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METABOLISM AND PHYSIOLOGICAL ACTIVITY OF ASCORBYL-2-PHOSPHATE IN FISH*1

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CONTENTS

ABBREVIATION

AA	L-ascorbic acid
AA-Na	sodium L-ascorbate
acetyl-CoA	acetyl coenzyme A
ADP	adenosine diphosphate
AG	L-ascorby1-2-glucoside
AMP	adenosine monophosphate
AP	L-ascorby1-2-monophosphate
APM	L-ascorby1-2-monophosphate magnesium salt
ApP	L-ascorby1-2-polyphosphate
APt	L-ascorby1-6-palmitate
AS	L-ascorby1-2-sulfate
ATP	adenosine triphosphate
BUN	blood urea nitrogen
CAT	carnitine acetyltransferase
CoASH	coenzyme A
DAA	dehydro L-ascorbic acid
DNP	2, 4-dinitrophenol
EDTA	ethylenediaminetetraacetate
GOT	glutamic-oxaloacetic transaminase
GPT	glutamic-pyruvic transaminase
HPLC	high-performance liquid chromatography
HR	Holmes-Stott Ringer
Hyp	L-hydroxyproline
Pro	L-proline
TCA	trichloroacetic acid

INTRODUCTION

L-Ascorbic acid (AA), known as an anti-scorbutic factor or vitamin C, is widely used in various fields such as medicines, cosmetics, food stuffs and feeds as an antioxidant or a medicable compound. Nowadays there is a boom in AA-enriched foods, *e.g.* snacks, candies and soft drinks. The sales of vitamin tablet containing AA are going up skyrocketing these days as people desire for better health. AA is thought, but not yet established indeed, to prevent or cure several disease; cold, cancer, allergy, aging and so on. AA is also useful for processing of food: as an antioxidant AA is used in juice, sliced vegetable, marine and dairy processed foods and other oxidizable foods to prevent coloration and oxidation. To the contrary AA has a function of oxidizing agent, using in bread and kamaboko production to improve the texture. As such, AA has been added to all sorts of food products to improve the quality and to add values to the products.

AA plays many physiological functions in animals on the basis of its strong reducibility derived from the endiol structure. This substance acts as a cofactor in various hydroxylation reactions in living tissues.[1] AA is indispensable for collagen hydroxylation, biosynthesis of carnitine and catecholamine, metabolism of tyrosine and corticosteroids, *etc.* Due to its reducing and chelating properties, AA has an effect on the metabolism of metal ions; enhancement of iron intake from intestine, reduction of heavy metals such as copper, cadmium, lead, *etc.* AA quenches the free radical derivatives of oxygen where exposed to the danger of radical attack as in the eye and lung. A role of AA during interferon production has been reported, and recently AA is thought to promote immune response.[2] Most of the animals synthesize AA, but the guinea pig, monkey, man[3], invertebrates[4], insects[4, 5], some species of birds[6] and most of fishes[4, 7] are incapable to synthesize AA. They lack L-gulono-γ-lactone oxidase that catalyzes the last step of AA synthesis. It is thus well known that they require AA in their diet to prevent scurvy.

Yamamoto *et al.*[7] found no activity of L-gulono-γ-lactone oxidase in rainbow trout, *amago*, *ayu*, eel, red sea bream, yellowtail, *kawahagi* and tilapia and weak activity in carp, *gengorobuna*, *ugui* and catfish. AA deficiency symptoms have been reported for several cultured fishes *in situ*[1, 8]; rainbow trout[9-13], brook trout[14], coho salmon[11], channel catfish[15-19], Japanese eel[20], red seabream[21], shrimp[22] and other fish. The symptoms of AA deficiency reported so far are spinal curvature, abnormally protruding eyes, contained hemorrhagic areas, distorted gill filaments, poor growth and high rates of mortality in salmonoids and other fishes and whitened or blackened lesions, poor wound repair and incomplete molting in shrimp. To prevent such AA deficiency, enough AA is recommended to feed the culturing fish and shellfish.

Recent studies of AA in fish have been focused on the possibilities to reduce negative impacts caused by stress and environmental factors upon health and disease resistance[1], and various positive results were reported. Lovell and Lim[23] indicated that the requirement of AA is increased in channel catfish stocked at high densities. Durve and Lovell[24] and Li and Lovell[25] showed that high level (150mg/kg) of AA supplementation to the diet increased the resistance against bacterial infection in channel catfish and the megadose (3000mg/kg) significantly enhanced antibody production and complement activity. Navarre and Halver[26] indicated that rainbow trout may survive bacterial challenge and improve antibody production when the diet contains more than 500mgAA/kg. Ishibashi *et al.*[27] reported that exposure to intermittent hypoxic stress induced AA-de-

ficiency disease early and increased the AA requirement and that high doses of AA (300mg/100g) increased the resistance against the stressor. Thus high dose of AA is recommended to intensive fish culture to nurse healthy fish.

AA is stable in pure crystal, but susceptible to oxidative and thermal degradation in solution or under moist conditions. Typically, over one half of AA added to fish feeds is oxidized during feed processing and storage.[28] The oxidized form (dehydroascorbic acid) is known to have the same vitamin C activity as AA, in guinea pigs[29, 30]; humans[31-33] and rainbow trout[34], with an exception in vitamin C-deficient guinea pigs.[35] Dehydroascorbic acid taken from feeds has no vitamin C activity in itself, possibly being readily reduced to AA by specific reductases and cofactors in the body and exhibit the activity. Dehydroascorbic acid is rapidly and irreversibly hydrolyzed to 2, 3-diketogulonic acid, which has no activity, during processing and storage of feeds.

There have been several approaches to overcome the problem of AA instability in fish feeds, *e. g.* microencapsulation, coating of crystalline AA[23, 36-39], and application of AA spraying on the surface of fish feeds after processing.[40] However, these contrivances are not economically and practically feasible.[1] Another approach is to use the stable AA derivatives, such as L-ascorbyl-2-sulfate (AS), L-ascorbyl-6-palmitate (APt), L-ascorbyl-2-glucoside (AG), L-ascorbyl-2-polyphosphate (ApP) and L-ascorbyl-2-monophosphate (AP). Most studies showed that AS was inferior to AA as a dietary vitamin C source in rainbow trout[41, 42], channel catfish[43-45], tilapia[46, 47] and Atlantic salmon[48]. AS dosed to fish is accumulated in the body and the safety of the accumulation has never been discussed. APt showed vitamin C activity in catfish[43], trout[49] and mammalians.[50] The stability of APt, however, may not be expected because the 2,3-endiol group is not protected. AG was newly synthesized by enzymatic procedures and showed vitamin C activity in rat and guinea pigs.[51, 52] Yamamoto *et al.*[51, 52] have discussed the metabolism of AG in mammalians and tried mass-production of AG. ApP synthesized by Seib and Liao[53] had a vitamin C activity in channel catfish[45], trout and other fishes[28]. However, ApP is a compound containing tri- (96%), di- (3%), monophosphate (1%)[54] and the dehydration products of AA as impurities. Effects of such impurities in ApP on animals have never been discussed. AP has been shown to be efficacious as a vitamin C source in guinea pigs[55-57], rhesus monkey[57], channel catfish[43] and penaeid shrimp[22].

Efficacy of AP was discussed as such in several animals, but little study has been made thus far on the metabolism of AP especially in fish. This study was designed to evaluate the efficiency of AP as a vitamin C source in fish feeds and discussed on the metabolism of AP in fish.

CHAPTER 1

Stability of Ascorbyl-2-phosphate in Fish Feeds

L-Ascorbic acid (AA) is believed to be a nutrient essential for fish feeds.[9, 11, 14, 15, 17, 19, 25, 58] AA is readily oxidized to ineffective forms as a function of temperature, pH, oxygen, concentration, heavy metals, light and time during processing and storage of feeds. Eva *et al.*[59] reported that the loss of AA in feeds' processing and 6 weeks' storage at room temperature were 20% and 65%, respectively. There was a report that almost all the AA added to the feeds was lost during processing and 6 weeks' storage.[13] One of the attempts to improve the retention of ascorbate in fish feeds is to use stable form of AA derivatives such as L-ascorbyl-2-monophosphate (AP). The endiol group of AP is protected by a phosphate and AP is thought to be stable.[56] There have been only a few reports available on stability of AP. In the present chapter, the stability of AP was compared with that of AA during processing and storage of the three types of fish feeds.

Materials and Methods

Reagents

AP was obtained from Showa Denko Inc. (Tokyo) as magnesium-L-ascorbyl-2-monophosphate. AA and other reagents were obtained from Nacalai Tesque Inc. (Kyoto).

Determination of AA and AP

AA and AP were determined by high-performance liquid chromatography (HPLC). The equipment for AA analysis was: a pump, Hitachi 655 liquid chromatograph; an electrochemical detector, Shimadzu L-ECD-6A set at +0.8V *vs.* Ag/AgCl; a processor, Hitachi 833 Data Processor; a reversed-phase column, Cosmosil $5C_{18}$-AR, 4.6 × 150mm (Nacalai Tesque Inc.). The mobile phase was a 20mM NaH_2PO_4 solution containing 1mM ethylenediaminetetraacetate (EDTA), 1-octanesulfonic acid sodium salt 6.5% (w/v) and 0.015% (w/v) metaphosphoric acid, the pH of which was adjusted to 3.00 with H_3PO_4. The flow rate was 0.8m*l*/minute. The column and detector temperature was 40℃. AA was extracted with 5% metaphosphoric acid and injected onto the column after filtration (0.45μm).

To analyze dehydroascorbic acid (DAA), the extract was mixed with dithiothreitol and neutralized with NaOH. The mixture was allowed to stand for 10 minutes under cooling in an ice bath, and filtered through a syringe filter. Total ascorbate (AA + dehydroascorbic acid) of the filtrate was analyzed by HPLC. Dehydroascorbic acid content was determined by subtracting the AA value from total AA value.

The equipment for AP analysis was: a pump, Hitachi 655 liquid chromatograph; a detector, Hitachi L-4000 u.v. Detector set at 254nm; a processor, Hitachi 833 Data Processor, a reversed-phase column, Cosmosil $5C_{18}$-AR, 4.6 × 250mm (Nacalai Tesque Inc.). The mobile phase was a 50mM NaH_2PO_4 solution and the flow rate was 0.7m*l*/minute. AP was extracted with 5% metaphosphoric acid and injected onto the column after filtration (0.45μm). Extraction of AP and AA was repeated 4 times.

Stability of AA and AP in solution

AA and AP were dissolved in distilled water at a concentration of 1% AA equivalent. Each solution was sealed in a transparent tube of polypropylene and exposed to the sun light for 180 weeks. The concentrations of AA and AP were determined at definite intervals.

Stability of AA and AP in pelleted diet

Vitamin C free basal powder diet (Table 1.1) was mixed well with AA or AP (50 or 500mg AA eq./100g powder) and water

Table 1.1. Composition of basal diet

Ingredients	%
Brown fish meal	55
Wheat	10
α-Starch	15
Dextrin	8
Soybean oil	3
Pollack liver oil	2
Mineral mixture*1	5
Vitamin mixture (AA-free)*2	2

*1 The formula of mineral mixture was the same as reported by Halver *et al.* (1969).

*2 Supplied the following amounts per 100g diet: thiamine-HCl 5mg, riboflavin 20mg, nicotinic acid 75mg, calcium pantothenate 50mg, vitamin B_{12} 0.01mg, menadione 4mg, biotin 0.5mg, pyridoxine-HCl 5mg, inositol 200mg, choline chloride 500mg, folic acid 1.5mg, vitamin A acetate 2000 IU, α-tocopherol acetate 60mg. α-Cellulose was used as a carrier for the vitamin mixture.

(60m*l*/100g powder) and extruded through a die (3mm-diameter) in a food grinder. The extrusion was cut into pellets (about 8mm-length). It was within 10 minutes from the water addition to the cutting process. The pellets formulated are referred to AA 50 (AA 50mg /100g), AA 500 (AA 500mg /100g), AP 50 (AP 50mg AA eq./100g) and AP 500 (AP 500mg AA eq./100g). To obtain dry pellets, these moist pellets were dried by electric furnaces at 120℃ with air blowing for about one hour to contain about 5% moisture. These moist and dried pellets were stored at 20℃ in the sealed polyethylene bags. AA, DAA and AP contents and moisture were determined before and after pelleting, after drying and during the storage.

Stability of AA and AP in minced fish

Sardine (bone and all), purchased from local market, was minced with a meat grinder. The mince was mixed with AA or AP (100mg AA eq./100g mince) and stored at 20℃. These two kinds of diet are referred as AA 100 and AP 100 diets, respectively. Contents of AA, DAA and AP were determined by the methods described above.

Results

Stability of AA and AP in solution

Figure 1.1 shows the stability of AP and AA in solution during storage. AA content after 20 weeks' storage was 39% of the initial level and the solution was slightly colored

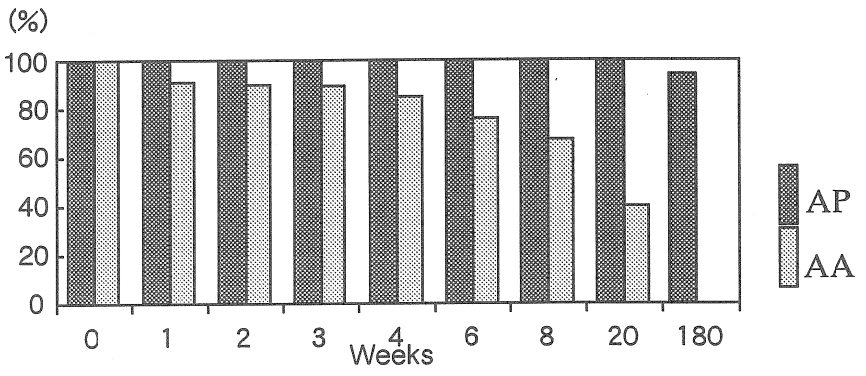

Fig. 1.1. Stability of AP and AA in solution. AP or AA was dissolved in distilled water and was stored in a plastic tube. AP and AA contents were determined by HPLC.

brown. No AA was detected after 180 weeks' storage exhibiting dark brown in color. In contrast the AP in solution was highly stable throughout the storage period. AP remained at 100% after 20 weeks and at more than 94% even after 180 weeks without coloring.

Stability of AA and AP in moist pellets during processing and storage

The retention of AA and AP in moist pellets during processing and storage was listed in Table 1.2. The percentage values were obtained from 3 determinations on a basis of mole per dried weight calculated from the moisture values. AA in the diet AA 50 was degraded rapidly after mixing with water and the retention after pelleting was 57.2% as AA and 28.8% as oxidized form, DAA. After one day storage at 20℃, no AA was detected and only 7.5% remained as DAA; no ascorbate was detected after 5 days' storage. To the contrary the degradation of AP was slow. The retention of AP after 5 days' storage were 72.5 and 88.4% in the diet AP 50 and AP 500, respectively, indicating that the degradation of AP was very slow. Neither AA nor DAA was found in AP diets throughout the period.

Stability of AA and AP in dried pellets during drying process and storage

Table 1.3 shows the retention of AA and AP in dried pellets during processing and storage. Most of AA in the diets AA 50 and 500 was destroyed during the drying process. Only 5.5 and 38.2% of the initial amounts of AA in the diets AA 50 and 500, respectively, remained undestroyed after the processing. The degradation of AA occurred also during storage:

Table 1.2. Retention (%) of AA and AP in moist pellets during processing and storage

	Storage period				Storage period			
	Before pelleting	After pelleting	1 day	5 days	Before pelleting	After pelleting	1 day	5 days
	AA 50*1				AA 500*2			
Moisture(%)	14.3	43.8	43.9	41.8	13.1	42.8	42.1	41.3
AA	100	57.2	N. D.*5	N. D.	100	94.0	20.4	N. D.
DAA	N. D.	28.8	7.5	N. D.	N. D.	6.0	15.1	N. D.
Total*6	100	86.0	7.5	0	100	100	35.5	0
	AP 50*3				AP 500*4			
Moisture(%)	14.3	43.8	43.1	43.3	13.1	42.5	42.6	42.8
AP	100	92.5	84.5	72.5	100	100	90.7	88.4
AA	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.
DAA	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.
Total	100	92.5	84.5	72.5	100	100	90.7	88.4

Each value was mean of 3 determinations. Retention values were obtained on the basis of mole/dried weight.

*1 AA was added at a ratio of 50mg/100g basal diet.

*2 AA was added at a ratio of 500mg/100g basal diet.

*3 AP was added at a ratio of 50mg AA eq./100g basal diet.

*4 AP was added at a ratio of 500mg AA eq./100g basal diet.

*5 Not detected.

*6 Total ascorbate contains AP, AA and DAA.

Table 1.3. Retention (%) of AA and AP in dried pellets during processing and storage

	Storage period (days)					Storage period (days)					
	Before pelleting	After drying	1	5	10	Before pelleting	After drying	1	5	10	30
	AA 50*1					AA 500*2					
Moisture (%)	14.3	3.6	3.8	5.0	5.0	13.1	4.4	5.2	6.2	9.0	10.0
AA	100	5.5	5.5	4.0	3.8	100	38.2	29.3	28.4	21.4	16.0
DAA	N. D.*5	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.	0.1	1.4	1.4
Total*6	100	5.5	5.5	4.0	3.8	100	38.2	29.3	28.5	22.8	17.4
	AP 50*3					AP 500*4					
Moisture (%)	14.3	4.8	4.7	4.4	6.5	13.1	6.3	6.9	7.7	9.0	10.0
AP	100	71.8	71.3	68.9	69.1	100	95.5	94.9	94.0	91.8	90.2
AA	N. D.	7.5	7.0	4.4	trace	N. D.	1.9	2.5	1.9	1.4	0.9
DAA	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.	N. D.
Total	100	79.3	78.3	73.4	69.1	100	97.4	97.4	95.9	93.2	91.1

Each value was mean of 3 determinations. Retention values were obtained on the basis of mole/dried weight.
*1AA was added at a ratio of 50mg/100g basal diet.
*2AA was added at a ratio of 500mg/100g basal diet.
*3AP was added at a ratio of 50mg AA eq./100g basal diet.
*4AP was added at a ratio of 500mg AA eq./100g basal diet.
*5Not detected.
*6Total ascorbate contains AP, AA and DAA.

in the diets AA 50 and 500, 30.9% (from 5.5 to 3.8% of supplemented amounts) and 44.0% (38.2 to 21.4%) of AA remained at the beginning of storage was lost during the following 10 days' storage, respectively. The lost of AA during the 30 days' storage was more than 50% in the diet AA 500. No DAA was detected after drying process and the subsequent storage except for the diet AA 500 after 5 days' storage (0.1 to 1.4%). AP showed much higher stability than AA in drying process and storage. The loss of AP in the diets AP 50 and 500 during drying process was only 28.2 and 4.5%, respectively. The degradation of AP in the storage was slow and the degradation ratios during 10 days' storage were 3.8% (71.8% to 69.1%) in the diet AP 50 and 3.9% (95.5% to 91.8%) in the diet AP 500. The lost of AP during the 30 days' storage was only 5.6% (95.5% to 90.2%) in the diet AP 500. AA was detected in AP 50 and 500 diets after the drying process but AA in such diets was diminished during the storage period.

