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Ascorbate-2-sulfate as a dietary vitamin C source for Atlantic salmon (*Salmo salar*): 2. Effects of dietary levels and immunization on the metabolism of trace elements

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Abstract

Atlantic salmon fingerlings were fed a vitamin C deficient diet for four months. The fish were then provided a dry, practical fishmeal based diet supplemented with 0, 500 or 5000 mg vitamin C/kg as L-ascorbic acid or equivalent amounts of ascorbate-2-sulfate. After six weeks on these diets ten fish in each group were injected with a soluble antigen (NIP_{11}-LPH). Six weeks thereafter blood, liver, kidney, spleen and vertebrae were examined for trace elements. The livers were also analysed for metallothionein.

The vitamin C deficient fish were anemic despite the significantly elevated iron concentrations in the liver. Vitamin C had no positive effect in lowering tissue levels of cadmium. The highest level of dietary vitamin C given as ascorbic acid reduced the liver selenium concentrations. In response to antigen injection, the fish in all groups showed increased levels of hepatic metallothionein, copper, zinc and cadmium, while hepatic selenium and iron levels were less affected. The elemental composition in other organs was affected by the antigen injection to a minor extent.

Introduction

Sufficient dietary levels of essential minerals and trace elements are important for optimum health and growth in fish. Specific requirements for the minerals phosphorus, calcium and magnesium as well as the trace elements iron, zinc, copper, selenium, manganese and iodine have been reported (Lall 1989). The exact dietary requirements are difficult to quantify (NRC 1981) but essential minerals and trace elements are supplemented in fish feeds.

Several studies have shown that ascorbic acid (AA) plays a role in the absorption, metabolism and excretion of certain essential and non-essential elements in both terrestrial animals and fish (Hilton 1984, 1989; Sandnes *et al.* 1984). AA can affect the absorption and distribution of iron both at the absorptive and metabolic level (Hornig *et al.* 1984), and has been reported to reduce the toxicity of both cadmium (Fox 1975) and copper (Yamamoto *et al.* 1981; Yamamoto and Inoue 1985).

As discussed by Herlyn and Glaser (1976), contradictory results have been found regarding the influence of AA on humoral resistance factors in animals. However, high dietary doses of AA have been reported to exert positive effects upon non-specific and specific immune mechanisms in fish (Li and Lovell 1985; Navarre and Halver 1989). This has raised the question of using high doses of vitamin C in fish feeds. Ascorbate-2-sulfate (AS) has been suggested as a stable vitamin C source in fish diets (Tucker and Halver 1986) and has been used commercially. Recent studies have shown that the bioactivity of this compound is limited in Atlantic

salmon, *Salmo salar* (Sandnes *et al.* 1990) and in rainbow trout, *Oncorhynchus mykiss* (Dabrowski and Köck 1989).

One of the major problems in the fish farming industry is the loss caused by infectious diseases. To avoid excessive use of antibiotics, the development of effective vaccines and search for active agents capable of enhancing the defence mechanisms in fish are of interest. In this study we examined the relationship between the humoral response and trace element metabolism in Atlantic salmon.

Since trace elements are important for fish health and growth and little is known of factors affecting their metabolism in Atlantic salmon we have investigated (1) the effect of vitamin C deficiency and high doses of AA and AS on trace element metabolism in Atlantic salmon and (2) the effect of antigen injection on the trace element metabolism in the fish.

Materials and methods

Experimental protocol

After an initial feeding period of four months on a dry, vitamin C-deficient, practical salmonid diet with fish meal as the main protein source (Albrektsen *et al.* 1988) the fish were randomly distributed into separate tanks and fed the experimental diets (each in duplicate) for twelve weeks. These were the same basal diet with: a) no supplementation of vitamin C (0); b) 500 mg crystalline L-ascorbic acid (AA)/kg (500 AA); c) 5000 mg AA/kg (5000 AA); d) ascorbate-2-sulfate (AS) equivalent to 500 mg AA/kg (500 AS) and e) AS equivalent to 5000 mg AA/kg (5000 AS). Fish weight at the start of the experimental period was 19.9 ± 5.5 g (n = 477) and the water temperature was 7.2 ± 3.1°C (recorded daily) during the feeding period. The natural mineral content of the diets was: iron 134 mg/kg, zinc 42 mg/kg, copper 3.4 mg/kg, selenium 0.77 mg/kg and cadmium 0.11 mg/kg.

