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# Hematin and sulfide removal in hemolymph of the hemoglobin-containing bivalve *Scapharca inaequivalvis*

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ABSTRACT: The hemolymph of the bivalve *Scapharca inaequivalvis* (Bruguière) contains in addition to hemoglobin the unusual heme compound hematin. The variation in total heme concentration in the hemolymph was best accounted for by the variation in hematin. In the hemolymph the hematin is effectively separated from the hemoglobin. The compound is present *in vivo* in the hemolymph. The sulfide removal in the hemolymph is high and caused only by hematin (>90 %). The results are discussed in terms of adaptations to sulfide in hemoglobin-containing invertebrates from sulfide-rich environments.

## INTRODUCTION

Hydrogen sulfide is extremely toxic to aerobes because it inhibits ATP production by blocking the oxidative phosphorylation at Site III (cytochrome c oxidase) (National Research Council 1979). However, the discovery of the fauna associated with hydrothermal vents (Corliss et al. 1979) initiated a new perception of sulfide in the marine environment (reviewed e.g. by Somero et al. 1989, Fisher 1990, Vismann 1991a). It is now recognized that several species, ranging from ciliates to giant vestimentiferan worms, harbor sulfide-oxidizing bacteria as symbionts and thrive only in a mixture of oxygen and hydrogen sulfide (e.g. Cavanaugh et al. 1981, Fenchel & Finlay 1989). During the last decade physiological adaptations in nonsymbiotic animals from sulfide-rich habitats have also been recognized. These adaptations render the species sulfide tolerant to a varying degree and thus increase the species' ecological potential (e.g. Vismann 1990). Adaptations to sulfide identified in nonsymbiotic animals include partial exclusion by sulfide trapping in external mucus layers (Giere et al. 1988), sulfide immobilization by precipitation with iron compounds (Oeschger & Janssen 1991, Vismann 1991b), sulfide detoxification by special pigments in the hemolymph (Patel & Spencer 1963, Powell & Arp 1989, Vismann 1990), and sulfide detoxification by mitochondria (Powell & Somero 1985, Bagarinao & Vetter 1990, Oeschger & Vetter 1992).

Focusing on hemolymph, a sulfide-oxidizing pigment was demonstrated in the polychaete *Arenicola marina* by Patel & Spencer (1963). The pigment is an oxidized heme compound, and was termed 'brown pigment'. In a study on *Abarenicola affinis* Wells & Pankhurst (1980) were not able to demonstrate the presence of brown pigments and thus refuted the biological significance and *in vivo* presence of brown pigments. In annelids, oxidized heme compounds have been suggested to be an artifact and to occur only in individuals kept under poor laboratory conditions (Mangum 1976). However, Powell & Arp (1989) demonstrated the presence of a pigment catalyzing sulfide oxidation in the hemolymph of healthy specimens of the echiurid *Urechis caupo* and of the bivalves *Solemya reidi*, *Calyptogena magnifica*, and *Lucinoma annulata*. Powell & Arp (1989) showed the brown pigment to have an absorbance spectrum similar to that of oxidized (ferric) heme compounds, previously identified as hematin (Baumberger & Michaelis 1931). Powell & Arp (1989) concluded that the hematin present in *U. caupo* was contained in granules and not associated with proteins. The sporadic *in vivo* occurrence of hematin in polychaetes and bivalves from

sulfide-rich habitats (Mangum & Dales 1965, Powell & Arp 1989) suggests that it is a sulfide detoxifying factor in the hemolymph of hemoglobin-containing marine invertebrates. Furthermore, the brown pigment in *A. marina* (Patel & Spencer 1963) and in *Nereis diversicolor* (Vismann 1990) is most likely hematin, since the absorbance spectrum of hematin corresponds with the spectrophotometric analysis used in these studies (see Vismann 1990). Hematin has been demonstrated in several marine species belonging to different phyla, and the heme components of the hemolymph include both hematin and hemoglobin. The species shown to have hematin all live in habitats characterized by environmental sulfide.

