58 Kandel, E.R. (1979) *Aplysia* among the molluscs II: the nervous system. In *Behavioral Biology of Aplysia: A Contribution to the Comparative Study of Opisthobranch Molluscs*, pp. 107–147, W.H. Freeman

59 Geraerts, W.P.M. *et al.* (1988) The peptidergic neuroendocrine control of egg-laying behavior in *Aplysia* and *Lymnaea*. In *Endocrinology of Selected Invertebrate Types* (Laufer, H. and Downer, R.G.H., eds), pp.141–231, Alan R. Liss

60 Whittal, R.M. *et al.* (1998) Nanoliter chemistry combined with mass spectrometry for peptide mapping of proteins from single mammalian cell lysates. *Anal. Chem.* 70, 5344–5347

61 Annan, R.S. and Carr, S.A. (1996) Phosphopeptide analysis by matrix-assisted laser desorption time-of-flight mass spectrometry. *Anal. Chem.* 68, 3413–3421

62 Stimson, E. *et al.* (1997) Enhancement of charge remote fragmentation in protonated peptides by high-energy CID MALDI–TOF MS using cold matrices. *Int. J. Mass Spectrom. Ion Processes* 169/170, 231–240

63 Dickler, S. *et al.* (1997) Improving mass spectrometric sequencing of arginine-containing peptides by derivatization with acetylacetone. *J. Mass Spectrom.* 32, 1337–1349

64 Pfeifer, T. *et al.* (1999) A strategy for rapid and efficient sequencing of Lys-C peptides by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry post-source decay. *Rapid Commun. Mass Spectrom.* 13, 362–369

65 Keough, T. *et al.* (1999) A method for high-sensitivity peptide sequencing using postsource decay matrix-assisted laser desorption ionization mass spectrometry. *Proc. Natl. Acad. Sci. U. S. A.* 96, 7131–7136

66 Preston, L.M. *et al.* (1993) Reproducibility and quantitation of matrix-assisted laser desorption ionization mass spectrometry: effects of nitrocellulose on peptide ion yields. *Biol. Mass Spectrom.* 22, 544–550

67 Nicola, A.J. *et al.* (1995) Application of the fast-evaporation sample preparation method for improving quantification of angiotensin II by matrix-assisted laser desorption/ionization. *Rapid Commun. Mass Spectrom.* 9, 1164–1171

68 Stoeckli, M. *et al.* (1999) Automated mass spectrometry imaging with a matrix-assisted laser desorption ionization time-of-flight instrument. *J. Am. Soc. Mass Spectrom.* 10, 67–71

69 Wei, J. *et al.* (1999) Desorption-ionization mass spectrometry on porous silicon. *Nature* 399, 243–246

Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin

R. Todd Lorenz and Gerald R. Cysewski

As a result of high production costs, commercial products from microalgae must command high prices. Astaxanthin produced by *Haematococcus* is a product that has become a commercial reality through novel and advanced technology. Cultivation methods have been developed to produce *Haematococcus* containing 1.5–3.0% astaxanthin by dry weight, with potential applications as a pigment source in aquaculture, poultry feeds and in the worldwide nutraceutical market.

Microalgae are a potential source of single-cell protein, a means of sequestering carbon dioxide from stack gas, and can be used in effluent purification and for producing biofuels. However, microalgae are expensive to produce, with direct production costs of US$5–20 kg dry weight^{-1}. High production costs preclude the use of microalgae as commercial sources of commodity products that can be produced inexpensively on a vast scale by plants. Nevertheless, small microalgae industries have been developed for the production of high-value products[1,2]. *Spirulina* is produced for the health and nutrition industry in the USA, China, India and Cuba. In Australia and Israel, the carotenoid β-carotene is produced from *Dunaliella*.

Astaxanthin is another high-value carotenoid produced from microalgae that is achieving commercial success. Astaxanthin is ubiquitous in nature, especially in the marine environment, and is probably best known for eliciting the pinkish-red hue to the flesh of salmonids, as well as shrimp, lobsters and crayfish.

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Because these animals are unable to synthesize astaxanthin *de novo*, carotenoid pigments must be supplied in their diet[3,4]. In the marine environment, astaxanthin is biosynthesized in the food chain, within microalgae or phytoplankton, as the primary production level. Microalgae are consumed by zooplankton, insects or crustaceans that accumulate the astaxanthin and, in turn, are ingested[5,6].

The major market for astaxanthin is as a pigmentation source in aquaculture, primarily in salmon and trout. Astaxanthin sells for ~US$2500 kg^{-1} with an annual worldwide market estimated at US$200 million. Although >95% of this market consumes synthetically derived astaxanthin, consumer demand for natural products makes the synthetic pigments much less desirable and provides an opportunity for the production of natural astaxanthin by *Haematococcus*.

Before *Haematococcus* became commercially available, natural sources of astaxanthin included krill oil and meal, crawfish oil and *Phaffia* yeast. However, these sources have low astaxanthin concentrations ranging from 0.15% in the oils to 0.40% in *Phaffia* yeast. As a result, the quantities required in the feeds for efficient pigmentation add deleterious bulk and ash to the final feeds. By contrast, *Haematococcus* contains between
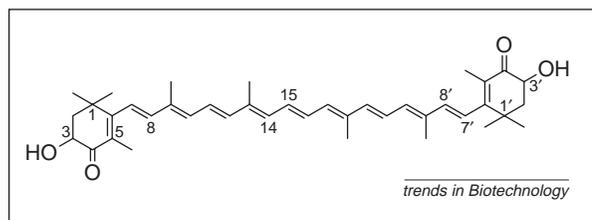
Figure 1
Structure of astaxanthin with a numbering scheme.

1.5–3.0% astaxanthin and has gained acceptance in aquaculture and other markets as a 'concentrated' form of natural astaxanthin.

Chemistry of astaxanthin

Carotenoids are a group of over 700 natural lipid-soluble pigments that are primarily produced within phytoplankton, algae and plants. These pigments are responsible for the broad variety of colours seen in nature; the most conspicuous are the brilliant yellow, orange and red colours of fruits, leaves and aquatic animals. Amongst all of the numerous classes of natural colours, the carotenoids are the most widespread and structurally diverse pigmenting agents. Only plants, algae and some fungal and bacterial species synthesize carotenoids, thus they must be supplied in the diet of animals[3,4,7]. Carotenoids are absorbed in animal diets, sometimes converted into other carotenoids and are incorporated into various tissues. Some fish species, such as koi and various crustaceans (*Penaeus japonicus* and *Penaeus monodon*), have the enzymatic mechanisms required to convert carotenoids into other forms such as astaxanthin[7,8].

