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Sulfide-hemoglobin interactions in the sulfide-tolerant salt marsh resident, the California killifish *Fundulus parvipinnis*

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Summary. Sulfide can potentially damage hemoglobin or be detoxified by hemoglobin. In the sulfide-tolerant California killifish neither seems to be the case at environmentally realistic (micromolar) and physiologically relevant (millimolar) sulfide concentrations. An 8-h exposure of killifish to 5 and 8 mmol sulfide $\cdot l^{-1}$ results in 50–100% mortality, but not due to sulfhemoglobin (where sulfide covalently binds to the porphyrin) nor ferric hemoglobin (Hb^+), both dysfunctional hemoglobin derivatives. Killifish hemoglobin converts to sulfhemoglobin in vitro only in the presence of 1–5 mmol sulfide $\cdot l^{-1}$. The amount of sulfhemoglobin formed increases with time and heme concentration but decreases with pH. Hb^+ binds sulfide as ferric hemoglobin sulfide (Hb^+S, an unstable complex where sulfide ligates to the iron), and also as sulfhemoglobin. Killifish blood does not catalyze the oxidation of 10–500 μmol sulfide $\cdot l^{-1}$ to any appreciable extent. Radiolabeled sulfide incubated with oxyhemoglobin or whole blood disappears at rates greater than in buffers, but only minimal amounts of thiosulfate and no sulfate nor sulfite are formed (elemental sulfur and bound sulfide not quantified). Sulfide disappearance rates increase linearly with initial sulfide concentration. Hb^+ does catalyze the oxidation of sulfide to thiosulfate in vitro. Similar experiments on another sulfide-tolerant species, the long-jawed mudsucker *Gillichthys mirabilis*, produced similar results.

Key words: Sulfhemoglobin – Sulfide oxidation – Blood – Sulfide tolerance – Cyprinodontidae

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Abbreviations: ANOVA, analysis of variance; BV, benzyl viologen; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; RBC, red blood cells; SHb, sulfhemoglobin

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Introduction

Hemoglobin and sulfide affect one another in several ways, and the interaction has been examined from different points of view. One is the impairment of O_2 transport due to the formation of SHb, a derivative with lower O_2 affinity (Carrico et al. 1978a, b; Wells and Pankhurst 1980). Another is the amelioration of sulfide toxicity by the blood. The blood has been implicated in sulfide metabolism, oxidation and detoxication in dogs, rats and cats (Haggard 1921; Sörbo 1958; Evans 1967; Curtis et al. 1972; Bartholomew et al. 1980), marine worms (Patel and Spencer 1963b; Powell and Arp 1989; Vismann 1990) and marine fishes (Bagarinao and Vetter 1989). Also, the immobilization or binding of sulfide to ferrous and ferric hemoglobin or other blood proteins reduces toxicity (Smith and Gosselin 1964, 1966; Smith et al. 1977; Torrans and Clemens 1982), or sulfide may be transported to internal bacterial symbionts (Arp et al. 1984, 1985, 1987). It should be noted that the above categories are not mutually exclusive. SHb formation is a form of immobilization, where sulfide adds across a pyrrole double bond in the porphyrin as an episulfide (Morell et al. 1967; Nichol et al. 1968; Berzofsky et al. 1972; Brittain et al. 1982). Formation of ferric hemoglobin-sulfide complex likewise immobilizes sulfide, but the complex is unstable and eventually yields an oxidized sulfur product (Coryell et al. 1937).

The major site of sulfide toxicity is cytochrome c oxidase, which is inhibited by sulfide at nanomolar to low micromolar levels (National Research Council 1979; Bagarinao 1992). If hemoglobin readily converts to SHb in the presence of sulfide, O_2 transport stops and sulfide toxicity is aggravated. However, if hemoglobin has sufficiently high binding and/or oxidation capacity for sulfide, such that it can rapidly bring the free sulfide concentration to low nanomolar levels, then it can potentially "protect" cytochrome c oxidase.

This paper describes experiments on the California killifish *Fundulus parvipinnis*, and to a lesser extent the long-jawed mudsucker *Gillichthys mirabilis* to determine

the role of hemoglobin in aggravating or ameliorating sulfide toxicity. These two species are remarkably tolerant to sulfide and experience potentially high levels of sulfide (millimolar levels in sediment pore water, micromolar levels in the water column) in their salt marsh habitat (Bagarinao and Vetter 1989; Vetter et al. 1989; Bagarinao 1991). Bagarinao and Vetter (1989) found that the blood of these two species, as well as several other marine fishes, had high sulfide-oxidizing activity, based on a spectrophotometric assay using the artificial electron acceptor BV (Powell and Somero 1985). In addition, sulfide-exposed fishes contained low sulfide and high thiosulfate concentrations in the tissues, indicating that sulfide was oxidized and detoxified in vivo. Thiosulfate concentrations were highest in the blood. These results raised the question of whether the blood itself oxidized sulfide to thiosulfate or simply acted as depot for thiosulfate formed elsewhere. For example, thiosulfate was produced by liver mitochondria incubated with radiolabeled sulfide (Bagarinao and Vetter 1990).

The specific questions addressed in the present study are: (1) Is fish mortality during acute sulfide exposure due to SHb formation? (2) Does sulfide bind to hemoglobin and form SHb in vitro; what factors affect SHb formation? (3) Does fish blood, specifically oxyhemoglobin and ferric hemoglobin, catalyze sulfide oxidation? At least for the California killifish and the long-jawed mudsucker, the answers are essentially negative at environmentally realistic and physiologically relevant sulfide concentrations: the blood neither aggravates nor ameliorates sulfide toxicity. Our results are important for the contrasts and clarification they provide relative to previous studies.

Materials and methods

Exposure of fish to sulfide. The killifish and the mudsucker were exposed to relatively high concentrations of sulfide to determine the acute lethal level and to see whether changes in the blood accompany mortality of fish. Newly caught fish from the salt marsh were exposed to sulfide in flow-through aquaria (Bagarinao and Vetter 1989). Duplicate aquaria were stocked with 12 killifish each and sulfide was delivered to achieve constant concentrations of 0.2, 1, 2, 5 and 8 mmol $\cdot l^{-1}$ for 8 h, with controls not receiving any sulfide. The long-jawed mudsucker was tested only at 2 and 5 mmol $\cdot l^{-1}$ sulfide. At 2-h intervals three fish were removed from one aquarium and examined for hemoglobin spectra.