The present study was initiated during a comparative study of anaerobic metabolism and sulfide resistance in 4 species of bivalves from the Adriatic Sea, when the distinct brown color of the hemolymph in the bivalve *Scapharca inaequivalvis* (Bruguière) was noticed. *S. inaequivalvis* is one of the few bivalve species containing hemoglobin. The hemoglobin has an oxygen half-saturation value ($p_{50}$) < 1.3 kPa (Weber 1990) and the species is known to be tolerant to hypoxia and anoxia (Brooks et al. 1991, de Zwaan et al. 1991). *S. inaequivalvis* is periodically exposed to hypoxia and sulfide (de Zwaan et al. 1991, Ab de Zwaan pers. comm.). The aim of the present study was to study the hemolymph of the bivalve *S. inaequivalvis* in order to reveal the presence of any hematin and to assess the activity of this compound on hydrogen sulfide.

## MATERIALS AND METHODS

*Scapharca inaequivalvis* was collected in the Adriatic Sea 5 km off Bellaria, Italy, at water depths of 10 m. At the time of collection the bottom water had a temperature of 19 °C and a salinity of 34.2 ppt. The experiments were carried out in both Italy and Denmark. In Italy the mussels were kept in filtered seawater (temperature = 20 °C, pH = 8.2, salinity = 35 ppt) aerated with atmospheric air in a large (3500 l) storage tank. Every second day about 20 % of the water was exchanged. In Denmark the mussels were kept in seawater (temperature = 17 °C, pH = 7.9, salinity = 35 ppt) aerated with atmospheric air in a 50 l tank. The mussels were fed daily with a mixture of *Pavlova lutheri, Rhodomonas* sp., and *Isochrysis* var. *thahitian.*

**Sample preparation.** The bivalves were carefully pried open and water in the mantle cavity was drained off. The blood-filled sinuses at the apices of the shells were perforated and a sample of hemolymph was taken with a pipette. Samples were immediately analyzed for heme compounds and protein.

**Heme compounds.** The concentration of hemoglobin was analyzed in subsamples (100 µl) diluted (1:10) in HEPES (10 mM, pH = 7.5). The absorbances at wavelengths 500, 577, and 600 nm were measured using a spectrophotometer (LKB, Ultrospec Plus). According to Powell & Arp (1989), the hemoglobin concentration in a mixture of hemoglobin and hematin is proportional to the absorbance at 576 nm minus the absorbance at 576 nm calculated from a line drawn between the absorbance values at 500 and 600 nm. The samples were compared to human hemoglobin standards using a hemoglobin kit (Sigma Diagnostics, No. 525-A) and expressed on a monomeric basis (tetrameric:dimeric ratio as in Weber 1990). In the present study the peak of hemoglobin absorbance was found at 577 nm, instead of at 576 nm as found by Powell & Arp (1989). Total heme concentration was analyzed on subsamples (100 µl), which were extracted in 1 ml acetone with 1 % concentrated HCl for 20 min (Falk 1964). After extraction the samples were centrifuged at 2000 × *g* for 2 min. The absorbance (635 nm) of the supernatants was measured spectrophotometrically and compared to human hemoglobin standards according to the method of Powell & Arp (1989). Hematin concentration was calculated from the concentration of hemoglobin and total heme using the formula: hematin = total heme – hemoglobin.

**Total protein.** The concentration of protein was assayed on 25 µl aliquots using a total protein kit (Sigma Diagnostics, No. 541-2). Protein concentrations were determined at 540 nm using a spectrophotometer (LKB, Ultrospec Plus) and compared to human albumin/globulin standards (Sigma Diagnostics, No. 540-10).

**Iron.** The iron concentration was measured using atomic absorption spectrophotometry. Samples were stored at −80 °C until analysis. After thawing the samples (100 µl) were dissolved in 2 ml concentrated $HNO_3$ and evaporated at 70 °C for 24 h and at 150 °C for 24 h. $HNO_3$ (0.2 %) was added and the absorbance of the samples was measured at 248.3 nm using an atomic absorption spectrophotometer (Philips Unicam, PU 9200). The iron concentration of the samples was calculated from standard curves.

**Hemolymph fractionation.** Hemoglobin and hematin were isolated from whole hemolymph using the following procedure of Powell & Arp (1989). The hemolymph was centrifugated twice for 5 min at 1000 × *g* followed by resuspension of the pellet in a volume of filtered seawater equal to the supernatant volume. The pellet from the third centrifugation was resuspended in 10 mM buffer (HEPES, pH = 7.5) and centrifugated for 20 min at 27 000 × *g*. The supernatant of this centrifugation contained the hemoglobin fraction. The pellet was washed twice by resuspension in

buffer and centrifugation for 10 min at 15 000 × *g*. The final pellet was resuspended in buffer and contained the hematin fraction. All operations were carried out on ice and centrifugations at a temperature of 3 ± 1 °C using a high-speed cooling centrifuge (Sigma, 3K3).

