A C T A U N I V E R S I T A T I S L O D Z I E N S I S
FOLIA BIOCHIMICA ET BIOPHISICA 7, 1990

Maria Adamska

PHYSICO-CHEMICAL PROPERTIES OF THE HEMOLYMPH AND HEMOCYANIN OF ASELLUS AQUATICUS L.

Physico-chemical properties of the hemolymph and hemocyanin of Asellus aquaticus L. have been studied. In the hemolymph, the contents of cations (Ca, Mg, Na, K, Cu), lactate and protein were determined. pH of the hemolymph was found to be 7.5. Absorption spectra of the hemolymph and of the hemocyanin were measured, finding peaks at 280 and 338 nm. Hemolymph proteins were subjected to analytical ultracentrifugation which yielded 3 fractions. The 16 S fraction was dominant, representing over 70% of the total. The 24 S and 5 S fractions were present in small amounts. Electrophoretic separations performed under different conditions illustrated properties of hemolymph proteins in the electric field. From oxygen dissociation curves, affinity parameters p_{50} and n were determined. The Hill coefficients for the hemolymph of Asellus aquaticus was 2.6.

Hemocyanin was isolated by preparative ultracentrifugation in a MOM3170B ultracentrifuge. Lyophylized hemocyanin samples were subjected to automatic amino acid analysis in a Jeol 6AH analyser.

1. INTRODUCTION

In invertebrates, hemoglobins and hemocyanins are the most widely spread respiratory proteins. No general rule has been found governing the presence of any individual pigment in relation to the taxonomic position of animals, their biology or habitat. Terrestrial animals, like aqueous ones, may contain hemoglobin or hemo-

cyanin in the blood (hemolymph). Many invertebrates have no pigment in the blood (hemolymph) but the pigment is found in the muscular or neural tissues. These proteins may also be localized in other tissues, of high oxygen demand. While myoglobin (a hemoprotein) occurs always in muscles of invertebrates, concomitant blood (hemolymph) pigments are different [1, 2].

Most frequently, respiratory pigments occur in pairs in invertebrates: myoglobin - hemoglobin (some annelids, gastropods), myoglobin - hemocyanin (some molluscs, crustaceans), myoglobin - chlorocruorin (some polychaets), or myohemoerithrin - hemoerythrin (some Sipunculoidea). Depending on ecological conditions, in particular on the extent of water oxygenation, the presence or absence of respiratory pigments is established. There are also species in which two or three respiratory proteins, of different properties, occur simultaneously. E.g., in some polychaets, one hemoglobin was found dissolved in blood, while another, different one, in the coelom. In the same phylum, some species have two chemically different pigments (hemoglobin and chlorocrorin) in their blood.

It is also unexplained why hemoglobin occurs in the hemolymph of one representative (Planorbis corneus) and hemocyanin in another representative (Limnaea stagnalis) of the same order, living under identical conditions. It may be also possible that one species (Solen legumen) contains hemoglobin in the hemolymph while other species have no pigment.

In invertebrates, hemoglobin is concentrated in erythrocytes or dissolved in the tissue. Hemocyanin, however, occurs always in the dissolved form. While hemoglobins are present, with variable frequency, in almost all phyla of the animal world, hemocyanin occur only in some Mollusca and Arthropoda.

Among molluscs, the presence of hemocyanin was established in representatives of three classes: Polyplacophora, Gastropoda (mainly in the subclasses of Prosobranchia and Pulmonata and Cephalopoda). In Opistobranchia, hemocyanin occurs only in Scaphander lignaris. Up to date, hemocyanin has not been revealed in the classes of Monoplacophora, Scaphopoda and Bivalvia [3].

Among arthropods, hemocyanin is present in representatives of the class Crustacea in the orders of Decapoda, Amphipoda, Iso-

poda and Stomatopoda. Moreover, this protein occurs in the phyla: Merostomata and Arachnida (in the orders od Scorpionidea and Araneida). To date, the occurence of hemocyanin has been established in one representative of the order of Pedipalpi and in Scutigera longicornis, belonging to the class Myriapoda. Hemocyanin does not occur however, in Diplopoda, and Insecta [1, 2, 10].

2. GENERAL PROPERTIES OF HEMOCYANIN

A characteristic feature distinguishing hemocyanins of Arthropoda and Mollusca from among other respiratory proteins is the way of oxygen binding. Only hemocyanins are copper proteins. O_2 molecule is bound to two copper atoms in the "active center" of the macromolecule. In the deoxygenated proteins, both copper atoms are at the +1 oxidation state while upon oxygen binding the valency of two Cu atoms changes, i.e. in oxygenated protein Cu occurs at the +2 oxidation state. Oxygenated hemocyanin has a blue color, due to the $Cu-O_2$ bond, and the deoxygenated form is colorless.

The hemocyanins of Arthropoda are heterogenous proteins, occuring in the form of aggregates of molecular weight ranging from 500 000 up to 3 5000 000 Da.

The arthropod hemocyanins are hexamers or oligohexamers, composed of subunits of molecular weight of 75 000 Da. Hemocyanin molecules associate into components of sedimentation coefficient of 60 S, 24 S, 35 S, 16 S and 5 S. The 16 S component is a hexamer [16, 24].

In vivo, hemocyanins occur predominantly as hexamer agregates of the 5 S component. The extent of aggregation is characteristic for a given species. In Crustacea, mostly hexamers (1 x x 6) and dodecamers (2 x 6) are present. In some shrimps, hemocyanin occurs in the form of tetramers of hexamers (4 x 6 = 24). In Chelicerata, octamers (8 x 6), dimers (2 x 6) are found in Limulus polyphemus while tetramers (4 x 6) are revealed in Arachnida.