Preparation of hemolysates. The California killifish were small and yielded only 30–100 µl blood per fish. Blood was taken from individual fish by cutting off the tail with a scalpel and allowing the blood to drain into heparinized capillary tubes or directly into a cuvette containing a buffer with 290 mmol NaCl $\cdot l^{-1}$, 1 mmol TRIS $\cdot l^{-1}$, pH 7.35, and 64 USP-STK-1 units heparin $\cdot ml^{-1}$ [method modified from Riggs (1981)]. After stirring, a spectral scan was immediately made of each whole blood suspension. These suspensions were always turbid but showed typical oxyhemoglobin spectra. Each blood sample was then transferred from the cuvette to a microcentrifuge tube and spun at 13 000 × *g* for 2 min to pellet the RBC from the plasma proteins. The pellet was resuspended in buffer with 150 mmol NaCl $\cdot l^{-1}$, 1 mmol TRIS $\cdot l^{-1}$, pH 7.35 and no heparin, then washed by spinning for 2 min. The RBC were lysed in distilled water, then spun again for 2 min to pellet membranes. The clear hemolysates were then used for spectral scans (within 10 min of blood withdrawal), sulfide-binding experiments, and sulfide oxidation assays. Some hemolysates were left to autoxidize in the refrigerator (5 °C) and used 2 weeks later for in vitro experiments with ferric hemoglobin.

Heme concentration was determined by adding known volumes of hemolysates to 2 ml Drabkin's reagent (1 g $NaHCO_3$, 0.2 g $K_3[Fe(CN)_6]$, 0.05 g KCN in 1 l distilled water, plus 0.5 ml BRIJ 35; Sigma assay kit #525), waiting for 5 min and reading the absorbance of the ferric hemoglobin-cyanide derivative at 540 nm. Heme (mmol $\cdot l^{-1}$) was calculated using the extinction coefficient 11 $mmol^{-1} \cdot l \cdot cm^{-1}$ (Salvati and Tentori 1981) and allowing for the dilution of the hemolysates.

Spectral scans of hemolysates. Scans were made at the wavelength range 375–675 nm using a Perkin-Elmer Lambda 4A spectrophotometer with software from Softways (Moreno Valley, CA, USA). Distilled water and all buffers used in the scans had a flat absorbance of about 0.037 (which was corrected for) throughout this wavelength range. All scans were done at 20 °C. In these scans, the particular form of hemoglobin was noted: ferrous oxyhemoglobin (oxyHb, with bands at 540 and 576 nm in addition to the Soret band), ferrous deoxygenated (Hb, with single band at 555 nm), ferric hemoglobin or methemoglobin (Hb^+, with bands at 500 and 630 nm), ferric hemoglobin-sulfide or *sulfmethemoglobin* (Hb^+S, with band at 545 nm and a shoulder at 575 nm), ferrous sulfhemoglobin (SHb, with band at 613–623 nm, depending on ligand), ferric sulfhemoglobin (SHb^+, with bands at 590 and 715 nm at pH 5.5 or 620 and 665 nm at pH 10), denatured ferric hemichrome (with bands at 535 and 565 nm), or denatured ferrous hemochrome (with bands at 527 and 557 nm) based on the literature [summarized by Salvati and Tentori (1981); Tentori and Salvati (1981)]. SHb spectra, ferric and ferrous, with and without various ligands, appear in several papers (van Assendelft 1970; Dijkhuizen et al. 1977; Carrico et al. 1978b; Brittain et al. 1982). Spectra of Hb^+S are shown by Keilin (1933), Doeller et al. (1988) and Kraus and Wittenberg (1990).

In the present study, SHb formation was monitored as the ratio of the absorbance at 618 nm and the absorbance at 576 nm (A_{618}/A_{576}), based on the observation that in the presence of sulfide the 576-nm band decreased as the 618-nm band increased. This relative measure was necessitated by the large numbers of samples that had to be scanned and assessed promptly. The purity and concentration of SHb solutions may be determined using the ratio $A_{620}/A_{580} = 2.6$ for a 100% pure deoxygenated SHb sample, and an extinction coefficient of 21.5 $mmol^{-1} \cdot l \cdot cm^{-1}$ at 620 nm (Nichol et al. 1968; Carrico et al. 1978b; Brittain 1981). Marchant et al. (1974) used A_{620}/A_{561}, with ratios greater than 2 indicating 80% purity or better. Other methods of SHb measurement have also been described by Evelyn and Malloy (1938) and Dijkhuizen et al. (1977).

A few observations were made of the in vitro reaction of sulfide with killifish Hb^+ to form Hb^+S. Hb^+S was never observed in sulfide-exposed killifish or mudsucker, and the assays were made mostly for comparison with the literature.

Assays of sulfhemoglobin formation and sulfide binding in vitro. The effects of sulfide on hemoglobin spectra and SHb formation in vitro were determined in the California killifish, long-jawed mudsucker and three other marine fishes. In the killifish experiments, a hemolysate was prepared from the RBC of five individuals and assayed for heme content. An appropriate volume of hemolysate was added to either distilled water (pH around 6.7) or saline buffer (150 mmol NaCl $\cdot l^{-1}$, 1 mmol TRIS $\cdot l^{-1}$, pH 7.35) in a cuvette to achieve the same concentration of 30 µmol heme $\cdot l^{-1}$ in all spectral assays. Stock solutions of sulfide were added in microliter amounts to the cuvettes to achieve various concentrations from 0.2 to 15 mmol $\cdot l^{-1}$. Spectral scans were made at different times up to 1 h following sulfide addition, with different heme concentrations (30, 60, 90 and 120 µmol $\cdot l^{-1}$) in the cuvette, and at different pH. In the pH experiment, buffer containing (mmol $\cdot l^{-1}$) 150 KCl, 25 potas-

sium phosphate and 20 HEPES and adjusted to 0.5 pH unit intervals between pH 6.5 and pH 8.0 was used. After addition of aliquots of hemolysate, the new pH was determined prior to the spectral scans.

In a related sulfide-binding experiment, hemoglobin samples of 3–4 ml in dialysis tubings were immersed in 1 l 50 mmol HEPES · l^{-1} (pH 7.4) with 1 mmol sulfide · l^{-1} initial concentration for 12 h at 5 °C (without stirring), and in another run for 24 h at room temperature (with stirring). Spectral scans and heme determinations were made of these samples before and after the immersion period. The samples were then dialyzed in 20 mmol HEPES · l^{-1} (pH 7.4) for 24 h and the spectra again determined. Duplicate samples of blood and buffers were analyzed by the (monobromo)bimane-HPLC method (Vetter et al. 1987, 1989). Sulfate and elemental sulfur were not determined.

Assays of sulfide oxidation by blood. Initial studies of sulfide oxidation by blood and blood components were made using BV in an indirect assay where the rate of BV reduction was assumed to be proportional to sulfide oxidation (Powell and Somero 1985). The assay used 5 mmol sulfide · l^{-1} and 2 mmol BV · l^{-1} under anaerobic conditions at pH 9.0. In one experiment, plasma, RBC and membrane fractions were made from walleye surfperch *Hyperprosopon argenteum* (a large-sized species that yielded good amounts of blood). All the activity was found in the hemolysate fraction. In another experiment, 13 RBC hemolysates from individual California killifish were assayed and the relationship between the rates of BV reduction (= sulfide oxidation?) and heme concentrations was determined.