Stability of AA and AP in fish mince

Table 1.4 shows the retention of AA and AP in minced fish. Initial AA and AP were measured after 5 minutes mixing of each ascorbate with the mince. About 7% of AA added was oxidized to DAA even in the 5 minutes mixing. DAA contents in the diet AA 100 showed 10.9% after 1.5 hours and declined to 3.6% after 24 hours. The loss of AA in diet AA 100 was intense and more than 50% of AA was destroyed to inactive forms during the 24 hours after the beginning of storage. After 6

Table 1.4. Retention (%) of AA and AP in minced fish

	After mixing	Hours 1.5	6	24	After mixing	Hours 1.5	6	24
	AA 100*1				AA 100*2			
AP	—	—	—	—	100	87.2	72.7	23.3
AA	93.3	73.1	55.3	45.7	N. D.*3	8.8	17.6	60.5
DAA	6.7	10.9	9.2	3.6	N. D.	2.0	1.5	2.0
Total	100	84.0	64.5	49.3	100	98.0	91.8	85.8

Each value was mean of 3 determinations.
*1 AA was added at a ratio of 100mg/100g.
*2 AP was added at a ratio of 100mg AA eq./100g.
*3 Not detected.

hours, 72.7% of AP remained in the diet AP 100, while AP was rapidly hydrolyzed within the next 18 hours. Most of AP (60%) was converted to AA during the 24 hours' storage. The retention of total ascorbate (AP + AA + DAA) in the diet AP 100 was 85.7% after 24 hours' storage and this value exceeded the value (49.3%) of the diet AA 100.

Discussion

It is difficult to define the rate of AA degradation in feeds uniformly, because the factors that define the degradation are varied such as time, moisture, oxygen, light, pH, temperature, heavy metals, raw materials, the way of processing, *etc.* However, it has been said that AA is very unstable in feeds.[13, 38, 39, 59] The present findings indeed showed the instability of AA; in contrast, AP showed remarkable stability.

Mima *et al.*[56] showed that AP is more stable than AA in solution as the endiol structure is protected by phosphate (Fig. 1.2). Indeed, AP showed much higher stability than

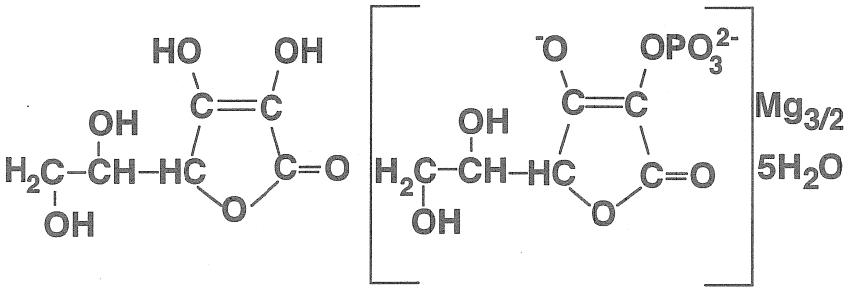

Fig. 1.2. Structure of L-ascorbic acid and magnesium-L-ascorbyl-2-phosphate.

AA in solution in the present investigation. AA solution colored brown as time passed but AP solution did not even after 180 weeks' storage. The coloration may have resulted from the formation of degraded compounds derived from AA.

As shown in Table 1.2, considerable amount of AA was oxidized to DAA during processing. Degradation of AA was very rapid in moist pellets especially in low level supplementation (AA50) and the retention of AA after 1 day was extremely low. Therefore it may be recommended not to leave moist pellets for a long time. AP showed excellent retention in moist pellets during processing and storage and was degraded slowly. Judging from the no detection of AA and DAA in the most AP diets, the release of AA from AP may have not occurred or been very slow even if any. Extraction was repeated 4 times in each sample in the present investigation; nevertheless, AP seemed to be left in the residue of extract since trace amount of AP was detected in the 5th to 7th extractions. These findings suggest that AP was tightly combined to some ingredients in the diet. Shigueno and Itoh[22] also suggested the absorption of AP to the ingredients of shrimp diet. High polarity of the phosphate group in AP may participate in the combination of AP to other components. Prolonged storage caused the slight decrease of AP, possibly resulting from the decreased recovery of AP from the diets.

The decrease in AA levels during the storage in the dried diets AA 50 and 500 was slow but drying process destroyed AA intensively (Table 1.3). The retentions of AA in the diets AA50 and 500 after the drying process were 5.5 and 38.2%, respectively. Soliman *et al.*[38] showed the retention of AA after drying was 33.5% in the diet supplemented AA 125mg per 100g of basal diet. Shigueno and Itoh[22] reported that the AA retention was about 9.7% after processing of shrimp diets supplemented 300mg AA per 100g diet. The retention after feed processing depended on the conditions employed. From the results, more than 10 times of AA is needed to sustain practical level of ascorbate (50 to 100mg /100g) after processing and storage.

AP in the dried pellets was much more stable than AA during processing and storage. AA was detected in AP diets after processing and decreased as the storage time prolonged. Upon considering the no increase of AA in the dried AP diets, the degradation of AP during storage seems to be zero or trace. It is possible that the slight decrease of AP during storage was the result of the combination of AP to the components of the ingredients.

The oxidation of AA occurred rapidly in the minced fish and half of AA was diminished after 24 hours' storage in the present investigation. Sueki *et al.*[60] also reported that the retention of AA in moist pellets was 50% and 10% after 3 and 9 hours' storage, respectively. Therefore, moist pellets containing raw fish should be used up immediately after mixing with additional vitamins.

The stability of AP itself in minced fish was rather poor. The decline of AP was high after 6 to 24 hours and considerable amount of AA was formed instead. It seems that the decline of AP in the mince was brought by the activity of phosphatase in the mince, since phosphatases are ubiquitous in the body of animals. Some participation of bacterial enzyme is also considered, because it took several hours to accelerate the degradation. There have been a few reports on hydrolysis of AP by phosphatases: *e. g.* Mima *et al.*[56] found the hydrolysis of AP by the phosphatases in homogenate of guinea pig liver and intestine. The hydrolysis of AP by phosphatases is discussed in detail in the CHAPTER 3. AA converted from AP is expected to degrade to inactive forms, if the storage period is prolonged. In spite of the

susceptibility of AP to hydrolysis, addition of AP postponed the retention of ascorbate by the time lag of AP hydrolysis to AA.

These findings, showing the stability of AP during processing and storage of feeds, indicated that AP is more profitable than AA to use as one of ascorbate sources if AP has a vitamin C activity. The activity of AP as a source of vitamin C is discussed in the next chapter. It should take care of the ingredients of feeds such as raw fish enriched phosphatases when AP is used because AP was found to be very susceptible to phosphatases in the present investigation.

CHAPTER 2

Physiological Activity of Ascorbyl-2-phosphate in Rainbow Trout

L-Ascorbyl-2-monophosphate (AP) was found to show excellent stability in solution and fish feeds as described in the previous chapter. In this chapter, the author evaluated the efficiency of AP as a vitamin C source in rainbow trout feed on a basis of deficiency symptoms and discussed the effect of massive intake of AP on the blood character of fish. Further investigation was carried out to confirm a physiological activity of AP as a vitamin C from such a novel point of view as carnitine and lipid metabolism.

Section 1. Utilization of Ascorbyl-2-phosphate in Rainbow Trout as a Dietary Vitamin C Source

It has been well established that many species of cultured fish require a dietary source of L-ascorbic acid (AA) for normal growth.[9, 11, 14, 15, 17, 19, 25, 58, 61] AA is readily oxidized to an irreversible form, diketogulonic acid and its degraded products, during processing and storage of feeds because of its chemical instability. Therefore, it should be included in feeds at levels in excess of the stated requirement to compensate for the considerable losses during processing and storage.

Several AA derivatives, which are more stable than AA, have been tested for their biological activity as a vitamin C source. For instance, L-ascorbyl-2-sulfate, L-ascorbyl-6-palmitate, L-ascorbyl-2-polyphosphate and AP prevented scurvy in channel catfish.[43, 45] AP has also been reported to have biological activity in kuruma prawn *Penaeus japonicus* and to be more stable than AA in prawn diets.[22] The author also confirmed the stability of AP in fish feed (CHAPTER 1). There have been no reports on the evaluation of AP activity as a vitamin C in rainbow trout. If AP is hydrolyzed to AA in the fish body, it certainly becomes a useful source of vitamin C. The author describes an evaluation of the efficiency of AP as a vitamin C source for rainbow trout in this section.

Materials and Methods

Fish and feeding methods

Rainbow trout (*Oncorhynchus mykiss*) eyed eggs were obtained from Shiga Prefectural Trout Station, Samegai and hatched out in the laboratory. The fry were supplied initially with ground beef liver and a commercial crumble diet (Nihon Nosan Kogyo Inc., Tokyo). After 8 weeks, the fish (average body weight 0.65g) were divided into 9 groups of 130 individuals each and each group was kept in an aquarium (50*l*) provided with continuous supply of well water (16℃) flowing at a rate of 1.5*l*/minute. The tested vitamin C sources were L-ascorbic acid (Nacalai Tesque, Inc., Kyoto) and magnesium L-ascorbyl-2-phosphate (Showa Denko Inc., Tokyo) referred as AA and AP, respectively. The experimental diets (Table 2.1) contained either no vitamin C (AA-free basal control diet) or were supplemented with either 10, 20, 50 or 200mg of AA or equimolar amounts of AP (22, 43, 108 and 431mg, respectively) per 100g of dry diet. Each of the diet powders (100g) was mixed well with 60m*l* of water and made into pellets. Each pelleted diet was sealed in a plastic bottle and stored in a freezer at -80℃. All diets were prepared every 2 weeks.

Before initiation of the experiment, each group of fish was given the AA-free diet for one week to accustom them to the experimental diet. Then each group was fed their prescribed experimental diet to satiation, 3 times a day, 6 days per a week. Every 2 weeks, each group was weighed and the aquarium was cleaned. After 9 weeks, each group was separated and kept in the duplicate aquaria to prevent overcrowding and cancel the effect of aquarium

Table 2.1. Composition of basal diet

Ingredients	%
Milk casein (vitamin-free)	53
Dextrin	16
α-Starch	15
α-Cellulose	5
Soybean oil	3
Pollack liver oil	2
Mineral mixture*1	4
Vitamin mixture (AA-free)*2	1
L-Tryptophan	0.5
L-Methionine	0.5

*1 The formula of mineral mixture was the same as reported by Halver *et al.* (1969).

*2 Supplied the following amounts per 100g diet; thiamine-HCl 5mg, riboflavin 20mg, nicotinic acid 40mg, calcium pantothenate 50mg, vitamin B_{12} 0.01mg, menadione 4mg, biotin 0.5mg, pyridoxine-HCl 5mg, inositol 200mg, choline inositol 200mg, choline chloride 500mg, folic acid 1.5mg, vitamin A acetate 2000 IU, α-tocopherol acetate 20mg. α-Cellulose was used as a carrier for the vitamin mixture.

position. At the end of the feeding experiment, 10 fish were sampled randomly from each group for the analysis of AA concentration in plasma and liver. The other fish were killed by an overdose of MS-222 and stored at -80℃ for the analysis of collagen and other components.

Determination of AA

AA was determined by the high-performance liquid chromatography (HPLC) using a reversed-phase column (NUCLEOSIL $5C_{18}$, 4.6 ×150mm, Waken, Kyoto) and an electrochemical detector (model L-ECD-6A, Shimadzu). The detector was set at +800mv *vs.* Ag/AgCl. The mobile phase was a 0.1M NaH_2PO_4 solution containing 1mM ethylenediaminetetraacetate (EDTA) and 0.15% (w/v) metaphosphoric acid, the pH of which was adjusted to 3.7 with H_3PO_4. The flow rate was 0.4m*l*/minute. The column and detector temperature was 40℃.

Rainbow trout were killed and their livers were immediately removed and weighed. A weighed portion of each liver (0.1 to 0.2g) was suspended in 3m*l* of cold 5% metaphosphoric acid and homogenized with an ultrasonic cell disrupter (model MS-50, Microson) for 30 seconds under cooling in an ice bath. After centrifugation of the homogenate, the resulting supernatant was filtered through a 0.45 μm syringe filter (DISMIC-13cp, Advantec). The filtrate was injected into the HPLC system. Each blood sample was obtained by severing the caudal fin and collected in a heparinized capillary tube for hematocrit measurement. Plasma was obtained after centrifugation. The plasma (15 to 20 μ*l*) was mixed well with 5 to 10-fold of cold 5% metaphosphoric acid and filtered through a 0.45 μm syringe filter. The filtrate was injected into the HPLC system.

Determination of hydroxyproline (Hyp) and proline (Pro) in collagen

Acid soluble collagens from the skin and bone were isolated by the method of Sato *et al.*[62]. The bone was decalcified in 0.5M EDTA (pH 7.5) for 48 hours before collagen extraction. The purified collagen was hydrolyzed in a sealed tube with 6N HCl at 130℃ for 3 hours. Hyp and Pro in the hydrolysate were determined by the methods of Woessner[63] and Troll and Lindsley[64], respectively.

Determination of glycogen concentration

Concentrations of glycogen in liver and ordinary muscle were determined by the methods described by Yoshinaka and Sato[65].

Statistical analysis

The data were analyzed statistically by Student's t test, Duncan's multiple range test and paired t-test on personal computers (PC-9801, NEC and Macintosh LCII, Apple).

Results

The fish fed on the AA-free diet began to show typical signs of scurvy, such as lordosis, scoliosis, hypodermal bleeding and exophthalmus, at the 9th week of rearing. In addition, the fish of this group began to show a loss of appetite at the end of the 12th week of rearing, resulting in cessation of their growth. In contrast, the fish fed AP-supplemented diets as well as those fed AA-supplemented diets showed excellent growth and absolutely no vitamin C deficiency signs.

Weight gain, feed efficiency, hepatosomatic index and hematocrit value are presented in Table 2.2. The AA-free group showed a lower weight gain and feed efficiency and a significantly ($p<0.05$) higher hepatosomatic index than any of the other groups. Hematocrit values of the AA 20mg- and AP 43mg-supplemented groups had a tendency to be lower than those of the other groups.

The proximate composition of the muscle was determined in each group after 15 weeks. There were no remarkable differences in the percentages of moisture, crude protein, crude lipid and crude ash among the groups (Table 2.3).

The AA concentrations in the liver and plasma are presented in Table 2.4. No detectable amount of AP was found in the liver and plasma of AP-supplemented groups. The fish fed the AA-free diet had a very low AA concentration in the liver. The AA concentration in the liver of the AP 22mg-supplemented group was significantly ($p<0.01$) higher than that of

Table 2.3. Proximate composition of ordinary muscle of rainbow trout fed on the experimental diets for 15 weeks (%)

Experimental group	Moisture	Crude protein	Crude lipid	Crude ash
AA-free	78.6	18.7	2.3	1.2
AA-10mg	77.2	20.2	2.3	1.3
AP-22mg	77.4	20.3	2.1	1.2
AA-20mg	77.7	19.8	2.7	1.2
AP-43mg	77.3	20.4	2.3	1.2
AA-50mg	78.0	19.9	2.0	1.2
AP-108mg	77.8	20.8	1.9	1.2
AA-200mg	78.0	20.0	1.8	1.2
AP-431mg	77.5	20.7	1.9	1.2

Each value is the mean of 3 determinations of a pooled sample consisted of 10 fish.

Table 2.2. Weight gain, feed efficiency, hepatosomatic index and hematocrit value of rainbow trout fed on the experimental diets for 15 weeks

Experimental group	Initial stage		Final stage		Weight gain (%)	Feed efficiency (%)	Hepato-somatic index* (%)	Hematocrit value* (%)
	No. of fish	Av. body weight (g)	No. of fish	Av. body weight (g)				
AA-free	130	0.64	121	7.53	1030	69.3	1.5 ± 0.3^{a}	$41.7\pm4.1^{a,b}$
AA-10mg	130	0.56	116	9.90	1478	72.5	1.3 ± 0.3^{b}	$45.3\pm6.4^{b,c}$
AP-22mg	130	0.65	119	9.26	1263	73.9	1.2 ± 0.2^{b}	$41.3\pm6.3^{a,b}$
AA-20mg	130	0.60	113	10.57	1440	76.5	1.2 ± 0.3^{b}	39.0 ± 2.4^{a}
AP-43mg	130	0.65	120	9.37	1245	71.4	1.3 ± 0.3^{b}	$39.9\pm2.6^{a,c,d,e}$
AA-50mg	130	0.62	120	9.65	1341	74.0	1.3 ± 0.3^{b}	45.5 ± 5.1^{b}
AP-108mg	130	0.68	113	9.66	1134	68.5	1.3 ± 0.2^{b}	$43.4\pm5.5^{a,b}$
AA-200mg	130	0.63	126	8.84	1260	71.0	1.4 ± 0.2^{b}	$43.1\pm3.1^{b,d}$
AP-431mg	130	0.68	116	9.76	1196	71.1	1.4 ± 0.2^{b}	$43.6\pm4.0^{b,e}$

* Values are means ± S. D. of 10 fish. Means in the same column with fifferent superscripts are significantly different ($p<0.05$).

Table 2.4. Concentration of ascorbic acid in the liver and plasma and of glycogen in the muscle and liver of rainbow trout fed on the experimental diets for 15 weeks

Experimental group	Liver AA (μg/g)*	Plasma AA (μg/ml)*	Muscle glycogen (mg/g)*	Liver glycogen (mg/g)*
AA-free	0.5± 0.1	trace	9.7±4.2^{a}	24.4±11.2^{a}
AA-10mg	107.5±20.3^{a}	5.7± 2.2^{a}	4.5±1.4^{a}	39.0±13.1^{b}
AP-22mg	136.7±19.0^{b}	5.9± 2.3^{a}	5.2±3.5^{a}	54.0± 5.3^{b}
AA-20mg	201.1±25.7^{c}	15.2± 3.9^{b}	3.3±2.0^{a}	60.5±14.0^{b}
AP-43mg	224.4±39.4^{c,d}	26.9± 8.7^{c}	2.5±1.2^{a}	60.9±21.6^{b}
AA-50mg	222.9±48.2^{c,d}	34.8± 8.8^{c,d}	3.5±2.8^{a}	50.3±18.0^{b}
AP-108mg	230.0±39.8^{c,d}	28.9±11.6^{c,d}	2.5±2.7^{a}	44.7±17.7^{b}
AA-200mg	235.6±42.5^{c,d}	34.3±10.1^{c,d}	5.0±2.8^{a}	54.5±19.1^{b}
AP-431mg	241.7±34.2^{d}	42.5±13.2^{d}	4.3±1.4^{a}	68.2±16.4^{b}

* Values are means±S. D. of 10 fish. Means in the same column with different superscripts are significanntly different ($p<0.01$).

AA 10mg-supplemented group. However, no significant differences were observed in the AA concentrations in the liver among AA 20, 50 and 200mg-, and AP 43 and 108 mg-supplemented groups. Similarly, the AA concentrations in the plasma of fish fed AA-free diet were below detectable limits. However, the plasma AA concentrations were raised according to the increase in the dietary AP or AA concentrations. There were no significant differences in the plasma AA concentration between the AA-supplemented group and the group supplemented with an equimolar amount of AP except for the AA 20mg- and AP 43mg-supplemented groups. The plasma AA concentration of the AP 43mg-supplemented group was significantly ($p<0.01$) higher than that of the AA 20mg-supplemented group.

Glycogen concentrations in the ordinary muscle and liver are also listed in Table 2.4. Liver glycogen concentrations in AA-free diet group were significantly ($p<0.01$) lower than that of other groups reflecting the poor appetite in the group.

The molar ratios of Hyp to Pro in acid soluble collagens extracted from the skin and bone are presented in Fig. 2.1. The molar ratios in collagens of the skin and bone were significantly ($p<0.01$) lower in the AA-free group than noted in the other diet groups. No differences in the ratios were observed among the other 8 groups.

Discussion

No signs of scurvy were found in any of the AP-supplemented groups. The AA concentrations in the liver and plasma of AP-supplemented groups were slightly higher than or almost the same as those of the AA-supplemented groups. No AP could be detected in the liver and plasma of fish fed AP-supplemented diets. This result indicated that AP was readily converted to AA in the rainbow trout body and that it had a physiological activity equivalent to AA. The ratio of Hyp to Pro in collagens isolated from the skin and bone was used as an indicator of AA status in the fish body together with the concentration of AA, because the ratio decreased before the fish develop the apparent AA-deficiency signs.[58, 61] Statistical differences in the ratios were not found among AP- and AA-supplemented groups. The ratios for the AA-free group, which exhibited scurvy signs, were lower than those for the other groups. This result indicates that sufficient AA to biosynthesize normal collagen was supplied in the AP-supplemented groups.

