

Sulphide tolerance and adaptation in the California killifish, *Fundulus parvipinnis*, a salt marsh resident

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Hydrogen sulphide is a toxicant naturally produced in hypoxic marine sediments, hydrocarbon and brine seeps and hydrothermal vents. The California killifish, a salt marsh resident, is remarkably tolerant of sulphide. The 50% lethal concentration is 700 μM total sulphide in 96 h, and 5 mM in 8 h (determined in flow-through, oxygenated sea water). Killifish exposed to sulphide produce thiosulphate which accumulates in the blood. The cytochrome *c* oxidase (a major site of toxicity) of the killifish is 50% inhibited by < 1 μM sulphide. Killifish liver mitochondria are poisoned by 50–75 μM sulphide but can oxidize 10–20 μM sulphide to thiosulphate. Sulphide causes sulphhaemoglobin formation (and impairment of oxygen transport) at 1–5 mM *in vitro* and to a small extent at 2 mM *in vivo*. Killifish blood neither catalyses sulphide oxidation significantly nor binds sulphide at environmental (low) sulphide concentrations. Exposure to 200 μM and 700 μM sulphide over several days causes significant increases in lactate concentrations, indicating shift to anaerobic glycolysis. However, individuals with the most lactate die. In terms of diffusible H_2S , the killifish can withstand concentrations two to three orders of magnitude greater than would poison cytochrome *c* oxidase. The high sulphide tolerance of the killifish, particularly of concentrations typical of salt marshes, can be explained chiefly by mitochondrial sulphide oxidation. Sulphide tolerance and mitochondrial sulphide oxidation in the killifish have a constitutive basis, i.e. do not diminish in fish held in the laboratory in sulphide-free water for 1–2 months, and are improved by prior acclimation.

Key words: *Fundulus parvipinnis*; sulphide tolerance and detoxication.

I. INTRODUCTION

Sulphide is an important environmental factor and toxicant for aquatic plants, invertebrates and fishes (Theede *et al.*, 1969; Smith & Oseid, 1974; Ingold & Havill, 1984; Bagarinao & Vetter, 1989; Levitt & Arp, 1991; Bagarinao, 1992). In the marine environment, sulphide is produced mostly from bacterial sulphate reduction in sediments (Fenchel & Riedl, 1970; Jorgensen, 1984). Salt marshes, enclosed bays and estuaries and areas of pollution such as sewage outfalls are characterized by micromolar to millimolar sulphide concentrations in sediments (Bagarinao, 1992, for table of measured values). Effects of sulphide on various enzymes, cellular structures and physiological functions are well documented in the biomedical literature (National Research Council, 1979; Beauchamp *et al.*, 1984; Bagarinao, 1992). The major biochemical lesion caused by sulphide appears to be the inhibition of cytochrome *c* oxidase, the terminal enzyme in the electron transport chain of mitochondria. Another quite colourful effect of

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sulphide is the formation of sulphhaemoglobin, a green derivative with a much reduced affinity for oxygen and therefore non-functional in oxygen transport (Carrico *et al.*, 1978a, 1978b).

Many species of fish spend all or part of their lives in shallow waters and may experience sulphide. Mass fish kills in dredged or impounded salt marshes, or in earthen culture ponds are often attributed routinely to hypoxia/anoxia, salinity and temperature fluctuations, or low pH (Waggoner & Feldmeth, 1971; Inland Fisheries Project, 1974), but it is quite likely that sulphide is responsible for some fish kills (Torrans & Clemens, 1982). Fishes also occur in the sulphidic water column of the Cariaco Trench (Baird *et al.*, 1973), at the deep-sea hydrothermal vents (Cohen & Haedrich, 1983; Rosenblatt & Cohen, 1986; Cohen *et al.*, 1990; Geistdoerfer, 1991), and hydrocarbon and brine seeps (Brooks *et al.*, 1979; Kennicutt *et al.*, 1985). Yet, sulphide has been almost totally overlooked as an environmental factor for marine fishes, except for our work.

The California killifish, *Fundulus parvipinnis* Girard, is a small, ecologically and physiologically versatile cyprinodontiform that spends its entire life in bays, lagoons and salt marshes from Morro Bay southward to Magdalena Bay in Baja California (Fritz, 1975; Allen, 1982). Earlier comparative studies have shown that the killifish is more tolerant to sulphide than several other species of shallow-water marine fishes (Bagarinao & Vetter, 1989), has remarkable sulphide-oxidizing activity in the mitochondria (Bagarinao & Vetter, 1990), but negligible sulphide-oxidizing and sulphide-binding capacity in the blood under physiological conditions (Bagarinao & Vetter, 1992).

This paper will present a model of sulphide tolerance in the California killifish, based on several sets of data, most of which are presented here, and some already published. The specific questions addressed are:

- (i) How much sulphide can the killifish tolerate at the whole-animal level?
- (ii) What physiological and biochemical changes occur during exposure to sulphide?
- (iii) How much sulphide is inhibitory to killifish cytochrome *c* oxidase?
- (iv) What accounts for the high sulphide tolerance of the killifish?
- (v) Is anaerobic metabolism an important mechanism in sulphide tolerance?
- (vi) Is sulphide tolerance constitutive? Is it improved by acclimation?

II. MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

The California killifish used in the experiments consisted of juveniles and adults 50–150 mm in total length and 3–8 g in body weight. They were collected by minnow traps mostly from a creek in the Kendall-Frost salt marsh reserve in the northeast corner of Mission Bay in San Diego, California during winter and spring in 1988–1991.

SULPHIDE EXPOSURE EXPERIMENTS

The killifish were exposed to sulphide in flow-through aquaria in which sulphide and fully aerated sea water from reservoirs flowed in continuously by peristaltic pumps. Seawater flow rate was 100 ml min⁻¹. Stock solutions of sulphide, prepared from thoroughly washed and dried crystals of Na₂S·9H₂O dissolved in nitrogen-bubbled distilled water, was diluted 100-fold in the aquaria. Turnover time for water in the aquaria was 40 min, and oxygen concentrations at the outflows were 50–80% of saturation depending on treatment.

Control fish in the aquarium not receiving sulphide suffered no mortality. Water temperature during the tests was 14–18°C.

Long-term sulphide tolerance was determined by exposure of groups of 10 killifish to different concentrations up to 800 µM sulphide for 96 h. The 96-h LC_{50} , the lethal concentration for 50% of the fish was computed by probit analysis. Short-term tolerance was determined by exposure to different concentrations up to 8 mM sulphide for 8 h. In one experiment, 10 California killifish were exposed to each of the following sulphide treatments: 500 µM for 72 h, 1 mM for 8 h, 2 mM for 8 h, and 5 mM until death of 50% of the fish. In a subsequent experiment, duplicate aquaria were stocked with 12 killifish each and exposed to 200 µM, 1 mM, 2 mM, 5 mM and 8 mM sulphide for 8 h. At 2-h intervals, three fish were removed from one aquarium and analysed for haemoglobin spectra, haematocrit, haem concentration, and for sulphide and sulphide oxidation products in the blood (below).

