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Administration of gulonolactone does not evoke ascorbic acid synthesis in teleost fish

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Abstract

Evidence is presented that for common carp (*Cyprinus carpio* L.), intraperitoneal injection of L-gulonolactone, a precursor of ascorbic acid synthesis in the D-glucuronic acid pathway, does not result in an increased concentration of ascorbate in tissue. Control fish injected with an equimolar amount of ascorbic acid have shown a significant increase in ascorbic acid concentration in the kidney, hepatopancreas, plasma and spleen. The ascorbate status in the carp body, *i.e.*, the ascorbate nutritional history, produced significant differences in ascorbate withdrawal from circulation and probably in the catabolic rate. Acute fasting decreased ascorbate uptake into tissues as compared to fish fed a diet lacking ascorbate. Intraperitoneally injected ascorbate affects common carp being fed a diet containing 295 mg of total ascorbic acid kg^{-1} by causing tissue to become saturated with vitamin C, similar to the tissues in the group undergoing acute fasting. There was no gulonolactone oxidase activity in the hepatopancreas of the common carp. These results suggest that the metabolic rate induced by feeding is the primary factor regulating ascorbate requirement.

Introduction

Controversy surrounds the ability of lower vertebrates to synthesize ascorbate. It was shown that common carp (*Cyprinus carpio* L.) (Teleostei; Pisces), after injection with D-glucose and D-glucuronolactone, have ^{14}C-labelled ascorbic acid (Ikeda and Sato 1964). Following this observation, the biosynthetic pathway of ascorbic acid in common carp was examined further by gulonolactone oxidase activity studies (Yamamoto *et al.* 1978; Soliman *et al.* 1985). Feeding experiments, in which a diet lacking ascorbate was offered to young carp, resulted in no induction of scurvy or depression of ascorbate concentration in carp tissues (Sato *et al.* 1978). However, the growth rate of the fish was extremely slow. Recent studies (Soliman *et al.* 1985; Thomas *et al.* 1985) reported the presence of gulonolactone oxidase activity in other teleostean fishes systematically distinct from carp. However, inconsistency regarding whether gulonolactone oxidase activity occurs in specific fish species exists (Soliman *et al.* 1985; Thomas *et al.* 1985). The enzyme seems to be absent from the microsomal fractions of cells (Yamamoto *et al.* 1978), which differs from all higher vertebrates having the ascorbic acid synthesizing capacity (Sato *et al.* 1976; Kiuchi *et al.* 1982).

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Table 1. Initial and final individual body weights and ascorbate intake

	Experimental group			
	Fasting	Diet 1	Diet 2	Diet 3
Initial weight[1]	81.1 ± 19.0	78.1 ± 6.7	62.5 ± 10.g	66.5 ± 12.3
Final weight[1]	65.7 ± 15.4	128.5 ± 12.3	108.3 ± 27.0	111.0 ± 16.8
Ascorbate[2] AA	00	0.044	5.15	0.113
intake AS	0.0			5.30

[1]Data as shown as mean [g] ± SD (n = 10); [2]data are shown as mg kg $^{-1}$ wet body weight day $^{-1}$ on ascorbate molar basis.

Furthermore, Kitamura *et al.* (1965) demonstrated ascorbic acid deficiency in juvenile common carp manifested by lordosis. Dabrowski *et al.* (1988), and Dabrowski (1990b) found that tissue ascorbate concentration decreased in carp fed diets deficient in ascorbate, and this seems to be the most reliable indicator of ascorbate status in fish (Hilton 1984).

In order to resolve the controversy on ascorbate synthesis in common carp and other teleost fish, a new approach has been used in the present study. Two procedures, ascorbate and gulonolactone intraperitoneal injections, were applied simultaneously.

Intravenous injection of ascorbic acid in guinea pigs resulted in peak concentration of ascorbate in liver and kidney after 5–30 min, whereas it took 2–4 h to obtain peak concentrations in the spleen or adrenal gland (Hammerstrom 1966). On the other hand, the increase in dehydroascorbate concentration in the blood took several days in guinea pigs offered large doses of dietary ascorbate (Chatterjee *et al.* 1975). It seems, therefore, that the method of administering ascorbic acid influences both the time of tissue response and the catabolic rate. Intraperitoneal injection results in the steady increase of ascorbate in tissues (Reid 1948) and ascorbate reabsorption from the intestine as it occurs in fish (Dabrowski and Köck 1989). Thus it would seem unlikely that destruction of ascorbate would occur to any great extent in the digestive tract.

