ASCORBIC ACID AND STABLE ASCORBATE ESTERS AS SOURCES OF VITAMIN C IN AQUACULTURE FEEDS

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Essentiality

Vitamin C is known to perform numerous biochemical and physiological functions in both plant and animal metabolism (Tolbert, 1979). Most animals can synthesize this vitamin in the form of ascorbic acid in amounts sufficient to prevent the clinical symptoms of deficiency collectively known as scurvy. However, primates, guinea pigs, fish, shrimp, and some insects, bats, and birds require a dietary source of vitamin C to prevent or reverse scorbutic symptoms. Among these species, dietary essentiality of vitamin C in fish and shrimp probably results from an absence or insufficiency of L-gulonolactone oxidase (Wilson, 1973; Yamomoto et al., 1978). This enzyme is required for biosynthesis of ascorbic acid from glucose or other simple precursors (Lehninger, 1971).

Function

Ascorbic acid is a strong reducing agent that provides electrons to functional groups of other biochemicals and free radicals found in the aqueous phase of biologic fluids. Two biochemical reactions commonly associated with the function of ascorbic acid in animals are hydroxylation and reduction. There may also be other as yet undetermined functions of ascorbic acid as suggested by Tolbert (1979).

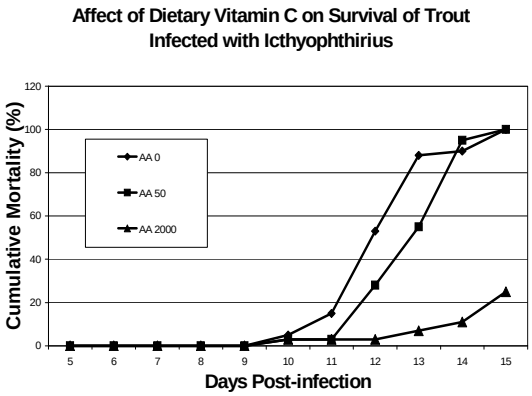
Over the past 30 years a great deal of research has been conducted to study the function of ascorbic acid in aquatic species. Effects of dietary ascorbic acid on growth, morphogenesis, reproduction, and adaptation have been studied extensively in carp (Sato *et al.*, 1978), catfish (Lovell, 1973; Wilson and Poe, 1973; Mayer et al., 1978; Lovell and Lim, 1978; Lim and Lovell, 1978; Li and Lovell, 1985; Lovell, 1982; Launer *et al*., 1978), trout and salmon (Hilton *et al*., 1977a, 1977b, 1978 & 1979; Sandnes *et al*., 1984; Wahli *et al*., 1977, 1985 & 1986; Grant *et al*., 1989), shrimp (Lightner *et al*., 1977; Magarelli *et al*., 1979; Magarelli and Colvin, 1978; Lightner *et al*., 1979), tilapia (Jauncey *et al*., 1985; Soliman *et al*., 1986) and even snake heads (Mahajan and Agrawal, 1980).

In all of these species the most studied, and perhaps best understood, function of ascorbic acid is its role as a cofactor in hydroxylating lysine and proline of collagen. This protein is the major component of connective tissue, including bone and cartilage. Impaired collagen formation results in the classical vitamin C deficiency symptoms of scurvy. These include lordosis and scoliosis, as well as poor growth, anorexia, reduced wound healing efficiency, and hemorrhaging.

Tucker and Halver (1984) cited other evidence supporting a similar role of ascorbic acid in hydroxylation reactions in carnitine synthesis. They suggested that early vitamin C deficiency symptoms of lethargy and fatigue may be due to depleted muscle carnitine. These symptoms have been reported in trout (Grant et al., 1989) and described similarly as prolonged periods of torpor.

Ascorbic acid also serves as a cofactor in hydroxylation reactions involved in excretion of drugs and toxicants. Its role in detoxification of organochlorine pesticides was investigated by Wagstaff and Street (1971). It was shown that ascorbic activity was required to perform specific detoxification reactions in the liver. Mayer *et al*. (1977) found that exposure to the pesticide toxaphene resulted in reduced levels of whole body vitamin C activity and decreased backbone collagen in fathead minnows and channel catfish. This lead to the hypothesis that hydroxylation reactions may compete with one another for available vitamin C activity, thereby increasing the requirement for ascorbic acid.