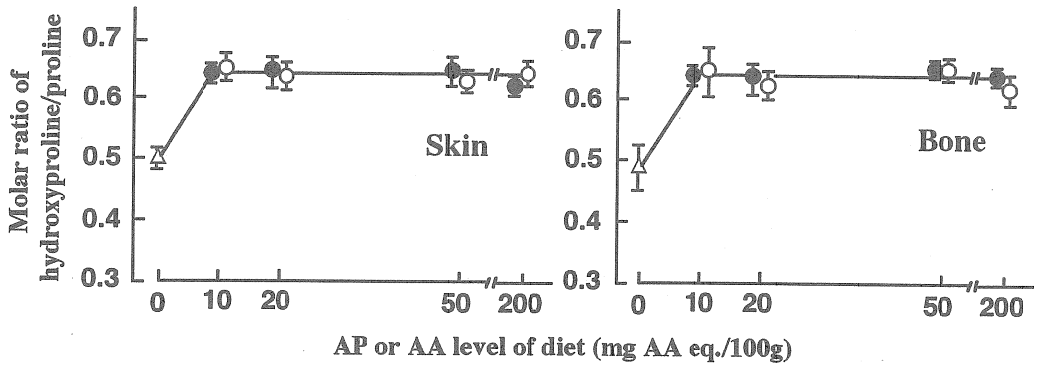

Fig. 2.1. Molar ratios of hydroxyproline to proline in collagens extracted from the skin and bone of the fish fed diets contaning various levels of AP or AA. ●, AP; ○, AA; △, AA-free. Each value is the mean±S. D. of 5 fish.

The AA concentrations in the liver and plasma of AP-supplemented groups had a tendency to be higher than those of AA-supplemented groups. From these findings, it was postulated that the AA in the diet was partially lost during diet processing and storage. The author therefore evaluated the contents of AA or AP in the diets formulated for the present feeding experiment by the HPLC method described in the previous chapter. When AA was added to the diet at a level of 50mg per 100g of diet, about 50% of the added AA was oxidized to dehydroascorbic acid (DAA) during diet preparation but total amount of AA and DAA was retained almost completely (>98%). After the storage at -80℃ for 2 weeks, AA and DAA in the diets remained unchanged. In contrast, when AP was added to the diet at a level of 108mg (50mg AA eq.) per 100g of the diet, more than 99% of AP was retained as AP in the diet after 2 weeks. These results indicate that the AA-supplemented groups received a considerable amount of DAA in the present feeding experiment. DAA is thought to be equivalent to AA for humans[32] and rainbow trout[34]. It seems therefore that the degradation of AA to DAA in the diet did not affect the AA concentration in the fish body. Juvenile rainbow trout was used in the present experiment, but Sato *et al.*[34] used adult rainbow trout for the evaluation of DAA. Efficacy of DAA in young fish may be inferior to that in adult fish. Consequently AP is superior to AA as a vitamin C source especially in a situation where high rate of AA oxidation to DAA is expected such as a low level supplementation to feeds.

Weight gain and feed efficiency had a tendency to be high in AA-supplemented groups compared to the AP-supplemented groups and a tendency to decrease with the increase of supplemented AA or AP level. But Brandt *et al.*[43] reported that the average weight gain was higher in channel catfish fed diets with AP than in those fed diets with AA. Further study is necessary to clarify this situation. The poor growth and feed efficiency of the AP 108mg-supplemented group resulted from the accidental loss of 10 fish during the feeding experiment.

In conclusion of this section, AP was readily converted to AA in the rainbow trout body and exhibited a physiological activity equivalent to AA.

Section 2. Effect of Massive Intake of Ascorbyl-2-phosphate on Rainbow trout

There are many reports that showed positive effect of high level supplementation of AA to the feeds on terrestrials and fish. Massive intake of AA enhances antibody production, complement activity and resistance to disease and stress of fish.[24-27] AA requirement of cultured fish increases as the culture density rises[23]; this is the reason why high doses of AA have been so far recommended to nurse healthy fish.

AP exhibited the excellent stability and physiological activity equivalent to AA (CHAPTER 1 and CHAPTER 2; Section 1) and is expected to be used as an alternate source of vitamin C. It is important to evaluate the safety of AP supplemented at high level to feed. Acute toxicity of AP magnesium salt and AA were investigated for rat and mouse; LD_{50} of AP and AA per os was more than 10g/kg for both animals.[56] Chronic toxicity of AP for mouse fed on the diet contained massive AP (2g/kg·day) was not observed.[56] But there are no data available on the massive dose of AP for fish. In this section, the author investigated the effect of massive intake of AP on rainbow trout.

Materials and Methods

Fish and feeding methods

Rainbow trout *(Oncorhynchus mykiss)* of 2 years old, hatched out and reared by feeding on a commercial diet (Nihon Nosan Kogyo Inc., Tokyo) in the laboratory tanks, was used. The fish were divided into 4 experimental groups of duplicated aquariums (50*l* each). Ten fish were accommodated to one aquarium. Each aquarium was provided with continuous supply of well water (16℃) flowing at a rate of 1.5*l*/minute. L-Ascorbyl-2-monophosphate (AP) magnesium salt and AA used were obtained from Showa Denko Inc., Tokyo and Nacalai Tesque, Inc., Kyoto, respectively. The experimental diets were formulated from the basal casein diet (Table 2.5) supplemented with either 10 or 1000mg of AA or equimolar amounts of AP (22 and 2156mg, respectively) per 100g of dry diet. Each diet powder (100g) was mixed well with 60m*l* of water and made into pellets. Each pelleted diet was sealed in a plastic bottle and stored in a freezer at -80℃. All diets were prepared every 2 weeks. Each group was fed on the prescribed experimental diet once a day, 6 days per a week. Feeding amount was 2g/fish (till 20 days) or 2.5g/fish (after 20 days) and the feeding period was 44 days. At the end of the feeding experiment, 5 fish were sampled randomly from each group to measure AA and AP concentrations, blood parameters and activity of acid phosphatase in the liver.

Table 2.5. Composition of basal diet

Ingredients	%
Milk casein (vitamin-free)	52
Dextrin	15
α-Starch	15
α-Cellulose	5.14
Soybean oil	3
Pollack liver oil	2
Mineral mixture*1	5
Vitamin mixture (AA-free)*2	1.56
L-Cysteine	0.9
L-Arginine	0.4

*1 The formula of mineral mixture was the same as reported by Halver *et al.* (1969).

*2 Supplied the following amounts per 100g diet: thiamine-HCl 5mg, riboflavin 20mg, nicotinic acid 40mg, calcium pantothenate 50mg, vitamin B_{12} 0.01mg, menadione 4mg, biotin 0.5mg, pyridoxine-HCl 5mg, inositol 200mg, choline chloride 500mg, folic acid 1.5mg, vitamin A acetate 2000 IU, α-tocopherol acetate 20mg. α-Cellulose was used as carrier for the vitamin mixture.

Determination of AA and AP

AA and AP were determined by the high-performance liquid chromatography (HPLC) us-

ing a reversed-phase column (COSMOSIL $5C_{18}$-AR, 4.6×300mm, Nacalai Tesque). The detector was Hitachi L-4000 UV Detector set at 254nm. The mobile phase was a 0.1M phosphate buffer (pH 3.0) and the flow rate was 0.4m*l*/minute. The liver sample (about 0.2g) was homogenized with 1m*l* of 5% metaphosphoric acid by ultrasonic cell disrupter (MS-50, Microson) under cooling in an ice bath. After centrifugation of the homogenate, the resulting supernatant was filtered through a 0.45μm syringe filter. The filtrate was injected into the HPLC system described above. Each blood sample was obtained by severing the caudal fin and collected in a heparinized capillary tube for hematocrit measurement. Plasma was obtained after centrifugation. The plasma was mixed with cold 5% metaphosphoric acid solution and filtered through a filter. The filtrate was injected into the HPLC system.

Blood analysis

The blood collected from the severed caudal fin was centrifuged without heparin and the serum sample was obtained. Serum analysis was carried out using a commercial kit (RaBA 3010 UNIKIT, CHUGAI, Tokyo) on concentration of hemoglobin, BUN-S, total protein, iron, phosphorus and calcium and the activity of alkaline phosphatase, GPT and GOT.

Acid phosphatase activity

The liver sample was homogenized with 5 volumes of 0.9% sodium chloride solution by glass homogenizer for 5 minutes and ultrasonic disrupter for 1 minute. Reaction mixture (pH 5.0, 3.05m*l*) contained 1mM *p*-nitrophenylphosphate disodium salt, 200mM acetic acid and 50μl of the liver homogenate. The mixture was incubated at 30℃ for 10 minutes, added with 10m*l* of 0.4N sodium hydroxide to stop the reaction and monitored the absorbance at 405nm.

Proximate composition

Lipid was estimated by the method of Bligh and Dyer[66]. Micro-Kjeldahl's method was used for the determination of nitrogen and multiplied by 6.25 to measure protein content.

Statistical analysis

The data were statistically analyzed by Duncan's multiple range test after an analysis of variance (ANOVA).

Results and Discussion

Table 2.6 shows the growth, the condition factor and the feed efficiency after the 44 days feeding. No statistical differences were found among the four groups on these values. All fish of all groups showed normal growth without any abnormal appearance.

Table 2.7 shows the concentrations of AA and AP in the plasma and liver. AA concentrations of AA-10mg and AP-22mg groups were significantly ($p<0.01$) lower than those of AA-1000mg and AP-2156mg groups, but there were no statistical differences ($p>0.05$) of AA concentration between the groups supplemented with AA and equimolar amounts of AP except the plasma AA concentration of AA-10mg and AP-22mg groups. Plasma AA level of AA-10mg group was slightly lower ($p\leqq0.05$) than that of AP-22mg group. This tendency, plasma AA concentration of AP supplemented (at low level) group was slightly higher than that of equimolar amounts of AA, was observed in the previous investigation as well (this CHAPTER; Section 1). There is no possibility that AA in the AA-10mg diet was oxidized to inactive compounds such as 2, 3-diketogulonic acid (this CHAPTER; Section 1). The cause of low plasma AA concentration in AA-10mg group is not clear from the results of this feeding experiment, however, it seems profitable to use AP instead of AA especially at low level supplementation to feed.

Table 2.6. Growth, feed efficiency and condition factor of the fish after 44 days feeding

Experimental group	Initial weight (g)	Final weight (g)	Final body length (cm)	Feed efficiency (%)	Condition factor
AA 10mg	102±10[1)]	148±42	21.1±1.2	60	15.7±3.7
AP 22mg	103±10	143±31	20.8±1.1	62	15.9±2.5
AA 1000mg	103±11	150±21	21.2±1.1	63	15.9±1.8
AP 2156mg	102±11	146±40	21.8±1.8	63	15.4±1.2

[1)]Mean±S. D., Each value is the mean of 10 fish.

Table 2.7. Concentration of AA and AP in the plasma and liver

Experimental group	AA consentration		AP concentration	
	Plasma (μg/ml)	Liver (μg/g)	Plasma	Liver
AA 10mg	[1)]1.4± 1.2[a,b]	140.5±28.2[a,b]	N. D.[2)]	N. D.
AP 22mg	4.2± 2.6[c,d]	160.6±26.7[c,d]	N. D.	N. D.
AA 1000mg	121.1±13.1[a,c]	405.3±45.9[a,c]	N. D.	N. D.
AP 2156mg	126.7±25.1[b,d]	343.5±40.9[b,d]	N. D.	N. D.

[1)]Mean±S. D.,[2)] Not detected (below about 0.1 μg/ml or g)
Each value is the mean of 5 fish.
Means with same superscripts in the same column are significantly different ($p<0.01$).

No AP (below the detectable limit, about 0.1 μg/ml or g) was found in the plasma and liver of all groups including the group received massive amount of AP (Table 2.7). This indicates the ready hydrolysis of AP to AA and no accumulation of AP in the fish body. Activity of AP hydrolysis will be discussed in the CHAPTER 3.

Results of blood analysis and activity of acid phosphatase in the liver are listed in Table 2.8. Concentrations of hemoglobin and iron and hematocrit value of AA-10mg group were significantly ($p<0.01$) lower than those of AA-1000mg group. It is well-known that AA enhances iron absorption from intestine in terrestrial animals.[1)] Dabrowski and Kϕck[67)] suggested that AA enhanced the absorption of iron in rainbow trout. Decrease of hemoglobin and iron concentrations and of hematocrit value in AA-10mg group may be due to the decreased absorption of iron from the intestine caused by the lower AA concentration in the body. Requirement of AA in rainbow trout for normal growth was reported to be 10mg/100g diet.[11, 58)] Considering the iron absorption parameters, requirement of AA in rainbow trout might be above 10mg/100g diet.

AA deficiency has been said to be associated with a decreased absorption of calcium in fish[68, 69)] but a negative data was also proposed[70)]. Effect of AA on calcium and phosphorus absorption and skeletal mineralization in fish is unclear.[1, 71)] Since analysis was not performed on the content of phosphorus in the diet used in the present feeding experiment, the value was estimated from the diet composition. Phosphorus content in 100g basal diet was about 800mg (600mg from mineral mixture and 200mg from casein). AP-2156mg diet exceeded AA-1000mg diet in phosphorus content about 180mg/100g derived from AP. Phosphorus from AP seemed to be not negligible in massive dose of AP, therefore the serum phosphorus content was measured. Serum phosphorus content of AP-22mg group was significantly ($p<0.01$) higher than that of other 3

groups and the ratio of calcium to phosphorus of AA-1000mg group was significantly ($p<0.01$) greater than that of other 3 groups (Table 2.8). These results can not be explained by the massive dose of phosphorus. Therefore the increases in serum phosphorus content in AP-22mg group and in Ca/P ratio in AA-1000mg group could be due to other factors unknown.

Hepatic acid phosphatase activity of AA-10mg group was significantly ($p<0.01$) higher than that of other groups (Table 2.8). AA deficiency enhances the activity of acid phosphatase in the liver of guinea pig.[72] This increase in phosphatase activity in AA-10mg group may be due to the latent deficiency of AA reflected in the tendency of decreased plasma AA concentration (Table 2.7).

There were no remarkable differences among the groups on the serum alkaline phosphatase, GPT and GOT activities and on the concentrations of BUN-S and total protein (Table 2.8). No remarkable differences of proximate composition of ordinary muscle were found among the groups (Table 2.9).

The above results indicate that massive dose of AP has no malign effect on rainbow trout as regards to growing, serum parameters and proximate composition of ordinary muscle and that rainbow trout accumulates no AP in the body even if the fish intakes massive AP. Moreover it is concluded that addition of AP instead of AA at low level supplementation to feed is more profitable.

Table 2.8. Results of blood analysis and activity of acid phosphatase in liver

Experimental group	Hgb (g/dl)	Ht (%)	ALP[2] (KA-U)	GPT (Karmen)	GOT (Karmen)	BUN-S (mg/dl)
AA 10mg	[1]9.6±0.7[a]	34.0±5.5[a]	7.1±4.8	19± 2	482±100	3.4±0.7
AP 22mg	10.6±2.3	37.5±1.7	7.8±2.2	30± 8	636±171	3.7±0.3
AA 1000mg	11.8±0.4[a]	42.0±6.2[a]	5.6±1.3	31±10	542±100	3.7±0.5
AP 2156mg	11.3±1.2	36.9±5.0	5.7±1.0	25± 6	606± 68	3.1±0.4

Experimental group	Protein (g/dl)	Fe (mg/dl)	P (mg/dl)	Ca (mg/dl)	Ca/P	Hepatic ACP[3] (n mol/mg·minute)
AA 10mg	3.9±0.2	87±29[a]	9.9±0.6[a]	14.9±1.9	1.4±0.1[a]	21.2±3.5[a,b,c]
AP 22mg	4.5±0.4	145±92	11.1±0.3[a,b,c]	16.0±0.5	1.4±0.1[b]	14.5±1.0[a]
AA 1000mg	4.3±0.3	167±32[a]	9.6±0.7[b]	15.8±1.4	1.6±0.1[a,b,c]	14.8±1.0[b]
AP 2156mg	4.0±0.4	140±45	9.7±0.8[c]	13.6±1.3	1.4±0.1[c]	12.1±2.4[c]

[1]Mean±S. D. [2]Alkaline phosphatase. [3]Acid phosphatase.
Each value is the mean of 5 samples except for Ht values (n=10).
Means with same superscripts in the same column are significantly diffrernt ($p<0.01$).

Table 2.9. Proximate composition of the ordinary muscle of the fish fed on the experimental dirts for 44 days

Experimental group	Moisture (%)	Crude ash (%)	Crude protein (%)	Crude lipid (%)
AA 10mg	77.3	1.2	21.8	1.3
AP 22mg	77.8	1.2	21.8	1.2
AA 1000mg	77.5	1.3	21.7	1.2
AP 2156mg	77.5	1.2	21.7	1.3

Each value is the mean of the triplicate determinations of a pooled sample composed of 5 fish of each group.

Section 3. Effect of Ascorbyl-2-phosphate and Ascorbic Acid on Lipid and Carnitine Metabolism in Rainbow Trout

Carnitine is essential for the β-oxidation of long-chain fatty acids in animal tissues. Long-chain fatty acids are transferred from an extramitochondrial site to the intramitochondrial site of oxidation in the form of carnitine esters.[73-77] Carnitine is synthesized from lysine and methionine in animals.[78-81] The biosynthesis is accomplished by a sequence of five enzymes. Two enzymes, ε-*N*-trimethyllysine hydroxylase and γ-butyrobetaine hydroxylase, require ascorbate as a cofactor for activity.[82-84] There has been several reports that indicated decreased carnitine level in scorbutic guinea pigs[85-92], but these results were not consistent with the site whose carnitine level was decreased. However, the participation of ascorbate in carnitine metabolism of terrestrials is strongly suggested.[93] There are no reports available about the effect of ascorbate on carnitine metabolism in fish. In this section, the author describes the effect of AA and AP on carnitine and lipid metabolism in rainbow trout.

Materials and Methods

Reagents

AP magnesium salt was obtained from Showa Denko (Tokyo). Acetyl coenzyme A (acetyl-CoA) sodium salt, L-carnitine and carnitine acetyltransferase (CAT)(EC 2.3.1.7) were obtained from Sigma Chemical Co. (USA). Other reagents without any remarks were of guaranteed grade, obtained from Nacalai Tesque (Kyoto).

Fish and feeding methods

Young rainbow trout *(Oncorhynchus mykiss)*, weighing 61g on average, were used. The fish were divided into 6 acrylic aquaria of 10 individuals each, and each aquarium (50*l*) was supplied with well water (16℃). Three experimental groups, AA-free, AA-supplemented and AP-supplemented groups, were set up. The experimental diets were prepared from the AA-free basal diet (Table 2.10) supplemented with 10mg of AA or equimolar amounts of AP (22mg) per 100g of dried basal diet. These diets are referred to as AA and AP, respectively. An AA-free diet is referred to as Free. Each diet powder (100g) was mixed well with 60m*l* of water and made into pellets. Each pelleted moist diet was stored at -80℃ until use. These diets were prepared every 10 days.

Before initiation of the experiment, each group of the fish was given the Free diet for a week. Then each group was fed the prescribed diet to satiation twice a day. After 60 days feeding, the fish were starved for 60 days. Before and after the starvation, five fish were sampled randomly from each group to analyze carnitine, AA and lipid.

Table 2.10. Composition of basal diet

Ingredients	%
Milk casein (vitamin-free)	52
Dextrin	15
α-Starch	15
α-Cellulose	5.14
Soybean oil	3
Pollack liver oil	2
Mineral mixrure*1	5
Vitamin mixture (AA-free)*2	1.56
L-Cysteine	0.9
L-Arginine	0.4

*1The formula of mineral mixture was the same as reported by Halver *et al.* (1969).

