

Developmental Toxicity of the Dithiocarbamate Pesticide Sodium Metam in Zebrafish

Melissa A. Haendel,* Fred Tilton,† George S. Bailey,‡ and Robert L. Tanguay§¹

**Linus Pauling Institute and the Marine/Freshwater Biomedical Sciences Center, 571 Weniger Hall, Oregon State University, Corvallis, Oregon 97331;* †*Environmental and Molecular Toxicology, the Environmental Health Sciences Center, 1007 Ag and Life Science Bldg, Oregon State University, Corvallis, Oregon 97331;* ‡*Linus Pauling Institute, Environmental and Molecular Toxicology, the Environmental Health Sciences Center and the Marine/Freshwater Biomedical Sciences Center, 435 Weniger Hall, Oregon State University, Corvallis, Oregon 97331;* and §*Environmental and Molecular Toxicology, the Environmental Health Sciences Center and the Marine/Freshwater Biomedical Sciences Center, 1007 Ag and Life Science Bldg, Oregon State University, Corvallis, Oregon 97331*

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Sodium metam (NaM), a dithiocarbamate, is a general agricultural biocide applied prior to planting for the elimination of nematodes, soil pathogens, and weeds. There is a remarkable paucity of information about the mechanism of action and the risk that dithiocarbamates may pose to developing vertebrates. We have characterized NaM toxicity during early life stage exposure in zebrafish. Zebrafish embryos are most sensitive to NaM exposure during gastrulation and early segmentation (4–14 hours post fertilization, hpf). For mortality, the dose response curve is steep with an LC₅₀ estimate of 1.95 μ M (248 ppb) at 48 hpf. The most notable malformation among surviving embryos was a severely twisted notochord, which became evident by 24 hpf. Surprisingly, this notochord defect was not immediately lethal and the animals continued to grow despite delays in hatching, apparent paralysis, and an inability to feed. We have characterized the notochord malformation using histological and *in situ* hybridization techniques. *collagen 2a1* mRNA expression is normally localized to the notochord sheath cells at 24 hpf, whereas in NaM-exposed embryos it is misexpressed in the notochord cells. Histological staining and *myoD* expression indicate that the myotomes of the NaM-exposed embryos are less defined, compacted and block-shaped compared to controls. The degradation product of NaM, methyl isothiocyanate (MITC), causes similar malformations at similar concentrations as NaM, suggesting that MITC or another common product may be the active toxicant. Our results indicate that developing zebrafish are sensitive to NaM and MITC and we believe that this model is ideal to elucidate the molecular mechanism(s) and etiology of NaM toxicity in vertebrates.

Key Words: sodium metam; methyl isothiocyanate; zebrafish; developmental toxicity; notochord.

Dithiocarbamates and their disulfides have many uses in agriculture, manufacturing, and in medicine (Eneanya *et al.*, 1981;

Haley, 1979). There is concern that acute levels of dithiocarbamate exposure could occur in an occupational setting, and low chronic exposures could potentially occur through the consumption of contaminated foods. In medicine, dithiocarbamates are used for alcohol aversion therapy and to treat patients for nickel intoxication (Brewer, 1993; Jones and Jones, 1984). Sodium metam (NaM) is a pesticide used prior to planting to eliminate nematodes, soil pathogens, and weeds. The U.S. EPA reported NaM as the third largest quantity of agricultural pesticide used in the United States in 1998–1999 (EPA, 2002c). The complete phase-out of an alternate biocide, methyl bromide, from use in developed countries by 1 January 2005 (EPA, 2003) will likely result in substantial increases in NaM application. Nationally, the EPA estimates that approximately 59 million pounds of NaM were used in 1997 (EPA, 2002c). This remarkably high value is due partially to the recommended application rate of NaM ranging from 60 to the more typical 320 pounds per acre (CEPA, 2002). This far exceeds the amounts used for most other pesticide applications (Tomlin, 1997). The levels of MITC in agricultural run-off are unknown as no methods exist for measuring MITC at these low levels. Under normal and appropriate applications it would be expected that the majority of NaM would be converted to MITC and dissipate into the air as intended. Potential greater risk would involve subsurface movement of NaM and MITC where soil and environmental conditions exist for movement. Significant run-off could occur if large rain events occur following NaM application. Several studies have measured NaM and MITC in aqueous solutions (e.g., groundwater), however there are significant technical challenges limiting environmentally relevant detection of these compounds (Yu *et al.*, 2003). NaM is perhaps most well known from a 1991 train derailment in which 19,000 thousand gallons of NaM were released into the Sacramento River. Within days nearly every living organism was killed within the 45 miles downstream of the spill site. It was reported that the average MITC concentrations 17 days after the spill to be approximately 260 ppb and on the day of the spill five miles downstream there was a concentration of

¹ To whom correspondence should be sent at Marine/Freshwater Biomedical Sciences Center, 435 Weniger Hall, Oregon State University, Corvallis, OR 97331. Fax: (541) 737-7966. E-mail: robert.tanguay@oregonstate.edu.

97 ppm (Alexeeff *et al.*, 1994). Models suggested that the concentration of NaM and MITC within the first 48 h ranged from between 100 to 6000 ppm (Kreutzer *et al.*, 1994). Currently, drinking water standards for NaM or its degradation products have not been established by the U.S. EPA. NaM was not part of the analyte list of USGS National Water Quality Assessment (NAWQA) program and therefore was not monitored as a part of their national reconnaissance in the 1990's. Therefore, the potential risk for human exposure to NaM from drinking water sources is undefined and may be significant in areas near agricultural communities.

One of the most remarkable facts regarding NaM is that the mechanism(s) of its widespread biocidal activities is unknown. NaM degrades into multiple breakdown products following application, including methylisothiocyanate (MITC), carbon disulfide (CS₂), and methylamine (Fig. 1) (Tomlin, 1997). In soils, the decomposition occurs rapidly with a half-life of as little as 30 min, but this has been shown to vary widely dependent on temperature, concentration, pH, and water content of the soil (Frick *et al.*, 1998; Gerstl *et al.*, 1977; Joris *et al.*, 1970; Saeed *et al.*, 1996; Turner and Corden, 1963). In aqueous solutions exposed to light, NaM degrades into MITC with a half-life of less than 10 min (Draper and Wakeham, 1993). Since MITC is thought to be the active biocide, it is important to consider the rate of degradation of NaM in a given system (Tomlin, 1997; Pruett *et al.*, 2001). However, it has been challenging to develop analytical methods of detection for NaM and MITC at ppb concentrations, which has made it difficult to ascertain the biological or ecological consequences of NaM (Yu *et al.*, 2003).

