HAEMOCYANIN OF THE CRAWFISH (*JASUS LALANDII*)

by

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As summarised by SVEDBERG AND PEDERSEN[1] in *The Ultracentrifuge*, a large number of haemocyanins has been studied by ultracentrifuge techniques. A wide range of molecular weights was found, from 397,000 for *Pandalus borealis* to 6,680,000 for *Helix pomatia*, but, on the whole, haemocyanins from animals of the same group showed similar molecular weights. As will appear in the discussion of the present paper, this is well confirmed by the haemocyanin of the South African crawfish (*Jasus lalandii*). This is also the Australian crawfish, and RAWLINSON[2] has made a previous study of this haemocyanin from the viewpoint of its copper content, and the behaviour of its copper on oxidation.

Apart from ultracentrifugal methods, osmotic pressures of haemocyanin solutions have been measured[3,4], but, as osmotic pressures are small, the molecular weights obtained are of doubtful accuracy. PUTZEYS AND BROSTEAUX[5] used light scattering, and obtained results in reasonably good agreement with those by the ultracentrifuge, but their measurements were not carried out on the absolute basis which has characterised light-scattering measurements of the last decade. Their molecular weights were obtained by comparison with the protein amandin, assumed to be 330,000. As no ultracentrifuge was available, the present work had to place reliance on the light-scattering method. This has been repeatedly shown to be reliable for proteins[6,7,8], provided sufficient care has been taken in preparation of solutions and in calibration of the photometer to absolute standards. In the case of heterodisperse systems, it gives a weight average molecular weight, which is similar to the sedimentation equilibrium value, M_e, obtained with the ultracentrifuge. In addition to molecular weight determinations, measurements have also been made of diffusion, viscosity, electrophoresis, osmotic pressure and absorption spectrum.

METHODS

A Brice-Phoenix light-scattering photometer[7] was used for the measurements of the light scattered at 45°, 90° and 135° to the incident beam. The blue mercury line of wavelength 4358 A and the green 5461 A were used for turbidity measurements. The solutions were prepared dust free by centrifugation at 20,000 r.p.m. for an hour and a final gravity filtration through a No. 4 Pyrex sintered disc. Using these methods, dissymetry values (I_{45}/I_{135}) between 1.05 and 1.08 were obtained. It was assumed that these were residual effects resulting from forward scatter by a very small amount of high scattering material which has escaped removal during preparation of the solution. For this reason, (i) the 90° scatter was not corrected for dissymmetry, as would be the case if it resulted from the size of the haemocyanin molecule; and (ii) it was also assumed that though the impurity

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was sufficient to affect 45° scatter, the solutions were clean enough for its effect at 90° to be neglected. As discussed by GORING AND JOHNSON[8], a dissymmetry of the order found in this case is not unusual for protein solutions and was attributed by them to small amounts of aggregates. Another possibility is incomplete removal of traces of high molecular weight substances associated with the protein in the organism from which it is prepared.

For absolute calibration of light-scattering intensities at 90°, it was decided to use liquids of known turbidity in the scattering cell. The first standards chosen were pure benzene and a standard polystyrene which has been distributed to a large number of laboratories by Debye and Bueche, Cornell University. For benzene, the Rayleigh ratios or reduced intensities of $48.5 \cdot 10^{-6}$ at 4358 A and $16.3 \cdot 10^{-6}$ at 5461 A were assumed. These values due to CARR AND ZIMM[9] are higher than earlier values and have been questioned[10], but the balance of present evidence is in their favour[11]. The turbidity of polystyrene as a 0.5% solution in toluene at 4358 A has been found to be 0.0035 (cm^{-1}) in several laboratories[7], though, unfortunately, an earlier value was 0.0027 and this has led to some confusion in the literature[12]. For the turbidity at 5461 A a value of 0.00131 (cm^{-1}) was used[7].