To determine whether fish hemoglobin can remove sulfide from free solution at fast enough rates under near-physiological conditions (without BV, with O_2, lower sulfide concentrations, and at pH 6.7–7.4 in vitro), time-series assays were done on RBC hemolysates prepared from killifish and mudsucker. In the low-sulfide assays, samples were placed in an all-glass chamber with an O_2 electrode. In each assay, either radiolabeled (^{35}S) or unlabeled sulfide was added in microliter amounts to achieve concentrations between 10 and 500 µmol · l^{-1} in a reaction volume of 1.0–1.5 ml. Then, 100-µl aliquots were withdrawn by Hamilton syringe after 0, 2, 5, 10, and 15 min, fixed in 10 mmol bimane · l^{-1} and analyzed for sulfide and its oxidation products by HPLC with fluorescence detection and on-line scintillation counting (Vetter et al. 1987, 1989).

Killifish hemolysates were made from single fish or pooled from 2–5 fish. Only 2–4 time-series assays at different sulfide concentrations could be done on any hemolysate sample. A total of 22 assays were made of oxyHb in 14 hemolysate samples from 20 killifish, and 7 assays of Hb^+ in 5 samples from 5 fish. Three assays were made of a sample of mudsucker oxyHb. For each assay, the concentrations of sulfide and its oxidation products were plotted against time, and linear regressions were fitted to obtain rates from the slopes (µmol · l^{-1} · min^{-1}, recalculated as nmol · min^{-1}). The rates were normalized to nanomoles heme in the assays, then to milligrams Hb (1 nmol heme = 0.016 mg Hb) to account for differences in heme concentration in the assays. Rates were then plotted against initial sulfide concentration to determine the kinetics, analogous to enzyme-catalyzed reactions where rates are analyzed as a function of substrate concentration.

Whole blood of killifish (pooled from 15 individuals) was also assayed for oxidation of high concentrations of sulfide in 2-ml heparinized glass vials at room temperature (about 20 °C) without stirring. Radiolabeled sulfide was added to 250 µl blood or NaCl buffer control to a concentration of 5 mmol · l^{-1} (this concentration being acutely lethal to the killifish and the one used in the BV assay). Aliquots of 50 µl reaction mixture were fixed at 0, 2, 5 and 10 min, and analyzed by the bimane-HPLC method.

Statistical analysis. Where appropriate, data and treatments were analyzed for statistical significance by Student's *t*-test, ANOVA, and Tukey's test, according to Zar (1984). Figures were drawn by the Cricket Graph computer program.

Results

SHb (non-formation) in fish exposed to sulfide

No killifish died in the control or exposure treatments at concentrations between 0.2 and 2 mmol sulfide · l^{-1}. However, 50% of killifish exposed to 5 mmol sulfide · l^{-1} died in 6–8 h, and all died in 2–4 h in 8 mmol sulfide · l^{-1}. Among the mudsuckers, 50% died in 4–6 h in 5 mmol sulfide · l^{-1}, and 17% succumbed in 2 mmol sulfide · l^{-1}. Exposure of killifish to sulfide at all concentrations up to 8 mmol · l^{-1} in seawater for 2–8 h did not result in the formation of high levels of SHb, nor any other hemoglobin derivative such as Hb^+ and Hb^+S. Almost all hemolysates showed strong alpha (576 nm), beta (540) and Soret (413 nm) bands typical of oxyHb, except those of two killifish and three mudsuckers. The two killifish with SHb were an individual that died after 6 h in 5 mmol sulfide · l^{-1} (A_{618}/A_{576} ratio = 0.142), and an individual still alive after 6 h in 2 mmol sulfide · l^{-1} (ratio = 0.172). Seven others that died in 5 mmol sulfide · l^{-1} did not have SHb in the bloodstream. In the 8 mmol sulfide · l^{-1} treatment, 12 dead fish showed no SHb probably because death was more immediate. The two mudsuckers with SHb died in 4–6 h in 2 mmol sulfide · l^{-1}. No mudsucker in 5 mmol sulfide · l^{-1} was examined for SHb. These data indicated that SHb was not formed in significant amounts during in vivo sulfide exposure, and that death of fish during the experiments was not due to SHb. Control killifish showed A_{618}/A_{576} ratios of 0.024 ± 0.017 (mean ± SD, $n = 25$), and sulfide-exposed

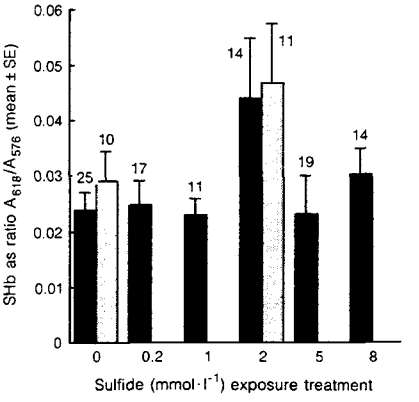


Fig. 1. Sulfhemoglobin (SHb) levels as ratio A_{618}/A_{576} in *Fundulus parvipinnis (black bars)* and *Gillichthys mirabilis (stippled bars)* exposed to different sulfide treatments. Bars show mean ± SE, with sample sizes indicated. Samples from each treatment include fish bled at 2, 4, 6 and 8 h (no significant difference between sampling periods). All fish from the controls and the 0.2, 1 and 2 mmol sulfide · l^{-1} treatments were alive; 50% of those from 5 mmol sulfide · l^{-1} and 75% of those from 8 mmol sulfide · l^{-1} were dead at sampling. No significant difference in mean SHb levels with sulfide concentration (one-way ANOVA, $F = 1.685$, $p > 0.05$)

fish had mean ratios not significantly different from the controls (Fig. 1). No significant difference was found in SHb levels with duration of exposure from 2 to 8 h. In another study, killifish exposed to 200 μM sulfide for 4 days had SHb levels of 0.026 ± 0.012 ($n = 12$).

Although the activity of cytochrome c oxidase was not assayed during the above experiment, it was highly likely that inhibition of the enzyme was responsible for the mortality observed in 2–5 mmol sulfide · l^{-1} over 4–8 h. In a separate experiment, exposure of killifish to 700 μmol sulfide · l^{-1} caused significant inhibition (50–90% over 2–72 h) of cytochrome c oxidase in the gill and brain (Bagarinao 1991).

Effects of sulfide on fish hemoglobin in vitro

Spectral changes. Hemoglobin solutions of the California killifish, long-jawed mudsucker and three other species of fish showed absorbance spectra typical of vertebrates and were affected by sulfide in the same manner. In no case was Hb^+ nor Hb^+S generated when sulfide was added to oxyHb. Instead, when the spectral assays were done at pH 6.5–7.0, with 30 μmol heme · l^{-1} and after 5 min of sulfide addition, absorbance at the alpha, beta and Soret bands decreased with increasing concentrations of sulfide between 0.02 and 10 mmol · l^{-1}. The reductions in absorbance were steeper at concentrations greater than 1 mmol sulfide · l^{-1}. The decreases of the alpha and beta bands were on the order of 0.01 absorbance units per mmol sulfide · l^{-1}. The absorbance at 618 nm, indicative of SHb formation, increased very slightly (0.01 absorbance unit per mmol sulfide · l^{-1}) through three orders of magnitude increase in sulfide concentration and became noticeable only at greater than 1 mmol · l^{-1}. Figure 2A shows four spectra of killifish hemoglobin with increasing A_{618}.