Dietary intake of ascorbate results in peak concentrations in plasma within 3–4 h in humans (Vinson and Bose 1988), 6–8h in chicks (Frigg 1987) and 4h in guinea pigs (Bush and Verlangieri 1987). Intraperitoneal injections of common carp during winter fasting allowed Shanta and Motelike (1962) to conclude that there is a linear relationship between injected doses (10–150 mg ascorbate kg^{-1}) and the increase in ascorbate concentrations of some tissues (kidney, ovary), while there is no response in other tissues (brain, hepatopancreas).

Sato and Grahn (1981) injected L-gulonolactone into guinea pigs to monitor formation of ascorbate in animals simultaneously injected with gulonolactone oxidase. In control animals, doses of 50 mg L-gulonolactone alone (in seven injections) did not change ascorbate plasma concentration.

The purpose of the present study is to develop an *in vivo* assay to determine the potential of ascorbate synthesis by comparing the effect of injecting equimolar amounts of ascorbic acid or its precursor, L-gulonolactone, in the D-glucuronic acid pathway (Eisenberg *et al.* 1959; Sato *et al.* 1976).

Materials and methods

The feeding experiment

Common carp (*Cyprinus carpio* L.) yearlings were offered three different diets, and one group was not fed during the 30-day period. Each group consisted of 10 fish (Table 1). Fish were acclimated to 22°C, and dissolved oxygen (polarographic sensor) was maintained close to saturation by water flow and aeration. The basal diet was prepared using a formula identical to that used by Dabrowski and Köck (1989). Diet 1 was not supplemented with ascorbic acid, whereas diets 2 and 3 were supplemented with

500 mg of L-ascorbic acid and 1084 mg ascorbic sulfate (dipotassium ascorbic-2-sulfate dihydrate) kg^{-1}, respectively. The diets were freeze-dried and stored in sealed bags at −18°C until required for feeding.

At the termination of the feeding trial, four fish from each experimental group were anesthetized and blood was obtained with a caudal vessel puncture. Blood was centrifuged (11,000 × g, 3 min) and the plasma fraction separated and frozen immediately. The fish were killed, and specific organs (hepatopancreas, kidney, spleen and gall bladder) were removed, quick-frozen, and kept at −70°C before analysis.

In a separate experiment with another lot of common carp, five fish were injected with 0.5 ml of physiological saline (0.65%) and ascorbate concentration was examined in tissues. Forty-eight hours after injection, concentrations of total ascorbic acid in kidney and hepatic tissues were not significantly ($p < 0.01$) different from those of fish before injection. It is therefore concluded that injection and anesthetizing of fish did not contribute to the differences in ascorbate concentrations in the major experiment.

Administration of ascorbate and gulonolactone

Six fish from each group were anesthetized (1:10000,MS 222) – and three were injected intraperitoneally with 2.5 mg of L-gulonolactone and three with 2.5 mg of ascorbic acid. These substances were diluted in 0.65% NaCl solution immediately before injection and each fish received 0.5 ml of the solution.

Each fish was marked and replaced in their original tanks for recovery. After 40 h they were anesthetized and blood and tissue samples were taken again.

Ascorbate assay

Frozen tissues, 100–200 mg, were homogenized in a cold extraction solution (5% trichloroacetic acid in 250 mM $HCLO_4$ containing 0.08% EDTA). Total ascorbic acaid and dehydroascorbic acid were analyzed according to a method described previously (Dabrowski and Hinterleitner 1989). Briefly, the dehydroascorbate forms color complexes with dinitrophenyl hydrazine (DNPH) at an acidic pH. Modification of the original method included background level determinations of interfering substances. It was confirmed that all interference was excluded by monitoring the absorbance of dinitrophenyl osazones on a Beckman DU-6 spectrophotometer. The difference in absorbance at 524 nm was measured directly on the scans.