*2Supplied the following amounts per 100g diet: thiamine-HCl 5mg, riboflavin 20mg, nicotinic acid 75mg, calcium pantothenate 50mg, vitamin B_{12} 0.01mg, menadione 4mg, biotin 0.5mg, pyridoxine-HCl 5mg, inositol 200mg, choline chloride 500mg, folic acid 1.5mg, vitamin A acetate 2000IU, α-tocopherol acetate 60mg. α-Cellulose was used as a carrier for the vitamin mixture.

Determination of carnitine

Free carnitine, acid-soluble total carnitine and long-chain acylcarnitines in the liver and ordinary muscle were determined by the high-performance liquid chromatography (HPLC) method based mainly on the procedures of Arakawa *et al.*[94], Williamson and Corkey[95] and Pearson *et al.*[96].

Extraction from tissues. Weighed (about 0.3g) portions of ordinary muscle and liver were homogenized in 8 volumes of 6% perchloric acid containing 20mM 2-mercaptoethanol with an ultrasonic disrupter. The homogenate was centrifuged at 5000×g for 10 minutes. The supernatant fraction was collected and 8 volumes of 6% perchloric acid was added to the residue. The residue was homogenized and centrifuged. The precipitated residue was used for determining long-chain acylcarnitines. The pooled supernatant was neutralized (pH 7.5) with potassium carbonate (3M) containing triethanolamine (0.5M) and centrifuged. The supernatant was filtered and used to determine free carnitine and acid soluble total carnitine.

Enzymatic reaction. The sample solution was filtered through a 0.45 μm filter and applied to the reaction. The reaction mixture (1.0ml) contained acetyl-CoA (0.1mM), CAT (1.3U), phosphate buffer (10mM, pH 7.5), EDTA (1mM), 2-mercaptoethanol (10mM) and the sample (0.5ml). The mixture was incubated at 25℃ for 30 minutes. The reaction was stopped by adding 11N phosphoric acid (0.1ml). The mixture was filtered through a 0.45 μm filter and 20 μl of it was applied to the HPLC for the assay of coenzyme A (CoASH) released. The reaction was performed twice per each sample. One was the complete reaction and the other was a blank test added with 10mM KH_2PO_4 (pH 7.5) instead of the CAT solution. Each reaction mixture was applied to the HPLC.

Free carnitine analysis. The filtered supernatant was reacted with the enzyme and the released CoASH was analyzed by the HPLC.

Acid soluble total carnitine analysis. The supernatant was adjusted to alkaline (pH 13) and incubated at 25℃ for 30 minutes to release free carnitine from acetylcarnitine and short-chain acylcarnitines on alkaline hydrolysis. The incubated solution was neutralized and analyzed for total free carnitine in the solution. This value contains free carnitine, acetylcarnitine and short-chain acylcarnitines (C_3-C_{10}) in the tissues.

Long-chain acylcarnitine analysis. The precipitated residue obtained from the perchloric acid extraction was suspended in 1N KOH and incubated at 55℃ for two hours. It is then cooled with ice, re-acidified with perchloric acid and centrifuged. The supernatant was neutralized by 3M K_2CO_3 containing 0.5M triethanolamine and assayed for free carnitine released from long-chain acylcarnitines ($C_{12}<$).

HPLC analysis. CoASH converted stoichiometrically from carnitine was analyzed on HPLC. The equipment for HPLC consisted of a pump, Hitachi 655 Liquid Chromatograph; a detector, Hitachi L-4000 U.V. Detector set at 254nm; and a processor, Hitachi 833 Data Processor. The column used was Cosmosil $5C_8$ 4.6 ×250mm (Nacalai Tesque; Kyoto) with 190mM KH_2PO_4-methanol (75:25, v/v) as solvent. The flow rate of the solvent was 0.7ml/minute. The amount of CoASH released in the reaction mixture was obtained by subtracting the amount of CoASH derived from the tissue (the value obtained from the enzyme blank test) from the amount of CoASH contained in the complete reaction mixture.

Determination of AA

Concentrations of AA in the liver and plasma were determined by the HPLC equipped an electrochemical detector described in the CHAPTER 2; Section 1.

Lipid analysis

Lipid contents of viscera and eviscerated whole body were estimated according to the method of Bligh and Dyer[66]. The extracted total lipid was fractionated into phospholipid + monoglyceride, diglyceride + cholesterol, free fatty acid, triglyceride and cholesterol esters. The extracted lipid was dissolved in chloroform-methanol (1:1) and spotted on a pre-coated thin-layer chromatography SIL G-25 (Macherey-Nagel). The spots were developed with a solvent of *n*-hexane: ethyl ether: acetic acid (85 : 15 : 1, v/v/v) The developed fractions of the lipid were quantified by a Shimadzu dual-wavelength thin layer chromato scanner CS-930 after coloration by copper acetate and phosphoric acid.

Protein content

Micro-Kjeldahl method was applied for the determination of nitrogen and multiplied by 6.25 to get protein content.

Statistical analysis

The data were statistically analyzed by Duncan's multiple range test after an analysis of variance (ANOVA) on personal computers (PC-9801, NEC and Macintosh LC II, Apple).

Results

Table 2.11. shows the change of body weight in the experimental period. There were no statistical differences ($p>0.7$) of growth among the three groups.

Table 2.11. Change in body weight in the experimental period (g)

Group	Initial[1)]	Before starvation[1)]	After starvation[2)]
Free	61.0±7.4	133.3±25.3	105.8±22.8
AA	61.0±7.9	133.9±20.7	105.2±21.5
AP	61.0±7.2	135.8±17.3	103.7±14.8

Values are the mean±S. D. ([1)], n=20;[2)], n=10).
There are no significant differences among the group ($p>0.7$, by Student's t-test).

Hematocrit value, condition factor and hepatosomatic index are listed in the Table 2.12. No changes in hematocrit values were observed before and after the starvation and no statistical differences were detected among the groups. Both condition factor and hepatosomatic index decreased ($p<0.01$) after the starvation, reflecting the emaciation by the starvation, but there were no statistical differences among the groups before and after the starvation.

The concentrations of AA in the liver, ordinary muscle and plasma of the Free diet group were significantly ($p<0.01$) lower than that of AA- or AP-supplemented groups (Table 2.13). The starvation resulted in the decrease of AA concentrations in the liver and muscle, but the differences were not significant. AA concentrations in the liver and muscle of AP-supplemented groups were slightly higher on average than those of AA groups, however, there were no statistical differences ($p>0.07$) between the groups. A tendency of lower AA level in AA group than in AP group was observed also in the experiments of the previous sections (CHAPTER 2; Section 1 and 2).

Proximate composition was listed in Table

Table 2.12. Hematocrit value, condition factor and hepatosomatic index

Group	Hematocrit value (%)	Condition factor	Hepatosomatic index (%)
Before starvation			
Free	40.7±4.8[a]	18.3±1.8[a]	1.7±0.3[a]
AA	42.1±3.7[a]	18.8±1.7[a]	1.5±0.4[a]
AP	46.4±2.6[a]	18.7±1.6[a]	1.6±0.2[a]
After starvation			
Free	44.9±3.5[a]	14.1±1.2[b]	0.8±0.1[b]
AA	40.6±9.0[a]	15.1±1.4[b]	0.7±0.1[b]
AP	42.1±8.3[a]	15.5±1.8[b]	0.7±0.04[b]

Figures bearing different superscript in the same column are significantly different ($p<0.01$).
Values are the mean±S. D. (n=5).

Table 2.13. Concentration of AA in liver, ordinary muscle and plasma

Group	Liver (μg/g)	Ordinary muscle (μg/g)	Plasma (μg/ml)
Before starvation			
Free	7.8± 3.9[a,b,c,d]	1.9± 1.7[a,b,c,d]	4.2±1.1[a]
AA	101.7±20.6[a,e]	15.7± 5.0[a,e]	10.2±3.6
AP	142.0±55.6[b,f]	20.9±10.7[b,f]	13.5±4.6
After starvation			
Free	9.1± 1.5[e,f,g,h]	2.3± 0.6[e,f,g,h]	trace
AA	70.1±14.4[c,g]	9.9± 4.0[c,g]	15.7±9.6
AP	90.2±16.2[d,h]	11.4± 4.1[d,h]	14.5±3.8[a]

Figures bearing common superscript in the same column are significantly different ($p<0.01$).
Values are the mean±S. D. (n=5).

2.14. There found no marked differences in crude protein and crude ash contents. Moisture increased a little during the starvation, but the differences among the group were not observed. Visceral lipid contents had a tendency to decline during the starvation, but the differences were not significant. Lipid contents in the eviscerated whole body were significantly ($p<0.01$) decreased in AA and AP groups during the starvation on the basis of g/body as well as g/100g wet; the decrease in Free diet group was not significant ($p>0.05$). This feature of change in lipid contents during the starvation is

Table 2.14. Proximate composition

Group	Visceral lipid		Eviscerated whole body lipid		Total whole body lipid (g/body)
	(g/100g wet)	(g/body)	(g/100g wet)	(g/body)	
Befor starvation					
Free	14.1±4.6	1.9±0.9	7.2±0.7[a]	7.7±1.2[a,b]	9.6±2.2[a,b]
AA	14.9±4.1	1.8±0.8	6.8±0.7[b]	8.0±1.9[c,d]	9.9±2.2[c,d]
AP	17.4±1.2	2.1±0.3	6.9±0.8[c,d]	8.1±0.7[e,f]	10.2±0.6[e,f,g]
After srarvation					
Free	12.6±4.7	1.3±0.8	5.9±0.9	6.2±1.2	7.5±1.9[e]
AA	13.8±5.1	1.2±0.2	5.4±0.6[a,b,c]	5.6±1.3[a,c,e]	6.8±1.5[a,c,f]
AP	12.2±4.2	1.4±0.3	5.2±0.8[d]	5.3±0.6[b,d,f]	6.6±0.5[b,d,g]

Group	Moisture (g/100g wet)		Crude protein (g/100g wet)		Crude ash (g/100g wet)	
	Viscera	Eviscerated whole body	Viscera	Eviscerated whole body	Viscera	Eviscerated whole body
Befor starvation						
Free	69.7	73.0	14.5	18.8	1.2	2.3
AA	70.2	73.4	14.6	19.6	1.2	2.6
AP	71.7	72.8	14.5	19.6	1.1	4.0
After srarvation						
Free	74.4	74.5	14.6	18.6	1.5	3.3
AA	74.9	74.3	14.6	19.5	1.4	2.6
AP	74.3	74.4	14.5	19.6	1.4	3.6

Figures bearing common superscript in the same column of lipid content are significantly different ($p<0.01$).
The calues of lipid content are the mean±S. D. (n=5).
The values of moisture, crude protein and crude ash are the mean of 3 determinatioons of a pooled sample consisted of 5 fish from each group (C. V. values are: Crude protein; <0.5%, Moisture; <1%, Crude ash; <2%).

indicated typically in the column of total lipid content in the whole body . Among the three groups, differences of lipid contents were not significant.

Composition of the lipid extracted from the viscera and the eviscerated whole body are shown in Table 2.15. The ratio of free fatty acid to the visceral lipid was high in AP group before the starvation. The ratio of cholesterol ester to the visceral lipid was significantly lower in Free group after the starvation than in other groups. The ratio of cholesterol ester to the lipid extracted from the eviscerated whole body of AP group had a tendency to be lower before the starvation than those of Free and AA groups, and was significantly increased during the starvation. No remarkable differences were found in the other values of lipid composition.

Carnitine concentrations in the liver and the ordinary muscle are shown in Table 2.16 and 2.17, respectively. Difference was not significant among the experimental groups in the free and acid soluble total carnitine concentrations in the liver. The starvation raised the free and acid soluble total carnitine concentrations on the basis of μg/g scale but reduced them on the basis of μg/liver scale. Long chain acylcarnitine concentration in the liver before the starvation was significantly ($p<0.01$) lower in the Free group than in the AA- and AP-supplemented groups. During the starvation, long chain acylcarnitine concentration in the liver decreased more than 50% except for the Free diet group calculated on the basis μg/g. Free and acid soluble total carnitine concentrations in the ordinary muscle had a tendency to increase during the starvation, but there were no differences among the experimental groups. Long chain acylcarnitine concentration in the muscle of the AP-group decreased significantly ($p<0.01$) during the starvation, but in the other groups no significant

Table 2. 15. Composition of the lipid extracted from the viscera and the eviscerared whole body (%)

	Phos.[1)]+Mono.[2)]	Di.[3)]+Chol.[4)]	Free fat.[5)]	Tri.[6)]	Chol. ester	Unknown
Viscera						
Before starvation						
Free	12.5±2.6	20.0±4.8	8.5±3.0^{a}	54.8±4.6	4.2±1.6^{a}	0.2±0.4
AA	11.3±1.7	20.0±2.0	8.5±3.3^{b}	55.0±3.2	5.3±1.5^{b}	0.0±0.0
AP	9.8±1.0	17.3±4.2	13.3±1.7^{a,b,c,d}	56.5±7.0	3.4±1.8	0.1±0.2
After starvation						
Free	15.5±5.4	24.3±14.8	7.3±3.4^{c}	51.8±15.5	1.1±1.1^{a,b,c}	0.3±0.4
AA	18.5±5.5	20.8±6.7	8.5±1.7^{d}	49.0±11.1	2.9±1.6	0.3±0.3
AP	15.8±6.8	19.5±4.7	10.5±2.5	49.5±11.2	3.9±2.0^{c}	0.5±0.6
Eviscerated whole body						
Befor starvation						
Free	12.8±2.1	16.8±4.2	1.3±0.5	64.3±6.5	4.2±1.8^{a}	0.1±0.3
AA	15.3±3.1	18.8±2.6	1.8±0.5	59.0±5.5	5.7±1.6^{b}	0.1±0.2
AP	13.8±2.8	15.5±3.7	1.8±0.5	66.5±4.4	2.4±1.1^{b,c,d}	0.1±0.3
After starvation						
Free	14.3±3.6	15.0±3.4	1.3±0.5	64.5±5.8	4.5±1.3^{e}	0.6±0.5
AA	13.5±4.7	15.3±5.0	2.0±0.2	61.5±9.1	7.5±1.4^{a,c,e}	0.6±0.2
AP	14.5±2.9	17.5±5.0	2.0±0.8	60.5±7.9	5.5±1.0^{d}	0.4±0.2

Figures bearing common superscript in the same column are significantly different ($p<0.01$).
Values are the mean ± S. D. (n=5).
[1)], phospholipid; [2)], monoglyceride; [3)], diglyceride; [4)], cholesterol; [5)], free fatty acid; [6)], triglyceride.

Table 2.16. Carnitine contents in the liver

Group	Free		Acid soluble		Long chain acyl	
	μg/g	μg/iver	μg/g	μg/iver	μg/g	μg/iver
Before starvation						
Free	$64.3\pm14.4^{a,b,c}$	158.0 ± 42.1^{a}	122.9 ± 4.5	$282.5\pm74.1^{a,b,c}$	$14.6\pm8.3^{a,b}$	$36.8\pm14.2^{a,b,c,d,e}$
AA	$71.0\pm12.4^{d,e,f}$	$174.0\pm55.5^{b,c}$	113.6 ± 13.4	$288.0\pm93.6^{d,e,f}$	35.6 ± 7.2^{a}	$59.0\pm15.1^{a,f,g,h}$
AP	$76.0\pm15.1^{g,h,i}$	$187.5\pm 9.6^{d,e,f}$	122.2 ± 5.3	$302.0\pm60.6^{g,h,i}$	33.4 ± 4.6^{b}	$72.3\pm 7.9^{b,i,j,k}$
After starvation						
Free	$122.4\pm16.3^{a,d,g}$	$100.3\pm18.3^{b,d}$	172.8 ± 46.9	$134.3\pm26.6^{a,d,g}$	16.0 ± 9.6	$18.2\pm11.8^{c,f,i}$
AA	$154.2\pm52.0^{b,e,h}$	118.8 ± 51.1^{e}	204.5 ± 43.2	$131.0\pm43.7^{b,e,h}$	18.1 ± 3.4	$15.3\pm 3.1^{d,g,j}$
AP	$113.6\pm27.1^{c,f,i}$	$88.0\pm32.9^{a,c,f}$	187.8 ± 46.7	$128.0\pm29.0^{c,f,i}$	15.4 ± 6.8	$16.2\pm11.9^{e,h,k}$

Figures bearing common superscript in the same column are significantly different ($p<0.01$).
Values are the mean±S. D. (n=5).

Table 2.17. Carnitine content in the ordinary muscle (μg/g)

Group	Free	Acid soluble	Long chain acyl
Before starvation			
Free	$85.2\pm13.7^{a,d,e}$	145.2 ± 14.3^{a}	18.3 ± 7.9
AA	$92.7\pm10.1^{b,f}$	188.0 ± 59.9	15.5 ± 4.2
AP	$93.5\pm12.3^{c,g}$	164.2 ± 29.7	$23.3\pm3.5^{a,b}$
After starvation			
Free	$145.7\pm33.6^{a,b,c}$	189.0 ± 64.5	12.9 ± 6.0^{a}
AA	$123.5\pm36.2^{d,h}$	228.1 ± 48.2^{a}	17.5 ± 6.2
AP	$170.8\pm 4.3^{e,f,g,h}$	186.8 ± 54.5	13.1 ± 3.1^{b}

Figures bearing common superscript in the same column are significantly different ($p<0.01$).
Values are the mean±S. D. (n=5).

change in the values were observed.

Discussion

Methods for carnitine determination reported recently are based on enzymatic reactions. Acetylcarnitine and CoASH are formed from carnitine and acetyl-CoA in the presence of CAT. The acetylcarnitine or CoASH formed was determined by radioisotopic method[97] or by HPLC[98, 99]. The HPLC method is based on the direct determination of the CoASH released stoichiometrically from the reaction. A gradient system, which needs more time for an analysis to be done, was adopted in most of the HPLC methods reported.[98, 99] Arakawa *et al.* proposed an isocratic time-saving system to determine free carnitine and acid-soluble total carnitine but not long-chain acylcarnitines.[94] It is important to measure the concentration of long-chain acylcarnitine for the assessment of fatty acid metabolism activity, because it needs carnitine as a intermediator for long-chain fatty acids to enter mitochondria where they are oxidized to provide metabolic energy.[93] Thus the author investigated a method of carnitine determination by HPLC be-

fore the feeding experiment. Modifying the procedures of Arakawa *et al.*[94], Williamson and Corkey[95] and Pearson *et al.*[96], the method mentioned above was established for the determination of long-chain acylcarnitines besides free carnitine and acid-soluble total carnitine. CoASH was successfully isolated from other compounds and the standard curve for carnitine vs. the peak area of the CoASH was clearly in proportion from 0.1 μM to 100 μM. Recovery of free carnitine added to the homogenate of ordinary muscle tissue from rainbow trout was 98.9 ± 4.7% (1 μmol/g added) and 99.8 ± 6.2% (0.05 μmol/g added). The addition of 2-mercaptoethanol, which was omitted in the method of Arakawa *et al.*[94], to the reaction mixture prevented the oxidation of CoASH during the analysis, and such reducing reagent should therefore be added.

Long-chain acylcarnitine content in the liver was significantly lower in Free diet group than in AA- and AP-supplemented groups before the starvation (Table 2.16). Carnitine is essential as a carrier for long-chain fatty acid to be transported across the mitochondrial membrane.[73] Reduced long-chain acylcarnitine level is considered to mean that the activity to utilize fatty acid as energy is depressed. Lipid content in the whole body was significantly decreased during the starvation in AA- and AP-supplemented groups but not in the Free diet group (Table 2.14). This result seems to indicate that the fish of Free diet group could not utilize body lipid efficiently during the starvation.