In the presence of (excessive) 15 mmol sulfide · l^{-1}, hemoglobin became deoxygenated within 2–5 min, its absorbance band shifting to 555 nm. This was immediately followed by denaturation, with the bands shifting to 527 and 557 nm (hemochrome, Fig. 2B), and the Soret band coming back up. A hemochrome also formed after about 10 min in 10 mmol sulfide · l^{-1}. The hemochrome bands increased while the absorbance at 618 nm decreased with time. No hemochrome was formed in sulfide up to 1 mmol · l^{-1}.

The spectral changes caused by sulfide were different from those caused by cyanide and nitrite at the same 10 mmol · l^{-1} concentration (Fig. 2C). Cyanide had little effect on oxyHb spectra. Nitrite produced Hb^+ as expected. Addition of cyanide to SHb did not eliminate A_{618}.

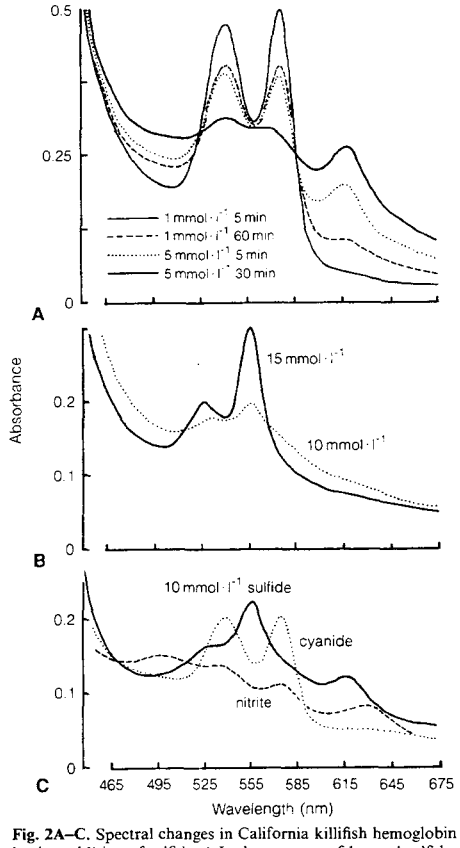
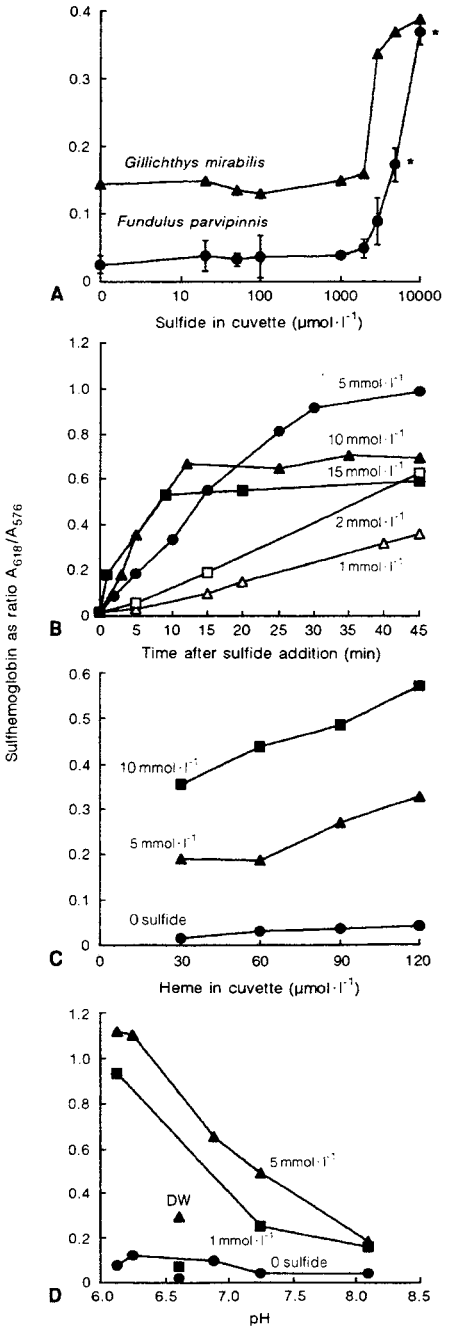


Fig. 2A–C. Spectral changes in California killifish hemoglobin following addition of sulfide. **A** In the presence of 1 mmol sulfide · l^{-1} after 5 min *(thin solid line)*, and 60 min *(dashed line)*, 5 mmol sulfide · l^{-1} after 5 min *(dotted line)*, and 30 min *(bold solid line)*. Note decrease in absorbance at 540 nm and 576 nm and increase in absorbance at 618 nm, indicative of sulfhemoglobin (SHb). The Soret peak also decreases but is omitted in figure. The spectrum for 1 mmol · l^{-1} sulfide at 5 min differs little from that of the control hemolysate, i.e., typical oxyhemoglobin. **B** In the presence of 10 and 15 mmol sulfide · l^{-1}, hemoglobin is deoxygenated (a single peak at 555 nm momentarily appears), then becomes denatured, i.e., a hemochrome is formed with absorbance at 527 nm and 557 nm. **C** The effect of sulfide on hemoglobin differs from that of cyanide and nitrite at the same 10 mmol · l^{-1} concentration. All scans were made of hemolysates with 30 μmol heme · l^{-1} in distilled water pH 6.7

Sulfhemoglobin formation in vitro. Red hemoglobin solutions with 1–5 mM sulfide turned green with time and showed increasingly larger bands at 618 nm. SHb levels expressed as the ratio A_{618}/A_{576} increased with sulfide concentration, time, and heme concentration, and decreased with pH (Fig. 3). OxyHb (30 μmol heme · l^{-1}) is distilled water (pH 6.75) formed SHb in 5 min only in the presence of sulfide concentrations in excess of 1 mmol · l^{-1}. This was true for the five species of fish examined, only two being shown in Fig. 3A. At any sulfide concentration, SHb levels increased with time, and more rapidly in high sulfide (Fig. 3B). At 5 mmol sulfide · l^{-1} an asymptote SHb level was reached in 45 min near a ratio of 1.0. At 10 and 15 mmol sulfide · l^{-1}, SHb ratios remained near 0.6 due to the onset of denaturation and hemochrome formation. Given



enough time, sulfide at concentrations less than 1 mmol · l^{-1} also produced SHb. For example, in one hemolysate, 500 μmol sulfide · $^{-1}$ produced SHb of ratio 0.5 in 2 h (not shown).

