Efficacy of Dehydro-L-Ascorbic Acid as a Dietary Vitamin C Source in Rainbow Trout

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Dehydro-L-ascorbic acid (DAsA), an oxidized form of L-ascorbic acid (AsA), was tested for efficacy as a vitamin C source and safety in young rainbow trout *Onchorhynchus mykiss*. The fish, weighing about 100 g on average, were fed with experimental diets containing various levels of DAsA or AsA for 6 weeks. The fish receiving the AsA-deficient diet had very low concentrations of AsA in several tissues and plasma. In the fish fed with the DAsA-supplemented diet, similar to those fed with the AsA-supplemented diet, only the AsA level rose markedly, whereas DAsA did not increase. There were no statistical differences in the AsA and DAsA concentrations in the various tissues and plasma between AsA- and DAsA-supplemented groups. The glutathione levels of tissues and hematological parameters did not vary between the AsA- and DAsA-supplemented groups. In addition, no cell disruption was brought about by DAsA administration.

These results suggest that dietary DAsA is readily reduced to AsA in the fish body and can be effectively utilized by the rainbow trout as a vitamin C source.

L-Ascorbic acid (AsA) is readily oxidized to dehydro-L-ascorbic acid (DAsA) during processing and storage of feeds because of its instability. It is possible that cultured fish are fed with diets containing considerable amounts of DAsA.

The vitamin C action of AsA in animals is based on the oxidation process of AsA to DAsA via mono-DAsA, while DAsA itself has no vitamin C activity.¹⁾ DAsA is known to be reversibly converted to AsA in animal tissues. Various studies have so far been undertaken to evaluate the antiscorbutic activity of DAsA in guinea pigs^{2,3)} and humans.⁴⁻⁶⁾ Tsujimura *et al.*⁵⁾ concluded that DAsA had the antiscorbutic activity equivalent to AsA, on the basis of the results of urinary AsA excretions of healthy men who were fed diets supplemented with DAsA. Otsuka *et al.*⁷⁾ showed that the antiscorbutic activity of DAsA in vitamin C-deficient guinea pigs was less than that of AsA. There are few reports on vitamin C activity of DAsA in fishes.

The present study was designed to clarify the conversion of dietary DAsA to AsA in the fish body and to evaluate the efficacy of DAsA as a vitamin C source for rainbow trout.

Materials and Methods

Reagents

AsA was obtained from Nacalai Tesque Inc. (Kyoto). DAsA was prepared by the method of Tsujimura.⁸⁾

Fish, Diets, and Feeding Methods

Young rainbow trout *Onchorhynchus mykiss*, weighing about 100 g on average, were used. The fish were divided into 5 groups of 10 individuals each, and each group was kept in an aquarium (50 l) supplied with well water at 16°C. The basal diet (AsA-free) was the same as that reported previously.⁹⁾ The experimental diets were prepared from the AsA-free basal diet supplemented with either 50 or 1,000 mg of AsA or equimolar amounts of DAsA (49 and 989 mg, respectively) per 100 g of dry basal diet. These diets are referred to as A-50, A-1000, and D-50, D-1000, respectively. As a control, an AsA-free basal diet is referred to as F. Each diet powder (100 g) was mixed well with 60 ml of water and made into pellets at room temperature. Each pelleted moist diet was sealed in a plastic bottle and stored at -80°C until use. These

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Table 1. Stability of AsA and DAsA during pelleting and storage of the experimental diets

Diet	Storage -80°C (day)	Moisture (%)	Total AsA* ¹ (μg/g of dry)	Yield (%)	Composition (%)		
					AsA	DAsA	DKG
A-50	Pre-pelleted diet powder	0	9.3	551.3 ± 2.8* ²	100	0	0
	Pellets	0	41.6	515.8 ± 9.6	93.6	71.6 ± 2.3	27.6 ± 4.4
	"	5	41.5	516.4 ± 8.8	93.7	71.3 ± 3.6	27.8 ± 3.5
	"	10	41.6	515.5 ± 4.9	93.5	71.5 ± 3.2	26.3 ± 3.0
D-50	Pre-pelleted diet powder	0	9.2	540.2 ± 4.2	100	0	0
	Pellets	0	41.6	494.4 ± 18.6	91.5	0	97.8 ± 0.8
	"	5	41.6	486.6 ± 26.3	90.1	0	97.9 ± 0.9
	"	10	41.3	482.3 ± 26.0	89.3	0	92.0 ± 1.6

*¹ Total AsA (AsA + DAsA + DKG) was determined by the hydrazine method.¹⁰⁾ DKG content was determined by the hydrazine method after the reduction of DAsA with dithiothreitol to AsA.

