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ASPECTS OF ASCORBIC ACID (VITAMIN C) NUTRITION IN *OREOCHROMIS NILOTICUS* AND *O. MOSSAMBICUS*

by

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AFFECTIONATELY DEDICATED TO

MY

PARENTS, SISTERS & BROTHERS

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ABSTRACT

Various aspects of the ascorbic acid (vitamin C) nutrition of *Oreochromis niloticus* and *O. mossambicus* are considered in this treatise.

The activity of L-gulono-γ-lactone oxidase was assessed in liver and kidney of 14 teleosts of 3 genera, histochemically (qualitatively) and biochemically (quantitatively). Activity of this enzyme was only detected in liver and kidney of common carp, *Cyprinus carpio*, and kidney of *O. spilurus* and *O. aureus*. No activity was detected in the species considered herein.

The quantitative dietary ascorbic acid requirements of juvenile *O. niloticus* and *O. mossambicus* were determined by feeding diets containing graded levels of the vitamin (0-400mg/100g) and were based on growth response, food utilization, gross body composition data, tissue and biochemical changes and ability to prevent signs of ascorbic acid deficiency. The recommended level of supplementation of dietary ascorbic acid is 125mg/100g and the net requirement 42mg/100g diet.

Long-term ascorbic acid deprivation in *O. niloticus* and *O. mossambicus* resulted in poor performance in terms of growth, food utilization and survival. Other parameters evaluated included hepatosomatic index, liver and muscle glycogen content, blood parameters, tissue ascorbate concentrations, collagen contents, hydroxyproline and proline contents, and serum transaminase and cholesterol levels. Signs of ascorbic acid

deficiency were severe and included haemorrhage, opercular deformity, tail erosion, exophthalmia, cataract and spinal deformity (lordosis and scoliosis). Histologically scorbutic fish showed evidence of generalized bone changes associated with excessive production of chondrocytes and failure of ossification of growing bone areas. Eye lesions were associated with scleral collapse and also observed was hyperplasia of gill secondary lamellae epithelial cells and pronounced steatitis.

Tissue ascorbate concentrations were correlated with dietary ascorbic acid levels and both species exhibited highest concentrations in the ovary, brain and testis, followed by heart, liver, gut, gills, eyes and the lowest levels in muscle and gall-bladder. The physiological role for ascorbic acid in each tissue is discussed.

Eight week growth studies were conducted to evaluate the utilization of L-ascorbic acid (AA), the sodium salt of L-ascorbic acid (NaAA), glyceride coated L-ascorbic acid (GCAA), the barium salt of L-ascorbic acid 2-sulphate (AA2S) and ascorbyl palmitate (AP) in diets for O. niloticus and O. mossambicus. All five forms were added to the basal diet, containing no ascorbic acid (AAF), on an equimolar basis to supply 125mg ascorbic acid/100g diet. All forms performed well in terms of growth, food utilization, and prevented signs of deficiency.

Retention of ascorbic acid in diets after processing was increased by increasing dietary ascorbic acid level. AA2S and GCAA were more stable than AA and NaAA during processing and storage. The stability of AA, NaAA, GCAA and AA2S under different storage conditions was in

descending order as follows: Freezer (-20°C), Fridge (5-8°C), room temperature in black bags (22-24°C) and room temperature in clear bags (22-24°C). Leaching of dietary ascorbic acid increased with increasing immersion time and water temperature. Stability and price of each form evaluated suggested that GCAA is to be preferred for use in fish feeds.

The antioxidant effects of ascorbic acid were investigated. The results showed that ascorbic acid was not as effective as butylated hydroxy toluene (BHT) in preventing *in vitro* oxidation.

An experiment was conducted to compare the performance of a commercial trout diet with the same diet supplemented to a level of 125mg ascorbic acid/100g diet (Diet 2) when fed to *O. niloticus*. Fish fed the supplemented diet performed significantly better in terms of growth and food utilization concomittant with significantly increased tissue ascorbate concentrations.

Sex differences in relation to dietary ascorbic acid nutrition were investigated. Females of both species exhibited significantly higher gonado- and hepatosomatic indices than males. Females in both species exhibited higher total ascorbate concentrations in gonad, gills, spleen, brain and blood than males whereas the reverse was true for the eyes. Dehydroascorbic acid (DHAA) levels were very low in tissues of both species.

Ascorbic acid depleted *O. niloticus* fingerlings were fed on each of three diets providing nil, adequate (125mg ascorbic acid/100g diet),

and luxus (400mg/100g diet) of the vitamin after small surgical incisions had been made in dorsolateral musclature. Fish from each group were sampled regularly over 16 days and histological evaluation of the lesion area carried out as well as measurement of the tissue ascorbate levels. Epithelial elements of the healing process developed irrespective of the vitamin level but although fibroblast activity was marked in all three groups, collagenisation was very much slower in the deficient group, and in these the lesion was not mature, even at the termination of the experiment.

The role of ascorbic acid in reproduction of tilapias was investigated in three experiments. The first showed that ascorbic acid supplementation of broodstock feed improved both hatchability and fry condition. In the second experiment fry produced from fish fed an unsupplemented diet and subsequently fed the same diet performed poorly in respect of growth and food utilization. Fry produced from broodstock fed the supplemented diet and subsequently fed the unsupplemented diet performed better than the previous group. This indicates transfer of ascorbic acid from the ovary to the eggs thence to the fry providing some protection against ascorbic acid deficiency during the early stages of life.

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CHAPTER 1

INTRODUCTION

SECTION 1.1 : PREFACE

The fact that a purified diet of protein, carbohydrate, fat and minerals is insufficient to maintain life was first established by Lunin in 1881 (Hawk et al., 1947). Supplementing such a purified diet with certain natural foods, growth was restored to the experimental animals. From these facts, the importance of nutrients derived from vegetables and fruits was established (Johnson, 1979).

Although the discovery of the vitamins dates from the beginning of the twentieth century, the association of certain diseases with dietary deficiencies had been recognised earlier. In 1753 Lind, a British naval physician, indicated that scurvy could be prevented in human beings by consumption of salads and fruit. Eijkman, the Dutch physician observed in 1897 that beri-beri could be cured by giving patients brown rice rather than polished rice. It was also known that cod liver oil has a significant role in preventing rickets (McDonald et al., 1980).

The discovery and isolation of many of the vitamins was originally achieved through studies on rats which had been fed purified diets. In this way, Hopkins in 1912 discovered that purified diets were not adequate for normal growth of rats and that by addition of small quantities of milk to these diets normal growth was restored. This observation led to the discovery of what were called 'Accessory Food Factors' by Osborne and Mendel in 1913 in milk (Hawk, 1965a).

The word vitamine was coined by Funk in 1912 because he believed the substance that he isolated possessed the properties of an amine

and he thought that all of the accessory factors contained amino-nitrogen. With the discovery and isolation of many of these accessory factors it was noticed that few of these factors contained an amine and subsequently the terminal 'e' was dropped and the word 'vitamin' is now used for all such accessory factors whether or not they contain an amine (Johnson, 1979).

Vitamins have been defined in various ways:

> "A vitamin is an organic substance of a nutritional nature which is present in low concentrations as a natural component of enzyme systems and which catalyses specific reactions and may be derived externally to the tissues or by intrinsic biosynthesis"
>
> (Folkers, 1969)

Another definition is:

> "A vitamin is an organic compound, other than alpha-amino acids, that is necessary in relatively small amounts unless a similar compound is available to the bodily processes of growth, metabolism or repair"
>
> (Johnson, 1979)

From a general point of view the vitamins are defined as organic compounds that are required in minute amounts for normal growth, reproduction, health and general maintenance of metabolism(NRC, 1981).

The vitamins are classified by their chemical solubility into two groups:

1. <u>The fat-soluble vitamins</u>:

   A (Anti-othalmic vitamin; D (Anti-rachitic vitamin); E (Anti-sterility vitamin) and K (Anti-haemorrhagic vitamin).

2. The water-soluble vitamins:

B_1 (Thiamine or Anti-neuritic vitamin); B_2 (Riboflavin or vitamin G); Niacin (Nicotinic acid or Anti-pellagra vitamin); B_6 (Pyridoxine or Anti-acrodynia vitamin); Pantothenic acid (Chick-anti-dermatitis vitamin); Biotin (vitamin H or Anti-egg white injury vitamin); Pteroyl-glutamic acid (Folic acid or Anti-anaemia vitamin); B_{12} (Cyanocoabalamin); Choline (Sinkaline or Bilineurine); Myo-inositol (Mouse Anti-alopecia vitamin) and ascorbic acid (vitamin C or Anti-scorbutic factor) (Hawk, 1965a).

The last three water-soluble vitamins are required in appreciable quantities in diets and are occasionally not referred to as vitamins but as major dietary nutrients (Halver, 1980).

There are differences between the terms vitamin activity and vitamin content, and hypovitaminosis (avitaminosis) and hypervitaminosis. Vitamin activity refers to specific biological potency whereas vitamin content refers to chemical entities of which several may exhibit similar biological activity. Hypovitaminosis is the term used when deficiency symptoms are related to absence of a single vitamin, but if these symptoms are related to a deficiency of more than one vitamin the term is changed to polyavitaminosis. Hypervitaminosis refers to exceeding the dose of vitamin required and generally relates to the group of fat-soluble vitamins.

The major discoveries of vitamins in the 1930's and recognition of the importance of vitamins in human nutrition have prompted fish

experimentalists to examine the importance of these organic substances in fish diets. Early research on the vitamin requirements of fish was conducted by Jewell et al. (1933) with channel catfish (Ictalurus punctatus). These studies were hampered by lack of information on the total number and identity of vitamins and lack of a satisfactory vitamin test diet (Dupree, 1966). The first report of specific avitaminosis in rainbow trout was related to thiamine deficiency and appeared in 1939. Deficiency symptoms were relieved by injection of thiamine or addition of thiamine-rich yeast to the diet (NRC, 1973).

The ascorbic acid requirements of fish were not investigated until several years later. McCay & Tunsion (1933) reported that brook trout (Salvelinus fontinalis) fed meat preserved in formalin developed lordosis (anterior-posterior curvature of the spine) and scoliosis (lateral curvature of the spine) after one year but did not relate this to ascorbic acid deficiency. The possible essentiality of ascorbic acid in diets for fish was first demonstrated by McLaren et al. (1947) when rainbow trout fed a diet devoid of ascorbic acid developed haemorrhages in their intestine, liver and kidney. A more recent development of vitamin nutrition is analysis of the vitamin content of storage tissues used for assessment of the vitamin status of fish, for example, kidney, liver and blood (Halver et al., 1975; Hilton et al., 1977a; Mahajan & Agrawal, 1979).

Vitamin requirements can be stated in either of two ways, firstly as a function of body weight (mg vitamin/kg body weight/day) and secondly as a function of feed intake (mg vitamin/kg feed) with the second method of expression being easier to use from a practical point of view (NRC, 1973).

SECTION 1.2 : ASCORBIC ACID (Vitamin C)

Section 1.2.1 History of Scurvy

Scurvy (Scorbutus) is the term used to indicate the occurrence of signs of ascorbic acid deficiency. Even the chemical name, ascorbic acid, of this vitamin is derived from this term. According to Anderson (1977) the chemical name ascorbic acid is derived from the fact that scorbutus, or scurvy, means for the skin to be covered with scale or scurf perpetuating the relationship of this vitamin to scurvy.

Scurvy was known even in ancient times. Ebbell (1939) reported that scurvy was known to the ancient Egyptians (1550 B.C.) as indicated from the hieroglyphs in the Ebers papyrus. Hippocrates (460-370 B.C.) described symptoms similar to those of scurvy (Vilter, 1967). However, Hirsch (1885) conducted a critical analysis of the writings of ancient physicians such as Hippocrates, Celsus and others who described a disease called 'Lines magni' which in his opinion has been interpreted by some authors, incorrectly, as scurvy.

Scurvy was well known among soldiers and sailors and in the thirteenth century scurvy broke out among the Crusades army of King Louis IX near Cairo (Bourne, 1944; Sharman, 1974). Vasco Da Gama lost one hundred men out of one hundred and sixty of his crew (Harris, 1937) to this condition. The symptoms of scurvy in human beings as reviewed by Sharman (1974) and Anderson (1977) are as follows: swelling of legs and arms; softening of the gums with haemorrhage and gums becoming so decayed that the flesh peels off down to the roots of the teeth

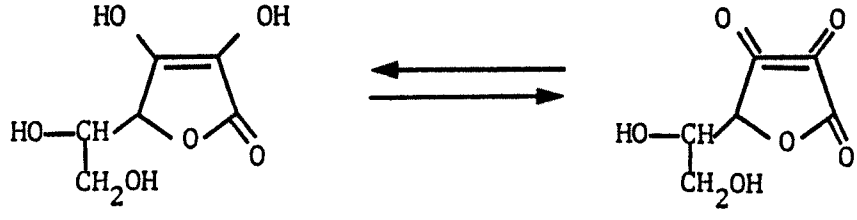
resulting in loose teeth; haemorrhage under the skin and foulness of the breath.

The first documented treatment for scurvy appears to be in 1535 when Red Indians showed scorbutic sailors how to make a decoction of swamp spruce which was a successful remedy (Bigger, 1924). Subsequently the importance of scurvy grass and oranges in curing scurvy was appreciated (Lind, 1757). In 1906, Hopkins stated that scurvy is a malnutrition disease.

Man and monkey were the only animals recorded as scurvy prone until 1907 when Holst and Frölich produced scurvy in guinea pigs by feeding them restricted diets and these authors demonstrated the importance of vegetables and fruits in preventing this condition. Burns and his co-workers (Burns et al., 1956; Burns, 1957) confirmed these findings using in vitro techniques. Roy and Guha (1958) reported that the fruit-eating bat (Pteropus medicus) and the red-vented bulbul (Pycnonotus cafer) also required dietary ascorbic acid, using an in vitro technique. In fish, feeding studies and in vitro techniques have shown that many freshwater and marine species are also dependent on exogenous sources of ascorbic acid (Kitamura et al., 1965; Halver et al., 1969; Sakaguchi et al., 1969; Arai et al., 1972; Chatterjee, 1973b; Wilson, 1973; Wilson & Poe, 1973; Lim & Lovell, 1978; Yamamoto et al., 1978; Mahajan & Agrawal, 1979, 1980a; Soliman et al., 1985).

Section 1.2.2 Isolation of L-ascorbic Acid

After recognising the importance of oranges and lemons in the prevention of scurvy (Lind, 1753) the interest of biochemists centered on investigation and identification of the antiscorbutic factor in these materials. Zilva, in a series of studies (1923, 1924a, b, 1927, 1928) evaluated several different aspects of the antiscorbutic factor in lemon juice. By 1928 Szent-Györgyi had independently succeeded in isolation of the antiscorbutic factor from the adrenal gland of ox and from cabbages, oranges and paprika (Hungarian pepper). This author proposed the term hexuronic acid ($C_6H_8O_6$) for this substance since it was a highly reactive isomer of glycuronic acid and was highly reducing. In 1932 Waugh and King demonstrated that the hexuronic acid isolated by Szent-Györgyi was identical to 'vitamin C' which they isolated from lemons and oranges. This was later confirmed by Svirbely and Szent-Györgyi (1932). Subsequently Svirbely and Szent-Györgyi demonstrated the antiscorbutic activity of hexuronic acid in guinea pigs (1933) and in the same year the structure of ascorbic acid was proposed by Haworth et al. (1933) as follows:



L-ascorbic acid Dehydro-L-ascorbic acid

FIGURE 1. Structural formulae of L-ascorbic acid (reductant) and dehydro-L-ascorbic acid (oxidant)

In a later investigation Banga and Szent-Györgyi (1934) successfully prepared ascorbic acid from the Hungarian red pepper (Capsicum annuum) in large quantities and in addition Haworth et al. (1934) demonstrated that synthetic ascorbic acid had the same antiscorbutic activity as the natural substance. This initiated production of synthetic ascorbic acid commercially on a large scale. Bauernfeind (1982) reported that one modern factory can produce 30 tons of pure L-ascorbic acid daily which is equivalent to the vitamin contained in half a billion large oranges.

Section 1.2.3 Physical and Chemical Properties of L-Ascorbic Acid

The physical and chemical properties of L-ascorbic acid with its nomenclature are presented in Table 1.

TABLE 1. Nomenclature and physical and chemical properties of L-ascorbic acid

1. NOMENCLATURE

L-ascorbic acid (L-threo-2,3,4,5,6-pentahydroxy-1-hexenoic acid-4-lactone) formerly known as vitamin C, cevitamic acid and hexuronic acid, is a six carbon molecule with the empirical formula $C_6H_8O_6$.

2. PHYSICAL PROPERTIES

White; colourless, odourless; crystalline component from coarse to ultra fine crystals with a molecular weight 176.13 Daltons and melting at 190-192°C.

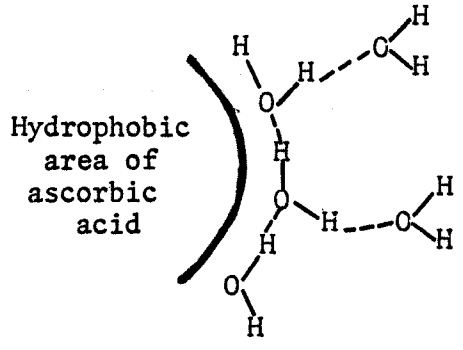
3. CHEMICAL PROPERTIES

The vitamin is water soluble, one gram of this compound dissolves in about 3ml of water (sodium salt more soluble) or 50ml of absolute ethanol or 100ml of glycerol; rotation $(\alpha)D^{20}$ + 23° in water and 48° in methanol; possesses pK_1 of 4.17 and pK_2 of 11.57; pH of 10% aqueous solution is 2.1-2.5 (corresponding values for its sodium salt were 7.4-7.9); decomposes in aqueous acid under nonoxidative conditions to furfural, carbon dioxide, L-xylose and several organic acids; powerful reducing agent; its acidity is sufficient to form neutral salts with bases; exposure to heat, air or alkaline medium hastens its oxidation; possesses several hydrophilic loci with a limited hydrophobic area.

Birch & Harris (1933); Harris (1967a); Hay *et al.* (1967); Hodges & Baker (1973); Hornig (1975a); Lewin (1975); Johnson (1979); Bauernfeind (1982).

Johnson (1979) suggested that ascorbic acid possesses a unique character where the acidity of this compound is derived from the enediol group not from the carboxyl group as in normal acids and the author supported this view by the fact that the oxidized form (dehydro-L-ascorbic acid) has no acidic properties due to loss of the enediol group.

The solubility of ascorbic acid in water is related to the existence of several hydrophilic loci with limited hydrophobic area. Lewin (1975) reported that ascorbic acid and its entities have several hydrophilic loci, namely the charged group itself or its hydroxyl forerunner and the free hydroxyl groups present in C-5 and C-6 whereas the hydrophobic area was limited and composed of the adjoining CH groups as follows:



Section 1.2.4 The Biosynthesis of L-ascorbic Acid in Animals

With a few exceptions L-ascorbic acid is synthesised by the majority of living organisms, both plant and animal (Sato & Udenfriend, 1978). The ability to synthesise this vitamin has been lost in primates, some sub-primates, guinea pigs, insects, certain other invertebrates, fishes and certain bats and birds (Table 2).

There has been much speculation about the site of ascorbic acid synthesis in animals and its significance. Ascorbic acid synthesis occurs in the kidney of amphibians, reptiles and less evolved birds and is transferred to the liver in mammals and highly evolved birds (Grollman & Lehninger, 1957; Chatterjee et al., 1961a,b; Ray Chaudhuri & Chatterjee, 1969; Chatterjee, 1973a; Dutta Gupta et al., 1973; Chatterjee et al., 1975). The transition of ascorbic acid synthesis from the kidney of reptiles to the liver of mammals appears to correspond to the evolution of the temperature regulating mechanism and change from cold blooded forms to warm blooded species. A move to synthesis in the liver of terrestrial animals may be initiated by life on dry land where there is increased physiological demand for ascorbic acid to regulate urea, calcium, phosphate and other ions (Chatterjee et al., 1975). The relatively small kidneys of terrestrial animals have many other functions and the liver is large enough to accommodate larger scale synthesis (Stone, 1965; Chatterjee et al., 1975).

The inability of scurvy-prone animals to synthesise ascorbic acid has occurred during the course of evolution (Stone, 1965; 1966a,b; Jukes & King, 1975) which has lead to loss of the gene (Gluecksohn-

Waekch, 1963; Sato & Udenfriend, 1978) responsible for synthesising the enzyme, L-gulono-γ-lactone oxidase, a key enzyme in synthesising ascorbic acid (Burns et al., 1956; Burns, 1957; Grollman & Lehninger, 1957; Chatterjee et al., 1961a, b; Wilson, 1973; Yamamoto et al., 1978; Soliman et al., 1985). In its absence synthesis of ascorbic acid cannot take place even though the other enzymes required may be present (Grollman & Lehninger, 1957).

Isherwood et al. (1954) were the first to propose the sequence of biosynthetic steps for ascorbic acid production in rats. Synthesis of ascorbic acid in animals appears to be from D-glucose via D-glucuronic acid (Douglas & King, 1953; Horowitz & King, 1953; Isherwood et al., 1954). Burns (1959) proposed the following biosynthetic pathway (Fig. 2) for ascorbic acid in rats after using 1-C^{14}D-glucose as a substrate.