In the presence of 5 and 10 mmol sulfide · l^{-1}, more SHb formed with more hemoglobin (Fig. 3C), 120 μmol heme · l^{-1} being the upper limit for spectrophotometric resolution and about 1/20 of the heme concentration of whole blood. Samples with 30, 60, 90 and 120 μmol heme · l^{-1} and 5 mmol sulfide · l^{-1}, fixed in bimane after being scanned at 5 min, showed 1774, 1565, 1443 and 925 μmol sulfide · l^{-1}, and 98, 61, 52 and 57 μmol thiosulfate · l^{-1}, respectively (data not shown). Although these were single determinations they suggested that more heme bound more sulfide, such that less sulfide was oxidized to thiosulfate or was free and available to bimane binding.

Finally, acid pH favored SHb formation (Fig. 3D), with greater amounts being formed in KCl buffer than in distilled water at the same pH. The SHb values plotted in Fig. 3A–C were of hemolysates in distilled water of pH around 6.7 and probably underestimated SHb formation under KCl-buffered conditions.

Reaction of sulfide with ferric hemoglobin. California killifish hemoglobin autoxidized in the refrigerator at a rate of about 3% per day. Addition of 50, 100, 500, and 1 mmol sulfide · l^{-1} to Hb^+ resulted in the immediate (within 1 min) appearance of Hb^+S, a band at 540–541 nm with a shoulder at 574–576 nm, and the reduction or disappearance of the characteristic band of Hb^+ at 630 nm. At the two lower concentrations (50 and 100 μmol sulfide · l^{-1}), Hb^+S dissociated within 30 min back to Hb^+ with the band at 630 nm. At the two higher concentrations Hb^+S dissociated more slowly, and a band at 616–618 nm, indicative of SHb, appeared and became stronger with time. Figure 4 shows spectra of Hb^+ before addition of sulfide, and Hb^+S formed 1 min, 30 min and 1 h after addition of 1 mmol sulfide · l^{-1} to Hb^+. Addition of dithionite caused a shift to typical alpha and beta bands (i.e., reduction of Hb^+) and persistence of the SHb band. Cyanide at 1–10 mmol · l^{-1} converted Hb^+ to Hb^+CN with a band at 540 nm.

Fig. 3A–D. Sulfhemoglobin formation in vitro in California killifish hemolysates, expressed as the ratio A_{618}/A_{576}, as a function of various factors: **A** sulfide concentration (note that scale is logarithmic); **B** time after sulfide addition; **C** heme concentration; and **D** pH. All scans were made 5 min after sulfide addition except in **B**, with 30 μmol heme · l^{-1} except in **C**, in distilled water (DW) pH 6.7 except in **D**. For interspecies comparison, a curve for the long-jawed mudsucker is also given in panel **A**; panels **B–D** are for the killifish only. Variability between two hemolysate preparations (each preparation from 5 killifish) is indicated by the mean ±SD curve in panel **A**. Asterisks indicate significantly high SHb values at 5–10 mmol sulfide · l^{-1} (ANOVA, $F=52.83$, $p<0.005$ and Tukey's test, q values of $P<0.05$). All other points plotted are from single scans. Inter-scan variability about 5–6% for the same sample and same treatment. The results in **B** are duplicated for other species *(not plotted)*. **C** is from one hemolysate, and **D** is a composite of two hemolysates

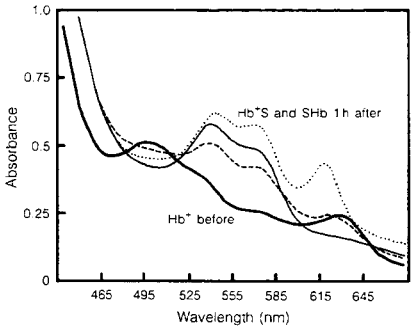


Fig. 4. Effect of sulfide on ferric hemoglobin (Hb^+) in vitro. Addition of 1 mmol sulfide · l^{-1} to Hb^+ with the band at 630 nm *(bold solid line)* produces ferric hemoglobin sulfide (Hb^+S) and no sulfhemoglobin (SHb) in 1 min *(thin solid line)*. With time, the SHb band at 618 nm appears (30 min, *broken line*) and becomes stronger (1 h, *dotted line*)

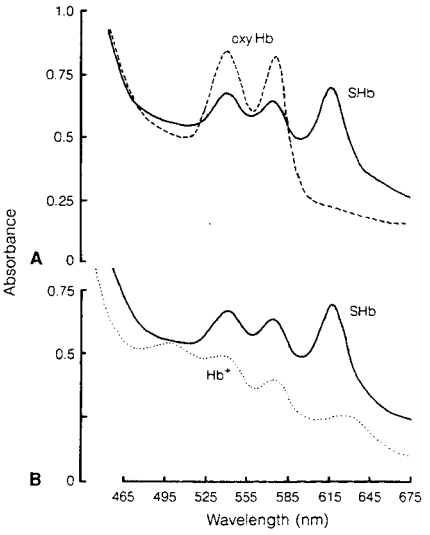


Fig. 5A, B. Sulfhemoglobin (SHb) formation in California killifish hemolysates incubated for 12 h in 1 mmol sulfide · l^{-1} at 5 °C. **A** Ferrous oxyhemoglobin (oxyHb) at the start, converted to SHb 12 h later. **B** Ferric hemoglobin (Hb^+) at the start, converted to SHb 12 h later. Dialysis of the SHb samples for 24 h does not remove the bound sulfide

Sulfide binding. The hemolysates of both killifish and mudsucker bound sulfide as SHb but did not concentrate it. Immersion of dialysis tubings with hemolysates in 1 mmol sulfide · l^{-1} for 12 h at 5 °C without stirring resulted in solutions with less than equilibrium concentrations of sulfide, around 500 µmol · l^{-1} instead of greater than 700 µmol · l^{-1} as in the buffers (Table 1, *middle panel*). More thiosulfate appeared in the Hb^+ sample than in the oxyHb sample and in the buffers. Both Hb^+ and oxyHb produced SHb of A_{618}/A_{576} ratio about 1.0 (Fig. 5). Another hemolysate (oxyHb 2) run at room temperature with stirring for 24 h resulted in a buffer bath with sulfide reduced from 800 to 15 µmol · l^{-1}, and in its place 530 µmol thiosulfate · l^{-1} and 30 µmol sulfite · l^{-1}. The buffer in the tubing reached the same concentrations as the buffer bath, while the hemolysate in the tubing showed 15 µmol sulfide · l^{-1} and 370 µmol thiosulfate · l^{-1}.