**Reduced sulfur compounds.** Samples were analyzed for reduced sulfur compounds using the monobromobimane technique (see e.g. Fahey & Newton 1987, Vetter et al. 1989). Sample treatment and HPLC analysis were according to Vismann (1991b).

***In vivo* exposure to normoxia and hypoxia.** A number of *Scapharca inaequivalvis* were placed in an aquarium (30 l) containing seawater (temperature = 17 °C, pH = 7.9, salinity = 35 ppt). The mussels were allowed 1 d of acclimation at atmospheric oxygen tension ($p_wO_2$). The $p_wO_2$ was then lowered to 2 kPa and kept constant. After 10 d of exposure the hemolymph of the mussels was analyzed for heme compounds and protein. In the aquarium the $p_wO_2$ was monitored using a Clark type oxygen electrode (Radiometer, E5047-0) connected to a pH/blood gas monitor (Radiometer, PHM 73). The pH/blood gas monitor was connected to a regulator (i.e. a comparator circuit), which kept the $p_wO_2$ at the set point. The regulator manipulated the $p_wO_2$ via 2 magnetic valves, supplying in the open position a flow of atmospheric air or $N_2$. The $p_wO_2$ was continuously recorded on a Philips PM8250A recorder. In this setup the $p_wO_2$ was controlled to ±0.13 kPa. As a control the experiment was repeated without lowering the $p_wO_2$.

***In vitro* sulfide exposure.** Samples (1 ml) were drawn into 10 ml syringes containing small magnetic bars. A $H_2S$ stock solution (in 10 mM HEPES, pH = 7.5, $pO_2$ = 20 kPa) was then drawn into the syringes giving a final sulfide concentration of 200 µM. Syringes added HEPES buffer instead of samples were used as a control. The syringes were sealed, placed on a magnetic stirrer and incubated. During incubation subsamples (100 µl) were taken through the outlet of the syringes using a Hamilton 100 µl syringe. The samples were analyzed for reduced sulfur compounds ($H_2S$, $SO_3^{2-}$, and $S_2O_3^{2-}$). The terms sulfide and $H_2S$ are throughout the rest of the paper referring to total sulfide (i.e. $H_2S$, $HS^-$, $S^{2-}$).

## RESULTS

### Hemolymph components

The concentration of heme compounds and protein in *Scapharca inaequivalvis* whole hemolymph is shown in Table 1. In Fig. 1 hematin and hemoglobin are shown as a function of total heme and protein. The variation in total heme (1.24 to 7.20 mM) was accounted for mostly by the variation in hematin (0.12 to 4.41 mM; $R^2$ = 0.54), but some of the variation (0.77 to 4.61 mM; $R^2$ = 0.20) was also due to hemoglobin. There was no correlation between hemoglobin and hematin ($R^2$ = 0.08) (not shown). Hemoglobin (Fig. 1D) was strongly correlated with protein ($R^2$ = 0.93) whereas hematin (Fig. 1C) was not ($R^2$ = 0.08).

Table 1. *Scapharca inaequivalvis*. *In vivo* concentration of heme compounds and protein in whole hemolymph. Data: mean ± SE, sample number in parenthesis

| Compound | | Concentration |
|---|---|---|
| Total heme | (n = 44) | 3.89 ± 1.34 mM |
| Hemoglobin | (n = 44) | 2.23 ± 0.95 mM |
| Hematin | (n = 44) | 1.66 ± 1.25 mM |
| Protein | (n = 9) | 54.32 ± 14.88 mg $ml^{-1}$ |

### Hemolymph fractionation

Table 2 shows the heme and protein concentrations of whole hemolymph and of the associated isolated heme compounds; 67 % of the total heme present in the hemolymph is recovered. In the isolated heme compounds 70 and 65 % of the hemoglobin and hematin is recovered. The isolated heme compounds are shown in Fig. 2 as a function of protein. The relation between the heme compounds and protein in whole hemolymph (Fig. 1C, D) and in fractionated hemolymph (Fig. 2) is relatively unchanged. The hematin and hemoglobin fractions both contained iron (Table 2). This supports the hypothesis that the isolated material is hematin and that the isolation method is satisfactory (see also Fig. 3). The hematin:iron molar ratio was not different from 1:1 ($0.30 < p < 0.40$), but the hemoglobin:iron ratio showed a significant deviation from unity ($p < 0.0001$).