A third method of calibration was the use of Ludox, as described by MOMMAERTS[13], to standardise the scattering intensity at 90° with the turbidity obtained by transmission. This was applied at both 4358 A and 5461 A. The results obtained by the three methods were in good accord. In order to check their application to protein solutions, the light-scattering molecular weight was found for a sample of crystalline bovine serum albumin supplied by Armour Laboratories. This protein has been frequently reported[6,7,8,14,15] and the most reliable results, both by osmometry and by light scattering give values close to 70,000. Serum albumin solutions were prepared in the same way as the haemocyanin and were also found to give a persistent dissymmetry of about the same magnitude. The molecular weights obtained, using the three calibrating methods in turn, are summarised in Table I and are seen to agree well with one another and with other determinations. Concentrations of the serum albumin solutions were obtained by the micro-kjeldahl method, assuming a nitrogen content of 15.6%[16]. Values of refractive index increments assumed were 0.196 for 4358 A and 0.189 for 5461 A.

TABLE I

MOLECULAR WEIGHT OF SERUM ALBUMIN WITH DIFFERENT CALIBRATING LIQUIDS

Calibration liquid	*Molecular weight*	
	4358 A	*5461 A*
Benzene	72,300	69,800
0.5% Polystyrene in toluene	70,400	70,900
Ludox solutions	70,600	69,900

Haemocyanin concentrations were also determined by micro-kjeldahl, the nitrogen content having been determined as 16.02 ± 0.14%. Refractive index increments were measured using a modified Pulfrich refractometer with a divided cell. Refractive index increments were measured at 5780 A, and values of 0.196 at 4358 A and 0.189 at 5461 A were calculated by using PERLMANN AND LONGSWORTH'S[17] equation.

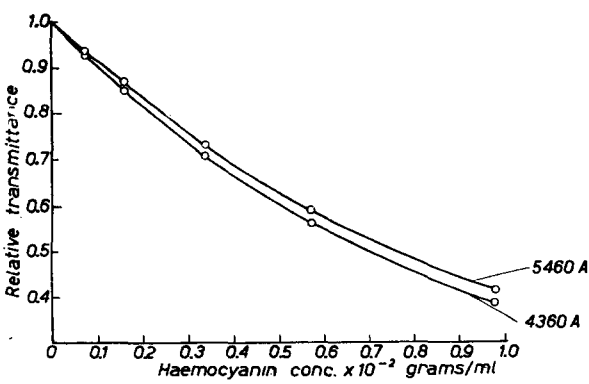

Fig. 1.
Absorption of haemocyanin at 4358 A and 5461 A.

Haemocyanin solutions are blue-green in colour. To find out whether there is any preferential absorption at either of the two wavelengths employed in light scattering, transmission measurements were carried out on the light-scattering photometer at different protein concentrations. As shown in Fig. 1, relative transmittances at the two wavelengths were of the same order. Correction for absorption for a Brice-Phoenix light-scattering photometer has been worked out by BRICE *et al.*[18], but, as this correction, in the protein concentration range studied, amounts to less than 2%, no absorption correction was applied to turbidities.

Electrophoresis was carried out in a

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Tiselius electrophoresis apparatus manufactured by Hilger Watts Ltd., using a medium U-tube equipped with a long centre section.

Diffusion coefficients have been determined by the free boundary method using the Tiselius U-tube for boundary formation. A plunger type of compensator was used to move the boundary between protein and buffer solution into the observation channel without disturbances.

Relative viscosities were measured using a Cannon-Fenske viscometer in a water bath thermostatically controlled to 25 ± 0.02° C.

Osmotic pressures were measured in an ADAIR[19] type of osmometer, and were corrected for capillary rise observed in the same osmometer with the same solutions.

Absorption bands in the visible and ultraviolet were carried out on a Beckman ultraviolet spectrophotometer.

Preparation of haemocyanin

The haemocyanin was prepared from crawfish blood obtained by puncturing the vascular system of the animal at the tail root. A medium size animal gave *ca.* 30 ml blood of 5.0–5.5% haemocyanin concentration. When left in air the blood sets to a firm gel. To prevent this, the blood was immediately diluted with an equal volume of phosphate buffer of ionic strength (I) of 0.15 and pH = 6.5 and dialysed against the same buffer. Changes of ionic strength and pH were routinely performed by dialysis of the blood in a slowly rotating cellophane bag against a large volume of the required buffer solution. Dialysis was carried out for 24 hours with 3 or 4 changes of buffer.

Occasionally a blood of a dark brown colour was obtained. This was particularly noticed with animals during the moulting period. The present examination was, however, limited to blood samples of the desired blue-green colour.