Animal studies suggest that NaM or MITC are potential carcinogens, immunological toxicants, and developmental toxicants (reviewed in Pruett *et al.*, 2001). The immunological consequence of NaM exposure in adults has been studied most thoroughly (Keil *et al.*, 1996; Padgett *et al.*, 1992). However, there have been few peer-reviewed citations investigating the developmental effects of dithiocarbamates as a class, and none looking at the developmental toxicity of NaM specifically. Studies submitted for pesticide registration provide some insight as to the developmental effects of NaM. In a rat study, NaM was delivered by gavage (50 to 60 mg/kg) during gestational days 7–16 and the pups were analyzed at gestational day 22. The author reports an increase in malformations including hydrocephaly, anophthalmia, and skeletal developmental delays (Tinston, 1993). In rabbits receiving NaM by gavage (up to 60 mg/kg/day) on gestation days 8–20, a number of adverse effects were reported. At doses exceeding 20 mg/kg/day there were skeletal variations and at the 60 mg/kg/day dose there was an increase in cleft palate and meningocele (Hodge 1993). However, these studies are of limited value in assessing the toxicity of NaM on the earliest stages of vertebrate development such as gastrulation and cell fate determination. Zebrafish are an excellent animal model to assess risk from water-soluble compounds to free-living vertebrates such as fish and amphibians, as well as in pre-implantation mammals.

Zebrafish (*Danio rerio*) share many cellular, anatomical, and physiological characteristics with other vertebrates. For developmental toxicology studies, zebrafish are especially useful in that they rapidly develop externally, their transparency allows

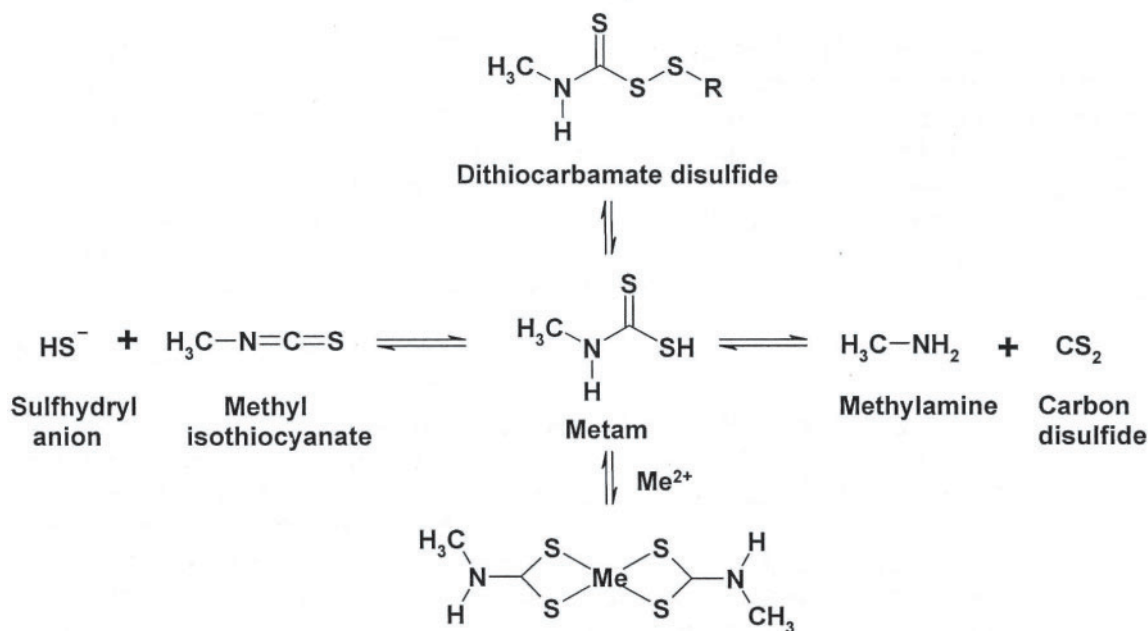


FIG. 1. The potential decomposition products of NaM are depicted. Decomposition is dependent on concentration, pH, temperature, and oxygen content.

observation during development, and hundreds of animals are easily obtained from the large clutch sizes. We have utilized zebrafish in order to characterize the toxicity and effects of NaM on the earliest stages of development. Our data suggest that both NaM and MITC are teratogenic to zebrafish embryos and cause notochord and muscle malformations when exposed during gastrulation and early somitogenesis. We have described the effects on the notochord and muscle using histological and molecular techniques. Because zebrafish are vertebrates, it will be critical to determine if other organisms, including mammalian vertebrates, are sensitive to NaM exposure prior to organogenesis. By utilizing the molecular and genetic tools available in the zebrafish, we will be able to investigate the mechanism of toxicity for NaM and possibly other dithiocarbamates, as well as whether NaM poses a significant health risk.

MATERIALS AND METHODS

Zebrafish maintenance and collection of embryos. Adult AB strain zebrafish (*Danio rerio*) were raised and kept at standard laboratory conditions of 28°C on a 14 h light/10 h dark photoperiod (Westerfield, 2000). Fish water consisted of reverse osmosis water supplemented with a commercially available salt solution (0.6% Instant Ocean). Embryos were collected from group spawns and staged as previously described (Kimmel *et al.*, 1995). Embryos were photographed live using a Nikon SMZ1500 microscope and a Nikon Coolpix 5000 digital camera.

Stock solutions and exposure protocols. Sodium metam (NaM; CAS # 137-42-8) and methyl isothiocyanate (MITC; CAS # 556-61-6) were both purchased from Chem Service, Inc. (West Chester, PA). A sodium metam stock solution of 0.29 M in water at pH 9 was stored at 4°C until further dilution. Stock solutions of MITC were prepared at 40 mM in fish water and stored in aliquots at -20°C. Embryos were waterborne exposed in their chorions to varying concentrations of NaM or MITC in 20 ml glass vials sealed with Teflon-lined lids (VWR International, West Chester, PA) to prevent loss by volatilization. Twenty embryos per vial were used as one replicate. At the end of the exposure period, the embryos were washed two times in excess fish water and allowed to grow until sampled.

In situ hybridization. Embryos were fixed overnight in 4% paraformaldehyde at specific hours or days post-fertilization (hpf or dpf). *In situ* hybridization was performed as described with minor modifications (Westerfield, 2000). Briefly, embryos were stored in 100% methanol at -20° until ready for use. The embryos were rehydrated in PBS + 0.1% Tween-20 (PBST) and treated with proteinase K at 2 µg/ml in PBST for varying lengths of time depending on the stage. The embryos were prehybridized in 50% formamide, 5X SSC, and 0.1% Tween (cheap hyb) for 1 h, and hybridized overnight at 70°C with digoxigenin-labeled antisense probe in cheap hyb + 500 µg/ml yeast RNA and 50 µg/ml heparin at pH 6.0. The embryos were first washed at 70°C in 2X SSC, 0.2X SSC, and 0.1X SSC, and then at 25°C in PBST. Digoxigenin was detected with an anti-DIG-AP Fab fragments antibody (Roche, Indianapolis, IN) in a blocking solution containing 1% DMSO, 2% sheep serum, and 2 mg/ml bovine serum albumin in PBST. Finally, the embryos were developed with 20 µl NBT/BCIP per ml (Roche) in color buffer containing 100 mM Tris-Cl, pH 9.5, 50 mM MgCl₂, 100 mM NaCl, and 0.1% Tween-20. The *collagen 2a1* and *myoD* antisense RNA probes have been described (Weinberg *et al.*, 1996; Yan *et al.*, 1995).