*² Mean ± S.D. (n = 3).

diets were prepared every 10 days. As shown in Table 1, when AsA was added to the diet at a level of 50 mg per 100 g of diet, about 6% of the added AsA was lost during diet preparation and about 28% of the remaining total AsA was oxidized to DAsA. Only a small amount of diketo-L-gulonic acid (DKG) was detected in the pellets. When DAsA was added to the diet at a level of 49 mg (50 mg AsA eq.) per 100 g of the diet, about 8% of the added DAsA was lost during diet preparation. After storage at -80°C for 10 days, AsA, DAsA, and DKG in diet A-50, as well as DAsA and DKG in diet D-50 remained unchanged.

Before initiation of the experiment, each group of fish was given the AsA-free diet for 2 weeks. Then each group was fed their prescribed experimental diet twice a day at a level of 2% of body weight. After 3 and 6 weeks of feeding, 3-5 fish were sampled randomly from each group to analyze AsA, DAsA, glutathione (GSH), and hematological parameters.

Determination of AsA, DAsA, and GSH

AsA, DAsA, and GSH were determined by high-performance liquid chromatography (HPLC) using a reversed-phase column (COSMOSIL 5C₁₈, 4.6 × 150 mm, Nacalai Tesque) and an electrochemical detector (model L-ECD-6A, Shimadzu). The detector was set at +800 mV vs. Ag/AgCl. The mobile phase was 20 mM NaH₂PO₄ containing 1 mM EDTA, 3 mM sodium 1-octanesulfonate, and 0.015% (w/v) metaphosphoric acid, the pH of which was adjusted to 3.00 with H₃PO₄. The flow rate was 0.8 ml/min.

The column and detector were kept at 40°C.

The preparation of tissue samples to determine AsA and GSH was carried out as described previously.¹¹⁾ For the determination of blood GSH, each blood sample (500 μl) was mixed well with 100 μl of 6% acetic acid, and then mixed with 400 μl of cold 5% metaphosphoric acid, and filtered through a 0.45 μm syringe filter. The filtrate was injected into the HPLC system.

DAsA was reduced with dithiothreitol to AsA. Each sample solution (900 μl) was mixed with 2% dithiothreitol (100 μl) and neutralized with 5 N NaOH. The mixture was allowed to stand for 10 min under cooling in an ice bath, and filtered through a 0.45 μm syringe filter. Total AsA (AsA + DAsA) of the filtrate was analyzed by HPLC. DAsA content was determined by subtracting the AsA value from total AsA value.

Determination of Blood Glucose and Activities of GPT and GOT in Serum

Blood was collected into test tubes by cutting the caudal peduncle and kept at room temperature for 1 h. The blood was centrifuged at 700 × g for 10 min to obtain serum, which was then kept at 0°C and analyzed within 4 to 5 h. Blood glucose and activities of GPT and GOT in serum were estimated by a Rapid Blood Analyzer (RaBA-3010, Chugai).

Statistical Analysis

The data were analyzed statistically by Student's *t* test.

Results

The AsA and DAsA concentrations in the plasma are presented in Table 2. The fish in group F had a very low AsA concentration. The plasma AsA concentration of group D-50 was not significantly different from that of group A-50. The plasma AsA concentrations of groups D-1000 and A-1000 were very high after 3 weeks

of feeding. The AsA concentrations of groups D-1000 and A-1000 were significantly higher ($P<0.05$) than those of groups D-50 and A-50. However, no significant difference was observed between group D-1000 and group A-1000. The DAsA concentrations in the plasma were below detectable limits even in the DAsA 1000 mg-supplemented group, except for group F.

