

ENZYMATIC HYDROLYSIS OF
ASCORBATE-2-MONOPHOSPHATE AND
ASCORBATE-2-SULPHATE *IN VITRO*, AND BIOACTIVITY OF
ASCORBATE-2-MONOPHOSPHATE IN
ATLANTIC SALMON (*SALMO SALAR*).

By

KJARTAN SANDNES and RUNE WAAGBØ

Institute of Nutrition, Directorate of Fisheries
P. O. Box 1900 N-5024 Bergen Norway

ABSTRACT

An *in vitro* study indicated that the phosphate moiety of Ca ascorbate-2-monophosphate (AP) is enzymatically hydrolyzed to ascorbic acid and phosphate in the gastrointestinal tract of Atlantic salmon (*Salmo salar*). A feeding experiment demonstrated significant vitamin C bioactivity of AP in this species, and by comparing the results with other studies it is suggested that the bioactivity of AP is equivalent to ascorbic acid (AA). The reported low vitamin C bioactivity of ascorbate-2-sulphate (AS) may probably be explained by lack of sulphatase activity in the gastrointestinal tract of the fish. It is suggested that fish have selective absorption mechanisms for AA, and that the derivatives tested are only absorbed after they have been hydrolysed in the intestine.

INTRODUCTION

Recent studies have shown that phosphate derivatives of ascorbic acid are more stable than free ascorbic acid (AA) in fish feeds, and possess vitamin C activity in aquatic animals. A polyphosphorylated form of AA, L-ascorbyl-2-polyphosphate, had vitamin C activity in channel catfish (*Ictalurus punctatus*) (Grant *et al.*, 1989; Robinson *et al.*, 1989; Wilson *et al.*, 1989), in fathead minnow (*Pimephales promelas*) and guppy (*Poecilia reticulata*) (Grant *et al.*, 1989) and in Atlantic salmon (*Salmo salar*) (Sandnes *et al.*, 1989). Ascorbate-2-phosphate was found to have vitamin C activity in catfish (Brandt *et al.*, 1985) and in shrimp (*Penaeus japonicus*) as reported by Shigueno and Itoh (1988), who demonstrated it to be stable i shrimp feed.

The first AA derivative tested as a vitamin C source for fish was ascorbate-

2-sulphate (AS). It was first detected in the brine shrimp (*Artemia salina*) and postulated to be a storage form of AA (Mead and Finamore, 1969; Bond *et al.*, 1972). Studies on AS in fish were undertaken by Halver and his group. In a series of reports AS was suggested to be a storage form of vitamin C and to be readily absorbed and equivalent to AA as a vitamin C source in rainbow trout (*Oncorhynchus mykiss*) (Halver *et al.*, 1975; Benitez and Halver, 1982; Tucker and Halver, 1984; 1986). Other studies indicated a lower bioactivity of AS than of AA in channel catfish (Murai *et al.*, 1978), tilapias (*Oreochromis niloticus*) (Soliman *et al.*, 1986) and in Atlantic salmon (Sandnes *et al.*, 1990).

In terrestrial animals AA appears to be absorbed by specific Na mediated transport processes from the intestine (Rose, 1981). Studies on the absorption mechanisms of AA in fish are not known, and data are scarce on how AS or phosphate derivatives of AA are affected by intestinal enzymes.

In order to acquire more knowledge on the absorption of these compounds in Atlantic salmon, the present *in vitro* experiment was conducted to study possible enzymatic hydrolysis of Ca ascorbate-2-monophosphate (AmP) and AS by intestinal extracts. A further aim was to estimate the bioactivity of AP by means of liver retention of AA during a feeding experiment. The bioactivity of AS as a vitamin C source in this species was reported previously (Sandnes *et al.*, 1990).

MATERIALS AND METHODS

In vitro study

The gastrointestinal tract was taken from Atlantic salmon of approx 250 g which had been fed a commercial dry diet. The pyloric caeca and the intestine (including the contents) from two fish were used for extract preparation. The pyloric caeca and the intestine were cut in small pieces, of which 5 g was extracted for ten minutes in 50 ml 0.2 M Na-acetate buffer, pH = 7, using a magnetic stirrer. After centrifugation for 20 minutes at 3000 rpm at 4 °C, 4 ml of the supernatant was incubated with AmP or AS in 94 ml acetate buffer at 10 °C using magnetic stirring. The initial substrate concentration in the assay medium was 20 µg AA equivalents/ml. Total volume of the incubation medium was 100 ml.

Ascorbate-2-monophosphate in the medium was analysed at different intervals according to a method described given by Showa Denko K.K. (personal communication) using an HPLC method with a NH₂ - silica gel column. Ascorbate-2-sulphate was analysed using an HPLC method as described by Schüep *et al.* (1989). Total protein was determined according to Sandnes *et al.* (1988).

Feeding experiment

Duplicate groups of Atlantic salmon weighing about 600 g were fed two experimental diets in 1,5 m x 1,5 m tanks for four weeks with 32 fish in each tank. The mean water temperature and salinity were 10,6 °C and 16 g/L, respectively, throughout the feeding period. The fish were fed by automatic feeders, and the amount of feed was increased daily according to fish size, fish growth and water temperature using conventional feed tables for this species.

The basal feed used was a practical diet with fish meal (Norse LT, 70 g/100 g diet), modified maize starch (Suprex, 16 g/100 g), capelin oil (Norsalmoil, 13 g/100 g), and vitamins (without AA) and minerals added according to NRC (1981). This diet was fed to one group of fish (group I) and compared to a group fed the basal feed supplemented with Ca ascorbate-2-monophosphate (F. Hoffmann La-Roche) equivalent to 500 mg AA per kg (group II). The experimental diets were cold pelleted (5 mm) and dried in the laboratory. Feed samples were analysed for AP.