Histology. Embryos were fixed in 2% paraformaldehyde, 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4 overnight. The embryos were embedded in 1% low temperature agarose, processed in epoxy resin on a Lynx EL automatic tissue processor, and sectioned at +1 µm. The sections were stained with paragon (basic fuchsin-toluidine blue in sodium borate) stain for 30 s (Bourne and StJohn, 1978).

Statistics. Data is illustrated as the mean with standard error of the mean (SEM). Sigmoidal regression analysis and LC₅₀ estimation was completed using

SigmaPlot 2001 for Windows (SPSS, Inc., Chicago, IL). For lowest observed adverse effect levels (LOAELs), ANOVA statistical analysis was performed using a *p*-value of 0.05 for significance. This was tabulated with SigmaStat Version 2.03 for Windows software (SPSS, Inc., Chicago, IL).

RESULTS

Sodium Metam Is Developmentally Toxic to Zebrafish

To determine if zebrafish are sensitive to NaM during gastrulation, somitogenesis, and organogenesis, embryos were waterborne exposed to a range of NaM concentrations from 4 hpf until 24 hpf (20 h exposure), transferred to chemical free water and allowed to develop. The most notable malformation, observed in 100% of the exposed animals at 0.8 µM, was related to the notochord (Fig. 2). The notochord was severely twisted compared to stage matched controls, but the animals otherwise developed normally (albeit delayed, see below).

We also evaluated the concentrations at which NaM is lethal to zebrafish embryos (Fig. 3A). The mortality observed at higher NaM concentrations generally occurred during the first 24 hpf, before notochord defects were observable. The mortality curve is plotted for embryos at 48 hpf following the 4 to 24 hpf exposure; the 48 hpf LC₅₀ is calculated to be 1.95 µM (248 ppb). At lower NaM concentrations, there is a clear relationship between notochord defects and chemical concentration (Fig. 3B). In the 0.05 µM exposure group, the animals were indistinguishable from controls, whereas in the 0.2 µM group, 25% of the animals displayed notochord malformations. In addition to the notochord response, NaM exposure also resulted in a concentration dependent reduction in hatching (Fig. 3B). At 120 hpf, 98% of the

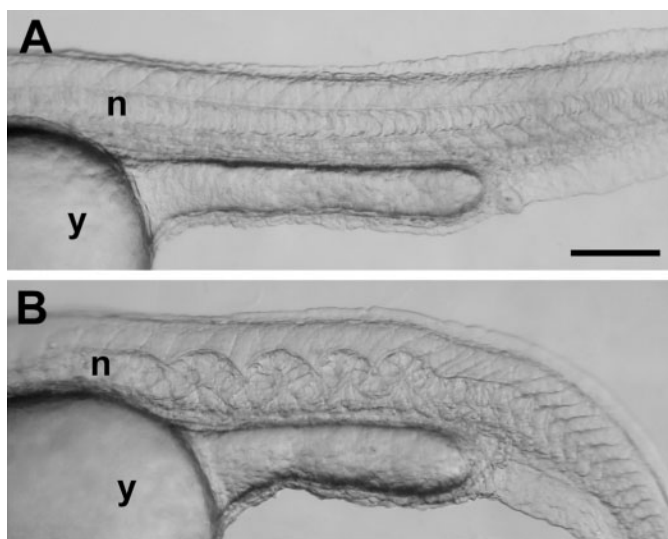


FIG. 2. Notochord defect following exposure to sodium metam. Embryos were exposed from 4 hpf to 24 hpf to 0.8 µM NaM. (A) Lateral view of control embryos. (B) Lateral view of exposed embryo. n, notochord; y, yolk; scale bar, 150 µm.

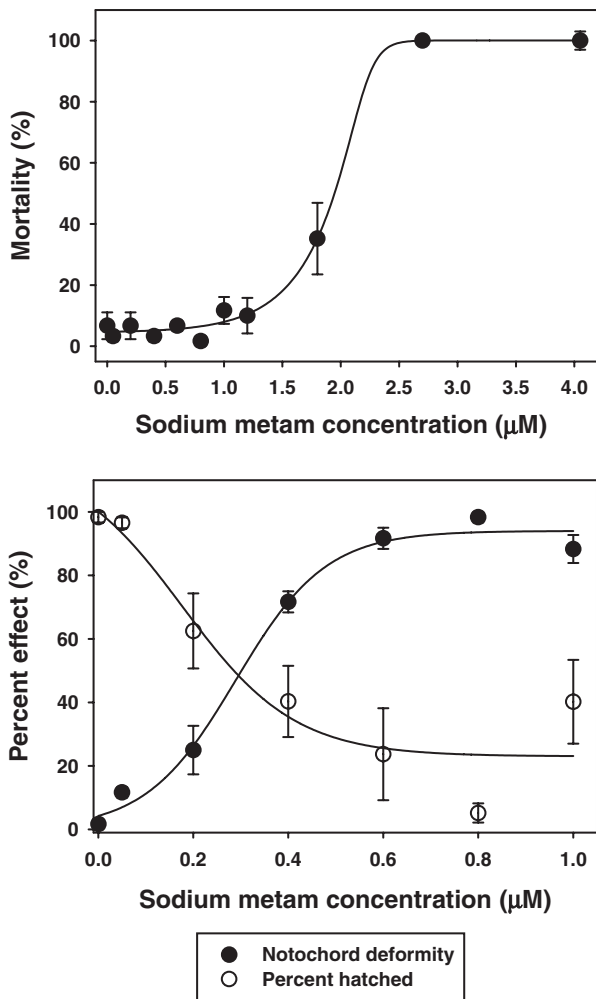


FIG. 3. Mortality, notochord malformation, and hatching rate exhibit a graded response following sodium metam exposure. Embryos were exposed from 4 hpf to 24 hpf to varying concentrations of NaM. (A) 48 hpf mortality curve. The LC₅₀ is estimated to be 1.95 μM. (B) Five dpf effects of exposure on notochord malformation (black circles) and hatching rate (open circles). The EC₅₀ for notochord defects is 0.30 μM and the EC₅₀ for reduced hatching is 0.28 μM. Three replicates of twenty embryos each were observed for each data point.

control animals hatched from their chorions, compared to 5% at 0.8 μM. The failure to hatch may be related to behavioral deficits; specifically, the animals appear to have delayed and weakened spontaneous motion (data not shown). The lowest observed adverse effect level (LOAEL) for both notochord defects and decreased hatching rate was 0.2 μM (26 ppb; $p < 0.05$). It is important to note that there were very few observable defects besides the notochord malformation, suggesting that NaM may specifically target this structure.