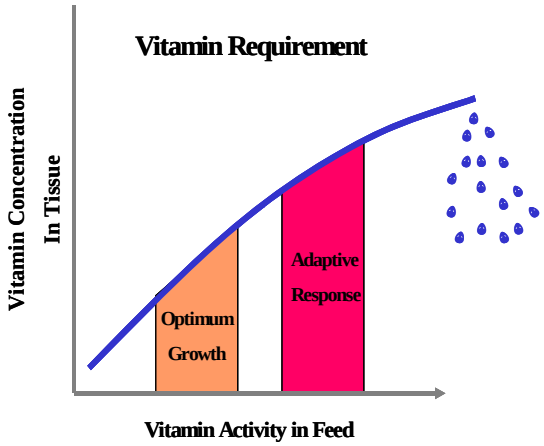
Studies with trout showed that ascorbic acid also plays a role in iron metabolism. Hilton *et al*. (1978) reported increased iron levels in the spleens of scorbutic fish, along with liver iron levels and hematocrit readings that were positively correlated to dietary levels of supplemental ascorbic acid. These data lead to the conclusion that ascorbic acid may control the release of iron within spleen tissue, thereby affecting the redistribution of iron stores.



Aside from these biochemical processes affecting growth and morphogenesis of various species of fish, vitamin C activity also has been linked conclusively to reproduction as well as adaptive responses such as disease resistance. Sandnes *et al*. (1984) and Soliman *et al*. (1986) showed that fish transfer ascorbic acid to eggs just before spawning, where it is used in larval development. Other researchers have observed increased resistance to bacterial infections by channel catfish (Lovell, 1982, and Li and Lovell, 1985) and reduced mortality in trout caused by the protozoan Ichthyophthirius multifiliis (Wahli et al., 1985, 1986 and 1995).

Requirement

A fish's requirements for vitamin C, like any other vitamin, are actually the amounts of vitamin activity required per kg of body weight per day to achieve specific physiological responses. At any response level, these requirements are affected by the size of the fish and its physiological state, as well as by nutrient interrelationships and environmental factors (NRC).



In general, relatively low vitamin C activity levels are sufficient for optimum growth and feed conversion, which is usually the desired response for fish that are cultured for food. Maximal adaptive response, such as disease resistance and tolerance to environmental stress, require slightly higher levels. For maximum tissue storage, as may be appropriate for broodstock or smolting salmon, loading levels would have to approach the point of excretion.

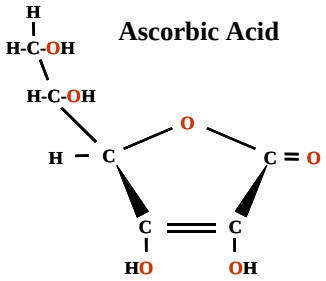
The nutritionist must take into account the desired response level and the whole body or indicator tissue concentration associated with that response, and calculate a concentration in the feed that will deliver that amount of vitamin C within the expected feeding range.

Different stability characteristics of various sources of vitamin C add to the difficulty of providing the amount required. Careful consideration must be given to losses of vitamin C activity that may be sustained during feed manufacturing and during the storage time before feeding.

Stability

Crystalline L-ascorbic acid is reasonably stable when dry and in pure form. However, it is easily oxidized in neutral or alkaline conditions. Oxidation by molecular oxygen, transitional metals, or other oxidants is accelerated by moisture, heat, and light.

Ascorbic acid is first oxidized to dehydroascorbic acid (DHA), which retains vitamin potency because it can be recycled to ascorbic acid by specific reductases and cofactors. However, in a second step DHA is rapidly and irreversibly converted to 2,3-diketogulonic acid, which has no vitamin C activity.

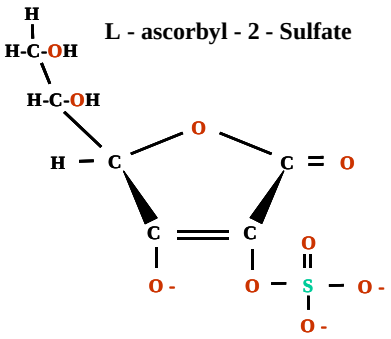


These oxidation steps occur quite rapidly in fish feeds, especially in formulations with high water activity (Putnam, 1976; Crawford et al., unpublished; and Grant, et al., 1989). Economic losses are substantial in spite of manufacturing and storage methods implemented within the feed manufacturing industry to reduce rapid loss of vitamin C activity. Processing and storage methods that remove oxygen, reduce heat, and avoid contact with iron, copper, and other metal salts measurably improve retention of vitamin C activity in feed. However, truly effective ascorbic acid stability has been achieved only by physically or chemically protecting it from oxidizing agents.