After six weeks five fish from each tank were intraperitoneally injected with 100 µl of a carrier-hapten antigen, NIP_{11}-LPH in Freunds complete adjuvant (50/50). Antibody production against this antigen was measured at the end of the experiment. A detailed description of the experimental design, antigen injection and antibody detection is given by Sandnes *et al.* (1990). The copper, zinc and cadmium content in each injected dose was 55 ng, 6 ng and < 0.15 ng, respectively.

Tissue samples

Samples of liver, spleen, vertebrae and kidney were collected at the end of the experiment. Five fish injected with antigen and five untreated fish were collected from each dietary group. Analyses of iron, copper, zinc, cadmium and selenium were carried out in all organs except the spleen, and metallothionein was determined in liver. Iron and zinc concentrations in spleen were analysed in pooled samples from five fish.

Analyses

Elemental analyses of the feed and organs were performed on freeze-dried samples which were digested according to Julshamn and Andersen (1982). Iron, zinc and copper were analysed by flame atomic absorption on a Perkin-Elmer 3030 AAS, cadmium and selenium were analysed by graphite furnace AAS (Perkin-Elmer 5000 and Zeeman 5000 AAS). The accuracy and precision of the elemental analyses were tested in an intercalibration study arranged by ICES (Berman 1984) as well as by analyses of standard reference materials from the National Institute of Standards and Technology. The methods applied were found satisfactory with regard to both tests.

Metallothionein was determined by a polarographic method (Olafson and Sim 1979) modified according to Olsson *et al.* (1987).

Statistical analyses were performed by means of Student's t-test and correlation analysis using a Luxor 806 computer equipped with an IDA 800 statistical program.

Results

The liver analyses presented in Table 1 show that fish fed the diet without supplementation of vita-

Table 1. The concentrations of trace elements and metallothionein in liver of Atlantic salmon given different dietary levels and forms of vitamin C, and after antigen injection

Treatment	Dietary vitamin C (mg/kg ascorbic acid, AA, or equivalent ascorbate-2-sulfate, AS)				
	0	500 AA	500 AS	5000 AA	5000 AS
Iron					
C	181 ± 71^{abc}	99 ± 12^{a}	89 ± 38^{b}	89 ± 39^{c}	121 ± 38
AG	252 ± 115^{abcd}	99 ± 17^{ae}	123 ± 28^{bf}	77 ± 19^{cef}	116 ± 15^{cd}
Zinc					
C	96 ± 8^{ab}	77 ± 4^{a}	69 ± 17^{bc}	86 ± 17	93 ± 9^{c}
AG	*117 ± 16^{abc}	*85 ± 2^{ad}	*92 ± 9^{b}	95 ± 6^{cd}	99 ± 17
Copper					
C	74 ± 41	78 ± 33	48 ± 28	57 ± 36	92 ± 41
AG	109 ± 21	98 ± 43	88 ± 51	78 ± 32	122 ± 47
Selenium					
C	4.3 ± 0.7^{ab}	4.3 ± 0.8^{c}	3.6 ± 0.3^{ad}	3.2 ± 0.4^{bcd}	4.8 ± 0.6^{d}
AG	*3.4 ± 0.5^{a}	4.0 ± 1.0	3.8 ± 0.6	3.4 ± 0.2^{b}	4.8 ± 1.2^{ab}
Cadmium					
C	0.9 ± 0.2	1.2 ± 0.4	1.1 ± 0.1	0.9 ± 0.4	1.1 ± 0.2
AG	1.2 ± 0.5^{abc}	*1.9 ± 0.7^{ad}	*1.8 ± 0.2^{be}	*1.3 ± 0.2^{def}	*2.4 ± 1.0^{cf}
Metallothionein					
C	16 ± 4^{a}	17 ± 3^{bc}	15 ± 5^{d}	8 ± 5^{abd}	10 ± 11^{c}
AG	22 ± 8	*30 ± 7^{a}	*25 ± 8	*21 ± 5^{a}	*28 ± 10

Data are shown as mean ± SD (n = 5); C = Control, AG = Antigen injected fish; Common superscript letters within one line indicate significant differences ($p < 0.05$); Antigen-injected groups significantly different ($p < 0.05$) from uninjected fish given the same diet are marked by *; Trace element concentrations given as mg/kg dry weight; Metallothionein concentrations given as nmol/g wet weight.

min C contained significantly ($p < 0.05$) higher hepatic iron concentrations than the other groups, except the group fed the 5000 AS diet. This pattern was observed both in antigen-injected and control fish. Further, the hepatic zinc concentration was elevated in the vitamin C deficient fish.

