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Calmodulin Concentration in Mucus of Rainbow Trout, *Salmo gairdneri*, Exposed to Combinations of Acid, Aluminum, and Calcium

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As a result of increasing acidification in various watersheds elevated levels of aluminum have been observed in soil and surface water (Cronan and Schofield 1979). In both field and controlled laboratory experiments aluminum has been shown to be highly toxic to fish (Baker and Schofield 1982; Henriksen et al. 1984). The toxicity of Al to fish has been shown to be positively correlated with the concentration of inorganic monomeric Al (Driscoll et al. 1980; Baker and Schofield 1982). The exact mechanism(s) of Al toxicity is not fully understood. It appears that the gill is a crucial target organ for the toxic action of Al. Of the organs analyzed in smallmouth bass (*Micropterus dolomieui*) exposed to high Al levels, the gill filaments exhibited the highest concentration of Al (Brumbaugh and Kane 1985). Visible signs of Al toxicity include coughing response, hyperventilation, and excessive mucus clogging of the gills (Muniz and Leivestad 1980). Accompanying these visible signs are more specific responses such as rapid loss of sodium and chloride from plasma and lowered blood oxygen tension (Muniz and Leivestad 1980). At the cellular level the toxic mode of action remains elusive. Cleveland et al. (1986) observed a reduction in RNA synthesis and RNA/DNA ratios in early life stages of brook trout (*Salvelinus fontinalis*) exposed to pH 5.5 in the presence of Al, suggesting disruption or deactivation of metabolic pathways. Reduced carbonic anhydrase and Na-K-ATPase activity in gills of salmonids exposed to Al-containing waters has also been reported (Staurnes et al. 1984).

Recently, the presence of calmodulin (CaM), a calcium-regulating protein, has been reported in fish gills and mucus (Flik et al. 1983; 1984). Calmodulin selectively binds inorganic monomeric Al causing conformational changes in the protein (Siegel and Haug 1983; Suhayda and Haug 1984). Aluminum-induced conformational changes cause a reduction in the ability of calmodulin to mediate Ca-dependant phosphodiesterase and ATPase activity (Siegel and Haug 1983; Siegel et al. 1983). Calmodulin also plays a key role in coordinating the effects of secondary messenger systems in response to cellular stimulation (Rasmussen 1981). Given the involvement of calmodulin in numerous biochemical pathways, its interaction with aluminum may be a key lesion in the broadly defined syndrome of aluminum toxicity (Siegel and Haug 1983). The present study was undertaken to establish a relationship between Al concentration in aqueous solution and the quantity and activity of CaM in the mucus of adult rainbow trout (*Salmo gairdneri*). Fish were exposed to various levels of pH, Ca, and Al. Mucus was collected and the amount of CaM was determined. The ability of the Al-exposed CaM to activate the phosphodiesterase enzyme system was also evaluated.

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MATERIALS AND METHODS

The exposure was conducted at the University of Wyoming's Red Butte Environmental Biology Laboratory, Laramie, WY. Details of exposure conditions appear in Mount (1988). This preliminary investigation was part of an on-going study consisting of seven combinations of pH, Ca, and Al. Table 1 presents the nominal and measured levels of pH, Ca, and Al in these seven treatments. Test fish were 2-1/2 year old rainbow trout (*Salmo gairdneri*) (Belaire strain) obtained from the Colorado Division of Wildlife's Crystal River Hatchery on April 15, 1986. Mean weight at the start of exposure was 1089 g. Floating trout chow (Purina 5106) was fed once daily at 0.8% body weight/day, and all tanks were cleaned daily.

Mucus samples were collected after 147 days of exposure (12 January 1987) from 1-5 fish in each of seven exposure combinations (Table 1), after the method of Flik et al. (1984). Fish were gently netted from the tank and anesthetized with MS-222 (Sigma Chemical Co., St. Louis, MO) in ambient tank water which was titrated with KOH back to ambient pH levels. Once anesthetized, each fish was grasped by the lower jaw and held over a 3,000-mL beaker while the body surface was rinsed with 30 to 50 mL of buffer solution (0.0137 M Tris, 0.12 M NaCl, and 0.003 M KCL, pH 7.4). Samples were transferred to polypropylene centrifuge bottles, chilled to 4°C, and shipped on ice to Michigan State University's Pesticide Research Center for CaM determinations.