Table 3 shows the concentrations of AsA and

Table 2. Concentration of AsA and DAsA in plasma of rainbow trout fed varying levels of DAsA and AsA for 3 and 6 weeks

Group	(nmol/ml)			
	After 3 weeks		After 6 weeks	
	AsA	DAsA	AsA	DAsA
F	30.1 ± 10.2 ^{*1}	0.6 ± 0.5	31.2 ± 8.0	7.5 ± 1.1
A-50	167.6 ± 35.2	n.d. ^{*2}	218.7 ± 38.1	n.d.
D-50	176.7 ± 76.7	n.d.	181.8 ± 77.8	n.d.
A-1000	897.2 ± 84.7	n.d.	430.7 ± 120.5	n.d.
D-1000	933.5 ± 109.7	n.d.	562.5 ± 164.2	n.d.

^{*1} Mean ± S.D. Each value is the mean of 3 fish.

^{*2} n.d.; not detected (below 0.2 nmol/ml).

Table 3. Concentration of AsA and DAsA in tissues of rainbow trout fed varying levels of DAsA or AsA for 3 and 6 weeks

Tissue	Group	(nmol/g)			
		After 3 weeks		After 6 weeks	
		AsA	DAsA	AsA	DAsA
Liver	F	146.6 ± 38.6 ^{*1}	4.0 ± 4.0	150.0 ± 9.1	43.7 ± 10.3
	A-50	1129.0 ± 439.2	36.2 ± 37.9	1510.8 ± 246.0	24.7 ± 43.1
	D-50	1360.2 ± 616.5	17.2 ± 16.1	1273.9 ± 659.1	31.0 ± 54.0
	A-1000	1585.8 ± 284.6	2.9 ± 5.2	927.8 ± 46.6	23.0 ± 33.9
	D-1000	1547.2 ± 260.8	23.6 ± 20.7	1115.3 ± 461.4	27.6 ± 33.9
Kidney	F	251.1 ± 10.2	36.8 ± 8.6	284.7 ± 10.2	82.8 ± 20.7
	A-50	1483.5 ± 298.3	86.2 ± 74.7	1259.1 ± 163.1	62.1 ± 51.1
	D-50	1442.0 ± 298.3	64.4 ± 56.3	1503.4 ± 158.0	68.4 ± 93.7
	A-1000	1281.8 ± 213.6	39.7 ± 24.1	1021.6 ± 263.1	73.0 ± 125.9
	D-1000	1226.1 ± 396.6	64.4 ± 33.3	1179.5 ± 211.9	106.9 ± 106.9
Spleen	F	180.1 ± 80.7	110.3 ± 13.2	182.9 ± 49.9	169.0 ± 43.7
	A-50	1128.4 ± 388.1	203.4 ± 179.3	1259.7 ± 149.4	256.9 ± 101.1
	D-50	1372.7 ± 520.5	220.7 ± 161.5	1580.7 ± 867.0	241.4 ± 209.2
	A-1000	1476.1 ± 306.2	201.7 ± 185.1	1618.7 ± 33.5	369.0 ± 23.6
	D-1000	1643.2 ± 824.4	130.5 ± 141.9	1650.0 ± 134.1	152.9 ± 134.5
Brain	F	1082.4 ± 92.0	n.d. ^{*2}	989.2 ± 147.2	34.5 ± 31.0
	A-50	1641.5 ± 230.1	17.2 ± 30.5	1875.6 ± 226.7	2.3 ± 3.4
	D-50	1979.0 ± 82.4	9.2 ± 15.5	2083.0 ± 497.7	5.2 ± 2.3
	A-1000	1664.2 ± 277.3	n.d.	1788.6 ± 444.9	15.5 ± 26.4
	D-1000	1882.4 ± 538.1	n.d.	1725.0 ± 328.4	6.9 ± 13.8
Muscle	F	29.0 ± 10.8	1.1 ± 2.9	41.5 ± 2.8	19.0 ± 1.7
	A-50	97.2 ± 32.9	9.2 ± 3.4	160.8 ± 29.5	28.2 ± 6.3
	D-50	117.0 ± 14.8	7.5 ± 3.4	174.4 ± 71.6	6.3 ± 7.5
	A-1000	198.3 ± 38.6	5.2 ± 5.2	185.2 ± 49.4	2.9 ± 0.6
	D-1000	223.3 ± 76.1	0.6 ± 0.6	167.0 ± 51.1	8.0 ± 13.8

^{*1} Mean ± S.D. Each value is the mean of 3 fish.