Stimulation of the immune system resulted in a general increase in the concentrations of copper, zinc and cadmium in the liver of fish from all dietary treatments. There was a difference between the fish fed megadoses of vitamin C (5000 AA or 5000 AS) in that the selenium concentrations in liver and bone were significantly higher ($p < 0.05$) in the fish fed 5000 AS than in the fish fed 5000 AA.

Immunized fish showed significantly higher levels of metallothionein ($p < 0.05$) compared to the control fish in all groups except the vitamin C-deficient fish. Correlation analyses of all fish showed significant positive correlations between liver metallothionein and copper ($r = 0.52$, $p < 0.01$), zinc ($r = 0.36$, $p < 0.05$) and cadmium ($r = 0.51$, $p < 0.01$).

Irrespective of diet or stimulation of humoral defence the gross composition and the relative liver weight of the fish (hepatosomatic index, HSI) did not vary among the groups (results not shown). Thus, the values which refer to hepatic elemental analyses are given and discussed on a tissue concentration basis.

The vitamin C-deficient fish injected with antigen showed significantly higher kidney concentrations of iron than the other antigen-injected fish (Table 2). In kidney there was no significant differences in cadmium concentration between the groups fed different levels or forms of vitamin C. The antigen injection showed only minor effects on the concentrations of trace elements in kidneys and vertebrae (Tables 2 and 3).

Table 2. The concentration of trace elements in kidney of Atlantic salmon given different dietary levels and forms of vitamin C and after antigen injection

Treatment	Dietary vitamin C (mg/kg ascorbic acid, AA, or equivalent ascorbate-2-sulfate, AS)				
	0	500 AA	500 AS	5000 AA	5000 AS
Iron					
C	242 ± 26	231 ± 42	199 ± 65	207 ± 42	245 ± 27
AG	284 ± 45^{abc}	197 ± 16^{a}	223 ± 56^{b}	200 ± 53^{c}	238 ± 56
Zinc					
C	115 ± 17	113 ± 22	107 ± 11	121 ± 18	106 ± 12
AG	109 ± 9	120 ± 12	106 ± 18	115 ± 16	112 ± 11
Copper					
C	5.2 ± 1.8^{a}	4.2 ± 1.0^{be}	3.0 ± 0.8^{abcd}	7.5 ± 2.6^{ce}	5.6 ± 1.5^{d}
AG	5.0 ± 1.2^{a}	4.2 ± 0.9^{b}	3.2 ± 0.9^{ac}	*4.2 ± 0.9^{d}	5.5 ± 1.0^{bcd}
Selenium					
C	3.6 ± 0.4^{abc}	4.3 ± 0.5^{a}	4.1 ± 0.3^{b}	4.5 ± 0.6^{c}	4.2 ± 0.7
AG	*4.1 ± 0.3^{a}	4.7 ± 0.3^{ab}	3.9 ± 0.3^{bc}	4.3 ± 0.5	4.7 ± 0.7^{c}
Cadmium					
C	5.0 ± 1.7	6.6 ± 1.3	6.7 ± 2.6	6.4 ± 3.3	5.8 ± 1.4
AG	5.6 ± 1.5	6.8 ± 1.7	6.8 ± 2.4	5.6 ± 0.5^{a}	7.3 ± 1.6^{a}

Data are shown as mean (mg/kg dry weight) ± SD (n = 5); C = Control, AG = Antigen injected fish; Common superscript letters within one line indicate significant differences ($p < 0.05$); Antigen injected fish significantly different ($p < 0.05$) from uninjected fish given the same diet marked by *.

