Ascorbyl-2-Sulfate Compared with Ascorbic Acid in Atlantic Salmon: Uptake and Distribution Confirmed by Mass Spectroscopy (44135)

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Abstract. **This paper reports an in-depth approach that identifies ascorbyl-2-sulfate (AS) in gastric, blood, liver, and muscle tissues of Atlantic salmon (*Salmo salar*). To insure the identity of the AS, the study utilized the latest high-performance liquid chromatography (HPLC) technology plus electron ionization mass spectrometry (EIMS). Just before saltwater adaptation stage, juvenile Atlantic salmon were force-fed AS, ascorbic acid (AA), and a molecular-equivalent combination of the two. After tissue analyses for AA and AS were performed by HPLC separation, the HPLC peaks were identified by EIMS.**

The data collected in this study indicate that Atlantic salmon can absorb AS through the gastric tissue when forced-fed AA and AS as described. The data also indicate that AS is transported through the blood to the liver. There is evidence to indicate that AS is converted to AA in the livers of these salmon. In addition, the muscle tissue contained a large portion of AA and AS. [P.S.E.B.M. 1997, Vol 215]

In recent years, many scientists have explored the uptake and utilization of ascorbyl-2-sulfate (AS) as a dietary source of ascorbic acid (AA) in salmonids (1–6). Their findings seem to run the gamut. Some observed no uptake of AS in Atlantic salmon, while some found small uptake, and others, considerable uptake and distribution.

McLaren *et al.* (7) have been credited with first establishing the dietary requirement of AA in rainbow trout. In 1969, Halver et al. (8) established AA requirements for coho salmon, and in 1984 Tucker and Halver (9) reported the distribution of AS in rainbow trout. Since these earlier papers there have been numerous reports on the subject of AS in salmonids and other fish. Sandnes *et al.* (3) reported that they could not detect AS in the livers of Atlantic salmon that had been fed a diet using AS as the ascorbate source. Dabrowski *et al.* (10) suggested that released ascorbic acid in AS preparations might be sufficient "2–4%" to supply fish with needed AA for growth and development. Halver *et al.* (5) reported no differences in growth rates after 7-month feeding trials in rainbow trout fed 100 mg/k AA compared with fish fed 100-, 200-, and 300-mg/k amounts of AS.

In some cases, these reports have not confirmed one another's findings. A possible explanation for these diverse observations could be the diversity of methodologies employed in the analyses. Some investigators have used less direct methods for their analyses—that is, conversion of AS to AA by chemical hydrolysis and the determination of AA by colorimetry (11). The reduction of the dye was followed spectrophotometrically. Such an assay method is dependent upon screening out all reducing substances other than ascorbic acid as well as a complete hydrolysis of the AS. Dabrowski *et al.* (10) compared two chemical assays with two high-performance liquid chromatography (HPLC) assays. The HPLC assays utilized two different detectors—ultraviolet (UV) and electrochemical (EC), the EC being more specific for AA. It was concluded from this comparison that the HPLC was specific and the chemical assays were not specific.

There have been some problems with using an HPLC to separate ascorbic acid from ascorbic acid esters. Mainly,

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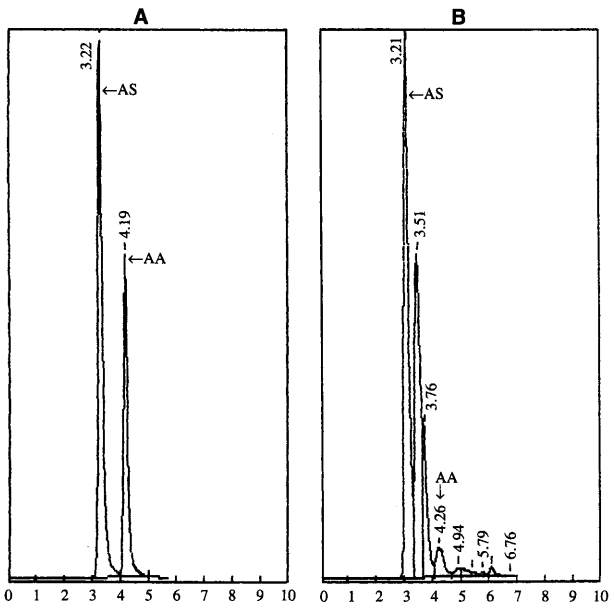

Figure 1. (A) A typical HPLC profile of standards ascorbate-2-sulfate and ascorbic acid eluted with the 0.1 *M* ammonium acetate, pH 5.0, mobile phase. Concentrations are 1 µg and 0.5 µg with retention times (RT) of 3.2 and 4.16 min, respectively. The *y* axis scale is 0–200 mV. (B) A HPLC profile of a 5% TCA extract of a well-washed gut tissue excised from a fish force-fed AS with a post-feeding time of 24 hr. Note the small amount of AA which may have been formed in the gut tissue at RT 4.26 min. Same mobile phase as in Panel A. The *y* axis scale is from 0–200 mV.