Sodium Metam Alters Notochord Differentiation

We have utilized *collagen 2a1* mRNA to label the notochord in whole animals to aid in the determination of developmental

progress (Yan *et al.*, 1995). In 18 hpf control and 0.8 μM NaM exposed embryos, *collagen 2a1* is expressed in both the notochord and sheath cells surrounding the notochord (Figs. 4A and 4B). However, by 24 hpf, the expression becomes limited to the sheath cells in control embryos; whereas in NaM-exposed embryos, *collagen 2a1* mRNA continues to be expressed in the notochord cells (Figs. 4C, 4D, 4E, and 4F). This misexpression continues in the posterior notochord as late as 36 hpf. We have also examined the expression of the myogenic bHLH transcription factor, *myoD*, in control and NaM exposed embryos. *myoD* labels myogenic precursor cells in the paraxial mesoderm (Weinberg *et al.*, 1996). At 24 hpf, the anterior-posterior extent of expression is comparable; however, the myotomes are less defined, irregularly arranged, and more block-shaped in NaM exposed animals compared to stage-matched controls (lines, Figs. 4G and 4H). Careful staging of control and NaM exposed embryos and analysis of gene expression patterns has allowed us to follow developmental progression. Control embryos staged at 12 hpf had between three and five somites while embryos exposed to 0.8 μM NaM had between one and three somites, approximately a 1 h developmental delay. When the embryos were staged at 18 hpf, the controls had 17–18 somites while embryos exposed to 0.8 μM NaM had 14–18 somites, suggesting that some embryos were delayed by as much as 2 h while others were more comparable to controls. Interestingly, at 18 hpf, notochord defects were not yet observable by light microscopy. At 24 hpf, control embryos were still approximately 2 h ahead of NaM exposed embryos. Thus, developmental staging indicates that the embryos may be delayed by as much as 2 h at 24 hpf but that they do not appear to become more delayed.

Notochord Sensitivity to Sodium Metam is Stage Specific

In order to ascertain which stage of notochord development is most sensitive to NaM, we further restricted the developmental window during which we exposed the embryos. The embryos were waterborne exposed from 4 to 14 hpf and from 14 to 24 hpf of development and compared to embryos exposed for the full duration (4–24 hpf). This experiment was performed at multiple concentrations to account for possible decreased response to NaM (Table 1). At all concentrations analyzed, 85–95% of the embryos exposed from 4–14 hpf exhibited wavy notochords compared to 74% of the embryos exposed from 4–24 hpf. In contrast, 0% of the embryos exposed to multiple concentrations of NaM from 14–24 hpf had wavy notochords. While these embryos did not have wavy notochords, 50–95% exhibited posterior malformations wherein notochord cells were seen to extend beyond the normal boundaries in small region(s) of the posterior trunk (Fig. 5). When the developmental windows were further restricted (Table 1, experiment 2) no observable notochord defects were detected. Thus, the notochord is affected by NaM during its initial specification and differentiation; however, shorter exposures of 4 to 5 h are not sufficient to induce notochord malformations at this concentration.

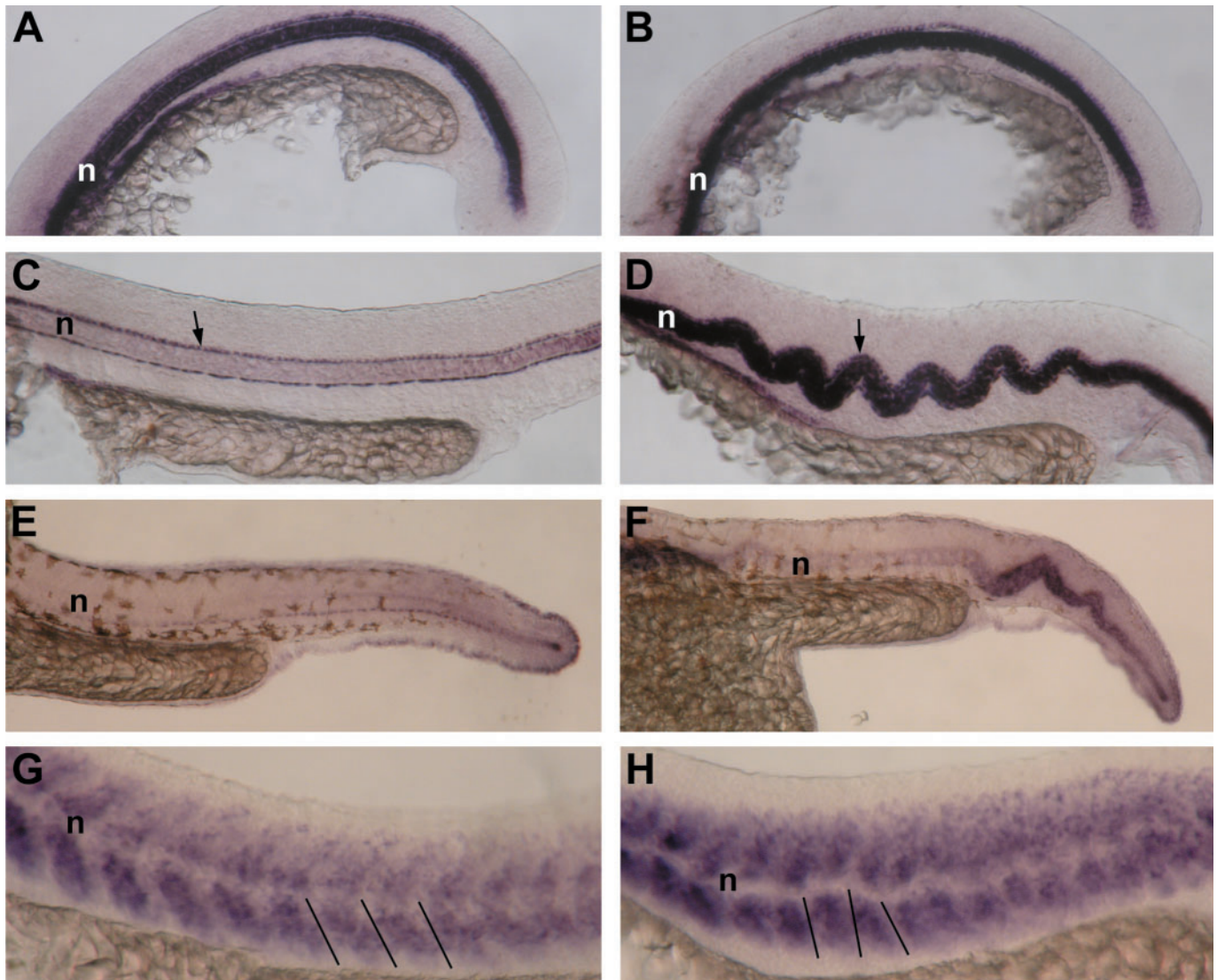


FIG. 4. *In situ* hybridization comparison of control and embryos exposed to 0.8 μM NaM from 4 to 24 hpf. (A–E) *Collagen 2a1* antisense mRNA; (F, G) *myoD* antisense mRNA. (A) 18 hpf control; (B) 18 hpf NaM exposed; (C, G) 24 hpf control; (D, H) 24 hpf NaM exposed; (E) 36 hpf control; (F) 36 hpf NaM exposed. Fifteen or more embryos were hybridized for each probe and developmental stage. n, notochord; arrows, notochord sheath; lines, somite boundaries.