^{*2} n.d.; not detected (below 0.2 nmol/g).

DAsA in the liver, kidney, spleen, brain, and muscle of rainbow trout fed with the experimental diets for 3 and 6 weeks. The concentrations of AsA in the tissues of the groups fed with the DAsA-supplemented diet were significantly higher than those of group F. However, no significant difference was observed between AsA-supplemented and DAsA-supplemented groups for the liver, kidney, spleen, brain, and muscle AsA concentrations after 3 and 6 weeks of feeding. In all of the groups, DAsA concentrations of the spleen and kidney were high compared with those of the liver, brain, and muscle. But no significant difference in the tissue DAsA concentrations was observed between DAsA-supplemented and AsA-supplemented groups. Moreover, within each tissue no significant difference in the DAsA concentration was observed among the groups fed with diet F, A-50, D-50, A-1000, and D-1000 for 3 and 6 weeks. These results suggest that dietary DAsA was readily reduced in the fish body and utilized as AsA by rainbow trout.

Table 4 shows the GSH concentrations in the liver, kidney, spleen, brain, muscle, and blood of rainbow trout fed with the experimental diets

for 3 and 6 weeks. The GSH concentrations of none of the tissues examined except liver and blood in groups A-50 and D-50 after 3 weeks of feeding were significantly different from those of group F. The GSH concentrations of all the tissues except muscle and blood in groups A-1000 and D-1000 were significantly lower ($P<0.05$) than those of group F. The GSH concentrations of the tissues and blood in group F remained almost constant until 6 weeks of feeding. The GSH concentrations of groups fed with the diets supplemented with AsA or DAsA (groups A-50, D-50, A-1000, and D-1000) were significantly lower ($P<0.05$) than those of the AsA-deficient group. No significant differences in GSH concentrations were observed in any of the tissues examined among groups A-50, D-50, A-1000, and D-1000 after 6 weeks of feeding.

Table 5 shows the hematocrit values, blood glucose levels, and serum GPT and GOT activities of rainbow trout fed with the experimental diets for 6 weeks. In groups F, blood glucose level was slightly higher than those of the AsA-supplemented and DAsA-supplemented groups, although the level was not significantly different.

Table 4. Concentration of GSH in blood and tissues of rainbow trout fed varying levels of DAsA or AsA for 3 and 6 weeks

Feeding period	Group	Blood (nmol/ml)	Liver (nmol/g)	Kidney (nmol/g)	Spleen (nmol/g)	Brain (nmol/g)	Muscle (nmol/g)
3 weeks	F	1240±100*	2900±250	2800±460	3680±460	1500±40	390±30
	A-50	1170±80	3380±160	3280±610	3810±420	1510±10	430±100
	D-50	1160±120	3670±160	2910±150	3720±90	1160±390	350±40
	A-1000	540±70	1450±420	1370±110	1830±650	610±20	270±70
	D-1000	570±150	1150±60	1610±460	2220±160	740±60	270±60
6 weeks	F	1130±230	2140±240	2590±300	3210±710	1150±410	320±70
	A-50	690±110	1200±270	1330±220	1630±280	570±100	290±210
	D-50	820±70	1090±440	1640±80	2310±230	630±190	230±40
	A-1000	590±240	1270±140	1370±20	1590±110	650±100	310±80
	D-1000	590±80	1440±370	1240±420	1290±730	610±190	230±50

* Mean±S.D. Each value is the mean of 3 fish.