One method of physically protecting ascorbic acid from oxidation is encapsulation. Ethylcellulose, a water-soluble “coating” typically used as a compressible tableting compound, has been somewhat effective in providing increased stability of pure crystalline ascorbic acid during storage. However, when blended with other ingredients and subjected to all of the processes involved in feed manufacturing, ascorbic acid with this type of coating is only slightly protected. Hilton *et al.* (1977) reported manufacturing losses of vitamin C activity ranging form 74 to 100% in feeds supplemented with increasing levels of ethylcellulose-coated ascorbic acid. As with crystalline ascorbic acid, they showed that absolute losses of vitamin C activity from the coated product increased as supplement levels increased. However, when expressed as percent, the greatest losses were in feeds with supplement levels below 320 ppm.

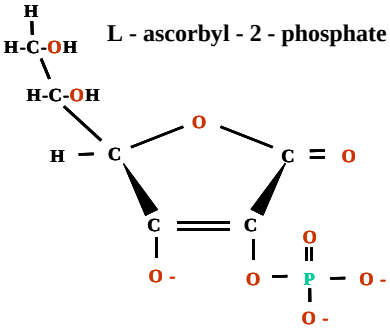
High melting point fats or waxes have also been used to protect supplemented ascorbic acid, particularly in feeds for aquatic species. Feeding trials have shown that these coating materials are highly digestible and do not adversely affect ascorbic acid bioavailability. Fat and wax coated products have been demonstrated to be more effective to varying degrees than ethylcellulose. Still, relatively high losses of vitamin C activity occur if shear forces and heat disrupt the coating. This tends to limit the effective application of these coated ascorbic acid products in extruded feeds.

As an alternative to encapsulation, several chemical methods of stabilizing ascorbic acid (against oxidation) have been developed to maintain vitamin C activity in aquaculture feeds. The most effective derivatives developed to date are 2-sulfate and 2-phosphate esters. In these compounds esterification protects the 2,3-enediol of AA from oxidation by substituting the 2-hydroxyl group with electron-dense phosphate or sulfate groups.



L-ascorbyl-2-sulfate (AS) is perhaps the most stable derivative of ascorbic acid yet discovered. It is a natural metabolite of ascorbic acid, occurring in the urine of primates, guinea pigs, and fish (Baker et al., 1971). AS is not a biologically available source of vitamin C in the guinea pig (Kuenzig, 1974) or the rhesus monkey (Machlin et al., 1976), but at high levels it has been shown to prevent vitamin C deficiency symptoms in rainbow trout and channel catfish (Halver, 1975). Based on growth data with channel catfish, the bioavailability of vitamin C activity in AS has been estimated to be almost one-fourth that of the of ascorbic acid on an equimolar basis (Murai et al., 1978).

The most recently developed ascorbic acid derivatives to be used in aquaculture feeds are phosphate esters. In 1987 Seib and Liao developed a process for producing a mixture of mono-, di- and tri-phosphorylated esters of ascorbic acid, called L-ascorbyl-2-polyphosphate (APP). This product is presently sold by Hoffmann-LaRoche under the brand name of Stay-C™. BASF Corporation markets a similar mono-phosphate (AMP) product as Aqua-Stab™. Both of these esterified forms of ascorbic acid are extremely stable under harsh feed manufacturing and storage conditions. Like AS, the number 2-carbon in the ascorbic acid molecule is chemically protected from oxidation. Unlike AS, however, both APP and AMP have been proven to be equivalent to ascorbic acid on an equimolar basis when fed to catfish (Brandt *et al*., 1985 and Robinson *et al*., 1989), rainbow trout (Grant *et al*., 1989) and shrimp (Shigueno and Itoh, 1988).



Bioavailability

In attempting to protect any labile nutrient from destruction, the objective is to stabilize the compound prior to consumption without compromising its biologic activity within the animal. Bioavailability of a micronutrient such as vitamin C from a physically altered or chemically derived form is evaluated on its ability to support growth, maintain tissue levels, and support other physiologic functions relative to ascorbic acid.

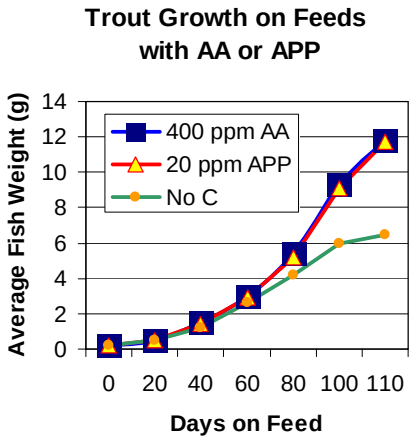
Presently available coated or encapsulated ascorbic acid products, although not very stable in many applications, have been shown to be good sources of vitamin C activity (Hilton et al., 1977). Bioavailability of vitamin C from AS, most likely depends on the presence of the enzyme sulfatase (Benitez and Halver, 1982) and the animal's ability to produce enough sulfatase activity to release required quantities of ascorbic acid

at all times. Likewise, APP and AMP require phosphatase activity to release their protecting phosphate groups.