A separate experiment was conducted to see whether killifish shifted to anaerobic metabolism during sulphide exposure. Fish that had been held in the laboratory for 6 months were stocked in the flow-through aquaria in four groups of 25 fish each: (i) fish exposed to 200 µM for 6 days; (ii) fish exposed to 700 µM sulphide for 3 days, and two control groups, one run side by side with each treatment. Fish from treatment and control aquaria were sampled 2 h into the exposure and every day for 6 days in 200 µM, and for 3 days (when all fish died) in 700 µM. During each sampling period, three control and three sulphide-exposed fish were removed, and assayed for blood lactate and enzyme activities in tissue homogenates (below).

Another experiment was made to determine the 'nature/acclimation' basis of sulphide tolerance in the killifish. Sulphide tolerance may be due to a heritable genetic program (a constitutive basis), or to adjustments individuals make during their lifetimes (an acclimation effect), or to both. The approach taken to determine a constitutive basis was to remove killifish from the salt marsh, keep them in sulphide-free water in the laboratory for a period of months ('de-acclimation', a term provisionally used for brevity's sake to mean 'acclimation to sulphide-free water in laboratory holding tanks'), and test them for *whole-animal* sulphide tolerance and for *mitochondrial* sulphide oxidation capacity. Conversely, killifish were acclimated in low sulphide (below the previously determined 96-h LC_{50} but probably higher than typical field levels) and tested similarly. Thus, three groups of killifish were exposed to 5 mM sulphide for 8 h: (i) fish newly caught from the salt marsh; (ii) fish de-acclimated for 1.5 months in the laboratory; and (iii) fish acclimated in 200 µM sulphide for 7 days. The control group consisted of newly-caught fish also placed in the aquaria for 8 h but not exposed to 5 mM sulphide. The first two groups were tested with three replicate aquaria with 20 fish each, the third and the control groups with two replicates. After 6–8 h when most sulphide-exposed fish were dying, five dying fish were removed from each group, and blood and other tissues were fixed in monobromobimane and analysed by HPLC (below).

In this paper, 'sulphide' refers to total dissolved sulphide (the sum of H_2S , HS^- and S^{2-}), unless otherwise specified. At environmental and physiological pH values, the HS^- anion and the gaseous H_2S forms are the important forms. Uncharged H_2S freely diffuses across membranes, and sulphide toxicity to animals can best be approximated by the H_2S concentration. Thus, the toxic sulphide concentrations will all be recalculated as H_2S in the discussion.

MEASUREMENT OF BLOOD PARAMETERS

Killifish from the 8-h tolerance tests at different sulphide concentrations were bled via the caudal vein into heparinized microcapillary tubes (length 75 mm, internal diameter 1.15 mm). The tubes were spun for 5 min in an IEC Model MB microhaematocrit centrifuge at $13\,460 \times g$, and the haematocrit (packed RBC volume as percent of total blood volume) determined using a reader (microcapillary tubes, centrifuge and reader all from Fisher Scientific). The volume of the packed RBC was calculated to be $\pi r^2 h$ or $1.0387 h$, where h is the height in the capillary tubes. The packed RBC were lysed in 0.2 or 1 ml distilled water and the clear supernatant was scanned for haemoglobin spectra and assayed for haem concentration. Formation of sulphhaemoglobin (SHb) and ferric haemoglobin

(Hb⁺), both indicators of impairment of oxygen transport, was monitored with a Perkin-Elmer Lambda 4A spectrophotometer with software from Softways (San Diego, California) using methods described earlier (Bagarinao & Vetter, 1992). Haem concentration was determined by adding known volumes of haemolysates to 2 ml Drabkin's reagent (Sigma assay kit #525), reading the absorbance of the ferric haemoglobin-cyanide derivative at 540 nm after 5 min, and calculating with the extinction coefficient $11 \text{ mm}^{-1} \text{ cm}^{-1}$.

CHROMATOGRAPHY OF SULPHUR COMPOUNDS

Sulphide is the most reduced form of sulphur and can be oxidized to elemental sulphur, thiosulphate, sulphite, and/or sulphate depending on the conditions and the organisms involved. The monobromobimane-HPLC method is able to fix and measure sulphide and other reduced sulphur compounds in the same samples (Fahey & Newton, 1987; Vetter *et al.*, 1987, 1989), and has been crucial to our studies of sulphide metabolism by whole animals, mitochondria and blood (Bagarinao & Vetter, 1989, 1990, 1992). During the 8-h tolerance experiments on the killifish, blood, heart/spleen (blood-rich tissues, combined because of small mass) and liver (not as blood-rich) were removed from sulphide-exposed individuals as well as controls and analysed by monobromobimane-HPLC. Various tissues from killifish in the 'nature/acclimation' experiment were similarly analysed.

ENZYME ASSAYS

The activities of two key enzymes, cytochrome *c* oxidase (EC 1.9.3.1; ferrocycytochrome *c*: oxygen oxidoreductase) and lactate dehydrogenase (LDH; EC 1.1.1.27; NAD⁺: L-lactate oxidoreductase) were determined spectrophotometrically at 20° C. Tissues of killifish were homogenized in 4 or 9 volumes of 10 mM Tris pH 7.2 and centrifuged for 5 min at 2000 × *g* at 5° C. Each tissue homogenate was assayed in three replicates. For cytochrome *c* oxidase, the 2-ml assay mixture contained 100 mM potassium phosphate pH 6.0, 0.5 mM EDTA and 50 μM reduced cytochrome *c*. Addition of 25 μl of the tissue supernatants initiated the reaction. Activities were calculated from the linear rate of decrease in absorbance at 550 nm within the first 1–2 min, using the extinction coefficient $18.5 \text{ mm}^{-1} \text{ cm}^{-1}$. Sulphide concentrations inhibitory to cytochrome *c* oxidase in tissue homogenates (*in vitro*) were determined by adding microlitre amounts of sulphide to the assay mixture to achieve final concentrations up to 5 μM. The 50% inhibitory concentration, IC₅₀, was estimated by transforming activity data into percent inhibition, plotting them against the logarithm of sulphide concentration and fitting linear regression lines. In addition, inhibition of cytochrome *c* oxidase *in vivo* was monitored during the experiment on anaerobic metabolism.

During the latter experiment, LDH was also assayed in control fish and fish exposed to sulphide for different lengths of time. LDH activity was assayed in 2 ml buffer containing 80 mM imidazole pH 7.0, 200 μM NADH, 5 mM pyruvate and 10 μl tissue homogenate, by following the decrease in absorbance at 340 nm, and calculating with the extinction coefficient $6.22 \text{ mm}^{-1} \text{ cm}^{-1}$.