Table 5. Hematocrit values, blood glucose levels and serum GPT and GOT activities of rainbow trout fed varying levels of DAsA or AsA for 6 weeks

Group	Hematocrit value (%)	Blood glucose (mg/100 ml)	GPT (karmen unit)	GOT (karmen unit)
F	37.6±3.8*	80.6±5.3	20.3±1.5	475.0±70.5
A-50	36.4±3.4	72.6±8.7	17.5±4.8	413.3±102.5
D-50	36.7±5.1	68.8±18.8	31.0±7.1	530.0±74.7
A-1000	37.9±5.2	65.0±9.1	19.0±3.5	475.0±184.3
D-1000	38.1±3.9	63.6±9.8	33.5±8.4	463.3±46.5

* Mean±S.D. Each value is the mean of 5 fish.

Also, no significant difference in the hematocrit values was observed between AsA-supplemented and DAsA-supplemented groups. The activities of GPT and GOT in serum were slightly higher in the DAsA-supplemented groups than those in the AsA-supplemented groups, but the difference was not significant ($P > 0.05$).

Discussion

The present study indicated that there were no significant differences in AsA concentrations in the tissues and plasma between groups supplemented with DAsA and those with equimolar amounts of AsA. This result suggests that orally administered DAsA is readily reduced to AsA in the fish body and could be effectively utilized by the rainbow trout as a vitamin C source.

DAsA has been shown to prevent scurvy in guinea pigs^{2,3)} and humans.⁴⁻⁶⁾ Torbert¹²⁾ suggested that the vitamin C activity of DAsA is about 70% of the AsA in guinea pigs. Tsujimura *et al.*⁵⁾ concluded that DAsA had the antiscorbutic activity equivalent to AsA on the basis of results of urinary AsA excretions of healthy men who were fed diets supplemented with DAsA. Otsuka *et al.*⁷⁾ showed that the antiscorbutic effect of DAsA in guinea pigs was less than that of AsA administered intravenously. It is apparent from the present study that the antiscorbutic activity of DAsA is equivalent to AsA in rainbow trout under these experimental conditions.

Patterson¹³⁾ observed that injection of large doses of DAsA intravenously in rats caused temporary diabetes mellitus, and large doses of repeated injections caused permanent atrophy of the β -cells of the islets of Langerhans. Chatterjee *et al.*¹⁴⁾ also reported that as the blood DAsA levels increased in guinea pigs fed with high doses of AsA, there was a concomitant increase in blood sugar levels. In the present study, after feeding large doses of AsA or DAsA to rainbow trouts for 6 weeks, no increase in plasma DAsA levels was observed while there was also no increase in blood glucose levels.

GSH is a well-known intracellular tripeptide that acts as a reducing agent and a free radical scavenger. It plays an important role in preventing oxidative damage in liver, endothelial tissue, and heart.¹⁵⁾ Both AsA and GSH act as homeostatic systems; a positive correlation between AsA and GSH exists in some tissues. Martenson and Meister¹⁶⁾ showed that the depletion of GSH

led to depletion of tissue AsA in newborn rats. Dabrowski *et al.*¹⁷⁾ reported that the GSH concentration in viscera of rainbow trout decreased significantly when the fish were fed with AsA-deficient diets. In the present study, however, no decrease in the GSH concentrations of the tissues was observed in rainbow trout fed with AsA-free diets for 6 weeks. Furthermore, no significant differences in AsA and DAsA concentrations in various tissues and blood were observed between AsA-supplemented and DAsA-supplemented groups. If GSH was the main reducing agent of DAsA, DAsA-supplemented groups would reduce GSH concentration more than AsA-supplemented groups. Basu *et al.*¹⁸⁾ postulated that the reduction of DAsA in human erythrocytes was coupled with the GSH-regenerating system. Recently, Choi and Rose¹⁹⁾ reported that DAsA reduction was catalyzed by a soluble enzyme that transferred hydrogen from NADPH to GSSG. Accordingly, such a system may be coupled to a greater extent with the reaction involving DAsA and GSH in rainbow trout resulting in little decrease in the GSH level. Further investigation is needed to elucidate the reduction mechanisms of DAsA in the fish body.

From the results obtained in this experiment, it is suggested that the DAsA present in fish feed is readily reduced to AsA in the fish body and can be effectively utilized by the rainbow trout as a vitamin C source.

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