Components of Arthropoda hemocyanins are stable in the pH

range of 5-8. Alkaline dissociation results in monomeric 5 S subunits. The rate of the dissociation process is affected by: pH, some chelates and some ions. E.g., hemocyanin of Carcer pagurus (Decapoda) does not dissociate in Tris-HCl buffer, pH 9, but dissociates into hexamers in Tris-HCl-EDTA, pH 9, or in Tris-borate-EDTA, pH 8,2. Further dissociation of the hexameric form into the 5 S monomeric subunits was observed at pH up to 10.6 (glycine-NaOH-EDTA buffer) [27, 28, 30].

A single polypeptide chain (monomer) has a sedimentation coefficient of 5 S and molecular weight of 68 000 to 71 000 Da. The 16 S component is a hexamer of the 5 S monomers. Its molecular weight is about 470 000 Da. The 24 S component is a dodecamer of the monomers, and a dimer of the 16 S component; its molecular weight amounts to about 1 000 000 Da. The 34 S component is built of 24 polypeptide chains, and the 60 S component of 48 polypeptide chains. Its molecular weight is about 3 320 000 Da. The 60 S component occurs only in the class of Merostomata, the 34 S component predominates in Arachnida, and the 16 S and 24 S components in Crustacea.

Quarternary structure of the molecule of Chelicerata hemocyanin has been determined in 1981 using the method of electron microscopy. Also, amino acid sequence has been deciphered for two polypeptide chains (d and e) of hemocyanin of Eurypelma californicum and for a part of the a-subunits of hemocyanin of Tachypleus tridentatus. Moreover, amino acid sequence of hemocyanin of four representatives of Crustacea is elucidated. From comparison of known amino acid sequences of hemocyanins, the following general regularities have been established:

1) Polypeptide chains of hemocyanins are similar in length (about 625 amino acids in Chelicerata, and 660 in Crustacea).

2) Identical sequences occur usually in the neighbourhood of histidyl residues of copper - binding ligands.

3) There is a high similarity between various hemocyanin chains with respect to the number of hydrophilic and hydrophobic residues [7, 23].

3. CHARACTERISTICS OF ASELLUS AQUATICUS RACOV. (CRUSTACEA, MALACOSTRACA, ISOPODA, ASELLIDAE)

Isopoda are an order of Crustacea in which terrestrial forms have the highest shave. From among 42 species occuring in Poland, only 8 inhabit waters, and of this number only ane Asellus aquaticus, occurs in surface fresh waters [14, 29].

Asellus aquaticus belongs to gonochoric animals, males being usually bigger (17-20 mm) than females (12-15 mm). Eggs are deposited to the egg pocket and embryos develop there. Diameter of eggs is about 0.3-0.4 mm. Female produces several tens of eggs per year, on the average. The number of eggs varies in different individuals, and depends on their size which correlates closely with the body weight.

Embryo development in the egg pocket of the female lasts usually 1 to 2 months, depending on environmental conditions (mainly on the temperature of water). During this time, young crustaceans molt three times. Small completely formed animals of the length of about 1 mm leave the egg pocket.

Reproduction period begins in early spring, usually at water temperature of about 10°C. Animals which hatched of these eggs are capable of reproduction already in autumn and produce the second generation in the same year.

Maximal life span of Asellus aquaticus amounts to about 2.5 year, and its ecological longevity is several months up to one year.

Asellus aquaticus has an open circulatory system, composed of heart, blood vessels and sinuses of the coelom. Its hemolymph is almost colorless (pale yellow).

Fallen leaves are the main food of Asellus aquaticus. Also animal carcasses (under laboratory conditions dead animals of the same species) may serve a food [6, 8].

This species of very tolerant to changcable environmental conditions and may rapidly adapt to diverse habitat. It occurs in lake littoral, and in running waters, on muddy bottom and on plant detritus. Asellus aquaticus is frequent in organically polluted waters, including the α-β and β-mesaprobic zones of

sewage receivers (breweries, cellulose factories). It survives easily oxygen deficiency and requires oxygen concentration in water of about 0.4-0.5 mg/l O_2 i.e. approx. 10-fold lower than the saturating oxygen concentration. This is in contrast with the behavior of some crustaceans as the oxygen concentration of 2 mg/ml is critical for crayfish: in other crustaceans (Daphnia, Artemia) an increase in the concentration of hemoglobin was constated with diminishing oxygen level in the medium [14, 17].

Lately (1983) it has been demonstrated, using the respirometric method, that oxygen consumption by males of Asellus aquaticus was significantly higher when the animals were grown in polluted water from the Vistula river than when they were grown in equilibrated tap water. Oxygen consumption increased with increasing pollution water used for animal culture. Up to date, this phenomenon has not been explained. It has been suggested that toxic compounds uncouple electron transport from oxidative phosphorylation [18, 34, 35, 36].

4. AIM OF THE STUDY

Our interest in Asellus aquaticus is due to its exceptional susceptibility to oxygen deficiency in water. Asellus aquaticus withstands oxygen deficiency exceptionally easily and even does not avoid polluted reservoirs. It has been also observed that this crustacean increased its oxygen consumption with increasing water pollution [36].

Asellus aquaticus is a typical saprophyte, being thus different from its distant relative, the crayfish Astacus astacus which is sensitive to even slightest pollution of its environment [20].

For estimation of the pollution of water ecosystems, apart from physico-chemical studies (evidencing the situation in the moment of sampling), a multigrade scale is employed, based on the presence or absence of some bioindicator species. Such a bioindicator scale commonly used for estimation of water pollution is based on a saprobe system in which Asellus aquaticus is employed mainly in test analysis [32].

In crustaceans, either hemoglobin (Hb) or hemocyanin (Hc) serves as a respiratory protein. Both the aggregation state of hemocyanin and its oxygen affinity are dependent on concentrations of some ions (H, Ca, Mg, lactate). However, strange and hard to explain phenomena can be observed in nature. E. g., an aqueous snail Planorbis corneus contains hemoglobin while another representative of this order, also inhabiting freshwaters, Lymnaea stagnalis, has hemocyanin.