The effect of AA deficiency on the tissue and plasma carnitine concentration has been investigated in guinea pigs. Ciman *et al.*[85] first reported that carnitine concentration in cardiac muscle was reduced in scorbutic guinea pigs. There are many subsequent reports[86-92], but the results were not consistent. The two enzymes, ε-N-trimethyllysine hydroxylase and γ-butyrobetaine hydroxylase, which catalyze hydroxylation reaction in the pathway of carnitine biosynthesis require Fe^{2+} and AA as cofactors such as prolyl and lysyl hydroxylases that participate in the pathway of collagen synthesis.[82-84] Rebouche[93] concluded that these results provided compelling evidence for participation of AA in carnitine biosynthesis. There are no reports available on the effect of AA on carnitine metabolism in fish. The concentration of long-chain acylcarnitine in the Free diet group, whose AA level was significantly lower than in AA- and AP-supplemented groups, was significantly lower than other groups before the starvation; lipid was not utilized efficiently during the starvation in Free diet group. These findings strongly suggest the participation of AA in carnitine and lipid metabolism in fish. No statistically significant differences were found among the groups on the concentrations of free and acid soluble total carnitine before the starvation in the present experiment. Free and acid soluble total carnitine level might have decreased if young fish, which is more susceptible to scurvy than adult fish, was used or the feeding period was more prolonged, since the AA level of Free diet group was low, but not scarce after the 60 days feeding of the present experiment.

There were little differences in the free and acid soluble total carnitine contents in the liver (Table 2.16) among the groups, but after the starvation, the values were raised on the basis of μg/g scale and reduced on the basis of μg/liver scale. The reason expected for the difference after the starvation is the atrophy of the liver during the starvation; the livers of all groups were atrophied more than half (Table 2.12). Rise of free and acid soluble total carnitine concentrations was observed in the ordinary muscle after the starvation (Table 2.17). It has been shown that the level of long-chain acylcarnitine was elevated under the conditions

where high rates of fatty acid oxidation are exhibited, such as in starvation[100], high fat feeding, glucagon administration[101] and cold acclimation[102] in terrestrial animals.[98] Takeyama *et al.*[98] reported that free and acid soluble total carnitine besides long-chain acylcarnitine level were elevated after the starvation in rat liver. In contrast, long-chain acylcarnitine concentration had a tendency to decrease after the starvation (Table 2.16). This decline of long-chain acylcarnitine level may be attributed to the adaptation of the fish to fall their metabolism for the starvation, which was due to the longer period of starvation (60 days). Short time starvation might have elevated the long-chain acylcarnitine level in fish.

Guinea pig fed on AA-free diet becomes scurvy shortly with a loss of appetite. It is important to use pair-fed control in a guinea pig experiment, since diminished appetite reduces the carnitine content of tissues.[90] On the contrary, it takes long time to become scurvy in adult fish. Sixty days' feeding in the present experiment decreased the AA level in Free diet group, but did not cause the scurvy symptoms such as loss of appetite. No statistical differences were found among the diet intake of each group in the present experiment on adult rainbow trout, in spite of the satiation feeding. A pair-fed control group should be set up, if young fish is used for the experiment, since young rainbow trout showed scurvy symptoms within 9 weeks' feeding and loss of appetite at the 12th week (CHAPTER 2; Section 1).

Some differences of lipid composition were found in free fatty acid and cholesterol ester in the lipid, although the reason is unclear.

In the present experiment, the addition of AP as well as AA prevented abnormal metabolism of carnitine and lipid, such as reduced long-chain acylcarnitine level and depression of lipid utilization during starvation, which were caused by scorbutic condition. These findings confirmed that AP is effective as a vitamin C source in rainbow trout.

CHAPTER 3

Conversion of Ascorbyl-2-phosphate to Ascorbic Acid in Rainbow Trout

L-Ascorbyl-2-monophosphate (AP) has been found to be much more stable than L-ascorbic acid (AA) in solution and also in fish feeds.[CHAPTER 1, 22, 28, 56] Several phosphate derivatives of AA have been demonstrated to possess marked vitamin C activity in aquatic animals.[22, 43, 45] The author also has demonstrated in the previous chapter that AP has a physiological activity equivalent to that of AA in rainbow trout. In the previous chapter, rainbow trout were fed on experimental diets containing different amounts of AA or AP. AA concentrations in the liver and plasma were slightly higher in the AP-supplemented groups than in the groups supplemented with equimolar amounts of AA and no AP could be detected in the liver or plasma of fish fed AP-supplemented diets (even in the 1000mg AA equivalent/100g diet group). Presumably, AP was readily converted to AA in the rainbow trout body. In the present chapter, the author investigated the conversion of AP to AA in vivo in rainbow trout administered with AP orally and intraperitoneally and the activity of enzymatic hydrolysis of AP.

Section 1. Oral and Intraperitoneal Administration of Ascorbyl-2-phosphate to Rainbow Trout

Imai *et al.*[55] reported the ready conversion of AP to AA in guinea pig. However, very little is known about the conversion in fish. In the present section, AP was administered to rainbow trout orally and intraperitoneally to chase the change of AP to AA in the fish body.

Materials and Methods

Reagents

AP was obtained from Showa Denko Inc. (Tokyo) as magnesium salt. Other reagents were of analytical grade, obtained from Nacalai Tesque Inc. (Kyoto).

Oral administration

Rainbow trout *(Oncorhynchus mykiss)* with an average body weight of 170g were fed for 3 days on an AA-free diet prepared from the casein basal diet described in the CHAPTER 2; Section 1. The fish were fasted for 5 days and used as the experimental fish. AP or AA (200 μmol) in a 2g diet, prepared from the AA-free casein basal diet, was force-fed once to each experimental fish. After 3, 6, 12, 25, 50, 75 and 100 hours, the fish were taken out. The caudal fin was cut and the blood was collected in a heparinized capillary tube. After centrifugation, the plasma was deproteinized with 5% metaphosphoric acid solution and the plasma AA and AP concentrations were measured by the high-performance liquid chromatography (HPLC) described in the CHAPTER 2; Section 2. The alimentary canal was removed from the fish taken out 3, 6, 12, 25 and 50 hours after the administration. The alimentary canal was dissected into 3 parts: stomach, pyloric caeca and intestine. The contents of each part were washed with 5% trichloroacetic acid (TCA) and collected. The contents in the TCA solution (10ml in total) were homogenized by glass homogenizer to extract AA, dehydro L-ascorbic acid (DAA) and AP. The extract (0.4ml) was neutralized by 3M K_2HPO_4 and added to 0.1ml of 3 % dithiothreitol to reduce DAA to AA. After 5 minutes at room temperature, it was diluted with the mobile phase and the total AA (AA + DAA) and AP were determined by HPLC. The equipment for HPLC was: a pump, Hitachi 655 liquid chromatograph; a detector, Hitachi 638-41 UV detector set at 254 nm; a processor, Hitachi 833 Data Processor; and a column, Shodex RS pak DE-613 (Showa Denko Inc., Tokyo). The solvent was 50mM

KH_2PO_4 containing 5% (v/v) acetonitrile and 0.0475% (v/v) *n*-octylamine, and its pH was adjusted at 4.6 by acetic acid. The flow rate was 0.7m*l*/min. Each sample (10-20 μl) was injected onto the column. Standard curves for AP and AA were demonstrated by plotting peak area vs. authentic AP and AA amounts.

Intraperitoneal administration

Rainbow trout (40-70g) fed on the AA-free diet (described in the CHAPTER 2; Section 1.) for 3 days and fasted for 5 days were used as the experimental fish. AP (10 μmol) dissolved in 0.2m*l* of 0.9% sodium chloride solution was administered intraperitoneally to each fish. AP and AA concentrations of the plasma, liver and kidney were measured 1 and 3 hours after the administration. AP and AA were extracted with 5% metaphosphoric acid solution and measured by the HPLC method reported previously.

Results

Oral administration

The time course of AA and AP concentrations in the plasma after the oral administration of AP is shown in Table 3.1. The AA concentration in the plasma increased gradually and reached a maximum level 50 hours after the AP administration, and then began to decline. There was no significant difference in the plasma AA concentration between the AP- and AA-administered groups, except for that at 12 hours after the administration. AA concentration in the plasma was higher in the AA-administered group than in the AP-administered group at 12 hours. AP concentration in the plasma was below the detectable limit (about 2.6nmol/m*l*) throughout the experimental period. Table 3.2 shows the time course of AA + DAA and AP in the contents of the alimentary canal (stomach, pyloric caeca and intestine portions) of the fish administered with 200 μmol of AP orally. In the stomach contents, the amount of total AA (AA+DAA) had been kept at a low level throughout the experimental period (1.6 to 2.4 μmol) and the amount of AP decreased with time. In the contents of pyloric caeca and intestine, the amount of total AA increased and reached a maximum at 12 hours (43.0 and 56.8 μmol, re-

Table 3.1. Plasma AA and AP concentrations after oral administration of AP and AA to rainbow trout

Time (hours)	AP (200 μmol) administration		AA (200 μmol) administration
	AA concentration (nmol/m*l*)	AP concentration (nmol/m*l*)	AA concentration (nmol/m*l*)
0	38± 16*1		
3	76± 44	n. d.*2	111± 27
6	93± 53	n. d.	245±100
12	248± 68	n. d.	450± 86
25	617± 29	n. d.	635± 87
50	730±116	n. d.	662±143
75	469± 44	n. d.	498±135
100	268±145	n. d.	239±115

*1 Mean±S. D. Each value is the mean of 5 fish.
*2 Not detected (below 2.6 nmol/m*l*).

Table 3.2. Time course of AA and AP in the contents of alimentary canal of rainbow trout given 200 μmol of AP orally (μmol)

Time (hours)	Stomach		pyloric caeca		Intestine	
	AA+DAA	AP	AA+DAA	AP	AA+DAA	AP
3	2.4±0.4*	135.6± 9.6	13.0± 6.6	8.6±6.1	7.8± 8.5	5.6± 4.9
6	2.4±0.7	87.3±25.8	24.0±14.5	8.8±6.9	30.0± 5.2	9.7±10.3
12	1.9±0.2	52.7± 9.4	43.0±24.5	1.7±1.4	56.8±10.3	2.5± 0.9
25	2.1±0.6	35.0±22.1	33.6± 7.0	0.5±0.3	44.9± 7.3	0.2± 0.06
50	1.6±0.6	1.2± 1.2	15.3±13.2	1.1±0.3	17.9± 9.0	0.4± 0.06

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

Table 3.3. Concentration of AA and AP in plasma, liver and kidney of rainbow trout given 10 μmol of AP intraperitoneally

Time (hours)	Plasma (nmol/ml)		Liver (nmol/g)		Kidney (nmol/g)	
	AA	AP	AA	AP	AA	AP
0	76.5±35.2*1	n. d.*2	338.8± 27.5	n. d.	548.7±151.3	n. d.
1	726.4±159.7	24.6±35.2	553.3± 88.0	Trace	1275.0±250.9	9.7±14.2
3	619.1±207.2	n. d.	866.7±162.5	n. d.	1299.1±315.7	n. d.

*1 Mean±S. D. Each value is the mean of 5 fish.
*2 Not detected (below 2.6nmol/ml or g).

spectively), and then decreased gradually. In the contents of pyloric caeca and intestine, 5.6 to 9.7 μmol of AP was found 3 and 6 hours after the administration, but only a small amount of AP was detected after 12 hours.

Intraperitoneal administration

Table 3.3 shows the changes in AA and AP concentrations in the plasma, liver and kidney of the fish after injection of 10 μmol of AP intraperitoneally. AA concentrations of the plasma, liver and kidney increased apparently within 1 hour after the AP injection, and were kept at a high level by 3 hours. At 1 hour after the injection, 24.6nmol/ml and 9.7nmol/g of AP were detected in plasma and kidney, respectively; after 3 hours, the AP was below the detectable level in the plasma, liver and kidney.

Discussion

There were no statistical differences in change in plasma AA concentration between the AA and AP groups in the oral administration test throughout the experimental period, except 12 hours after the administration. A little time lag was observed on change in plasma AA concentration between the AA and AP groups. The plasma AA concentration in the AP group increased slower than that in the AA group. The AP concentration in the plasma of the AP group was below the detectable limit throughout the experiment period. These findings suggest that most AP administered orally seems to be converted to AA in the alimentary

canal and absorbed in the form of AA. The time lag might come from the time needed to hydrolyze the AP in the alimentary canal. This was clearly shown by the change in concentration of AA+DAA and AP in the contents of alimentary canal (Table 3.2). The amounts of AP in pyloric caeca and intestine contents reached about 7 to 9% of administered AP after 3 and 6 hours, but decreased below 1% after 25 hours. Amounts of AA + DAA in the pyloric caeca and intestine reached a maximum level at 12 hours, while the plasma AA concentration 50 hours after administration. These results indicate that most of the administered AP was converted to AA in the pyloric caeca and intestine, then moving to the blood stream in the form of AA. AP was not detected in the plasma in spite of the existence of AP in the pyloric caeca and intestine. AP may be converted to AA by the phosphatases, which exist in the intestine, while it was transported through the intestine tissue, or the AP transported through the intestine may immediately be hydrolyzed to AA in the blood and in such organ as the liver where some phosphatase activity exists.

The amounts of AP in the contents of the stomach decreased as the volume of the stomach contents decreased. Only a small amount of AA + DAA in the contents of the stomach was detected throughout the experimental period. This means that the activity of AP hydrolysis to AA is lower in the stomach than in the pyloric caeca and intestine in rainbow trout. It is well-known that the brush border membrane of the intestine has a high activity of alkaline phosphatase, which shows low substrate specificity.[103] This alkaline phosphatase might play a considerable part in AP hydrolysis; the enzyme of some intestinal bacteria may also be considered.

In this oral administration test, a megadose of AP (35mg of AA equivalent per single dose) did not produce any detectable AP in the plasma. If a small amount of AP was administered, little AP would be found in the plasma and much smaller amounts of AP would be detected in the pyloric caeca and intestine contents, because the activity of AP hydrolysis to AA is very strong.

The intraperitoneal administration test showed the rapid disappearance of AP from the body and the rapid appearance of AA. AP administered intraperitoneally was easily hydrolyzed to AA in this experiment. Acid phosphatase is a ubiquitous enzyme in blood and in lysosomes of all cells and therefore, if being absorbed in such a form through the intestine, AP seems to be hydrolyzed rapidly to AA.

Imai *et al.*[55] reported that the blood AA level of guinea pigs after oral administration of AP (25mg AA equivalent) did not differ significantly from the level after the administration of AA (25mg). A ready hydrolysis of AP to AA in rainbow trout was also found in this investigation. This high potency of AP, a readily available source of vitamin C, is universal among animals as phosphatase activity is ubiquitous in animals. But some difference in availability may be found among species especially fish with a large degree of variety. Although Imai *et al.*[55] reported the activity of AP hydrolysis in the homogenates of small intestine and liver of guinea pigs, little study has been made on the participation of phosphatases in the hydrolysis of AP to AA. The author investigated the activity of AP hydrolysis by the homogenate of several tissues of rainbow trout and the result will be described in the next section.

Section 2. Activity of Enzymatic Hydrolysis of Ascorbyl-2-phosphate in Rainbow trout

In the previous section, in vivo investigation showed the hydrolysis of AP in the pyloric caeca and intestine but not in the stomach and the rapid disappearance of AP from the plasma after intraperitoneal injection in rainbow trout. From the results, high activity of AP hydrolysis was expected in the trout tissues, especially in pyloric caeca and intestine. In the present section, activity of enzymatic hydrolysis of AP was investigated in several tissues of rainbow trout.

Materials and Methods

Reagents

AP was obtained from Showa Denko Inc. (Tokyo) as magnesium salt. Other reagents were of analytical grade, obtained from Nacalai Tesque Inc. (Kyoto).

Measurement of AP and AA

AP and AA were measured by high-performance liquid chromatography (HPLC). The HPLC condition was the same as described in the previous section (CHAPTER 3; Section 1).

Homogenate of tissues

Stomach, pyloric caeca, intestine and liver were excised from rainbow trout *(Oncorhynchus mykiss)* reared in the laboratory. The contents of alimentary canal were washed out with 0.9% sodium chloride. Each organ was homogenized in 5 volumes of 0.9% sodium chloride in a glass homogenizer. The homogenate was used as the crude enzyme.

Assay of AP hydrolysis

The reaction mixture (6.0m*l*) for the time course analysis contained 2.0m*l* of the homogenate, 50mM acetate buffer (pH4.0) and AP magnesium salt (25mM). The reaction mixture was incubated at 30℃. At definite intervals, 0.5m*l* of the reaction mixture was sampled and 1.0m*l* of 5% trichloroacetic acid (TCA) was added to the mixture to stop the reaction. To reduce dehydroascorbic acid (DAA) to AA, 0.4m*l* of the stopped reaction mixture was taken into a test tube and mixed with 0.1m*l* of K_2HPO_4 solution (3M) and 0.1m*l* of 2.5% dithiothreitol. The mixture was incubated for 10 minutes at room temperature and added with 10 m*l* of phosphoric acid (50mM). After filtration, the mixture was applied to the HPLC.

The activity of AP hydrolysis was compared among the stomach, pyloric caeca, intestine and liver at various pHs. A reaction mixture (1.5m*l*) contained AP magnesium salt (25mM), buffer solution (100mM) and 0.5m*l* of the homogenate. The buffer consisted of glycine-hydrochloric acid (pH2.0-4.0), acetic acid-sodium hydroxide (pH3.6-6.1), phosphate (pH6.0-8.1) and glycine sodium hydroxide (pH7.7-10.5). The reaction mixture was incubated at 30℃ for 30 minutes. The reaction was stopped by adding 5% TCA (1.5m*l*) and the AA produced from AP was measured by the HPLC described above. The Km values for AP in AP-hydrolysis activity were estimated from the Michaelis-Menten's equation. Protein concentration was determined by the method of Lowry *et al.*[104] using bovine serum albumin as a standard.

Results and Discussion

Figure 3.1 A shows the typical chromatogram of authentic AP. Figures 3.1 B and C illustrate the typical chromatograms of the complete reaction mixture containing the crude enzyme from the intestine of rainbow trout and the control reaction mixture containing the homogenate inactivated by heating at 100℃ for 30 minutes, respectively. AA was identified in the complete reaction mixture from the retention time indicated in Fig. 3.1 B (peak 2). No corresponding peak was found in the case of in-

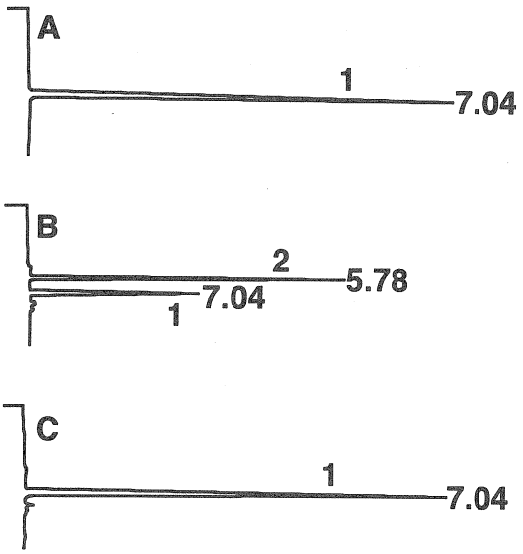

Fig. 3.1. Typical HPLC profiles of the AP hydrolysis by the homogenate of intestine. A, chromatogram of authentic AP; B, chromatogram of the complete reaction mixture; C, chromatogram of the control reaction mixture containing inactivated homogenate. Peaks 1 and 2 are AP and AA, respectively.

activated enzyme (Fig. 3.1 C) confirming the enzymatic reaction. The AA peak 2 (Fig. 3.1 B) continued to increase in proportion to the incubation time till 30 minutes.

The AP-hydrolysis activities of several tissues as a function of incubation pH are illustrated in Fig. 3.2. Intestine and pyloric caeca showed high activities around pH9.5 and moderate around pH3.0 to 5.0. Liver had the highest activity around pH5.0 and high around pH 3.2. Stomach showed low activity at all pHs (2.0 to 10.5).