Table 1. Results of the sulfide-binding experiment on fish blood. Sulfhemoglobin levels are given as the ratio A_{618}/A_{576}. See text for explanation of oxyHb2

Blood sample	pH	Heme (µmol · l^{-1})	A_{618}/A_{576} ratio	Sulfide (µmol · l^{-1})	Thiosulfate (µmol · l^{-1})
At start of immersion in 50 mmol HEPES · l^{-1} buffer, pH 7.4, with 1 mmol sulfide · l^{-1}					
Fundulus parvipinnis					
OxyHb	7.22	132	0.24		
Hb^+	6.75	113	0.59		
OxyHb 2	7.30	168	0.06		
Gillichthys mirabilis					
OxyHb	6.77	170	0.12		
Buffer bath (with sulfide)				925	116
Buffer tubing (without sulfide)				0	0
After 12 h immersion in buffer with sulfide at 5 °C without stirring					
F. parvipinnis					
OxyHb	7.45	94	1.04	448	230
Hb^+	7.48	108	1.08	545	337
OxyHb 2	nd	nd	1.00		
G. mirabilis					
OxyHb	7.45	115	0.70	454	204
Buffer bath (with sulfide)				793	200
Buffer tubing (with sulfide)				727	204
After 24 h dialysis in 20 mmol HEPES · l^{-1} buffer, pH 7.4, at 5 °C without stirring					
F. parvipinnis					
OxyHb	7.30	91	0.88	43	131
Hb^+	7.35	nd	0.89	44	153
OxyHb 2	nd	nd	0.88		
G. mirabilis					
OxyHb	7.27	nd	0.70	34	129
Buffer bath (without sulfide)				1	139
Buffer tubing (with sulfide)				1	138

nd, not determined. Sulfide and thiosulfate values are averages of duplicate samples

SHb remained stable through 24 h dialysis, with the A_{618}/A_{576} ratio remaining about 0.9 (Table 1, *bottom panel*). Other hemolysates (30 μmol heme · l^{-1}) with SHb ratios of 0.89 and 0.94 (30 min after treatment with 1 and 5 mmol sulfide · l^{-1} in KCl-HEPES buffer) also retained SHb levels of 0.82 and 0.89 after 24 h dialysis in sulfide-free buffer (not shown in table). Green but turbid solutions usually resulted from the 24-h dialysis, presumably due to SHb plus elemental sulfur and some denatured protein. Sulfide-free hemolysates (control) in tubings remained bright red in the same dialysis buffer.

Effect of hemoglobin on sulfide oxidation

In the indirect assay of sulfide oxidation using BV and 5 mmol sulfide · l^{-1} no oxygen present, a positive linear relationship existed between nanomoles BV reduced or sulfide oxidized (y) and nanomoles heme (x) in killifish hemolysates: $y = 1.74 + 0.78x$, $r^2 = 0.83$ (data for 26 assays not shown; regression significant by ANOVA, $F = 117.74$, $P < 0.0005$).

However, the finding that hemoglobin was responsible for sulfide oxidation in the BV assays was not corroborated by the direct time-series assays with ^{35}S-sulfide. Killifish and mudsucker hemolysates incubated with 10–500 μmol ^{35}S-sulfide · l^{-1} in oxygenated buffer of pH 7.4 showed little oxidation to thiosulfate. Figure 6 shows some of the time-series assays, comparing two different initial sulfide concentrations *(panels A and B)*, ferrous oxyHb and ferric Hb^+ *(panels B and C)*, and killifish and mudsucker *(panels A and D)*. Sulfide concentrations decrease with time, faster at 140 than at 40 μmol initial sulfide · l^{-1}, faster in the presence of Hb^+ than oxyHb, and at comparable rates in the two species under similar conditions. Sulfide disappearance was generally not matched by the appearance of stoichiometric amounts of soluble product(s), except in the presence of Hb^+ where thiosulfate was produced *(panel C)*.

The rates for killifish obtained from 22 assays using oxyHb, 7 assays using Hb^+, and 12 control assays without hemoglobin are plotted against initial sulfide concentrations in Fig. 7A–C. Sulfide disappearance rates increased with initial sulfide concentration, and linear regression provided the best fit to the kinetics plots in the range of sulfide concentrations considered. The parameter estimates are summarized in Table 2; all individual regression coefficients are significant except for the thiosulfate rates in buffer. Significant differences were found between oxyHb and Hb^+ in the regressions for both

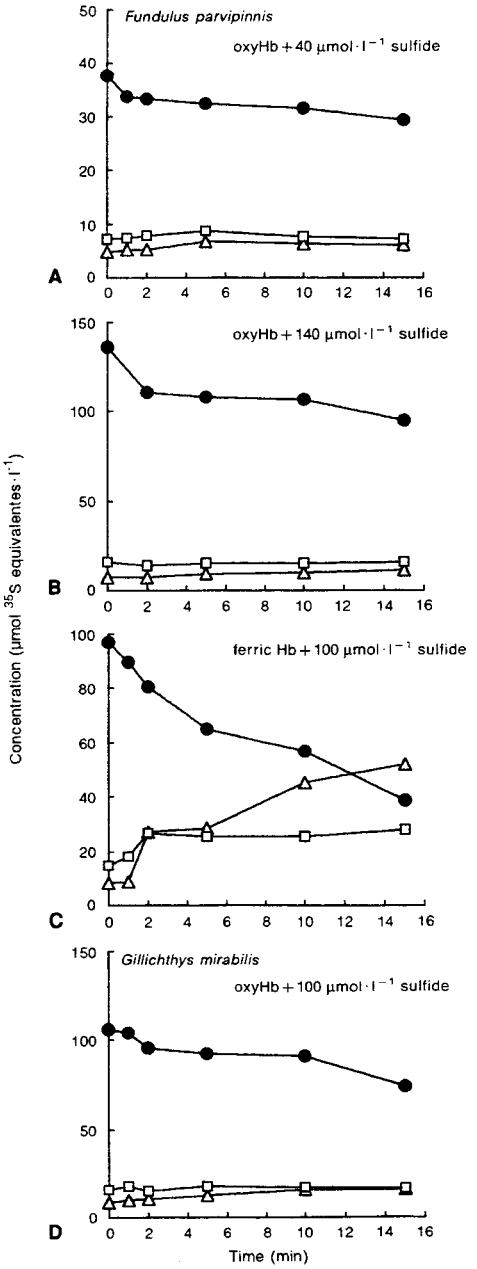


Fig. 6A–D. Changes in the concentrations of ^{35}S-sulfide *(circles)* incubated with red blood cell hemolysates. Thiosulfate *(triangles)* and sulfate *(squares)* present at time zero as contaminant in the ^{35}S-sulfide stock solution. **A** and **B** California killifish oxyhemoglobin (293 μmol heme · l^{-1}). **C** California killifish ferric hemoglobin (30 μmol heme · l^{-1}). **D** Long-jawed mudsucker oxyhemoglobin (300 μmol heme · l^{-1}). ^{35}S was calculated as the μmol · l^{-1} equivalents from chromatographic peak areas of 200 μmol sulfide · l^{-1} standards analyzed by bimane-HPLC. Regression lines were fitted to these four graphs and others to calculate the slopes = rates of sulfide disappearance and rates of product formation. For control rates in buffer only, refer to Figure 7