Fig. 3 shows representative absorbance spectra of whole and fractionated hemolymph. From the absorbance spectra it is evident that a mixture of compounds exists in the hemolymph (Fig. 3A) and that the fractionation procedure used is effective in separating the compounds (Fig. 3B, C).

### *In vivo* exposure to normoxia and hypoxia

The difference in whole hemolymph components after *in vivo* exposure to 20 and 2 kPa oxygen tension for 10 d is seen in Fig. 4. The total heme concentration increased in hypoxia ($p < 0.0001$). This increase is due solely to an increase in hemoglobin

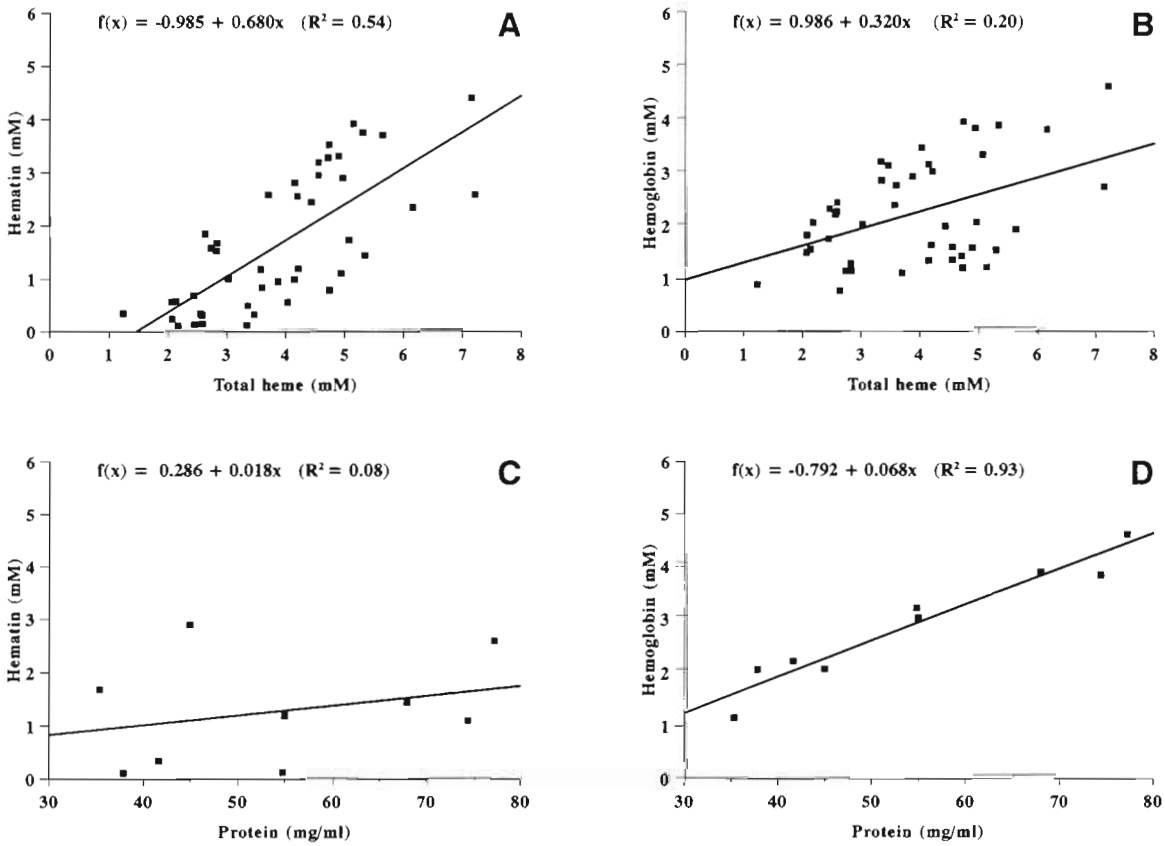


Fig. 1. *Scapharca inaequivalvis*. Heme compounds and protein in whole hemolymph: (A) hematin (n = 44) as a function of total heme; (B) hemoglobin (n = 44) as a function of total heme; (C) hematin (n = 9) as a function of protein; (D) hemoglobin (n = 9) as a function of protein. Data presented with regression and correlation coefficients

($p < 0.0001$). In fact, the hematin concentration decreased during hypoxia ($0.01 < p < 0.05$). The protein concentration also decreased significantly in hypoxia ($p < 0.0001$).