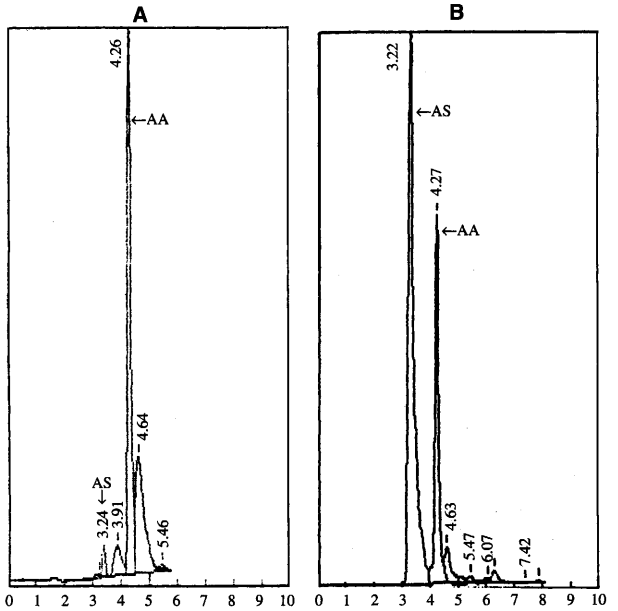

Figure 2. (A) A typical HPLC profile of a 5% TCA extract of blood sampled from a fish force-fed AA with a post-feeding time of 24 hr. Retention time (RT) of AA is 4.26 min. Same mobile phase as Figure 1. The *y* axis scale is 0–200 mV. (B) A typical HPLC profile of a 5% TCA extract of liver tissue from a fish at a post-feeding time of 24 hr. Fish was force-fed AS and AA in equivalent amounts. Note large peaks at a RT of 3.22 min and at RT of 4.27 min, compared with Figure 1A for the standards, indicating no competition for gastric transport. Mobile phase same as in Figure 1. The *y* axis scale is 0–200 mV.

these problems have come from the use of ion-pairing reagents in the mobile phase. Differing concentrations of ion-pairing reagents can produce different retention times for AS and AA (12), introducing yet another variable.

Investigators in this laboratory recently developed a method utilizing a new type of reverse-phase column which does not require the use of ion-pairing reagents (13). The method used an Altima C18 column developed by Alltech and Associates. The column used an ammonium acetate mobile phase which gave a fortuitous separation of AS and AA. The AS fraction elution, which preceded that of the AA, eluted immediately after the solvent front. The method used a UV monitor with an in-tandem EC detector. UV sensitivity of the assay was 58 pmol for AA and AS. Using this assay technique has allowed for a straightforward collection of AS and AA fractions for electron ionization mass spectrometry (EIMS).

This paper reports the next step in pursuing the hypothesis that AS is transported across the gastric membrane as an intact molecule, and through the blood stream to the liver, where it is reportedly converted to useful ascorbic acid (14). The gastric membrane transport was documented in 1995 (6). The current study was designed to ascertain if AS, and/or converted AS, would be deposited in the muscle tissue. Another goal of this study was to test whether or not ascorbic acid and ascorbyl-2-sulfate would compete with one another for transport at the gastric membrane level.

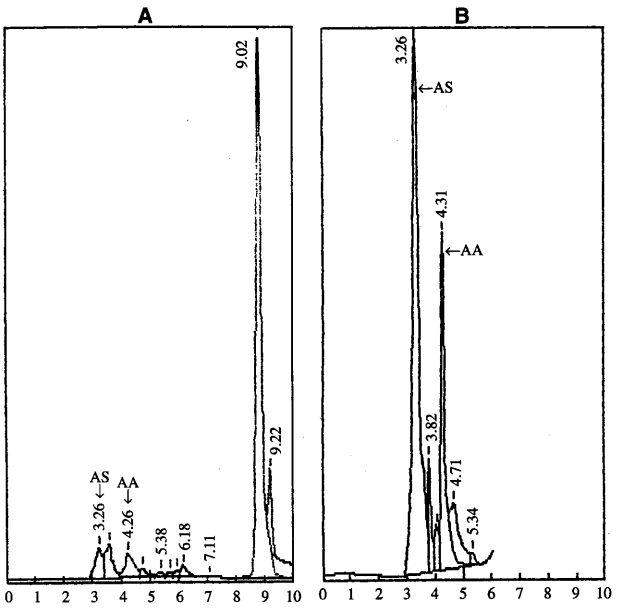

Figure 3. (A) This HPLC profile, taken from a fish force-fed AS only, shows a 5% TCA extract of muscle tissue at a post-feeding time of 48 hr. Note the presence of AS and AA at RTs of 3.26 and 4.26 min, respectively. Mobile phase same as in Figure 1. The *y* axis scale is 0–200 mV. (B) This HPLC profile is of a 5% TCA extract of muscle tissue from fish force-fed equivalent amounts of ascorbic acid in the form of AS and AA (24 hr post feeding). Mobile phase same as in Figure 1. The *y* axis scale is 0–200 mV.

Table I. Tissue Concentrations of AS and AA in Control Fish after 6 Weeks on a Abernathy Diet Containing AS as the Dietary Source of Ascorbic Acid

	AS control fish			AA control fish		
	Wet wt	AS ($\mu g\ g^{-1}$)	AA ($\mu g\ g^{-1}$)	Wet wt	AS ($\mu g\ g^{-1}$)	AA ($\mu g\ g^{-1}$)
Fish 1						
Gut	1.69	1.78	33.84	0.304	—	—
Blood	0.5	2.93	—	0.312	—	94.55
Liver	0.588	22.29	143.62	0.48	—	233.02
Muscle	1.87	—	36.73	1	—	21.93
Fish 2						
Gut	2.69	0.73	46.99	0.476	—	8.22
Blood	1.04	—	3.17	0.262	Trace	—
Liver	0.48	15.35	47.82	0.362	—	164.99
Muscle	1	—	18.73	1.42	—	38.77

Note. On the right are comparisons of AS and AA in tissues of AA diet control fish under similar conditions. In both control groups the fish were starved 7 days prior to beginning the force-feeding experiment.

Table II. The Concentrations of AS and AA Found in Atlantic Salmon Tissues 24 hr after the Force-Feeding with AS

AA found in AS-dosed fish in 24 hr				AS found in AS-dosed fish in 24 hr			
Gut	Blood	Liver	Muscle	Gut	Blood	Liver	Muscle
0	0	118.89	16.29	60.76	94.06	626.37	74.55
6.51	9.24	149.29	20.68	51.57	294.7	621.36	121.39
0	10.17	112.39	27.72	140.51	159.73	912.08	94.96
5.19	4.51	58.67	23.26	127.71	239.21	1146.2	125.25
0	0	37.03	0.00	167.98	1010.5	1919.11	225.2
0	0	40.75	16.73	80.53	237.54	1006.56	77.72
18.14	0	137.37	34.26	430.95	360.21	2085.46	176.59
0	10.52	105.01	0	171.25	644.45	1441.79	170.15
0.00	0	57.38	46.69	56.93	745.45	2364.99	258.58
35.66	0	83.63	37.41	111.25	183.26	818.28	110.44
Mean							
6.55	3.44	90.04	22.30	139.94	396.91	1294.22	143.48
SD							
11.74	4.73	40.33	15.13	111.52	300.91	629.01	62.43

Note. Concentrations are expressed as $\mu g\ g^{-1}$ of wet tissue wt.