RESULTS

The dialysed blood examined by electrophoresis (Fig. 2(a) and Table II) in phosphate buffer of $I = 0.15$ and pH = 6.5, gave a major fast electrophoretic component and a small quantity (*ca.* 5%) of a slow component.

Various methods were tried for the storage of the haemocyanin. To the dialysed blood ammonium sulphate was added to 50% saturation, the precipitated haemocyanin was then stored for a month under its mother liquor at 5° C. With this method of storage no change in the electrophoretic mobilities of the two components (Table II) was observed, but the diagrams of Fig. 2(b) seem to indicate that a slight increase of the area of the slow component occurred. Fig. 2(c) contains diagrams of a freeze-dried sample of the haemocyanin. On both ascending and descending sides two components of about equal concentration were observed. Mobilities of these components (Table II) seem to agree with previous values. Hence, the concentration of the low component was increased considerably.

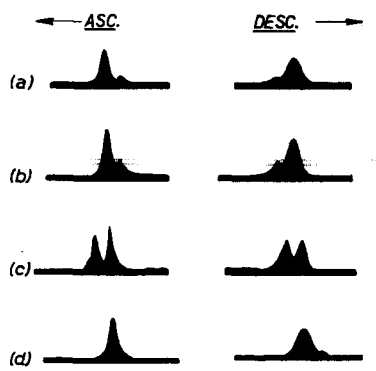

Fig. 2. Electrophoresis diagrams of the haemocyanin.

As no satisfactory method of storage was obtained, freshly prepared haemocyanin was used in this examination.

It has been observed that the haemocyanin, being a globulin, is readily precipitated at pH values close to its iso-electric point. A 1% solution of the haemocyanin in phosphate buffer of $I = 0.15$ and pH = 6.5 was dialysed overnight against acetate buffer of $I = 0.2$ and pH = 5.0, the precipitate which formed was dissolved in phosphate buffer of $I = 0.15$ and pH = 6.5. When examined by electrophoresis (Fig. 2(d)), single boundaries on both ascending and

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descending sides were obtained. Mobilities of these boundaries (Table II) correspond to mobilities of the major component of the original solution. This method therefore affords a means of fractionating the major component from the small amount of the slow electrophoretic component.

TABLE II

HAEMOCYANIN ELECTROPHORETIC MOBILITIES AT 1° C

Experimental conditions	*Mobility* × 10^{-4} *cm² sec⁻¹ volt⁻¹*			
	Descending		*Ascending*	
Dialysed blood				
at $I = 0.15$, pH = 6.5	0.52*	0.64	0.55*	0.66
$(NH_4)_2SO_4$ stored blood				
at $I = 0.15$, pH = 6.5	0.53	0.63	0.55	0.66
Freeze-dried blood				
at $I = 0.15$, pH = 6.5	0.49	0.64	0.51	0.65
Blood ppt. at $I = 0.2$, pH = 5.0				
examined at $I = 0.15$, pH = 6.5	—	0.64	—	0.66

* Electrophoretic slow component.

In phosphate buffer of $I = 0.15$ and pH = 6.5, the haemocyanin can probably be regarded as electrophoretically homogeneous. However, when examined in barbiturate buffer of $I = 0.1$, pH = 7.8 (Fig. 3), somewhat different electrophoretic results were obtained. On the descending side a major fast and small slow component were again observed, but on the ascending side in addition to the small slow component separation into four probable components with very similar mobilities occurred. Further work with these buffers was not carried out.

Fig. 3. Electrophoresis diagram of haemocyanin in barbiturate buffer of $I = 0.1$, pH = 7.8.

In Fig. 4 the average electrophoretic mobilities from descending and ascending sides, which were determined in buffers of $I = 0.2$ at 1° C. (haemocyanin concentration 0.3%) are plotted as ordinates against pH values as abscissae. The iso-electric point of the haemocyanin studied is at pH 4.65; which agrees well with RAWLINSON'S[2] value of 4.60, by sedimentation volume method, the slope of the mobility curve in this region $dU/d(pH) = 5.5 \cdot 10^{-5}$.

RAWLINSON[2] found the absorption spectrum possessed a very diffuse band centered at 5580 A and others at 3350 A and 2780 A, and this has been experienced by the present observations.