Zebrafish Embryos Respond Similarly to MITC

Because NaM degrades into MITC, we sought to determine if MITC could cause similar effects on notochord development. Zebrafish embryos waterborne exposed to MITC from 4 to 24 hpf also developed wavy notochords (Fig. 6). From comparative dose-response studies, it became evident that the MITC concentration at which notochord defects were observed was nearly identical to that of NaM. When mortality is monitored and plotted for embryos at 48 hpf following 4 to 24 hpf exposure to MITC (Fig. 7A), the estimated the LC_{50} is 1.87 μM (137 ppb). Notochord defects and hatching rates are also correlated with chemical concentration with a notochord defect EC_{50} of 0.35 μM and a reduced hatching EC_{50} of 0.22 μM (Fig. 7B). The LOAEL for both notochord malformation and decreased hatching rate

was 0.4 μM (29 ppb; $p < 0.05$). Following exposure to either NaM or MITC, the embryos similarly develop a wavy notochord that becomes increasingly debilitating as they mature (Fig. 8). In addition to the aforementioned defects, the embryos also appear to exhibit decreasing mobility and touch responsiveness, as well as a diminished ability to feed (data not shown). However, NaM and MITC exposure did not lead to common signs of toxicity that have been reported in zebrafish exposed to a number of xenobiotics, such as pericardial and yolk sac edema.

Characterization of the Notochord Defect

To further investigate the malformations observed following exposure to NaM or MITC, we analyzed epoxy sections with

TABLE 1
Sodium Metam Exposure during Smaller Windows of Development Differentially Affects Notochord Development

NaM (μ M)	Exposure window (hpf)	Notochord malformation				Mortality
		Normal	Wavy	Posterior	Other	
Experiment 1						
0.0	4 to 24	100%	0%	0%	0%	0%
0.8	4 to 24	5	74	11	11	0
0.8	4 to 14	0	95	0	0	5
1.2	4 to 14	11	88	0	0	0
2.0	4 to 14	0	85	0	15	0
0.8	14 to 24	45	0	50	0	5
1.2	14 to 24	0	0	95	0	5
2.0	14 to 24	0	0	95	5	0
Experiment 2						
0.0	4 to 14	100	0	0	0	0
0.8	4 to 14	5	90	0	0	5
0.8	4 to 9	85	0	0	0	15
0.8	9 to 14	85	0	0	5	10
0.8	6 to 10	95	0	0	0	5

Note. Wavy indicates that the notochord was wavy along its full length, while posterior indicates a malformation only in the posterior portion of the animal. Other malformations include notochord malformations that were not easily categorized as wavy or posterior, such as a single kink. Analysis was performed at 48 hpf. One replicate of twenty embryos was observed for each dose and time period combination.

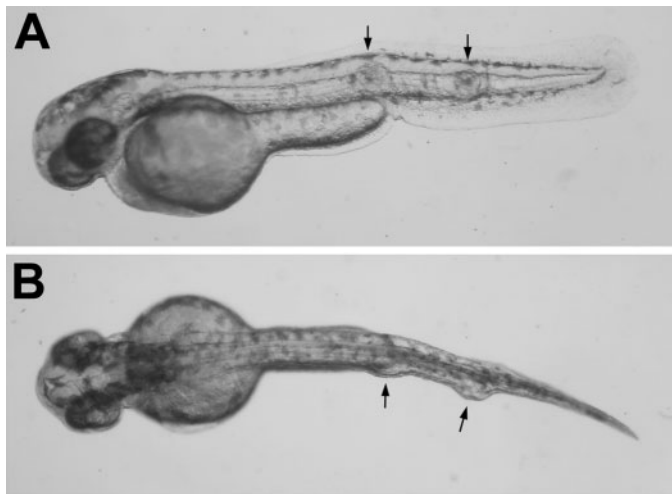


FIG. 5. Posterior malformation resulting from exposure during 14–24 hpf. (A) lateral view; (B) dorsal view. Arrows, local expansion of the notochord.

paragon staining at different time points following exposure from 4 to 24 hpf (Fig. 9). Analysis at 24, 48, and 72 hpf reveals abnormal characteristics that are indistinguishable between NaM and MITC exposed embryos (data not shown for NaM exposed embryos). It is obvious from these sections that the notochord of MITC exposed embryos contain regions where the notochord cells are irregularly packed and are not

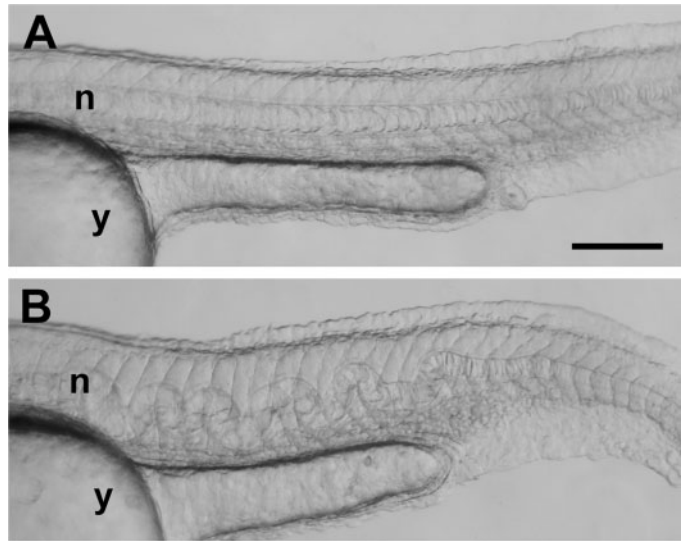


FIG. 6. Notochord defect following exposure to MITC. Embryos were exposed from 4 hpf to 24 hpf to 0.8 μ M MITC. (A) Lateral view of control embryo; (B) lateral view of exposed embryo. n, notochord; y, yolk; scale bar, 150 μ m.

homogeneous in size. There are also noticeable “globules” present along the inside of the plasma membrane of the notochord cells in exposed embryos (Fig. 9D, arrow). The notochord cell membranes are “rumpled” compared to the smooth appearance of control cells (compare Fig. 9C to 9D). The notochord sheath is also thicker and darker staining in MITC exposed embryos (arrowheads, Figs. 9E and 9F). Another obvious feature that can be visualized in these sections is the muscle of the somites. In 72 hpf control embryos, the somites form distinct chevron-shaped muscles with the muscle cells extending from one somite boundary to the next. In MITC exposed embryos, the muscle appears compacted (compare distance between asterisks in Fig. 9G and H) and has a wavy structure, the somites are block-shaped, and somite boundaries are not always evident. As described above, these alterations could be responsible for the observed behavioral changes.