***In vitro* sulfide exposure**. Fig. 5 shows sulfide, sulfite, and thiosulfate concentrations as a function of time in isolated hematin (0.38 ± 0.05 mM) and hemoglobin (0.15 ± 0.02 mM) incubated with sulfide (200 µM). The sulfide was removed significantly only by hematin. Within 3.5 min the hematin removed about 150 µM sulfide giving a minimum removal rate of 112.8 µM sulfide $min^{-1}$ (mM hematin)$^{-1}$. In contrast, the hemoglobin fraction removed only about 20 µM sulfide in 12 min, giving a removal rate of 11.1 µM sulfide $min^{-1}$ (mM hemoglobin)$^{-1}$. Hematin was, therefore, responsible for more than 90 % of the sulfide removal. The $SO_3^{2-}$ concentration remained constant in the control, tending to decrease in the hematin fraction and to increase in the hemoglobin fraction. The $S_2O_3^{2-}$ concentration in the hematin fraction increased about 20 µM in 12 min, giving a $S_2O_3^{2-}$ production rate of 4.4 µM thiosulfate $min^{-1}$ (mM hematin)$^{-1}$. No $S_2O_3^{2-}$ was produced in the hemoglobin fraction or the control.

Table 2. *Scapharca inaequivalvis*. *In vivo* concentration (n = 10) of heme compounds, iron, and protein in whole hemolymph and in associated isolated heme compounds. Data: mean ± SE

| Sample | Total heme (mM) | Hemoglobin (mM) | Hematin (mM) | Iron (mM) | Protein (mg $ml^{-1}$) |
|---|---|---|---|---|---|
| Whole hemolymph | 3.14 ± 0.32 | 1.56 ± 0.25 | 1.58 ± 0.37 | – | 66.86 ± 6.25 |
| Hemoglobin | 1.08 ± 0.27 | – | – | 0.51 ± 0.16 | 13.91 ± 2.57 |
| Hematin | 1.03 ± 0.42 | – | – | 0.88 ± 0.25 | 4.37 ± 2.45 |

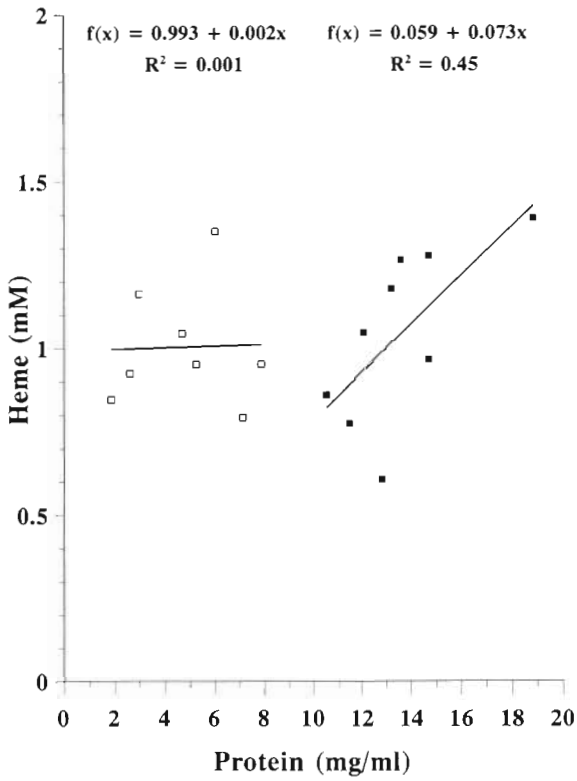


Fig. 2. *Scapharca inaequivalvis*. Fractionated hemolymph; (□) hematin and (■) hemoglobin as a function of protein. Data presented with regression and correlation coefficients