The order of Isopoda includes also the family of Oniscoidea (millipedes) containing terrestrial crustaceans, most of which inhabits moist, darkened environments [14, 16]. Different habitats (water vs earth) of so dosely related animals make them a useful model for studies of the organism - environment relationship [16].

Till now, data on physico-chemical properties of the hemolymph of Asellus aquaticus are lacking. Also, there is no information on the type of respiratory protein present in the hemolymph of Asellus aquaticus. This study has been aimed at elucidation of these questions.

5. MATERIAL AND METHODS

5.1. Hemolymph

Hemolymph of Asellus aquaticus was used in this study.

The animals have been collected from March to September in the "Niebieskie Źródła" springs by Tomaszów Mazowiecki, in water ponds at the Rogowska street in Łódź and in running waters in Łagiewniki. After transportation of the animals, their hemolymph was taken immediately, or the crustaceans were kept for a short time in aquaria.

Hemolymph was taken from the dorsal vessel using a glass capillary, the puncture being done between the last segment of the thorax and the first segment of the abdomen. Approx. 1-1.5 µl of the hemolymph was obtained from one animal. Hemolymph was stored in a refrigerator at the temperature of $+4^{\circ}C$.

5.2. pH determination

pH of the hemolymph was determined using a Beckman pH-meter with a conjugated microelectrode enabling measurements of samples of the volume limited to about 0.1 ml.

5.3. Protein estimation

The protein content of the hemolysate was estimated using a slight modification of the microbiuret method [19].

Bovine serum albumine of stock concentration of 250 µg/ml in 0.5 M NaOH - 0.9% NaCl was used as a standard. For determination of a standard curve the absorbance of two sets of tubes, A and B, was measured.

Set A: Into five tubes, 50, 100, 150, 200 and 250 µg protein was added (0.2; 0.4; 0.6; 0.8 and 1.0 ml of the stock albumin solution). Then 0.5 ml of 0.21% $CuSO_4 \cdot 5H_2O$ in 30% NaOH was pipetted to each tube and the content of each tube was made up to 1.5 ml with 0.5 M NaOH. Absorbance was measured against a blank in a 2 cm cuvette.

Set B: Procedure was similar to that used for set A but only 0.5 ml of 30% NaOH was added instead of 0.5 ml 0.21% $CuSO_4 \cdot 5H_2O$ in 30% NaOH.

Solutions in the tubes were stirred and left for 15 min. at room temperature. Absorbance was read at the wave length of 310 nm. Differences in the absorbance of the tubes in the two sets at the same protein concentration (A-B) were used for determination of the absorbance vs concentration relationship.

For determination of protein concentration, 2 µl of fresh hemolymph were taken, added with 0.5 ml of 0.21% $CuSO_4 \cdot 5H_2O$ in 30% NaOH and made up with 0.5 M NaOH up to the volume of 1.5 ml. After 15 min. absorbance was measured against a blank, at the wavelength of 310 nm. In parallel, absorbance of a sample in which 0.21% $CuSO_4 \cdot 5H_2O$ in 30% NaOH was substituted for 30% NaOH, was measured.

Protein concentration of the hemolymph was read from the standard curve, on the basis of the value of difference between the two absorbance measurements.

5.4. Determination of metal ion concentrations

The cation content of the hemolymph was determined by atomic absorption spectrophotometry, using an AAS 1 spectrometer (Carl Zeiss, Jena). Sample volume was 1,5-2 ml.

5.5. Lactate concentration

Lactate was estimated using a Lactat UV-test (Boehringer Mannheim GmbH Diagnostica). The test is based on the reaction: L--lactate + $NAD^+ \rightarrow$ pyruvate + NADH + H^+. The lactate content is proportional to changes in the absorbance of the solution at 340 nm after incubation with LDH [25].

5.6. Analytical ultracentrifugation of hemolymph proteins

Analytical ultracentrifugation of hemolymph proteins was performed in a MOM 3170B ultracentrifuge at the temperature of $+4^{\circ}C$. Rotation speed of the A-60 rotor was 48 000 rpm. Protein position was monitored using a Phillpot-Svenson automatic optical system. On the basis of photograms, the rate of protein dislocation was determined. The value of the sedimentation constant was calculated using the formula.

$$S = \frac{2.303 \ tg\alpha}{\left(\frac{2 \ xrpm}{60}\right)^2 60}$$

5.7. Electrophoretic separation

a) Standard electrophoresis at pH 7.6, with the addition of $CaCl_2$ [5].

Electrophoresis was performed in 5% polyacrylamide gel. For the induction of polymerization, TEMED and ammonium persulfate was used. The composition of the electrode buffer, pH 7.6, was the following: 12.1 g Tris, 0.2 g potassium azide, 4.4 g $CaCl_2$ in 100 ml. Before sample application, preelectrophoresis was run for 6 hrs at the curvent of 15 mA.

Samples for electrophoresis were diluted with the electrode buffer and charged with an equal volume of the bromphenol blue: glycerol mixture (1 : 1). The separation was run in the cathode → anode direction at the current of 30 mA for 60 hrs. After separation, the protein fractions were visuolized by Coomassie Brilliant Blue R-250 staining. Then, the stain was removed and the gel was destained with a washing solution of methanol : acetic acid : water (10 : 25 : 175) up to the complete destaining.

b) Standard electrophoresis at pH 9 without $CaCl_2$

The electrophoretic method was as above, with the following exceptions: electrode buffer without $CaCl_2$, 1-hr preelectrophoresis, 6-hr eletrophoresis.

c) SDS - electrophoresis at pH 8.3 [5.21]

Composition of the electrode buffer, pH 8.3, per 500 ml solution, was as follows: Tris-HCl, 1.5; glycine, 7.2 g; SDS, 0.5 g.

Samples were prepared using 10 volumes of the hemolymph, 9 volumes of the dissolving solution, and 1 volume of β-mercaptoethanol.