As the haemocyanin was not very stable at pH values below its iso-electric point, the light-scattering examination was limited to pH values above the iso-electric point. In Table III the molecular weight found for the haemocyanin under

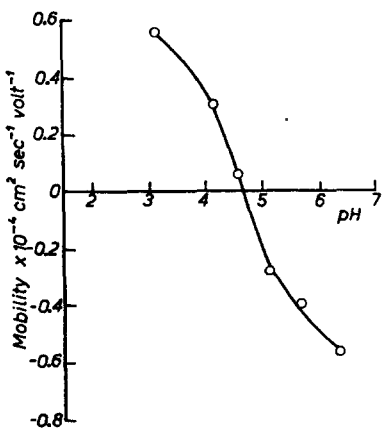

Fig. 4. pH against electrophoretic mobility in buffers of $I = 0.2$ at 1° C.

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different conditions of pH values and ionic strength are given and a typical graph of c/τ against c is shown in Fig. 5.

TABLE III

LIGHT-SCATTERING MOLECULAR WEIGHTS OF HAEMOCYANIN

Experimental conditions		*Molecular weight*		
Ionic strength	*pH*	*4358 A*	*5461 A*	*Average*
0.1	5.5	457,000 440,000	459,000 446,000	450,000
0.1	6.5	488,000 478,000	508,000 493,000	492,000
0.1	7.6	504,000 501,000	511,000 508,000	506,000
0.1	9.0	303,000	315,000	309,000
0.3	5.5	482,000 492,000	494,000 511,000	495,000
0.5	5.5	485,000 480,000	494,000 501,000	490,000

At $I = 0.1$ and pH = 9.0 a definite lower molecular weight was obtained. This pH value probably falls outside the stability range of the protein and the lower molecular weight indicates that the haemocyanin is dissociated into smaller units. A 10% lowering in molecular weight was observed at $I = 0.1$ and pH = 5.5, but when the salt was increased to $I = 0.3$, a molecular weight which agrees with the other values was obtained.

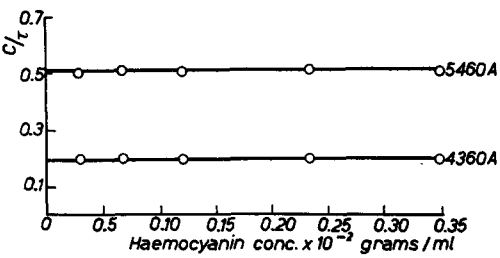

Fig. 5. c/τ against c for haemocyanin at 4358 A and 5461 A (pH = 5.5, $I = 0.3$).

In Table IV diffusion coefficients, calculated by the maximum height, $D^0_{20}(H_{max})$, and the standard deviation, $D^0_{20}(\sigma^2)$ methods, for different haemocyanin concentrations are listed. These measurements were made in phosphate buffer of $I = 0.15$ and pH = 6.5 at 20° C.

TABLE IV

DIFFUSION COEFFICIENTS OF HAEMOCYANIN

Concentration (g./100 ml)	D^0_{20} $(H_{max}) \cdot 10^7$ cm²/sec	D^0_{20} $(\sigma^2) \cdot 10^7$ cm²/sec
2.180	2.91 2.90	3.19 3.25
1.567	2.88 2.87	3.19 3.14
1.139	2.69 2.76	3.04 3.23

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As shown (Table IV) no trend of diffusion coefficients with concentration was observed, but the two methods of calculation gave considerably different diffusion coefficients. This difference indicates a certain degree of heterogeneity[20].

In Fig. 6 the viscosity increment (η sp/c) is plotted against haemocyanin concentration. The intrinsic viscosity $(\eta \text{ sp}/c)_{c \to 0}$ of the haemocyanin is 5.5. From the intrinsic viscosity using POLSON'S[21] equation and assuming a partial specific volume of 0.74[1], an axial ratio of 5.9 was calculated for the haemocyanin. By substitution of this value into PERRIN'S[22] formula, a frictional ratio (f/f_0) of 1.31 was obtained. Together with the diffusion coefficient $D^0_{20}(\sigma^2)$, this gives a molecular weight of 455,000. Conversely, a f/f_0 value of 1.28 was obtained from the diffusion coefficient and light-scattering molecular weight. As viscosity measurements are rather uncertain, the light-scattering measurements which are based on an accurate calibration must be regarded as the more accurate.