## DISCUSSION

The blood clam *Scapharca inaequivalvis*, a native of the Indo-Pacific (Moore 1969), was recently introduced to the Adriatic Sea where it is rapidly increasing in abundance. The 2 native species *Mytilus galloprovincialis* and *Venus gallina* in the same area are decreasing in abundance (Ghisotti & Rinaldi 1976). The demise of native species is related to seasonal periods of low environmental oxygen concentrations (Marchetti et al. 1989). *S. inaequivalvis* and *M. galloprovincialis* have similar tolerance to anoxia, but *S. inaequivalvis* is superior to *M. galloprovincialis* in terms of tolerance to hypoxia, which explains the success of the former (de Zwaan et. al 1991). The tolerance to hypoxia in *S. inaequivalvis* is due to the hemoglobin-containing erythrocytes, which secure the oxygen transport and also act as an oxygen store so that the bivalve can maintain aerobic metabolism at lower $p_wO_2$ values than is the case in the native species (Weber et al. 1990, Brooks et al. 1991, de Zwaan et. al 1991). Under these circumstances protection against hydrogen sulfide in the hemolymph might be important since sulfide is likely to be present at such low oxygen tensions.

Hematin concentrations of *Scapharca inaequivalvis* are comparable to those of the echiuran worm *Urechis caupo* (Powell & Arp 1989). The hemoglobin corresponds to what Weber et al. (1990) found present in *S. inaequivalvis*. The relationships between hemoglobin and protein and between hematin and total heme all agree with the study by Powell & Arp (1989). The hematin is present *in vivo* and is not an artifact (i.e. caused by denaturation of hemoglobin during handling). Thus there is no correlation between hemoglobin and hematin in whole hemolymph and the hemoglobin concentration is equal to what has been found in the species by Weber et al. (1990). In the fractionated hemolymph the hematin, if originating from hemoglobin, must result from a separation of heme and globin. In this case the separation of heme

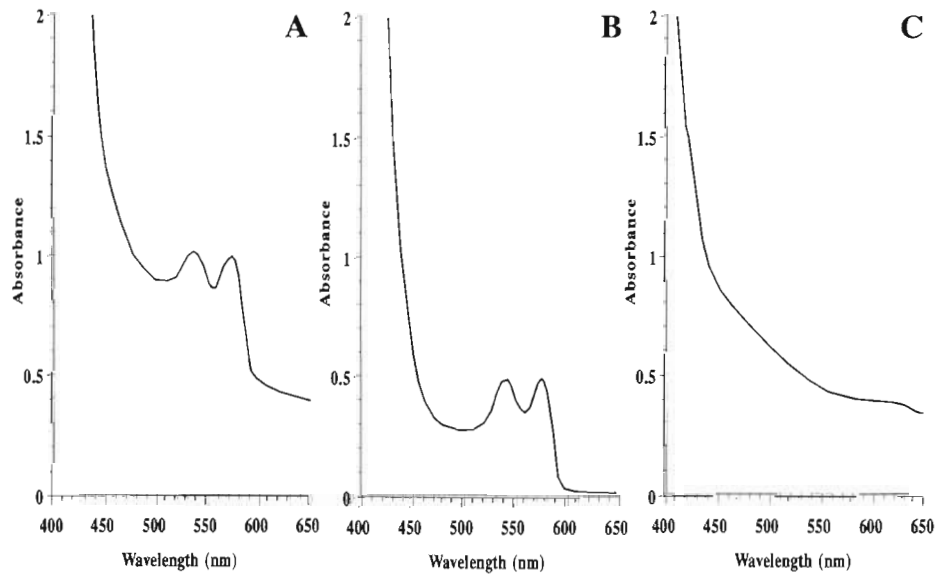


Fig. 3. *Scapharca inaequivalvis*. Representative absorbance spectra of whole and fractionated hemolymph diluted (1:10) in HEPES (10 mM, pH = 7.5). (A) Whole hemolymph, (B) hemoglobin, and (C) hematin