Composition of the dissolving solution was the following: 1 ml of 1.25 M Tris-HCl, pH 6.8; 2 ml of 20% SDS; 2 ml glycerol; 0.1 ml of pyronine Y, 2.5 mg/ml; H_2O, up to 9 ml. So prepared samples were incubated for 24 hrs at room temperature. Prior to application, the samples were heated for 5 min. in a boiling water bath.

Electrophoresis was run from cathode to anode at the current of 10 mA, for 6 hrs.

For the staining, the gel was immersed in 0.05% Coomassie Brilliant Blue R-250 in 25% isopropanol and 10% acetic acid for 12 hrs. Then the stain was removed and the gel was destained in 0.005% Coomassie Brillant Blue R-250 in 25% isopropanol - 10% acetic acid, and then in 0.0025% Coomassie Brilliant Blue in 10% acetic acid.

5.8. Immunochemical localization of the glycoproteins

Immunochemical localization of CoA - dependent glycoproteins was performed according to Glass et al. [9] and to Towbin et al [31].

5.9. Isolation of hemocyanin

Hemocyanin was obtained by preparative ultracentrifugation of the hemolymph, using a MOM 3170B ultracentrifuge run at 50 000 rpm for 6 hrs.

5.10. Spectrum of Hc

Absorption spectra of the hemolymph (hemocyanin) were read in the range of 230-700 nm in a Pye-Unicam SP-1700 spectrophotometer.

5.11. Analysis of amino acid composition of Hc

The hemocyanin preparation obtained was lyophylized and 1 ml aliquots were sealed in ampoules with 1 ml methanosulphonic, acid after deaeration with helium. Hydrolysis was performed at 115°C for 24 hrs. Then the acid was precipitated with 1 ml 3 N NaOH. The samples were filtered and made up with distilled water up to 4 ml. So prepared aliquots were subjected to amino acid analysis in a Jeol 6AH analyser.

Basic amino acids were separated in a column, of a bed height of 15 cm. Before apllication, the samples were eluted with 0.35 M buffer, of pH 5.28.

Acid and neutral amino acids were eluted with 0.2 M buffer of increasing pH in the range of 3.28-4.25, in a column of a bed height of 45 cm. Color reaction with ninhydrin was performed in the column eluate. Color intensity was read at two wavelengths: 440 nm for proline and hydroxyproline, and 570 nm for the remaining amino acids.

5.12. Determination of parameters of oxygen affinity and allostery

Oxygen affinity was determined by the spectrophotometric method, absorbance being measured at 340 nm. For these measurements, a Volvekamp tonometer in the modification of Konings [11, 33] was used.

2 ml of approx. 10 ml diluted hemolymph (hemocyanin), of absorbance of 0.400-0.700 was introduced into the tonometer. Then the hemolymph was deaerated with argon for about 50 min. and the absorption spectrum was read in the range 300-400 nm. After conversion of the hemocyanin into the deoxy form, appropriate air volumes were introduced with a syringe, the solution was mixed for 5-10 min, and absorbance was measured.

From the oxygen dissociation curves, parameters of oxygen affinity (p_{50} and n) were determined. Functional properties of the protein were compared with the MWC model using program for the Amstrad CPC 464 microcomputer [12].

5.13. Staining for copper fractions

Identification of copper protein fractions in the gel was based on the selective copper staining according to Gould and Karolus [13] and comparison with electrophoresis of fractions stained for protein.

6. RESULTS

6.1. Physico-chemical properties of the hemolymph

The content of the components determined occuring in the hemolymph of Asellus aquaticus is summarized in Tabl. 1.

The sequence of cations in the hemolymph shown corresponds to the rising sequence of their concentrations. Sodium is preponderant, and magnesium in present in the lowest amounst.

The ratio of individual mineral components of the hemolymph

(Tabl. 1) differs from the average content of sea water or fresh water [26].

Table 1

The contents of components determined in the hemolymph (mg/l)

Nr	Parameter	♂	♀	♂ + ♀
1.	Cu	23.7 ±1.2	51.9 ±2.1	37.8 ±1.7
		31.0 ±1.0	34.0 ±1.1	33.1 ±1.05
				48.8 ±1.5
				29.0 ±0.9
				55.0 ±2.3
$\bar{x}$				40.74
2.	Mg	12.0 ±0.4	16.1 ±0.5	14.05 ±0.7
				26.40 ±0.8
				26.00 ±0.75
				34.00 ±10.5
$\bar{x}$				25.11
3.	K			208.8 ±7.2
				180.3 ±5.4
				140.0 ±4.4
$\bar{x}$				176.37
4.	Ca	277 ±0.5	146 ±4.5	196.5 ±3.2
		495 ±15.2	730 ±25	612.5 ±20.6
				362.0 ±12.1
				867.0 ±26.2
				780.0 ±24.3
$\bar{x}$				563.6
5.	Na			2552,8 +93,1
				2420,0 +75,2
$\bar{x}$				2486,4

Tabele 1 (cd.)

Nr	Parameter	♂	♀	♂ + ♀
6.	Protein g/100	1.3 ±0.065	2.5 ±0.12	1.9 ±0.09
				2.9 ±0.07
				2.47 ±0.05
				2.50 ±0.06
$\bar{x}$				2.44
7.	Lactate mM	0.6 ±0.020	1.31 ±0.033	0.955 ±0.015
		0.49 ±0.010	0.56 ±0.02	0.520 ±0.010
		0.35 ±0.004	0.42 ±0.001	0.385 ±0.009
				0.711 ±0.009
$\bar{x}$				0.643
8.	pH			7.6 ±0.05
				7.5 ±0.05
$\bar{x}$				7.55

On this basis it can be hypothesized that Asellus aquaticus has not only the capacity for controlling osmotic pressure, but possesses also a mechanism allow the maintenance of quantitative ratios of individual components different from those in the external medium. This is typical also for majority of invertebrates.

The final product of aerobic metabolism, lactic acid, has been proved to be an allosteric moderator of hemocyanin, increasing its oxygen affinity. The concentration of lactate in the hemolymph of Asellus aquaticus is 0.6 mM and is higher than in the hemolymph of millipedes [16].