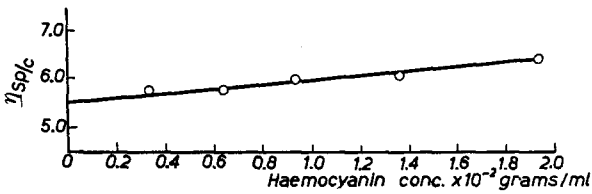

Fig. 6. ηsp/c against c for haemocyanin (pH = 6.5, I = 0.15).

An osmotic pressure molecular weight of 540,000 was obtained for the haemocyanin using Adair osmometers. This determination cannot be regarded as very accurate, because it involves a correction due to capillary rise of uncertain magnitude. At a haemocyanin concentration of 1.3%, an osmotic head of 1.54 cm water was obtained, which had to be corrected for a capillary rise of 0.98 cm. As osmotic pressure gives number average molecular weights, the obtained molecular weight seems at least to indicate that the haemocyanin was free from any low molecular weight impurities.

DISCUSSION

The relationship to haemocyanins from similar species will first be considered. In SVEDBERG AND PEDERSEN[1] (p. 365, Table 44), ultracentrifuge data on haemocyanins have been assembled and grouped biologically. Unfortunately, though sedimentation constants are given for each species, molecular weights have been measured for only a relative small number. In the class Malacostraca, sedimentation constants, $s_{20} \cdot 10^{13}$, can be divided into two groups, one about 16.5, associated with molecular weights between 400,000 and 450,000, and the other about 24, associated with molecular weights of about 800,000. This class includes the order of Decapoda, and in Table V examples have been selected from the table of SVEDBERG AND PEDERSEN and arranged in sub-orders of Decapoda. It is seen that sedimentation constants of the major haemocyanin constituents in a given sub-order belong to a single group of sedimentation constants may be present.

Light-scattering molecular weights by PUTZEYS AND BROSTEAUX[5] averaged 462,000 for *Palinurus vulgaris* and 624,000 and 711,000 for two haemocyanin preparations from *Homarus vulgaris*. These agree reasonably with Table V, though the methods used cannot be regarded as of comparable accuracy to either the ultracentrifugal or the present light-scattering method.

Jasus lalandii is shown in the sub-order of *Palinura*, and its molecular weight by light scattering has been entered in the column of molecular weights by sedimentation equilibrium. The value entered in Table V for the sedimentation constant is an approxi-

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mate value reported by POLSON[23], and obtained with a preparative ultracentrifuge. With the diffusion coefficient in Table IV, it gives a molecular weight of 512,000, which has been entered under M_s in Table V.

TABLE V

HAEMOCYANINS ARRANGED IN SUB-ORDERS OF DECAPODA

Sub-order	*Species*	$s_{20} \times 10^{13}$	$D_{20} \times 10^{7}$	M_S	M_e
Caridea (prawns and shrimps)	*Pandalus borealis*	17.4	—	—	397,000
	Palaemon fabrici	16	—	—	—
Palinura (crawfish)	*Palinurus vulgaris*	16.4	3.4	446,000	447,000
	Jasus lalandii	17.1	3.2	512,000	496,000
Astacura (lobsters and crayfish)	*Nephrops norvegicus*	24.5	2.79	812,000	—
	Homarus vulgaris	22.6	2.78	752,000	—
	Astacus fluviatilis	23.3	—	—	—
Anomura (hermit crabs)	*Pagurus striatus*	16	—	—	—
	Eupagurus bernhardus	17 (22)*	—	—	—
Brachyura (crabs)	*Hyas araneus*	23	—	—	—
	Maja squinado	27	—	—	—
	Cancer pagurus	23.6 (16.4)*	—	—	—
	Carcinus maenas	23.3 (16.7)*	—	—	—

* Minor constituents shown in brackets.

Its molecular weight, though somewhat higher than for *Palinurus vulgaris*, is of the expected order. As the light-scattering molecular weight is a weight average, it exaggerates the effect of a high molecular weight constituent, and it is possible that the somewhat high molecular weight is due to a small proportion of a constituent with a molecular weight of 800,000–900,000. The difference between the two calculations of the diffusion coefficient in Table IV gives evidence for a certain degree of heterogeneity. It is possible that the high molecular weight constituent tends to dissociate near the iso-electric point and that the true molecular weight of the major constituent is that at pH = 5.5, I = 0.1 in Table III. Ultracentrifugal examination of the haemocyanin from *Palinurus vulgaris*[24] showed no signs of any second constituent between pH 3.6 and 9.4. Above this, it showed progressive and more or less irreversible dissociation to a constituent with a much lower molecular weight. Presumably the low molecular weight at pH = 9 in Table III is due to a similar dissociation.