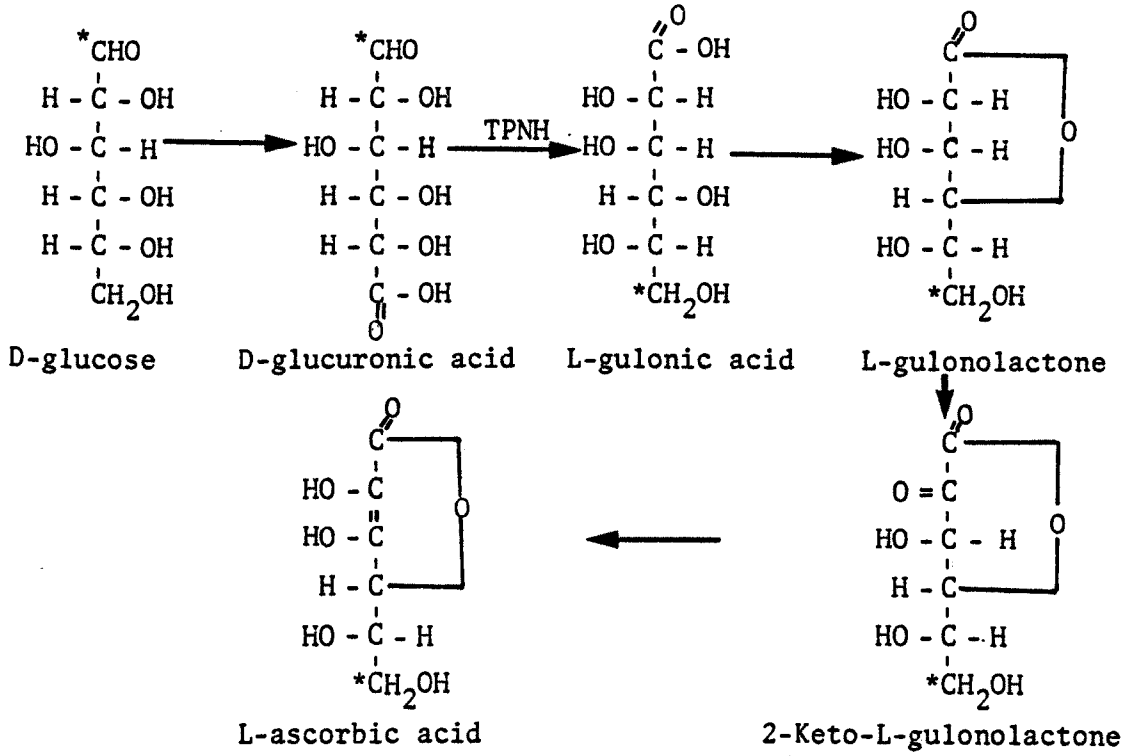


FIGURE 2. Biosynthetic pathway of L-ascorbic acid in rats

*The asterisks denote the fate of C-1 of D-glucose

TABLE 2. Ascorbic acid biosynthesis in different species of animals

Animal	Ability (A) or inability (B) to synthesise ascorbic acid	Reference
1. INVERTEBRATES		
Fresh water snail (*Pila* sp.)	B	1
Spider (*Spider* sp.)	B	1
Prawn (*Palamenon* sp.)	B	1
Crab (*Scylla* sp.)	B	1
Earthworm (*Pheretima* sp.)	B	1
Centipede (*Myriapod* sp.)	B	1
Leech (*Hirudinaria* sp.)	B	1
2. INSECTS	B	2
3. FISHES		
3.1 Salmonids		
Rainbow trout (*Salmo gairdneri*)	B	3 & 4
Brown trout (*S. trutta*)	B	4
Brook trout (*Salvelinus fontinalis*)	B	4
3.2 Cyprinids		
Labeo rohita	B	1
Catla catla	B	1
Labeo calbasu	B	1
Cirrhina mrigala	B	1
Gengorobuna (*Carassius carassius cuvieri*)	A(L)[a]	3
Ugui (*Tribolodon hakonensis*)	A(L)	3
Common carp (*Cyprinus carpio*)	A(L&K[b])	3 & 4
Grass carp (*Ctenopharyngodon idellus*)	B	4

TABLE 2 (cont'd)

Animal	Ability (A) or inability (B) to synthesise ascorbic acid	Reference
3.3 Ictalurids		
Catfish (*Parasilurus asotus*)	A(L&K)	3
Channel catfish (*Ictalurus punctatus*)	B	5
Blue catfish (*Ictalurus frucatus*)	B	5
Catfish (*Clarias batrachus*)	B	1
Catfish (*Heteropneustes fossilis*)	B	1
Catfish (*Mystus bleekeri*)	B	1
3.4 Cichlid		
Oreochromis niloticus	B	3
O. mossambicus	B	4
O. macrochir	B	4
O. aureus	A(K)	4
O. spilurus	A(K)	4
O. niloticus x *O. mossambicus*	B	4
Tilapia zillii	B	4
T. buttikoferi	B	4
Sarotherodon galilaeus	B	4
3.5 Others		
Amago (*Oncorhynchus masou macrostomus*)	B	3
Ayu (*Plecoglossus altivelis*)	B	3
Eel (*Anguilla japonica*)	B	3
Red sea bream (*Pagrus major*)	B	3
Yellow tail (*Seriola quinqueradiata*)	B	3
Kawahagi (*Stephanalepsis cirrhifer*)	B	3
Climbing perch (*Anabas testudineus*)	B	1
Murrels (*Ophicephalus striatus*)	B	1
Murrels (*Ophicephalus punctatus*)	B	1

TABLE 2 (cont'd)

Animal	Ability (A) or inability (B) to synthesise ascorbic acid	Reference
Indian tarpon (Megalops cyprinoides)	B	1
Gold fish (Apocryptes lanceolatus)	B	1
Milk fish (Chanos chanos)	B	1
Mullet (Mugil persia)	B	1
Perth (Lates calcarifer)	B	1
Feather back (Notopterus notopterus)	B	1
Lungfish (Neoceratodus forsteri)	A(K)	6
4. AMPHIBIANS		
Common Indian toad (Bufo melanosticitus schneider)	A(K)	7
Frog (Rana pipiens)	A(K)	8
Frog (Rana tigrina)	A(K)	1, 9 & 10
Toad (Bufo melanosticitus)	A(K)	1, 9 & 10
5. REPTILES		
Blood sucker (Calotes versicolor daudin)	A(K)	1, 7, 9 & 10
Common Indian monitor (Varanus monitor)	A(K)	1, 7, 9 & 10
Turtle (Lissemys punctata)	A(K)	1, 9, 10 & 11
Tortoise (Testudo elegans)	A(K)	1, 9 & 10
House lizard (Hemidectylus flaviviridis)	A(K)	9 & 10
Snake (Natrix piscator)	A(K)	1, 9 & 10
Angani (Mabuya carinata)	A(K)	9
6. BIRDS		
6.1 Primitive Species		
Common pochard (Aytha ferina)	A(K)	7 & 10
Chicken (Gallus gallus)	A(K)	7, 10 & 11

TABLE 2 (cont'd)

Animal	Ability (A) or inability (B) to synthesise ascorbic acid	Reference
Grey partridge (*Francolinus pondicerianus*)	A(K)	7 & 10
Pigeon (*Columba livia*)	A(K)	7, 10 & 11
Owl (*Otus bakkamoena pennant*)	A(K)	7, 10 & 11
6.2 *Passeriform Species*		
House crow (*Corvus splendens splendens*)	A(L&K)	7, 10 & 11
Common myna (*Acridotheres tristis*)	A(L&K)	7, 10 & 11
Munia (*Lonchura malacca*)	A(L)	7, 10 & 11
Red-vented bulbul (*Pycnonotus cafer*)	B	7, 10 & 11
7. *MAMMALS*		
7.1 *Flying Mammals*		
Indian fruit bat (*Pteropus medicus*)	B	7, 9 & 10
Indian pipistrelle (*Vesperugo abramus*)	B	9 & 10
7.2 *Land Mammals*		
Goat	A(L)	1, 9 & 10
Cow	A(L)	1, 9, 10 & 12
Sheep	A(L)	1, 9 & 10
Rat	A(L)	1, 9, 10, 12 & 13
Mouse	A(L)	1, 9, 10 & 11
Squirrel	A(L)	1, 9 & 10
Rabbit	A(L)	9 & 12
Cat	A(L)	1, 9 & 10
Dog	A(L)	1, 9, 10 & 12
Pig	A(L)	11
Guinea pig	B	1, 8, 9, 10, 12 & 14

TABLE 2 (cont'd)

Animal	Ability (A) or inability (B) to synthesise ascorbic acid	Reference
8. PRIMATES		
Man	B	9, 12 & 14
Monkey (Macaca mulatta)	B	9 & 10
Rhesus monkey	B	12
Cynomolgus monkey	B	12

L. Liver

K. Kidney

1. Chatterjee (1973a)
2. Dutta Gupta et al. (1972)
3. Yamamoto et al. (1978)
4. Soliman et al. (1985)
5. Wilson (1973)
6. Dykhuizen et al. (1979)
7. Roy & Guha (1958)
8. Cohen (1961)
9. Chatterjee (1973b)
10. Dutta Gupta et al. (1973)
11. Ray Chaudhuri & Chatterjee (1969)
12. Grollman & Lehninger (1957)
13. Ul Hassan & Lehninger (1956)
14. Burns et al. (1956)

Chatterjee and his co-workers (Chatterjee et al., 1961a, b; Chatterjee, 1970) proposed the following route (Fig. 3) for biosynthesis of ascorbic acid in animal tissues.

More recent suggestions have been made by Chatterjee et al. (1961a) that D-glucurono-γ-lactone reductase is also absent (in addition to L-gulono-γ-lactone oxidase) in animals unable to synthesise ascorbic acid thus providing two genetically determined blocks to the biosynthesis of ascorbic acid.

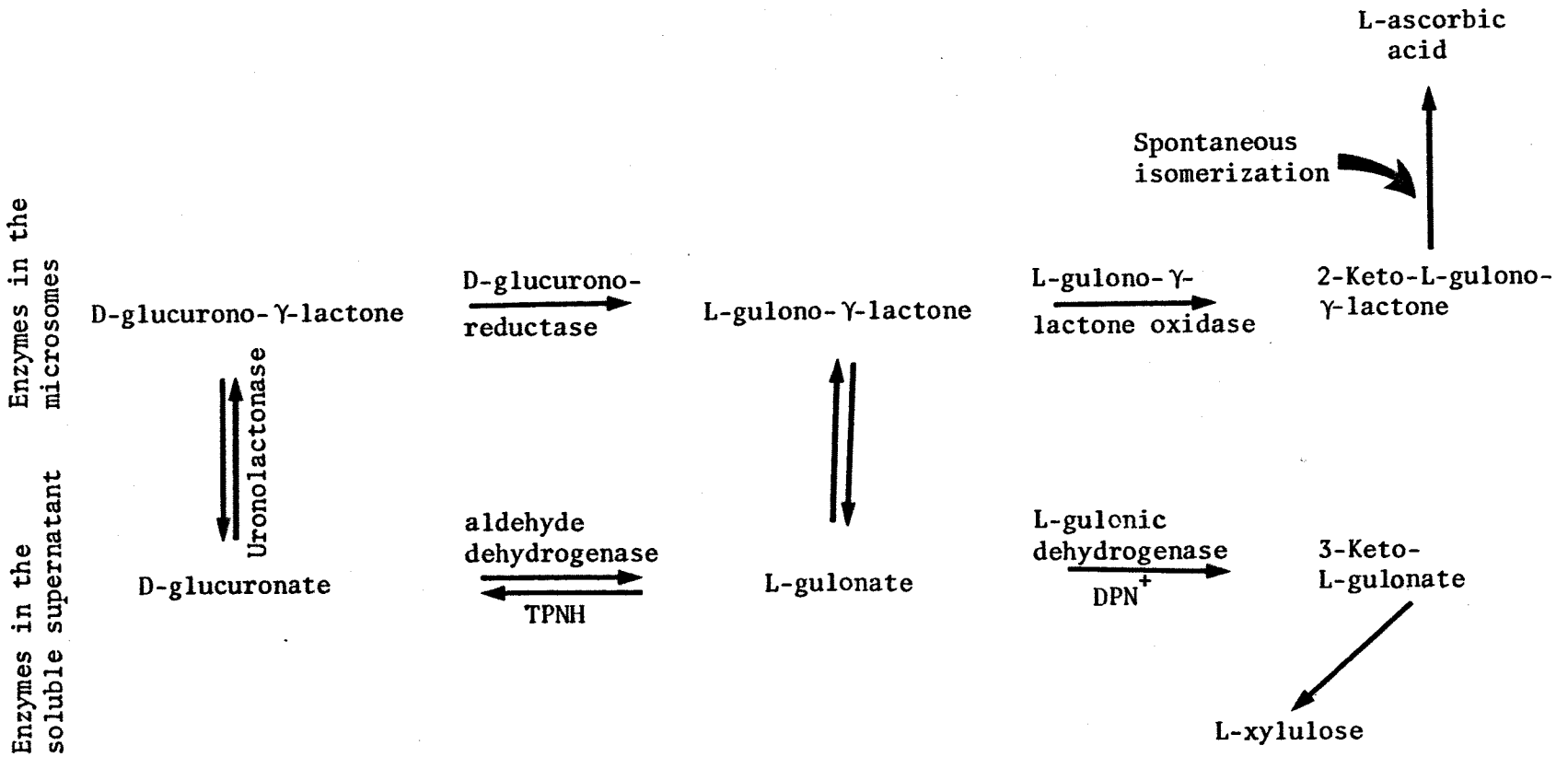


FIGURE 3. Biosynthetic pathway for L-ascorbic acid in microsomes

Section 1.2.5 Metabolism of L-ascorbic Acid

Section 1.2.5.1 Absorption

In guinea pigs ascorbic acid has been shown to be absorbed in the small intestine by an active transport system that has characteristics of Na^+-dependency (Stevenson & Brush, 1969). In guinea pigs the site of absorption was located in the duodenal and proximal small intestine wall with the highest rates of absorption being recorded between the ileum and jejunum with no absorption in the duodenum (Hornig, 1975a). Sadoogh-Abasian and Evered (1979) reported that ascorbic acid is absorbed from the human buccal cavity in a similar way to absorption from the small intestine.

Section 1.2.5.2 Transport

Biological transport can be classified into:

1. Diffusion transport where the solutes will be transferred from higher concentrations to lower concentration (i.e. down the concentration gradient) motivated by a decrease in free energy.

2. Active transport where the solutes are transferred from lower concentrations to higher concentration (i.e. up the concentration gradient) and this process requires energy.

Harris (1938) reported that ascorbic acid is found in considerably higher concentrations in the aqueous humour of the eye of guinea pigs than in the plasma and Sharma et al. (1963) reported that the uptake of ascorbic acid in the brain cortex and adrenal cortex of guinea pigs was energy-dependent. These observations led Lewin (1975)

to conclude that the transport of ascorbic acid is by an active mechanism where ascorbic acid is transferred from the plasma to the erythrocytes and by the leucocytes to other tissues. Hornig (1975a) suggested that free ascorbic acid is taken by several tissues via an energy-dependent and sodium-ion-sensitive process, whereas transport of the oxidized form (dehydro-L-ascorbic acid) follows the principle of diffusion.

It is well known that ascorbic acid oxidized by the loss of one electron to form the ascorbic acid free radical (AFR) subsequently loses a further electron and one hydrogen ion to form dehydro-L-ascorbic acid (DHAA). Thus, biologically ascorbic acid exists in two readily interconvertible forms, the reduced form of the vitamin, L-ascorbic acid (AA) which is relatively strong acid (pK 4.17) and the oxidized form (DHAA) which is nonionic and more lipid-soluble. Martin (1961) and Martin and Mecca (1961) concluded from that DHAA may be the transportable form of the vitamin as improved lipid solubility would facilitate its crossing cell membranes. Martin (1961) confirmed this suggestion by injection of the oxidized form into hearts of rats and he observed that the level of ascorbic acid in the brain and eye approached the equilibrium state with the serum more rapidly than when the free acid was injected.

Martin (1961) also demonstrated in <u>in vitro</u> studies that DHAA penetrated the cell membranes of erythrocytes more rapidly than the reduced form (AA). Martin and Mecca (1961) showed that administration of D-isoascorbic acid, D-ascorbic acid, D-glucoascorbic acid and their respective dehydroascorbic acid forms did not increase the

level of ascorbic acid in the brain indicating that transport of ascorbic acid is structurally specific. The inability of these optical analogues to penetrate cellular barriers could explain the low antiscorbutic activity of these forms. Bigley and Stankova (1974) and Hornig (1975a) confirmed the findings of Martin and his colleague when they reported that the reduced form is (AA) the preferred form for transport of the vitamin in the pituitary gland, adrenal gland, lungs, liver, kidney, bone, skin and nasal mucosa whereas the oxidized form (DHAA) is the preferred form of uptake by neutrophils, erythrocytes and lymphocytes.

Section 1.2.5.3 Distribution

Distribution of L-ascorbic acid in tissues is considered to be an important estimate of the involvement of this vitamin in metabolism of specific tissues (Hornig, 1975b). There are two theories concerning the distribution of L-ascorbic acid in the animal body. Firstly Martin (1961) proposed that the kidney plays an important role in distribution of the vitamin when he observed that nephrectomy caused a lag in the accumulation of the vitamin by various tissues and he suggested that there was a defect in the transport of ascorbic acid from serum to the tissues in nephrectomized animals. In addition, this author supported his theory by stating that the kidney has the ability to oxidize ascorbic acid to dehydroascorbic acid and in this way facilitate its distribution to other tissues and organs, e.g. brain, eye and erythrocytes (Martin & Mecca, 1961). Alternatively, Hughes and Maton (1966) reported that erythrocytes make an important contribution to distribution of vitamin C in the whole body by

reduction of dehydroascorbic acid to L-ascorbic acid. It was found by these authors that the rate of release of L-ascorbic acid from the erythrocytes was much less rapid than the rate of uptake of dehydroascorbic acid.

An enzyme system exists for reduction of dehydroascorbic acid to L-ascorbic acid. Staudinger et al. (1961) proposed that the enzyme NADH monodehydroascorbic acid transhydrogenase mediates the transfer of hydrogen from NADH to oxygen producing NAD^+ and regenerating ascorbic acid. This activity of this enzyme has been shown to be affected by ascorbic acid concentration in the tissues. Devine and Rivers (1968) found that the activity of this enzyme was decreased by increasing tissue ascorbate concentrations. Glutathione (GSH) seems to play a role in reduction of the oxidized form of the vitamin. Kanazawa et al. (1981) reported that GSH is considered to be a co-factor of the enzyme dehydro-L-ascorbic acid reductase (EC 1.8. 5.1) as follows:

$$DHAA + 2GSH \longrightarrow AA + GSSG$$

$$GSSG + NADPH_2 \longrightarrow 2GSH + NAD$$

The enzyme dehydroascorbic acid reductase has been reported in hepatopancreas of common carp (Cyprinus carpio), yellow tail (Seriola quinqueradiata), black sea bream (Mylio macrocephalus), eel (Anguilla japonica), rainbow trout (Salmo gairdneri) and rats (Yamamoto et al., 1977a).

L-ascorbic acid occurs in almost every tissue in the body, however it is principally concentrated in tissues of high metabolic activity

(Ikeda et al., 1963a; Kirk (1962) and Lewin (1975) suggested that the tissue ascorbate concentrations will be in descending order approximately as follows:

Adrenals > Leucocytes / Pituitary > Brain > Eye-lens / Pancreas > Kidney / Liver / Spleen > Heart / Muscle > Plasma

Adrenal glands contain the highest concentration of ascorbic acid in animal tissues. Kitabachi (1967) explained this on the following bases:

1. Ascorbic acid in the resting state exerts a 'braking influence' on steroid biosynthesis and this inhibition is principally effected through the adrenal hydroxylase systems.

2. Under conditions of adreno-corticotrophic hormone (ACTH) stimulation, the adrenal releases a considerable amount of ascorbic acid and therefore facilitates steroidogenesis.

Lewin (1975) reported that ascorbic acid is required in the formation of adrenaline and noradrenaline in the adrenal medulla and for protection of these hormones from oxidation. The high concentration of ascorbic acid in the pituitary might be necessary for maintenance of the redox status in these cells (Martin & Mecca, 1961). Ascorbic acid was found at high levels in leucocytes and Lewin (1975) proposed the following reasons for this;

1. ascorbic acid is required for production of antibodies (formation of γ-globulin);

2. ascorbic acid is required in production of lysozymes (phagocytosis);

3. ascorbic acid is for formation and transport of cyclic 3'5' adenosine monophosphate (cAMP);

4. ascorbic acid exerts an anti-histamine effect.

In addition, Abt et al. (1963) reported that leucocytes play an important role in extraction of ascorbic acid from the plasma.

The islets of Langerhans (β-cells) of the pancreas are responsible for formation of pro-insulin which contains a number of disulphide bonds (Lewin, 1975). Banerjee (1943a) reported that scorbutic guinea pigs exhibited a lowered insulin content of the pancreas and the same author (1944) demonstrated, through histological studies, that the β-cells of the pancreas of scorbutic guinea pigs were degranulated as indicated from an increased number of α-cells in proportion to β-cells. In a previous report Banerjee (1943b) correlated the diminished glycogen content of the liver of scorbutic guinea pigs with diminished insulin content and the author confirmed this when he observed that the liver glycogen content was decreased in partially pancreatectomized guinea pigs and that injection of vitamin C promoted rapid deposition of liver glycogen. Ascorbic acid, therefore, plays an indirect role in carbohydrate metabolism due to its effects on the β-cells of the islets of Langerhans. The role of ascorbic acid in spleen and kidney is a result of its direct and indirect effects on erythropoiesis (Agrawal & Mahajan, 1980b). The role of ascorbic acid in brain, liver, heart, muscle, gall-bladder, gut and gonads is reviewed in the light of the results of Chapters 6 and 11 of this thesis.