Gulonolactone oxidase assay

A portion of kidney and hepatopancreas (0.3 g) was homogenized in a glass homogenizer in 2 ml of 50 mM phosphate buffer, pH 7.4, containing 1 mM EDTA and 0.2% of sodium deoxycholate. The homogenate was centrifuged for 30 min at 4°C at 30,00 × g. An aliquot of 200 µl of enzyme was incubated in 650 µl of 50 mM sodium phosphate buffer, pH 7.4, 50 µl of 50 mM gluthatione (GSH) and 100 µl of 100 mM L-gulonolactone at 25°C. The reaction was stopped at 0 and 2 h time by transferring 400 µl of the mixture to 400 µl of the protein precipitating solution mentioned above. Ascorbate was determined as described (Dabrowski and Hinterleitner 1989).

Student's t-test was used to evaluate the significance of differences.

Results

Diet 1 contained 2.7 µg g^{-1} of DNPH-reactive substances, which were identified as dehydroascorbate. An analysis of dietary ingredients did not reveal the source of this impurity. Diet 2 contained 295 µg g^{-1} of total ascorbate and diet 3, 316.5 µg g^{-1} of total ascorbic acid in the form of ascorbic sulfate. A small proportion of ascorbic sulfate (AS) was apparently hydrolyzed during storage and diet processing and this amounted to 6.7 µg g^{-1} of free ascorbate.

The fed fish increased in weight during the 30

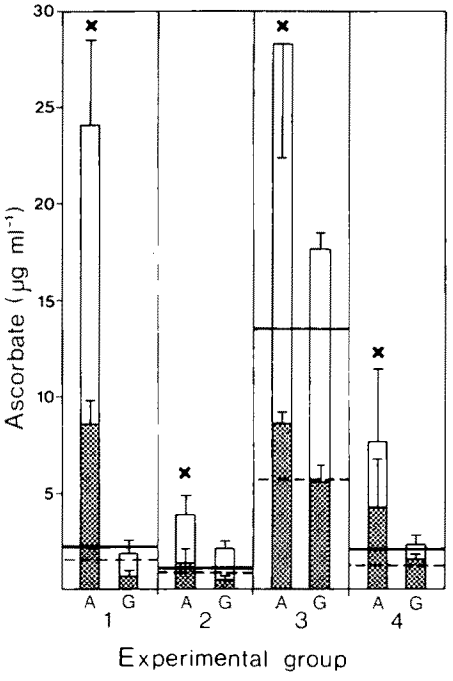

Fig. 1. Concentration of the dehydroascorbate (dashed columns) and the total ascorbate (whole columns) in common carp plasma following the ascorbic acid (A) or L-gulonolactone (G) injection. Experimental groups refer to (1) fasting fish; (2) scorbutic diet lacking vitamin C fed fish; (3) fish fed ascorbic acid supplemented diet; and (4) fish fed ascorbic sulfate supplemented diet. Solid and dashed lines refer to the pre-injection concentrations of the total and dehydroascorbic acid, respectively. Vertical bar represents SD of three experimental animals and an asterisk indicates the significant difference ($p < 0.05$) from pre-injection situation (total ascorbate).

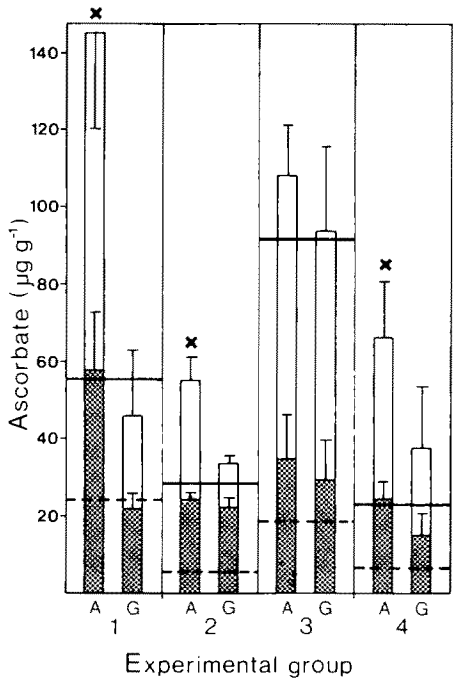

Fig. 2. Concentrations of the total ascorbate and dehydroascorbate in carp hepatopancreas. See legend to Fig. 1 for further details.

days, but the differences were not significant (Table 1). Fasting carp lost 19% body weight.

The effects of feeding fish a diet without ascorbate or diets supplemented with ascorbic acid (AA) or ascorbic sulfate (AS) were manifested in differences in ascorbic acid concentrations in fish plasma and tissues (solid and dashed lines in Figs. 1–4). The total ascorbate concentration decreased to half in fish fed diet 1 as compared to the fasting fish. However, this may result from the difference in an increase in weight in the group fed diet 1 and the group that fasted (Table 1).