LACTATE ASSAY

Lactate accumulation was used as an indicator of the shift to, and the extent of, anaerobic metabolism in the California killifish during sulphide exposure. Whole blood was obtained by microcapillary tubes and promptly prepared for lactate determination. Samples were transferred into microcentrifuge tubes, diluted with three volumes of ice-cold 6% perchloric acid to precipitate the proteins, and stored frozen until assay at the end of the experiment. Lactate was assayed spectrophotometrically, using Sigma Diagnostics procedure #826-UV but scaled down to 750 μl assay volume. The assay mixture contained 197 mM glycine pH 9.2, 164 mM hydrazine, 12.3 units lactate dehydrogenase, 1.85 mM NAD, and 25 μl supernatant of the precipitated blood samples (4 × dilution). The mixture was incubated at 37° C for 30 min, and the absorbance at 340 nm was recorded. Lactate was estimated from the absorbance of NADH, using the extinction coefficient $6.22 \text{ mm}^{-1} \text{ cm}^{-1}$, and allowing for the dilution of the blood sample. Each blood sample was assayed in three replicates.

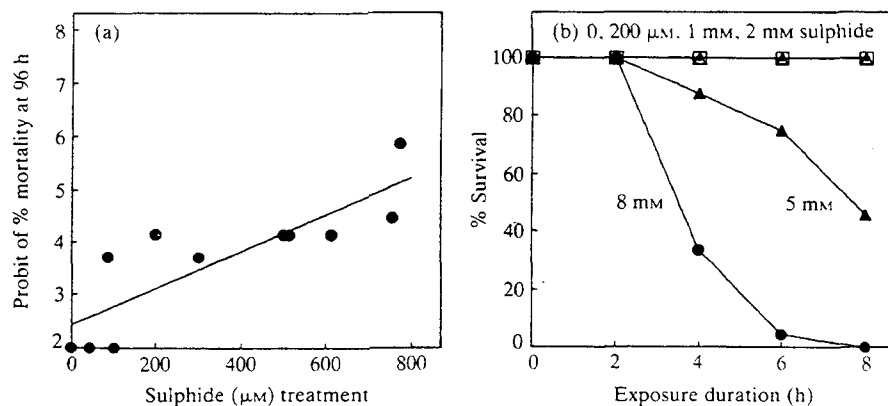


FIG. 1. Tolerance of California killifish to sulphide, determined in a flow-through oxygenated aquarium system. (a) 96-h tolerance; each point is per cent survival among 10 fish. The regression equation is $y = 2.4 + 0.0037x$, $r^2 = 0.72$, where y is the probit value and x is μM sulphide. (b) Tolerance of fish exposed to high sulphide concentrations for up to 8 h; each point is the average survival in two aquaria with 12 fish each.

ASSAY OF SULPHIDE OXIDATION CAPACITY OF MITOCHONDRIA

Killifish mitochondria have previously been shown to rapidly oxidize low micromolar concentrations of sulphide exclusively to thiosulphate, a reaction that is stoichiometrically and obligately linked to oxygen consumption (Bagarinao & Vetter, 1990). Mitochondrial oxygen consumption in the presence of sulphide is therefore a direct measure of the sulphide oxidation capacity of killifish mitochondria. To determine whether mitochondrial sulphide oxidation was constitutive and/or improved by prior acclimation, mitochondria were isolated from five groups of killifish and assayed for oxygen consumption in the presence of sulphide. The five groups consisted of 40 fish each: (i) newly caught; (ii) de-acclimated for 1 month; (iii) de-acclimated for 2 months; (iv) acclimated to $200 \mu\text{M}$ sulphide for 10–12 days (with 20% mortality); and (v) acclimated to $500 \mu\text{M}$ sulphide for 10–12 days (with 50% mortality). Three mitochondrial preparations were made on three consecutive days from each group, each preparation from the livers of 10 killifish (seven fish for group 5). Each mitochondrial preparation was assayed three times in the presence of 3 mM succinate (a control substrate) and 10, 20, 50 and $75 \mu\text{M}$ sulphide according to methods described by Bagarinao & Vetter (1990).

STATISTICAL ANALYSIS

Analysis of variance (ANOVA) and related tests were done by hand according to Zar (1984), and probit analysis with the BMDP computer program.

III. RESULTS

SULPHIDE TOLERANCE OF WHOLE FISH

Mortality after 96-h exposure to different concentrations of sulphide is shown in Fig. 1(a). The 96-h LC_{50} calculated from probit transformation of the data was $700 \pm 364 \mu\text{M}$ (95% confidence limits) total sulphide in oxygenated sea water at $14\text{--}18^\circ\text{C}$. In the short-term tolerance experiments, no killifish died in the controls and treatments up to 2 mM [Fig. 1(b)]. However, 50% of killifish exposed to 5 mM sulphide died in 3 h in one experiment, and in 6–8 h in the other. Half of the fish exposed to 8 mM sulphide died in 2–4 h. In the course of acute sulphide exposure, the fish at first huddled together with increased opercular and pectoral fin

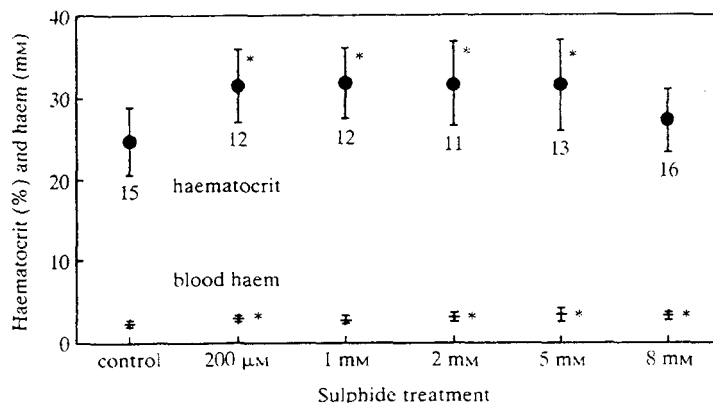


Fig. 2. Haematocrit and haem concentration (mean \pm s.d.; sample sizes indicated on the haematocrit curve; same sample sizes for the haem curve) in fish from different sulphide treatments. Asterisks indicate significant differences from control values (ANOVA and two-tailed Dunnett's test, q values of $P < 0.05$).

movement. This was followed by laboured ventilation, swimming to the surface and gulping air, and by frenzied attempts to leave the water. Poisoned fish lost equilibrium, swam awkwardly, ventilated long and deep, and finally turned over on their backs. Fish turned a darker colour during sulphide exposure.

Survival of killifish in 5 mM sulphide was improved by prior acclimation to 200 μ M sulphide for 7 days. Newly-caught killifish suffered $75 \pm 27\%$ mortality (mean \pm s.d., $n = 3$ replicate aquaria, with a total of 64 fish); de-acclimated fish, $86 \pm 5\%$ ($n = 3$, total 63 fish); and acclimated fish, only $23 \pm 4\%$ ($n = 2$, total 40 fish). Mortality in the acclimated group was significantly different from the de-acclimated group, but neither was significantly different from the newly-caught group (one-way ANOVA and Tukey's test, q values of $P < 0.05$).

