some of the original molecules which have escaped disintegration or may be possibly a more stable denaturation product of the same molecular weight as the protein present in the more concentrated solutions.

The progressive nature of the disintegration change due to dilution is indicated also by an effect noted in the latter part of some of the runs in dilute solutions and not noted in the case of other proteins. In the more dilute runs the successive curves tend to overlap each other in such a way as to indicate that during a given run non-centrifugible material is formed in increasing amounts. In each successive curve the light absorption increases in the region between the protein boundary and the meniscus. That a similar overlapping occurs also in the region in which the concentration is uniform indicates that the sum of the light absorp-

tion due to the presence of some large and some small molecules is greater than that due to the same amount of protein when present entirely as large, non-disintegrated molecules. The light absorption experiments reported in a later section point to the same possibility.

If the mean diffusion constant, 0.0139, of only those runs (two at 0.06% and one at 0.98%) in which no marked drift with time in the values occurred is used, together with mean specific sedimentation velocity obtained from all of the experiments, a molecular weight of 1.90×10^6 is found. If only the diffusion constant obtained at 0.98% is used, the value is 2.04×10^6.

B. Sedimentation Equilibrium Method.—This method is based upon the attainment of an equilibrium in the cell between the centrifugation of the protein toward the bottom and its diffusion toward the top. A series of values for the molecular weight, M, is calculated by the equation

$$M = \frac{2RT \ln (c_2/c_1)}{(1 - V\rho)\omega^2(x_2^2 - x_1^2)}$$

for a series of portions $(x_2 - x_1)$ of the column of liquid. R, T, ρ and ω have their usual meanings and c_1 and c_2 are the concentrations at the

TABLE III

SEDIMENTATION EQUILIBRIUM RUNS OF *Limulus* HEMOCYANIN

Part A

Concentration, 0.063%; PH, 6.63; length of column of solution, 0.4 cm.; thickness of column, 1.4 cm.; distance of bottom of cell from the axis of rotation, 5.95 cm.; T, 293.2°; speed, 1929 r.p.m. ($\omega = 64.3\pi$). Exposures were 20, 40 and 60 seconds.

Distances, cm.		Mean concentration, %		
x_2	x_1	c_2	c_1	M
5.95	5.90	0.1503	0.1425	4.04×10^5
5.90	5.85	.1425	.1159	15.90×10^5
5.85	5.80	.1159	.0911	18.63×10^5
5.80	5.75	.0911	.0761	14.04×10^5
5.75	5.70	.0761	.0672	9.79×10^5
5.70	5.65	.0672	.0591	10.20×10^5
5.65	5.60	.0591	.0531	7.98×10^5

Part B

Concentration, 0.15%; PH, 6.63; length of column of solution, 0.5 cm.; thickness of column, 0.8 cm.; distance of the bottom of the cell from the axis of rotation, 5.95 cm.; T, 293.2°; speed, 1929 r.p.m.; time exceeded 90 to 100 hours. Exposures were 50, 100 and 150 seconds.

x_2	x_1	c_2	c_1	M
5.95	5.90	0.1553	0.1496	2.84×10^5
5.90	5.85	.1496	.1413	4.38×10^5
5.85	5.80	.1413	.1290	7.05×10^5
5.80	5.75	.1290	.1189	6.36×10^5
5.75	5.70	.1189	.1086	7.13×10^5
5.70	5.65	.1086	.0983	7.91×10^5
5.65	5.60	.0983	.0878	9.05×10^5
5.60	5.55	.0878	.0790	8.53×10^5
5.55	5.50	.0790	.0703	9.52×10^5