DISCUSSION

The aim of this study was to ascertain whether NaM was developmentally toxic to zebrafish. Our initial experiments have shown that NaM causes notochord malformations which become apparent by 24 hpf in zebrafish embryos when exposed during gastrulation and early segmentation periods. NaM is toxic to zebrafish embryos at low ppb levels, as evidenced by the steep mortality curve. At non-lethal concentrations, the hatching rate and notochord malformation are affected by NaM in a dose-dependent manner. We have determined that embryonic development may also be delayed following exposure to NaM using both morphological and molecular criteria. We have also shown

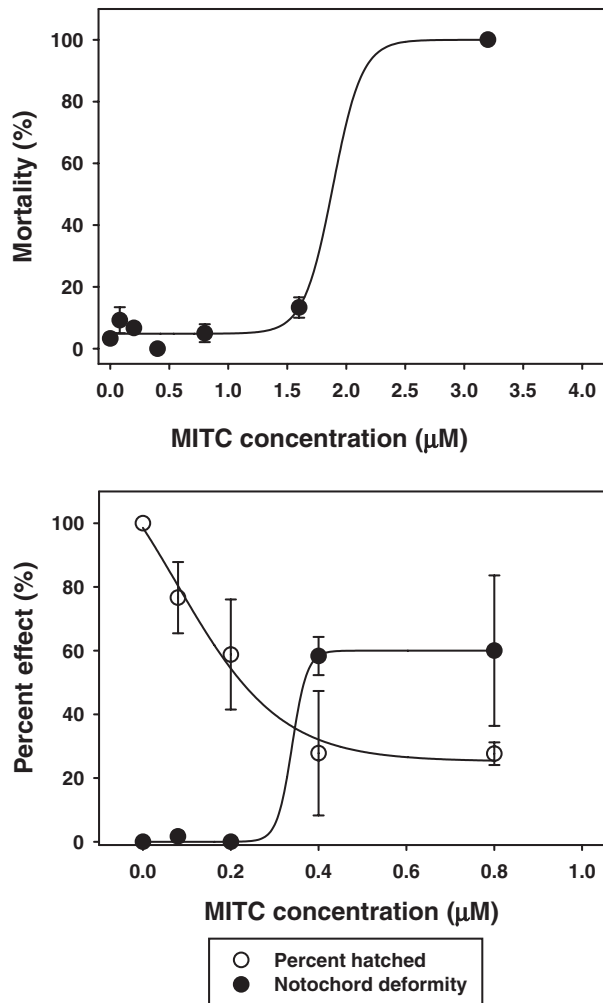


FIG. 7. Mortality, notochord malformation, and hatching rate exhibit a graded response following MITC exposure. Embryos were exposed from 4 hpf to 24 hpf to varying concentrations of MITC. (A) 48 hpf mortality curve. The LC_{50} is estimated to be $1.87 \mu\text{M}$. (B) Five dpf effects of exposure on notochord malformation (black circles) and hatching rate (open circles). The EC_{50} for notochord defects is $0.35 \mu\text{M}$ and the EC_{50} for reduced hatching is $0.22 \mu\text{M}$. Three replicates of twenty embryos each were observed for each data point.

that a degradation product of NaM, MITC, causes similar effects at similar concentrations, suggesting that MITC or other common breakdown products or metabolites may be the active toxicants following NaM exposure. Alternatively, NaM, MITC, or other products may have similar mechanisms of action. Importantly, under our experimental conditions the active water constituent appears to be stable. For instance, NaM and MITC solutions previously exposed to different temperature and light conditions resulted in similar rates of notochord deformities and mortalities in our standard assay (data not shown).

Embryos that survive the initial exposure to NaM or MITC continue to develop despite notochord malformations. At 5 dpf, the embryos from both exposure groups appear indistinguishable, and are similarly unable to feed, abnormally respond to

touch and exhibit decreased motor activity (data not shown). These results indicate that NaM and MITC are potent teratogens during early zebrafish development. This study is especially timely given that NaM is one of the proposed replacements for the agricultural biocide, MeBr, after the 2005 phase-out (EPA 2002a,b, 2003). Since no studies have yet been published examining the effects of NaM or MITC on similar stages of mammalian development, it is imperative not only that these studies be undertaken but also that the mechanism of action of these compounds be further elucidated.

Despite the fact that there are few studies regarding NaM toxicity, a number of different hypotheses have been proposed to explain its mechanism of action. We hypothesize that NaM or MITC may be targeting a specific reactive site, protein, or biological pathway. Because the effective concentration is very low, this suggests that the cellular targets are limited in number. The dose response curves for NaM and MITC are both very steep, which also indicates that there are molecular sites of action that become saturated. MITC has the capacity to form protein adducts, which may contribute to its toxicity (Valentine *et al.*, 1995). MITC and NaM are both metabolized via glutathione conjugation (Lam *et al.*, 1993). It has been proposed that the MITC-glutathione conjugate may serve as a source of MITC elsewhere in the body at a later time point (Slatter *et al.*, 1991). Zebrafish exhibit functional glutathione-S-transferase activity throughout all of development (Wiegand *et al.*, 2000). Alternatively, S-methylation of NaM or MITC to S-methyl metam could potentially inhibit aldehyde dehydrogenase, thereby causing toxicity (Staub *et al.*, 1995). MITC has been shown to be cytotoxic and cause DNA strand breaks in cultured human hepatoma cells (Kassie *et al.*, 2001). These investigators suggest that the DNA damage may be due to the production of reactive oxygen species, which could damage other macromolecules in addition to DNA. Because dithiocarbamates are known to chelate metals, it is possible that they may act via sequestering bioactive metals away from proteins. However, since MITC is not a dithiocarbamate and is not known as a metal chelator, it is unlikely that its mechanism of toxicity is mediated by chelation (Tomlin, 1997). Therefore, it is clear from the diversity of targets and hypotheses that the mechanism of action of NaM or MITC remains unknown.