Samples of fish were collected at start ($n = 8$) and at the end of the experiment (5 fish from each replicate tank). Fish weights and liver weights were recorded. Ascorbic acid was analysed in the liver by an automated fluorometric method described by Roy *et al.* (1976).

RESULTS AND DISCUSSION

In vitro experiment

The results demonstrated that AP was hydrolysed by the an extract prepared from the pyloric caeca and the intestine of Atlantic salmon (Table 1). Without

Table 1. *In vitro* hydrolysis of Ca ascorbate-2-monophosphate (AP) and Na ascorbate-2-sulphate (AS) by intestinal extracts from Atlantic salmon.

Substrate	Extract	Incubation time (min)	Remaining substrate (%)
AP	native	0	100
		180	97
		420	92
		1550	39
AP	boiled	0-1550	100
AP	without	0-1550	100
AS	native	0-1550	100
AS	boiled	0-1550	100
AS	without	0-1550	100

incubation with the extract, or using boiled extract, the concentration of AP in the medium was not reduced. These results indicate the presence of enzyme activity in the gastrointestinal tract able to split off the phosphate moiety of the AP molecule.

The presented data refer to an incubation assay at 10 °C, but tests were also run at 5 °C and 15 °C (data not shown). Slight differences in enzyme activities were seen, but the amount of substrate removed from the medium was not significantly different. A preliminary test incubation at pH = 6 using AmP as substrate was also carried out, but the results showed reduced phosphatase activity at this pH.

The concentration of protein in the enzyme extract used was 1.8 g/L. Thus 0.11 µg AmP (AA equivalents)/minute/mg protein was hydrolyzed in average during the course of incubation lasting about 26 hours (1550 min).

There was no hydrolysis of AS in the incubation medium (Table 1). This finding supports earlier studies (Sandnes *et al.*, 1990) which reported low bioactivity of AS as a vitamin C source in Atlantic salmon.

Several methods could be used for preparing the enzyme extract, of which a simple extraction using small cut fragments of the pyloric caeca and the intestine including the contents was applied in the present study. This approach was used in order to obtain an extract with the least possible contribution of intracellular enzymes with phosphatase activity, which would otherwise have been extracted if the tissues were homogenized. The extraction method applied, which is assumed to be more like the situation *in vivo*, predicted that hydrolysis of AP takes place in the intestinal cavity and on the brush border, and not intracellularly. By means of an *in vitro* assay using homogenized intestine samples from carp and rainbow trout as enzyme sources,

Table 2. Ascorbic acid (AA) in liver and head kidney (ug/g w.w, mean sd) of Atlantic salmon fed no dietary vitamin C (I) or Ca ascorbate-2-monophosphate (II) equivalent to 500 mg AA/kg diet for four weeks (n = 10).

	Group		
	At start n = 8	I n = 10	II n = 10
Liver	56 ± 8	27 ± 8	171 ± 25
Head kidney	-	<5	196 ± 17

Dabrowski (1990) demonstrated liberation of AA, and found that alkaline phosphatase activity did not correlate with phosphatase activity when AmP was the substrate.

In order to evaluate the vitamin C bioactivity of AP in Atlantic salmon, a feeding study was performed. The AA concentration in liver at start of the experiment (8 fish) was $56 \mu\text{g/g}$ w.w.. Fish fed without supplementation of any vitamin C form contained $27 \mu\text{g AA/g}$ in the liver (10 fish) after 4 weeks. Salmon fed the AmP supplemented diet (group II) showed an AA liver concentration of $171 \mu\text{g/g}$ at the end of the experiment. Head kidney AA concentrations were <5 (I) and $196 \mu\text{g/g}$ (II) at the end of the feeding period. The weight gains were 48% and 50% for the groups I and II during the course of the experiment, respectively. There were no macroscopic signs of fish disease or nutritional deficiency symptoms in any group.

Analysis of AmP in the supplemented feed (three replicates) showed a value of $503 \pm 7 \text{ mg/kg d.w.}$ after pelleting (AA equiv.). No AP could be detected in the unsupplemented feed.

The vitamin C status of salmonids can be evaluated by the concentration of AA in the liver (Hilton *et al.*, 1977; Sandnes, 1982). The ratio feed/liver with respect to AA equivalents may be useful in evaluating the bioactivity of vitamin C compounds, provided the fish is in a steady state with respect to vitamin status and kept under normal conditions. Experience with vitamin C studies has shown that long time is needed to deplete the liver AA reserves, especially in larger fish (Waagbø *et al.*, 1989). However, four weeks feeding of fish which is depleted or partly depleted of AA, with a diet which increases the liver AA concentration, is sufficient to obtain a steady state with regard to AA status (Sandnes, 1982). In the present study a feed/liver ratio with respect to AA equivalents showed a value of 2.9 (group II). In a previous study (Sandnes *et al.*, 1990) values of 3.0 and 18.5 were found for AA and AS, respectively, in Atlantic salmon smolt fed the same dietary levels as in the present experiment. No comparable data on other forms of vitamin C are known for this species.

Halver *et al.* (1975) suggested that AA in the head kidney reflects the vitamin C status in rainbow trout, but in channel catfish (*Ictalurus punctatus*) no correlations between dietary AA and AA in the head kidney were found (Lim and Lovell, 1978). The present results demonstrate that the head kidney AA concentration is affected by dietary AmP in Atlantic salmon.

In summary, the present *in vitro* study indicates that AmP is enzymatically hydrolyzed to AA and phosphate in the intestine of Atlantic salmon, and that the bioactivity of AmP in this species seems to be equivalent to AA. The low bioactivity of AS reported (Sandnes *et al.*, 1990) may be explained by lack of sulphatase activity in the gastrointestinal tract of the fish.

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