Materials and Methods

Juvenile Atlantic salmon were secured from Global Aqua Co. (Bainbridge Island, WA). The fish averaged about 60–80 g and were still residing in freshwater.

Chemicals and Equipment. L-Ascorbic acid 2-sulfate dipotassium dihydrate (Astos) was furnished by Pfizer Food Science Group. Ascorbic acid was obtained from Sigma Chemical Co. (Saint Louis, MO). Reagent grade glacial acetic acid ammonia hydroxide and trichloroacetic acid were purchased from J. T. Baker Inc. (Phillipsburg, NJ). Tricaine methanesulfonate (MS 222) was supplied by Argent Chemical Co. (Redmond, WA). The 25-mm 0.45-micron syringe filters were obtained from Alltech Associates (Deerfield, II).

The solvent system for the mobile phase was made as follows: a 0.1 *M* acetic acid solution was titrated topH 5.0 with isothermally distilled concentrated ammonium hydroxide.

The following equipment was used for the study: a Perkin-Elmer (PE) Model 250 binary LC pump with a P&E Model LC 290 UV/Vis detector set at 254 nm, linked in tandem to a Bioanalytical Systems Inc. (BAS) electrochemical detector set at +0.72 V. The data system was linked to a PE Nelson 950 interface and an Epson III+ computer. The software used was the PE Nelson 2100.

Mass Spectroscopy. The mass spectroscopy was performed and confirmed by two different departments, Biochemistry and Medical Chemistry, at the University of Washington. Two different instruments were employed: first, a liquid chromatograph/mass spectrograph (LC/MS),

Table III. The Concentrations of AS and AA Found in Atlantic Salmon Tissues 48 hr after the Force-Feeding with AS

AA found in AS-dosed fish in 48 hr				AS found in AS-dosed fish in 48 hr			
Gut	Blood	Liver	Muscle	Gut	Blood	Liver	Muscle
1.31	7.79	103.52	109.76	18.03	75.3	231.12	76.22
0	0	149.38	23.6	41.33	186.38	285.71	88.14
4.33	0	178.5	37.63	44.29	159.1	1039.79	126.21
12.58	0	65.13	30.66	571.58	62.91	511.07	16.57
0	5.29	91.67	28.95	179.26	331.77	652.13	73.38
6.37	9.72	206.24	28.49	23.23	233.49	743.33	54.02
13.23	11.46	113.04	51.22	776.76	75.18	1411.08	82.82
40.86	0	95.77	18.96	72.28	285.19	1165.96	126.65
48.27	0	103.15	0	350.15	106.75	1287.46	175.33
28.67	0	104.73	27.94	771.18	244.3	1425.41	231.32
Mean							
15.56	3.43	121.11	35.72	284.81	176.04	875.31	105.07
SD							
17.62	4.68	43.39	29.09	312.36	95.71	451.36	62.22

Note. Concentrations are expressed as μg g^{-1} wet tissue wt.

Table IV. The Concentrations of AS and AA Found in Atlantic Salmon Tissues 24 hr after the Force-Feeding with AA

AA found in AA-dosed fish in 24 hr				AS found in AA-dosed fish in 24 hr			
Gut	Blood	Liver	Muscle	Gut	Blood	Liver	Muscle
70.87	5.83	284	36.68	0		0	0
124.47	6.11	148.42	41.24	0	1.1	4.25	0
160.55	123.96	573.88	21.27	0	0.58	0	0
401.38	55.23	305.68	71.51	0	3.87	64.08	0
42.36	8.16	530.9	37.6	0.92		0	0
103.84	49.45	639.71	96.36	0	0.55	0	0
157.06	565.11	1030.05	263.59	0		0	0
308.2	0	37.33	83.6	0.35	8.1	12.56	0
4.69	4.21	215.37	63.31	0.74	1.57	15.77	0
264.2	4.58	517.97	73.02	0		0	0
Mean							
163.76	82.26	428.33	78.82	0.20	2.63	9.67	0
SD							
125.29	174.07	290.39	69.12	0.35	2.95	19.99	

Note. Concentrations are expressed as μg g^{-1} wet tissue wt.

MEGA Flow Electrospray/VG QUATTRO II Triple Quadrupole (Micro Mass, VG Fisons, Alprincham, Manchester, United Kingdom); and, second, a triple-quadrupole Sciex API III instrument (Perkin-Elmer/Sciex, Thornhill, Ontario, Canada). Instruments were used in the following modes: mass spectroscopy (MS), mass spectroscopy/mass spectroscopy (MS/MS), multiple reaction monitoring (MRM), and centroid mode. MS and MS/MS were described previously (6). The MRM mode was used because of its specificity. The first quadrupole (Q1) was set to pass only ions of *m/z* 255.5 (ascrobyl-2-sulfate) through to Q2. Q3 was set to scan only ions of *m/z* 175.5, ascorbic acid, the daughter ion of AS. Thus, only those ions of *m/z* 255.5 that yielded a fragment ion at *m/z* 175.5 would produce a trace. Q1/MS scan was from 100 Daltons over 2 sec in centroid mode. Flow injection analysis was by 5 μl loop injections. Selected mass chromatogram of *m/z* 175.5 was used to locate the ion. Combined spectra, with subtracted background, was used over the located peak to obtain mass spectrum.

HPLC Conditions. The HPLC conditions and equipment were as previously described (13). Briefly, the analytical column used was an Altima (Alltech Associates) C_{18} reverse phase, 5 μm, 250 × 4.6 mm, with a hand-packed Altima guard column. Flow rate was not allowed to exceed 1.0 ml/min.