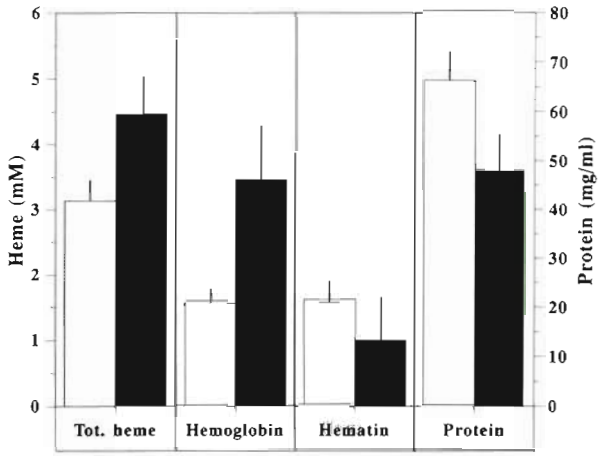


Fig. 4. *Scapharca inaequivalvis*. Concentration of heme compounds and protein in whole hemolymph (n = 9) after incubation at water oxygen tensions of 20 and 2 kPa for 10 d. Shaded bars = 20 kPa; solid bars = 2 kPa. Data: mean + SE

and globin would have to take place at pH = 7.5, which according to Powell & Arp (1989) is highly unlikely, as such separation normally requires a pH outside the range 2 to 12.

In the lysate of whole hemolymph, centrifugation effectively separates hemoglobin and hematin. This shows the hematin to be associated with proteins or to be contained in granules. If hematin in *Scapharca inaequivalvis* is associated with proteins this dictates (at a molar ratio of 1:1) that the protein should be approximately 4200 dalton (see Table 2), or that the molar ratio be different from 1:1. Irrespective of the value of the molar ratio any association should turn up as a high coefficient of correlation between the 2 compounds. However, in both whole and fractionated hemolymph hematin is not correlated to protein. The present results seem to indicate that hematin is not associated with proteins. If so then the hematin must be contained in granules. This conclusion was reached in the case of *Urechis caupo* (Powell & Arp 1989). However, compounds interacting with a gas (e.g. oxygen or hydrogen sulfide) are very unlikely to be contained in granules which have a minimum surface-to-volume ratio. Therefore I conclude that the hematin is most likely associated with protein at a molar ratio higher than 1:1. The low correlation between hematin and protein is thus most likely due to the presence of residual proteins in the pellet (i.e. proteins not associated with hematin). The hemoglobin (monomeric) molecular weight can be calculated from the isolated hemoglobin and the associated protein concentration using a heme (i.e. Fe-protoporphyrin IX) molecular weight of 615 dalton. The hemoglobin weight is in the range of 11 115 to 15 915 dalton, which is close to the weight of *S. inaequivalvis* hemoglobin (17 000 dalton) found by others (R. Weber pers. comm.).

The exposure to 2 kPa oxygen for 10 d resulted in a significant increase in hemoglobin concentrations. This effect of hypoxia on oxygen carriers is well known (e.g. Dejours 1975). Sulfide exposure in nature occurs during periods of hypoxia. To my knowledge, a chemosensory response to oxygen and sulfide has only been studied comparatively in some ciliated protozoa, which all respond only to oxygen (Finlay et al. 1986, Fenchel & Finlay 1989, Fenchel et al. 1989). Therefore, assuming a similar chemosensory response in *Scapharca inaequivalvis*, adaptation of hematin to sulfide can be expected in hypoxia. However, in the experiment the hematin decreased. Hematin is the ferric form of protoheme with an attached hydroxyl ion (Falk