The author showed high activity of AP hydrolysis at alkaline pHs in the homogenate of intestine and pyloric caeca but not in stomach at all pHs. These findings coincided with the results of the *in vivo* investigation that AP administered orally was hydrolyzed to AA in the pyloric caeca and intestine but not in the stomach as given in the previous section (Table 3.2). Noda and Tachino[105, 106] investigated the activity of several phosphatases in various organs of several kinds of fish including rainbow trout. They found high activity of alkaline phosphatase in the kidney, intestine and pyloric caeca of rainbow trout. It is well-known that brush border of intestine is rich in alkaline phosphatase and the enzyme has relatively low specificity for substrate such as β-glycerophosphate, α-naphtyl phosphate, p-nitrophenyl phosphate, AMP, ATP and so on.[103] AP would be hydrolyzed by this alkaline phosphatase in pyloric caeca and intestine whose pHs are kept relatively high.

The results of kinetic analysis are listed in Table 3.4. The Km values for AP were 1.48 to 5.90mM and 5.37 to 17.1mM at the pH9.6 and 5.0, respectively. The Km values for various phosphatase substrates have been reported to vary from 0.09 to 35mM according to the pH and the origin of the enzyme.[107-110] The values obtained here were not so different from such values as reported by these investigators.

As a conclusion of this chapter, AP is readily converted to AA by alkaline phosphatase in pyloric caeca and intestine of rainbow trout.

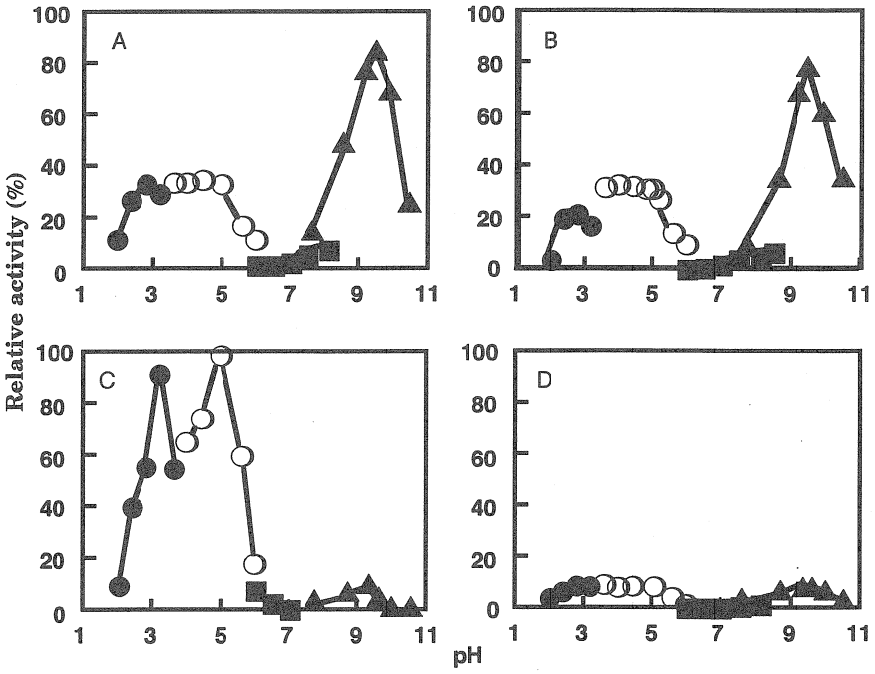

Fig. 3.2. Activities of AP-hydrolysis in several organs of rainbow trout at various pHs. The rate for liver at pH5.0 was taken as 100, while the other values are indicated as percentages of this value on the basis of AA produced from AP per reaction mixture. A, pyloric caeca; B, intestine; C, liver; and D, stomach.

Table 3.4. Comparison of AP hydrolysis activity

Tissues	pH 9.6			pH 5.0		
	Km*1	Vmax*2	Total*3 activity	Km	Vmax	Total activity
Stomach	1.48	8.1	0.48	5.37	6.6	0.39
Pyloric caeca	5.90	69.9	4.16	—	—	—
Intestine	4.65	56.4	3.07	—	—	—
Liver	—	—	—	17.1	23.4	2.37

*1 mM.
*2 nmol/mg protein per minute.
*3 μnmol/g tissue per minute.

CHAPTER 4

Intestinal Absorption of Ascorbyl-2-phosphate in Rainbow Trout

In the previous chapter the author showed that L-ascorbyl-2-monophosphate (AP) administered orally was hydrolyzed to L-ascorbic acid (AA) in the pyloric caeca and intestine but not in the stomach in rainbow trout. It was also shown that intraperitoneal administration of AP raised the body AA level rapidly. Research in vitro confirmed the high activity of AP hydrolysis by phosphatases in the trout tissues, especially in the pyloric caeca and intestine. In spite of oral megadose of AP, no AP was found in the plasma. Such results raised a question whether AP is absorbed from intestine. Absorption mechanism of AA and dehydro L-ascorbic acid (DAA) has been investigated on terrestrial animals by many investigators[110], while there are no reports on absorption of AP from intestine. In the present chapter, *in vitro* absorption of AP in intestine is described for rainbow trout.

Materials and Methods

Reagents

AP was obtained from Showa Denko Inc. (Tokyo) as magnesium salt. Other reagents were of analytical grade, obtained from Nacalai Tesque Inc. (Kyoto).

Measurement of AP and AA

AP and AA were measured by high-performance liquid chromatography (HPLC). The HPLC conditions were the same described in the previous chapter (CHAPTER 3) with the exception of a UV detector. The detector equipped was Hitachi L-4000 UV detector set at 254nm.

Procedure of absorption test

The method for intestinal absorption test was that of the modification of Tsujimura *et al.*[111]. An intestine was removed from rainbow trout *(Oncorhynchus mykiss)* weighing 180 to 200g and was rinsed with Holmes-Stott Ringer (HR) solution. Whole intestine was used without turning inside out. One end (near pyloric caeca) of the intestine was closed with a ligature. HR solution (0.5m*l*) containing 26μmol of AP magnesium salt (APM) or sodium L-ascorbate (AA-Na) as control was injected into the intestine (mucosal side). The open end was closed with a ligature. This intestine was rinsed with the HR solution. Then it was suspended in the HR solution (15m*l*) in an experimental apparatus with aeration (Fig. 4.1) and the apparatus was maintained at 16℃. At intervals, a sample of 20μl each was withdrawn from the serosal fluid and diluted with cold dis-

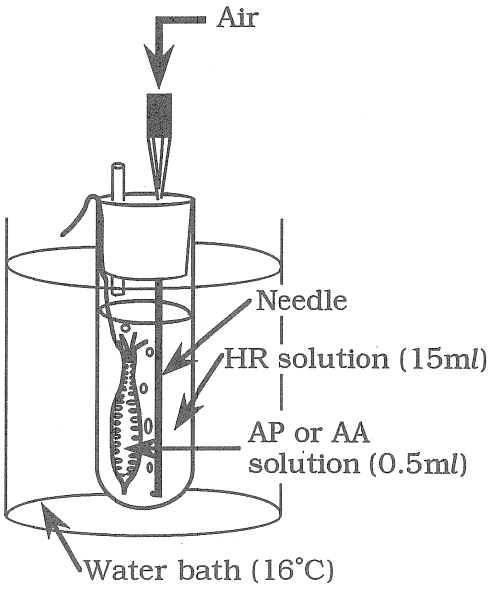

Fig. 4.1. Schematic illustration of experimental apparatus for the examination of intestinal absorption of L-ascorbyl-2-phosphate (AP) and L-ascorbic acid (AA).

tilled water. Immediately, it was filtered through 0.45 μm membrane filter and applied to the HPLC to measure the contents of AA and AP.

Uncoupler treatments

To examine the effect of 2, 4-dinitrophenol (DNP) on the absorption of AP and AA, the DNP treatment was performed on the intestine. The intestine removed from the fish was incubated in the HR solution containing 1mM DNP for 15 minutes at room temperature before use. DNP was also added at the concentration of 1mM to the AP or AA solution injected into the intestine of the mucosal side and to the solution of serosal side.

Results and Discussion

We tested the stability of AA before the absorption test was performed since the degradation of AA in the HR solution was presumed. AA-Na dissolved in HR solution (2.5 μmol/15ml and 1.3 μmol/15ml) was placed in the test tube of the experimental apparatus (Fig. 4.1) followed by aeration at 16℃, and AA was determined by the HPLC at intervals. Unexpectedly, almost all AA (more than 98%) remained in reduced form after 240 minutes incubation under these conditions.

Figure 4.2-a shows the time course of AP and AA transported from the mucosal side to the serosal fluid when AP was injected into the mucosal side. AA derived from the intestine tissues was subtracted and the real quantity of

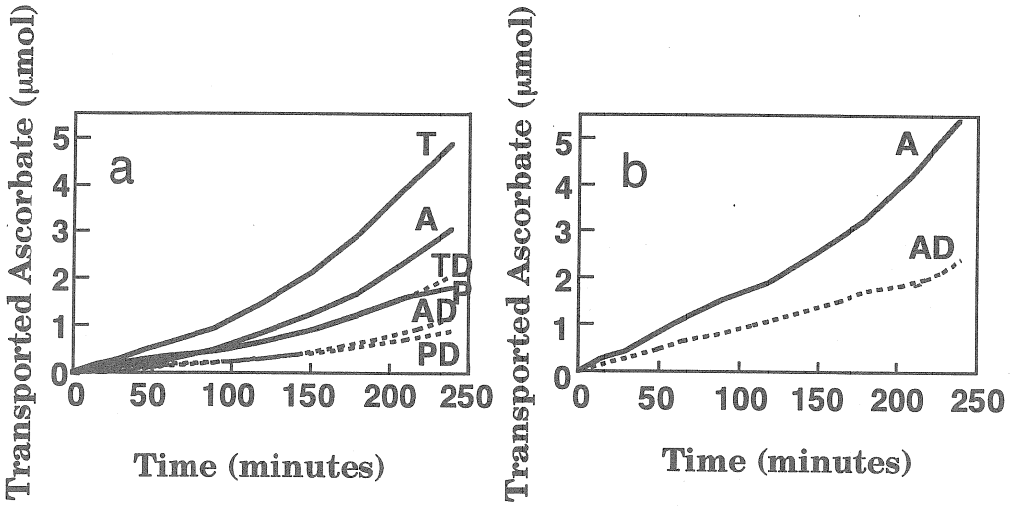

Fig. 4.2. Time course of AP and AA transported from the mucosal side to the serosal side. a, AP magnesium salt (26 μmol) was injected into the mucosal side; b, AA sodium salt (26 μmol) was injected into the mucosal side; T, total ascorbate (AA+AP); A, AA; P, AP; D, DNP treatment. DNP treatments are drawn in dotted lines. Each line was drawn by connecting the points (omitted in the figures) obtained from every 15 minutes analyses. The value of each point was the mean calculated from 5 fish intestines.

AA or AP passed through the intestine was obtained from the mean of 5 fish intestine. AP was partly transported in the form of AP from the mucosal side to serosal fluid. Amounts of AA in the serosal fluid were found to increase gradually after 15 minutes. AP and AA concentrations in the mucosal side of the intestine injected AP without DNP were 12.0 μmol/ml and 16.0 μmol/ml, respectively, in average at 240 minutes. Possibly, AP was hydrolyzed by phosphatases in the intestine. No AP was found in the plasma and liver of rainbow trout in spite of the oral megadose of AP as described in the previous chapter. The present *in vitro* absorption test indicated that AP passed through the intestine in an AP form but part of which was hydrolyzed to AA (Fig. 4.2). Presumably, the AP passed through the intestine without hydrolysis was readily hydrolyzed to AA in the blood or other tissues, which were abundant in phosphatases. High activity of AP hydrolysis was found in the liver at acidic region (CHAPTER 3; Fig. 3.2). There have been found various kinds of acid phosphatases with low substrate specificity in animal tissues, especially in lysosome.[112-114] Rainbow trout have high activity of acid phosphatase in the kidney and liver.[106] The AP passed through the intestine would readily be hydrolyzed to AA by these phosphatases in such tissues. The results of CHAPTER 3 also showed high activity of AP hydrolysis in the liver tissue at acidic pHs. Alkaline phosphatase might also participate in the AP hydrolysis after intestinal absorption occurs because alkaline phosphatase is known to be activated in vivo near neutral pHs when the substrate concentration is low[115-117]. In the *in vivo* investigation (CHAPTER 3), concentrations of AP in the plasma and liver of rainbow trout were below detectable limit in spite of the megadose of AA. In any case, AP passed through the intestine would readily be hydrolyzed to AA and the concentration of AP would become below detectable level in every tissue.

DNP is one of the uncouplers that inhibit the ATP synthesis. Tsujimura *et al.*[32] reported that *in vitro* absorption of AA and dehydroascorbic acid (DAA) in rat intestine was inhibited by DNP (less than 30%). They postulated that the mechanisms of vitamin C absorption in rat intestine are based on mainly passive diffusion and partly active transport. Rose[110] reported that, in terrestrial animals, AA was absorbed by specific Na-mediated transport processes from the intestine. DNP inhibited the absorption of AP and AA (Fig. 4.2 a; PD and AD) in rainbow trout about 50% and 60% (240 minutes), respectively. The absorption of AA (Fig. 4.2 b) was also inhibited approximately 50% at 240 minutes by DNP, suggesting that active transport mechanisms are involved in the absorption of AP and AA in the fish intestine.

CHAPTER 5

Enzymatic Synthesis of Ascorbyl-2-phosphate

L-Ascorbyl-2-monophosphate (AP) showed excellent stability during feed processing and storage and was readily converted to L-ascorbic acid (AA) in fish body as described in the previous chapters. AP seems to be a good source of ascorbate as fish feed, but AP is too expensive to use for mass consumption nowadays. One of the reason is the high cost of manufacturing with chemical synthesis. There has been a report on AP synthesis by bacterial enzyme *in vitro*[118] but the synthesis has never been in practice for mass production. In this part, the author describes the possibility of AP synthesis by enzyme of fish origin and discusses the possibility of the occurrence of AP in animal tissues.

Section 1. Synthesis of Ascorbyl-2-phosphate by Liver Enzyme of Rainbow Trout

AP is now synthesized by chemical methods in industries. Recently, Tani and Hattori[119, 120] and Maruyama *et al.*[118] reported that AP was formed by a bacterial enzyme. There are, however, no reports indicating the occurrence of AP in nature or the enzymatic formation of AP in animals. In this chapter the author investigated the possibility of AP synthesis by liver enzyme of rainbow trout.

Materials and Methods

Reagents

AP magnesium and sodium salts were obtained from Showa Denko (Tokyo). Other reagents were of analytical grade, obtained from Nacalai Tesque (Kyoto).

High performance liquid chromatography

The assay of AA and AP was carried out by high performance liquid chromatography (HPLC). The equipment for HPLC was: a pump, Hitachi 655 liquid chromatograph; a detector, Hitachi L-4000 u.v. detector set at 254nm; a processor, Hitachi 833 data processor; a column, Cosmosil $5C_{18}$-AR 10 × 250mm (Nacalai Tesque; Kyoto). The solvent was 50mM H_3PO_4 (pH 3.0) and the flow rate was 1.5m*l*/minute. Each sample (10-20 μl) was injected onto the column. Standard curves for AA and AP were demonstrated by plotting peak area *vs.* amounts of authentic AA and L-ascorbyl-2-monophosphate magnesium salt (APM).

Preparation of crude enzyme

Rainbow trout *(Oncorhynchus mykiss)* (300-500g) were killed by decapitation. The liver was excised and immediately homogenized in 4 vols. of 0.1M sodium phosphate buffer (pH 7.0) with a glass homogenizer. The homogenate was centrifuged at 10,000 × g for 30 minutes. The supernatant was dialyzed overnight against 0.01M sodium phosphate buffer (pH 7.0) and used as the crude enzyme.

Enzymatic reaction

The reaction mixture contained 0.25m*l* of the crude enzyme and 1.75m*l* of substrate solution. The substrate solution consisted of 206mM sodium L-ascorbate (AA-Na) and 200mM sodium pyrophosphate. The pH of the substrate solution was adjusted at 5.0 by hydrochloric acid. The reaction mixture was incubated at 30 for 3 hours. Then it was diluted with 20% metaphosphoric acid to stop the reaction. After filtration through a 0.45 μm membrane filter, 20 μl of the filtrate was injected onto the HPLC column.

Isolation of the AP-like product

The crude enzyme (10m*l*), 200mM sodium pyrophosphate (10m*l*) and AA-Na (2 g) were mixed in a flask. After adjusting the pH at 5.0

by hydrochloric acid, the mixture was incubated at 30℃ for 12 hours to form large amounts of AP-like product. After centrifugation at 10,000 ×g for 30 minutes, the supernatant was filtered through a 0.8 μm membrane filter. The filtrate was applied to a column (2.5 × 10cm) of QAE Sephadex A-25 (OH^- form). The column was washed with water (about 50ml) and with 0.1M sodium hydrogen carbonate (about 500ml) to elute AA. After washing the column with water (about 50ml), the AP-like product was eluted with 0.5M ammonium formate (about 200ml). The eluate was concentrated to about 12ml with a rotary evaporator. After filtration with a 0.8 μm filter, the concentrated solution was put on a column (2.5×96cm) of Sephadex G-10 for further purification. The column had previously been equilibrated with distilled water and the separation was developed with distilled water. The AP-like product fraction was collected, lyophilized, dissolved in 5ml of distilled water, and applied to the Sephadex G-10 column again. Sephadex G-10 treatment was repeated three times. The lyophilized AP-like product was used for further identification. Detection of the AP-like product at the column separation was performed by measuring the change in absorbance at 260nm.

Identification of the AP-like product

HPLC analysis. The AP-like product was dissolved in distilled water and applied to the HPLC column described above.

Ultraviolet (u.v.) spectral analysis. The AP-like product thus prepared was dissolved in 0.1M phosphoric acid-sodium hydroxide buffer (pH 2.0) or in 0.1M sodium phosphate buffer (pH 7.0). Ultraviolet absorption spectra of the sample at 20℃ were monitored between 200 and 300nm by a Shimadzu u.v.-visible recording spectrophotometer UV-160.

Reducing activity. The reducing activity of the AP-like product towards 2, 6-dichlorophenolindophenol (DCIP) was determined. The sample solution (1.5ml) was mixed with a 20 μl of 0.05% DCIP dissolved in 50% ethanol. The change of absorbance at 522nm was immediately measured.

TLC analysis. The AP-like product dissolved in distilled water was spotted on a precoated TLC plate SIL G-25 (Macherey-Nagel) and developed with a solvent of 1-propanol : water : 25% ammonia solution (6:6:1, v/v/v) and 1-butanol : acetic acid : water (2:1:3, v/v/v). The product was detected by a Shimadzu dual-wavelength thin layer chromato scanner CS-930 at 260nm and by spraying 0.5% ferric chloride ethanol solution on the TLC plate.[121]

Acid hydrolysis. The AP-like product was hydrolyzed in 6M HCl at 100℃ for 1 hour and analyzed on the HPLC.

Hydrolysis by acid phosphatase. The AP-like product (42.9nmol) and three units of purified acid phosphatase (EC 3.1.3.2) from wheat germ (Sigma) were incubated for 10 minutes at 25℃ in 1ml of 50mM acetate buffer (pH 5.0). Before and after the incubation, the AP-like product and the AA released were measured using HPLC.

Liquid chromatography mass (LC mass) spectral and nuclear magnetic resonance (NMR) analysis. The LC mass spectrum was obtained on a DX-303 mass spectrometer (Nippon Denshi) in glycerol matrix (Xe 1kV, m/z 100-1000). ^{13}C-NMR analysis was performed on a Burker AMX 400 NMR spectrometer in D_2O at 100MHz.

Kinetic analysis.

The K_m values for AA and pyrophosphate were estimated from the Michaelis-Menten's equation.