Total protein content of the hemolymph is about 2.5%. It is known (from the literature data) that hemocyanin accounts for over 90% of the hemolymph proteins. Therefore, the ratio of protein (B) to divalent cations (and other components) shown in Tabl. 2 represents the ratio of hemocyanin to the components determined. There would be an only slight error in the assumption that

so calculated ratios correspond to the physiological (in vivo) conditions.

Table 2

Ratios of some components in the hemolymph of Asellus aquaticus

Cations (mM)		Cations/protein (mg/l)	
Na/Ca	7.6	Cu/B	0.0017
Na/Mg	103.9	Ca/B	0.023
Na/K	23.9	Mg/B	0.001
Ca/Mg	13.4	Ca+Mg/B	0.024
Ca/K	3.13	Na/B	0.102
Mg/K	0.23	K/B	0.0072
Na + K		Na+K/B	0.1092
-----	7.43	lactate/B	0.002
Ca + Mg			

In the hemolymph of Asellus aquaticus, the Cu content was found to be 40 mg/l, and the ratio of Cu to protein was 0.17%. This value is similar to the Cu content of the hemocyanins. A next indirect proof for the presence of the respiratory protein, hemocyanin, in the hemolymph of Asellus aquaticus is its absorption spectrum. The maximum occuring at 339 ± 2 nm is characteristic for HcO_2 as the so-called "copper band". The spectrum of hemoproteins, with its Soret band (405-415 nm) and maxima at 530 and 560 nm for the deoxygenated form is different from that presented in Fig. 1.

Saturation of the hemolymph with argon (fremoval of oxygen from the solution) resulted in a decrease of the copper maximum. Its reappearance was observed upon some time after passing air through the hemolymph.

Hemolymph proteins subjected to analytical ultracentrifugation were separated into 3 fractions (Fig. 2). After determination of their sedimentation coefficient, the 16 S fraction was found to be dominant, accounting for over 70% of the total. Frac-

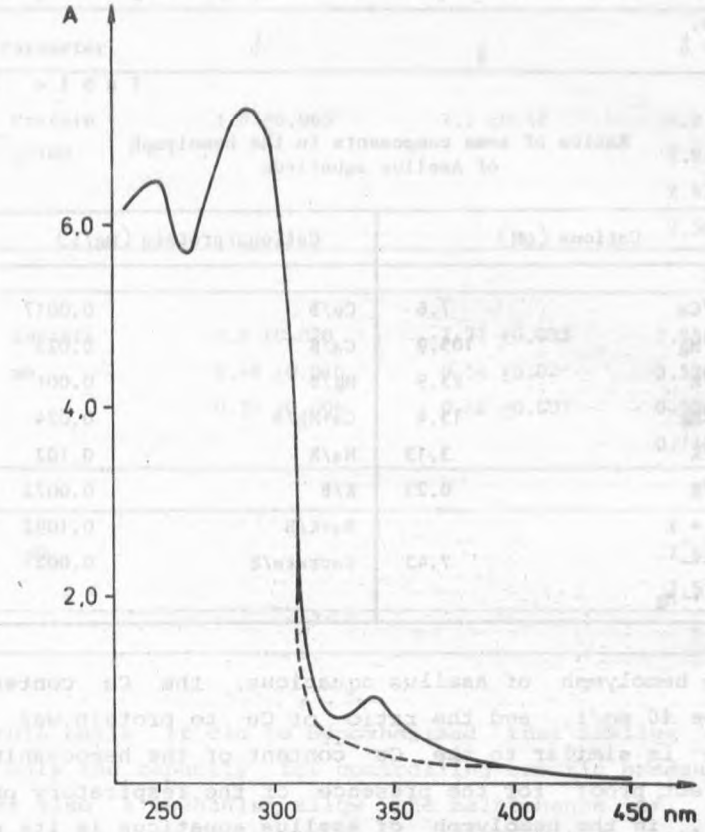
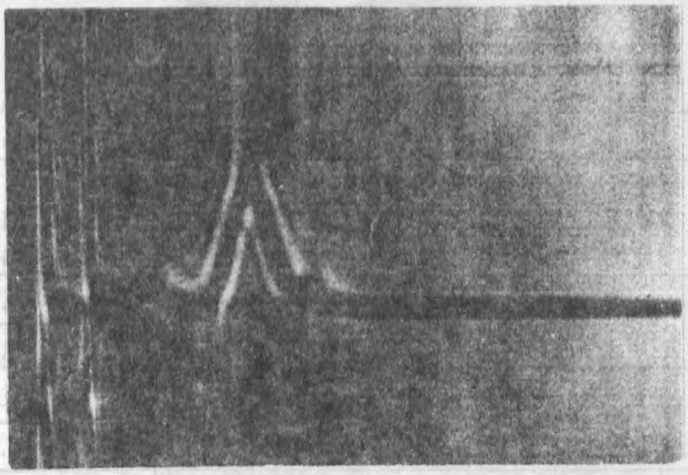


Fig. 1. Spectrum of the hemolymph (Hc)

tions of 5 S and of 24 S were present is small amounts (10 and 15%, respectively; Tabl. 3). The 5 S band seems inhomogenous. It can be assumed on the basis of the obtained data that hemocyanin in vivo occurs mainly in the form of a hexamer, of sedimentation constant of 16 S, and of the molecular weight of 500 000 Da.