HAEMATOCRIT AND BLOOD HAEM CONCENTRATIONS

The haematocrit was $24.7 \pm 4.1\%$ and whole blood haem concentration was 2.4 ± 0.4 mm (mean \pm s.d., $n = 15$) in control killifish not exposed to sulphide. Significant increases in these parameters were observed in sulphide-exposed fish (Fig. 2). These increases occurred within the initial 2 h of sulphide exposure, and did not significantly change thereafter. Killifish dying in 8 mM sulphide did not show the haematocrit response.

SHb OR Hb⁺ FORMATION

Only two of 60 killifish (including 20 dead ones) examined after exposure to 200 μ M to 8 mM sulphide showed some sulphhaemoglobin (SHb) in the bloodstream (A_{618}/A_{576} ratio = 0.142–0.172). None of the fish examined showed any ferric haemoglobin (Hb⁺) nor ferric haemoglobin sulphide (Hb⁺S). Thus, death of killifish within 2–8 h in 5 mM and 8 mM sulphide was not due to significant SHb formation or impairment of oxygen transport.

Earlier experiments *in vitro* showed that killifish haemoglobin converted to SHb within minutes in 5 mM sulphide and within hours in 1 mM sulphide (Bagarinao &

Vetter, 1992). Thus, 1–5 mM sulphide is possibly the threshold concentration for impairment of oxygen transport in the killifish.

SULPHIDE AND THIOSULPHATE IN TISSUES

In terms of the diffusible form of sulphide in sea water of pH 8.3, the concentrations used in the short-term tolerance experiments (200 μM to 8 mM) were equivalent to about 12–480 μM H_2S . If the blood equilibrated with the ambient sulphide and no oxidation occurred, it would have contained about this much sulphide. Figure 3 shows the sulphide and thiosulphate concentrations in the blood (a), spleen/heart (b) and liver (c) of fish from different sulphide treatments. Since the blood was used for other determinations (above), HPLC analysis of blood was done only for control fish, fish 6–8 h in the lethal 5 mM sulphide, and fish 11 days in non-lethal 200 μM sulphide from another experiment. Some sulphide (3 ± 1 μM , mean \pm s.d., $n=8$) was detected in blood from control fish, and this increased only two- to three-fold in fish from both the short-term 5 mM and the long-term 200 μM sulphide treatments. In contrast to the low sulphide concentrations, thiosulphate measured 71 ± 23 μM in controls, 303 ± 230 μM in the 200 μM treatment, and significantly higher at 853 ± 539 μM in the 5 mM treatment.

The heart/spleen and liver were analysed for more of the high-sulphide treatments [Fig. 3 (b, c)]. In these tissues, as in the blood, sulphide remained relatively low while thiosulphate increased much more with sulphide exposure. Fish in the lethal 5 mM and 8 mM sulphide treatments had, respectively, 30 ± 21 μM and 46 ± 24 μM sulphide in the heart/spleen, and 6 ± 9 μM and 35 ± 39 μM sulphide in the liver. Maximum thiosulphate concentrations noted were 956 μM in the heart/spleen and 680 μM in the liver in the 8 mM sulphide treatment.

Sulphide and thiosulphate concentrations in the blood and other tissues of fish from the 'nature/acclimation' experiment are shown in Table I. The brain had the highest sulphide concentration among the tissues examined. Such high sulphide level in the brain probably contributed to death of the fish. Blood sulphide concentrations were low and not significantly different among the three groups exposed to 5 mM sulphide nor between these and the controls. Thiosulphate concentrations were high and not significantly different among the three groups exposed to 5 mM sulphide; those from the newly-caught and acclimated groups were significantly elevated relative to the controls (one-way ANOVA and Tukey's test, q values of $P < 0.01$). Other tissues had lower thiosulphate levels than the blood. Statistical tests were not made for other tissues because of small sample sizes.

INHIBITION OF CYTOCHROME C OXIDASE BY SULPHIDE

Figure 4(a) shows *in vitro* inhibition of cytochrome *c* oxidase of the killifish, as assayed in tissue homogenates. Inhibition of the enzyme was concentration-dependent over a range up to 5 μM sulphide. The IC_{50} estimates ranged from 426–464 nM in the heart and brain, to 750 nM in muscle [Fig. 4(b)]. These inhibitory concentrations were very low, and showed that even a sulphide-tolerant fish had a sulphide-sensitive enzyme.

In vivo inhibition of cytochrome *c* oxidase was also observed during the experiment to determine shift to anaerobic metabolism. There was no mortality among the killifish during the 6-day 200 μM sulphide treatment, but 50% mortality during the 3-day 700 μM treatment. Cytochrome *c* oxidase was inhibited 50–90%

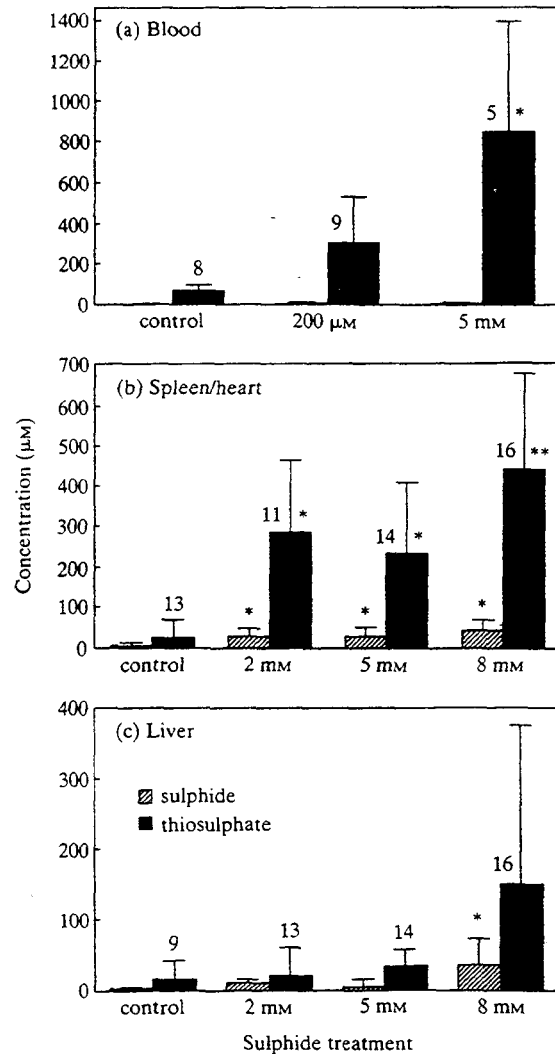


FIG. 3. Sulphide and thiosulphate concentrations in tissues of California killifish from the sulphide treatments (mean \pm s.d.; sample sizes indicated on the bars). (a) Blood. Sulphide concentrations very low and not different between control and sulphide-exposed fish. Thiosulphate concentrations significantly elevated in fish from the 5 mM sulphide treatment. (b) Spleen/heart. Sulphide and thiosulphate concentrations significantly higher in fish from all treatments than in controls. (c) Liver. Sulphide concentration in fish from the 8 mM treatment significantly higher than in controls and the other sulphide treatments. No significant differences in thiosulphate concentration. Based on ANOVA and Tukey's test, q values of $P < 0.05$ (*) and $P < 0.01$ (**).

in the gill, 10–50% in the brain, and 0–50% in muscle (Table II). In fish that died in 700 µM sulphide, only the gill showed nearly complete inhibition (by 90%) to account for death. Liver activity did not seem inhibited and was even 50% stimulated in low sulphide. It must be noted that at least some reactivation of the inhibited enzyme may have taken place during preparation of homogenates and the 2–6 h between sampling and assay.