Tissues ascorbate concentrations will be influenced by various factors:

1. Rate of uptake (fixation) of ascorbic acid: Penney & Zilva (1946); Hughes et al. (1971a); Murai et al. (1978); Sato et al. (1978a); Hilton et al. (1979b) reported that tissue ascorbate concentrations were correlated with dietary levels of ascorbic acid for guinea pigs, channel catfish (Ictalurus punctatus) and rainbow trout (Salmo gairdneri).

2. Metabolic role of ascorbic acid: Tissues in which ascorbic acid has metabolic roles will have developed more efficient mechanisms for its abstraction and retention (Hughes & Hurley, 1969). These include adrenal glands, ovary, leucocytes, pituitary gland and brain (Ikeda et al., 1963a; Lewin, 1974, 1975; Wilson, 1977; Hilton et al., 1979a, b; Seymour, 1981a). Lewin (1975) attributed the concentration of ascorbic acid in these tissues to convenience of site for storage, active utilization site for biochemical interactions and transport activity itself.

3. Sex of animals: The eye-lens of male guinea pigs has a significantly higher concentration of ascorbic acid than that of females (Hughes & Jones, 1971). In animals capable of synthesis of ascorbic acid Stubbs and McKernan (1967) reported that sex influences hepatic biosynthesis of ascorbic acid in rats where higher values of ascorbic acid were found in plasma, liver, lungs, kidney, spleen and muscle of males than in females.

4. Age of the animal: Kirk and Chieffi (1958) reported that the ascorbic acid concentration in blood tends to be lowered in

in older, compared with young, individuals and Kataria and Roe (1965) reported that vitamin C status decreases with increased age as indicated by the decrease in vitamin C concentration of leucocytes with increased age.

5. Physical stress: Hughes et al. (1971b) reported that guinea pigs subjected to the physical stress of forced swimming exhibited decreased ascorbic acid concentrations in adrenal glands, spleen and brain. Toads, mice and rats exposed to low temperatures (5°C) also exhibited a significant decrease in testicular ascorbic acid (Roy Chowdhury & Mukerjee, 1976a).

6. Physiological stress: Cigarette smoking causes a significant reduction in the ascorbic acid content of adrenal glands in guinea pigs (Hughes et al., 1970) and leucocytes in humans (Brook & Grimshaw, 1968). Hornig (1981) suggested that lowered ascorbate concentrations in smokers related to epiderminological factors and certain physiological effects on the absorption, metabolism and excretion of ascorbic acid. Hypophysectomy has also been shown to cause decreased ascorbic acid concentrations in liver, spleen, kidney, brain and heart muscle (Kovtunyak et al., 1971).

7. Infection and disease: During infection there is generally a greatly increased metabolic demand for vitamin C which may result in lowering of tissue ascorbate concentrations (Abbasy et al., 1936; Wilson, 1974, 1977). Chakrabarty and Banerjee (1955) and Banerjee (1977) reported that blood ascorbate concentrations

decreased markedly in patients suffering from infectious diseases. Lewin (1975) suggested that this decrease was due to utilization of ascorbic acid in antibody production (formation of γ-globulin) and the production of lyzozymes (phagocytosis).

8. Drugs: Certain drugs have been shown to initiate biosynthesis of L-ascorbic acid in animals not dependent on an exogenous source of this vitamin. Burns et al. (1960) and Conney et al. (1961) reported that drugs such as chloretone and barbital stimulated the biosynthesis of ascorbic acid in rats via the glucuronic acid pathway.

9. Hormones: Administration of some steroids has been shown to affect tissue ascorbate concentrations in certain species. Intravenous injection of mare serum gonadotropin resulted in a rapid reduction in ascorbic acid concentration of the interstitial gland of rabbit ovary (Claesson et al., 1949). Injection of chorionic gonatotrophin into female rats resulted in a signficant decrease in ovarian ascorbic acid concentration (Noach & Van Rees, 1958) as did injection of luteininizing hormone (LH) (Goldstein & Sturgis, 1961).

10. Pollution: Pollution of the environment by residues of organochlorine pesticides and heavy metals represents a health hazard to humans and animals. During metabolism of such toxic substances there is increased mobilization and utilization of ascorbic acid which may result in decreased tissue ascorbic acid concentrations. Hodson et al. (1980) reported that in newly hatched rainbow trout

exposure to water borne lead, of fish fed diets either deficient in or supplemented with ascorbic acid, caused severe reductions in ascorbate concentrations in the whole carcass, liver, kidney and brain. Yamamoto et al. (1981) reported that rainbow trout exposed to 0.035ppm of copper exhibited a marked reduction in liver ascorbate concentration of fish fed a diet not supplemented with ascorbic acid. In addition, Thomas et al. (1982) reported that mullet (Mugil cephalus) exposed to water borne cadmium exhibited severe reduction of ascorbic acid concentration in their livers. In contrast to these studies Diwan et al. (1978) reported that Clarias batrachus exposed to industrial effluent exhibited a slight increase in ascorbic acid content of the brain in comparison to those kept in tap water. This apparent discrepancy may be due to the short experimental period employed in the latter study.

Organochlorine pesticides such as DDT, dieldrin, toxaphene, lindan, aldrin and polychlorinated biphenyls (PCBs) are very toxic substances particularly to fish and these pesticides are used extensively in agricultural areas where fish ponds may become contaminated. Mayer et al. (1978) reported that toxaphene caused a severe reduction in the ascorbic acid concentration of the back bone in channel catfish (Ictalurus punctatus) which explained the incidence of a high percentage of spinal deformity due to impairment of collagen synthesis. Eyed eggs of brook trout (Salvelinus fontinalis) exposed to the polychlorinated biphenyl aroclor 1254 exhibited low vitamin C values in yolk-sac fry (Mauck et al., 1978). Rainbow trout and brook trout are unable to synthesise

ascorbic acid (Yamamoto et al., 1978; Soliman et al., 1985) and exposure to such toxins result in a decrease in tissue ascorbate concentrations, possibly as a result of the vitamin acting in some kind of detoxification system. Conversely in rats, which are able to synthesise vitamin C (Ul Hassan & Lehninger, 1956; Cohen, 1961) exposure to organophosphorus insecticides (parathion and malathion) resulted in increased L-gulono-γ-lactone oxidase activity, increased ascorbic acid synthesis and increased excretion of this vitamin indicating, again, a role for ascorbic acid in detoxification of these toxic compounds (Chakraborty et al., 1978).

Section 1.2.5.4 Half-life of L-ascorbic acid

The half-life of L-ascorbic acid in animals appears to be affected by species, tissue type and exposure to certain drugs. Hodges et al. (1969, 1971) reported that the half-life of ascorbic acid in man was 16 days whereas in guinea pigs it was only 3.5 days so that vitamin C deficiency appears in guinea pigs earlier (after 20 days) than in man (after 60 days). In fish there is an interesting difference in the half-life of ascorbic acid between common carp (a warm water fish) and rainbow trout (a cold water fish). Ikeda and Sato (1965) reported that the half-life of C^{14}-labelled ascorbic acid was 3.8 days in carp whereas the corresponding value in the head kidney of rainbow trout was 42 days (Tucker, 1983). It is possible to relate the short half-life of ascorbic acid in certain species of animals to existence of the enzyme dehydro-L-ascorbatase which is responsible for catalyzing or delactonization of dehydroascorbic acid. Yamamoto

et al. (1977b) reported that dehydro-L-ascorbatase is present in the hepatopancreas of common carp whereas no trace of activity of this enzyme was detected in rainbow trout. Different tissues from the same species may show differences with respect to the half-life of ascorbic acid. Pelletier (1969) reported that the half-life of ascorbic acid was 2-3 days for liver, heart, kidney, adrenal and spleen of guinea pigs whereas it was 5 days in the brain. Exposure to drugs may affect the half-life of ascorbic acid, for example chloretone reduced the half-life of ascorbic acid (Conney et al., 1961) in rats.

Section 1.2.5.5 Catabolism of L-ascorbic acid

The metabolic fate of L-ascorbic acid relies on a number of factors such as species, route of ingestion, level of uptake and nutritional status (Hornig, 1981). There are fundamental differences in the metabolism and fate of ascorbic acid in primates as compared to rats and guinea pigs. Guinea pigs convert ascorbic acid to carbon dioxide (66%) and excrete it via expired air and a little is excreted in the urine (10%) with less than 1% excreted in the faeces (Burns et al., 1951; Burns, 1960). In contrast, in man no carbon dioxide derived from labelled ingested ascorbic acid was expired (Burns, 1967). Hellman and Burns (1958) reported that in human ascorbic acid is converted to urinary oxalate. Tolbert (1979) related these differences to the fact that rats and guinea pigs have a metabolic pathway for degradation of ascorbic acid to CO_2 not present in primates and that this is due to absence of the enzyme dehydroascorbic acid lactonase, an enzyme which catalyzes the irreversible ring opening of dehydro-

ascorbic acid to L-2,3 diketogulonic acid which is then decarboxylated to L-carbohydrate and catabolized via D-glucuronate-L-gulonate pathways (Fig. 4). The route of ingestion also affects the metabolic fate of ascorbic acid. Tolbert (1979) reported that intravenous injection of labelled ascorbic acid in guinea pigs resulted in less than 1% of the label being detected in the CO_2 of expired air as compared to 66% when administered orally (Burns et al., 1951; Burns, 1960). Increased intake of dietary ascorbic acid results in increased catabolism. Kallner et al. (1979) suggested that ascorbic acid will be excreted when the body pool has been saturated and Tillotson and O'Connor (1981) reported that the percentage of C^{14} in the urine derived from labelled dietary ascorbic acid increased as the dietary level of ascorbic acid increased. Hornig (1981) related this to a decrease in the half-life of the vitamin with increased level of ingestion.

Section 1.2.5.6 Excretion of chemically intact ascorbic acid

Although the chemical structure of L-ascorbic acid is related to the carbohydrates, it is not reabsorbed or excreted by the same mechanism as glucose. This has been confirmed by Ralli et al. (1938) and Sherry et al. (1940) who reported that there was no impairment of the reabsorption of vitamin C by increased glucose intake in humans and dogs. Elevation of vitamin C clearance at high plasma concentrations is due to the fact that the renal tubules reabsorb the vitamin up to the same maximal limiting rate, after which any vitamin present in the glomerular filtrate is excreted. In addition there is an absolute limitation in the capacity of renal tubules to reabsorb the

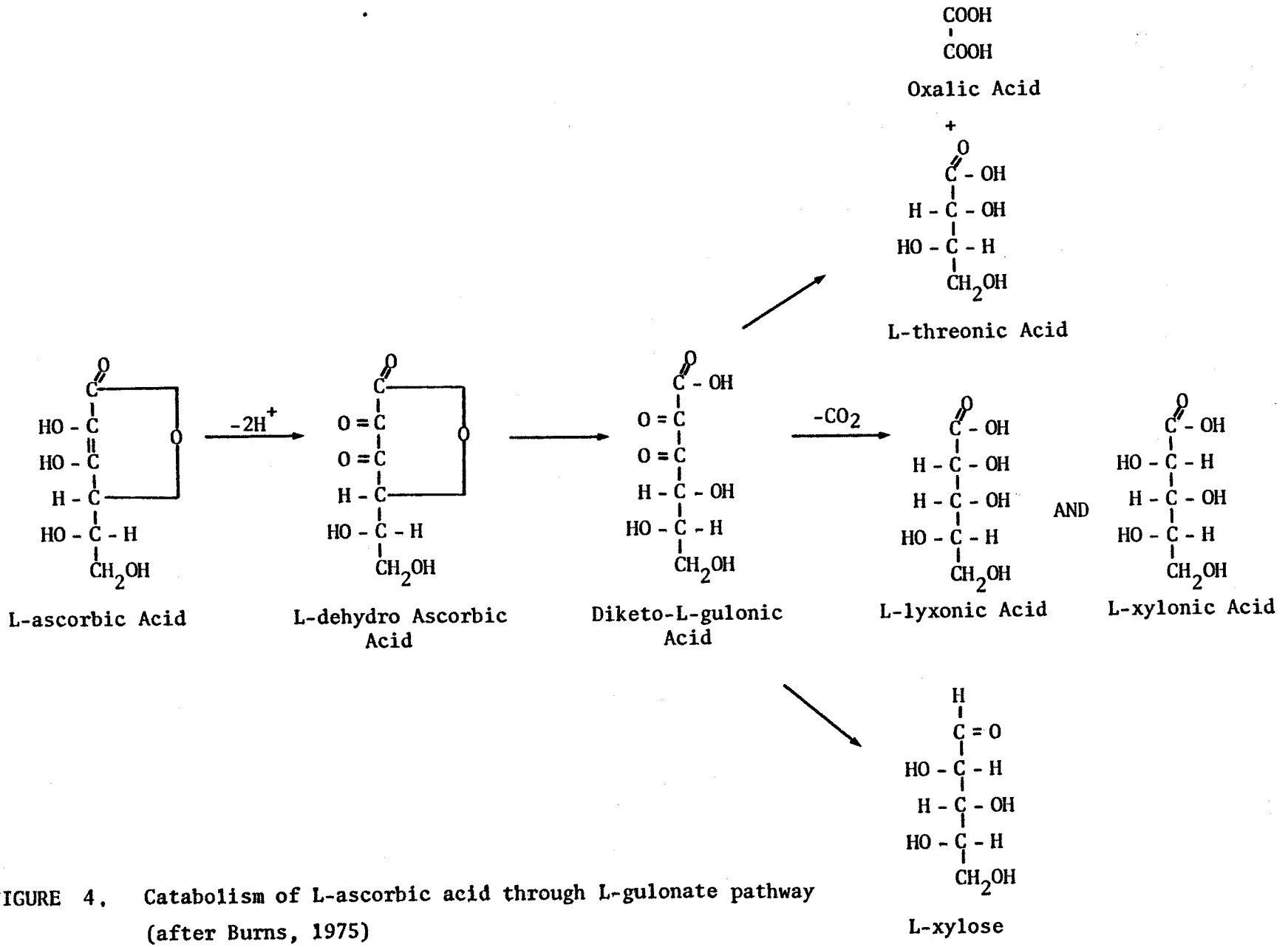


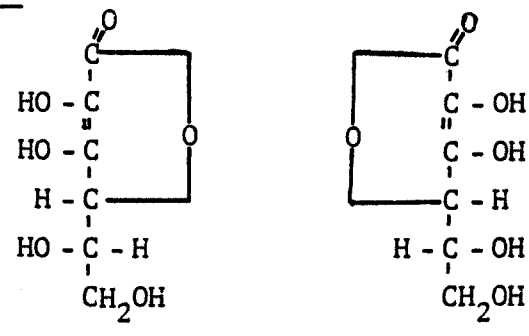
FIGURE 4. Catabolism of L-ascorbic acid through L-gulonate pathway (after Burns, 1975)

vitamin (Ralli et al., 1938; Sherry et al., 1940). These authors suggested that vitamin C is excreted in dogs and humans by filtration and active tubular absorption (1.2-2.1mg per 100 C^3 of glomeruli in humans and 0.77mg/100 C^3 of glomeruli in dogs).

Section 1.2.6 Ascorbic Acid Forms

In addition to the established structure of L-ascorbic acid (Haworth et al., 1933) there are apparently a number of chemically related compounds (Harris, 1967b). Due to the instability of ascorbic acid during processing and storage of diets for humans or animals which require this vitamin protected forms have been produced commercially such as ascorbidan 50 (mono-, di- and triglyceride coated ascorbic acid), ascorbyl palmitate, ethyl cellulose coated ascorbic acid, ascorbic acid 2-sulphate and the sodium salt of ascorbic acid (see Chapter 7 in this thesis). The nature of these other forms of this vitamin is considered below.

1. D-ascorbic acid



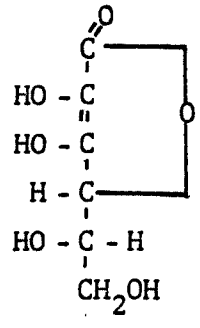
L-ascorbic acid D-ascorbic acid

D-ascorbic acid failed to show any antiscorbutic activity in guinea pigs (Demole, 1934). Dayton and Burns (1958) suggested that the lack of antiscorbutic activity of this form is due to poor retention of

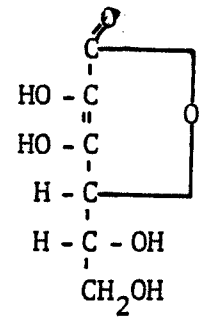
this compound in animal tissues. No reports appear in the literature concerning the antiscorbutic activity of this compound in fish. Requirement for ascorbic acid may well exhibit stereospecificity.

2. D-isoascorbic acid

D-isoascorbic acid is also known as D-araboascorbic acid or erythorbic acid (Kadin & Osadca, 1959). The close chemical relationship between L-ascorbic acid and D-araboascorbic acid is indicated by the fact that the dye-titration method fails to differentiate between these forms. D-araboascorbic acid differs from L-ascorbic acid in the relative position of the hydrogen and hydroxyl group on the fifth carbon atom of the molecule.



L-ascorbic acid



D-araboascorbic acid

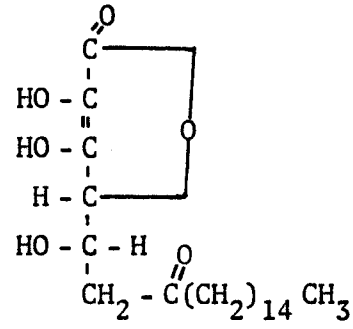
There is some controversy concerning the antiscorbutic activity of this form. Several workers (Demole, 1934; Yourga et al., 1944; Pelletier, 1969; Pelletier & Godin, 1969) have reported that D-araboascorbic acid possesses one-twentieth of the antiscorbutic activity of L-ascorbic acid when guinea pigs are used as the test animal. However, very low activity (1% of L-ascorbic acid) was reported by Reiff and Free (1959). Fabianek and Herp (1967) reported that D-isoascorbic acid was able to replace vitamin C when

given in significant doses (10-200mg daily) to guinea pigs. In later experiments Rivers et al. (1963) suggested that the lack of anitiscorbutic activity of this compound was due, primarily, to its molecular structure which limited its uptake by the tissues and this was supported by Hughes and Jones (1970) who reported that the absorption efficiency of D-araboascorbic acid in the gastrointestinal tract of guinea pigs was considerably lower than that of L-ascorbic acid.

In addition to an apparent lower biological activity D-araboascorbic acid also has an effect on the availability of ascorbic acid. Hornig and his colleagues (Hornig et al., 1974; Hornig, 1975a; Hornig & Weiser, 1976) have demonstrated that the availability and utilization of L-ascorbic acid was impaired by the presence of D-araboascorbic acid. The literature contains no reports concerning the antiscorbutic activity of this compound in fish.

3. Ascorbyl palmitate

Ascorbyl palmitate (L-ascorbic ester 6-hexa-decanoate) is a lipid-soluble antioxidant (Johnson, 1979) which has been used on a large scale to preserve human foods (Riemenschneider et al., 1944; Bauernfeind, 1982; Cort, 1982) and as a dietary source of vitamin C for channel catfish (Brandt et al., 1985). Use of this compound may result in reduced losses of ascorbate during processing, storage and immersion of fish feeds in water.

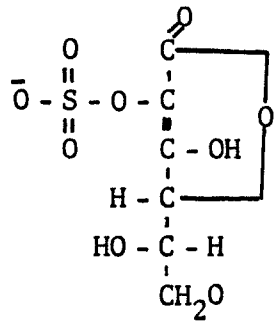


L-ascorbyl palmitate

High dietary levels of this compound (5%) were toxic in rats but lower levels (2%) were considered adequate for normal growth (Fitzhugh & Nelson, 1946). As rats are able to synthesise ascorbic acid (Ul Hassan & Lehninger, 1956) the observed toxicity might be related to increased tissue ascorbate concentrations or to the effects of high dietary levels of the palmitate portion of this compound.

4. L-ascorbic acid 2-sulphate

L-ascorbic acid 2-sulphate (AA2S) is considered to be the most chemically stable form of ascorbic acid (Quadri et al., 1973, 1975) and its natural occurence was first reported by Mead & Finamore (1969) in brine shrimp (Artemia salina). Subsequently many workers (Baker et al., 1971a, 1974; Mumma & Verlangieri, 1972; Hornig et al., 1973; Benitez & Coloso, 1984) have reported its occurrence in the liver, spleen, adrenals, bile, eyes, lymph, skin, muscle, heart, kidney, thyroid and urine of rat, guinea pig, dog, cow, monkey, human, rainbow trout and milk fish. Mead & Finamore (1969) suggest that this compound may have a dual metabolic role as a storage form of both ascorbic acid and sulphate.



L-ascorbic acid 2-sulphate

The antiscorbutic activity of ascorbic acid 2-sulphate is apparently species specific. Kuenzig et al. (1974), Machline et al. (1976), Tsujimura et al. (1976) and Tsujimura (1978) reported that ascorbic acid 2-sulphate failed to prevent scurvy in guinea pigs and rhesus monkey, whereas in fish this compound prevented scurvy and enhanced growth and food utilization (Halver et al., 1974, 1975, 1983; Murai et al., 1978; Tsujimura et al., 1978; Brandt et al., 1985). However, Murai et al. (1978) and Tsujimura et al. (1978) reported that channel catfish and rainbow trout fed diets supplemented with ascorbate 2-sulphate on an equimolar basis with L-ascorbic acid exhibited lower tissue ascorbate concentrations than fish fed diets supplemented with L-ascorbic acid. This suggests that this compound is only partially hydrolyzed to L-ascorbic acid in these species. However, Tucker (1983) later reported that this compound was completely hydrolyzed in rainbow trout.

Baker et al. (1974) and Omaye et al. (1982) suggested that this compound is excreted rapidly in the urine of guinea pigs, rats and mammals after its administration and Baker et al. (1974) suggested that the rapid excretion of this compound is related to malabsorption.

This might explain the inability of L-ascorbic acid 2-sulphate to prevent scurvy in guinea pig and rhesus monkey, although guinea pig have been shown to possess the enzyme ascorbate sulphatase (Baker et al., 1974).