distances x_1 and x_2 from the axis of rotation. If the molecules of the protein solution have uniform frictional resistances, the successive values of M obtained at successive fractions of the way from the top to the bottom of the solution fluctuate irregularly about a mean value. The presence of a second type of molecule makes itself evident by a progressive increase in these values as the bottom of the cell is approached. The indication given by the sedimentation velocity method of the presence of more than one kind of molecule in 0.06–0.09% solutions was confirmed by the finding of a drift in the molecular weight values in an equilibrium run A of Table III. In this run equilibrium was attained in a column of solution, 0.06%, 4 mm. in height after eighty hours of centrifuging at a speed of 1900 r.p.m. The second run in Table III was made by using a 5-mm. column of a somewhat more concentrated 0.15% solution. In this case even after ninety to one hundred hours equilibrium had not been attained, as the concentration–distance curves at seventy-eight, ninety-one and one hundred hours did not coincide. It is probable that as the solution toward the central end of the cell becomes more dilute (0.07%) as equilibrium is approached, a disintegration of the molecules there occurs. Since this continues as the heavier molecules diffuse into this region, new conditions are set up, delaying greatly the attainment of a final state of equilibrium.

The Light Absorption of the Protein.—The light absorption of *Limulus* hemocyanin was measured in the visible region by means of a König-Martens spectrophotometer in a 2.86% solution at P_H 6.74 and in the ultraviolet region by a modified Judd-Lewis spectrophotometer at a series of concentrations from 0.03 to 0.136%, corresponding to the range employed in the centrifuging experiments. In general the positions of the maxima of the absorption bands agree with those reported for *Helix* hemocyanin, although the values of ϵ/c are lower throughout than in the case of the latter protein. This is indicated in Fig. 1, which gives for comparison the light absorption curves of the two proteins. For the *Limulus* hemocyanin the values plotted in the ultraviolet were the mean of determinations at the concentrations 0.09 and 0.136%.

TABLE IV

LIGHT ABSORPTION OF THE HEMOCYANINS IN THE VISIBLE REGION

Wave length, $\mu\mu$	*Limulus* 2.86%, P_H 6.74	*Helix* 1.75%, P_H 3.8	*Helix* 1.69%, P_H 5.6	Wave length, $\mu\mu$	*Limulus* 2.86%, P_H 6.74	*Helix* 1.75%, P_H 3.8	*Helix* 1.69%, P_H 5.6
635	0.146	0.199	0.297	522	0.157	0.216	0.396
605	.158	.229	.339	506	.143	.206	.361
579	.178	.248	.377	492	.104	.200	.360
556	.176	.249	.395	479	.098	....	.349
538	.165	.238	.401	468	.093	.197	.346

The values of ϵ/c for the visible spectral region are given in Table IV and Fig. 2. It was shown by Svedberg and Chirnoaga that at P_H 3.8

the maximum for *Helix* was shifted to the red and that the values over the whole region became relatively lower than those obtained at P_H 5.6.

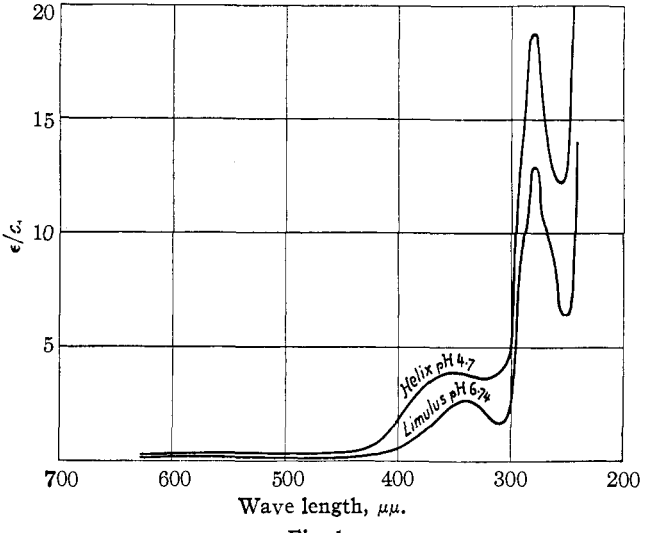

Fig. 1.