The notochord is an axial structure common to the Chordata phylum. In lower chordates and in larval stages of lower vertebrates it plays an important role as a structural element required for locomotion and coordinated movement. More importantly, the notochord is required for proper differentiation of adjoining tissues such as the neurectoderm, muscle, and vertebral elements in all vertebrates. This is accomplished in part by release of signaling molecules such as *sonic hedgehog (shh)* from the notochord. In zebrafish, Shh induces the formation of slow muscle fibers in the medial somite, which then migrate laterally and complete their differentiation (Blagden *et al.*, 1997). A Shh gradient is also thought to regulate the dorsal-ventral identity of neurons in the neural tube, such that neurons closest to the notochord form motoneurons, whilst neurons further away

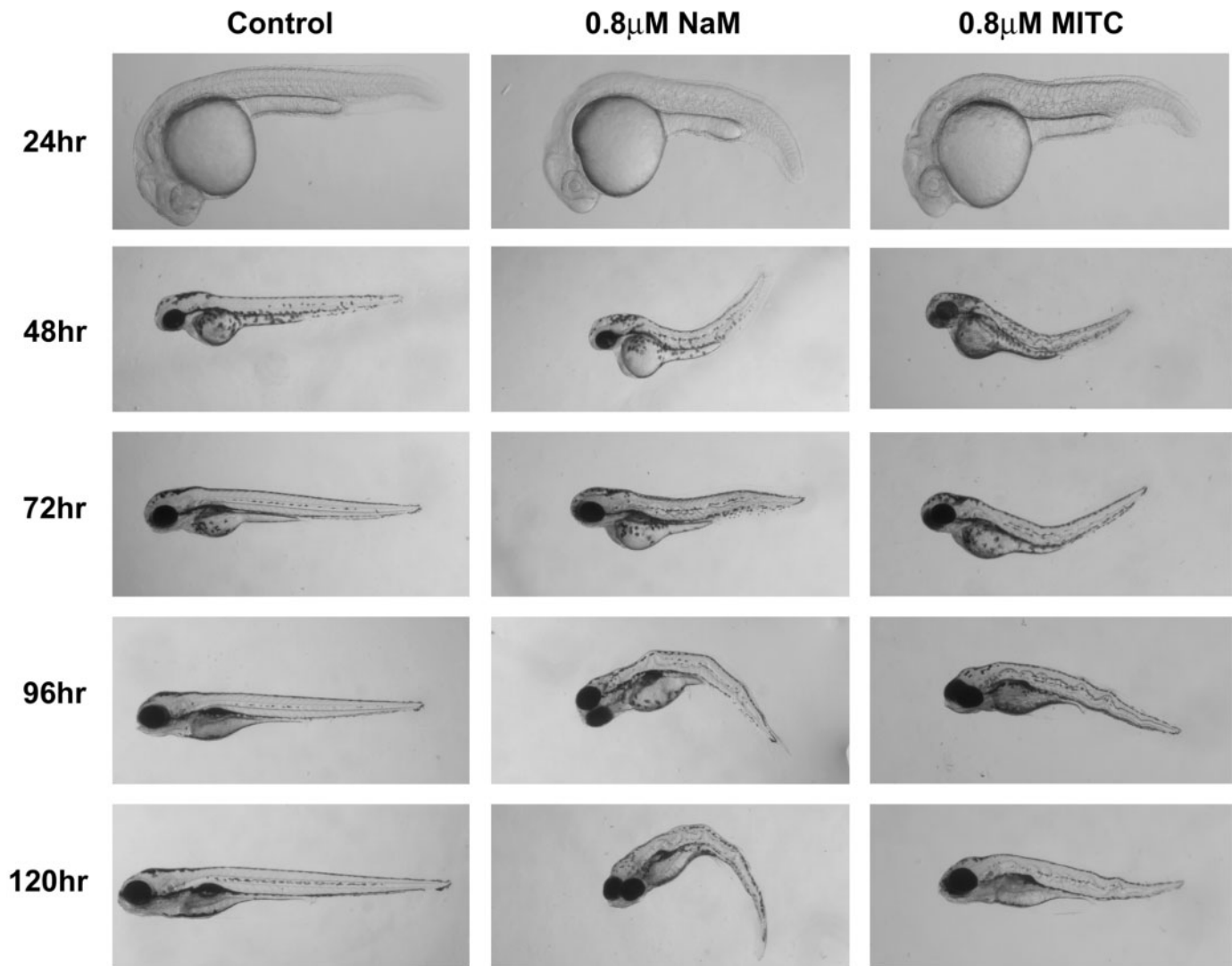


FIG. 8. Development of embryos from 24 to 120 hpf following 0.8 μM NaM or 0.8 μM MITC exposure from 4 to 24 hpf. Photographs at 24 hpf are shown at a higher magnification.

become interneurons or sensory neurons (for recent review see Jacob and Briscoe, 2003). Therefore, the notochord is the primary axial structure upon which many other tissues depend for their proper formation and differentiation. Toxicants that disrupt normal morphogenesis and differentiation of the notochord may therefore result in permanent skeletal deformities, muscle abnormalities, and neurological dysfunction.

A search of the literature has revealed a few examples of severe notochord malformations following early life stage exposure to various toxicants, including dithiocarbamates. Zebrafish exposed to cadmium exhibit notochord and skeletal deformities (Hen Chow and Cheng, 2003). A study in rainbow trout embryos revealed that a number of dithiocarbamates are teratogenic, with the notochord being particularly sensitive; however, the teratogenic effects of NaM were not specifically examined

(Van Leeuwen *et al.*, 1986). In this study, the notochord increased considerably in both length and diameter and therefore became twisted and distorted. The authors also observed ectopic osteogenesis. Among the animals that were raised to adulthood, compression and fusion of vertebrae and various twisted skeletal elements were observed. *Xenopus laevis* exposed to nabam (a dithiocarbamate) developed malformed notochords which contained larger and more numerous cells at concentrations greater or equal to 0.40 $\mu\text{g/l}$ (Birch and Prahlad, 1986b). Interestingly, embryos exposed to MITC and ethylene thiourea concurrently developed notochord malformations similar to nabam, while neither of these compounds alone resulted in malformations at any of the concentrations tested. Interactions between these and additional degradation products may be responsible for these results (Birch and Prahlad, 1986b). North African catfish