Benitez and Halver (1982) identified the enzyme L-ascorbic acid 2-sulphate sulphohydrolase (C_2-sulphatase) in rainbow trout liver; this enzyme is responsible for convertion of L-ascorbic acid 2-sulphate to L-ascorbic acid. It is possible this ascorbic acid form is not available to humans as Benitez and Halver (1982) suggest that humans possess the enzyme arylsulphatase which acts on cerebroside 3-sulphate and not on ascorbic acid 2-sulphate.

Section 1.2.7 Physiological Roles of Ascorbic Acid

Section 1.2.7.1 The role of ascorbic acid in prevention of anaemia

Anaemia is a reduction in the number of erythrocytes or haemoglobin content and/or a reduction in the packed cell volume of erythrocytes with a corresponding increase in the proportion of leucocytes (Wintrobe, 1946; DesMarais & McGraw, 1956).

During scurvy scorbutic animals appear to be anaemic (Banerjee & Pal, 1959; Halver, 1972a; Lovell, 1973; Andrews & Murai, 1975; Lim & Lovell, 1978; Agrawal & Mahajan, 1980a). Banerjee and Pal (1959) reported that in scorbutic monkeys anaemia was normocytic, normochromic and normoblastic and Agrawal and Mahajan (1980c), in a well controlled experiment, investigated the haematological changes due to vitamin C deficiency in Channa punctatus. The latter authors reported

the incidence of normochromic normocytic anaemia which developed into a normochromic macrocytic form by the end of the experiment.

The cause of anaemia in scurvy may be related to a defect in iron metabolism and to severe haemorrhages. Transferrin is plasma-bound iron, existing as an iron-protein complex, and ascorbic acid, which is a reducing agent (Szent-Györgyi, 1928), participates in one-electron transfer reactions such as $Fe^{++} \underset{-e}{\overset{+e}{\rightleftharpoons}} Fe^{+++}$. Ascorbic acid thus plays an important role in the reduction and release of ferric iron from transferrin and its subsequent incorporation into tissue ferritin (Mazur et al., 1960; Mazur, 1961; Brise & Hallberg, 1962; Banerjee & Chakrabarty, 1965; Monsen & Page, 1978).

Hepatic stores of iron are located not only in the hepatocytes but also in the cells of the reticulo-endothelial system. It has been reported by various workers (Brise & Hallberg, 1962; Bothwell et al., 1964; Hopping & Ruliffson, 1966; Lipschitz et al., 1971; El-Hawary et al., 1975) that ascorbic acid promotes the absorption and utilization of iron and facilitates its release from reticulo-endothelial stores. In addition, Banerjee & Chakrabarty (1965) suggested that ascorbic acid can be considered an essential factor in the utilization of iron in haemoglobin synthesis.

Haemorrhaging is a feature of severe ascorbic acid deficiency in animals (Barkhan & Howard, 1959; Nutrition Reviews, 1960). Lee (1961) suggested that the haemorrhage observed in scorbutic animals is related to an increased fragility of the blood vessels of the capillary bed. Increase in the capillary fragility could be related to:

1. Increase in hyaluronidase. Ascorbic acid inhibits such increases through its effect on the hyaluronidase-hyauronic system (Reppert et al., 1951);

2. Increased platelet counts with decreased platelet adhesiveness (Barkhan & Howard, 1959) and

3. Increase in histamine production during scurvy. Histamine acts as strong vasodilator and may increase capillary permeability resulting in blood leaching from the capillaries (Chatterjee et al., 1975).

Section 1.2.7.2 The role of ascorbic acid in biosynthesis of collagen and wound healing

1. Biosynthesis of collagen

Collagen is an important component of animal connective tissues comprising one-third of the body protein and it is considered to be most unusual animal protein due to the absence of cysteine and tryptophan, the high level (more than 30%) of glycine and the presence of such unusual amino acids as hydroxyproline and hydroxylysine (Udenfriend, 1966). It has been reported by Stone & Meister (1962) that hydroxyproline comprises 13% of the total amino acids of collagen.

The role of ascorbic acid in the biosynthesis of collagen is considered to be one of the principal biochemical roles of this vitamin (Barnes, 1975). The function of ascorbic acid in this respect has been demonstrated in many different types of experiment:

(i) Withdrawal of ascorbic acid from the diets of ascorbic acid dependent animals (e.g. guinea pigs, rainbow trout, channel catfish) (Robertson, 1950; Gould et al., 1960; Mitoma & Smith, 1960; Barnes et al., 1970; Wilson & Poe, 1973; Sen Gupta & Deb, 1978; Sato et al., 1978a) which results in impaired collagen biosynthesis in these animals.

(ii) Study of the effect of wound healing of withdrawal of ascorbic acid from diets of animals prone to scurvy result in delayed wounds healing (Pirani & Levenson, 1953; Gould & Woessner, 1957; Halver, 1972b; Lim & Lovell, 1978; Jauncey et al., 1985).

(iii) Studies of model systems, such as implanted polyvinyl sponges and carrageenan granulomes where collagen synthesis was markedly impaired (Gould, 1958; Robertson et al., 1959) by ascorbic acid deficiency.

In collagen synthesis there is more than one mechanism involved and Gould (1961) suggested the existence of two principal mechanisms:

(i) Ascorbic acid-independent collagen biosynthesis where collagen formation both in skin and in the carcass takes place in scorbutic animals. This was shown in new born guinea pigs suggesting that collagen synthesis can be ascorbic acid-independent.

(ii) Ascorbic acid-dependent collagen biosynthesis where collagen formation in skin wounds or in sub-cutaneously implanted

polyvinyl sponges in animals fed diets devoid of ascorbic acid was impaired.

The possible existence of an ascorbic acid-independent pathway for collagen biosynthesis as suggested above is refuted by the work of numerous authors in various animals including fish (Wilson & Poe, 1973; Lim & Lovell, 1978; Sato et al., 1978a; Sen Gupta & Deb, 1978; Yoshinaka et al., 1978; Sato et al., 1982a, b).

Two principal processes occur during collagen synthesis, namely protein synthesis and hydroxylation (Udenfriend, 1966). The studies of Robertson and Hiwett (1961) were the first to suggest the role of ascorbic acid in hydroxylation of proline which could be related to the earlier findings of Stetten (1949) who reported that N^{15}-labelled hydroxyproline fed to rats was not incorporated into collagen. In 1965 Petrofskey and Udenfriend (Nutrition Reviews, 1978a) suggested that ascorbic acid was a co-factor for the enzyme prolyl hydroxylase which is responsible for hydroxylation of proline to hydroxyproline during collagen formation. By 1967 Prockop & Kivirikko had proposed a detailed scheme for the biosynthesis of collagen (Fig. 5).

Free amino acids

⇓

Amino acyl-tRNAs

⇓

Assembly of proline and lysine-rich protocollagen polypeptides on ribosomes

⇓

Intracellular hydroxylation of appropriate proline and lysine residues in protocollagen

⇓

Extrusion of soluble collagen (Tropocollagen) into extracellular matrix

⇓

Formation of 'soluble collagen' fibres

⇓

Formation of insoluble collagen fibres

FIGURE 5. Biosynthesis of collagen and of collagen fibres

tRNA : transfer-ribonucleic acid

Cardinale and Udenfriend (1974) and Barnes (1975) reported that the prolyl and lysyl hydroxylases required, in addition to ascorbic acid, molecular oxygen, ferrous iron and 2-ketoglutarate as co-factors. Cardinale and Udenfriend (1974) proposed and described the mechanism by which proline hydroxylation occurs (Fig. 6) as follows:

The enzyme decarboxylates α-ketoglutarate stoichimetrically to CO_2 and succinate and ascorbic acid is required for reduction of a proportion of the enzyme-bound Fe^{2+}. The Fe^{2+} becomes oxidized to Fe^{3+} in a side reaction after the prolyl hydroxylation.

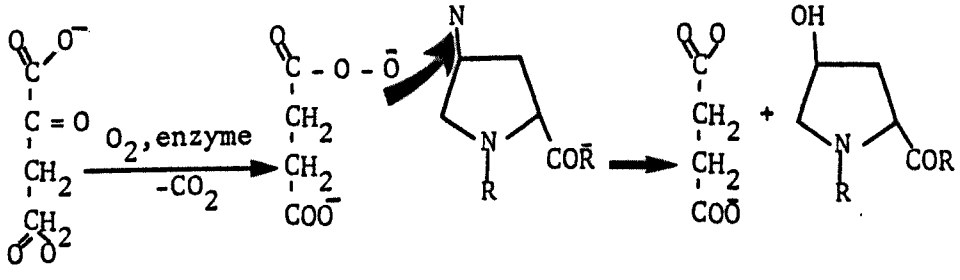


FIGURE 6. Prolyl hydroxylase mechanism, showing the formation of peroxysuccinic acid and the subsequent oxenoid attach on peptydyl proline

Ascorbic acid does not directly participate in the hydroxylation reaction but functions as a highly specific protective agent keeping the enzyme active by reversing the oxidation of the enzyme-bound iron. The hydroxylation process is very important since protocollagen cannot pass out through the membranes of the synthesis cells until hydroxylated, (Prockop & Kivirikko, 1967; Nutrition Reviews, 1978a,1979). A single polypeptide chain of collagen contains approximately 1000 amino acids with a molecular weight of 100,000 and when single polypeptides aggregate into three-strand complexes, these serve as a basic unit for collagen fibres which are then called tropocollagen (Prockop & Kivirikko, 1967; Fig. 5).

2. Wound healing

Ascorbic acid (vitamin C) is a very important nutrient associated with repair of wounds in animals (Hunt, 1940; Halver, 1972b).

Ascorbic acid is an excellent reducing agent (Szent-Györgyi, 1928) which acts with oxidation-reduction enzyme systems enabling deposition of reticulum, collagen, bone, enamel, dentine and possibly cartilage and elastin to take place (Hunt, 1940).

Wound healing has been shown to be delayed in scorbutic animals (Galloway et al., 1947; Editorials & Comments, 1953; Gould, 1961; Pirani & Levenson, 1953; Halver, 1972b; Lim & Lovell, 1978; Jauncey et al., 1985). Ascorbic acid is not only important in the healing of wounds but also in the maintenance of previously formed scar tissue (Editorials & Comments, 1953; Pirani & Levenson,

1953). Sufficient ascorbic acid in the diets is necessary for wound healing where cells build up a mature vascular scar, composed of fibrocytes, collagen and small blood vessels (Hunt, 1940).

Section 1,2.7.3 Role of L-ascorbic acid in protein metabolism

The role of L-ascorbic acid in protein metabolism can be related to its effects on different aspects of protein metabolism as follows:

1. Collagen

The role of ascorbic acid in the biosynthesis of collagen has been reviewed earlier (Section 1,2.7.2).

2. Non-protein nitrogen excretion and amino acid and protein levels in muscle and liver

Non-protein nitrogen (NPN) excretion in the urine is an index of the extent of protein catabolism. Rohatgi et al. (1958) reported that scorbutic monkeys excreted significant amounts of NPN in the urine which indicates a decrease in utilization of nitrogen or increase in catabolism of protein or both.

Amino acid and protein levels in certain tissues have been reported to be used as indicators in judging the involvement of ascorbic acid in protein metabolism. Christensen and Lynch (1948) reported that scorbutic guinea pigs exhibited a reduction in free glycine and glutamine contents of muscle cells and of glutamic acid in liver cells which could explain the restricted ability of scorbutic animals to synthesise proteins.

Liver protein has been reported to be diminished in scorbutic guinea pigs and monkeys (Fukuda & Sibatani, 1953; Rohatgi *et al.*, 1958). Rohatgi *et al.* (1958) explained this reduction on the basis that ascorbic acid is involved in utilization of circulating amino acids by the liver.

3. Tyrosine and tryptophan metabolism

A requirement for ascorbic acid to maintain normal tyrosine oxidation is considered to be the best example of the involvement of this vitamin in a specific biochemical reaction. Sealock and Silberstein (1940) first indicated the involvement of ascorbic acid in protein metabolism when they reported that scorbutic guinea pigs receiving large amounts of dietary tyrosine excreted increased amounts of 4-hydroxyphenyl pyruvate and homogentisate and that these excretions ceased after administration of ascorbic acid. Sealock and Goodland (1951) reported that this role of ascorbic acid is a result of its action as a specific coenzyme for the liver enzyme p-hydroxyphenyl pyruvate oxidase, the enzyme responsible for catalyzing oxidation of p-hydroxyphenyl pyruvate to homogentisate. This role of ascorbic acid in tyrosine metabolism is illustrated in Fig. 7 .

The role of ascorbic acid in tyrosine metabolism has been further elucidated by Yanaka and Okumura (1982) who reported that addition of 50g tyrosine per kg body weight to chicks (chickens able to synthesise ascorbic acid, see Table 2) resulted in decreased body weight and protein efficiency ratio and that addition of supplemental L-ascorbic acid to their diets improved these parameters.

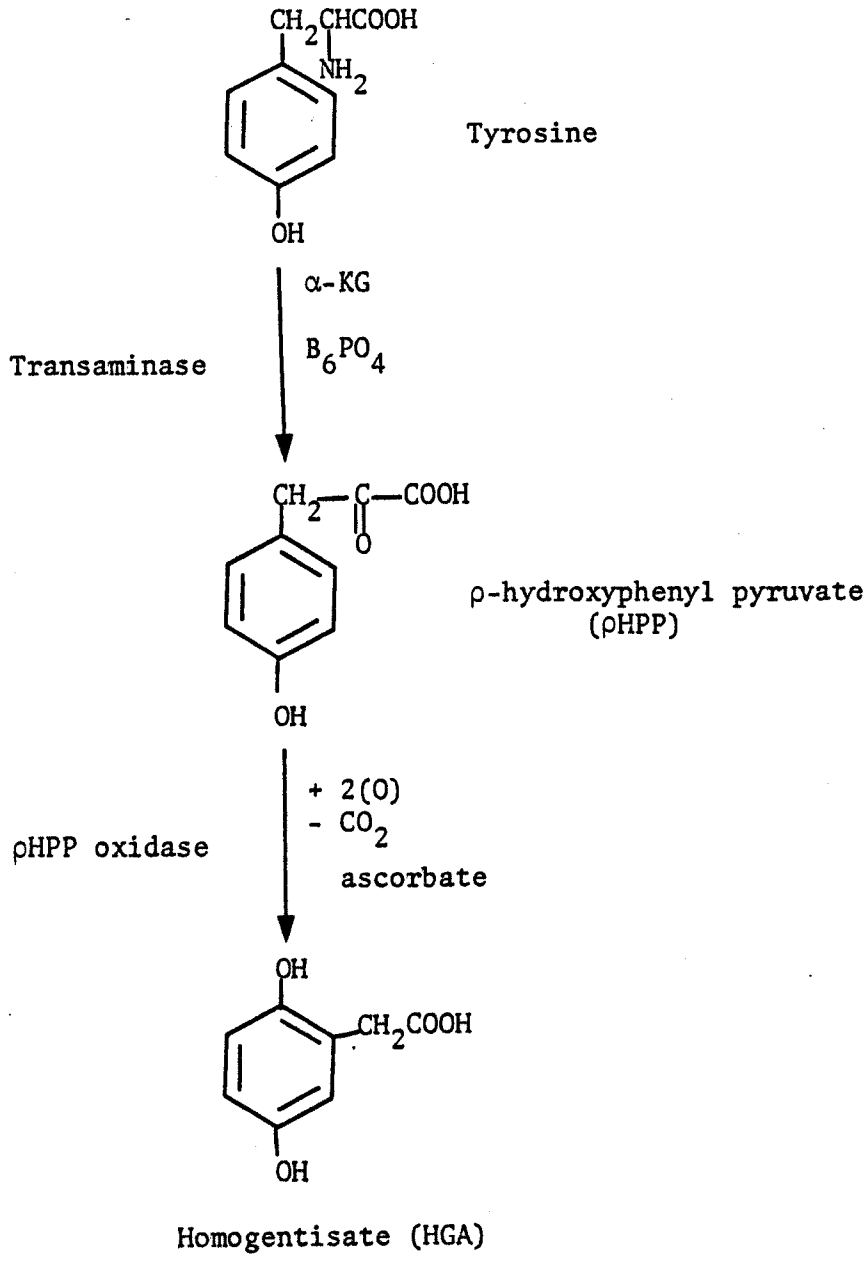


FIGURE 7. Role of ascorbic acid in tyrosine metabolism (after La Du & Zannoni, 1961).
B_6PO_4 : Pyridoxal phosphate, KG : α-ketoglutrate

The role of ascorbic acid in tryptophan metabolism was investigated by Cooper (1961) who showed that hydroxylation of tryptophan to 5-hydroxytryptophan required ascorbic acid, Cu^{++}, and the enzyme tryptophan-5-hydroxylase and this reaction is illustrated in Fig. 8.

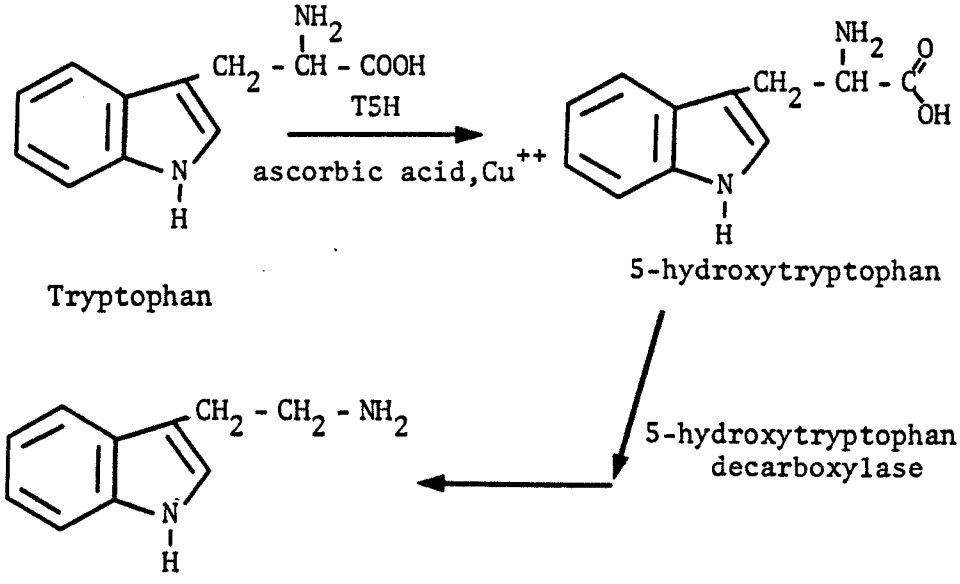


FIGURE 8. Role of ascorbic acid in the oxidation of tryptophan to 5-hydroxytryptophan. (T5H: Tryptophan-5-hydroxylase)

4. Plasma protein

Ascorbic acid deficiency results in alteration in plasma protein. Salmon and May (1952) reported that ascorbic acid is an important factor in controlling production of plasma fibrinogen since scorbutic monkeys exhibited elevated levels of plasma protein which were returned to normal by ascorbic acid therapy. Banerjee and Rohatgi (1958) reported that scorbutic guinea pigs showed a decrease in serum albumin:globulin ratio which might be related

to dysfunction of the liver in synthesis of these components. In addition, these authors reported increased non-protein nitrogen in the plasma of scorbutic guinea pigs which was possibly related to increased tissue catabolism of protein or to dysfunction of the kidney.

These changes in the various components of plasma protein in scurvy suggest the interrelationship between ascorbic acid and protein metabolism.

Section 1.2.7.4 Interrelationships of ascorbic acid with other nutrients

A relatively new approach in the nutritional sciences is the study of the interaction between and among nutrients. Ascorbic acid exhibits interaction with other nutrients, particularly with other vitamins and with minerals (Solomons & Veteri, 1982).

1. Interactions of ascorbic acid with other vitamins

a) Effect of vitamin status on biosynthesis of ascorbic acid

A deficiency of certain vitamins such as riboflavin, thiamine, folic acid, and vitamins A, D and E results in a decrease in biosynthesis of ascorbic acid in rat liver as indicated from tissue ascorbate concentrations and urinary ascorbate concentrations (Roy et al., 1946; Williams, 1951; Schwartz & Williams, 1952a, b; Carpenter et al., 1959; Ghosh et al., 1965). This impairment in biosynthesis of the vitamin may be related to disturbance of the microsomal enzymes responsible for synthesising this vitamin.

Hypervitaminosis A and D results in increased biosynthesis of ascorbic acid from D-glucuronolactone in rats (Ghosh et al., 1965).

In common carp (Cyprinus carpio), which can synthesise ascorbic acid (see Table 2), administration of α-tocopherol resulted in increased activity of L-gulono-γ-lactone oxidase in hepatopancreatic tissue in comparison with untreated carp (Ikeda et al., 1966a).

b) Interactions of ascorbic acid and other vitamins

The possible interrelationship between ascorbic acid and vitamin E in biochemical systems has been proposed by Tappel (1968). Both of these vitamins are recognized as antioxidant vitamins (Johnson, 1979) and are mutually sparing. Leung et al. (1981) suggested that vitamins C and E work cooperatively to protect cell membranes from peroxidation where vitamin C serves as a reservoir antioxidant while vitamin E functions as a specific membrane-bound free radical scavenger. Growth trials in guinea pigs have supported these hypotheses. Chen & Barnes (1976) and Hruba et al. (1982) reported that deficiency of vitamin C was ameliorated by administration of vitamin E and vice versa, however best growth was obtained when guinea pigs were fed diets containing optimal levels of both vitamins. Poston and Combs (1979) reported that vitamin C and E promote growth in Atlantic salmon (Salmo salar) fed diets deficient in selenium, Ascorbic acid reduced the incidence of exudative diathesis in chicken (Machlin & Gabriel, 1980) which might be

related to the ability of ascorbic acid to spare some vitamin E in the body or, as suggested by Machlin and Gabriel (1980), that ascorbic acid might be spare selenium.