These changes accompanied a decomposition of the protein which has since been studied in detail. The values obtained for *Limulus* hemocyanin near its isoelectric point correspond more closely with those for the smaller particles of *Helix* which are present at P_H 3.8 than with the values for the larger molecules present at P_H 5.6. The position of the maximum for *Limulus* hemocyanin also corresponds better with that of *Helix* at 3.8 than at 5.6.

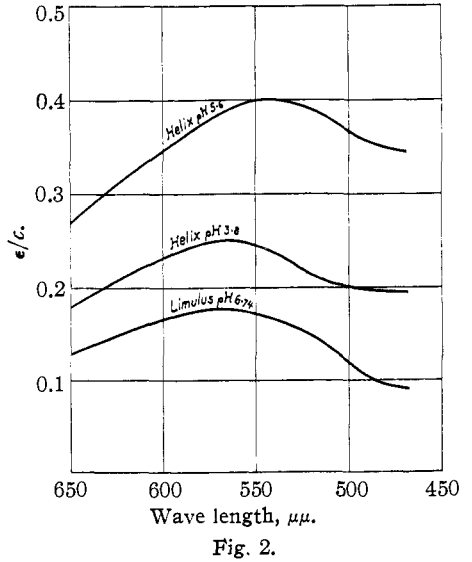

Fig. 2.

Although the accuracy of the measurements made with the Judd-Lewis apparatus in the ultraviolet is not as great as that of those in the visible made with the König-Martens spectrophotometer, two curves made at different concentrations of *Limulus* hemocyanin at P_H 6.63 are presented in Fig. 3, as significant differences are shown which are readily reproducible. The measurements plotted indicate that Beer's law does not hold

over this concentration range and that the light absorption increases as the concentration is lowered. This change in light absorption occurs in the same concentration range as that in which the sedimentation velocity and equilibrium runs had indicated the protein to undergo a change leading to the production of units of lesser magnitude. This change occurs over the entire ultraviolet region but is most evident on the broad maximum between 360–330$\mu\mu$. This region is shown on a larger scale in Fig. 4, in which values at several concentrations are plotted. It appears here that the minimum which occurs at about 300–315$\mu\mu$ in the more concentrated solutions is by dilution shifted toward the longer wave lengths.

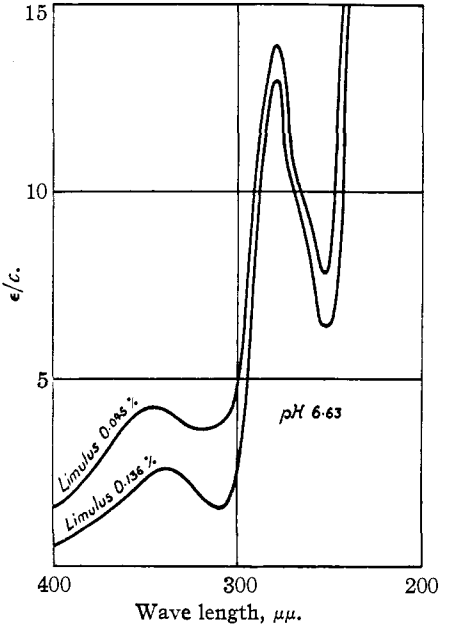

Fig. 3.

Reversibility of the Decomposition.—The light absorption of a portion of a 0.03% solution of *Limulus* hemocyanin was measured in a 20-mm. cell. After twenty-four hours the remainder of the solution was dialyzed against distilled water until a precipitate appeared. This was then dissolved to form an 0.088% solution at P_H 6.63 and the light absorption was again measured. Fig. 4 shows that the light absorption of this solution corresponded to about that which had been previously obtained by using a solution of 0.09%. In a repetition of this experiment the reprecipitated protein was dissolved to form a somewhat stronger solution, 0.13%. In this case the light absorption was that of about a 0.09% solution. The decomposition attending dilution thus appears to be at least in large part reversible.