In nature, algae synthesize the carotenoid pigment astaxanthin and concentrate it into the food chain through zooplankton and crustaceans, which are prey for salmon, trout and other aquatic animals[3,5,6]. The astaxanthin molecule has two asymmetric carbons located at the 3 and 3′ positions of the benzenoid rings on either end of the molecule. Different enantiomers of the molecule result from the way that the hydroxyl groups are attached to the carbon atoms at these centres of asymmetry (Fig. 1). When the hydroxyl group is attached so that it projects above the plane of the molecule, it is said to be in the 'R configuration', and when the hydroxyl group is attached to project below the plane of the molecule, it is said to be in the 'S configuration'. Thus, the three possible enantiomers are designated: 3R,3R′; 3S,3S′; and 3R,3S′ (meso).

Haematococcus primarily contains monoesters of astaxanthin linked to 16:0, 18:1 and 18:2 fatty acids. All of the free astaxanthin and its monoesters and diesters in *Haematococcus* have optically pure (3S,3′S) chirality[9,10]. Fatty acids are esterified onto the 3′ hydroxyl group(s) of astaxanthin after biosynthesis of the carotenoid, thereby increasing its solubility and stability in the cellular lipid environment. Studies now support a major role of astaxanthin accumulation in *Haematococcus* as a form of protection against high light and oxygen radicals.

Astaxanthin is biosynthesized through the isoprenoid pathway, which is also responsible for the vast array of lipid-soluble molecules such as sterols, steroids, prostaglandins, hormones and vitamins D, K and E. The pathway initiates at acetyl-CoA and proceeds through phytoene, lycopene, β-carotene and cantha-xanthin before the last oxidative steps to astaxanthin[4,7,10]. The carotenoid fraction of green vegetative cells consists mostly of lutein (75–80%) and β-carotene (10–20%), whereas in red cysts, astaxanthin accounts for ~80% of the carotenoid fraction[10,11]. The change in *Haematococcus* morphology can be dramatic when haematocysts accumulate astaxanthin (Fig. 2).

The composition of astaxanthin esters in *Haematococcus* is similar to that of crustaceans, the natural dietary source of salmonids. That is, the astaxanthin pool of encysted *Haematococcus* is approximately 70% monoesters, 25% diesters and 5% free. Wild rainbow trout caught in the alpine lakes of Austria are intensely pigmented with astaxanthin, and a qualitative analysis of the carotenoids in the skin and flesh reveals only the presence of the 3S,3S′ isomer. Because salmonids are unable to epimerize the 3-hydroxy groups, it follows that their crustacean diet also contained 3S,3′S-astaxanthin[12,13]. Another study revealed that fish caught
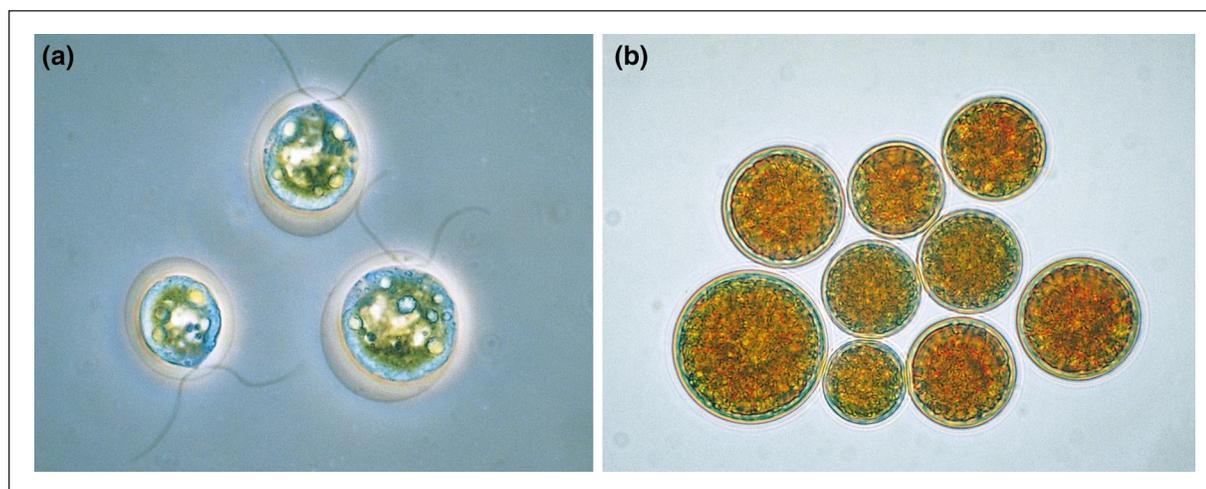

Figure 2
(a) Vegetative actively growing *Haematococcus* cells and **(b)** *Haematococcus* haematocysts that have accumulated astaxanthin as a result of nutrient starvation and sunlight. 400 × magnification.

from the wild in Scotland, Ireland and Norway contained >80% 3S,3′S astaxanthin in their flesh[14]. HPLC separation of astaxanthin isomers has also been used to identify the eggs of escaped salmon because wild fish contain ~80% 3S,3′S astaxanthin and farmed fish contain less than[15,16]. Thus, *Haematococcus* algae included in aquaculture feeds provide the same configuration of astaxanthin as their natural counterparts. By contrast, *Phaffia* yeast contain pure 3R,3′R astaxanthin; synthetic astaxanthin is a mixture of all three isomers[16].

Industrial applications

Haematococcus algae meal has been approved in Japan as a natural red food colour and as a pigment for fish feeds. In addition, the Canadian Food Inspection Agency has recently granted approval for *Haematococcus* algae to be used as a pigment in salmonid feeds, and approval from the US Food and Drug Administration (FDA) for its use as a colour additive in salmonid feeds is expected early in the year 2000. *Haematococcus* algae have been recently cleared by the US FDA for marketing as a dietary-supplement ingredient and have been approved in several European countries for human consumption.