Hilton et al. (1978a) reported no interrelationship between the metabolism of vitamins C and A since hypervitaminosis A did not affect the occurrence of ascorbic acid deficiency signs in rainbow trout fed diets devoid of ascorbic acid but supplemented with 4000 I.U or 124,000 I.U retinyl palmitate/kg diet. Launer et al. (1978) rather surprisingly reported that channel catfish (Ictalurus punctatus) fed diets containing no vitamin C, containing no vitamin D or containing both vitamins performed equally well in terms of body weight gain, food conversion ratio and survival rate with no incidence of vitamin C or vitamin D deficiency.

An interaction between folic acid (Pteroylglutamic acid) and vitamin C has been shown through biochemical and physiological studies, Nichol and Welch (1950) were the first to indicate that ascorbic acid is an important factor in the conversion of folic acid to folinic acid and May et al. (1950) reported that ascorbic acid was able to cure the megaloblastic anaemia which resulted from folic acid deficiency.

2. Interrelationships of ascorbic acid with minerals

Ascorbic acid represents a vitamin that undergoes several interactions with minerals. Ascorbic acid affects the uptake of dietary minerals due to its effect on gut pH; reducing action; its effects

on intraluminal solubility; the formation of intraluminal complexes and its effects on transumucosal transport (Solomons & Veteri, 1982).

Interrelationships between ascorbic acid and minerals have been investigated both from nutritional-physiological view or by studying the ability of ascorbic acid to affect elimination of toxic minerals including heavy metals.

a) Interrelationship between ascorbic acid and iron

The metabolism of ascorbic acid and iron is interrelated. Bothwell et al. (1964) reported that in the Bantu people of South Africa there was a relationship between plasma iron and tissue ascorbate level. If tissue ascorbate was depleted there was a severe decrease in plasma iron which indicates that the mechanism of iron release from endothelial and other stores of iron is impaired. Banerjee and Chakrabarty (1965) found similar results in guinea pigs where those deficient in ascorbic acid exhibited greater deposition of haemosiderin in their tissues than those receiving supplemental ascorbic acid and the soluble fraction of liver iron was higher in the latter group. Smith and Bidlack (1980) stated that ascorbic acid and iron are metabolically interrelated and that a high dietary intake of either nutrient was found to modify tissue ascorbate levels and iron distribution.

After absorption of iron by mucosal cells it combines with a specific protein called 'transferrin' leading to formation of

transferrin-bound iron (Bothwell et al., 1964) which will be stored in iron stores such as bone marrow. The iron in this complex exists in a ferric state and the role of ascorbic acid is to release the iron from this complex and combine it with apoferritin to form ferritin (Mazur et al., 1961). Vitamin C also has the ability to release iron from ferritin to plasma (Mazur et al., 1955; Bothwell et al., 1964).

b) Ascorbic acid and copper

Caution is required when high doses of ascorbic acid are administered since ascorbic acid may alter the metabolism of copper leading to depletion. Milne and Omaye (1979) reported that high doses of ascorbic acid (25mg ascorbic acid/100g body weight/day) decreased plasma and hepatic copper in male guinea pigs when compared to animals given lower levels of ascorbic acid (0.5mg/100g body weight/day). Evans et al. (1970) reported that the direct effect of ascorbic acid on hepatic copper is not understood but they suggested that the effect of ascorbic acid in reducing copper level may be due to alteration of the copper-binding site on metallothionein affecting the absorption of copper from the intestinal tract and decreasing storage of copper in the liver.

Reports of the interaction of ascorbic acid and copper are contradictory. Hunt et al. (1970a) reported that the condition of severely copper deficient animals is worsened when their diets are supplemented with ascorbic acid. Rabbits fed a diet deficient in copper and supplemented with ascorbic acid showed

signs of copper deficiency such as reduced growth, achromotrichia and alopecia, anaemia and gross alterations in the bones of the forelimbs. These signs were developed more rapidly in animals fed the ascorbic acid supplemented diet than in those deficient in copper only. Hunt et al. (1970b) also reported that chicken fed a diet deficient in copper and supplemented with 0.5% ascorbic acid showed reduced body weight gain and increased mortality concomittant with haemorrhage into the thorax or peritoneal cavity. The authors also found that chicken fed a diet supplemented with copper and ascorbic acid exhibited lowered liver copper levels. In contrast to these adverse effects of ascorbic acid on copper, ascorbic exerts beneficial effects by reducing copper toxicity. Gipp et al. (1974) reported that dietary ascorbic acid (0.5%) prevented the adverse effects of inclusion of high levels of copper (250ppm) in diets of guinea pigs by returning the blood parameters to the normal levels through its effect in reducing liver copper levels. Yamamoto et al. (1977c) reported that increased copper levels in the hepatopancreas caused reduced activity of L-gulono-γ-lactone oxidase in the hepatopancreas of common carp exposed to 0.05ppm of copper and supplementation of ascorbic acid (200mg AA/100g diet) resulted in decreased copper content in the gills, kidney, intestine and vertebrae.

c) Interrelationship between ascorbic acid and calcium and phosphorous

The role of ascorbic acid in bone calcification has attracted some research interest. Thornton (1968) reported that mobili-

zation of bone salts is usually regarded as being a parathyroid hormone function and secretion of this hormone occurs when fluid ionic calcium levels are reduced and ascorbic acid has the ability to stimulate parathyroid gland activity indicating its direct role in this respect. The author suggested that increased ascorbic acid in the tissues could cause a calcium chelating effect which results in increased parathyroid gland secretion and reduced calcium in the blood. Ascorbic acid thus influences bone matrix formation and has a role in bone salt mobilization. Ascorbic acid also influences the metabolism of phosphorus as injection of ascorbic acid reduces the relative retention of phosphorous indicating a role in phosphorus deposition and bone formation (Thornton, 1970).

d) Ascorbic acid and selenium

Selenide, the reduced form of selenium, appears to be the most usable form of the mineral (Underwood, 1977), and vitamin E has the ability to increase the proportion of the selenide form in the liver of rats (Diplock et al., 1971). Ascorbic acid has been reported to 'spare' selenium (Machlin & Gabriel, 1980) and it is possible that it may behave like vitamin E in increasing the selenide level. Poston and Combs (1979) fed Atlantic salmon (Salmo salar) diets containing 0, 0.05, 0.1 or 0.15mg of selenium per kg of diet with supplements of either 80 IU/kg of vitamin E, 1000 mg/kg of vitamin C or both. Salmon exhibited faster growth when fed diets containing 0.15mg selenium and 1000mg ascorbic acid/kg diet compared to those on other dietary regimes.

e) Ameliorative effects of ascorbic acid on heavy metal toxicity

The following examples demonstrate some roles of ascorbic acid in prevention of the toxic effects of heavy metals:

(i) Lead

Holmes et al. (1939) reported that administration of 100mg ascorbic acid daily to humans exposed to factory lead hazards was a successful therapy in prevention of the toxic effects of lead. These authors suggested that the protective effect of the vitamin was due to its formation of a poorly ionic salt (lead ascorbate) thus preventing lead from interfering with body functions. Pal et al. (1975) also reported that ascorbic acid gave slight protection against lead toxicity and prevented anaemia in rats. In contrast to these studies, Hodson et al. (1980) reported that ascorbic acid failed to prevent signs of lead toxicity in rainbow trout.

(ii) Cadmium

Cadmium is considered to be the most important heavy metal contaminant of the marine environment (Ketchum et al., 1975) although its effects on teleosts remain unclear (Thomas et al., 1982).

Ascorbic acid can ameliorate the toxic effects of cadmium in mammals and birds. Chatterjee et al. (1973) reported that ascorbic acid prevented reduction in growth and anaemia in rats exposed to toxic levels of cadmium. In birds, ascorbic acid eliminates the reduction growth and severe anaemia observed in Japanese quail (Coturnix coturnix japonica) exposed to

cadmium, however, the vitamin did not alter the low level of iron and high level of cadmium in the liver of this species (Fox & Fry, 1970). In fish, Thomas et al. (1982) reported that cadmium toxicity in mullet (Mugil cephalus) resulted in marked fluctuations of the ascorbic acid content of the gills, brain, liver and kidney. The authors suggested that the depletion of ascorbic acid, particularly in the liver, indicated that cadmium may reduce vitamin absorption or that the vitamin had been used in detoxification of cadmium.

(iii) Mercury

In guinea pigs high levels of ascorbic acid resulted in increased tissue deposition of mercury (Blackstone et al., 1974), an indication of the danger of using megadoses of ascorbic acid as therapy during mercury toxicity. Hill (1979) found that ascorbic acid (0,1%) failed to prevent mercury toxicity in chicks.

(iv) Cobalt and vanadium

Hill (1979) reported that ascorbic acid (0.1%) prevented the reduction in growth of chicks receiving 200ppm of cobalt or 25ppm of vanadium.

Section 1.2.7.5 The indirect biological role of ascorbic acid on cyclic nucleotide mediated hormonal activities

Ascorbic acid is required as a co-factor by dopamine-β-hydroxylase in the formation of noradrenaline from dopamine (Levin & Kaufman, 1961) (Fig. 9)

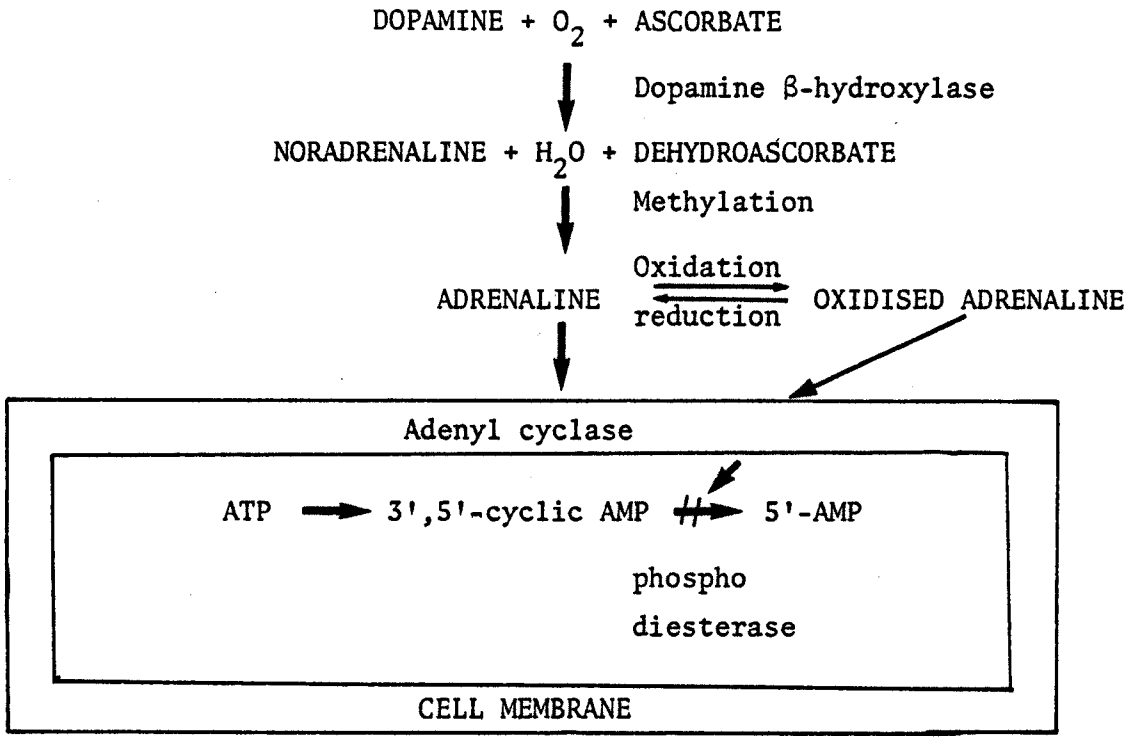


FIGURE 9. Ascorbic acid enhanced formation of cyclic AMP

Adrenaline in turn is formed from noradrenaline by methylation (Fig. 9). Ascorbic acid has also been reported (Park et al., 1956; Krall et al., 1964) as preventing oxidation of adrenalin to adrenochrome (oxidised adrenalin). Adrenalin potentiates adenyl cyclase which in turn activates the formation of c-AMP from ATP (Fig. 9). Ascorbic acid also reduces the breakdown 3,5 cyclic AMP to 5-AMP by inhibiting the enzyme phosphodiesterase (Fig. 9). Therefore, ascorbic acid results in increased tissue levels of c-AMP by stimulating its synthesis and by decreasing its degradation. Ascorbic acid thus has a significant role as c-AMP is involved in many physiological functions in the body (Table 3).

TABLE 3. Hormonal activities in which c-AMP is involved

Hormone	c-AMP involvement in
Adrenalin	Glycolysis; heart contraction; stimulation of enzyme secretions; lipolysis of fat cells; β-insulin secretion; β-melanophore stimulation
ACTH	Phosphorylase activation; protein kinase activation; steroid synthesis; lipolysis of fat cells
LH	Glucose uptake; glycolysis; steroid synthesis
TSH	Thyroid growth and cell division; release of thyroid hormone; phosphorylase activity; glucose uptake
Vasopressin	Transport of H_2O,Na^+ and urea
Oxytocin	Enzyme secretion
Glucagon	Glycolysis; heart contraction; transport of K^+ and urea

Lewin (1975)

Section 1.2.7.6 L-ascorbic acid and immune response

The immune system is one component of the protective system of all vertebrates which enables individuals to survive and maintain homeostais in an environment which is inately hostile (Ellis, 1978).

The immune response is believed to be affected by ascorbic acid status (Shilotri, 1977; Leibovitz & Siegel, 1978). Channel catfish (Ictalurus punctatus) fed diets deficient in ascorbic acid were more susceptible to bacterial infection than those fed diets supplemented with ascorbic acid

(Lovell, 1973; Lovell & Lim, 1978). Durve and Lovell (1982) demonstrated that high levels of dietary ascorbic acid, five times the required level for normal growth, increased resistance of channel catfish to infection by Edwardsiella tarda (the bacterium causing emphysematous putrefactive disease). These results encouraged Li and Lovell (1985) to use megadoses of ascorbic acid (3000mg/kg diet) in diets for channel catfish and to investigate their effects on resistance to E. ictaluri (the bacterium causing eneteric septicemia in catfish). These authors found that ascorbic acid deficiency resulted in reduced resistance of channel catfish to E. ictaluri infection whereas megadoses increased resistance.

The action of ascorbic acid in the immune response is postulated to be as follows:

1. Effect of ascorbic acid on macrophages

   Macrophages are large phagocytic cells found in the connective tissues and are considered to be important constituents of the effector arm of immunological functions. Ganguly et al. (1976) reported that in scorbutic guinea pigs, migration of macrophages was decreased concomittant with a reduction in size and yield of macrophage cells in the peritoneal exudate.

2. Ascorbic acid and leucocytes

   A role of ascorbic acid in the immune response is evident from the rapid consumption of ascorbic acid by leucocytes during infection (Wilson, 1974; Lewin, 1975). High leucocyte ascorbate concentrations have been related to the involvement of ascorbic acid in

antibody and lysozyme production (Lewin, 1975). Phagocytic and microbicidal activity of leucocytes are among the major defence mechanisms against infection (Shilotri & Bhat, 1977). The phagocytosis index (the number of bacteria engulfed by the peripheral phagocytes) was reduced in scorbutic channel catfish (Li & Lovell, 1985). A daily intake of 2g of ascorbic acid for 15 days improved the microbicidal activity of leucocytes in humans (Shilotri & Bhat, 1977). Fraser et al. (1980) reported that pharmacological doses of ascorbic acid stimulate lymphocytes (responsible for immune response) and that the antibody titre of guinea pigs was increased when diets were supplemented with ascorbic acid.

Ascorbic acid may affect the immune response of leucocytes on a biochemical basis. Leibovitz and Siegel (1978) reported that ascorbic acid enhanced neutrophil functions such as chemotaxis (directional migration of cells in response to chemical substances) and hexose monophosphate shunt activity. Ascorbic acid functions in preserving cell integrity by inactivating free radicals and oxidants produced during phagocytosis (Nutrition Reviews, 1978b). In addition, Shilotri (1977) reported that the leucocytes of scorbutic guinea pigs showed significantly lowered stimulation of enzyme NADPH-oxidase activity which resulted in impairment in bacteriocidal activity.

3. Serum complement activity (CH_{50})

Serum complement activity can be defined as one unit of CH_{50} representing hemolysis of 50% of sensitized sheep erythrocytes.

Scorbutic channel catfish exhibited a lowered CH_{50} in comparison to those fed diets supplemented with ascorbic acid (Li & Lovell, 1985).

Section 1.2.7.7 Role of ascorbic acid in cancer

Ascorbic acid (vitamin C) seems to have value in all kinds of cancer therapy although some researchers dispute this. Pauling (1979) reported in a review a role for vitamin C in cancer therapy in that megadoses (10-20g/day) of vitamin C were able to cure a reticulum cell sarcoma in humans. Basu (1979) reported in a study of the effect of vitamin C on cell lines from an epidermoid carcinoma of the mouth of a 54-year old caucasian, that addition of 600µg ml^{-1} or more of the vitamin to these cells resulted in an increased ratio of dead:live cells and decreased rates of DNA synthesis and this author concluded that megadoses of ascorbic acid may have anti-tumour activity.

The possible action of ascorbic acid in prevention and cure of cancer may be explained as follows:

1. Szent-Györgyi (1968) suggested that cell division may be controlled by two antagonistic substances, an inhibitor (retine) and a promotor (promine). He suggests that the inhibitor may be an electron acceptor containing a glyoxal group with the enzyme system glyoxalase acting as a promotor. On the basis of this Edger (1969, 1970) reported that ascorbic acid (L-ascorbic acid dehydroascorbic acid) may be involved in modulation of mitotic activity as dehydroascorbic acid is an electron acceptor structurally similar to glyoxal and high concentrations of dehydroascorbic acid prepare quiescent cells

for division by releasing and activating lysomal hydrolytic enzymes while inhibiting mitosis. This action is a result of maintenance of the essential -SH - in an oxidized form -S-S- and its rapid reaction with glutathione (GSH) which reduces dehydroascorbic acid to ascorbic acid. Barron (1951) suggested that GSH is a promotor (promine) of cell division. Edger (1970) also suggested that dehydroascorbic acid in high concentrations is able to promote catabolism of certain tissues. It is, therefore, possible that megadoses of ascorbic acid may have a role in prevention of cancer by increasing the concentration of dehydroascorbic acid which acts on cancer cells and destroys them due to its ability to stimulate and release the lysomal hydrolytic enzymes, reducing the glutathione level required for mitotic division of cancer cells.

2. Cameron and Pauling (1973) suggested that ascorbic acid is a good inhibitor of the enzyme hyaluronidase, which liquefies and breaks down tissues. This enzyme is required for cells to grow and proliferate as tissues release this enzyme which permits cells to divide, proliferate and migrate and that these processes cease when hyaluronidase is inhibited. Ascorbic acid has the potential to slow down or stop release of this enzyme, thus inhibiting cancer.

3. Stone (1976) states that administration of ascorbic acid might provide an effective means of permanently suppressing neoplastic cellular proliferation and invasion.

4. Basu (1979) suggests that the adverse action of ascorbic acid on cancer tumours may be related to increased production of c-AMP which participates in arresting tumour growth.

5. Basu (1979) also explains the action of ascorbic acid in preventing cancer on the basis of bio-availability of certain amino acids such as cysteine and certain minerals such as zinc. Ascorbic acid in orthomolecular doses utilizes these amino acids and minerals and therefore reduces the availability of lysine, cysteine and zinc which are required for tumour growth.

From the foregoing it can be concluded that ascorbic acid may be required to prevent abnormal cell and growth such as tumours and in megadoses the vitamin might have therapeutic value.

Section 1,2,8 The Experimental Methodology Employed in Ascorbic Acid Studies

The experimental methodology employed in estimation of ascorbic acid (vitamin C) can be broadly divided into two categories, namely biological methods and chemical methods.

1. Biological methods

   Biological methods for estimation of ascorbic acid are specific for antiscorbutic activity (Olliver, 1967) and rely on summation of chemical entities that possess only vitamin activity (Sauberlich et al., 1982). Such methods can be used in comparative studies to establish the biological specificity of individual products (Sauberlich et al., 1982).

Sherman et al. (1922) were the first to use this methodology which was applied to guinea pig feeding trials with diets containing graded levels of the vitamin. The degree of protection against scurvy was evaluated by looking for clinical signs of scurvy (autopsy examination) and measuring survival rate. The amount of test material sufficient to prevent scurvy was taken to be one Sharman unit which is equivalent to 0.5-0.6mg ascorbic acid. Subsequently one International Unit (IU) or one USP.XIV unit of vitamin was stated to be the antiscorbutic activity of 0.05mg of ascorbig acid, the approximate amount in 1ml of lemon juice, so that 1g of ascorbic acid is equivalent to 20,000 IU.

Various biological methods have been introduced by different workers. Harris and Olliver (1942) calculated the biological potency of unknown substances from a comparison of the resultant dose response (body weight gain) curves of guinea pigs. In 1942 Gould and Shwachman observed that serum alkaline phosphatase activity was reduced during scurvy and these authors (Gould & Shwachman, 1943) suggested that alkaline phosphatase activity be used as an indicator of ascorbic acid status.

Biological methods are subject to various disadvantages (Olliver, 1967; Sauberlich et al., 1982) such as:

(i) Special care should be taken in estimation of the ascorbic acid content of fresh fruits and vegetables to ensure uniformity of the sample administered.

(ii) They are time consuming, expensive and lack precision.

2. Chemical methods

These can be subdivided into qualitative and quantitative methods.