Properties of the hemolymph proteins in the electric field are illustrated by electrophoretic separations performed under various conditions. Separation in 5% polyacrylamide gel in a continuous system of pH 7.6 in the presence of $CaCl_2$, yielded 5 protein fractions (Fig. 3).

a



b

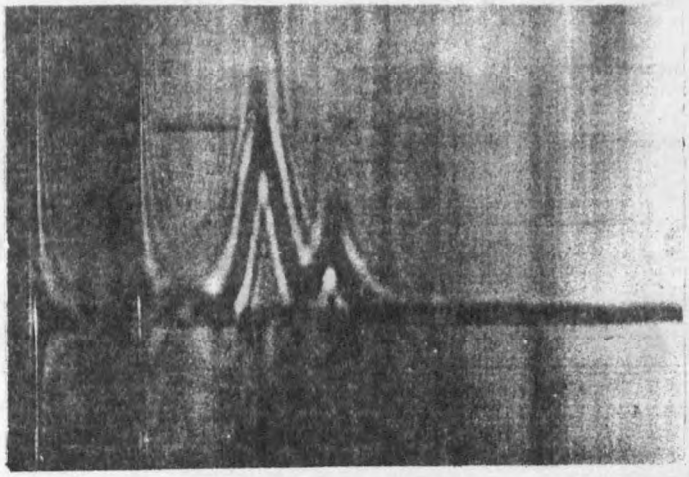


Fig. 2. Ultracentrifugal separation of hemolymph proteins: a)- males, b) - females

In a parallel staining for copper, 4 fractions were visualized (Fig. 3).

Therefore, copper proteins (hemocyanin) dominate among the hemolymph proteins.

Table 3

Per cent composition and sedimentation constans of hemolymph proteins

		Fraction I	Fraction II	Fraction III
♂	S	5.9 ±0.4	15.9 ±0.65	22.6 ±1.1
	%	15 ±0.30	75 ±1.5	10 ±0.3
♀	S	3.2 ±0.35	15.1 ±0.50	23.6 ±0.7
	%	10 ±0.2	65 ±1.6	25 ±0.5
♂ + ♀	S	4.5 ±0.37	15.5 ±0.82	23.0 ±1.3
	%	12.5 ±0.36	70 ±2.2	17.5 ±0.58

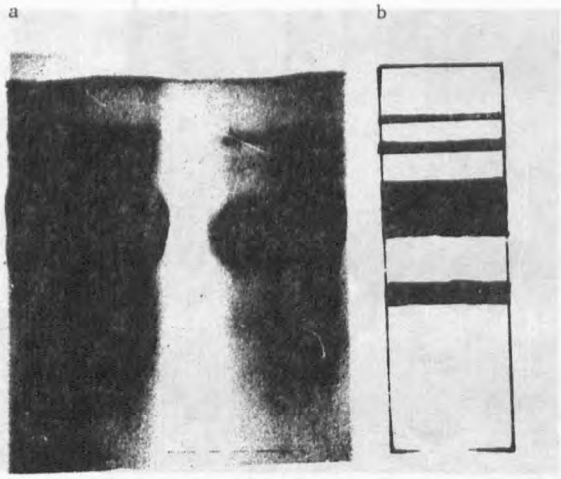


Fig. 3. Electrophoretic separation of hemolymph proteins at pH 7.6 a) staining for protein; b) staining for copper

Standard electrophoresis at pH 9 in the absence of calcium ions revealed 8 fractions (Fig. 4).

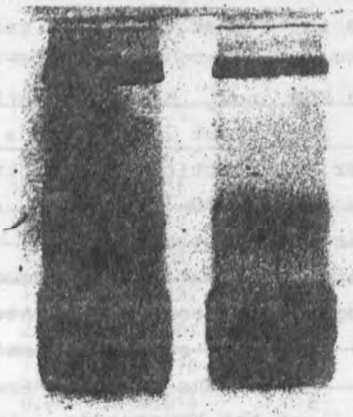


Fig. 4. Electrophoresis at pH 9 without $CaCl_2$

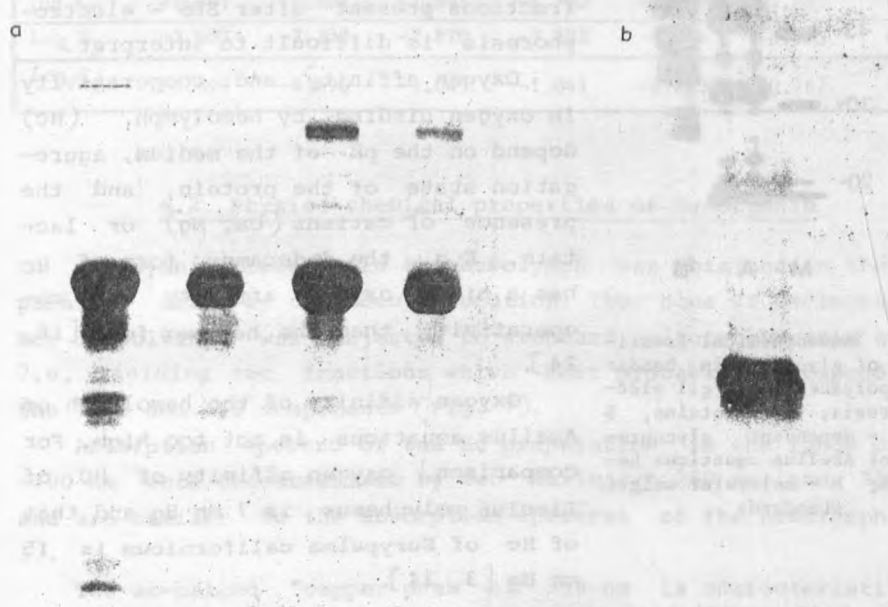


Fig. 5. a) SDS - Electrophoretic separation of hemolymph proteins; b) lower protein concentration

Alkaline dissociation of hemocyanin into smaller components in the absence of salts is a characteristic feature of this protein.

After treatment with SDS and β-mercaptoethanol, hemolymph proteins were separated into about 25 fractions (Fig. 5).

A considerable number of fractions, was obtained of molecular weights in the range of 20 000-100 000 Da as estimated by comparison with molecular weight standards.

It is known from literature data that hemocyanins are glycoproteins, of the carbohydrate component content of 1-10%.