The effect of body weight difference between fasting and fed fish might also account for the response in plasma ascorbate concentration (Fig. 1) after the ascorbic acid administration. However, the difference in the plasma ascorbate surge is by one order of magnitude and seems to be related to the rate of ascorbate transfer to tissues. Administration of L-gulonolactone did not provoke the significant increase of plasma ascorbate concentration. Ascorbic acid injection to carp increased the proportion of the reduced form in plasma.

Ascorbate concentration in hepatic tissue is essentially responding in a similar manner to that in plasma after the ascorbic acid or gulonolactone injection (Fig. 2). However, in combination with the results from plasma, it may be suggested that it is not only the transfer of ascorbate from plasma to tissues, but also the ascorbate catabolism in tissues that differs considerably between the fasting group and the group fed a scorbutogenic diet. Fasting carp accumulated more ascorbate in hepatopancreas than fish fed diet 1, as might be expected to result from the 'dilution effect'. One may also conclude, comparing group 3 and 4, that fish on an ascorbate supplemented diet catabolize more than fish on an ascorbic sulfate diet, considering the surge of ascorbate after injection. Differences between A and G subgroups (Fig. 2) indicate that L-gulonolactone has no value as a precursor in ascor-

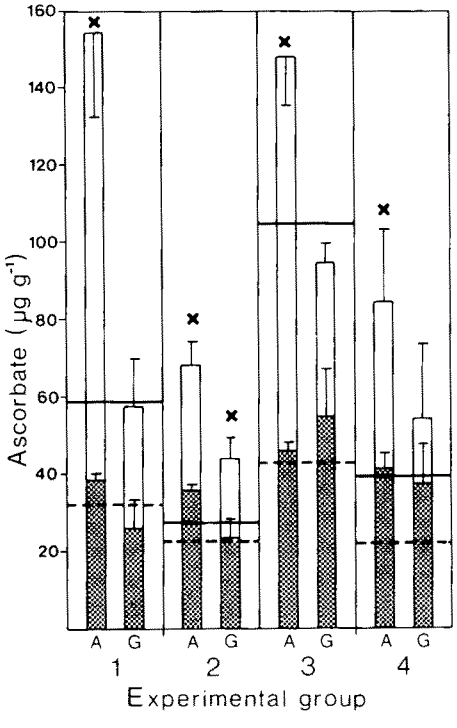

Fig. 3. Concentrations of the total ascobate and dehydroascorbate in carp kidney. See legend to Fig. 1 for further details.

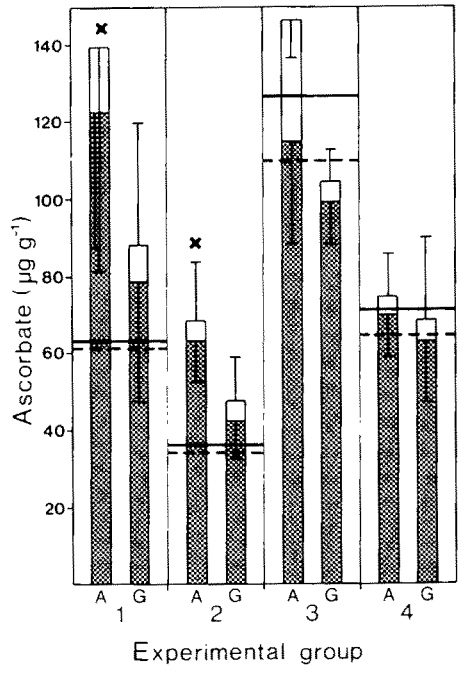

Fig. 4. Concentrations of the total ascorbate and dehydroascorbate in carp spleen. See legend to Fig. 1 for further details.

bate synthesis in carp hepatopancreas.

A redistribution of ascorbic acid might have occurred between the kidney and other tissues that have contributed to somehow obscure results in the former tissues (Fig. 3). The tendency is, however, the same, which supports the previous assumption that no ascorbate is synthesized after gulonolactone injection. Injection of ascorbate leads to a rise of the reduced ascorbate in the kidney. This is a marked contrast to ascorbate concentration in the spleen (Fig. 4). The dehydroascorbate predominates in the spleen in the total ascorbate pool and injection of the essentially reduced ascorbic acid intraperitoneally raised the concentration of the dehydroascorbate.