(i) Qualitative methods

Qualitative methods are generally used to determine the vitamin qualitatively by estimation of its distribution in tissues. Chiony (1969) reported that alcoholic acidic silver nitrate, which contains fixatives like acetic acid and alcohol, penetrates into tissues rapidly when the silver nitrate fixes ascorbic acid thus localizing the distribution of the vitamin in the tissue under investigation.

(ii) Quantitative methods

A. Colorimetric methods

2,6-dichlorophenolindophenol (DCPIP) method

In this method oxidized dye is titrated into a definite volume of strongly acidic solution containing an unknown amount of ascorbic acid and the persistence of the faintest pink colour for 30 seconds is the endpoint (AOAC, 1980). Although this method is satisfactory for estimation of the reduced form of the vitamin (L-ascorbic acid) in fruits and vegetables (Harris & Olliver, 1942), the method suffers from many disadvantages (Bessey, 1938; Mindlin & Butler, 1939).

1. The endpoint is not precise for the determination of small amounts of ascorbic acid.
2. It is difficult to determine vitamin concentration in the highly pigmented samples.

3. Turbid solutions and samples containing high levels of impurity lead to high and indistinct endpoints.

4. The method estimates only the reduced form of the vitamin.

Microbiological Assay

To overcome the inability of the previous method to estimate total ascorbate (reduced and oxidized forms) Gunsalus and Hand (1941) reported that certain bacteria such as Bacterium coli are able to reduce the oxidized form (dehydroascorbic acid) to the reduced form (L-ascorbic acid) such that subsequent use of DCPIP will estimate total ascorbate.

2,4-dinitrophenylhydrazine (DNPH) method

This method is widely used for determination of total ascorbic acid in biological substances and it is known as Roe's Method (Roe & Kuether, 1949). The procedure is described in section 2.4.4.1 of the Materials and Methods Chapter of this thesis. Inspite of the fact that the method is generally preferred (Inagaki et al., 1959) some workers (Schaffert & Kingsley, 1955; Kedo et al., 1960) have stated that the DNPH method is not convenient in practice due to the long times of reaction, particularly incubation time. The method has thus been modified by increasing the temperature. Schaffert and Kingsley (1955) increased the temperature of incubation to 100°C for 10 minutes instead of 37°C for 3hr and Kedo et al. (1960) increased the temperature to 50°C for 1hr. However, Roe (1961) has defended his method and proved its sensitivity and specificity by some excellent and well designed experiments in

which he demonstrated that the amount of interference from non-ascorbic acid substances such as glucose, fructose and glucuronic acid was increased significantly at 100°C as compared to 37°C.

Automated methods

Aeschbacher and Brown (1972) introduced an automated method for determination of total ascorbic acid. In this method ascorbic acid is oxidized to dehydroascorbic acid with chloramine-T and determined by reaction with 2,4-dinitrophenylhydrazine using an autoanalyzer.

Micro-methods

A micro-method (1µg of ascorbic acid) for determination of ascorbic acid in plasma and other biological tissues has been reported by Zannoni et al. (1974). Ferric ion (as ferric chloride) is reduced by ascorbic acid to ferrous ion which is coupled to α-ᾱ-dipyridyl forming chromogen which can be determined colorimetrically at 525nm.

B. Fluorometric methods

A fluorometric method (AOAC, 1980) has been proposed as follows:

Ascorbic acid is oxidized by norit to dehydroascorbic acid which reacts with O-phenylenediamine (OPDA) producing a fluorescent compound and the intensity of the fluorescence indicates the concentration of ascorbic acid.

C. Chromatographic methods

Chromatographic methods such as paper chromatography; column chromatography; thin-layer chromatography (TLC); anion-exchange liquid chromatography (LC); high performance liquid chromatography (HPLC) are valuable and useful tools in determination of ascorbic acid and its metabolites where:

1. The biological samples contain high amounts of interfering substances. Carr and Neff (1980) reported that the values of ascorbic acid obtained by the LC method were lower than those obtained by α-ᾱ-dipyridyl method of Zannoni et al. (1974) indicating interference from nonascorbic acid substances.

2. Separation of different forms of ascorbic acid is required. Baker et al. (1973) reported that ascorbic acid 2-sulphate interferes with ascorbic acid determination. Bigler and Kelly (1975) reported that the LC method was able to separate ascorbic acid from ascorbic acid 2-sulphate and determined each form independently.

3. Colorimetric methods (e.g. DCPIP) fail to differentiate between D-araboascorbic and ascorbic acids due to the close similarity in their structure. Miki et al. (1962) reported that paper chromatography succeeded in differentiating between these two forms and determined each form separately.

4. Investigation of the metabolites of ascorbic acid. Levandoski et al. (1964) detected the degradation products of ascorbic acid, such as diketogulonic acid and oxalate, by paper chromatography and Saari et al. (1967) detected, using TLC, oxalate

and dehydroascorbic acid as the oxidative products of ascorbic acid.

5. Experimental procedures may necessitate inclusion of certain interfering substances such as sucrose in the identification of the enzyme L-gulonolactone oxidase activity (Chatterjee, 1960). Ikeda et al., (1963b) therefore used column chromatography to separate ascorbic acid from interference substances.

Section 1.2.9 Aim of Research

The research programme detailed here was conducted to investigate the ascorbic acid (vitamin C) nutrition of Oreochromis niloticus and O. mossambicus, the principal cultured tilapias.

Various aspects of ascorbic acid nutrition have been already reviewed (Chapter 1). An important aspect of evaluation of dietary ascorbic acid requirements in animals is demonstration of the presence or absence of the enzyme L-gulono-γ-lactone oxidase which has a key role in synthesis of ascorbic acid. The activity of this enzyme was investigated in 14 species of fish of three genera (Chapter 3). In the absence of this enzyme, O. niloticus and O. mossambicus will be dependent on exogenous dietary sources of ascorbic acid and thus their quantitative requirements for this vitamin were determined (Chapter 4) as were the effects of long-term deprivation (Chapter 5).

It is well known that tissue ascorbate concentrations will be affected by dietary ascorbic acid levels and by the duration of exposure. In addition, tissue ascorbic acid levels under varying dietary regimes may indicate the physiological involvement of this vitamin in various body

functions and this aspect of ascorbic acid nutrition of tilapias was studied (Chapter 6).

Dietary ascorbic acid is subject of significant losses during processing and storage as well as during immersion of fish feeds in water prior to ingestion. Forms of the vitamin, other than the free acid, exhibiting improved stability were thus studied in tilapia feeds (Chapter 7). The stability of these forms during and after processing was evaluated as well as during storage under different conditions for various periods (Chapter 8). In addition, leaching of dietary ascorbic acid was evaluated at two different water temperatures for varying periods of immersion (Chapter 8).

Contradictory results concerning the antioxidant effect of ascorbic acid have been reported by different workers and for this reason the antioxidant effects of ascorbic acid were evaluated for tilapias (Chapter 9).

In contrast to the volume of literature concerning the nutritional requirements of salmonids, relatively little is known of those of tilapias. Because of this the practice of using the requirements of salmonids to formulate diets for other species of fish (e.g. tilapias) has evolved and therefore studies were conducted (Chapter 10) to evaluate the differences between such species in relation to dietary ascorbic acid nutrition.

Sex differences with respect to tissue ascorbic acid concentrations have been investigated in guinea pigs, rats and humans and there appears to be

no such information for fish, therefore such sex differences were investigated (Chapter 11).

The role of ascorbic acid in wound healing has been extensively reported in guinea pigs with much less information for fish so that the role of ascorbic acid in wound healing in tilapias was investigated (Chapter 12).

The existence of high levels of ascorbic acid in gonadal tissues and its role in steroidogenesis suggests a role for ascorbic acid in reproduction. The effect of dietary ascorbic acid on hatchability, fry condition, survival rate and growth of fry of tilapias was therefore investigated (Chapter 13).

Of the water-soluble vitamins ascorbic acid in those species unable to synthesise it de novo, is possibly the most important particularly in fish feeds, due to its lability. Successful intensification of tilapia culture depends upon supply of nutritionally complete artificial feeds and it is in this context that a detailed knowledge of their requirements for ascorbic acid is essential.

CHAPTER 2

GENERAL MATERIALS AND METHODS

SECTION 2.1 : THE EXPERIMENTAL SYSTEMS

Due to variations in length and aim of the experiments, several different experimental systems were used:

Section 2.1.1 Recirculation System 1

This system was established for short growth trials, wound healing and fry experiments (Chapters 4, 6, 7, 8, 12 and 13 respectively). It consisted of 32 x 10 l circular plastic tanks (each containing 9 l water and an air stone) fed from a 100 l header tank (containing four Interpet 200 watt combined heater/thermostats). The experimental tanks drained into the first of three 100 l filtration/sedimentation tanks (containing plastic rings) in series. The third of these drained, by gravity, into a 100 l sump tank from which water was pumped to the header tank. An overflow from the header tank drained through a 100 l filter tank (containing plastic rings, broken shells (for buffering) and filter wool) into the sump tank (Fig. 10 and Plate 1). The water was pumped from the sump tank to the header tank from where it was distributed by gravity to the circular tanks at a rate 740 cm^3/min/tank for fingerlings, reduced to 400 cm^3/min/tank for fry. The biological filter tanks were cleaned every two weeks to reduce the levels of nitrite and ammonia in the system. After each weekly weighing of fish the circular plastic tanks and stand pipes were cleaned to prevent algae growth which may have acted as an uncontrolled source of dietary nutrients. Water quality criteria are presented in Table 4.

Section 2.1.2 Recirculation System 2

This system was used for long-term experiments (Chapters 5, 10 and 13) and consisted of 12 x 150 l circular tanks, a 220 l header tank (which contained a 3 kilowatt heater with automatic temperature control which maintained the temperature 28°C), 2 x 200 l filter

B.F.S.T. - Biological filtration/sedimentation tank
C.P.T. - Circular plastic tanks
F.T. - Filter tank
H.T. - Header tank
S.T. - Sump tank
T. - Tap

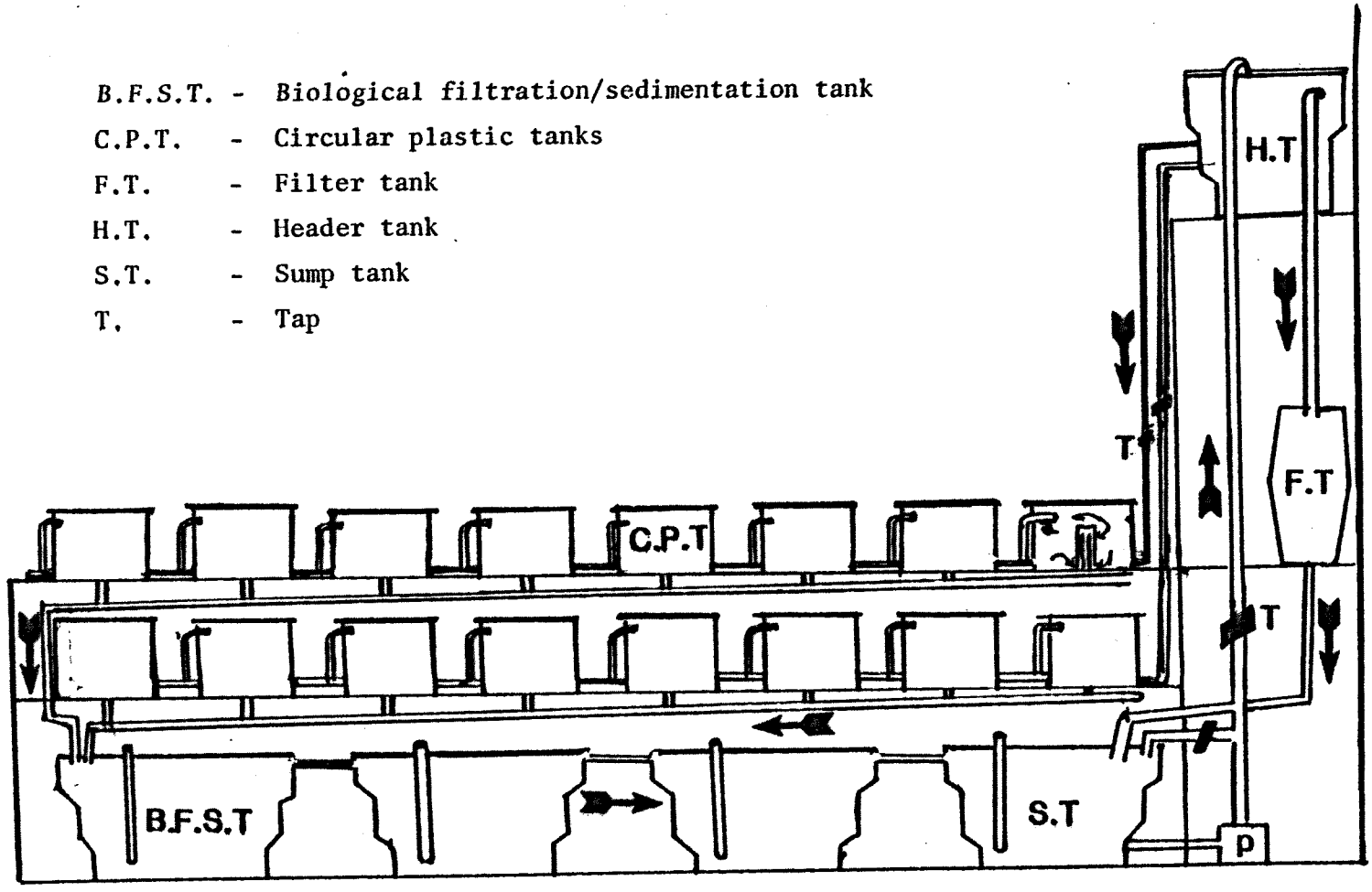


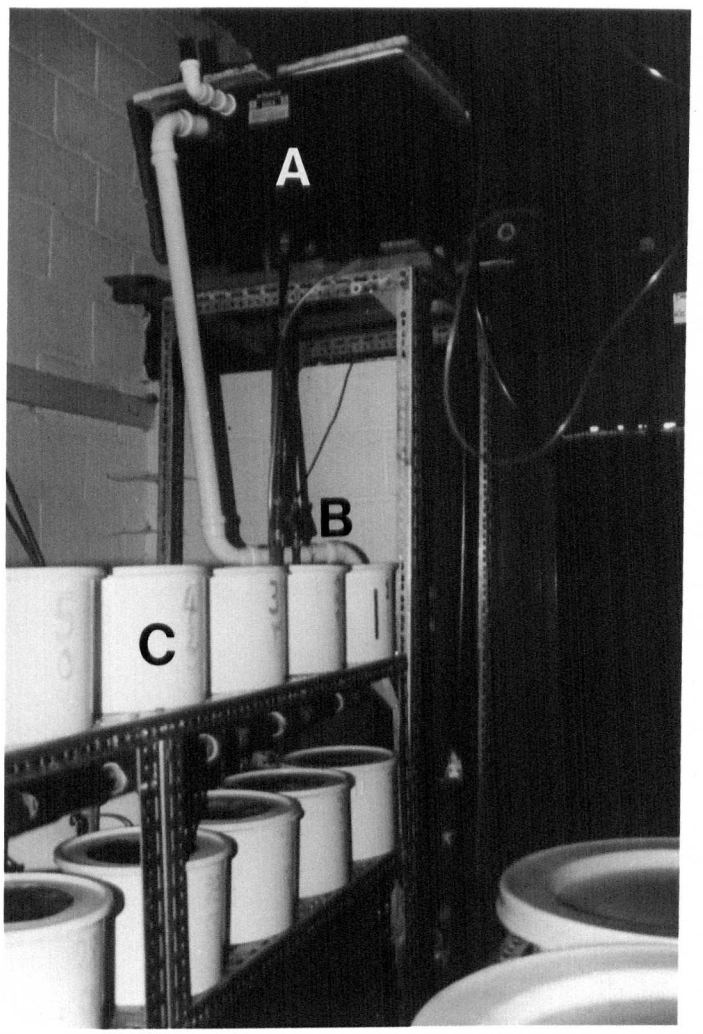
FIGURE 10. Layout of recirculation experimental system 1

PLATE 1 A photograph of recirculation experimental system 1 employed for growth and fry experiments

A - Header tank

B - Tap

C - Circular plastic tank


A
B
C

tanks which contained gravel and 2 x 100 l sump tanks (Plate 2). The mechanism of the system is similar to system 1 with a flow rate 6.4 l/minute per tank. The circular tanks were cleaned weekly as above. Water quality criteria are presented in Table 5.

Section 2.1.3 Recirculation System 3

This system was used for experiments with large tilapias and consisted of 4 x 220 l rectangular tanks, 2 x 200 l header tanks (one containing a 3 kilowatt heater with automatic temperature control and the other containing gravel), 4 x 200 l biological filter/sedimentation tanks and a 100 l sump tank (Plate 3). The mechanism is similar to that of the previous systems with a flow rate 9.6 l/minute/tank. Water quality criteria are presented in Table 6.

Section 2.1.4 Hatchery System

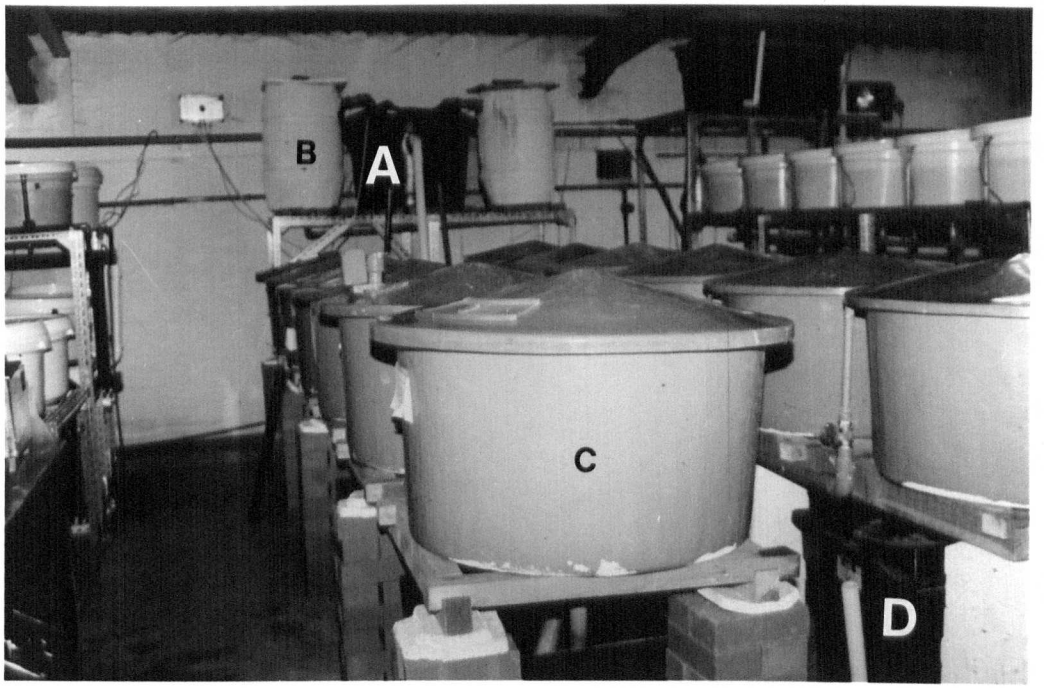
This system consisted of 18 incubation units (10 x 15 x 18 cm), a 100 l header tank, a 100 l biological filter/sedimentation tank containing filter trays (containing gravel and filter wool) and a 100 l sump tank containing an Otter submersible pump (Plate 4). The water was pumped from the sump tank to the header tank from where it passed through an ultra violet light (30 watts) sterilizer to produce pathogen-free water. By gravity the water was distributed to the incubation units at a rate 250 cm^3/minute/unit. Eggs collected from females were loaded into the plastic bottles (Plate 5), each bottle was placed in an incubation unit and supplied with water through two pipes, one of which was connected to a submerged tube (18 cm length, 0.4 cm diameter) which caused gentle stirring of the eggs during the incubation period. The second inlet pipe was only used when large numbers of eggs needed to be stirred. Water quality criteria are presented in Table 7.

PLATE 2. A photograph of recirculation experimental system 2 employed for long-term experiments.

A - Header tank B - Filter tank
C - 150 litre circular fibre glass tank
D - Biological filtration/sedimentation tank

PLATE 3. A photograph of recirculation experimental system 3 employed for experiments with large tilapias.