It appears that the use of carotenoids as pigments in aquaculture have broader functions, including as: (1) an antioxidant, (2) hormone precursor; and in: (3) immune enhancement, (4) provitamin A activity, (5) reproduction, (6) growth, (7) maturation and (8) photoprotection. Researchers stress the vital role that carotenoids play in physiology and in overall health, and suggest that carotenoids are essential nutrients that should be included in all aquatic diets at a minimum level of 5–10 parts per million (ppm).

Salmon and trout farming

Perhaps the largest potential use of natural astaxanthin produced by *Haematococcus* is for salmonid aquaculture feeds. The continued growth of salmonid farming has created an enormous demand for pigments. The flesh colour of salmonids is the result of the absorption and deposition of dietary astaxanthin; salmonids are unable to synthesize astaxanthin *de novo*, therefore carotenoid pigments must be supplied in their artificial aquaculture diet[3,4].

The harvesting of wild salmon has been declining over the past 10 years and has probably reached its maximum sustainable level at ~800 000 tons annually. Conversely, salmon and trout farming have increased substantially during this period. The world production of all farmed salmon (in 1999) is estimated to be >750 000 metric tons annually. These figures are expected to reach one million metric tons by the year 2001 and 1.3 million tons by the year 2005. Although for many years Norway has been a leading producer of farmed salmon, its share of the total production might decline, however, it is still expected to produce ~620 000 tons by 2005. More recently, Chile has entered into salmon farming and ideal conditions in Southern Chile will allow for significant expansion, estimated to be ~350 000 tons by 2005.

Interestingly, a recent study in Norway demonstrated that Atlantic salmon fry have a definitive growth and survival requirement for astaxanthin in their diet[17]. Fish fed astaxanthin <5.3 ppm in their diet were found to have marginal growth; those fed levels >5.3 ppm had normal growth and significantly higher lipid levels. When salmon fry were fed astaxanthin concentrations <1 ppm, survival rates decreased significantly. More than 50% of the fry that were fed diets with <1.0 ppm astaxanthin died; survival rates of those groups receiving higher concentrations were >90%. Thus, Atlantic salmon have the distinction of being the first species for which astaxanthin has been shown to be an essential vitamin, with minimum levels of ~5.1 ppm. However, higher astaxanthin levels of 13.7 ppm in the feed increased the fish lipid levels by 20% and gave a better overall performance compared with the 5.3 ppm feeds. These results also indicate a provitamin A function for astaxanthin over the same period[17].

There are several reports on the use of *Haematococcus* algae meal for pigmenting salmonids, although numerous studies have demonstrated equal or superior pigmentation compared with synthetic astaxanthin. One of the first investigations involved a 100-day feeding trial in which intact or homogenized *Haematococcus* algae meal was compared with synthetic astaxanthin. The mean total carotenoid and astaxanthin levels were measured and these demonstrated the necessity of cracked (homogenized) cells for bioavailability of the pigment; the bioavailability of astaxanthin from uncracked cells was poor. Unfortunately, the disruption of the homogenized spores was only ~60% and probably reduced its total bioavailability and absorption from the gut. No significant differences were seen in the growth rate or levels of mortality in the trout, and the carotenoid levels and ester distribution measured in both flesh and skin were consistent with values for wild and farmed trout. The study established that *Haematococcus* algae meal caused significant astaxanthin deposition in flesh and skin, as well as a visual enhancement of flesh colouration, and concluded that it is a safe and effective pigment source for rainbow trout[18].

Another study used non-homogenized *Haematococcus* cells fed to rainbow trout in order to compare the pigmentation of muscle tissue with synthetic carotenoids. Approximately 6% of the trout's diet consisted of algae and no major effect on growth or mortality was observed. The report reiterated the importance of cracking the algal cells to increase the bioavailability of the carotenoids and it established that astaxanthin and canthaxanthin, which represented 85% of total carotenoids, caused significant pigment deposition in the muscle tissue. The final muscle carotenoid was 6.2 mg kg^{-1}, which is considered acceptable for market production. Thus, it was similarly concluded that *Haematococcus* algae is a safe and effective source of pigment for rainbow trout[19].

It has also been demonstrated that there is little difference between astaxanthin esters from krill and free astaxanthin in their absorption and deposition by Coho salmon. Neither is there a significant difference between the optical isomers of astaxanthin in the deposition efficiency in the flesh of Coho salmon that are fed diets containing astaxanthin esters from Antarctic krill[20,21].

Sea bream

Sea bream (*Chrysophrys major*, *Pagrus major*, tai, red snapper) are highly prized for the pigmentation of their skin, which is primarily owing to astaxanthin[22]. It was

first discovered that the reduced colour of cultured sea bream was caused by the lack of astaxanthin in their artificial diet. Sea bream that are cultured without a supply of astaxanthin contain only 5% of the astaxanthin content of their wild counterpart. The stomach contents of wild sea bream were found to contain *Squilla oratoria* and other crustacea that supply the necessary astaxanthin[23]. Sea bream that were provided with other dietary carotenoids, such as β-carotene, lutein, canthaxanthin and zeaxanthin, were unpigmented and unable to convert these pigments to astaxanthin. Carotenoids are lost from the flesh and the skin as a result of insufficient dietary intake, metabolic degradation and excretion. Thus, to obtain the reddish pigmentation of cultured sea bream, it is necessary to provide a source of dietary astaxanthin[22–24].

In one investigation, sea bream were fed diets containing either β-carotene, zeaxanthin, lutein, canthaxanthin, free astaxanthin or astaxanthin ester. Feeding β-carotene or canthaxanthin resulted in a decrease in the carotenoid level of the integuments. It was concluded that one could reduce the intake of astaxanthin ester by ~50% and still obtain a 2.3-fold higher carotenoid deposition than free astaxanthin[25].

Another study compared sea bream fed 100 ppm free astaxanthin with those fed with astaxanthin ester. In the group fed free astaxanthin, the carotenoid content of the skin improved for one month, but reached a saturation point and did not increase further. In the group fed astaxanthin ester, the carotenoids in the skin were significantly higher after both sampling periods. After two months, the group fed astaxanthin esters had a 1.7-fold higher astaxanthin content in the skin than the group fed free astaxanthin (13.23 mg kg^{-1} compared with 7.94 mg kg^{-1}). Thus, it appears that dietary astaxanthin esters are more efficiently utilized in sea bream than free astaxanthin[26].