Table 1. Measured levels of pH, Ca, and Al during the 147 day exposure period. Values are means ± standard error mean. Numbers in parentheses are the number of observations.

	Nominal Chemistry						
pH (units)	6.50	5.20	5.20	5.20	5.20	4.80	4.80
Ca (mg/L)	2	2	4	2	1	2	2
Al (μg/L)	0	0	50	50	50	0	100
pH (units)	6.45±0.01 (157)	5.25±0.01 (158)	5.24±0.01 (158)	5.24±0.01 (158)	5.21±0.01 (158)	4.83±0.01 (158)	4.80±0.01 (158)
Ca (mg/L)	2.05±0.05 (35)	2.09±0.03 (35)	3.81±0.08 (35)	2.14±0.06 (35)	1.08±0.02 (35)	2.15±0.03 (35)	2.08±0.03 (35)
Total Al (μg/L)	1.8±0.3 (38)	1.8±0.4 (38)	39.8±1.4 (38)	41.7±1.4 (38)	41.3±1.5 (38)	1.9±0.4 (38)	97.3±2.6 (38)

Mucus samples in solution were centrifuged at 1,000xg for 10 min at 4°C. Protein content of mucus in the centrifugate was measured according to Bradford (1976), with bovine brain gamma globulin used as a standard. Protein assay reagents were purchased from Bio-Rad Laboratories (Richmond, CA). All other chemicals were of the highest quality available. The minimum detection limit of the method was 8 μg protein/mL. Error associated with the protein determination was estimated to be ±5%. Prior to quantitation of CaM, stimulation of cyclic AMP-dependent phosphodiesterase activity by CaM was determined by a one-step procedure (Teo et al. 1973) involving two enzymes, viz. activator-free 3':5'-cyclic nucleotide-phosphodiesterase and alkaline phosphatase, both obtained from Sigma Chemical Co. (St. Louis, MO). The phosphate released by the enzyme was quantitated by the Malachite green method (Penney 1976), with a detection limit of 0.01 mg phosphate/mL. Analytical error for this procedure was estimated to be ±10%.

A calmodulin [^{125}I]-radioimmunoassay kit (RIA) (New England Nuclear Co., Boston, MA) was used for CaM quantitation in fish mucus samples. The centrifugate was incubated at 100°C for 5 min. Calmodulin content in the incubated sample was determined by RIA with preheated bovine brain CaM as a standard. The minimum detection limit was approximately 3.1 ng CaM/mL solution. The upper limit of assay applicability was 200 ng CaM/mL solution. Error associated with the assay was approximately ±5%. The QA/QC protocol used in the procedure was that provided by the manufacturer. Other calcium-binding proteins, such as troponin C and parvalbumin, have been shown not to be reactive in the CaM assay procedure.

RESULTS AND DISCUSSION

Table 2 summarizes the results of mucus sample analyses performed on surviving fish from each of the seven treatments. The CaM content in mucus collected from fish exposed to aluminum was generally greater than that from those not exposed to aluminum. In mucus derived from Al-exposed fish, the total protein concentration was found to be less than in mucus from non-exposed fish, although the μg CaM per mg total protein was greater in exposed fish. In addition, CaM content tended to be higher at pH 6.5 (0 μg Al/L) compared to values obtained at pH 4.8 and 5.2 (0 μg Al/L), but these levels were still small in comparison to Al-exposed fish. Three levels of calcium can be compared with pH and Al levels fixed at 5.2 and 50 μg/L, respectively. Slightly less CaM was present in the mucus of fish held at low Ca levels (1 mg/L), however, only two fish were available from this treatment. At constant pH (5.2) and Ca levels of 2 and 4 mg/L levels, six of the eight fish exposed to 50 μg Al/L had large amounts of CaM in mucus. None of the four fish exposed the 0 μg Al/L, at pH 5.2 and 2 mg Ca/L treatment had detectable amounts of CaM in the mucus.