A - 220 litre rectangular fibre glass tank
B - Biological filtration/sedimentation tank


B
A
C
D

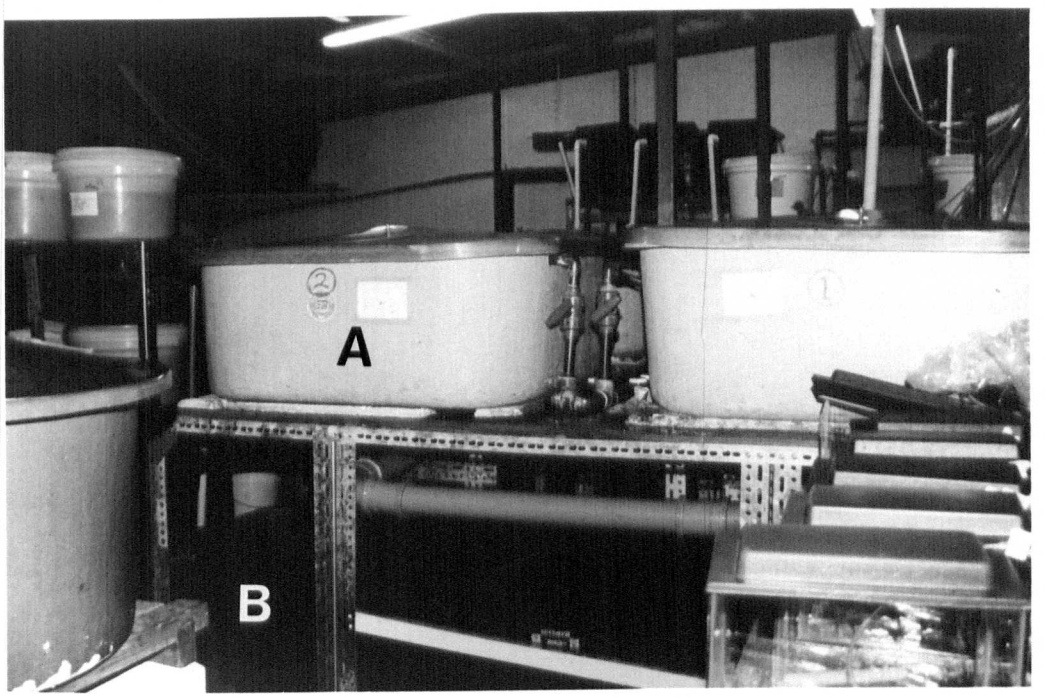

A
B

TABLE 4. Water Quality Criteria in the System 1

Dissolved oxygen	7-8 mg/l
pH	6.6-6.9
Temperature	27-28°C

TABLE 5. Water Quality Criteria in the System 2

Dissolved oxygen	7.5 mg/l
pH	6.4-6.7
Temperature	28°C ± 1

PLATE 4. A photograph of hatchery system employed for hatchability studies.

A - Incubation unit B - Filter tray
C - Biological filtration/sedimentation tank
D - Sump tank

PLATE 5. A photograph of a plastic bottle used for loading eggs from female tilapias in the incubation unit and showing newly hatched fry

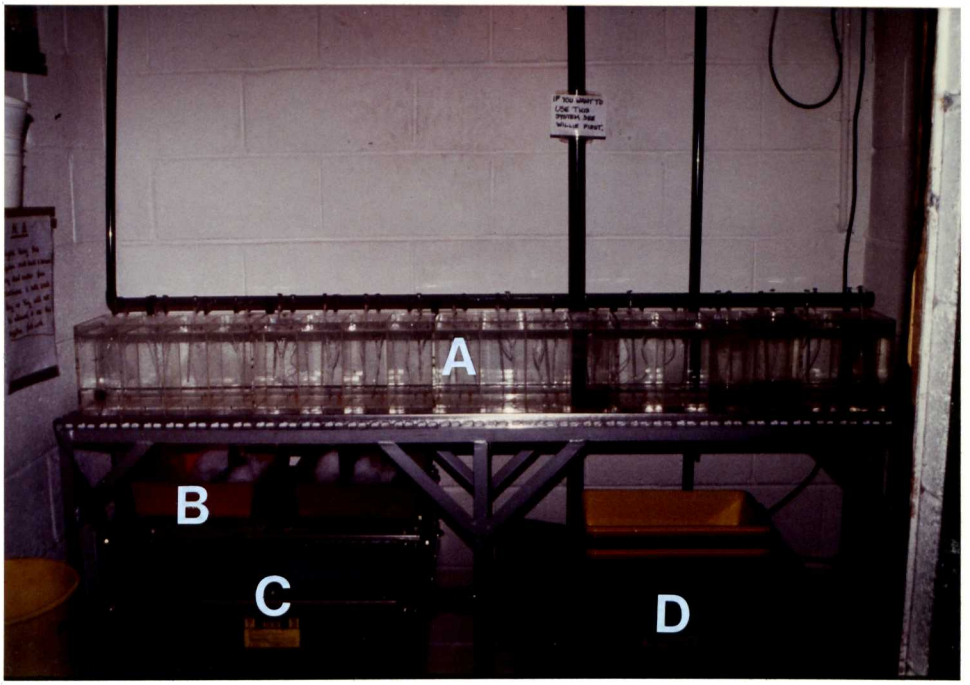

A
B
C
D

TABLE 6. Water Quality Criteria in the System 3

Dissolved oxygen	8 mg/l
pH	6.4-6.5
Temperature	28°C ± 1

TABLE 7. Water Quality Criteria in the Hatchery System

Dissolved oxygen	6.5 mg/l
pH	6.9
Temperature	27.5-28°C

SECTION 2.2 : FISHES

All species of tilapia used in the present studies were obtained from the Institute of Aquaculture, Stirling University, from genetically homogenous stocks (McAndrew & Majumdar, 1983; 1984). Since *Oreochromis niloticus* and *O. mossambicus* were used extensively in the present studies, it is necessary to consider the characteristics and environmental requirements of these, the most often cultured of tilapias (Table 8).

SECTION 2.3 : DIETS

Section 2.3.1 Diet Formulation

A basal diet, ascorbic acid free, was formulated to contain 40% crude protein (Chapters 4, 5, 6, 7, 8, 11, 12 and 13) and 30% crude protein (Chapter 11) using defatted brown fish meal as the sole dietary protein source and 10% crude lipid supplied by cod liver oil (4%) and corn oil (6%) as the sole dietary lipid sources. To all diets were added, 2% mineral mix. (Table 9), 2% vitamin mix. (Table10), 2% binder (Carboxymethylcellulose, sodium salt, high viscosity) and 0.5% chromic oxide (as an inert indicator for digestibility studies). The remainder of the diet was filled with a mixture of alpha-cellulose, dextrin and starch. The amount of ascorbic acid, or its forms, added to the experimental diets was calculated and substituted for alpha-cellulose in the basal diet.

Section 2.3.2 Diet Preparation

The amount of the diet required for each experiment was calculated according to the number of the fish used, growth expected, dietary

TABLE 8. Summary of the characteristics and environmental requirements of O. niloticus and O. mossambicus

Character	O. niloticus	O. mossambicus
Family	Cichlidae	Cichlidae
Common name	Nile tilapia	Java tilapia
Distribution	Freshwater fish, from Syria through Egypt, all East Africa to Zaire, brackish water also because commonly cultured in Israel and Thailand	Fresh and brackish water, East and Southern Africa, cultured widely in South East Asia, Near East Southern Africa and cultured experimentally in Japan, Latin America, USSR and USA
Food habits	Omnivorous, feeds on phytoplankton blue green algae, benthic fauna and pelleted feeds	Omnivorous, feeds on phytoplankton vegetation and benthic algae, zooplankton, small crustaceans and artificial feed
Temperature	Lower lethal below 12°C, upper lethal 42°C and opt. 24°C	Lower lethal 8-10°C, upper lethal 39°C and opt. 24-35°C
Salinity	0-35‰ and opt. 29‰	Lower lethal 0.1‰, upper lethal 69‰ and opt. 15-18‰
pH	5-11	5-11
Dissolved Oxygen	minimum 1.2 mg/l	minimum 0.1 mg/l

TABLE 8 (cont'd)

Character	O. niloticus	O. mossambicus
Growth	Males faster growth than females, maximum size in the wild 2.5 Kg, in cages 120-200 g in 4 months	Males faster growth than females, maximum size in the wild 1.75 Kg and 450 g per year
Reproduction	Mature at 4-5 months (200 gms), female spawns 3 times/year, 1500-2000 eggs at a time. 17,500 eggs per kg body wt.	Mature at 2-3 months (25-50 g), female spawns 6-11 times/year. 17,500 eggs per kg body wt.
Incubation	Mouthbrooder, maternal	Mouthbrooder, maternal
Incubation time	4-5 days	4-5 days

Balarin (1977); Balarin and Hatton (1979); Ben-Tuvia (1960); Bardach et al. (1972); Kirk (1972); Moriarty and Moriarty (1973) and Ross and Beveridge (1984).

TABLE 9. Mineral Supplement Composition/100 gram Mixture

Mineral	Weight in grams
$CaH_4(PO_4)_2\ 2H_2O$	72.78
$MgSO_4.7H_2O$	12.75
NaCl	6.00
KCl	5.00
$FeSO_4.7H_2O$	2.50
$ZnSO_4.7H_2O$	0.50
$MnSO_4.4H_2O$	0.25
$CuSO_4.5H_2O$	0.08
$CoSO_4.7H_2O$	0.05
$CaIO_3.6H_2O$	0.03
$CrCl_3.6H_2O$	0.01

Tacon et al. (1983)

TABLE 10. Vitamin Supplement Composition/100 gram diet

Retinal acetate (Vitamin A)	2000 I.U.
Cholecalciferol (Vitamin D_3)	1000 I.U.
Alpha-tocopherol acetate (Vitamin E)	10 mg*
Menadione (Vitamin K)	4 mg
Thiamine hydrochloride (Vitamin B_1)	5 mg
Riboflavin (Vitamin B_2)	5 mg
Pyridoxine hydrochloride (Vitamin B_6)	4 mg
Cyanocobalamin (Vitamin B_{12})	0.01 mg
Calcium pantothenate	10 mg
Folic acid	1.5 mg
Para aminobenzoic acid	5 mg
Myo-inositol	200 mg
Niacin	20 mg
Biotin	0.6 mg
Choline chloride	400 mg
Ascorbic acid (Vitamin C)	0
Alpha-cellulose as a carrier for the vitamin mixture	

Soliman* (1982), Tacon et al. (1983)

regimen, experimental period and losses due to processing and grinding of the experimental diets. The dry ingredients were weighed out together with the requisite quantity of ascorbic acid, or its forms, using a Mettler AC 100 electronic balance. The dry ingredients were thoroughly mixed for 10 minutes in a Moulinex mixer followed by addition of the corn oil and cod liver oil (in Chapter 9 BHT dissolved in unstabilized body oil was used) and further mixing for 10 minutes. Cold water was then added until a stiff dough resulted, this was passed through a mincer with a 3 mm die and the resulting 'spaghetti-like' strings were dried in a forced convection air-dryer at 35°C. After drying, the diets were broken up into convenient pellet sizes. Samples were taken for analysis and the remainder stored in containers kept in a deep freezer (-20°C) until required.

SECTION 2.4 : CHEMICAL AND BIOCHEMICAL ANALYSES

Section 2.4.1 Oil Extraction and Oil Oxidation Parameters

Section 2.4.1.1 Oil extraction

For obtaining samples of lipid for measurement of the degree of oxidation oil was extracted from the experimental diets,(Chapter 8), after drying and at the end of the experiment by the method of Korn & Macedo (1973).

Section 2.4.1.2 Oil oxidation parameters

Fresh oils and oils extracted from the experimental diets were subjected to the following analyses:

Peroxide value (POV meq/kg)

Peroxide value indicates what proportion of the double bond positions in a sample of lipid have been oxidized to form peroxides. POV was determined as described by AOAC (1980).

Thiobarbituric acid number determination (TBA, mg malonaldehyde/kg)

The 2-thiobarbituric acid (TBA) reactive material in autoxidized fat responsible for the red colour at 535 nm has been found to be malonaldehyde (MA) (Sinnhuber et al., 1958; Schmidt, 1959). The TBA number, or mg of MA per 1000 g of sample, has been proposed as a measurement of the degree of autoxidation of lipid and fat-containing foods. The method of Shibata and Kinumaki (1979) was adopted for the determination of the TBA value.

Anisidine value

The anisidine value (AV) is a measure of the alpha-beta unsaturated aldehydes of fats and oils. This was estimated using the method of Windsor and Barlow (1981).

Section 2.4.2 Methods of Proximate Analysis

Proximate analysis of the defatted fish meal used in the experimental studies, experimental fish and faeces were carried out as described by AOAC (1980) as follows:

Moisture

The moisture content was determined by air drying the samples in an oven at 105^{o}C for 24 hours.

Crude lipid

Crude lipid content was determined by extracting dried samples for 4 hours using a soxhlet apparatus and petroleum ether (40-60°C) and measuring, by weight difference, the amount of ether soluble material extracted. For extraction of oil from fish meal to be used in the experimental diets the extraction was carried out for 6 hours.

Crude protein

Crude protein was determined by Kjeldahl method which measures the total nitrogen content of the sample and converts to crude protein by multiplying the empirical factor 6.25.

Ash

Ash content was determined by heating samples in a muffle furnace overnight at a temperature of 450°C.

Section 2.4.3 Haematocrit and Haemoglobin Determinations

The methods of Baker et al., (1966a), were adopted for determination of haematocrit and haemoglobin.

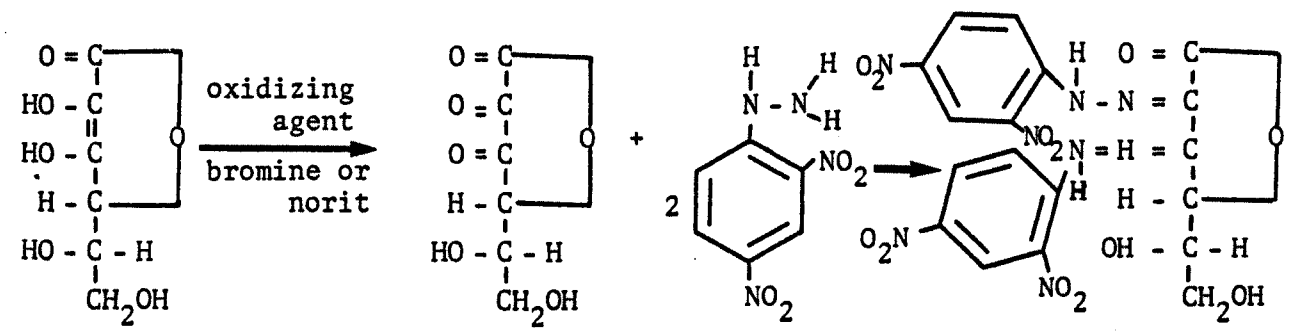
Blood was collected, using heparinized syringes, from the caudal vein. Haematocrit was determined by filling special capillary tubes with blood, heat sealing the end of the tubes and centrifuging, in a microhaematocrit centrifuge, 3000 G for 5 minutes. Finally the tubes were placed into scale which gave the packed cell volume (PCV) as a percentage. Haemoglobin was determined using Drabkins solution which converts haemoglobin, by the action of ferricyanide, to

methaemoglobin and the latter is converted to cyanmethaemoglobin by potassium cyanide. The color formed was read photometrically and the concentration of haemoglobin was calculated against the reading of a standard solution of cyanmethaemoglobin (545 nm).

Section 2.4.4 Total Ascorbic Acid, Dehydro L-Ascorbic Acid and L-Ascorbic Acid Determination

Section 2.4.4.1 Total ascorbate determination

Total L-ascorbic acid determination in both experimental diets and fish tissues was as described by Roe (1967) as follows: The method is based on oxidizing L-ascorbic acid with oxidizing agents such as bromine or norit to dehydroascorbic acid (DHAA). Spontaneously, DHAA changes slowly to diketogulonic acid (DKGA) in mildly acid solution. These two compounds react with 2,4-dinitrophenylhydrazine (DNPH) forming a derivative, a bis-2,4-dinitrophenylhydrazine (DNPH), which is coupled to carbon atoms 2 and 3.



L-ascorbic acid — Dehydroascorbic acid — 2,4-dinitrophenylhydrazine — Bis-2,4-dinitrophenylhydrazine derivative

This derivative yields a highly stable brownish red colour when treated with 85% H_2SO_4 and the colour developed can be photometrically measured at 540 nm. Since it has been suggested that norit has ability to remove

any pigment which may interfere with colour of the reaction above (Lowry et al., 1952) it was decided to use norit, instead of bromine, since the experimental diets contained potentially interfering pigments in the form of chromic oxide and derived from fishmeal.

Section 2.4.4.2 Dehydro L-ascorbic acid determination

Dehydro L-ascorbic acid was determined according to Roe (1957); Räiha (1958) with the following modifications; addition of norit or bromine was omitted and in order to prevent any minute oxidation of ascorbic acid to dehydroascorbic acid and to keep to a minimum spontaneous change to dehydroascorbic acid, anhydrous Stannous chloride was added (Roe et al., 1948).

Section 2.4.4.3 L-ascorbic acid estimation

L-ascorbic acid was estimated from the difference between total ascorbate and dehydroascorbic acid values.

Section 2.4.5 Ascorbic Acid 2-sulphate Determination

Ascorbic acid 2-sulphate was determined by the method of Roe (1967) with the modifications suggested by Baker et al. (1973).

Section 2.4.6 Hepatosomatic Index and Muscle and Liver Glycogen Determination

$$\text{Hepatosomatic index (HSI,\%)} = \frac{\text{Wet liver weight (g)}}{\text{Body weight (g)}} \times 100$$

Muscle and liver glycogen were determined by the method of Van Der Vies (1954) after extraction of glycogen from liver and muscle tissue

with 5% trichloroacetic acid followed by estimation with an iodine reagent.

Section 2.4.7 Collagen, Proline and Hydroxyproline Determination

Section 2.4.7.1 Preparation of vertebrae and tail for collagen extraction

The vertebral column was removed and brushed free of adhering tissue. The centra were separated by dissecting the ribs and spines away from the vertebral column. The vertebral column was defatted with a mixture of chloroform : methanol (2 : 1) by dipping the vertebral column, cut into small portions, in this mixture overnight, filtering then washing with another portion of the same mixture followed by filtering and drying at room temperature. The caudal fin was removed without defatting.

Section 2.4.7.2 Collagen extraction

The vertebral column was ground using pestle and mortar and the tail was cut into pieces using scalpel. The tissue samples were then subjected to collagen extraction (Sato et al., 1978a) by heating tissues in deionized water in sealed tubes at 125^{o}C for 3 hours which converted collagen of the sample to soluble gelatin. The extract was filtered and the filtrate was dialyzed overnight against deionized water. The collagen was then estimated gravimetrically after extraction and drying.

Section 2.4.7.3 Proline and hydroxyproline estimation

The dried gelatin was hydrolyzed in 6 N HCl in a sealed tube at 130°C for 3 hr. Proline and hydroxyproline in the hydrolyzate were determined by the method of Troll and Lindsley (1955) and the method of Woessner (1961) respectively.

Section 2.4.8 Cholesterol Determination

The method used was based on adding a fixed volume of concentrated sulphuric acid, glacial acetic acid and ferric chloride solution to 0.1 ml of plasma (after blood, collected by the method described in Section 2.4.3, had been centrifuged at 3000 G for 5 minutes to allow separation of the plasma) in 3 ml of glacial acetic acid. The colour which developed after one minute was measured spectrophotometrically at 560 nm (Zlatkis et al., 1953).

Section 2.4.9 Enzyme Studies

These studies involved determination of the activities of Glutamic Oxaloacetic Transaminase (GOT) and Glutamic Pyruvic Transaminase (GPT) in serum and identification of L-gulonolactone oxidase activity in samples of liver and kidney.

Section 2.4.9.1 Determination of GOT and GPT

GOT and GPT were determined by methods as described by Hawk (1965b). The two methods were similar except in the substrate components, for GOT the substrate contained aspartic acid whereas for GPT it contained alanine. The reactions of the two methods are as follows: serum was incubated with alpha-ketoglutarate for one hour at 37°C and the

reaction was stopped by adding dinitrophenylhydrazine reagent. The complex with the remaining alpha-ketoglutarate forms a dinitrophenylhydrazone which absorbs light at 505 nm.

Section 2.4.9.2. Identification of L-gulonolactone oxidase activity in liver and kidney

This was accomplished using a histochemical (qualitative) method and a biochemical (quantitative) method.

Histochemical method

The histochemical method demonstrates the oxidative enzyme activity by use of paranitrophenyl substituted ditetrazolium salt (Nitro-BT). Oxidation of L-gulonolactone causes a transfer of electrons through one or more intermediates and ultimate reduction of the nitro-BT to form a blue granular insoluble substantive diformazan at the site of enzyme activity. The method was used as described by Cohen (1961): Tissues from freshly killed animals were frozen using hexane and liquid nitrogen and were sectioned in a cryostat (-20^{o}C) at 8 μm thickness. Sections were put on slides and allowed to rinse for 5-10 minutes in cold 0.03 M KCl and then removed to an incubating mixture which consisted of 15 mg L-gulonolactone, 25 mg of the paranitrophenyl substituted dititrazolium salt in 4.75 cm^3 of 0.1 M phosphate buffer (pH 7.4). 0.25 cm^3 acetone was added to dissolve vitamin K thus the mixture was made up to 5 cm^3. The mixture was warmed to 37^{o}C before introducing the sections which were incubated at 37^{o}C for 20-40 minutes. At the end of the incubation period, the sections were removed and placed in 10% neutral formalin for 1 hr at room

temperature followed by mounting in glycerogel and examination.

Biochemical method

Tissues from freshly killed test animals were homogenized in cold 0.25 M sucrose with a Thyristor Regler homogenizer.

L-gulonolactone oxidase activity was assayed according to the method of Chatterjee et al. (1960) with some modifications developed by Yamamoto et al. (1978) as follows: The reaction mixture contained 150 µmol of sodium phosphate buffer (pH 7.2), 50 µmol of L-gulonolactone and 2 ml of enzyme solution in a total volume of 5 ml. The mixture was incubated at 37°C for 20 minutes with aeration. The reaction was stopped by adding 10 ml of metaphosphoric acid. The total ascorbate produced from L-gulonolactone and the amount of endogenous total ascorbate contained in the enzyme solution (under the same conditions as the enzyme assay) were determined by the method of Ikeda et al. (1963b) chromotographically using a 1.2 cm x 25 cm column containing acid alumina.

One unit of enzyme activity was defined as 1µg of total ascorbate formed enzymatically in 20 minutes.