Ornamental fish

The luminous carotenoid colours of tropical fish are not only the keys for species identification and mating signals but they might also have significant physiological roles. Ornamental fish obtain carotenoids from feeding upon algae, coral or prey that have accumulated these pigments. The copepod and euphausid microcrustaceans are by far the most abundant of all marine animals and act as the primary food for a wide variety of larger animals. Astaxanthin and tunaxanthin, either in a complexed or non-complexed form, are the most abundant carotenoids found in all marine animals.

One of the greatest challenges in the tropical marine ornamental industry is to accurately replicate the natural colour of fish that are maintained in captivity. Numerous operations that have mastered the art and science of propagation have failed to successfully market their fish as a result of the loss of pigmentation. Various products have been introduced to alleviate this problem, but none have performed as effectively and consistently as natural astaxanthin from *Haematococcus*.

On a commercial scale, an inclusion rate of 30 ppm astaxanthin is used to supplement live and flaked foods resulting in a significant colour improvement in most species of tetras, cichlids, gouramis, goldfish, koi and danios. In a recently published study, 100 ppm astaxanthin was incorporated into the diet of the clown anemone fish (*Amphripion ocellaris* and *Premnas biaculeatus*). Within a period of one week, the yellow, maroon and black pigmentation of the cultured clown fish intensified significantly. Subsequently, 1% *Haematococcus* algae meal was used to augment the diet of red velvet swordtails, rainbowfish, 24K mollies, topaz cichlids, discus, rainbow sharks, pink kissing gouramis and rosy barbs. Within one week, in each of the species, there were significant improvements in pigmentation, and some species had faster growth rates. Although a relatively high concentration of *Haematococcus* algae was used in the feed, many commercial producers prefer to use an acute pigmentation treatment to prepare fish for the market. Much lower dosages could effectively be used to maintain pigmentation[27].

Poultry

Natural astaxanthin derived from *Haematococcus* has been successfully used to pigment egg yolks. White leghorn hens were fed on a basal diet for two weeks to deplete their carotenoid levels; at the initiation of the feeding experiment, they had eggs with an average colour score of 4 on the Roche colour fan. New diets were then prepared that were supplemented with 0.5, 1.0, 1.5, 2.0 and 3.0 ppm of astaxanthin from homogenized *Haematococcus* algae meal; these were fed for a period of four weeks. After approximately seven days, the egg yolk pigments reached steady states of 5.8, 7.9, 9.4, 10.1 and 11.8 on the colour scale, respectively. The increased colour scores correlated with increased astaxanthin, zeaxanthin and lutein concentrations in the yolks. The hens were then returned to the carotenoid-free diet for an additional two weeks and each group decreased to the basal colour score of 4 within the period. It was shown that the deposition rate of the carotenoids, calculated from feed intake and retention in egg yolks, was ~16% with 2–20 ppm astaxanthin supplementation. In addition, the importance of feeding cracked algal cells (compared with intact cells), which allowed a 20-fold increase in bioavailability, was demonstrated[28].

In a study carried out to investigate the effects of *Haematococcus* algae meal on the efficacy and deposition of carotenoids in different broiler-chicken tissues, it was found that carotenoid concentrations increased in the liver, adipose tissue and breast muscle with increasing algae meal in the diets. In addition, the birds had increased yellow pigment in their skin, feet and beaks compared with the control group. The broilers on the algae meal diet gained weight faster, had significantly higher breast-muscle weights and higher feed efficiency, and there were no significant differences in mortalities or incidence of yolk sac infections. Further studies on the fertility of broiler breeders demonstrated a 5% increase in hatchability rates when *Haematococcus* algae meal was incorporated into the diets[29,30].

A trial at the University of New England (Armidale NSW, Australia) was conducted in 1998 to assess the suitability of NatuRose *Haematococcus* algae meal as a yolk pigment additive in a typical commercial wheat-based layer diet. The birds received feeds containing 0, 4, 8 or 12 ppm astaxanthin from *Haematococcus* algae meal in their feeds, and eggs were collected for one month to assess various quality parameters. The increase in egg yolk pigmentation became apparent after only 5 days and reached an equilibrium after 15 days with egg

yolk colour scores of 3.86, 7.68, 12.10 and 12.74 for the four groups, respectively. There were no significant differences in eggshell quality, egg weight, yolk weight, yolk percentage, shell reflectivity or breaking strength within the four groups, although observations revealed that eggs from the highest level of *Haematococcus* algae meal had a thicker perivitelline membrane compared with the control group. A subsequent study demonstrated that astaxanthin at 6 ppm resulted in average egg yolk colour scores of 10.75 after 15 days, which is the preferred range for the Australian consumer (J. Roberts, unpublished). These results have been corroborated by poultry trials utilizing natural esterified astaxanthin from crawfish waste products. In this study, astaxanthin produced a linear increase in egg yolk colouration and 1 ppm astaxanthin gave optimal egg yolk colour scores of 10–11. Astaxanthin concentrations of more than 5 ppm with any level of yellow corn in the feed produced yolks with a deep-red colour[31].

Shrimp farming

The kuruma prawn (*Penaeus japonicus*) and tiger prawn (*Penaeus monodon*) are cultured worldwide, and their demand and production have steadily increased. The market value of these shrimp is predominately based on the visual appeal of their body colour. Astaxanthin has been identified as the dominant pigment isolated from the *Penaeus* shrimp and is responsible for the desirable body colour that appears upon cooking. Specifically, the body colour of crustaceans is dependent on the qualitative and quantitative presence of carotenoids in the hypodermal chromophores and the pigmented layer of the epidermal exoskeleton[32]. The majority of the astaxanthin within the epidermal tissue is in the mono-esterified form; complexes of carotenoids and proteins (carotenoproteins and carotenolipoproteins) dominate in the exoskeleton. Astaxanthin appears as a red pigment, but when complexed with other proteins, the light-absorbance shifts and causes the crustaceans to vary in colour from blue-green to brown. Thus, despite the fact that astaxanthin is the chromophore prosthetic group of the different carotenoproteins, many different colours can be achieved. The red colour of cooked crustaceans is produced by the release of the individual astaxanthin prosthetic groups from the carotenoproteins when denatured by heat.