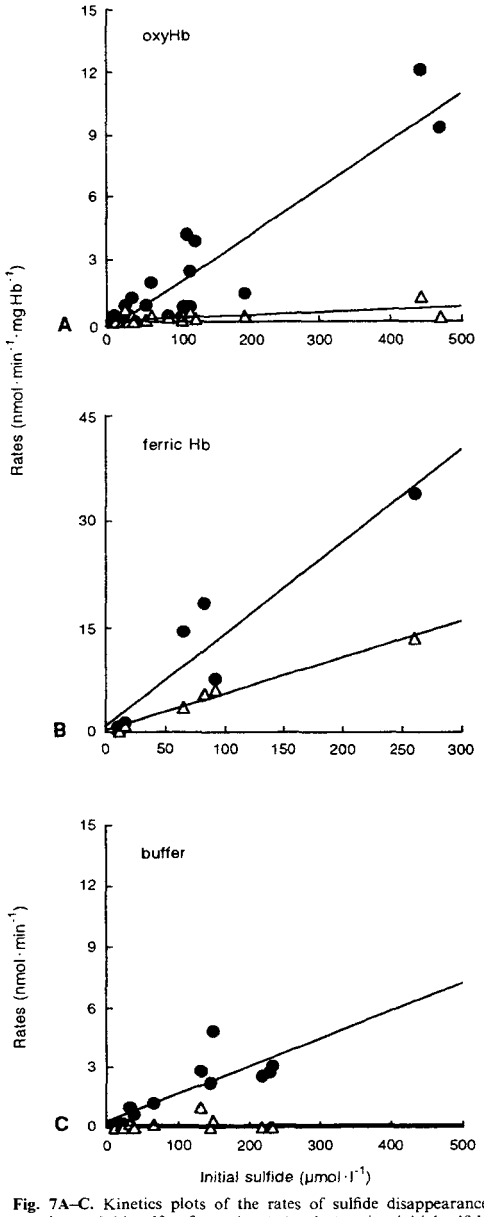


Fig. 7A–C. Kinetics plots of the rates of sulfide disappearance *(circles)* and thiosulfate formation *(triangles)* against initial sulfide concentration for California killifish ferrous oxyhemoglobin (**A**) and ferric hemoglobin (**B**). Rates in **A** and **B** normalized to hemoglobin concentration; note that ordinate scale of **B** *is* 3× *that of* **A**. **C** Control rates in buffer only. For parameter estimates of the regression equations, refer to Table 2

sulfide disappearance rates (Student's test, $t=9.28$, $P<0.001$) and thiosulfate formation rates ($t=28.09$, $P<0.001$). The rates in buffer were of slightly different units and not directly comparable to those in oxyHb and Hb^+ (normalized to milligrams Hb). Nevertheless, when the regressions for sulfide disappearance rates were tested together, oxyHb was different from Hb^+ but not from buffer, and Hb^+ was different from buffer (ANOVA, $F=60.65$, and Tukey's test, q values of $P<0.0005$). Sulfide disappeared at rates greater in the presence of oxyHb than in buffer alone, and even higher with Hb^+. The regression of thiosulfate formation rates against initial sulfide concentration was deemed significant in oxyHb; however, the actual rates were low, particularly in comparison with Hb^+ (Table 2, predicted values).

Assays using whole blood showed a sulfide disappearance rate of about 2 nmol · min^{-1} · mg Hb^{-1} at 3 mmol sulfide · l^{-1}, lower than expected from extrapolation of the regression equations in Fig. 7A. This result suggested that the kinetics plot reached an asymptote at higher than 500 μmol initial sulfide · l^{-1}, but no further study was made because of the difficulty of extracting enough whole blood.

Discussion

Sulfide-hemoglobin interactions have attracted a great deal of study, as the extensive literature cited indicates, and raised considerable confusion. To appreciate the multiplicity of hemoglobin-sulfide interactions it is important to realize that hemoglobin (and myoglobin) can participate in both ionic and covalent interactions with various molecules including sulfide. Coordination of the heme Fe, either ferric or ferrous, with ligands is an ionic interaction. For example, ferrous heme ligates O_2 and CO, but not sulfide; ferric heme ligates sulfide or cyanide, but not O_2. "Methemoglobin" is ferric hemoglobin with a ligated water molecule. When ferric hemoglobin ligates sulfide, "sulfmethemoglobin" is formed. Ferric derivatives can be reduced to the ferrous form by dithionite. On the other hand, nucleophiles can potentially attack any double bond in the heme or in the protein and be covalently bound; this is what sulfide does in SHb formation. SHb is a bright green derivative with sulfur atoms covalently bound to the porphyrin as an episulfide, and with the heme either ferrous or ferric and capable of ligating various compounds. Ferrous SHb reversibly binds O_2 and CO but with different properties and kinetics than ferrous hemoglobin (Marchant et al. 1974; Carrico et al. 1978b; Brittain 1981). Ferrous SHb treated with ferricyanide produces ferric SHb that can bind the same ligands as ferric hemoglobin (Dijkhuizen et al. 1977; Brittain et al. 1982).

Sulfhemoglobin formation

Fish and mammalian hemoglobins share many similarities in spectral and physico-chemical properties (Riggs 1970). The present study on the California killifish and

Table 2. Parameter estimates of the linear regression: $y = a + bx$ of sulfide disappearance rates (y_1, nmol sulfide · min^{-1} · mg Hb^{-1} in A and B, nmol sulfide · min^{-1} in C) and thiosulfate formation rates (y_2, nmol thiosulfate · min^{-1} · mg Hb^{-1} in A and B, nmol thiosulfate · min^{-1} in C) against initial sulfide concentration (x, μmol · l^{-1}) in Fig. 7, including some predicted values of y for selected values of x

Assay medium/ regression	Parameters of regression				Predicted values of y for x equals			
	a	b	r^2	F statistic	20	50	100	250
A: With oxyHb ($n = 22$ assays)								
y_1	−0.41	0.023	0.85	111.43**	0.44	0.73	1.86	5.34
y_2	0.08	0.001	0.38	12.11**	0.11	0.15	0.21	0.40
B: With Hb$^-$ ($n = 7$ assays)								
y_1	0.94	0.131	0.88	36.90**	3.56	7.49	14.05	33.71
y_2	0.34	0.052	0.98	257.76**	1.38	2.94	5.54	13.34
C: Buffer control ($n = 12$ assays)								
y_1	0.33	0.014	0.64	17.85**	0.60	1.02	1.71	3.77
y_2	0.16	0.000	0	0				

ANOVA of the regression coefficient, b, gives the F statistic; ** indicates $P < 0.0025$; r^2 is the coefficient of determination

other marine fishes shows that the formation of SHb is one of these similarities. Both the in vivo and the in vitro experiments show that fish hemoglobin is relatively insensitive to sulfide. The hemoglobin remains oxygenated in the presence of 20–100 μmol sulfide · l^{-1} (environmentally relevant for the killifish) and even high concentrations up to 1 mmol · l^{-1}. Only with 1 mmol sulfide · l^{-1} and greater is SHb formed within a reasonable time frame (minutes to hours). Time, pH, heme concentration and ionic strength also affect the amount of SHb formed. The covalently bound sulfur does not come off even after 24 h dialysis of fish SHb, consistent with the conclusion of Carrico et al. (1978b) that SHb is an irreversible derivative. Since SHb rarely occurs in the blood of fish exposed to and killed by sulfide, the present study is in agreement with the conclusion that SHb is not a significant factor in cases of sulfide poisoning (National Research Council 1979). Sulfhemoglobinemia in humans is more often induced by drugs such as phenacetin than by H_2S inhalation (Park and Nagel 1984; Curry and Gerkin 1987). For animals in very high sulfide concentrations, SHb is a two-edged sword. Binding of sulfide as SHb can potentially ameliorate toxicity by removing some free sulfide from solution. However, SHb is practically non-functional in O_2 transport (Carrico et al. 1978b), and O_2 is necessary for sulfide detoxication by mitochondria (Bagarinao and Vetter 1990).