Results

Formation of AP-like product

Figure 5.1A, B and C show typical chro-

matograms of the authentic APM, the complete reaction mixture containing crude enzyme and the control reaction mixture containing inactivated crude enzyme heated at 100℃ for 30 minutes, respectively. Peaks 1 and 3 were identified as metaphosphoric acid and ascorbic acid, respectively. The retention time of the peak 2' in Fig. 5.1B coincided with that of authentic APM (peak 2 in Fig. 5.1A). No corresponding peak was found in the case of inactivated enzyme (Fig. 5.1C). The peak area of the peak 2' increased in proportion to the incubation time. To identify the peak 2' in Fig. 5.1B, large amounts of this product (AP-like product) were formed and isolated from the reaction mixture. The isolated AP-like product was used for further identification.

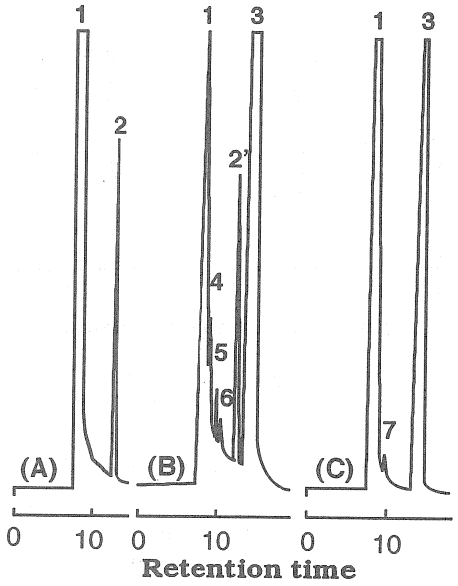

Fig. 5.1. HPLC profiles of the AP-like product formation with rainbow trout liver. Reaction mixture of L-ascorbic acid, pyrophosphate and the crude enzyme was incubated at pH 5.0 and 30℃ for 3 hours and analyzed by HPLC. Chart (A) shows the authentic APM in metaphosphoric acid. Charts (B) and (C) shows the complete reaction mixture and the reaction mixture that used inactivated enzyme heated at 100℃ for 30 minutes, respectively. Peaks 1 and 3 are metaphosphoric acid and AA, respectively; peaks 2 is the authentic APM (retention time: 12.45 minutes); peak 2' is the AP-like product (retention time: 12.42 minutes); peaks 4, 5, 6 and 7 are unknown.

Identification of the AP-like product

HPLC analysis. Figure 5.2A shows the chromatogram of the AP-like product dissolved in distilled water. Only one peak was observed, whose retention time coincided with that of the authentic APM (Fig. 5.2B). By HPLC analysis, it was estimated that about 40mg (equivalent to APM) of AP-like product was recovered from 2g of AA-Na after the third Sephadex G-10 treatment.

Ultraviolet spectral analysis. Figure 5.3 illustrates the u.v. absorption spectra of the AP-like product and the authentic AP and AA.

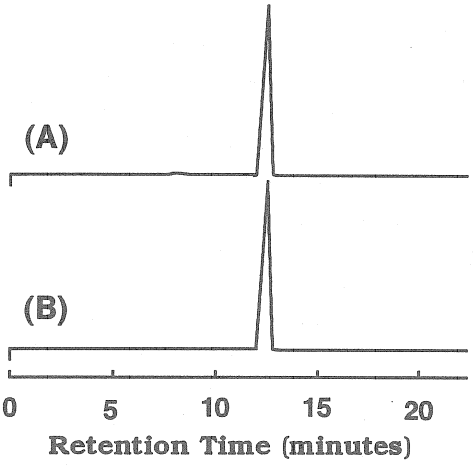

Fig. 5.2. HPLC profiles of (A) isolated AP-like product dissolved in water (retention time: 12.74 minutes) and (B) authentic APM dissolved in water (retention time: 12.77 minutes).

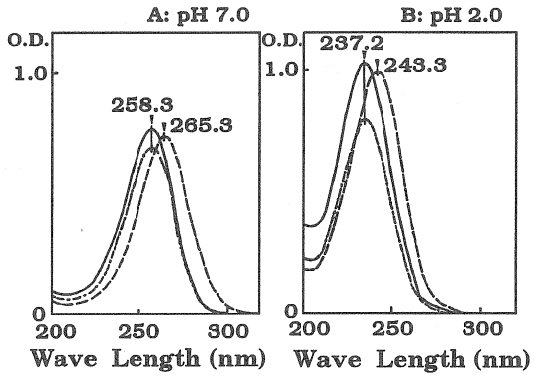

Fig. 5.3. Ultraviolet absorption spectra of the isolated AP-like product (——), the authentic AP (— - —) and the authentic AA (----) at pH 7.0 (A) and pH 2.0 (B).

Absorption maxima at pH 7.0 and 2.0 were: AP-like product, 258.3 and 237.2nm; authentic AP, 258.3 and 237.2nm authentic AA, 265.3 and 243.3nm, respectively. The absorption spectra and absorption maxima of the AP-like product were completely identical to those of authentic AP.

Reducing activity. Reducing activity toward DCIP was found only in AA, while AP and the AP-like product did not reduce DCIP.

TLC analysis. The Rf values of the AP-like product were identical to those of authentic AP (0.31 for propanol-water-ammonia, 0.64 for butanol-acetic acid-water). The detection of the AP-like product and AP was performed by scanning at 260nm. Also, they could be detected as a wine red spot (480nm) by spraying ferric chloride on the TLC plate. This coloration reaction is specific for several phosphorylated derivatives of AA.[121]

Acid hydrolysis. Figure 5.4A and B illustrate the HPLC profiles before and after acid hydrolysis, respectively. Peak 1 was the solvent peak. Peak 2 was the AP-like product corresponding to the authentic AP. After hydrolysis, the peak area of peak 2 decreased and a new peak (peak 3), whose retention time coincided with that of authentic AA, appeared. Measuring the inorganic phosphate before and after hydrolysis by the method of Nakamura[122], an increase of inorganic phosphate was found by hydrolysis.

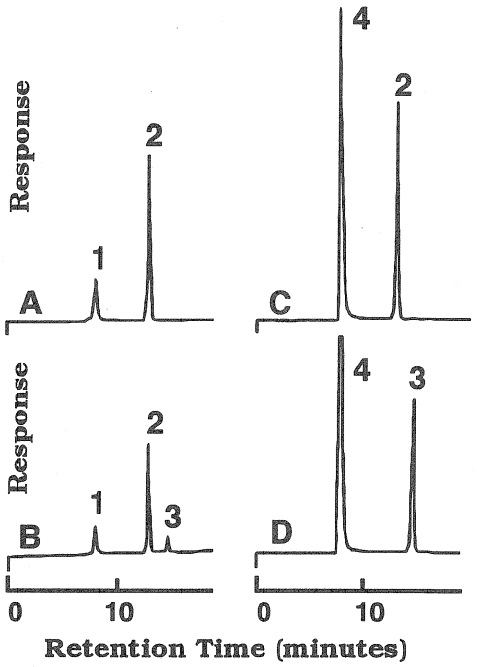

Fig. 5.4. HPLC profiles before and after hydrolysis of AP-like product. The isolated AP-like product was hydrolysed in 6M HCl at 100℃ for 1 hour and was hydrolyzed by purified phosphatase (EC 3.1.3.2) in 50 mM acetate buffer (pH5.0) at 25℃ for 10 minutes. Before and after the hydrolysis, an aliquot of each sample was assayed by the HPLC. The charts (A), (B), (C) and (D) are before acid hydrolysis, after acid hydrolysis, before phosphatase hydrolysis and after phosphatase hydrolysis, respectively. (Peak 1, solvent peak; peak 2, AP-like product; peak 3, AA; peak 4, metaphosphoric acid.)

Phosphatase hydrolysis. The AP-like product was hydrolyzed by phosphatase to examine whether it was sensitive for phosphatase. Figure 5.4 C and D illustrate the HPLC profiles before and after hydrolysis by acid phosphatase, respectively. Peak 4 was the peak of metaphosphoric acid used as deproteination reagent. Peak 2 was the peak of the AP-like product before the hydrolysis. After the hydrolysis, the AP-like product disappeared, while AA appeared (peak 3). In this case, 39.2nmol of AP-like product was hydrolyzed and 37.0nmol of ascorbic acid was detected.

Mass and NMR analysis. Figure 5.5 shows the mass spectrum of the AP-like product. The AP-like product gave three strong peaks at m/z 257, 274 and 279. The peaks of 110 and 115 are glycerol+NH_4^+ and glycerol+Na^+, respectively. In the ^{13}C-NMR spectra, as shown in Fig. 5.6, the AP-like product, the authentic APM and the authentic AP sodium salt gave similar doublet signals at the C-2 position. Only six signals of ^{13}C were found in all the preparations. From these results, it can be concluded that the AP-like product is ascorbyl-2-monophosphate itself.

Kinetic analysis The AP-forming activities as a function of incubation pH and temperature are illustrated in Figures 5.7 and 5.8, respectively. The optimum pH was around 5.0 and the optimum temperature was 30℃. The substrate specificity of AP-forming was examined for triphosphate, pyrophosphate, phosphoric acid, ATP, ADP, AMP and glycerophosphate as a phosphoryl donor. As shown in Table 5.1, pyrophosphate was the best substrate. The distribution of AA phosphorylating enzyme in the viscera of rainbow trout was examined (Table 5.2). The activity was very high in the liver and low in other organs. The liver enzyme had the Km values, 370mM for AA and 83mM for pyrophosphate.

Discussion

The AP-like product was produced in the reaction mixture consisting of AA, pyrophosphate and rainbow trout liver homogenate. The AP-like product isolated from the reaction mixture had the same properties as authentic AP on the analysis of HPLC, u.v. spectrum, TLC, LC mass and NMR spectra. The AP-like product was composed of AA and inorganic phosphate, because the AP-like product released these components by the hydrolysis.

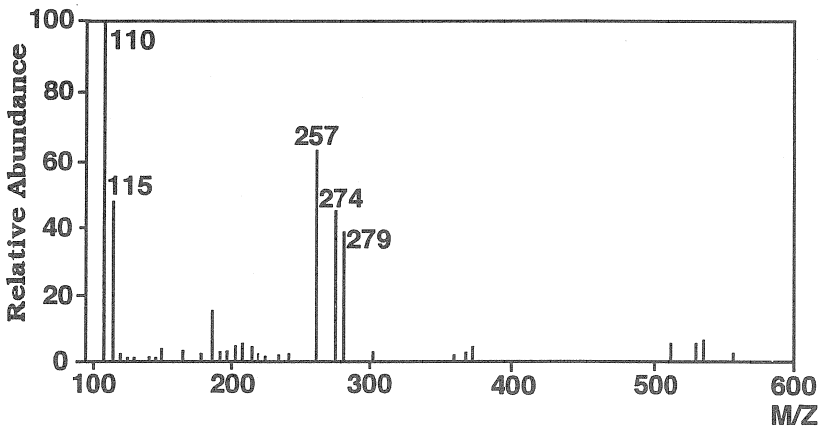

Fig. 5.5. Mass spectrum of the isolated AP-like product in glycerol matrix.

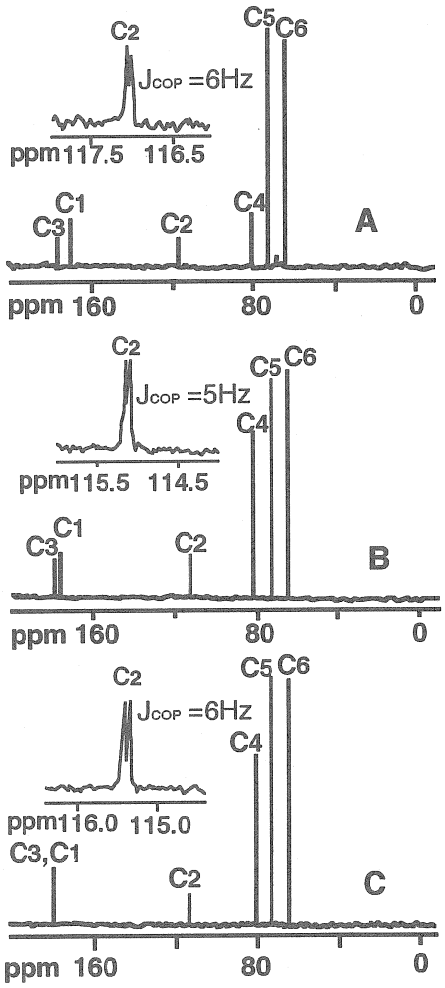

Fig. 5.6. ^{13}C-NMR spectra of the AP-like product isolated. [(A) AP-like product; (B) authentic magnesium L-ascorbyl-2-phosphate; (C) authentic sodium L-ascorbyl-2-phosphate.]

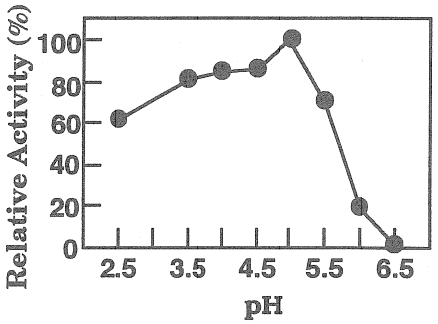

Fig. 5.7. Effect of pH on the AP-forming activity. The reaction mixture (1m*l*) contained 40mM pyrophosphate, 40mM L-ascorbic acid, 100mM acetic acid and 0.25m*l* of the crude enzyme from rainbow trout liver. It was incubated at 30℃ for 1 hour and AP formed was measured by HPLC. The activity at pH 5.0 was taken as 100%.

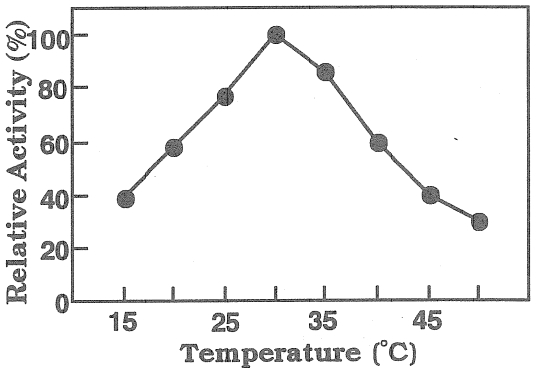

Fig. 5.8. Effect of temperature on AP-forming activity. The reaction mixture (1m*l*, pH 5.0) contained 100mM pyrophosphate, 500mM L-ascorbic acid, 100mM acetic acid and 0.5m*l* of crude enzyme from rainbow trout liver. It was incubated for 10 minutes and the AP formed was measured by HPLC. The activity at 30℃ was taken as 100%.

Table 5.1. Phosphoryl donor specificity of AP-forming enzyme

Substrates	Relative activity (%)
Triphosphate	49.9
Pyrophosphate	100.0
Phosphoric acid	1.1
ATP	9.5
ADP	19.7
AMP	0.6
Glycerophosphate	2.9

The reaction mixture (1m*l*, pH 5.0) contained 40mM of each phosphate substrate, 40mM L-ascorbic acid, 100mM acetic acid, 10mM $MgSO_4$ and 0.5m*l* of crude enzyme from rainbow trout liver. After incubation at 30℃ for 30 minutes, the AP formed was measured by HPLC to compare the relative activity of AP-forming for several substrates. The rate for pyrophosphate is taken as 100 and the other values are indicated as percentages of this value.

Table 5.2. Distribution of AA phosphorylating enzyme in viscera of rainbow trout

Organs	Relative activity (%)
Liver	100.0
Kidney	5.3
Spleen	1.1
Stomach	3.1
Pyloric caeca	2.4
Intestine	2.4

The reaction mixture (1m*l*, pH 5.0) contained 50mM pyrophosphate, 50mM L-ascorbic acid, 100mM acetic acid and 0.5m*l* of crude enzyme from rainbow trout organs. To obtain each enzyme, each organ was treated as liver described in the text. Ater the incubation at 30℃ for 30 minutes, the AP formed was measured by HPLC. The rate for liver is taken as 100 and the other values are indicated as percentages of this value on the basis of AP formed per gram of tissue.

The AP-like product gave three strong peaks in the mass spectrum, indicating that the isolated AP-like product is composed of three compounds. These compounds are considered to be ascorbyl phosphate ($C_6H_9O_9P$, mol. wt 256), ascorbyl phosphate ammonium salt ($C_6H_{12}O_9NP$, mol. wt 273) and ascorbyl phosphate sodium salt ($C_6H_8O_9PNa$, mol. wt 278), because sodium hydrogen carbonate and ammonium formate were used as eluents in the isolation process of the AP-like product. The results of ^{13}C-NMR analysis support the conjugation of the phosphate to only the C-2 position of ascorbic acid. From these results it can be concluded that the AP-like product formed in the enzymatic reaction is exactly ascorbyl-2-monophosphate.

Recently, Koizumi *et al.*[123] reported the purification and characterization of ascorbic acid phosphorylating enzyme from *Pseudomonas azotocolligans*. Their results coincided with the present results in several respects. They found ATP, pyrophosphate and tripolyphosphate can be used as phosphoryl donors but not phosphoric acid. In the present study, ADP, ATP, pyrophosphate and tripolyphosphate were good substrates for AP-forming activity, but phosphoric acid and AMP were extremely poor substrates. The optimum pH for pyrophosphate was around 5.0 in rainbow trout enzyme, but in the bacterial enzyme it was 4.0 for pyrophosphate and 5.5 for ATP.[123] The optimum temperature in rainbow trout enzyme was 30℃, although 50℃ in the bacterial enzyme.[123] The apparent Km values for AA and pyrophosphate were very high; 370 and 83mM, respectively, in rainbow trout enzyme. It was reported in the bacterial enzyme that the values were 147mM for AA and 10mM for pyrophosphate. With respect to the substrate specificity, the acidic optimum pH and the high Km values, the AP-forming enzyme of rainbow trout resembles that of the bacterial enzyme.

Some phosphatases from several species of bacteria were reported to have the phosphorylating activity.[124, 125] Koizumi *et al.*[123] reported that the purified AP-forming enzyme from *Pseudomonas* had phosphatase activity and they proposed that the formation of AP might be done by the reversed reaction of phosphatase activity. In the present study, the optimum pH of AP hydrolysis activity in rainbow trout liver homogenate was around 3 to 5 (CHAPTER 3; Section 2) and that of AP-forming activity was around 5. Moreover, the Km value for AP in AP-hydrolysis activity in the liver was around 17mM (CHAPTER 3; Section 2) and the Km for AA in AP-forming was extremely high (370mM). Therefore, the AP formation might be catalyzed by the enzyme which hydrolyzes AP to AA and phosphoric acid.

The occurrence of a bound form such as L-ascorbyl-2-sulfate (AS) in brine shrimp cysts[126], human urine[127] and rat[128] was found as well. Tucker and Halver[129] concluded that AS is a major storage form of vitamin C in fish. Sandnes *et al.*[48] could not detect AS in the liver of Atlantic salmon but found a bound form of AA. Wang and Seib[130] also detected the bound form of AA in fish tissues by methanolysis. A bound form of AA, ascorbic acid α-glucoside was reported to be formed by mammalian enzyme.[51] There is thus a speculation that these conjugated compounds may be stabilized forms in the body and be converted to the native vitamin when required to maintain the vitamin C level. It strongly suggests that some conjugated forms of AA exist and play some roles in tissues of animals including fish.

It is well known that phosphorylated vitamin B_6 occurs in animals but unknown whether phosphorylated vitamin C (AP) occurs in animals. In the present study, the author found the *in vitro* production of AP in rainbow trout liver. Considering the high Km value for AA, possibility of AP synthesis in rainbow trout body in situ seems low unless AA concentration is high enough in tissues. AA concentration was saturated in rainbow trout liver at a level of about 200 μg/g (about 1.1mM, CHAPTER 2). At this saturated level, it is expected that the rate of AP hydrolysis exceeds that of AP synthesis because of the high Km value (370mM). Since micro distribution of AA at high concentration in cells was proposed[131], it is impossible to eliminate a possibility for the wide occurrence of AP in animal tissues.