The lack of dietary astaxanthin in cultured *P. monodon* has been shown to be the cause of 'blue color syndrome' or 'blue disease'. After four weeks of feeding a diet containing 50 ppm of astaxanthin, prawn suffering from blue color syndrome resume their normal greenish-brown pigmentation. Analysis of their tissues revealed >300% increase in carotenoid content and a normal appearance; those fed on a commercial diet without astaxanthin had a carotenoid increase of only 14% and maintained a blue hue. A total carotenoid concentration of 26.3 ppm was isolated from the exoskeleton of wild and farmed shrimp supplemented with sufficient astaxanthin. By contrast, specimens displaying blue disease had carotenoid concentrations in the exoskeleton of 4–7 ppm. Upon cooking, shrimp with blue disease assume a pale-yellow colour and are not as marketable, compared with the bright-red colouration characteristic of wild shrimp[33].

The effect of dietary carotenoids on pigmentation has been studied by feeding 100 ppm of various carotenoids (β-carotene, canthaxanthin and astaxanthin) to *P. japonicus*. It was found that all three carotenoids were deposited in the tissues, however, those fed dietary astaxanthin had the highest levels of tissue astaxanthin (16.5 mg kg body weight^{-1}) This result was ~23% higher than those fed on canthaxanthin and 43% higher than those fed β-carotene, demonstrating that astaxanthin was the most effective carotenoid source for pigmentation. In addition, it was established that as the dietary astaxanthin increased up to 200 ppm, deposition also increased to a maximum of 29.1 mg kg body weight^{-1}. Additionally, there was a significant decrease in the mortality of adult shrimp fed a carotenoid-enriched diet, in comparison with individuals receiving carotenoid-free diets. A survival rate of 91% was observed with shrimp fed a diet supplemented with 100 ppm astaxanthin, compared with 57% in the control group that were not fed astaxanthin[34].

Chien and Jeng found that astaxanthin was the most effective pigment for colouration of *P. japonicus*, compared with β-carotene[8]. Survival was higher in prawns fed an astaxanthin diet, and a positive correlation between survival and the pigment concentration in tissues suggested that astaxanthin can serve as an intracellular oxygen reserve; this would enable the shrimp to survive under the hypoxic conditions that are common in pond cultures. Shrimp that were fed with 100 mg kg^{-1} astaxanthin in their diet had an average survival rate of 77%, in contrast to ~40% in shrimp supplemented with β-carotene[9].

Metabolic and health effects

There is an increasing amount of evidence to suggest that astaxanthin surpasses the antioxidant benefits of β-carotene, zeaxanthin, canthaxanthin, vitamin C and vitamin E. Studies have also shown that astaxanthin can protect skin from the damaging effects of ultraviolet radiation, ameliorate age-related macular degeneration, protect against chemically induced cancers, increase high-density lipoproteins and enhance the immune system. Epidemiological studies have demonstrated a correlation between increased carotenoid intake and reduced incidence of coronary heart disease and certain cancers, macular degeneration and increased resistance to viral, bacterial, fungal and parasitic infections. Studies indicate that the mechanism for this protective attribute is partly owing to the direct enhancement of the immune response by carotenoids. Anticarcinogenic effects of carotenoids are likely to be attributable to its antioxidant effect, insomuch as oxygen radicals are related to the process of cancer initiation and propagation.

Numerous studies have demonstrated the potent radical scavenging and singlet oxygen quenching properties of astaxanthin. As a result of its particular molecular structure, astaxanthin has a potent neutralizing or 'quenching' effect against singlet oxygen, as well as a powerful scavenging ability for free radicals, and it serves as an extremely potent antioxidant against these reactive species. Within the cell, it can effectively scavenge lipid radicals and effectively destroys peroxide chain reactions to protect fatty acids and sensitive membranes[35–37]. It has been demonstrated that astaxanthin is significantly more effective than β-carotene

in neutralizing free radicals and gives better protection against the peroxidation of unsaturated fatty acid methyl esters than canthaxanthin, β-carotene or zeaxanthin[31,38]. The antioxidant activities of astaxanthin have been shown to be approximately ten times greater than other carotenoids, such as zeaxanthin, lutein, canthaxanthin and β-carotene, over 500 times greater than alpha-tocopherol, and astaxanthin has been proposed to be the 'super vitamin E' (Refs 37,39,40).

Astaxanthin might also be useful in preventing age-related macular degeneration (AMD), which causes irreversible blindness. When high-energy blue light waves interact with the retina, they can cause peroxide damage of the lipids through photo-oxidation, which in turn creates singlet oxygen and free radicals. Carotenoids within the macula absorb the high-energy blue light thereby quenching these damaging oxygen species. Clinical studies have indicated that light injury is a cause of AMD because of the cumulative insult leading to a gradual loss of photoreceptor cells. Unlike β-carotene, astaxanthin is able to readily cross the blood-retinal–brain barrier and can protect the retina against photo-oxidation and loss of photoreceptor cells. Furthermore, astaxanthin has the ability to protect the neurons of the retina, as well as the central nervous system, especially the brain and spinal cord, from damage caused by free radicals.

It appears that astaxanthin can also decrease the oxidation of lipid carriers and thereby reduce the risk of atherosclerosis[41]. In rat kidney fibroblasts, the addition of astaxanthin confers greater protection against ultraviolet (UV-A)-light-induced oxidative stress compared with lutein and β-carotene. When cell cultures were grown in carotenoid-supplemented media and exposed to UV-A light, β-carotene at a level of 1000 nM and lutein at 100 nM returned catalase activity to control levels, whereas it only required 5 nM of astaxanthin[42].