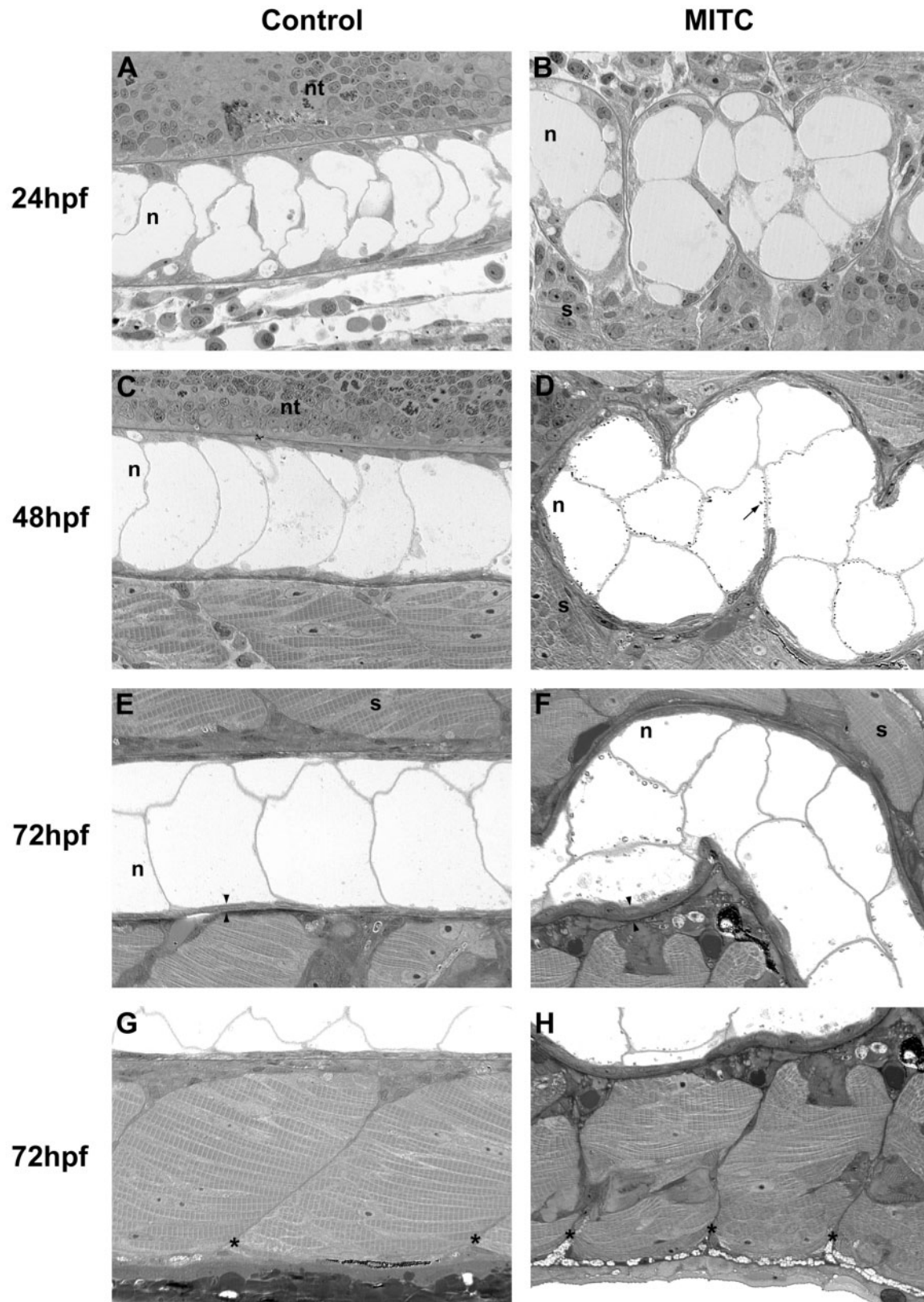


FIG. 9. Notochord histology. Sections of embryos exposed to 0.8 μ M MITC from 4 hpf to 24 hpf. (A, C, E, G) Control embryos at 24, 48, and 72 hpf. (B, D, F, H) Exposed embryos at 24, 48, and 72 hpf. (G, H) Views showing the muscle of the somites. Two embryos were sectioned per dose at each time point. nt, neural tube; n, notochord; s, somite; *Somite boundaries; arrows, globules; arrowheads, notochord sheath thickness.

(*Clarias gariepinus*) exposed to 2.5 and 5 mg/l of the organophosphate malathion also develop remarkably similar notochord malformations (Lien *et al.*, 1997). Lien *et al.* suggest that the notochord may become malformed by overactive muscle spasms. Teraoka *et al.* have recently proposed a similar explanation for notochord waviness following a 24 h exposure to the dithiocarbamate thiuram (Teraoka *et al.*, 2004). In this study, the notochord malformations could be prevented by inhibition of spontaneous muscle contractions by co-exposure to the anesthetic MS-222.

The data from the present study suggests that the notochord is sensitive to NaM at a period in its differentiation prior to spontaneous muscle contractions. The end of the developmental window during which exposure to NaM or MITC causes notochord malformations (4–14 hpf) is 3 h prior to the onset of spontaneous movement (17 hpf). It is conceivable that the notochord could be compromised during early development and the consequence of this earlier exposure becomes evident once muscle activity initiates. Alternatively, degradation products deposited in the tissue early could exert their effects later in development. Since exposure later in development does not result in notochord defects, the active toxicant must either be present earlier or create a degradation product that exerts its effects later. However, we believe that the notochord is affected by NaM and MITC because MITC is the major degradation product, is relatively stable, and both cause similar effects during the same window of development. In contrast to the malathion study noted above (Lien *et al.*, 1997), we have observed that zebrafish exhibit decreased touch responsiveness and movement following exposure to NaM or MITC. This indicates that either NaM and MITC exert similar effects by a different mechanism, or that notochord malformations result from alterations of other developmental processes besides muscle activity. Perhaps NaM and MITC affect notochord vacuolization and/or proliferation, which would distort the overall rigidity and axial length of the notochord, indirectly resulting in abnormal muscle structure. This concept is supported by studies examining ultrastructural changes in the notochord following dithiocarbamate exposure in *Xenopus* (Birch and Prahlad, 1986a; Prahlad *et al.*, 1974). Embryos exposed to nabam or a combination of ethylenethiourea and MITC both exhibit disruption of collagen fibers in the notochord sheath and surrounding tissue. Nabam exposed embryos also showed an absence of plasmalemmal vesicles normally found adjacent to the intercellular space of notochord cells (Prahlad *et al.*, 1974). Studies examining the effects of another dithiocarbamate, thiram, on development have shown that it is teratogenic to mice when administered between days 5 and 17. These embryos exhibited an increase in resorption as well as a syndrome of skeletal malformations including cleft palate, wavy ribs, curved long bones and micrognathia (Fishbein, 1976; Matthiaschk, 1973; Roll, 1971). These results suggest a conserved response and warrant more detailed examination of the effects of dithiocarbamates in general and NaM in particular on early mammalian development.

We believe that the zebrafish is a valuable system in which to elucidate the mechanism of action of these compounds specifically because of its molecular accessibility. For example, a zebrafish mutant, *leviathan*, has been identified in which a very similar notochord malformation has been noted (Stemple *et al.*, 1996). Given the large number of notochord mutants that have been found, it is interesting that only one has a phenotype similar to NaM exposed embryos (Stemple *et al.*, 1996). The determination of the mutation responsible for this phenotype may allow the molecular dissection of a regulatory cascade whose dysfunction leads to specific notochord malformations. Advances in micro array technology will also be useful in the molecular characterization of NaM or MITC effects during specific periods of development. This study has provided an initial morphological and molecular characterization of the effects of NaM and MITC which will allow more mechanistic questions regarding the effects of these compounds on tissue morphogenesis and gene expression to be addressed.

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