Only one specimen was available for CaM determination from the highest Al treatment (100 μg/L); held at pH 4.8 and a Ca concentration of 2 mg/L. It also exhibited elevated levels of CaM in mucus (0.6 μg CaM/g protein). The presence of CaM in fish mucus is further supported by the stimulation of CaM-free cyclic nucleotide phosphodiesterase activity (Table 2). The CaM-related stimulatory effect of the mucus was dependent upon the concentration of CaM, and was therefore more pronounced using mucus collected from Al-stressed fish. The mucus collected from the single specimen exposed to 100 μg Al/L released 8.0 μM/mg/hr of phosphate in the phosphodiesterase assay. The mucus collected from this fish was analyzed for Al by graphite furnace AA. While this fish had the greatest Al exposure, no Al (< 10 μg/L) was detected in the CaM fraction.

The presence of CaM in the mucus of certain rainbow trout is supported by two lines of evidence. Both the radioimmunoassay determination and the phosphodiesterase assay provided positive results for the presence of CaM in mucus. These findings support the observations made by Flik et al. (1984) that CaM is present at an extracellular location, mucus, as has been reported for adult tilipia (*Sarotherodon mossambicus*), catfish (*Clarias lazera*), and rainbow trout. With aluminum absent, at a Ca concentration of 2 mg/L, pH 6.5, an average CaM content of 0.2 μg/mg total protein in fish mucus in the present study seems to be of the same order of magnitude as that found in tilipia mucus preparations obtained from fish held in tapwater where the Ca concentration varied from 5 to 20 mg/L (Flik et al. 1984). No CaM values for rainbow trout were reported by the authors.

Flik et al. (1984) observed less CaM in tilipia mucus at lower Ca levels. At Ca levels of 1 and 2 mg/L, with pH held at 5.2 and Al concentration held at 50 μg/L, CaM levels in fish mucus at the lower Ca level were slightly lower than at the higher Ca level. However, the small sample size prohibit definitive conclusions. The lower pH level and

Table 2. Results of CaM [^{125}I] radioimmunoassay perfomed on mucus collected from rainbow trout held at various pH, Ca, and Al levels for 147 days.

pH (units)	Al (μg/L)	Ca (mg/L)	total Protein (μg/mL)	CaM (μg/mg protein)	phosphodiesterase Pi release (μM/mg • hr)
5.2	50	2.0	22	[1]ND	ND
5.2	50	2.0	8	1.65	12.2
5.2	50	2.0	[2]367	ND	ND
5.2	50	2.0	17	0.43	7.1
5.2	50	4.0	15	0.67	3.9
5.2	50	4.0	[3]16	0.68	5.8
5.2	50	4.0	17	1.14	4.3
5.2	50	4.0	18	0.61	ND
5.2	50	1.0	128	ND	0.4
5.2	50	1.0	17	0.4	3.1
4.8	100	2.0	17	0.6	8.0
4.8	0	2.0	[3]280	ND	ND
4.8	0	2.0	[3]455	ND	ND
4.8	0	2.0	[2]744	ND	ND
4.8	0	2.0	26	ND	ND
4.8	0	2.0	40	0.4	ND
6.5	0	2.0	40	0.3	2.3
6.5	0	2.0	173	0.1	0.5
6.5	0	2.0	55	0.4	0.4
6.5	0	2.0	77	0.1	0.4
6.5	0	2.0	59	ND	0.9
5.2	0	2.0	[3]602	ND	ND
5.2	0	2.0	[3]944	ND	ND
5.2	0	2.0	55	ND	ND
5.2	0	2.0	290	ND	ND

[1]ND = not detectable
[2]milt in sample
[3]eggs in sample

the presence of Al in the test solution may have caused an elevation in CaM content to values comparable to those observed by Flik et al. (1984) at higher Ca levels. Intraspecies variation may also account for the difference in CaM content in the mucus of rainbow trout and tilipia.