TABLE I. Sulphide and thiosulphate concentrations (mean \pm S.D., n = number of fish) in tissues of three groups of California killifish exposed to 5 mM sulphide for 6–8 h: newly-caught from the salt marsh, de-acclimated 1.5 months, and acclimated in 200 μ M sulphide for 7 days. Some tissue samples were pooled (e.g. 5 in 1)

Group	Tissue	n	Sulphide (μ M)	Thiosulphate (μ M)
Newly-caught, then 5 mM sulphide				
	gill	2	19 \pm 1	61 \pm 23
	blood	5	8 \pm 7	853 \pm 539
	heart	5 in 1	19	175
	spleen	5 in 1	10	427
	brain	3	85 \pm 58	157 \pm 104
	intestine	2	33 \pm 4	75 \pm 63
	liver	3	21 \pm 12	32 \pm 11
	kidney	5 in 2	9 \pm 6	140 \pm 4
De-acclimated, then 5 mM sulphide				
	gill	3	40 \pm 20	18 \pm 13
	blood	5	8 \pm 5	395 \pm 389
	heart	5 in 1	8	77
	spleen	5 in 1	9	219
	intestine	1	13	10
	liver	1	6	17
	kidney	2	6 \pm 1	75 \pm 37
Acclimated, then 5 mM sulphide				
	gill	3	18 \pm 8	190 \pm 107
	blood	5	10 \pm 6	958 \pm 516
	heart	5 in 1	30	329
	spleen	5 in 1	17	886
	brain	2 in 1	42	191
	intestine	1	16	111
	liver	3	24 \pm 10	94 \pm 54
	kidney	5 in 1	12	79
Controls, newly-caught and no sulphide				
	blood	8	3 \pm 1	71 \pm 23
	spleen	2	3 \pm 1	107 \pm 22
	kidney	3	1 \pm 1	21 \pm 2

ANAEROBIC GLYCOLYSIS DURING SULPHIDE EXPOSURE

Control unexposed killifish had 3.6 ± 1.4 mM lactate (mean \pm S.D., $n = 30$) in the blood. When killifish were exposed to 200 μ M sulphide for 6 days, anaerobic glycolysis came into play and blood lactate increased three-fold to a maximum of 10.8 ± 6.5 mM ($n = 3$) (Fig. 5). When killifish were exposed to 700 μ M sulphide for 3 days, blood lactate increased 10-fold to 37.3 ± 4.2 mM ($n = 3$) at 72, but the fish died. Metabolic acidosis probably compounded the inhibition of cytochrome oxidase and caused the death of killifish in the 700 μ M treatment. The lower lactate levels at 144 h in 200 μ M sulphide were measured 8 h after the sulphide supply ran out (Fig. 5), and showed that fish stopped producing lactate.

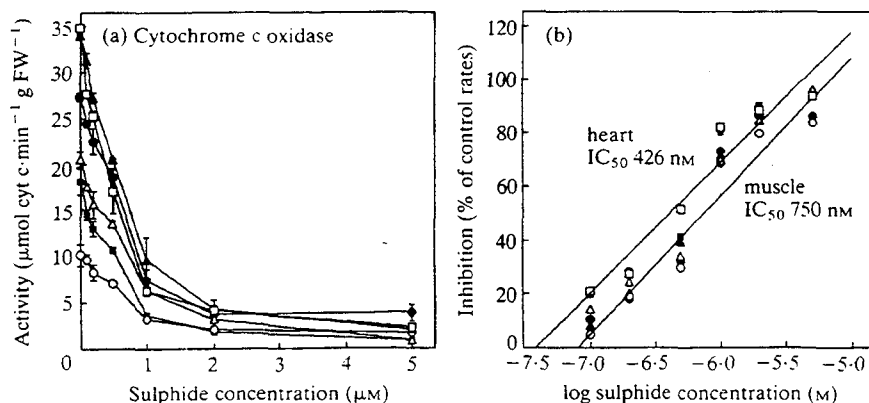


FIG. 4. Inhibition of cytochrome *c* oxidase in tissue homogenates of California killifish. (a) Enzyme activity against sulphide concentration [S], mean \pm s.d. of three assays at each sulphide concentration using pooled homogenates from three fish. (b) Same data transformed as % inhibition against log [S] for estimation of the IC_{50} , the 50% inhibitory sulphide concentration. Only the two most extreme curves for the heart and the muscle are drawn. —●—, Gill; —■—, brain; —▲—, liver; —○—, muscle; —□—, kidney; —□—, heart.

Lactate dehydrogenase activity in killifish tissues assayed *in vitro* showed no significant change during sulphide exposure (Table II), and could not explain the increased lactate concentration in the blood. White muscle LDH had by far the highest activity, consistent with its primarily anaerobic function. In the sulphide exposure experiment, LDH activity was measured 5–8 h after removal of tissues from the fish (because cytochrome *c* oxidase was assayed first) and the delay probably affected the rates. When measured more promptly, killifish LDH activities were higher (muscle, 698 ± 149 ; liver, 170 ± 37 ; gill, 88 ± 20 ; brain, 141 ± 12 ; heart, 252 ± 13 ; blood, $13 \pm 4 \mu\text{mol pyruvate min}^{-1} \cdot \text{g fresh weight}^{-1}$, $n=4-5$).

MITOCHONDRIAL OXIDATION OF SULPHIDE

Mitochondrial respiration was stimulated by 10–20 μM sulphide, with maximal rates around $15 \text{ nmol sulphide min}^{-1} \cdot \text{mg protein}^{-1}$, and by 50 μM sulphide at half-maximal rates (Fig. 6), similar to earlier results (Bagarinao & Vetter, 1990). The toxic level for killifish mitochondria is therefore 50 μM sulphide. Mitochondrial sulphide oxidation capacity was significantly higher in fish acclimated in 500 μM sulphide for 10–12 days, compared with those acclimated in 200 μM , newly-caught, or de-acclimated for 1–2 months (Fig. 6). Killifish de-acclimated for 1–2 months showed rates the same as for newly-caught fish.