In an effort to explain the redistribution of ascorbate in starving fish (all groups were not fed during the 40h postinjection), the concentration of ascorbate in the gall bladder was analyzed (Table 2). Only slight differences between the A and G subgroups in ascorbate concentration suggest the minor role of the bile in the redistribution of ascorbate in carp.

It has been shown that the gulonolactone oxidase activity is not present in carp hepatopancreas and kidney tissues, both in scorbutogenic diet-fed fish and fish fed a diet supplemented with ascorbate. Control assays with kidney from pigeons and liver from rats and beef confirmed the presence of gulonolactone oxidase activity in these tissues, 1.91 $\pm$ 0.04 and 1.13 $\pm$ 0.03 mg ascorbic acid g^{-1} wet tissue h^{-1} at 25°C (Dabrowski 1990a).

Discussion

The present studies contradict the existence of gulonolactone oxidase in common carp (Yamamoto *et al.* 1978; Soliman *et al.* 1985; Sato *et al.* 1978) and suggest that the ascorbic acid synthesis is not taking place in this species as proven in other teleostean fishes, specifically rainbow trout and catfish (Wilson 1973; Hilton *et al.* 1979). This study also confirmed earlier studies by Kitamura *et al.* (1967) leading to symptoms of vitamin C deficiency in carp and the most recent studies on carp larvae (Dabrowski *et al.* 1988).

Table 2. Ascorbate concentrations in bile of carp

Initial		Fasting		Diet 1		Diet 2		Diet 3	
		A	G	A	G	A	G	A	G
AA	14.1 ± 2.6	11.3 ± 1.6*	8.9 ± 1.6	11.8 ± 1.7*	8.2 ± 0.4	10.2 ± 1.3	10.4 ± 2.4	9.2 ± 1.6	8.1 ± 0.3
DA	11.7 ± 1.5	10.1 ± 1.5	6.3 ± 2.5	11.6 ± 1.7	7.2 ± 0.5	9.2 ± 0.7	10.1 ± 2.8	8.7 ± 2.0	7.5 ± 0.7
(%)	17.1	10.7	29.3	1.7	12.2	9.8	2.9	5.5	7.5

Values are given as $\mu g\ g^{-1}$ tissue; Percent of the reduced form in total ascorbate (AA) is indicated (%). * Significantly different ($p < 0.05$). DA – dehydroascorbic acid. A – ascorbic acid injected fish, G – gulonolactone injected fish.

The time chosen for tissue sampling after intraperitoneal injection of ascorbate or gulonolactone to carp appears to be appropriate. Catabolism of ascorbate is much slower in poikiloterms than homotherms. Ikeda *et al.* (1963) have shown that muscular or intraperitoneal injection in carp resulted in radioactive ascorbate level ten times higher than oral intake approximately one day after administration. This is a much slower rate of absorption than would be deduced from the appearance of the peak of ascorbate concentration in homotherms after dietary administration (Frigg 1987; Vinson and Bose 1988). Reid (1948) demonstrated that ascorbic acid administered intraperitoneally to the guinea pig, a scurvy-prone mammal, appears in the contents of the gastrointestinal tract. This suggests secretion of ascorbate into the gut lumen, but a decrease of ascorbate in the contents of the posterior intestine also implies that ascorbate is reabsorbed or degraded in the large intestine. The former process seems to dominate after the intraperitoneal injection of ascorbate, since a large proportion is recovered in the urine.

The present study demonstrates that the injection of the gulonolactone is an interesting *in vivo* assay to evaluate the capacity to synthesize ascorbic acid in animals. Although the doses of gulonolactone injected in guinea pigs were much higher (Sato and Grahn 1981) than used in the present study, the former authors followed exclusively the plasma level of ascorbate. Sato and Grahn (1981) demonstrated that the simultaneous injection of gulonolactone and the immuno precipitated chicken kidney gulonolactone oxidase to guinea pigs increased plasma ascorbate concentration more than 3 times during 4 h. This means that both guinea pigs and common carp are similarly inheriting the absence of the enzyme, gulonolactone oxidase. Studies by Sato and Grahn (1981) indicated that gulonolactone is absorbed into circulation and a degradation rate of gulonolactone is very low, allowing the synthesis of ascorbate.

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