A similar result was obtained when the reprecipitated, previously diluted hemocyanin was studied in the ultracentrifuge by the sedimentation velocity method. Precipitated hemocyanin obtained by the dialysis of a solution which had been only 0.03% for twenty-four hours was dissolved to form a solution of 0.127%. The light absorption of the non-centrifugible material corresponded to that of a solution about 17.5% as concentrated as that of the solution centrifuged. This was the value previously obtained in a solution of 0.09% (Table I). The effect of dilution was in this case partly reversed by the recombination which occurred during the dialysis.

which resulted in the production of a precipitate of the original protein. In the cases in which the reversibility was not entirely complete it is probable that dialysis had not been continued for a sufficient time; the suspension removed from the bag and brought into solution by the addition of phosphate solutions may have been contaminated by some as yet uncombined material.

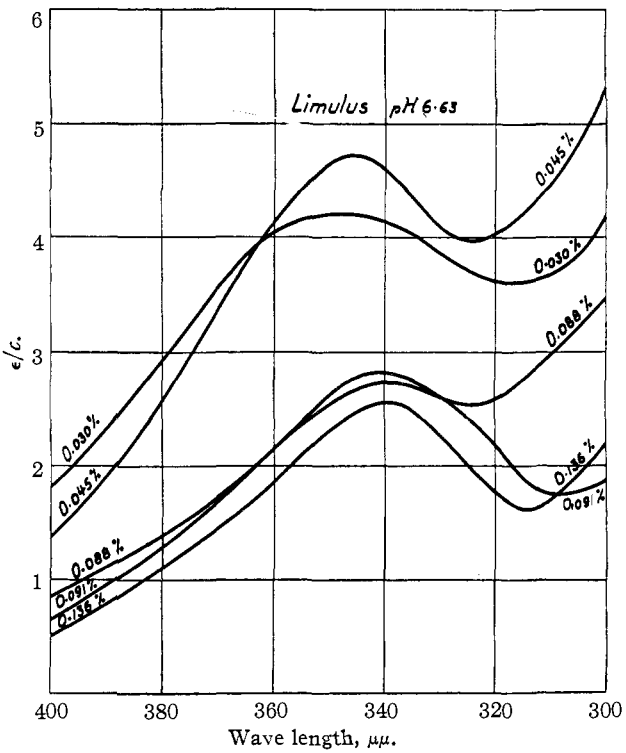

Fig. 4.

Discussion

Evidence has been presented from sedimentation velocity and equilibrium measurements in the ultracentrifuge and from measurements of the light absorption in the ultraviolet that a decomposition occurs in solutions of *Limulus* hemocyanin when diluted beyond 0.6%. The mechanism of the change (hydrolysis, disassociation or other type of splitting) has not been determined. The occurrence of such an effect of dilution upon the proteins is not unprecedented, for a similar change has been described in the case of serum albumin.[5] That the change occurs within the range of concentrations most suitable for the photographic recording of the motion of the protein in the centrifugal field complicates

[5] Svedberg and Sjögren, THIS JOURNAL, **50**, 3318 (1928).