Table 3. The concentration of trace elements in vertebrae of Atlantic salmon given different dietary levels and forms of vitamin C and after antigen injection

Treatment	Dietary vitamin C (mg/kg ascorbic acid, AA, or equivalent ascorbate-2-sulfate, AS)				
	0	500 AA	500 AS	5000 AA	5000 AS
Iron					
C	23 ± 10	23 ± 3	22 ± 3	25 ± 14	25 ± 4
AG	21 ± 7^{a}	27 ± 9	29 ± 8	23 ± 2^{b}	32 ± 11^{ab}
Zinc					
C	155 ± 33^{a}	149 ± 29^{b}	222 ± 35^{abcd}	171 ± 37^{c}	151 ± 33^{d}
AG	180 ± 38	169 ± 30	198 ± 52	130 ± 74	153 ± 14
Copper					
C	4.1 ± 1.5	4.3 ± 2.6	3.5 ± 1.3	3.4 ± 1.2	2.9 ± 0.7
AG	3.6 ± 0.9^{ab}	3.6 ± 1.4^{cd}	*2.2 ± 0.5^{ace}	2.4 ± 0.3^{bdf}	3.5 ± 1.2^{ef}
Selenium					
C	0.37 ± 0.08^{a}	0.26 ± 0.08^{ab}	0.40 ± 0.04^{bc}	0.28 ± 0.13^{c}	0.37 ± 0.11
AG	0.40 ± 0.19	0.28 ± 0.07^{a}	0.44 ± 0.10^{ab}	0.20 ± 0.17^{bc}	0.36 ± 0.07^{c}
Cadmium					
C	0.07 ± 0.03	0.09 ± 0.05	0.07 ± 0.02	0.08 ± 0.03	0.07 ± 0.03
AG	0.06 ± 0.01^{abc}	0.08 ± 0.02^{a}	*0.11 ± 0.04^{b}	0.08 ± 0.01^{c}	0.11 ± 0.09

Data are shown as mean (mg/kg dry weight) ± SD (n = 5); C = Control, AG = Antigen injected fish; Common superscript letters within one line indicate significant differences ($p < 0.05$); Antigen injected fish significantly different ($p < 0.05$) from uninjected fish given the same diet marked by *.

Table 4. Spleen weight, spleen somatic index, iron and zinc concentrations and total content of iron and zinc in Atlantic salmon given different dietary levels and forms of vitamin C and after antigen injection

	Dietary vitamin C (mg/kg ascorbic acid, AA, or equivalent ascorbate-2-sulfate, AS)				
Treatment	0	500 AA	500 AS	5000 AA	5000 AS
Mean spleen weight (mg)					
C	13.5	23.2	24.7	29.9	25.0
AG	34.8	17.1	30.4	40.8	33.9
Spleen somatic index					
C	0.042	0.062	0.067	0.079	0.068
AG	0.099	0.062	0.105	0.126	0.093
Iron concentration (mg/kg)					
C	510	264	449	245	271
AG	569	198	384	349	428
Total iron content (μg)					
C	6.9	6.1	11.1	7.3	6.8
AG	19.8	3.4	11.8	14.2	14.5
Zinc concentrations (mg/kg)					
C	36.7	28.3	26.2	26.1	34.3
AG	26.8	31.5	23.5	24.5	25.0
Total zinc content (μg)					
C	0.50	0.65	0.65	0.78	0.86
AG	0.93	0.53	0.71	1.00	0.85

Represents a mean of two pooled samples from five fish each; C = Control, AG = Antigen injected fish.

The concentration of cadmium was higher than the concentrations of copper and selenium in the kidney while the opposite was found in the liver and in the vertebrae.

The spleen somatic index (spleen weight as % of fish weight) showed a general increase in response to antigen stimulation (Table 4). A concomitant increase in the total content of iron was found in all groups except in fish fed the 500 AA diet.

Discussion

The concentration of the essential elements zinc, iron, copper and selenium in the practical diet used were found sufficient to meet the reported dietary requirements (Lall 1989), but the zinc concentration was lower than found in commercial salmon diets (Maage *et al.* 1989). The concentration of cadmium in the diet was in the same range as found in commercial salmon feeds (Maage 1990). In the present study the most significant effects of the test parameters were the accumulation of iron in the liver of fish fed no dietary vitamin C and the increase in the liver concentrations of copper, zinc, cadmium and metallothionein following stimulation of the humoral immune system.