Section 2. Distribution of Ascorbyl-2-phosphate Synthetase in Fish Tissues

AP was enzymatically formed from AA and phosphoryl donor by liver homogenate of rainbow trout, as described in the previous section. In this section, the author describes the distribution of the enzyme in the liver cells of rainbow trout and in several kinds of fish organs and discusses the correlation of AP synthetase activity to the activity of AP hydroxylase, phosphatase and pyrophosphatase.

Materials and Methods

Fish

Fishes and animals used were rainbow trout *(Oncorhynchus mykiss)*, lamprey *(Lampetra japonica)*, red skate *(Dasyatis akajei)*, gummy shark *(Mustelus manazo)*, eel *(Anguilla japonica)*, common carp *(Cyprinus carpio)*, mafuna *(Carassius auratus)*, cat fish *(Silurus asotus)*, sardine *(Sardinops melanostictus)*, horse mackerel *(Trachurus japonicus)*, *kijinu (Acanthopagrus latus)*, mackerel *(Scomber japonicus)*, yellowtail *(Seriola quinqueradiata)*, chicken, rabbit and beef. Rainbow trout reared in the fish tank of Department of Fisheries, Faculty of Agriculture, Kyoto University were used to fractionate liver cells. Other fresh fishes used were obtained from local markets.

Crude enzyme

Cell components of rainbow trout liver were fractionated by the methods of Hogeboom[132]. The fractions were suspended in sucrose solution (0.25M) and used as crude enzyme. A pooled sample, obtained from 3 individuals, of each tissue (liver, kidney or spleen) was homogenized in 5 vols. of 0.9% NaCl solution with a glass homogenizer for 5 minutes. The homogenate was disrupted by ultrasonic for 1 minute with Microson Ultrasonic Cell Disrupter MS-50 under ice cooling.

Assays for enzyme activities

AP synthetase. The reaction mixture (1.5m*l*, pH5.0) contained 0.1M pyrophosphate, 0.5M ascorbic acid and 0.5m*l* of crude enzyme. The mixture was incubated at 30℃ for 30 minutes and stopped the reaction by 20% metaphosphoric acid. The filtrate of the mixture was applied to the HPLC (Table 5.3) to determine AP formed.

AP hydrolase. The reaction mixture (1.1m*l*, pH5.0) contained 40mM AP-magnesium salt, 100mM acetic acid and 0.1m*l* crude enzyme. The mixture was incubated at 30℃ for 30 minutes and stopped the reaction by 20% metaphosphoric acid The filtrate of the mixture was applied to the HPLC (Table 5.3) to determine AA formed.

Acid phosphatase. The reaction mixture (3.05m*l*, pH5.0) contained 1mM *p*-nitrophenyl phosphate, 200mM acetic acid and 50μl of crude enzyme. The mixture was incubated at 30℃ for 30 minutes and stopped the reaction by 10m*l* of 0.4N NaOH. Absorbance at 405nm was observed to determine the release of the phosphate.

Pyrophosphatase. The reaction mixture (1.0m*l*, pH7.0) contained 200mM tris, 4mM sodium pyrophosphate, 5mM $MgCl_2$ and 0.2m*l* of crude enzyme. The mixture was incubated at 30℃ for 30 minutes and the reaction was stopped by 20% TCA. Remaining pyrophosphate was precipitated by adding 3M acetate buffer (pH5.0) containing 10mM $MnCl_2$ to the

Table 5.3. HPLC condition for the determination of AA and AP

Column	COSMOSIL $5C_{18}$-AR 4.6×300mm
Mobile phase	0.1M Phosphate buffer (pH3.0)
Flow rate	0.4m*l*/minute
Detector	Hitachi L-4000 UV Detector at 254nm
Processor	Hitachi 833 Chromato-Processor
Pump	Hitachi 655 Liquid Chromatograph

mixture. After centrifugation, supernatant was obtained to determine free phosphate by the method of Nakamura[122].

Results

Distribution of enzyme activities in the liver cells

Table 5.4 shows the distribution of the enzyme activities in the liver cell of rainbow trout. High activity of AP synthesis was found in the fraction of microsome. AP hydrolase activity was high in the fractions of microsome and mitochondria. There was little variation in the distribution of phosphatase activity in contrast to that of pyrophosphatase localized in supernatant fraction.

Distribution of Enzyme Activities in various tissues of fishes and domestic animals

Table 5.5 shows the comparison of AP synthetase, AP hydrolase, acid phosphatase and pyrophosphatase activity of various tissues of fishes and domestic animals. The four enzyme activities were found in all the tissues tested. High activity of AP synthetase was found in the liver of cat fish, beef, yellowtail and eel and the hepatopancreous of carp. There was a variation of the activity of AP synthetase among the species and tissues (0.008 to 5.7 nmol/mg·minute). Activity of AP hydrolase was high in the spleen of cat fish and the kidney of mackerel. Activity of acid phosphatase was high in the carp kidney and hepatopancreous. Pyrophosphatase activity was high in the kidney of yellowtail and in the liver of rainbow trout and yellowtail.

Discussion

Activity of AP synthetase was high in the fraction of microsome, which contains low molecular acid phosphatase derived from outer lysosome. Several kinds of the acid phosphatase derived from such location is known to have an activity of translocation of phosphate.[133] It is possible to consider that AP synthetase catalyzes the reverse reaction of phosphatase.

Pyrophosphatase activity was investigated in this study, because pyrophosphate was good substrate for AP synthetase as phosphoryl donor (described in the previous section). As shown in the Table 5.4, however, the distribution of AP synthetase was different from that of pyrophosphatase but rather similar to that of acid phosphatase.

Correlation coefficients among the four enzyme activities tested were calculated and listed in the Table 5.6. The values of AP synthetase with AP hydrolase and pyrophosphatase were high (0.904 and 0.847) in spleen and kidney, respectively. Correlation between AP synthetase and AP hydrolase activity was rather high in each tissue and the coefficient value calculated from all tissue activities was low. However, plotting the correlation between AP synthesis

Table 5.4. Distribution of AP synthetase, AP hydrolase, acid phosphatase and pyrophosphatase in fractionated liver cell of rainbow trout (n mol/mg protein minute)

Fractions	AP synthetase	AP hydrolase	Acid phosphatase	Pyrophosphatase
Homogenate	4.0	18.9	12.0	183.7
Nuclear	1.4	2.0	16.7	303.0
Mitochondrial	1.8	17.6	20.4	95.8
Supernatant	0.1	4.9	20.4	997.4
Microsomal	8.3	23.3	18.5	100.3

Table 5.5. Comparison of AP synthetase, AP hydrolase, acid phosphatase and pyrophosphatase activity of various fishes (n mol/mg protein minute)

Species	Tissue	AP synthetase	AP hydrolase	Acid phosphatase	Pyrophosphatase
Lamprey	Liver	0.008	24.1	25.9	56.9
Red skate	Liver	0.1	18.9	27.1	59.3
Gummy shark	Liver	0.1	23.4	34.0	55.9
Eel	Liver	3.4	41.1	24.7	88.5
Carp	Hep*	3.3	29.9	71.9	68.6
	Kidney	0.7	39.9	80.6	6.2
	Spleen	0.1	34.8	53.8	16.0
Mafuna	Liver	0.3	42.2	53.9	93.5
	Kidney	0.4	51.6	42.7	54.5
	Spleen	0.4	34.3	37.7	45.1
Cat fish	Liver	5.7	59.4	28.6	29.7
	Kidney	0.1	23.2	18.8	9.3
	Spleen	0.7	80.7	57.4	25.2
Rainbow trout	Liver	2.7	28.2	14.3	125.7
	Kidney	0.3	29.6	16.1	21.1
	Spleen	0.4	34.2	16.4	83.2
Sardine	Liver	0.2	21.9	19.5	34.0
	Spleen	0.1	6.0	5.8	2.9
Horse mackerel	Liver	0.1	8.8	11.4	26.2
Kijinu	Liver	0.5	32.5	46.4	35.1
Mackerel	Liver	0.6	62.5	21.3	79.6
	Kidney	1.3	94.4	57.0	117.2
	Spleen	0.4	48.8	28.1	39.4
Yellowtail	Liver	3.6	25.5	30.1	121.0
	Kidney	2.5	65.1	35.6	129.0
	Spleen	0.2	22.6	21.3	57.1
Chicken	Liver	0.3	28.3	26.4	30.8
Rabbit	Liver	2.3	16.1	15.7	45.9
Cattle	Liver	5.6	54.8	23.8	26.9

* Hepatopancreous.

and AP hydrolysis activities (Fig. 5.9), it could be classified into two groups with high correlation. Possibly, there exist the two kinds of enzyme possessing the both activities in fish tissues.

One of the reason why AP is now expensive is the high cost of manufacturing upon chemical synthesis. AP production with an enzyme of bacterial origin was reported[118], but it has never been in practice on commercial base. Recovery of AP from a reaction mixture with the enzyme of fish origin was not inferior to that with bacteria origin. The activity seems to occur ubiquitous among animals; AP synthetase activity was found in all class of the fish tested and terrestrial animals. It is needed to establish the mass production of AP with enzyme of animal origin as well as bacteria origin for utilizing the waste of fish guts.

Table 5.6. Corelation coefficient of AP synthetase, AP hydrolase, acid phosphatase and pyrophosphatase activities

	AP hydrolase	Acid phosphatase	Pyrophosphatase
All tissues tested (n=29)			
AP synthetase	0.281	0.010	0.272
AP hydrolase		0.458*	0.305
Acid phosphatase			0.032
Liver tissue (n=15)			
AP synthetase	0.508	0.032	0.089
AP hydrolase		0.148	0.045
Acid phosphatase			0.114
Kidney tissue (n=6)			
AP synthethase	0.635	0.182	0.847*
AP hydrolase		0.458	0.876*
Acid phosphatase			0.077
Spleen tissue (n=7)			
AP synthetase	0.904*	0.532	0.277
AP hydrolase		0.752*	0.045
Acid phosphatase			0.245

* Correlation is significant ($p < 0.05$).

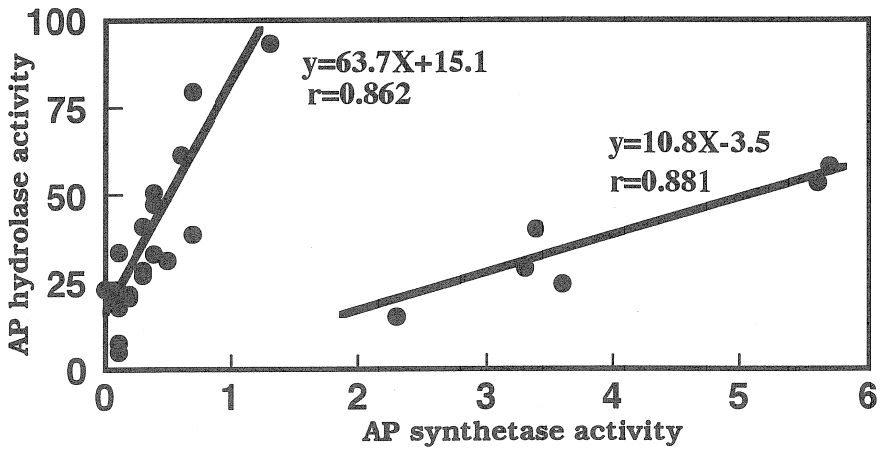

Fig. 5.9. Correlation between AP synthetase activity (x) and AP hydrolase activity (y) (n mol/mg·minute).

SUMMARY AND CONCLUSION

CHAPTER 1

The stability of ascorbyl-2-phosphate (AP) was compared with that of ascorbic acid (AA) in aqueous solution, in pelleted diet and in minced fish. AA and AP were dissolved in distilled water (1% AA equivalent) and stored in a transparent tube at room temperature. AA content after 20 weeks' storage was 39 % of the initial level; in contrast, AP remained at 100% after 20 weeks and at more than 94% after 180 weeks. Change of AP and AA content in moist and dried pellet during processing and storage was investigated. When AA was supplemented to the diet at a ratio of 50mg/100g, more than 40% of AA supplemented was rapidly oxidized to dehydroascorbic acid and inactive forms during the mixing and pelleting processes and no AA was detected in the moist pellet after one day storage; moreover, 95% of AA supplemented was destroyed to inactive forms during the drying process. To the contrary, AP showed excellent stability during the processing and storage. Retention of AP in moist pellet, which was supplemented with AP at a ratio of AA 50mg eq./100g diet, just after pelleting and after one day storage was more than 90% and 80%, respectively. More than 70% of AP supplemented was remained in the diet after the drying process in this case. Degradation of AP as well as AA occurred rapidly in minced fish. AA was degraded to inactive forms rapidly, but AP was first hydrolyzed to AA and then it was degraded to such forms. Therefore, the time lag of AP hydrolysis postponed the retention of ascorbate in the diet. These results indicate that AP is much more stable in feed processing and storage than AA, but susceptible to be hydrolyzed to AA by phosphatase occurring in such as fish mince.

CHAPTER 2

Section 1:

Rainbow trout fingerlings were fed on experimental diets containing different amounts of AP magnesium salt or AA for 15 weeks. No signs of scurvy were found in fish fed on AP- or AA-supplemented diet, though the fish of control group (AA free diet group) showed typical symptoms of scurvy. The liver and plasma AA concentrations were slightly higher in AP-supplemented groups than in the groups supplemented with an equimolar amount of AA. The molar ratios of hydroxyproline to proline of collagens isolated from the skin and bone of the fish fed on the AA-free diet were significantly lower than those from the fish fed on AP- or AA-supplemented groups. The foregoing results demonstrated that AP given is converted to AA stoichiometrically in the body and that AP has an equivalent physiological activity to AA for rainbow trout.

Section 2:

Adult rainbow trout was fed on the diet supplemented with massive amount (1000mg/100g) of AP. After 44 days feeding, determination of AA and AP, blood analysis and measurement of acid phosphatase activity in the liver were carried out for the fish. The results indicated that the massive dose of AP has no malign effect on rainbow trout as regards to growing, serum parameters, such as hemoglobin, protein, iron, phosphorus, calcium and BUN-S concentrations, activity of alkaline phosphatase, GPT and GOT, and to proximate composition of ordinary muscle and that rainbow trout accumulates no AP in the body even if the fish intakes massive AP. Moreover, it was concluded that the addition of AP instead of AA at low level to feed is more profitable, since several parameters such as hemoglobin and iron

concentrations, hematocrit value and hepatic acid phosphatase activity, indicated the reduced iron level in the body and a possibility of latent deficiency of AA in the fish fed on the diet containing low level AA (10mg/100g) but not in the fish given equimolar amount of AP.

Section 3:

Young rainbow trout was fed on the diet supplemented with AP or AA for 60 days and then starved for 60 days. Before and after the starvation, AA and carnitine concentrations and lipid contents were determined to examine the effect of ascorbate on the carnitine and lipid metabolism of the fish. Before the starvation, long-chain acylcarnitine concentration was significantly reduced in the control group (ascorbate free group) compared with the AP and AA supplemented groups. Lipid contents in AP and AA supplemented groups were significantly decreased during the starvation but not in the control group, that is, the fish whose AA level was reduced could not consume lipid efficiently as an energy source during the starvation. The results strongly suggested the participation of ascorbate in the carnitine and lipid metabolism in fish. AP as well as AA could prevent abnormal metabolism of carnitine and lipid, such as reduced long-chain acylcarnitine level and depression of lipid utilization during starvation, by raising the AA level in the fish body. The foregoing results confirmed that AP is effective as a vitamin C source in rainbow trout.

CHAPTER 3

Section 1:

The conversion of AP to AA was investigated *in vivo* in rainbow trout administered with AP orally and intraperitoneally. AP or AA was orally administered to the fish and the time course of the plasma AA concentration and the amounts of AP and AA in the contents of the alimentary canal were measured. There was no statistical difference in the plasma AA concentration between the AP-administered group and the AA-administered (control) group. The AP concentration in the plasma was below detectable level throughout the experimental period. Most of the AP administered was hydrolyzed to AA in the pyloric caeca and intestine but not in the stomach. AA concentrations in the plasma, liver and kidney increased rapidly after the intraperitoneal administration of AP. A little AP was detected in the plasma, liver and kidney 1 hour after the intraperitoneal injection; the AP concentration decreased below detectable levels after 3 hours. These findings indicated the ready conversion of AP to AA in rainbow trout body.

Section 2:

Activity of enzymatic hydrolysis of AP to AA was investigated using the homogenate of several tissues of rainbow trout. High activity was found in the pyloric caeca and the intestine at alkaline pHs and in the liver at acidic pHs. This *in vitro* result of high activity in the pyloric caeca and the intestine coincided with the results of *in vivo* investigation as described in the previous Section. The Km values for AP were not so different from the values for various phosphatase substrates. These results strongly suggested that the AP administered is hydrolyzed rapidly to AA by phosphatases which has relatively low specificity for substrates and which is ubiquitous in the body.

CHAPTER 4

Intestinal absorption of AP *in vitro* was investigated in rainbow trout. AP was injected into the intestine removed from the fish; and the intestine was ligatured at both ends and suspended in Ringer solution with aeration. AP

and AA transported from the mucosal side to the serosal side were measured by HPLC. AP passed through the intestine and part of the AP was detected as AA in the serosal fluid. The total quantity of ascorbate (AA + AP) transported was the same as that of the control injected sodium AA. The AP passed through the intestine without hydrolysis seemed to be readily hydrolyzed to AA in blood or other tissues, which were abundant in phosphatases. 2, 4-Dinitrophenol inhibited the transportation of AP and AA by more than 50%, suggesting the participation of active transport mechanism in AP and AA absorption from the intestine of rainbow trout.

CHAPTER 5

Section 1:

The possibility of AP synthesis by enzyme of fish origin was investigated. Enzymatic formation of AP was confirmed in the reaction mixture containing AA, pyrophosphate and the homogenate of rainbow trout liver by HPLC, ultraviolet absorption spectra, hydrolysis by phosphatase and acid, TLC, LC mass spectrum and ^{13}C-NMR analysis. Recovery of AP from 2g of AA-Na was about 40mg (APM eq.). The liver had the highest activity among the liver, spleen, kidney, stomach, pyloric caeca and intestine of rainbow trout. Pyrophosphate, triphosphate, ADP and ATP were good substrates as phosphoryl donors, but phosphoric acid and AMP were poor. The optimum pH and temperature of AP-forming activity in the liver were around 5.0 and 30℃, respectively. The Km values for AA and pyrophosphate were 370 and 83mM, respectively. Considering the high Km value for AA, possibility of AP synthesis in rainbow trout body *in situ* seemed low.

Section 2:

The distribution of AP synthetase and hydrolase activities in the liver cells of rainbow trout and in various tissues of fishes and domestic animals was investigated. Activity of AP synthetase was high in the fraction of microsome, which contains low-molecular-weight acid phosphatase having an activity of translocation of phosphate. AP hydrolase activity was high in the fractions of microsome and mitochondria. The activity of AP synthetase and hydrolase seemed to occur ubiquitous among animals, since the activities were found in all class of the fish tested and terrestrial animals. High activity of AP synthetase was found in the liver of cat fish, beef, yellow tail and eel and the hepatopancreous of carp. Correlation coefficient of AP synthetase with AP hydrolase was relatively high. It was strongly suggested that phosphatase, which hydrolyzes AP, catalyzes the AP synthesis reaction as well.

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魚類におけるアスコルビン酸-2-リン酸の代謝及び生理効果に関する研究

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ビタミンC(アスコルビン酸, AA)は元来不安定で，養魚飼料の製造・保存時にも速やかに分解される。そこで，本研究では安定型AAの一種，アスコルビン酸-2-リン酸(AP)の飼料中での有効性および魚体内での代謝を明らかにした。APは飼料中でAAに比べてはるかに安定であることを示した。またAPをニジマスに経口投与すると幽門垂および腸管内で速やかにAAに転換され，AAと同等のビタミンC効力を発揮することを認めた。APのAAへの加水分解活性は魚体内に広く分布し，ホスファターゼによるものと考えられた。さらに，魚類内臓に含まれる酵素を用いてAPの合成を容易に行い得ることを示した。