The green hemoglobin derivative was first observed and described as *Schwefelmethaemoglobin* by Hoppe-Seyler (1863), and later designated as *sulfhemoglobin* by Harnack (1898–99). Keilin (1933) generated sulfmethemoglobin from methemoglobin and sulfide, and showed it to be distinct from SHb. SHb has since then been produced and studied in vitro (Drabkin and Austin 1935–36; Evelyn and Malloy 1938; Michel 1938; Van Assendelft 1970; Carrico et al. 1978a, b; Brittain 1981; Brittain et al. 1982; Park and Nagel 1984; Park et al. 1986). Morell et al. (1967), Nichol et al. (1968) and Berzofsky et al. (1972) proposed similar reaction sequences for the generation of SHb and sulfmyoglobin and showed that one mole porphyrin binds one mole sulfide. The National Research Council (1979) discounted SHb as a real chemical entity, and several other workers did not find SHb during exposure of animals or blood to sulfide. The present study has shown that SHb formation is strongly concentration, time, and pH dependent. Thus, previous reports of non-formation of SHb in marine worms may have been due to the low sulfide concentrations used: low micromolar sulfide (concentration not specified) in *Arenicola* (Patel and Spencer 1963a, b), 40–100 μmol sulfide · l^{-1} in *Riftia* (Arp et al. 1985), and 417 μmol sulfide · l^{-1} in *Abarenicola* (Wells and Pankhurst 1980). The hemoglobins of all these phylogenetically and ecologically diverse groups are probably not fundamentally different in their ability to bind sulfide in the porphyrin ring, but they do so significantly only at high sulfide, at low pH, and given enough time.

Hemoglobin in sulfide detoxication

The results of the present study disproves the notion that fish blood oxidizes sulfide to a significant extent and acts as the first line of defense to protect internal tissues (Bagarinao and Vetter 1989). The time-series assays and bimane-HPLC analyses show that killifish oxyHb or whole blood incubated with 10–500 μmol sulfide · l^{-1} do not produce much thiosulfate nor any other soluble product. Thus, the high thiosulfate concentrations in the blood of sulfide-exposed fish (Bagarinao and Vetter 1989) can not be due to hemoglobin-catalyzed sulfide oxidation. Sulfide disappears from oxyHb solutions probably because of binding to hemoglobin and oxidation to elemental sulfur. However, both processes occur at rates too low to be meaningful in sulfide tolerance. In the presence of oxyHb, sulfide disappears at 0.4 nmol · min^{-1} · mg Hb^{-1} in 20 μmol sulfide · l^{-1} and 5 nmol · min^{-1} · mg Hb^{-1} in 250 μmol sulfide · l^{-1} (Table 2). In contrast, mitochondria oxidize sulfide to thiosulfate at

maximal rate of 20 nmol sulfide · min^{-1} · mg $protein^{-1}$ in the presence of 20 µmol sulfide · l^{-1} and at half-maximal rate in 50 µmol sulfide · l^{-1} (Bagarinao and Vetter 1990). In terms of removal kinetics, oxyHb has low affinity for sulfide and moderate capacity that extends into relatively high sulfide concentrations (i.e., the rates increase with no "saturation" between 10 and 500 µmol sulfide · l^{-1}, Fig. 7), whereas mitochondria have high affinity for sulfide and relatively high capacity that diminishes very rapidly at sulfide concentrations between 50 and 100 µmol · l^{-1} (Bagarinao and Vetter 1990).

Ferric hemoglobin is a better catalyst of sulfide oxidation. In the presence of Hb^+, sulfide is oxidized to thiosulfate at maximal rate of about 34 nmol · min^{-1} · mg Hb^{-1} in 250 µmol sulfide · l^{-1}. However, the contribution of Hb^+ to sulfide detoxication is probably small since there is normally only about 1% Hb^+ in circulation (Jaffe 1981). Hb^+ was not detectable in California killifish in sulfide-exposure tests nor the controls; neither was it generated in vitro when sulfide was added to oxyHb. Catalysis of sulfide oxidation by Hb^+ has been suggested by Beck et al. (1981) to be more important than Hb^+S complex formation. In the killifish, Hb^+S was unstable (Fig. 4).

The results of the BV assays on killifish hemolysates were not corroborated by the results of radiolabeling experiments. While it seems certain that electrons are transferred to BV in the presence of sulfide and hemoglobin, it is not clear that the electrons come from sulfide. Perhaps electrons are released from hemoglobin when sulfide binds to the porphyrin ring, and BV reduction is a measure of sulfide binding rather than oxidation. Whatever the mechanism is, the conditions of the BV assay are highly unrealistic, and the purported sulfide-oxidizing activities mediated by BV [widely distributed among animals: Powell and Somero (1985, 1986b); Vetter et al. (1987); Bagarinao and Vetter (1989); Powell and Arp (1989); Vismann (1990)] probably have no in vivo physiological significance. Hematin (ferric heme not associated with protein) is the effective agent in some invertebrates (Powell and Arp 1989). Ferritin (non-heme protein that binds ferric iron) possibly contributes to sulfide-oxidizing activity with BV since it has been shown to catalyze sulfide oxidation in rats (Baxter and Van Reen 1958b).

Conclusions

Impairment of O_2 transport through SHb formation is not involved in acute sulfide poisoning of the California killifish and the long-jawed mudsucker. The high thiosulfate concentrations in the blood of sulfide-exposed fishes (Bagarinao and Vetter 1989) is not due to sulfide oxidation by hemoglobin. Oxyhemoglobin does not oxidize sulfide to thiosulfate at significant rates but covalently binds sulfide as SHb at high sulfide concentrations. Ferric hemoglobin forms an unstable ligand complex with sulfide and eventually oxidizes sulfide to thiosulfate, but this mechanism is probably insignificant in vivo. Thus, the blood only acts as depot for thiosulfate formed elsewhere in the tissues, probably by mitochondria (Bagarinao and Vetter 1990). Future studies on the interactions of heme proteins and sulfide must clearly differentiate between binding of sulfide and catalysis of sulfide oxidation. Additionally, they must show that the assay conditions, concentrations and products formed are consistent with data from whole-animal (natural and experimental) exposures.

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