greatly the molecular weight determination. If the mean of all of the diffusion constants obtained in each of the sedimentation velocity runs is employed in determining the molecular weight, a value of 1.60×10^6 results, which multiplied by 3.12 gives 4.99×10^6, a value very close to the molecular weight 5.08×10^6, which has been determined for the *Helix* hemocyanin. Such a comparison is perhaps possible because the error introduced by the mean drift in the diffusion constants with time is about that observed in the sedimentation of *Helix* hemocyanin at P_H 4.7. It should, however, only be permissible to make such a comparison at either the isoelectric points of the two proteins or at regions so far removed from each of them that the ratio of charged to uncharged molecules is the same in each case. Only by so doing would it be possible to exclude the effects of the charges upon the ions which depress the diffusion constants, making the values of the molecular weights abnormally high. Since the reaction 6.63 employed in this work is probably less than 0.3 of a Sörensen unit alkaline to the isoelectric point, a comparison may be made of the molecular weight of the *Limulus* hemocyanin obtained by using only those diffusion constants free from drift, 2.04×10^6, with the apparent molecular weight of *Helix* hemocyanin at a P_H 0.3 unit alkaline to its isoelectric point, or 5.5. If the values 1.90×10^6 or 2.04×10^6 are multiplied by 3.12 the values 5.93×10^6 or 6.36×10^6 are obtained. The apparent molecular weight of *Helix* hemocyanin at 5.62 has been found to be 6.19×10^6. The molecular weight of *Limulus* hemocyanin may then be stated to be, within the limits of experimental error, one-third that of the hemocyanin of *Helix*.

An attempt was made to calculate the diffusion constant for this protein by the aid of the Einstein equation

$$D = \frac{RT}{N} \times \frac{1}{6\pi r\eta}$$

in which R, T and N have their usual meanings and η is the viscosity of water at 20° expressed in absolute units. The radius, r, of the particle is calculated to be 8.398×10^{-7} from the molecular weight and density of the protein on the assumption that the molecules are spherical. As the mean value for the diffusion constant in those runs in which there was no drift with time was 0.0139, while the value obtained by the calculation outlined is 0.0214, it is evident that there is a large discrepancy. It is therefore probable that the assumption upon which the calculation was made, that is, that the molecules are spherical, is without justification. There is thus an analogy between these two hemocyanins and the proteins phycoerythrin and phycocyan. In each case the molecules of greater molecular weight are spherical. Phycocyan with half the molecular weight and the same diffusion constant as phycoerythrin and *Limulus* hemocyanin with one-third the molecular weight of *Helix* hemocyanin

and a diffusion constant approximately that of the latter, each have molecules diverging widely from the spherical shape.

Summary

1. Measurements of the molecular weight of the hemocyanin of the horseshoe crab *Limulus polyphemus* have been made in dilute phosphate buffer solution at P_H 6.63 in concentrations of 0.03–0.09% of protein by the sedimentation velocity and the sedimentation equilibrium methods in the ultracentrifuge.

2. Both methods indicate that at concentrations of about 0.06–0.1% the protein undergoes decomposition.

3. The existence of this decomposition has been confirmed by measurements of the ultraviolet absorption, as the values of ϵ/c increase with dilution in this range of concentrations.

4. The decomposition is largely if not completely reversible and a protein of the same molecular weight and ϵ/c as that originally used may be precipitated from the dilute solutions by removing electrolytes by dialysis.

5. The sedimentation velocity method indicates for the hemocyanin of *Limulus polyphemus* a probable molecular weight of 2.04×10^6 under the conditions described.

UPSALA, SWEDEN

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

THE INFLUENCE OF THE HYDROGEN-ION ACTIVITY UPON THE STABILITY OF THE HEMOCYANIN OF HELIX POMATIA

BY THE SVEDBERG AND FRANCIS F. HEYROTH[1]

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The previously reported determinations by ultracentrifugal methods[2] of the molecular weight of the hemocyanin isolated from the blood of the vineyard snail, *Helix pomatia*, were made upon solutions buffered to P_H 4.7, which is not far removed from P_H 5.2, the isoelectric point of the protein. This reaction was adopted to obviate the disturbing variation in the diffusion constant which in preliminary experiments at P_H 8.0 had been found to vary approximately inversely as the centrifugal force applied. As it was also noted that at P_H 3.8 the hemocyanin molecule appeared to break up into smaller units of undetermined size, a further study of this protein was deemed advisable in order to determine the region within which its huge molecules (of molecular weight 5,000,000 at P_H 4.7 in solutions containing 0.09% of protein) are stable. A similar series

[1] Fellow in Medicine of the National Research Council.

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