IV. DISCUSSION

MODEL OF SULPHIDE TOLERANCE

Consider the schematic in Fig. 7. Cytochrome *c* oxidase is located in the inner membrane of the mitochondria, within the cell, bathed by the blood, in the fish. Sulphide in the water has to diffuse across several barriers to reach the enzyme. Toxicity depends on the balance between the rate at which sulphide diffuses

TABLE II. Cytochrome *c* oxidase activity and lactate dehydrogenase activity in tissues of the California killifish during sulphide exposure. Values are mean \pm S.D. ($n = 3$ fish, except control group $n = 6$). All fish were alive at sampling, except those in $700 \mu\text{M}$ sulphide at 72 h. Assays for cytochrome *c* oxidase were done 2–6 h after, and for LDH 5–8 h after removal of fish from aquaria

Sample	Cytochrome <i>c</i> oxidase activity ($\mu\text{mol cyt } c \text{ min}^{-1} \text{ g fresh weight}^{-1}$)				LDH activity ($\mu\text{mol pyruvate min}^{-1} \text{ g fresh weight}^{-1}$)			
	Brain	Gill	Liver	Muscle	Brain	Gill	Liver	Muscle
Control fish	12.5 \pm 1.3	20.3 \pm 3.6	11.5 \pm 2.3	7.2 \pm 0.9	89.8 \pm 23.1	64.5 \pm 9.9	114.7 \pm 4.9	348.2 \pm 95.6
Fish in 200 μM sulphide								
2 h	6.4 \pm 2.0	7.4 \pm 2.2	17.1 \pm 1.4	3.4 \pm 1.3	85.1 \pm 2.0	96.2 \pm 43.5	112.6 \pm 22.9	440.6 \pm 66.3
24 h	6.4 \pm 1.5	6.1 \pm 1.8	17.2 \pm 3.6	5.6 \pm 2.1	102.4 \pm 18.0	63.0 \pm 8.6	128.8 \pm 22.2	413.2 \pm 129.5
*Recovery	11.8 \pm 0.7	18.7 \pm 1.8	11.1 \pm 1.0	7.7 \pm 2.2				
Fish in 700 μM sulphide								
2 h	11.3 \pm 1.7	11.3 \pm 1.0	12.1 \pm 2.6	6.2 \pm 2.0	87.8 \pm 3.5	49.3 \pm 7.5	119.0 \pm 27.2	368.4 \pm 53.6
24 h	11.7 \pm 1.1	7.3 \pm 0.4	11.1 \pm 2.4	7.5 \pm 1.9	72.6 \pm 4.6	53.8 \pm 6.4	107.2 \pm 16.7	320.6 \pm 50.7
72 h	11.0 \pm 1.7	2.4 \pm 0.6	11.0 \pm 1.0	5.3 \pm 0.8	70.0 \pm 5.1	38.3 \pm 10.6	87.1 \pm 18.9	407.9 \pm 52.7

*Recovery 8 h after a 1.36-h sulphide exposure.

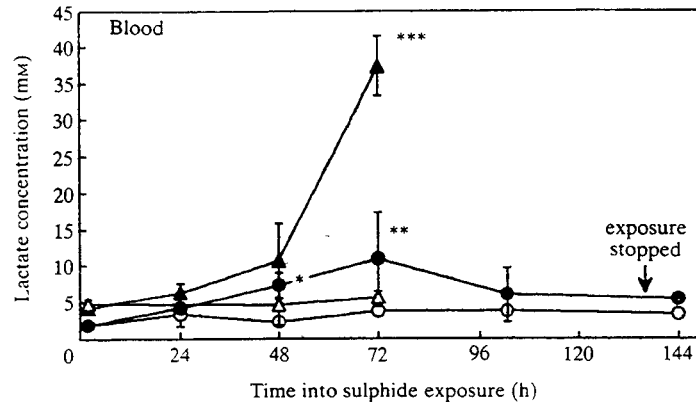


FIG. 5. Blood lactate concentrations in California killifish exposed to 200 and 700 μM sulphide. Control (unexposed) fish sampled at the same time and in the same manner as exposed fish to control for lactate production due to handling. Arrow indicates when the sulphide input stopped. Data for 144 h represent return to near-normal level. Asterisks indicate significant differences from respective controls and other time points, based on two-way ANOVA and Tukey's test, q values of $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***). —▲—, 700 μM ; —△—, control; —●—, 200 μM ; —○—, control.

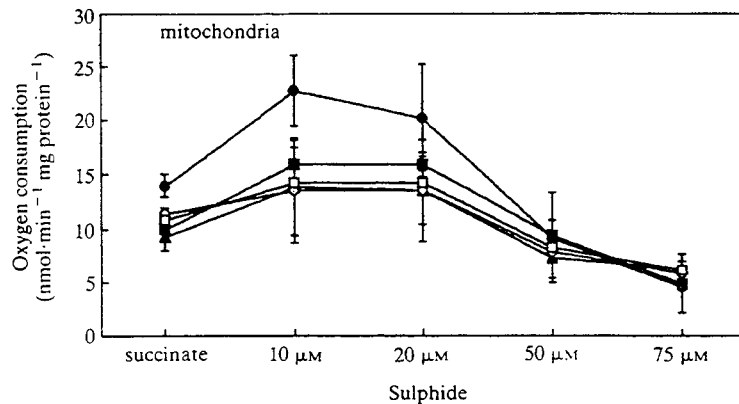


FIG. 6. Mitochondrial sulphide oxidation monitored as oxygen consumption rates (mean \pm S.D., $n=3$ mitochondrial preparations, each from 7–10 fish) as a function of de-acclimation and acclimation. Mitochondria were removed from five groups of fish: (i) acclimated in 500 μM sulphide for 10–12 days; (ii) acclimated in 200 μM sulphide for 10–12 days; (iii) newly-caught from salt marsh; (iv) de-acclimated for 1 month; and (v) de-acclimated for 2 months. Mitochondrial sulphide respiration was significantly higher in the group acclimated in 500 μM sulphide for 10–12 days than in the other four groups (two-way ANOVA and Tukey's test, q value of $P < 0.05$). —●—, acclimated 500 μM ; —■—, acclimated 200 μM ; —▲—, newly-caught; —○—, de-acclimated 1 mo; —□—, de-acclimated 2 mo.

towards the enzyme (which depends on external sulphide concentration and pH) and the rate at which sulphide is detoxified by agents external to the enzyme. Any physiologically important sulphide detoxication system must be able to oxidize sulphide at a rate equal to the flux rate and must have sufficiently high affinity to