Effects of vitamin C

It is generally assumed that vitamin C has a positive effect on the absorption of iron from the gastrointestinal tract. Further, it has been shown that this vitamin plays an important role in the release of ferritin-bound iron from the liver as well as in transfer of plasma iron to the liver and its incorporation into ferritin (Mazur 1960). Ferritin is a protein which stores cellular iron reversibly as ferric hydroxide or phosphate crystals. Excess ferritin is catabolized by lysosomal autophagy, which yields end-state hemosiderin complexes in intracellular

residual bodies. According to Bridges (1987) the role of AA is to inhibit the step of lysosomal autophagy. In the present study, iron accumulated in the liver, spleen and also to some extent in the kidney of the vitamin C-deficient fish. These fish also became anemic (Sandnes *et al.* 1990) which indicates that the hepatic iron was unavailable for utilization, probably because the iron was bound to hemosiderin. This theory is supported by the findings of Banerjee and Chakvabarty (1965) who found a decline in ferritin iron and a concomitant increase in hemosiderin in scorbutic guinea pigs. On the other hand, Hilton *et al.* (1978) reported a similar pattern in the spleen, but found an increase in the hepatic iron concentration with increasing dietary AA levels in rainbow trout.

In a study on Japanese quail (*Coturnix coturnix Japonica*), Fox *et al.* (1980) reported a protective effect of AA on cadmium toxicity by lowering the tissue concentration of cadmium in liver and kidney. This was also shown to occur at low levels of dietary cadmium. In the present work, even high dietary vitamin C levels had no apparent effect on cadmium levels of either the liver or kidney, key organs in cadmium metabolism. Since diet is the major cadmium source in fish (Dallinger and Kautzky 1985; Harrison and Klaverkamp 1989), the findings of the study reported here suggest that the protective effect of vitamin C against cadmium accumulation in fish is absent at low dietary cadmium levels.

There were significantly lower levels of selenium both in liver and vertebrae of fish fed megadoses of AA but not of those fed megadoses of AS. One possible explanation is that AA in the gastrointestinal tract reduces selenium making the element less readily absorbable and thus less bioavailable.

Effect of antigen injection

The levels of hepatic metallothionein, zinc and cadmium were significantly elevated in response to the injection of NIP_{11}-LPH in Freunds adjuvant. There was no increase in the concentrations of these metals in kidney and vertebrae. The low content of copper, zinc and cadmium in the injected antigen solution renders it unlikely that the increase in hepatic metallothionein, zinc, copper and cadmium was caused by the metals in the injection mixture.

Metallothionein is a small cysteine-rich protein that is thought to play a role in normal metabolism of the essential elements zinc and copper, and to act as a detoxifier of cadmium (Webb 1979; Hamer 1986). In rats, increased metallothionein levels have been reported in response to the release of glucocorticoids during stress (Hager and Palmiter 1981) and independently of glucocorticoids in inflammatory responses (Sobocinski *et al.* 1981). The increase in hepatic metallothionein concentration in response to the vaccination in this experiment could probably be explained by physiological effects caused by the stimulation of the immune system which resemble an inflammatory response. Glucocorticoids were not measured, and thus, it is not known to what extent the vaccination affected stress reactions resulting in the release of these hormones. Hager and Palmiter (1981) found that stress-induced metallothionein synthesis in mice and rats took place primarily in the liver and that other organs were less affected. However, it is unlikely that the effects reported in the present study were caused by handling stress during the vaccination procedure as sampling took place as long as six weeks after injection.

The increased concentrations of zinc, cadmium and copper in the liver of antigen-treated fish could be related to increased metallothionein synthesis. The exact function of increased metallothionein concentration following stimulation of antibody production is not known, but the results point at important aspects of element metabolism in fish which need further studies. The content of metallothionein in liver tissue differed somewhat according to dietary treatment, but the present data do not indicate that vitamin C plays a specific role in metallothionein metabolism.

The elevated hepatic iron concentration due to vitamin C deficiency limits general use of hepatic iron concentration as an iron status indicator in fish. Further investigations of the effects of antigen injection on trace element metabolism are need to elucidate the modes of action and the extent to which the efficiency of vaccination and fish health in general are affected.

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