Fish Housing and Transport. When fish were transported to the University of Washington School of Fisheries Laboratory, tank water was cooled with ice and aerated with medical oxygen. Fish were housed in lucite tanks, 15 × 15 × 30 in. at a density of five fish per tank, with a water

Table V. The Concentrations of AS and AA Found in Atlantic Salmon Tissues 48 hr after the Force-Feeding with AA

AA found in AA-dosed fish in 48 hr				AS found in AA-dosed fish in 48 hr			
Gut	Blood	Liver	Muscle	Gut	Blood	Liver	Muscle
58.87	280.49	56.87	164.8	0	0.54	0	0
139.58	5.01	139.58	23.66	0	0.34	0	0
45.71	1.73	45.71	42.94	0	0.19	0	0
10.68	19.04	223.53	90.61	0	0.43	0	0
248.73	3.95	471.63	39.18	0	0	0	0
135.41	3.15	522.93	46.8	0	0	40.8	0
595.25	4.72	288.63	64.6	0	0.58	0	0
462.06	4.36	167.19	74.44	0	0	8.82	0
269.89	6.53	394.63	67.34	0	2.39	11.37	0
194.2	4.68	345.37	45.28	0	0	0	0
Mean							
215.84	33.37	393.83	65.97	0	0.447	6.10	
SD							
187.86	86.96	190.05	39.84		0.721	12.91	

Note. Concentrations are expressed as μg g^{-1} wet tissue wt.

Table VI. The Concentrations of AS and AA Found in Atlantic Salmon Tissues 24 hr after Force-Feeding a Combination of AS and AA

AA found in AA/AS-dosed fish in 24 hr				AS found in AA/AS-dosed fish in 24 hr			
Gut	Blood	Liver	Muscle	Gut	Blood	Liver	Muscle
325.08	26.66	692.4	65.26	219.1	314.7	1235.54	68.72
180.55	20.08	941.1	66.5	272.23	651.85	1529.71	219.64
221.79	232.97	768.61	242.44	357.56	936	1181.69	237.77
44.49	35.78	1197.88	37.12	255.59	803.03	1156.99	314.71
224.78	6.97	600.97	45.87	180.53	345.68	1131.54	160.39
267.41	41.3	763.42	49.28	343.93	440.37	1601.58	190.78
521.09	7.2	173.42	46.93	33.04	76.47	651.76	190.9
108.3	2.4	386.77	43.85	170.12	112.91	944.24	91.58
84.68	50.79	1011.59	102.33	216.55	629.43	970.08	255.09
109.29	6.45	575.96	35.02	105.81	124.49	1153.11	90.23
Mean							
208.75	43.06	711.21	73.46	215.45	249.27	1155.62	181.98
SD							
140.68	68.77	300.23	62.56	95.33	302.17	274.76	79.95

Note. Concentrations are expressed as μg g^{-1} wet tissue wt.

source of chilled dechloronated city water. Temperature was held at 55°C.

Fish Force-Feeding. The Atlantic salmon had been reared on an Ewos (Vancouver, Canada) diet with "Stay C," as the ascorbic acid source. After travel, the fish were not fed for a period of 2 weeks, after which they were placed on an Abernathy diet with AS as the vitamin C source (150mg/k dry wt). The Atlantic salmon were fed this diet for 6 weeks before the start of the force-feeding experiment. The fish were then starved again for another 7 days before the actual force-feeding experiment began.

Each Atlantic salmon juvenile (weighing about 60–80 g) was force-fed AS and AA in the following manner: the fish was anaesthetized with MS-222 until it could be handled without struggling; then a polished-end glass tube (3.5 mm i.d.) was inserted through the mouth and esophagus into the stomach. A No. 5 gelatin capsule was filled with the appropriate amount and form of ascorbic acid plus serum albumin. The capsule was then forced through the glass tube with a small polished glass rod. The tube was removed, and the fish was returned to the tank. In order to insure that the capsule was retained, the fish was observed for 2 min.

Tissue Collection and Preparation. Sixty fish were separated into six groups. Groups 1 and 2 were fed AS. Groups 3 and 4 were fed AA. Groups 5 and 6 were fed both AS and AA. Groups 1, 3, and 5 were sacrificed at 24 hr post feeding. Groups 2, 4, and 6 were sacrificed at 48 hr post feeding. There were two control groups of two fish each—Group 7, which was fed AS, and Group 8, which was fed AA. The following tissues were removed: stomach tissue

Table VII. The Concentrations of AS and AA Found in Atlantic Salmon Tissues 48 hr after Force-Feeding a Combination of AS and AA

AA found in AA/AS-dosed fish in 48 hr				AS found in AA/AS-dosed fish in 48 hr			
Gut	Blood	Liver	Muscle	Gut	Blood	Liver	Muscle
10.27	0	278.45	14.11	48.01	115.93	1496.68	50.46
242.33	0	209.05	31.8	105.03	87.89	779.98	50.87
263.94	0	51.76	90.44	30.33	36.72	709.9	114.99
429.48	5.33	488.38	44.82	110.63	71.97	559.56	104.92
345.08	0	288.98	59.32	149.47	48.74	492.66	19.84
20.8	4.47	486.14	30.73	112.57	100.14	674.77	91.66
46.28	8.92	556.13	42.67	48.07	519.58	624.72	98.13
210.18	0	319.98	61.52	68.37	133.79	1147.02	25.88
242.93	7.12	334.29	20.34	391.07	31.68	364.43	9.73
483.2	6.7	854.01	38.42	457.17	510.02	1721.24	139.37
Mean							
229.45	6.51	386.72	43.42	152.07	165.65	857.10	70.59
SD							
164.93	3.62	220.99	22.38	148.76	186.99	449.54	44.91

Note. Concentrations are expressed as $\mu g\ g^{-1}$.