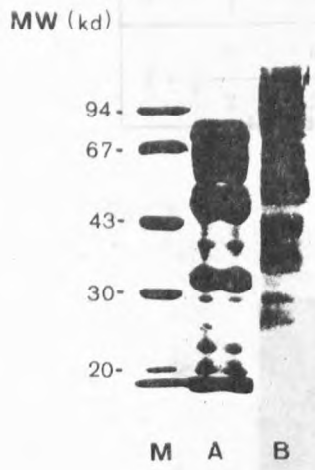


Fig. 6. Immunochemical localization of glycoprotein bands: SDS - polyacrylamide gel electrophoresis; A - proteins, B - ConA - dependent glycoproteins of Asellus aquaticus hemolymph; M - molecular weight standards

For eventual identification of fractions derived from the degradation of the hemocyanin, immunochemical identification of glycoproteins has been accomplished (Fig. 6). The presence of over 20 CoA - dependent glycoprotein fractions present after SDS - electrophoresis is difficult to interpret.

Oxygen affinity and cooperativity in oxygen binding by hemolymph (Hc) depend on the pH of the medium, aggregation state of the protein, and the presence of cations (Ca, Mg) or lactate. E.g., the dodecamer form of Hc has a higher oxygen affinity and cooperativity than the hexamer form [16, 24].

Oxygen affinity of the hemolymph of Asellus aquaticus is not too high. For comparison, oxygen affinity of Hc of Limulus polyphemus is 7 mm Hg, and that of Hc of Eurypelma californicum is 15 mm Hg [3, 24].

The Hill coefficient amounts to 2.6, i.e. is somewhat lower with respect to the values of n of other arthropods (3.8-5). Results of oxygen binding in the three states are shown in Tabl. 4.

Table 4

Parameters of oxygen affinity and allostery

	1	2	3	4	5	x	s
P_{50T}	113.61	56.54	51.31	58.31	30.05	62.01	12.64
P_{50}	18.48	18.39	18.12	17.83	20.87	18.74	0.50
P_{50R}	4.23	4.87	5.88	6.85	4.14	5.19	0.047
n_1	0.828	1.142	1.092	1.014	1.575	1.130	0.113
n_2	2.432	2.556	2.281	2.658	3.303	2.646	0.161
n_3	0.957	1.004	1.070	1.246	1.092	1.074	0.045
K_T	0.020	0.010	0.014	0.016	0.005	0.013	0.002
K_R	0.252	0.204	0.150	0.091	0.212	0.182	0.025
L_1	36.07	30.33	13.22	12.43	247.75	104.72	61.95
$L_2 x 10^3$	8.5	2.5	0.8	0.4	7.7	6.2	1.6
$\log K_T$	-1.701	-2.001	-1.867	-1.790	-4.318	-1.937	0.100
$\log K_0$	-3.081	-3.233	-2.870	-3.325	-4.358	-3.375	0.235
$\log K_R$	-0.599	-0.690	-1.041	-1.041	-0.673	-0.767	0.071

6.2. Physico-chemical properties of hemocyanin

Hemocyanin present in the hemolymph was obtained in the preparative scale by ultracentrifugation. The blue Hc sediment after dissolving, was subjected to standard electrophoresis at pH 7.6, yielding two fractions which most probably correspond to the 16 S and 4 S components (Fig. 7).

Absorption spectro of the Hc preparation in the range of 200--700 nm were characterized by two maxima at 280 nm and 338 nm, and are similar to the absorption spectrum of the hemolymph (Fig. 1).

The so-called "copper peak" at 338 nm is characteristic for the HcO_2 form. Removal of air (oxygen) from the solution resulted in a disappearance of that peak. In this way the deoxygenated form of hemocyanin (deoxyhemocyanin) was obtained.

On the basis of amino acid separation, the per cent amino

Table 5

Amino acid composition of Hc of Asellus aquaticus

Amino acid	%	Number of residues	Minimal molecular weight
Lysine	6.05	16.35	2390
Histidine	5.72	15.46	2399
Arginine	5.08	13.73	2391
Aspartic acid	15.31	41.38	5508
Threonine	5.39	14.57	1735
Serine	5.37	14.52	1525
Glutamic acid	9.91	26.79	3940
Proline	5.33	14.41	1658
Glycine	6.96	18.81	1412
Alanine	7.05	19.06	1697
Cystine	0.37	1	121
Valine	6.38	17.25	2019
Methionine	0.74	2	298
Isoleucine	3.82	10.33	1354
Leucine	7.23	19.54	2564
Tryptophan	3.52	9.51	1942
Phenylalanine	5.95	16.08	2656
	100,18		35607

Fig. 7. Electrophoretic separation of crude Hc

acid composition of Hc of Asellus aquaticus was established (Tabl. 5).

This amino acid composition does not differ from the composition of other crustacean hemocyanins. Dicarboxyl amino acids, and glutamate, are dominant (25%); less abundant are: alanine, glycine and leucine. Sulfur amino acids occur in small amounts. When estimating the molecular weight of the 5 S monomer as 70 000, the number of the residues obtained should be doubled.

7. CONCLUSIONS

1. On the basis of determinations of cation concentrations in the hemolymph of Asellus aquaticus it has been established that scdium is the dominant cation while magnesium occurs in lowest amounts.

2. The lactate concentration in the hemolymph of Asellus aquaticus is 0.6 mM.

3. The copper content of the hemolymph of Asellus aquaticus is close to that of other arthropods hemolymphs.

4. Absorption spectrum of the hemocyanin shows absorption maxima at 280 and 338 nm and is similar to the absorption spectrum of Hl.

5. Hc of Asellus aquaticus occurs in vivo as the 16 S hexamer of molecular weight of 500 000 Da. The 24 S and 5 S components occur in small amounts.

6. Amino acid composition of Asellus aquaticus Hc is dominated by aspartate and glutamate. Less frequent are: alanine, glycine and leucine. Sulfur amino acids occur in low amounts.

7. Oxygen affinity of the Hl of Asellus aquaticus is relatively low, as compared with other arthropods, and the Hill coefficient is 2.6.