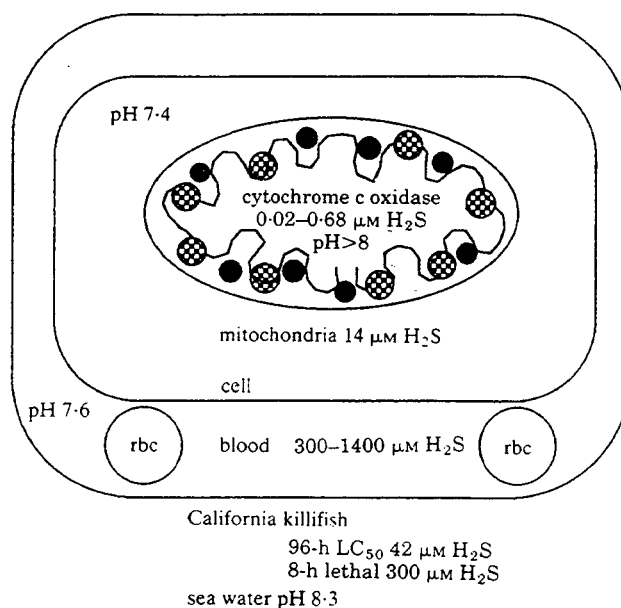


FIG. 7. Model of sulphide tolerance in the California killifish. Cytochrome *c* oxidase (larger circles) is located in the inner membrane of mitochondria. Cytochrome *c* (smaller circles) is located in the intermembrane space. The mitochondria are in cells, the cells are bathed by the blood, and the whole fish is exposed to sulphide in sea water. Sulphide has to diffuse across several barriers to reach the enzyme. Toxicity depends on the balance between the diffusion rate of sulphide towards the enzyme and the rate at which sulphide is detoxified by agents and mechanisms external to the enzyme. Drawn in the model are H_2S concentrations recalculated from the total sulphide concentrations experimentally derived for the killifish in this and other papers (Bagarinao & Vetter, 1989, 1990, 1992).

rapidly reduce sulphide concentration to below that inhibitory to cytochrome *c* oxidase.

The cytochrome *c* oxidase of the killifish is inhibited by nanomolar sulphide. If assayed in homogenates, i.e. with the enzyme in a relatively 'intact' cellular environment, the 50% inhibitory concentration is 426–750 nM sulphide (Fig. 4). If assayed in tissue supernatants, i.e. with the enzyme detached from membranes and other cell components, the 50% inhibitory concentration is 24–184 nM sulphide (Bagarinao & Vetter, 1989). Isolated killifish mitochondria are 50% inhibited by $50 \mu\text{M}$ sulphide (Fig. 6; also Bagarinao & Vetter, 1990). Killifish blood is damaged (converted to SHb) at 1–5 mM sulphide *in vitro* (Bagarinao & Vetter, 1992). At the whole-animal level, 50% of the killifish tolerate $700 \mu\text{M}$ sulphide for 96 h, and 5 mM sulphide for 8 h (Fig. 1).

Toxic sulphide concentrations are better expressed as H_2S since the latter is the diffusible form. H_2S constituted 6% of total sulphide in sea water, pH 8.3, at which the whole-animal experiments were conducted, 28% at pH 7.4 at which mitochondria and haemoglobin were assayed, and 90% at pH 6.0 at which cytochrome *c* oxidase was assayed. Therefore, the whole-animal 96-h LC_{50} for the California killifish is $42 \mu\text{M H}_2\text{S}$ and the 8-h lethal concentration is $300 \mu\text{M H}_2\text{S}$. The 50% inhibitory concentration for isolated mitochondria is $14 \mu\text{M H}_2\text{S}$. The

50% inhibitory concentration for cytochrome *c* oxidase is 0.022–0.166 μM H_2S in tissue supernatants and 0.383–0.684 μM H_2S in tissue homogenates. The threshold concentrations for damage to haemoglobin is 280 μM H_2S (over hours) or 1400 μM H_2S (immediately). These values are plotted in the model in Fig. 7.

Two main conclusions may be derived from Fig. 7. First is that the mitochondria allow the killifish to withstand H_2S concentrations two orders of magnitude greater than those that inhibit cytochrome *c* oxidase extracted from tissues. The sulphide concentration that inhibits mitochondria almost matches that which kills the fish. Thus, it appears that oxidative detoxication of sulphide by mitochondria is the chief mechanism behind the high sulphide tolerance of the killifish. If sulphide concentrations remain at relatively low concentrations in the water column, and they probably typically do in salt marshes, killifish mitochondria would be able to oxidize most of the sulphide before it reaches concentrations inhibitory to cytochrome *c* oxidase. Killifish mitochondria are capable of continuous detoxication of sulphide over several days (10–12 days in the 'nature-acclimation' experiment), and still do so upon subsequent isolation. Sulphide oxidation by killifish mitochondria is constitutive, and improved by acclimation (Fig. 6).

The sulphide-oxidizing mechanism in mitochondria probably consists of cytochrome *c* taking electrons from sulphide and passing them on to oxygen through cytochrome *c* oxidase. Based on the work of Moriarty & Nicholas (1969) and Nicholls & Kim (1981, 1982), we suggest that cytochrome *c* and cytochrome *c* oxidase are probably the 'sulphide oxidase', or components of the 'sulphide oxidase system' that some investigators have postulated (Powell & Somero, 1986; O'Brien & Vetter, 1990). The location of cytochrome *c* oxidase in the mitochondrial inner membrane, bathed in matrix fluid of $\text{pH} > 8$ fortuitously allows the enzyme to oxidize low concentrations of sulphide before being poisoned. The 0.02–0.68 μM H_2S that is inhibitory to the enzyme is reached only when the matrix contains 0.33–11.33 μM total sulphide at $\text{pH} 8.3$. These high sulphide concentrations in the matrix in turn can only be reached when the concentrations outside the mitochondria are higher still.

Second is that killifish haemoglobin (blood) is relatively insensitive to sulphide and 'protects' cytochrome *c* oxidase by allowing the continued delivery of oxygen to mitochondria. The high threshold concentrations (280–1400 μM H_2S) required for SHb formation may explain why very few killifish exposed to high sulphide concentrations show any SHb in the blood. In killifish exposed to 5 mM sulphide in sea water $\text{pH} 8.3$, 300 μM H_2S may be expected to occur in the blood by diffusion in the absence of sulphide uptake or detoxication. This concentration barely reaches the threshold for slow SHb formation and already equals the acute lethal concentration. Thus, the killifish die of sulphide poisoning not due to SHb formation.

Earlier studies have shown that killifish oxyhaemoglobin does not significantly oxidize sulphide, and that ferric haemoglobin, which does, occurs in negligible amounts (Bagarinao & Vetter, 1992). Other cellular factors may also be involved in the removal of free sulphide from solution, e.g. ferritin, disulphide-containing proteins, and S-methyltransferase (Baxter & van Reen, 1958; Cavallini *et al.*, 1970; Weisiger *et al.*, 1980). Their contribution is probably small because the reactions are non-specific. Although the killifish can shift to anaerobic metabolism

during sulphide exposure (Fig. 5), glycolysis is of limited utility and eventually contributes to death.

BIOCHEMICAL CHANGES DURING SULPHIDE EXPOSURE

Increases in the haematocrit and blood haem concentration in the killifish appear to be non-specific responses to stress induced by sulphide. Various studies suggest that haematological parameters may be useful indices of physiological response to environmental stress (Blaxhall, 1972). For example, teleosts respond to lowered dissolved oxygen or exercise by increasing the haem concentration (Cameron, 1970; Scott & Rogers, 1981). Such increases have been attributed to either water shifts from blood to tissue, or to compensatory haemoglobin production. Similarly, inhalation exposure of rats to $900 \text{ mg l}^{-1} \text{ H}_2\text{S}$ ($= 39 \mu\text{M}$ dissolved H_2S) for 5 min, and protracted exposure of rats to daily baths in $130\text{--}190 \mu\text{M}$ H_2S for 20–30 min elevated the red blood cell counts and haemoglobin levels (in Beauchamp *et al.*, 1984). The significance of such haematological changes is not known. However, an increased blood haem concentration would enhance oxygen delivery to, and facilitate sulphide oxidation by mitochondria.