In rats, azoxymethane (AOM) has been used to induce colon cancer, which can then be used to study the effect of anticancer agents. In a recent study, animals were treated with AOM by three weekly injections and then fed diets with or without astaxanthin at 100 and 500 ppm for a further 34 weeks. At the end of the 37-week study, 63% of the chemically induced group had cancerous intestinal neoplasms with an average 0.97 neoplasm per rat. However, the chemically induced group that were subsequently treated with 100 ppm astaxanthin had a 41% incidence of neoplasms with an average of 0.48 per rat. Groups treated with 500 ppm of dietary astaxanthin had a reduced cancer rate of 31% with an average of 0.41 neoplasms per rat. It was concluded that the administration of astaxanthin during the post-initiation phase significantly inhibited the appearance of AOM-induced colon carcinogenesis in a dose-dependent manner[43], reduced the appearance of bladder carcinomas in rats[44] and depressed the appearance of tongue neoplasms in rats[45]. It was speculated that the chemoprotective effects of astaxanthin might be partly owing to its antiproliferative effect on the epithelium cells, as well as enhancing the immune response. Additionally, an increase in the expression of a major gap-junction gene *connexin43* might explain the suppressive effects of astaxanthin on carcinogenesis[45]. Mice fed benzopyrene to induce stomach tumorigenesis and subsequently fed various concentrations of astaxanthin-enriched egg yolks developed ~66% less
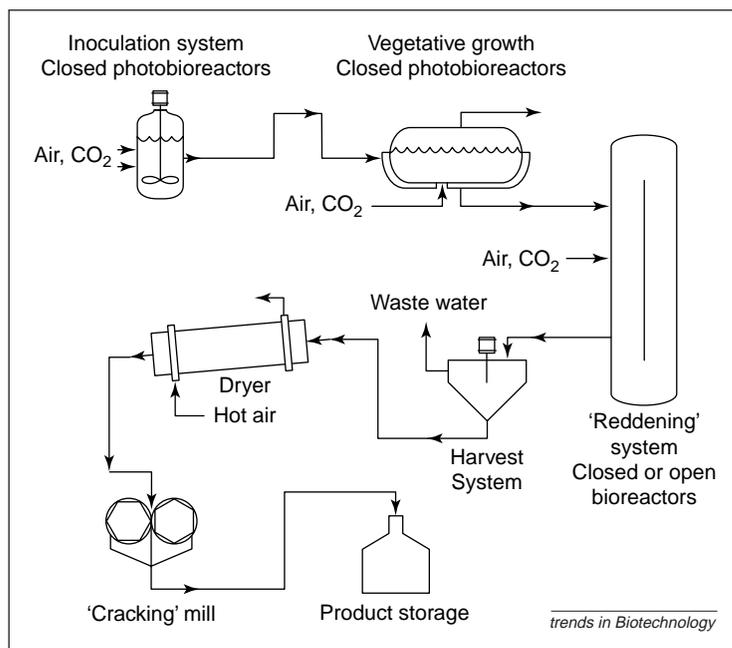

Figure 3

Typical process flowsheet for the commerical production of natural astaxanthin by *Haematococcus*.

neoplasms per animal and fewer incidences compared with the control animals[46].

There is also strong evidence to suggest that astaxanthin modulates the humoral and non-humoral immune system. Astaxanthin enhances the release of interleukin-1 and tumor necrosis factor α in mice, greater than canthaxanthin or β-carotene, and has the greatest cytokine-inducing activity[47]. Astaxanthin has a significant enhancing action on the production of immunoglobulins A, M and G, and on T-helper cell antibody production even when suboptimal amounts of antigen are present[48,49]. Consequently, at the initial stage of a pathogen invasion, doses of a particular antigen may be suboptimal for eliciting an effective immune reaction; astaxanthin appears to enhance this response.

Production technology

Recently, new techniques have been developed for the production of natural astaxanthin from *Haematococcus*. In Sweden, completely enclosed photobioreactors (with artificial light) are being used for astaxanthin production; in Hawaii, a combination of closed photobioreactors and open culture ponds are being successfully used to commercially produce *Haematococcus*.

In the large-scale, outdoor system, the production of astaxanthin-rich *Haematococcus* is a two-step process (Fig. 3). First, vegetative cells must be produced under near-optimal growth conditions with careful control of pH, temperature and nutrient levels. Regulation of pH can be controlled automatically with metered solenoids and carbon dioxide fed on demand. Temperature control can also be maintained automatically with metered solenoids and thermocouples. In Hawaii, deep ocean water at 14°C is utilized to cool culture systems using heat exchangers.

After a sufficient volume of vegetative cells is produced, the culture is subjected to environmental and nutrient stress. Commercial systems induce astaxanthin
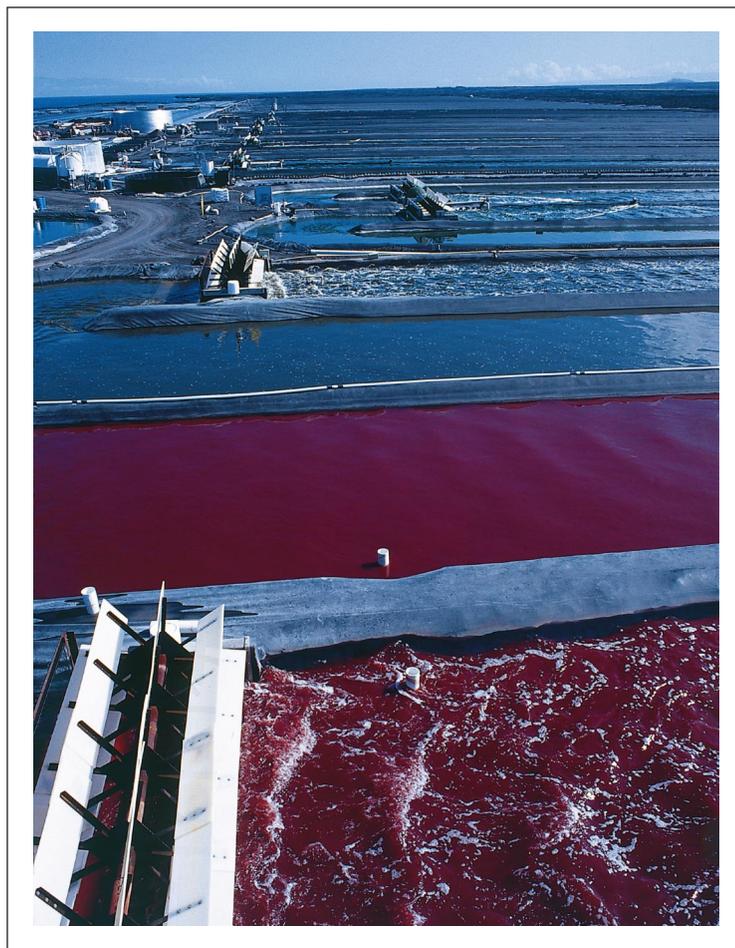
Figure 4

Culture ponds (500 000 litres) at Cyanotech Corporation (HI, USA). Pond in foreground has fully developed haematocysts and is ready to harvest; pond in background has just been inoculated with green vegetative cells.

production by deprivation of nitrate and phosphate, increasing temperature and light, or by the addition of sodium chloride to the culture medium[50]. Within 2 to 3 days after the culture is stressed, haematocysts are formed and begin accumulating astaxanthin; within 3 to 5 days following the formation of haematocysts (containing ~1.5–3.0% astaxanthin), they are ready for harvest. The change in a *Haematococcus* culture is striking when haematocysts accumulate astaxanthin (Fig. 4).