The assumption that heterogeneity would correspond to a second constituent of approximately double molecular weight follows on the conclusion of SVEDBERG AND PEDERSEN, that even the most complex haemocyanins appear to be built up by association of units with a molecular weight in the range 400,000–450,000. The more complex haemocyanins show a tendency to dissociate reversibly into simpler units, and eventually to the simplest unit in the above range. It may be noted, however, that though the simplest units possess a more or less common molecular weight, their electrophoretic mobilities, iso-electric points and frictional ratios may differ widely[24]. The frictional ratio for the haemocyanin of *Jasus lalandii* compares well with that for the haemocyanin

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of *Palinurus vulgaris*, but differs strongly from that of the simplest constituent of haemocyanin from *Helix pomatia*.

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SUMMARY

A physico-chemical study has been made of the haemocyanin from the South African crawfish (*Jasus lalandii*). Electrophoresis of freshly prepared haemocyanin in phosphate buffers gave single boundaries, indicating a single constituent, with an iso-electric point at pH = 4.65. The molecular weight by the light-scattering technique gave values between 450,000 and 496,000, which is in accord with values obtained by ultracentrifuge techniques for the common crawfish (*Palinurus vulgaris*). Though the haemocyanin was electrophoretically homogeneous, diffusion measurements showed some degree of heterogeneity, and this probably accounts for the variation in molecular weight, the true value for the main component being 450,000. The frictional ratio from molecular weight and diffusion measurements was 1.28 and, from viscosity measurements, was 1.31, values which are similar to those for *Palinurus vulgaris*.

RÉSUMÉ

Les auteurs ont effectué une étude physicochimique de l'hémocyanine de l'écrevisse d'Afrique du Sud (*Jasus lalandii*). L'électrophorèse en tampon phosphate de l'hémocyanine fraîchement préparée donne une seule frontière, correspondant à un constituant unique dont le point isoélectrique est de 4.65. La valeur du poids moléculaire, déterminée par diffusion de la lumière, est comprise entre 450,000 et 496,000, ce qui est en accord avec les valeurs obtenues par ultracentrifugation pour l'écrevisse commune (*Palinurus vulgaris*). Malgré l'homogénéité électrophorétique de l'hémocyanine, les mesures de diffusion indiquent un certain degré d'hétérogénéité, qui permet probablement de rendre compte de la variation du poids moléculaire, le poids moléculaire réel du constituant principal étant de 450,000. Le rapport de frottement, déterminé à partir du poids moléculaire et des mesures de diffusion, est de 1.28 et de 1.31, si on le détermine par viscosimétrie. Ces valeurs sont comparables à celles de *Palinurus vulgaris*.

ZUSAMMENFASSUNG

Es wurde eine physikalisch-chemische Untersuchung des Hämocyanins des Südafrikanischen Krebses (*Jasus lalandii*) durchgeführt. Die Elektrophorese des frisch hergestellten Hämocyanins in Phosphatpuffern ergab einfache Grenzlinien mit einem isoelektrischen Punkt bei pH 4.65 und zeigt so einen einzigen Bestandteil an. Das mit Lichstreuungsmethoden ermittelte Molekulargewicht ergab Werte zwischen 450,000 und 496,000; sie sind in Übereinstimmung mit Werten wie sie durch Untersuchungen mit der Ultrazentrifuge beim gewöhnlichen Krebs (*Palinurus vulgaris*) erhalten wurden. Obwohl das Hämocyanin elektrophoretisch einheitlich war, zeigten Diffusionsmessungen einen gewissen Grad von Uneinheitlichkeit. Dies ist wahrscheinlich der Grund für die Streuung des Molekulargewichts, dessen tatsächlicher Wert für die Hauptkomponente 450,000 beträgt. Der aus dem Molekulargewicht und den Diffusionsmessungen erhaltene Reibungsquotient betrug 1.28, der aus Viskositätsmessungen erhaltene 1.31. Beide Werte sind ähnlich denen für *Palinurus vulgaris* erhaltenen.

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