Occurrence of low sulphide and high thiosulphate concentrations in the tissues of fish exposed to 5 mM and 8 mM sulphide indicates that detoxication systems continue to work at these high concentrations, although for a limited time and at rates presumably lower than the sulphide influx rates. The highest thiosulphate concentrations are found in the blood, secondarily in blood-rich tissues such as the heart and spleen, and relatively low in the liver. Without evidence to the contrary, the blood may be assumed to oxidize sulphide. However, under nearly physiological conditions, the blood does not oxidize sulphide to thiosulphate at any appreciable rate, whereas liver mitochondria do so avidly (Bagarinao & Vetter, 1990, 1992). These results together with Fig. 3 and Table II suggest that thiosulphate produced in the liver (and probably other tissues) is readily transported into the blood. Accumulation of thiosulphate to high levels in the blood indicates that excretory mechanisms at best are slower than *in vivo* sulphide oxidation rates.

ANAEROBIC METABOLISM

When California killifish are exposed to $200 \mu\text{M}$ sulphide ($12 \mu\text{M}$ H_2S) for 6 days or to $700 \mu\text{M}$ sulphide ($42 \mu\text{M}$ H_2S) for 3 days, cytochrome *c* oxidase is partially inhibited, anaerobic glycolysis comes into play, and blood lactate concentrations increase. The highest blood lactate concentrations (mean 37 mM) are observed in fish that had died in sulphide. These results are consistent with the high sulphide tolerance of the killifish, but also suggest that the upper limits of sulphide tolerance may be set by factors secondary to inhibition of cytochrome *c* oxidase, e.g. acidosis. When the channel catfish *Ictalurus punctatus* Rafinesque are exposed to $3 \mu\text{M}$ H_2S for 30 min, cytochrome *c* oxidase in the brain and gills is inhibited 60–75%, and lactate concentrations in the blood increase more than three-fold (Torans & Clemens, 1982).

There is no evidence that the killifish has extraordinary anaerobic capacity. LDH activity per wet weight tissue does not increase during 3–6 days of sulphide exposure. The increase in blood lactate concentration during sulphide exposure is possibly due to a greater proportion of tissue mass shifting to anaerobic glycolysis

with longer exposure. LDH activities in the killifish are not much different from those of other coastal fishes, after allowing for assay temperature, use of homogenates versus supernatants, intraspecific scaling with size, and differences in swimming and feeding habits (Sullivan & Somero, 1980; Castellini & Somero, 1981). In the congener *Fundulus heteroclitus* L., serum lactate concentration is 3.5 mM, and LDH activities are 227 units g wet weight⁻¹ in liver and 300 units g wet weight⁻¹ in skeletal muscle supernatants assayed at 25° C (Greaney *et al.*, 1980).

Lactate accumulation during anaerobic glycolysis leads to tissue acidosis (Pörtner *et al.*, 1984; Fievet *et al.*, 1987), a problem particularly for white muscle, which generates most of the lactate and has generally poor capillary circulation. Thus, there is need for adequate buffering capacity in muscle. Indeed, Castellini & Somero (1981) found similarly high buffering capacities in skeletal muscles of 11 species of actively foraging pelagic fishes and 10 species of diving mammals. The killifish probably has comparable muscle buffering capacity with these animals, and is able to continue anaerobic glycolysis for a period of days.

Shift to anaerobic metabolism during sulphide exposure may be a more important physiological strategy for sulphide tolerance among benthic invertebrates, which employ a greater variety of adaptive strategies and metabolic pathways (Shumway *et al.*, 1983; Oeschger & Storey, 1990; Levitt & Arp, 1991).

CONSTITUTIVE BASIS OF SULPHIDE TOLERANCE AND EFFECT OF ACCLIMATION

The 'nature/acclimation' experiments on the California killifish have shown that survival in 5 mM sulphide, *in vivo* detoxication of sulphide to thiosulphate, and mitochondrial respiration on sulphide, are not different between newly-caught and de-acclimated fish, but are significantly improved by prior acclimation in 200 or 500 µM sulphide. In fact, killifish held in sulphide-free water for 6 months tolerate subsequent exposure to 700 µM sulphide for 3 days, and 200 µM sulphide for at least a week (in the separate experiment on anaerobic metabolism).

That de-acclimation or holding of killifish for months in the laboratory in sulphide-free water does not eliminate the ability of both whole fish and isolated mitochondria to oxidize sulphide is possibly because the sulphide-oxidizing enzyme system (cytochrome *c* and cytochrome *c* oxidase) is constitutive. The ability of mitochondria to oxidize sulphide has now been documented across four phyla (Bartholomew *et al.*, 1980; Lloyd *et al.*, 1981; Powell & Somero, 1986; Bagarinao & Vetter, 1990), likewise indicating a constitutive basis.

That acclimation improves fish survival in an otherwise lethal sulphide concentration, and enhances mitochondrial sulphide oxidation rates, is probably due to adjustments in the amounts or catalytic rates of cytochrome *c* and cytochrome *c* oxidase, or to a host of other factors that still have to be investigated. Acclimation is thus one more modifier of sulphide tolerance, which has been shown previously to vary with species, habitat, life stage or size, temperature, dissolved oxygen levels and pH (Theede *et al.*, 1969; Adelman & Smith, 1972; Smith & Oseid, 1974; Broderius *et al.*, 1977; Shumway *et al.*, 1983; Vetter *et al.*, 1987; Bagarinao & Vetter, 1989; Vismann, 1990; Levitt & Arp, 1991). Extensive research on other environmental factors (such as temperature, hypoxia and heavy metals) have shown that tolerance of animals to high levels is improved by prior exposure to lower levels. Cellular and molecular adjustments are made by the animal during

the acclimation period (Shaklee *et al.*, 1977; Greaney *et al.*, 1980; Hobson & Birge, 1989), usually months, such that it can survive subsequently an otherwise lethal exposure. The longest sulphide acclimation period in this study was 12 days because it was a difficult experiment to do in the long term (large amounts of sulphide are needed in the flow-through exposure system and sulphide solutions have to be made up fresh every day).

In conclusion, the remarkably high tolerance of California killifish to sulphide can be explained chiefly by mitochondrial sulphide oxidation. Killifish exposed to sulphide shift to anaerobic metabolism, but fish with the most lactate die. Killifish blood does not catalyse sulphide oxidation significantly, or binds sulphide at environmental (low) sulphide concentrations. Sulphide tolerance and mitochondrial sulphide oxidation in the killifish have a constitutive basis and are improved by prior acclimation.

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