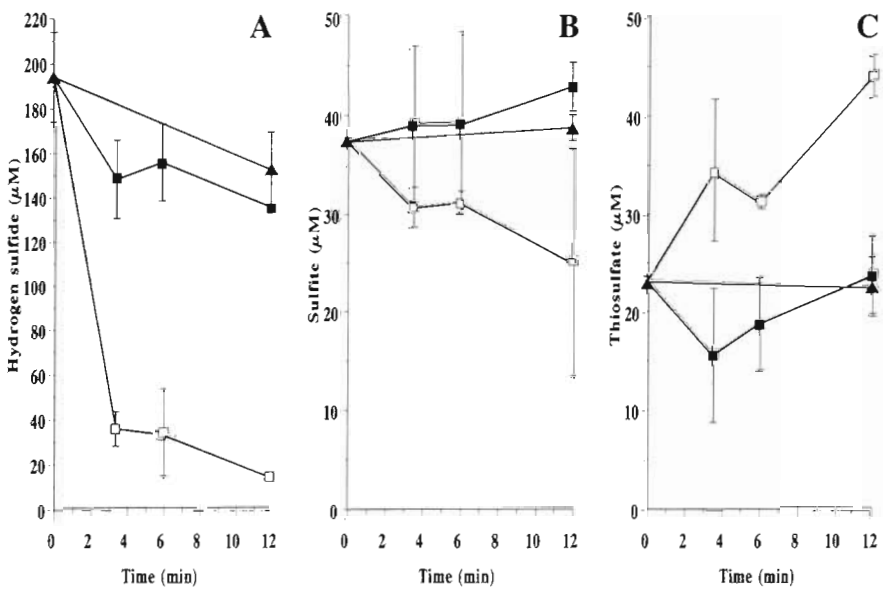


Fig. 5. *Scapharca inaequivalvis*. Fractionated hemolymph; hematin (0.38 ± 0.05 mM) and hemoglobin (0.15 ± 0.02 mM) incubated with 200 μM hydrogen sulfide. Concentration of (A) hydrogen sulfide, (B) sulfite, and (C) thiosulfate as a function of incubation time. (□) Hematin; (■) hemoglobin; (▲) control. Data points represent triplicates (mean ± SE)

1964). The compound is thus likely to be involved in hemoglobin synthesis and/or degradation (either directly or as an iron storage). It should be noted that the hematin fraction will probably include other lysosomally degraded ferric iron porphyrins if present. Although the decrease in hematin during hypoxia cannot explain the increase in hemoglobin, it seems to indicate that hematin is involved in hemoglobin recycling. The decrease in hematin might, however, also reflect a too small sample size, as hematin turned out to be individually highly variable. In this context it would be very interesting to follow the change in hematin concentration after *in vivo* exposure to hypoxia with sulfide present. Due to lack of animals, however, this experiment awaits an impending study.

The very low sulfide removal rate of the hemoglobin shows the separation to be effective, as was also seen in the absorbance spectra analysis. The removal of sulfide by the hematin produces $S_2O_3^{2-}$, but the majority of sulfide removed in the experiment is not recovered. In the hematin incubation only about 30% of the sulfide removed is refound as thiosulfate. The sulfide not refound might be oxidized to elemental sulfur, polysulfide, or sulfate or it can become insoluble due to binding, none of which is detectable in the HPLC analysis. The very rapid (instantaneous) initial sulfide removal strongly suggests that the greater part of the sulfide is removed by binding (to the ferric iron of hematin). The effect of hematin on sulfide thus resembles that of ferric hemoglobin (i.e. methemoglobin, which binds sulfide-forming sulfmethemoglobin (e.g. Smith & Gosselin 1966) and also oxidizes sulfide by nonenzymatical catalysis (e.g. Baxter & Van Reen 1958, Baxter et. al 1958, Sörbo 1958). Sulfate is produced by oxidation of $SO_3^{2-}$, which originates from a reduction of $S_2O_3^{2-}$ (Sörbo 1964, Koj & Frendo 1967, Koj 1968). Therefore the $SO_3^{2-}$ decrease seen in the hematin experiment seems to indicate $SO_4^{2-}$ production. Sulfate is the major oxidation product of sulfide detoxification in terrestrial animals, but in non-symbiotic marine animals detoxification of sulfide normally produces $S_2O_3^{2-}$ and not $SO_4^{2-}$ (see e.g. Powell et al. 1980, Vetter et al. 1987, Bagarinao & Vetter 1989, O'Brien & Vetter 1990, Vismann 1991a, b, Völkel & Grieshaber 1992). This together with the high oxygen tension of the incubations makes any $SO_4^{2-}$ production likely to be a methodological artifact.

In conclusion, hematin is shown to be present in the hemolymph of *Scapharca inaequivalvis* and to be an agent of sulfide removal. This offers an additional explanation for the increasing abundance of *S. inaequivalvis* in the Adriatic Sea. Further, the bivalve hematin exhibits characteristics similar to echiuran hematin. This suggests a similar mode of synthesis and function. The present study adds new evidence to the hypothesis that hematin is a widespread adaptation to sulfide in hemoglobin-containing invertebrates from sulfide-rich habitats. The questions of mode of synthesis and how the hematin level is controlled still need to be answered in future studies.

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