In mucus derived from Al-stressed fish the total protein concentration was found to be slightly less than in mucus from non-stressed fish. Therefore, the Al-induced enhancement of mucus CaM content does not seem to result from Al-related breakage of cells at the fish epithelial surface. This evidence suggests that the enhanced presence of calmodulin in mucus may be a response to Al stress for the purpose of maintaining membrane integrity and regulatory processes in epithelial cells. Increased mucus secretion by gills and skin is commonly reported for fish exposed to acid and aluminum stress (Jagoe and Haines 1983; Muniz and Leivestad 1980). Flik et al. (1984) proposed that the presence of CaM in the mucus may be related to enzymatic control

of integumental permeability. Therefore, interruptions in CaM activity could account for some of the ion-regulatory disturbances associated with acid/Al stress.

Calmodulin in fish mucus may serve as an extracellular chelator of Ca (Flik et al. 1984). Calcium plays a major role in membrane permeability by its interaction with phospholipids (Ebel and Gunther 1980). Acid-induced, and possibly aluminum-induced, secretion of mucus by fish gills and integument may be an adaptive mechanism, resulting in a reduction in epithelial ionic permeability by providing CaM complexation of external Ca (Marshall 1978; Shephard 1982). Since aluminum stress in fish is manifested by ionic imbalances (Muniz and Leivestad 1980), the effects of Al on the ability of CaM to mediate enzymatic control of membrane permeability cannot be discounted as a possible lesion in overall Al toxicity. Aluminum has been reported to inhibit the Na-K-ATPase system in fish (Staurnes et al. 1984). Inasmuch as CaM mediates this enzyme system, Al binding to CaM may be a primary cause for this inhibition. The binding of Al to CaM may induce the secretion of additional CaM into the mucus layer to compensate for the "apparent" lack of available CaM-regulated Ca. It is evident that Al-exposed fish exhibited elevated levels of CaM. However, the ability of Al-induced CaM to stimulate phosphodiesterase activity was expected to decrease due to conformational changes in the protein (Siegel and Haug 1983). This was not the case, however. Calmodulin collected from Al-exposed fish was still capable of mediating the phosphodiesterase enzyme system. One possible explanation is that the Tris component of the buffer used to rinse mucus from the fish body may contain potentially active binding sites for Al. Ganrot (1986) reported the tendency of Al to "migrate" from one binding site to another in biological systems. If the stability of the Al:Tris complex exceeds that of the Al:CaM complex, then Al may redistribute to Tris binding sites. This occurrence would partially return CaM to its normal conformation, thus restoring its ability to mediate phosphodiesterase activity. In future experiments an alternate buffer will be used to circumvent this occurrence.

Fairly low concentrations of Al were used in the present study (0-100 μg/L) in comparison to levels found in lakes and streams impacted by acidic deposition in the northeastern United States (Driscoll et al. 1984; Kanciruk et al. 1986). Nevertheless, a positive correlation was found between Al concentration and the amount of CaM in fish mucus. At higher concentrations of Al the increase in CaM content in mucus may be more pronounced. These are preliminary data and additional levels of pH, Ca, and Al must be tested before definitive conclusions can be drawn.

The presence of calmodulin in fish mucus may serve as an indicator of stress induced by biologically active Al in surface waters. Confounding the response of CaM to Al may be variations in mucus CaM content due to different calcium levels. Calcium has been reported to affect the concentration of CaM in fish mucus (Flik et al. 1984). However, the magnitude of increase in CaM over a given range of calcium may not be as significant as that observed for Al. Given the large increase in CaM at low concentrations of Al used in the present study, Ca may not act as a significant "interference" in the detection of Al-induced CaM.

Although these findings are inconclusive due to constraints imposed on the experimental design, the results suggest a possible relationship between Al concentration in solution and the amount of CaM in rainbow trout mucus. Despite the extremely low Al levels used in the experiment, the secretion of CaM into the mucus was significant. Given the involvement of CaM in membrane permeability and various enzyme systems, Al binding to this protein may be the first phase of action in Al toxicity syndrome in fish. These preliminary data indicate that CaM may serve as an indicator

of stress induced by the presence of biologically relevant Al in surface waters impacted by acidic deposition. Further study is in progress to verify these findings.

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