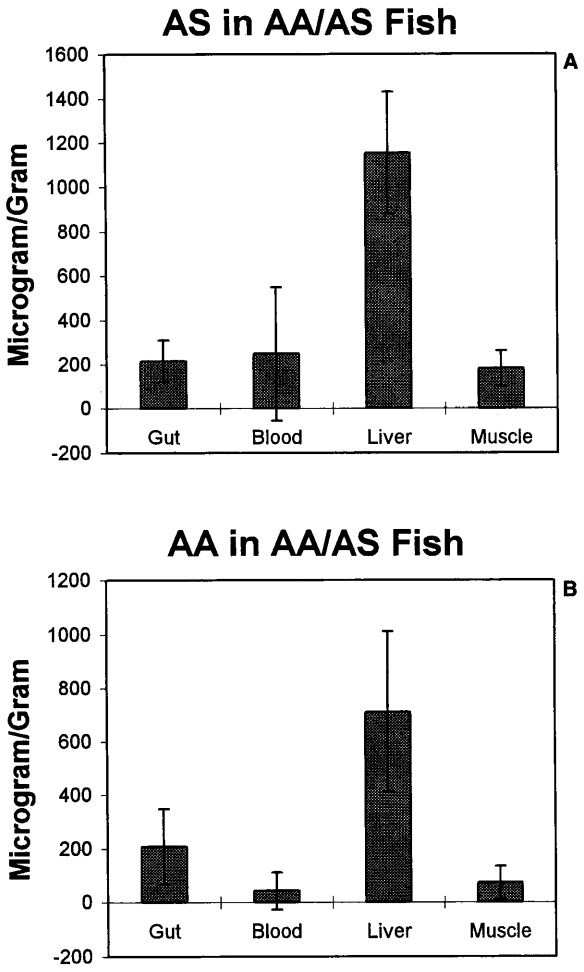

Figure 4. An illustration of the means and standard deviation of AS (A) and AA (B) found at 24 hr in tissues of fish force-fed AS/AA simultaneously.

from the esophagus to the intestine, the whole liver, blood collected *via* a caudal vein excision and a filet of muscle from just anterior of the adipose fin along the lateral line to the tail fin. The stomach was opened and rinsed 10 times with 10 volumes of glass distilled water, with the last rinse saved for any residual ascorbate analysis.

Tissue extracts were made as follows: The 5% trichloroacetic (TCA) extract was made by homogenizing one volume of tissue to four volumes of cold glass distilled water for 30 sec. While homogenizing the aqueous extract, another five volumes of 10% TCA were added, and homogenizing continued at top speed for an additional 45 sec. The homogenate was centrifuged at top speed in the microfuge for 4 min, decanted and filtered with the syringe filter. The filtrate was now ready for the HPLC.

Results and Discussion

As mentioned earlier, with this method the AS is the first to elute after the solvent front at a retention time (RT) of 3.2 min. AA elutes later, at RT of 4.15–4.2 min, Figure 1A shows a typical HPLC profile of AS and AA standards. It was fortuitous to have the AS elute first, making for a cleaner eluate to be run on the EIMS. When the AA peak elutes later, it creates a less pure eluate for the EIMS verification. However, the AA peak is also verified by the EC detector at a RT of 0.2–0.25 min after the AA peak appears on the UV detector. In Figure 1B it is noteworthy to point out the presence of AA in the gastric tissue extract from a fish force-fed AS only. This presence of AA might indicate some conversion of AS to AA is occurring in the gastric membrane. Also, it is interesting to note the converse in Figure 2A, where there is evidence of AS in the blood sampled from a fish force-fed AA only—findings that indicate some conversions of AS to AA and vice versa. Figure 2B clearly demonstrates high concentrations of AS and AA extracted from liver tissue of fish 24 hr post force-feeding of

Table VIII. A Summary of the Amounts of AS and AA Recovered in the Various Tissues (Including Total Eviscerated Carcasses) and Assuming a Bodily Volume of Blood at 5% of Body Weight

Treatment	Mean avg wt	Total AS absorbed (mg)	Total AA absorbed (mg)	Amount forced-fed (mg)	% AS recovered	% AA recovered	% total ascorbate conv. to AS or AA
AS uptake 24 hr	79.22 ± 13.54	12.6 ± 4.95	NC	45	28.0 ± 11.0		9.0 ± 4.0 to AA
AS uptake 48 hr	77.31 ± 19.06	8.6 ± 3.6	NC	45	19.0 ± 8.0		13.0 ± 9.0 to AA
AA uptake 24 hr	74.3 ± 16.4	NC	5.6 ± 3.3	25.5		22.0 ± 13.0	Trace of AS
AA uptake 48 hr	67.07 ± 13.45	NC	4.33 ± 2.04	25.5		17.0 ± 8.0	Trace of AS
AA/AS uptake 24 hr	67.56 ± 9.07	14.4 ± 4.4	6.0 ± 3.4	20 AA 40 AS	36.0 ± 11.0	30.0 ± 17.0	NC
AA/AS uptake 48 hr	62.11 ± 7.79	5.2 ± 2.4	3.0 ± 1.4	20 AA 40 AS	13.0 ± 6.0	15.0 ± 7.0	NC

Note. Weights are expressed in milligrams. Percent recovery is based upon no regurgitation of vitamin form. Last column is a percent conversion calculation of AS to AA (or AA to AS) in the fish force-fed AS and AA.