REFERENCES

[1] Adamska M., Gondko R., Kosmos, 3, 417 (1986).
[2] Adamska M., Gondko R., Przegl. Zoolog. XXXI, 3, 15 (1987).
[3] Aljakrinskaja O. J., Giemoglobiny i giemocianiny bezpozwonocznych, Nauka, Moskwa (1979).
[4] Bogucki M., Acta Biol. Expres., 8, 80 (1933).
[5] Catalog Hoefer Scientific Instruments (1983).
[6] Czeczuga B., Zoolog. Polon., 32, 73 (1985).
[7] Gaykema W. P. J., Jol W. G. J., Vereijken J. M., Soeter N. M., Bak H. J., Beintema J. J., Nature, 309, 23 (1984).
[8] Gemborek E., Ośliczka - Asellus aquaticus jako materiał do ćwiczeń w szkole, Warszawa 1924.
[9] Glass W. F., Brigss R. C., Hnilica L. S., Anal. Biochem, 115, 219 (1981).
[10] Gondko R., Alighan M. A., Leyko W., Zesz. nauk. UŁ, S. II, 19, 279 (1976).
[11] Gondko R., Acta Univ. Lodz., Folia biochim. biophys., 5, 79 (1986).
[12] Gondko R., Acta Univ. Lodz., Folia biochim. biophys., 7, 73 (1990).
[13] Gould E., Karolus J., Anal. Biochem., 67, 515 (1975).
[14] Grabda E. [red.], Zoologia, Bezkręgowce, Warszawa (1985).
[15] Grodzieński W., "Wszechświat", z. 7-8, 161 (1980).
[16] Helszer Z., Fizykochemiczne właściwości hemocyjaniny niektórych Oniscoidea - Ph. D. Thesis, Łódź 1986.
[17] Jażdżewski K., Zool. Polon., 20, 423 (1970).
[18] Kamiński A., Roczn. Wojsk. Inst. Hig. Epidem., 4 (3), No 2/3, 19 (1965).
[19] Kłyszejko-Stefanowicz L., [red.], Ćwiczenia z biochemii, Warszawa 1969.
[20] Kossakowski J., Raki, Warszawa 1966.
[21] Laemli U. K., Nature, 227, 680 (1970).
[22] Lewanidow W., Biologia i ekologia wodianych oslikow, [w:] Fauna SSSR - Raukobraznyje, Moskwa-Leningrad 7, 35 (1951).

[23] Linzen B., Soeter N. M., Riggs A. F., Schneider H. J., Schartau W., Moore M. D., Yokota E., Behrens P. Q., Nakashima H., Takagi T., Nemoto T., Vereijken J. M., Bak H. J., Beintema J. J., Volbeda A., Gaykema W. P. J., Science 229, 519 (1985).

[24] Michalak W., Heterogenność hemocyjaniny raka błotnego, Astacus leptodactylus. Ph. D. Thesis, Łódź 1985.

[25] Morris S., Bridges C. R., Grieshaber M. K., J. Experim. Zoology, 234, 151 (1985).

[26] Pinta M., Absorpcyjna spektrometria atomowa. Zastosowania w analizie chemicznej, Warszawa 1977.

[27] Rochu D., Fine J. M., Comp. Biochem. Physiol., 66, 273 (1980).

[28] Rochu D., Lambin P., Ghidalia W., Fine J. M., Comp. Biochem. Physiol., 59B, 117 (1978).

[29] Stańczykowska A., Zwierzęta bezkręgowe naszych wód, Warszawa 1979.

[30] Terwilliger N. B., Terwilliger R. C., Applestein M., Bonaventura C., Bonaventura J., "Biochemistry", 18, 102 (1979).

[31] Towbin H., Stachelin T., Gordon J., Proc. Natl. Acad. Sci., 76, 4350 (1979).

[32] Turoboyski L., Hydrobiologia techniczna, Warszawa 1979.

[33] Wolvekamp cyt. Koningsberger N. W., Thesis D., Rijksuniversiteit te Groningen (1969).

[34] Zimakowska D., Pol. Arch. Hydrobiol., 20, 469 (1973).

[35] Zimakowska-Gnoińska D., Pol. Arch. Hydrobiol., 24, 389 (1977).

[36] Zimakowska-Gnoińska D., Pol. Arch. Hydrobiol., 30, 165 (1983).

Praca wykonana w problemie PR.2.11.

Laboratorium of Biological Sciences
University of Łódź

Maria Adamska

WŁAŚCIWOŚCI FIZYKOCHEMICZNE HEMOLIMFY I HEMOCYJANINY ASELLUS AQUATICUS L.

W niniejszej pracy przedstawiono fizykochemiczne właściwości hemolimfy i hemocyjaniny Asellus aquaticus L. W hemolimfie Asellus aquaticus oznaczono zawartość kationów (Ca, Mg, Na, K, Cu) oraz mleczanu i białka. Zmierzono również pH hemolimfy, które wynosi 7.5. Wykreślono widmo absorpcyjne hemolimfy i hemocyjaniny przy 280 i 338 nm. Białka hemolimfy poddano ultrawirowaniu analitycznemu w wyniku, którego otrzymano 3 frakcje. Dominowała frakcja 16 S, stanowiąca ponad 70% całości, a w niewielkich ilościach obecne były frakcje 24 S i 5 S. W różnorodnych warunkach wykonano rozdziały elektroforetyczne, obrazujące właściwości białek hemolimfy w polu elektrycznym. Z krzywej dysocjacji tlenowej wyznaczono parametry powinowactwa p_{50} i n. Współczynnik Hilla dla hemolimfy Asellus aquaticus wynosi 2.6.

Hemocyjaninę otrzymano w wyniku ultrawirowania preparatywnego przy użyciu ultrawirówki typu MOM 3170B. Zliofilizowane próbki hemocyjaniny poddano automatycznej analizie aminokwasowej na analizatorze Jeol 6AH.