In studies designed to test bioavailability of vitamin C from AS, results seem to vary with species and perhaps with environmental conditions. On the basis of growth and tissue storage data, Halver et al. (1975) concluded that AS was equivalent to ascorbic acid on an equimolar basis in meeting vitamin C requirements of rainbow trout. However, catfish feeding trials showed that at levels below 200 ppm vitamin C activity from AS growth was much lower than for fish fed 50 ppm of ascorbic acid (Murai *et al*. 1978). Additionally, vitamin C activities in blood and liver tissues were higher in catfish fed ascorbic acid than in those fed molar-equivalent AS. Sandnes et al. (1989) observed similar differences in tissue levels of vitamin C activity in Atlantic salmon fed AS verses those fed ascorbic acid. These data on fish, along with other studies documenting the absence of anti-scorbutic effects in monkeys and guinea pigs raise serious questions about AS as a useful source of vitamin C activity.

Documented anti-scorbutic effects in a wide range of animal species are cause to consider phosphate esters of ascorbic acid to be the most potentially useful forms of stable vitamin C. They have been shown to be active in guinea pigs (Imai et al., 1967, and Machlin et al., 1979), the rhesus monkey (Machlin et al., 1979) shrimp (Shigueno and Itoh, 1988), and several other aquatic species.

Data from tests conducted by Grant *et al.* (1989) show equivalent growth of rainbow trout fed casein-based purified diets initially containing 400 ppm vitamin C activity from ascorbic acid or 20 ppm vitamin C from APP. In the same experiment, control fish fed no ascorbic acid exhibited all of the classical symptoms of vitamin C deficiency.



In another feeding trial with practical diets containing either ascorbic acid or APP, rainbow trout were grown for 252 days from first feeding swim-up fry to market size. No statistically significant difference in growth rates were observed between those two groups, whereas fish fed the same diet without a supplemental source of vitamin C exhibited scorbutic symptoms.

Apparent equivalent activity was evident from ascorbic acid and AS tissue storage data collected after feeding supplemental APP and ascorbic acid at various dietary levels (Table 1). Whole body, total vitamin C activity levels in fish fed APP as the source of vitamin C were approximately twice as high as those fed ascorbic acid. In fish fed graded levels of vitamin C activity up to 100 ppm from APP, liver and kidney levels of vitamin C activity appeared to increase up to the maximum amount fed. Maximum storage levels were not determined.

To test reproductive performance of fish fed APP as a source of vitamin C, Grant and coworkers raised fathead minnows through a complete egg-to-egg life cycle (Table2). Reproductive success, as measured by number of eggs laid with subsequent normal larval development, was statistically similar for fish fed ascorbic acid or APP.

Perhaps the most striking evidence of bioavailability of vitamin C activity from APP is the adaptive response observed by Satyabudhy *et al*. (1989). Resistance to infectious hematopoietic necrosis (IHN) virus in six-week-old trout was directly proportional to APP feeding levels over the range of 20 to 320 ppm ascorbic acid-equivalents. This dramatic response was observed in both vaccinated and non-vaccinated test groups of fish, indicating an effect on both native and conferred immune response.

Summary Recommendations

Nutrition research has shown that vitamin C has biochemical functions that are critical to life. Most animals can synthesize sufficient amounts of this vitamin in the form of ascorbic acid. However, fish and shrimp are among the few other species that cannot. They must consume adequate amounts of ascorbic acid almost daily.

The challenge in providing feeds supplemented with adequate levels of vitamin C activity is complicated by the instability of ascorbic acid. Oxidation is accelerated in the complex matrix of feed during manufacturing and storage. To address the need for more stable sources of vitamin C, several effectively protected ascorbic acid products have been developed. These include coated ascorbic acid as well as phosphorylated esters of ascorbic acid. Each of these has advantages and disadvantages associated with their use. However, all of them have been proven effective in providing essential vitamin C activity.

Based on the combination of experimental results and field experience, researchers and vitamin companies recommend the following levels of vitamin C activity in feed at the time that it is consumed:

Recommended Vitamin C Levels

Culture Conditions	Vitamin C (mg/kg Feed)
1st Feeding	250 - 500
Grow-out	75 - 125
Stress	150 - 300
Broodstock	500 +

Additionally, one vitamin company recommends a vitamin C level of 1000 mg per kg feed whenever the immune system of fish is challenged, such as handling and grading, vaccination, disease outbreaks and transfer of smolts to seawater. Their recommendation is to feed this high level for 2 to 4 weeks before and at least 2 weeks following these stressful events.

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