Because haematocysts are considerably denser than water, harvesting of the haematocysts is accomplished by settling and subsequent centrifugation. The haematocysts are then dried and cracked to ensure maximum bioavailability of the astaxanthin. For feed-grade applications, ethoxyquin or other antioxidants are added to the paste before drying, to minimize oxidation of the carotenoids. Milling can then be used to crack cells, although the exact details of the techniques are proprietary to companies producing *Haematococcus* algae.

In principle, the production of *Haematococcus* algae meal as a source of natural astaxanthin is relatively simple. Cultures grow readily in a simple nutrient media; however, *Haematococcus* grows in a neutral culture medium and contamination by other strains of microalgae, amoeba and protozoa can present problems, magnified as processes are scaled up, which require advanced technology to control.

Conclusion

Although the production of natural astaxanthin by *Haematococcus* requires advanced technology, it has already become a commercial reality. As a source of natural astaxanthin, *Haematococcus* algae meal has a large potential market in the salmonid and poultry feed industries, and as a nutraceutical in the dietary-supplement industry.

References

1 Borowitzka, M. and Borowitzka, L. (1988) *Micro-algal biotechnology*, Cambridge University Press
2 Stadler, T. *et al.* (1988) *Algal biotechnology*, Elsevier Applied Science
3 Steven, D.M. (1948) Studies on animal carotenoids. I. Carotenoids of the brown trout (*Salmo trutta* Linn.). *J. Exp. Biol.* 25, 369
4 Goodwin, T.W. (1984) *The Biochemistry of the Carotenoids* (Vols 1,2), Chapman & Hall
5 Kitahara, T. (1984) Carotenoids in the Pacific salmon during the marine period. *Comp. Biochem. Physiol.* 78B, 859–862
6 Foss, P. *et al.* (1987) Natural occurrence of enantiomeric and meso astaxanthin in crustaceans including zooplankton. *Comp. Biochem. Physiol.* 86B, 313–314
7 Davis, B.H. (1985) Carotenoid metabolism in animals: a biochemist's view. *Pure Appl. Chem.* 57, 679–684
8 Chien, Y. and Jeng, S. (1992) Pigmentation of kuruma prawn, *Penaeus japonicus* Bate, by various pigment sources and levels and feeding regimes. *Aquaculture* 102, 333–346
9 Chien, Y. (1996). Biological effects of astaxanthin in shrimp, a review (Hunter, B., ed.), *The 3rd annual Roche aquaculture centre conference on nutrition and disease*
10 Grung, M. *et al.* (1992) Algal carotenoids 51. Secondary carotenoids 2. *Haematococcus pluvialis* aplanospores as a source of (3S,3′S)-astaxanthin esters. *J. Appl. Phycol.* 4, 165–171
11 Renstrom, B. *et al.* (1981) Optical purity of (3S,3′S)-astaxanthin from *Haematococcus pluvialis*. *Phytochem.* 20, 2561–2564
12 Schiedt, K. *et al.* (1986) Astaxanthin and its metabolites in wild rainbow trout (*Salmo gairdneri*). *Comp. Biochem. Physiol.* 83B, 9–12
13 Storebakken, T. *et al.* (1985) Carotenoids in diets for salmonids. II. Epimerization studies with astaxanthin in Atlantic salmon. *Aquaculture* 44, 259–269
14 Schiedt, K. *et al.* (1981) Natural occurrence of enantiomeric and meso-astaxanthin. 5. Ex-wild salmon (*Salmo salar* and *Oncorhynchus*). *Helv. Chim. Acta* 64, 449–457
15 Lura, H. and Saegrov, H. (1991) A method of separating offspring from farmed and wild Atlantic salmon (*Salmo salar*) based on different ratios of optical isomers of astaxanthin. *Can. J. Fish. Aquat. Sci.* 48, 429–433
16 Turujman, S. *et al.* (1997) Rapid liquid chromatographic method to distinguish wild salmon from aquacultured salmon fed synthetic astaxanthin. *J. AOAC Int.* 80, 622–632
17 Christiansen, R. *et al.* (1995) Growth and survival of Atlantic salmon, *Salmo salar* L., fed different dietary levels of astaxanthin. First-feeding fry. *Aquaculture Nutrition* 1, 189–198
18 Sommer, T.R. *et al.* (1991) Utilization of microalgal astaxanthin by rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 94, 79–88
19 Choubert, G. and Heinrich, O. (1993) Carotenoid pigments of the green alga *Haematococcus pluvialis*: assay on rainbow trout, *Oncorhynchus mykiss*, pigmentation in comparison with synthetic astaxanthin and canthaxanthin. *Aquaculture* 112, 217–226
20 Mori, T. *et al.* (1989) Comparison between krill astaxanthin diester and synthesized free astaxanthin supplemented to diets in their absorption and deposition by juvenile salmon (*Oncorhynchus kisutch*). *Comp. Biochem. Physiol.* 93B, 255–258
21 Arai, S. *et al.* (1987) Pigmentation of juvenile coho salmon with carotenoid oil extracted from Antarctic krill. *Aquaculture* 66, 255–264
22 Tanaka, Y. *et al.* (1976) The carotenoids in marine red fish and the metabolism of the carotenoids in Sea bream, *Chrysophrys major* Temminch and *Schegel*. *Bull. Jap. Soc Sci. Fish.* 42, 1177–1182
23 Katayama, T. *et al.* (1972) The transformation of labeled astaxanthin from the diet of Sea bream, *Chrysophrys Major* Temminck and *Schegel*, to their body astaxanthin. *Bull. Jap. Soc. Sci Fish.* 38, 1399–1403

24 Katayama, T. *et al.* (1965) Carotenoids in Sea breams, *Chrysophrys major* and *schlegel*. *Bull. Jap. Soc. Sci. Fish.* 31, 947–952

25 Nakazoe, J. *et al.* (1984) Effects of supplemental carotenoid pigments on the carotenoid accumulation in young sea bream (*Chrysophrys major*). *Bull. Tokai Reg. Fish. Res. Lab.* 113, 29–41

26 Ito, Y. *et al.* (1986) Studies on the improvement of body color of red sea bream *Pagrus major* by astaxanthin and astaxanthin ester. *Suisanzoshoku* 34, 77–80.