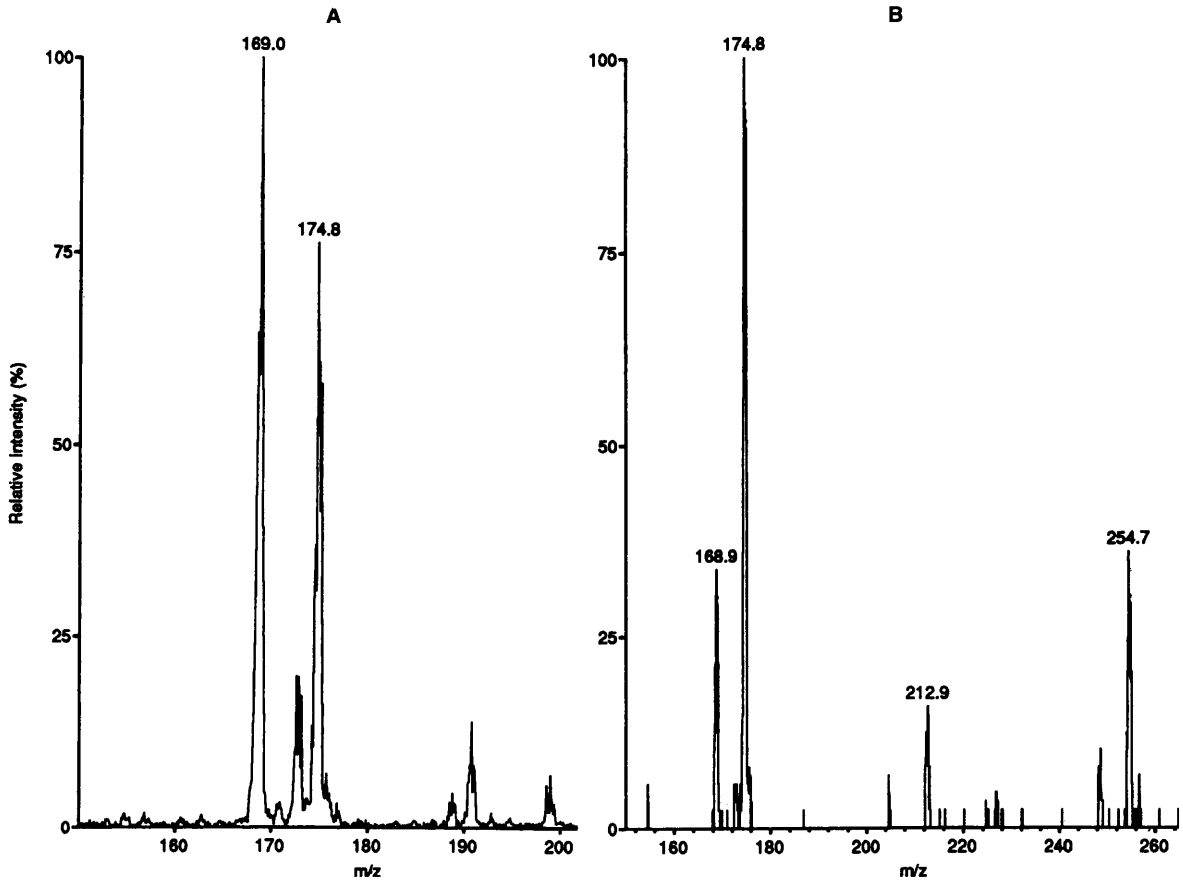

Figure 5. (A) An EIMS scan of standard ascorbic acid displaying a mass at 174.8 for AA and a fragmented mass at 169.0. (B) An EIMS scan of standard ascorbate-2-sulfate displaying a mass at 254.7 for AS, a mass of 174.8 of desulfated AS, and a fragmented mass of 168.9 for AA. *m/z,* mass to intensity.

molar-equivalent amounts of AS and AA. This figure shows that AA and AS did not compete with one another for transport at the gastric membrane level. Figure 3A shows the amounts of AS and AA found, 48 hr post feeding, in the muscle of fish force-fed AS only, another indication of AS conversion to AA. Figure 3B illustrates graphically the equivalent deposit of AS and AA in the muscle tissue of fish force-fed equivalent amounts of these forms of ascorbic acid. The lack of preferential absorption of one form over the other is observed with these equivalent concentrations of ascorbate found in the muscle tissue.

Table I shows the concentrations of AS and AA found in the gastric, blood, liver and muscle tissues of control fish. The AS control fish were on a diet containing 200 mg/k of Astos (AS) as the vitamin C source for 6 weeks. Both groups were starved 7 days prior to sacrifice and tissue

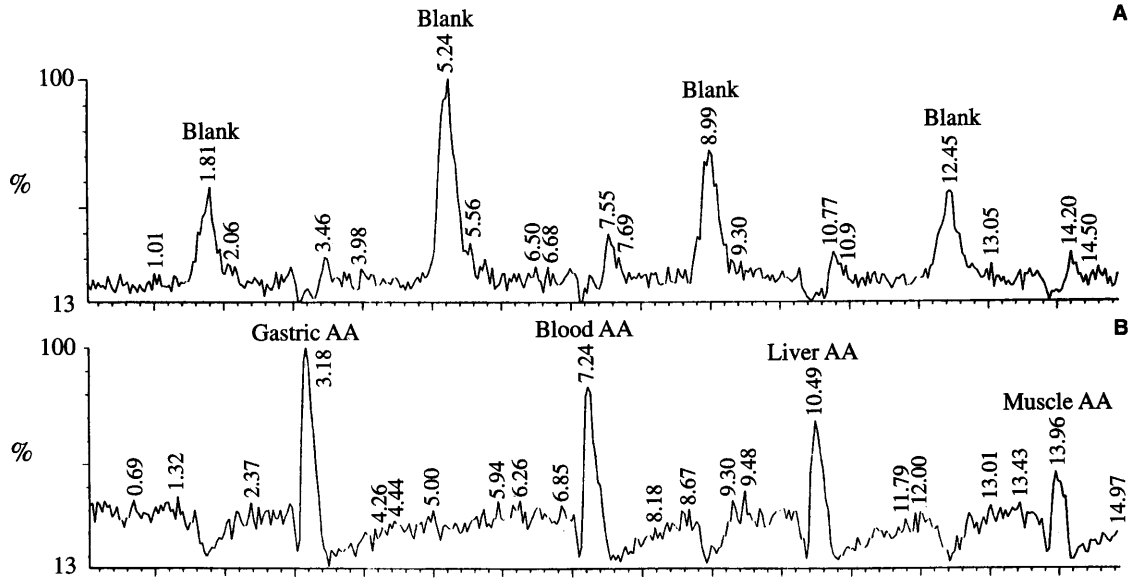

Figure 6. (A) Profile of blank solution injected into the mass spectrometer with the filter window set at a parent ion of 255.4 mass. The peaks at 1.81, 5.24, 8.99, and 12.45 are the injection peaks occurring due to ion contaminant. (B) Profile of peaks occurring due to injections of AA eluates from fish force-fed AA. There were no contaminating peaks with the filter window set at a parent ion of 175.5 mass. Peaks appearing at time periods 3.18, 7.24, 10.49, and 13.96 correspond to the AA found in the gastric, blood, liver, and muscle tissues of AA-fed fish.