27 Ako, H. and Tamaru, C.S. (1999) Are feeds for food fish practical for aquarium fish? *Intl. Aqua Feeds* 2, 30–36

28 Elwinger, K. *et al.* (1997) Astaxanthin rich algal meal (*Haematococcus pluvialis*) as carotenoid source in feed for laying hens. In *Proceedings of the VII European Symposium on the Quality of Eggs and Egg Products*, pp. 52–59, Poznan, Poland

29 Inborr, J. (1998) *Haematococus*, the poultry pigmentor. *Feed Mix.* 6, 31–34

30 Inborr, J. and Lignell, A. (1997) *Proceedings of XIII European Symposium on Poultry Meat Quality*, Poznan, Poland.

31 Lee, K.S. *et al.* (1986) Evaluation of crawfish astaxanthin as a natural red pigmenter of egg yolk. *Poultry Sci.* 65 (Suppl. 1), 177–178

32 Katayama, T. *et al.* (1972) The biosynthesis of astaxanthin in the prawn, *Penaeus japonicus* Bate (Part II). *Int. J. Biochem.* 3, 363–368

33 Menasveta, P. *et al.* (1993) Correction of black tiger prawn (*Penaeus monodon* Fabricus) coloration by astaxanthin. *Aquaculture Engineering* 12, 203–213

34 Yamada, S. *et al.* (1990) Pigmentation of prawn (*Penaeus japonicus*) with carotenoids. I. Effect of dietary astaxanthin, beta-carotene and canthaxanthin on pigmentation. *Aquaculture* 87, 323–330

35 Terao, J. (1989) Antioxidant activity of beta-carotene related carotenoids in solution. *Lipids* 24, 659–662

36 Kurashige, M. *et al.* (1990) Inhibition of oxidative injury of biological membranes by astaxanthin. *Physiol. Chem. Phys. Med. NMR* 22, 27–38

37 Miki, W. (1991) Biological functions and activities of carotenoids. *Pure Appl. Chem.* 63, 141–146

38 Jorgensen, K. and Skibsted, L. (1993) Carotenoid scavenging radicals. Effect of carotenoid structure and oxygen partial pressure on antioxidative activity. *Z. Lebensm. Unters Forsch.* 196, 423–429

39 Ranby, B. and Rabek, J.F. (1978) *Singlet Oxygen*, Wiley

40 Shimidzu, N. *et al.* (1996) Carotenoids as singlet oxygen quenchers in marine organisms. *Fisheries Science* 62, 134–137

41 Murillo, E. (1992) Hypercholesterolemic effect of canthaxanthin and astaxanthin in rats. *Arch. Latinoam Nutr.* 42, 409–413

42 O'Connor, I. and O'Brien, N. (1998) Modulation of UV-A light-induced oxidative stress by beta-carotene, lutein and astaxanthin in cultured fibroblasts. *J. Dermatol. Sci.* 16, 226–230

43 Tanaka, T. *et al.* (1994) Chemoprevention of mouse urinary bladder carcinogenesis by the naturally occurring carotenoids astaxanthin. *Carcinogenesis* 15, 15–19

44 Tanaka, T. *et al.* (1995) Suppression of azomethane-induced rat colon carcinogenesis by dietary administration of naturally occurring xanthophylls astaxanthin and canthaxanthin during the postinitiation phase. *Carcinogenesis* 16, 2957–2963

45 Tanaka, T. *et al.* (1995). Chemoprevention of rat oral carcinogenesis by naturally occurring xanthophylls, astaxanthin and canthaxanthin. *Cancer Res.* 55, 4059–4064

46 Lee-Sang, H. *et al.* (1997) Inhibition of benzo(a)pyrene-induced mouse forestomach neoplasia by astaxanthin containing egg yolks. *Agric. Chem. Biotechnol.* 40, 490–494

47 Okai, Y. and Higashi-Okai, K. (1996) Possible immunomodulating activities of carotenoids in *in vitro* cell culture experiments. *Int. J. Immunopharmacol.* 18, 753–758

48 Jyonouchi, H. *et al.* (1995) Astaxanthin, a carotenoid without vitamin A activity, augments antibody responses in cultures including T-helper cell clones and suboptimal doses of antigen. *J. Nutr.* 125, 2483–2492

49 Jyonouchi, H. *et al.* (1995) Effect of carotenoids on *in vitro* immunoglobulin production by human peripheral blood mononuclear cells: astaxanthin, a carotenoid without vitamin A activity, enhances *in vitro* immunoglobulin production in response to a T-dependent stimulant and antigen. *Nutr. Cancer.* 23, 171–183

50 Kakizono, T. *et al.* (1992) Effect of carbon/nitrogen ratio on encystment accompanied with astaxanthin formation in a green alga, *Haematococcus pluvialis*. *J. Ferment. Bioeng.* 74, 403–405

Genetic modulation of tumor antigen presentation

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An effective cancer-cell vaccine is created by expressing major-histocompatibility-complex (MHC) class II molecules without the invariant chain protein (Ii) that normally blocks the antigenic-peptide-binding site of MHC class II molecules at their synthesis in the endoplasmic reticulum. Such tumor-cell constructs are created either by the transfer of genes for MHC class IIα and β chains, or by the induction of MHC class II molecules and Ii protein with a transacting factor, followed by Ii suppression using antisense methods. Preclinical validation of this approach is reviewed with the goal of using this immunotherapy for metastatic human cancers.

Several approaches create effective tumor–cell vaccines for cancer patients. These methods include: genetically engineering tumor cells to express and secrete cytokines, engineering tumor cells to express costimulatory, cell-surface molecules to activate T lymphocytes, and creating DNA vaccines of tumor–associated antigens[1–5]. These methods either stimulate the host's immune system or modify tumor cells to express their tumor antigens directly for immune stimulation. Although early studies in this field focused on the activity of cytotoxic CD8^{+} T cells, more–recent studies have targeted CD4^{+} T-helper cells for recruitment in tumor immunotherapy. Indeed, studies using CD4^{+} T-helper

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