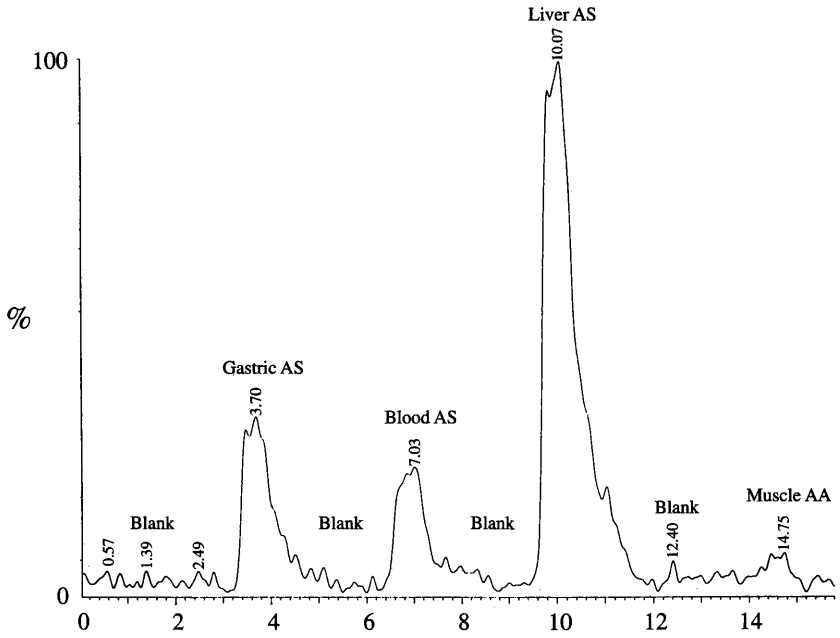

Figure 7. A multiple reaction monitoring (MRM) mode spectra of AS peaks eluted from HPLC runs of Atlantic salmon force-fed ascorbate-2-sulfate. Elutions were from gastric, blood, liver, and muscle tissue 5% TCA extracts separated on HPLC using the 0.1 *M* ammonium acetate, pH 5.0, mobile phase. Peaks at time periods of 3.70, 7.03, 10.07, and 14.75 represent parent ions *m/z* 255.5 which are fragmented to daughter ions *m/z* 175.5. Intermittent peaks are the result of blank solvent injections.

collection. The AA control fish were on the same Abernathy diet, but it contained 100 mg/k of AA as the vitamin C source. Analysis of tissues again points to evidence for conversion of AS to AA in the AS control fish. The finding of more AA in the tissues of these fish is not surprising in view of the starvation effect and lack of AS intake. Most of the AS has been converted to AA to serve the needs of the fish. Conversely, it is also important to note the absence of any AS in the tissues of the AA control fish. The lack of any AS in these fish could well reflect the effect of starvation and the moderate level of AA in the diet. Under these conditions it would have been surprising to find AS in the muscle because, according to earlier observations, excess AA is stored as AS (9).

Tables II–VII illustrate the concentrations of AS and AA found in selected tissues 24 and 48 hr post force-feeding. In Table II, it is again noted that there is evidence for AS to AA conversion even though there has been no AA in the force-feeding. Table III indicates a 34.5% increase in AS to AA conversion in the liver tissue and a 60% increase in muscle tissue. The variability among fish is great, as seen in the high standard deviation. This difference among fish may be due to differences in absorption among fish and/or partial regurgitation by some fish. When you examine the

same fish for AS uptake in Tables II and III, the same variability exists but with clear indication of high AS absorption. If you compare concentrations seen in the 24-hr period with the 48-hr period in the liver and muscle tissue, there is a concomitant decrease in AS with an increase of AA in these tissues. Conversions of AS to AA here seem to be indicated as well.

Tables IV and V represent the concentrations of AA and AS found in the selected tissues at 24 and 48 hr. There is little or no evidence that any sulfating has occurred in this time period. Small to trace amounts of AS are found in the gastric, blood, and liver tissue, which could indicate a beginning of sulfating even though no AS was found in the muscle at this time.

Tables VI and VII are the results of an experiment primarily designed to elucidate whether or not a synthetic ester of vitamin C would compete with the absorption of free ascorbic acid. The two forms of vitamin C were force-fed with AS in an amount representing an approximate molecular equivalent of AA. This translates on a weight basis to a ratio of about 2 to 1, AS to AA. The levels of each form found in the liver and muscle tissues reflect this same ratio. From this observation it appears that there is no competition for transport sites. Figure 4, A and B, summarizes Table VI, by showing the means and standard deviations as bar graphs. This finding is further substantiated in the uptake summary seen in Table VIII. Looking at the last two columns of the top row, you will notice that the uptake of AS did not change when fish were force-fed both AS and AA (as reported in the fifth row). In addition, it is interesting to note that there was more evidence of conversion of AS to AA than vice versa.

Panels A and B of Figure 5 are typical EIMS profiles of standards AA and AS respectively. In Figure 5A, ascorbic acid gives a major mass at 174.8 with a minor peak to the left at 172.5. The lesser peak represents dehydroascorbic acid which has formed in the aqueous standard solution of ascorbic acid. Dehydroascorbic is also found within the cell extracts, a similar find reported by Schorah (15). In Figure 5B, the major mass is as expected at 254.7 due to the additional sulfate moiety with a mass of 80. Also seen in Figure 5 is the fragmented daughter ion at 174.8 resulting from the loss of sulfate.

A different EIMS mode was employed for the profile of AA eluates collected from HPLC runs of specific tissues of fish force-fed AA. This mode, extremely sensitive, filters out all masses except the substance with a parent ion mass of 255.4 or 175.5 (as described in Materials and Methods). Figure 6A (at time periods 1.81, 5.24, 8.99, and 12.45) shows peaks appearing when pure water is injected, to illustrate the contaminating ion in the system (as mentioned in mass spectroscopy equipment section). Figure 6B (at time periods 3.18, 7.24, 10.49, and 13.96) shows peaks appearing when AA eluates from gastric, blood, liver, and muscle of fish force-fed AA. The small intermittent peaks are noise peaks that are produced in this mode of operation. However, the intensities of the peaks seen are relative to the concentrations found in the AA force-fed tissues.

To circumvent the background problem seen in Figure 6A, an even more specific mode of MS/MS was used—a mode that sees only the parent ion at 255.5, which is fragmented to the desulfated daughter ion 175.5 mass (Fig. 7). This mode, called multiple reaction monitoring (MRM), is employed using eluates with RTs corresponding to AS standards eluted from HPLC runs of AS force-fed fish tissues.

The peaks appearing at time periods 3.70, 7.03, 10.07, and 14.75 represent injections of these AS peak eluates eluted from the AS force-fed fish. The height of these peaks are indicative of the concentrations of AS found in the gastric, blood, liver, and muscle tissue of the AS force-fed fish. The intermittent peaks are blank solvent peaks indicating no contaminating ion interference when using this MRM mode. This MRM mode results in a very specific method for AS. The background resident ion (which produced the problem shown in Fig. 6A) would not respond to this technique, indicating it was not a true AS parent ion.

In conclusion, the data collected in this study indicate that Atlantic salmon can adsorb AS through the gastric tissue when fed ascorbic acid and ascorbate-2-sulfate as described. The data also indicate that AS is transported through the blood to the liver. There is evidence to indicate that AS is converted to AA in the livers of Atlantic salmon fed only AS. AA and AS accounting for a large portion of the two forms absorbed by the fish were found in the muscle tissue. Tissue extracts of AS and AA were separated by HPLC. Extracts were collected from muscle, liver, blood, and gastric tissues. EIMS confirmed the peaks with retention times identical to AS and AA standards.

These results show that AS was readily taken up and distributed through the system in Atlantic salmon and can be utilized as a source of ascorbic acid in their diets.

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1. Tucker BW, Halver JE. Distribution of ascorbate-2-sulfate and distribution, half-life and turnover rates of (1-14C) ascorbic acid in rainbow trout. J Nutr **114:**991–1000, 1984.
2. Dabrowski K, Lackner R, Doblander C. Effect of dietary ascorbate on concentration of tissue ascorbic acid dehydroascorbic acid, ascorbic sulfate and ascorbic sulfate sulfohydrolase in rainbow trout. Can J Fish Aquat Sci **47:**1518–1525, 1990.
3. Sandnes K, Hansen T, Killie J-EA, Waagbo R. Ascorbate-2-sulfate as a dietary vitamin C source for Atlantic salmon (*Salmo solar*): Growth, bioactivity, haematology and humoral immune response. Fish Physiol Biochem **8:**419–427, 1990.
4. Dabrowski K. Comparative bioavailability of ascorbic acid and its stable forms in rainbow trout. In: Wenk C, Fenster R, Volker L, Eds.

Ascorbic Acid in Animal Nutrition. Proceedings of the 2nd Symposium, Zurich Ittingen, Switzerland, pp344–356, 1992.
5. Halver JE, Felton S, Palmisano AN. Efficacy of ascorbyl-2-sulfate as a vitamin C source for rainbow trout. In: Kaushik SJ, Liquet P, Eds. Fish Nutrition in Practice. Paris: INRA, pp137–147, 1993.
6. Felton SP, Grace R. Authentication of L-ascorbyl-2-sulfate in salmonid gastric tissue: HPLC/electro-spray ionization mass spectroscopic verification. J Liq Chromatog **18:**1563–1581, 1995.
7. McLaren BA, Keller E, O'Donnell DJ, Elvehjem CA. The nutrition of rainbow trout. I. Studies of vitamin requirements. Arch Biochem Biophys **15:**169–178, 1947.
8. Halver JE, Ashley LM, Smith RR. Ascorbic requirements of coho salmon and rainbow trout. Trans Am Fish Soc **98:**762–771, 1969.
9. Tucker BW, Halver JE. Ascorbate-2-sulfate metabolism in fish. Nutr Rev **42:**173–179, 1984.
10. Dabrowski K, Matusiewicz M, Blom JH. Hydrolysis, absorption and bioavailability of ascorbic acid esters in fish. Aquaculture **124:**169–192, 1994.
11. Dabrowski K, Hinterleitner S. Simultaneous analysis of ascorbic acid, dehydroascorbic acid and ascorbate-2-sulfate in biological material. Analyst **114:**83–87, 1989.
12. Schuep W, Marmet J, Struder W. Stability of ascorbate-2-sulfate in trout feed measured by HPLC. Aquaculture **79:**249–258, 1989.
13. Felton SP, Grace R, Halver JE. A non-ion pairing HPLC method for measuring new forms of ascorbate and ascorbic acid. J Liq Chromatog **17:**123–131, 1994.
14. Benitez LV, Halver JE. Ascorbic acid sulfate sulfohydrolase (C_2 sulfatase): The modulator of cellular levels of L-ascorbic acid in rainbow trout. Proc Natl Acad Sci USA **79:**5445–5449, 1982.
15. Schorah CJ. The transport of vitamin C and effects of disease. Proc Nutr Soc **51:**189–198, 1992.