SECTION 2.5 : HISTOLOGICAL STUDIES

Histological studies were twofold investigating the effects of dietary ascorbic acid deficiency on:

1. Tissue changes
2. Wound healing

Section 2.5.1 Tissue Changes

At the end of the experiments 3 and 4 (Chapter 5) detailed post mortem was carried out on 4 fish from each treatment, plus any moribund fish. Tissue samples from all organs, plus areas of deformity, were fixed in buffered formal saline. Wax sections were cut at 5 µm and stained with haematoxylin and eosin (H & E), Gommori's trichrome and Van Gieson's method (Culling, 1974). For vertebral sections, blocks were decalcified with decalcification agent prior to cutting (Drury and Wallington, 1980). Deformed fry obtained from the hatchability experiment (Chapter 13) were also preserved in 10% formalin. After dehydration and wax impregnation sections were cut at 5 µm and stained with H & E.

Section 2.5.2 Wound Healing

Blocks of tissue encompassing the wound were immediately removed from freshly sacrificed fish and fixed in cooled 10% formal saline. Paraffin wax sections at right angles to the direction of wounding were cut at 5 µm and stained with haematoxylin and eosin (H & E), Marticus Scarlet Blue (MSB), Verhoeffs-Van Gieson, PAS/Alcian blue and Masson's trichrome methods (Culling, 1974).

SECTION 2.6 : X-RAY TECHNIQUE

X-rays were taken using a Watson 'Mobilix' mobile ward x-ray machine. Exposures were for 3 seconds at 2KVA using Kodak industrix 'C' film.

SECTION 2.7 : GROWTH AND NUTRITIONAL PARAMETERS MEASURED

Condition Factor (CF,%)

$$CF\ (\%) = \frac{\text{Body weight (g)}}{L^3} \times 100$$

where L is standard length of fish in cm

Specific Growth Rate (SGR, $\%d^{-1}$)

Specific growth rate is a parameter used to measure the change in weight of fish, expressed as percent per day (Brown, 1957). The following equation was used:

$$SGR\ (\%d^{-1}) = \frac{\log_e W_2 - \log_e W_1}{t} \times 100$$

where W_2 = Final mean weight of fish

W_1 = Initial mean weight of fish

t = Time interval in days

Food Conversion Ratio (FCR)

$$FCR = \frac{\text{Feed consumed in grams}}{\text{Total wet weight gain in grams}}$$

Protein Efficiency Ratio (PER)

This parameter measures the ability of the fish to utilize dietary protein (Osborne et al., 1919) and can be calculated using the following equation:

$$PER = \frac{\text{Total wet weight gain (g)}}{\text{Amount of crude protein fed (g)}}$$

Apparent Net Protein Utilization (ANPU)

Apparent net protein utilization was calculated using the method of Nose (1962).

$$APNU\ (\%) = \frac{B - B_o}{I} \times 100$$

where B = Total body protein of test fish at the end of trial

B_o = Total body protein at the beginning of trial

I = Protein intake on test diet

SECTION 2.8 : DIGESTIBILITY DETERMINATIONS

This was measured using the inert indicator chromic oxide by method of Furukawa and Tsukahara (1966). Faecal samples were collected by siphoning from the experimental tanks twice daily, drying at 105°C and pooling successive samples for each tank. Apparent protein digestibility was then calculated as follows:

$$APD\ (\%) = \frac{100 - (100 \times \%\ Cr_2O_3 \text{ in feed}) \times (\%\text{ protein in faeces})}{(\%\ Cr_2O_3 \text{ in faeces}) \times (\%\text{ protein in feed})}$$

The dry matter digestibility was calculated (Maynard and Loosli, 1969) as follows:

$$ADMD\ (\%) = \frac{100 - 100 \times \%\ Cr_2O_3 \text{ in feed}}{\%\ Cr_2O_3 \text{ in faeces}}$$

SECTION 2.9 : STATISTICAL ANALYSES

Analysis of variance and Duncan's multiple range and F tests were employed in evaluating the experimental results (Snedecor, 1966, and Duncan, 1955).

CHAPTER 3

QUALITATIVE AND QUANTITATIVE IDENTIFICATION OF L-GULONOLACTONE OXIDASE (E.C.1.1.3.8) IN SOME TELEOSTS

SECTION 3.1 : INTRODUCTION

L-ascorbic acid is an essential nutrient for normal metabolism in animals and the ability to synthesise this compound *in vivo* varies between families, genera and species (Fig.11) (Burns *et al.*, 1956; Chatterjee *et al.*, 1961a; Chatterjee, 1973a). Animals which are unable to synthesise L-ascorbic acid, and therefore rely on a dietary, exogenous, source include insects (Dutta Gupta *et al.*, 1972); some species of birds (Ray Chaudhuri & Chatterjee, 1969) and many fresh water and marine fish and invertebrates (Chatterjee, 1973b; Wilson, 1973; Yamamoto *et al.*, 1978). An inability to synthesise L-ascorbic acid is due to absence of the enzyme L-gulonolactone oxidase (E.C.1.1.3.8) which is responsible for conversion of precursor L-gulonolactone to L-ascorbic acid in kidney and/or liver tissue (Burns, 1957; Grollman & Lehninger, 1957; Chatterjee *et al.*, 1961a. Lack of this enzyme may be attributed to the loss of the relevant gene or its capacity to synthesise this enzyme. Gluecksohn-Waelsch (1963) explained the inability of man to synthesise ascorbic acid as a mutation which occurred in the course of evolution and he termed this 'a conditional lethal mutation'. Stone (1966a) on the other hand, termed this genetic defect 'hypoascorbemia' as low levels of ascorbic acid in the blood are pathognomonic of this condition.

The site of activity of L-gulonolactone oxidase appears to be related to evolutionary level with lower vertebrates (e.g. reptiles and amphibia) having activity in the kidney and higher vertebrates (e.g. mammals and highly evolved birds) exhibiting activity in the liver (Grollman & Lehninger, 1957; Dutta Gupta *et al.*, 1973).

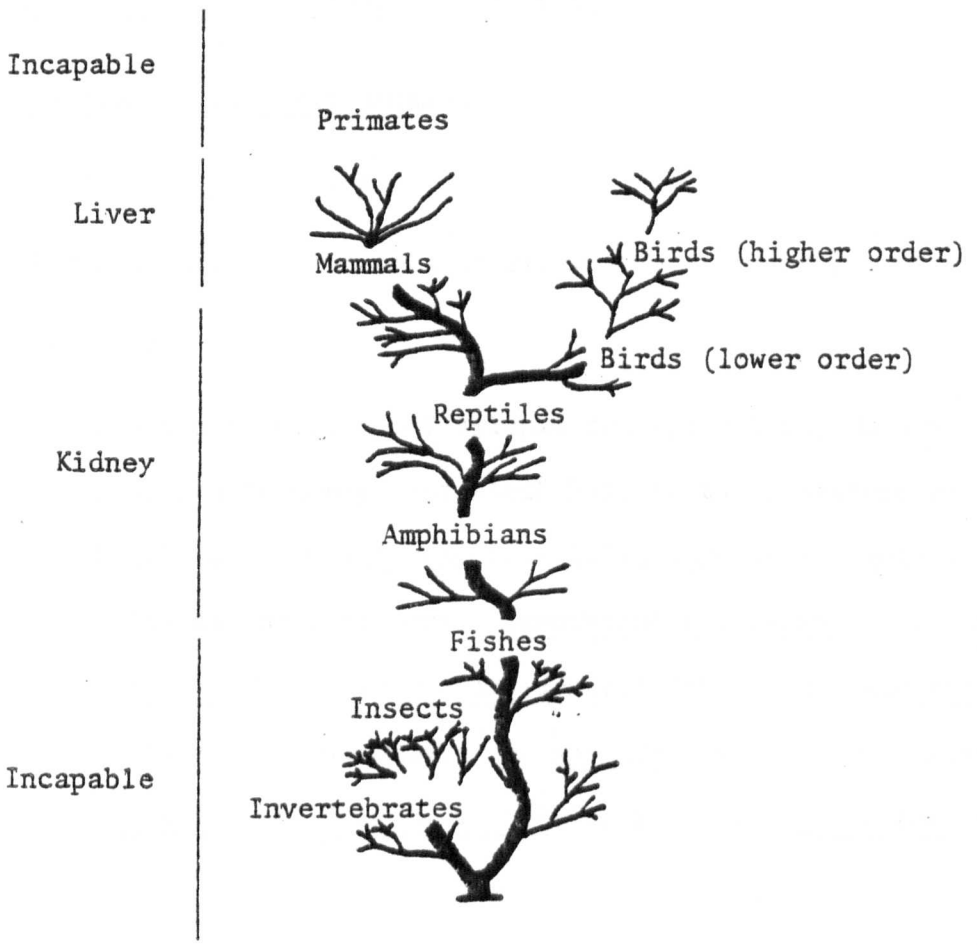


FIGURE 11. Ascorbic acid synthesizing abilities of various species of animals in relation to their phylogeny. (Chatterjee, 1973a)

The present study was undertaken to determine whether a range of teleosts representing different evolutionary levels possessed demonstrable L-gulonolactone oxidase activity in kidney and/or liver tissue.

SECTION 3.2 : MATERIALS AND METHODS

Section 3.2.1 Test Animals

Section 3.2.1.1 Fish

The fish used in the present study were as follows:

Tilapias

Eight pure species of tilapia and one hybrid, derived from genetically homogenous stock held at the Institute of Aquaculture, Stirling University (Section 2.2), were investigated. The tilapias employed were: *Sarotherodon galilaeus* L.; *Oreochromis niloticus* Trewavas; *O. mossambicus* Peters; *O. macrochir* Boulenger; *O. aureus* Steindachner; *O. spilurus* Gunther; *O. niloticus* x *O. mossambicus*; *Tilapia zillii* Gervais; *T. buttikoferi* Hurbrecht.

Salmonids

Three salmonid species were studied: rainbow trout (*Salmo gairdneri* Richardson); brown trout (*S. trutta* L.); brook trout (*Salvelinus fontinalis* Mitchill). Rainbow trout and brown trout were obtained from Howietoun Fish Farm, Stirling University, and brook trout from Solway Fisheries, Newabby, Dumfries, Scotland.

Cyprinids

Two cyprinids were investigated: grass carp (Ctenopharyngon idellus Valenciennes); common carp (Cyprinus carpio L.). Grass carp were obtained from Howietoun Fish Farm and common carp from Newhay Fisheries, Cliffe, Nr Selby, Yorkshire.

Section 3.2.1.2 Mammals

As controls rats and guinea pigs were obtained from a commercial supplier. Rats are known to be able, and guinea pigs unable, to synthesise ascorbic acid (Ul Hassan & Lehninger, 1956; Burns, 1957).

Section 3.2.2 Experimental Methodology

Section 3.2.2.1 Qualitative detection of L-gulonolactone oxidase activity

The enzyme activity was detected histochemically by the method of Cohen (1961) (Section 2.4.9.2) after samples of liver and kidney from fresh killed individuals had been frozen in hexane and liquid nitrogen and section at 8 μm in a cryostat at -20°C.

Section 3.2.2.2 Quantitative estimation of L-gulonolactone oxidase activity

Enzyme activity was assessed in samples of liver and kidney tissues using the method of Chatterjee et al. (1960) as modified by Yamamoto et al. (1978). The total quantity of ascorbic acid produced from L-gulonolactone and the amount of endogenous total ascorbic acid contained in the enzyme solution, under the conditions of enzyme assay, were determined by the hydrazine method of Ikeda et al. (1963b). (Section 2.4.9.2).

SECTION 3.3 : RESULTS

The results of both qualitative and quantitative identification of L-gulonolactone oxidase activity for the tested species are presented in Table 11.

Controls

Guinea pigs showed no histochemical (Plates 6 and 7) or biochemical (Table 11) activity of L-gulonolactone oxidase in either hepatic or renal tissue. In rats the histochemical stain detected enzyme activity in liver tissue (Plate 8) which was quantified by the biochemical technique (Table 11), with no detectable activity in renal tissue.

Cyprinids

Common carp showed enzyme activity in both hepatic and renal tissues (Plate 9 and Table 11) whereas grass carp showed no detectable enzyme activity.

Salmonids

The three species examined exhibited no detectable enzyme activity (Table 11).

Tilapias

Of the species and one hybrid investigated only *O. spilurus* and *O. aureus* showed enzyme activity and in both cases the enzyme was detected only in renal tissues (Plates 10 and 11 and Table 11).

TABLE 11. Histochemical Localization and Estimation of L-gulonolactone oxidase Activity in the Hepatic and Renal Tissues of Controls, Cyprinids, Salmonids and Tilapias

Species	No. of animals	Body weight (g)	Histochemical localization		Enzyme Activity (units/g tissue)(1)	
			Hepatic tissue	Renal tissue	Hepatic tissue	Renal tissue
Controls						
Rats	4	187-198	+(5)	-(4)	151.63 ± 8.84(2)	N.D.(3)
Guinea pigs	3	700-949	-	-	N.D.	N.D.
Fish						
Cyprinids						
Common carp (*Cyprinus carpio*)	5	289-382	+	+	59.20 ± 1.44	20.97 ± 0.34
Grass carp (*Ctenopharyngodon idellus*)	4	154-264	-	-	N.D.	N.D.
Salmonids						
Rainbow trout (*Salmo gairdneri*)	9	205-360	-	-	N.D.	N.D.
Brown trout (*S. trutta*)	9	190-225	-	-	N.D.	N.D.
Brook trout (*Salvelinus fontinalis*)	5	380-530	-	-	N.D.	N.D.

TABLE 11 (cont'd)

Species	No. of animals	Body weight (g)	Histochemical localization: Hepatic tissue	Histochemical localization: Renal tissue	Enzyme Activity (units/g tissue): Hepatic tissue	Enzyme Activity (units/g tissue): Renal tissue
Tilapias						
Oreochromis niloticus	25	214-315	-	-	N.D.	N.D.
O. mossambicus	16	110-260	-	-	N.D.	N.D.
O. spilurus	22	114-269	-	+	N.D.	58.93 ± 3.26
O. aureus	22	148-231	-	+	N.D.	46.75 ± 3.25
O. macrochir	2	352-373	-	-	N.D.	N.D.
O. niloticus x O. mossambicus	8	140-173	-	-	N.D.	N.D.
Tilapia zillii	2	217-498	-	-	N.D.	N.D.
T. buttikoferi	35	65-131	-	-	N.D.	N.D.
Sarotherodon galilaeus	2	404-447	-	-	N.D.	N.D.

(1) One unit of the enzyme activity is defined as 1 µg of total ascorbic acid formed per 20 minutes incubation

(2) Standard error of the means derived from analysis of variance

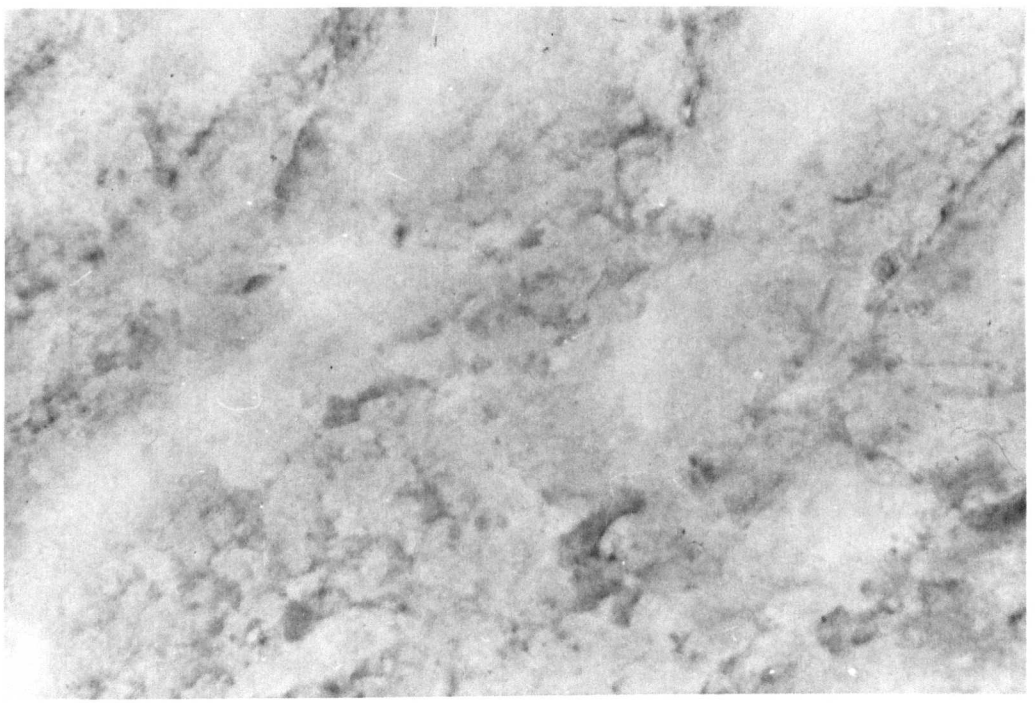
(3) Not detected

(4) Negative

(5) Positive

PLATE 6. A section of guinea pig liver showing no L-gulono-γ-lactone oxidase activity (x 600)

PLATE 7. A section through guinea pig kidney showing no detectable activity of L-gulono-γ-lactone oxidase (x 600)



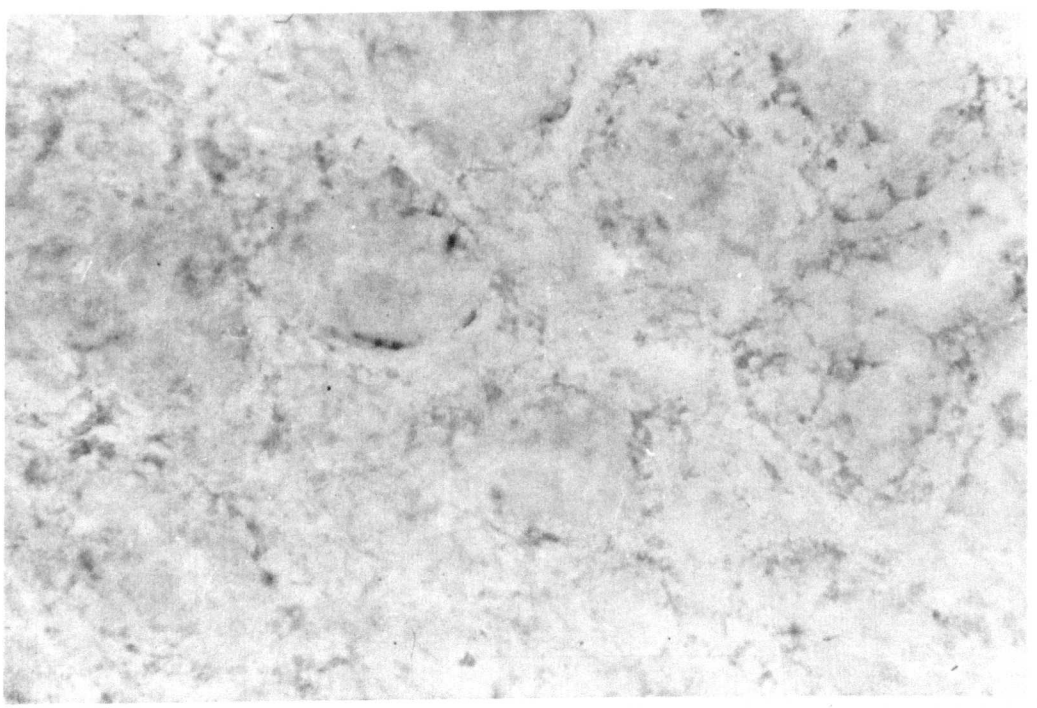
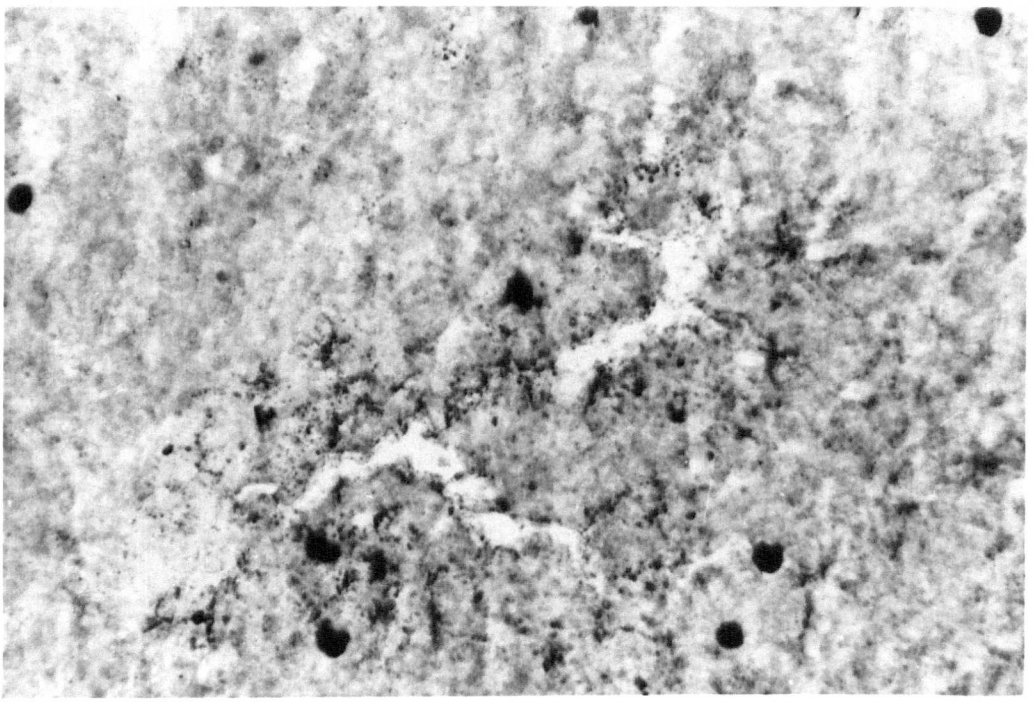


PLATE 8. A section of rat liver showing distribution of L-gulono-γ-lactone oxidase activity (x 600)

PLATE 9. A section of common carp liver (Cyprinus carpio) showing distribution of L-gulono-γ-lactone oxidase activity (x 600)



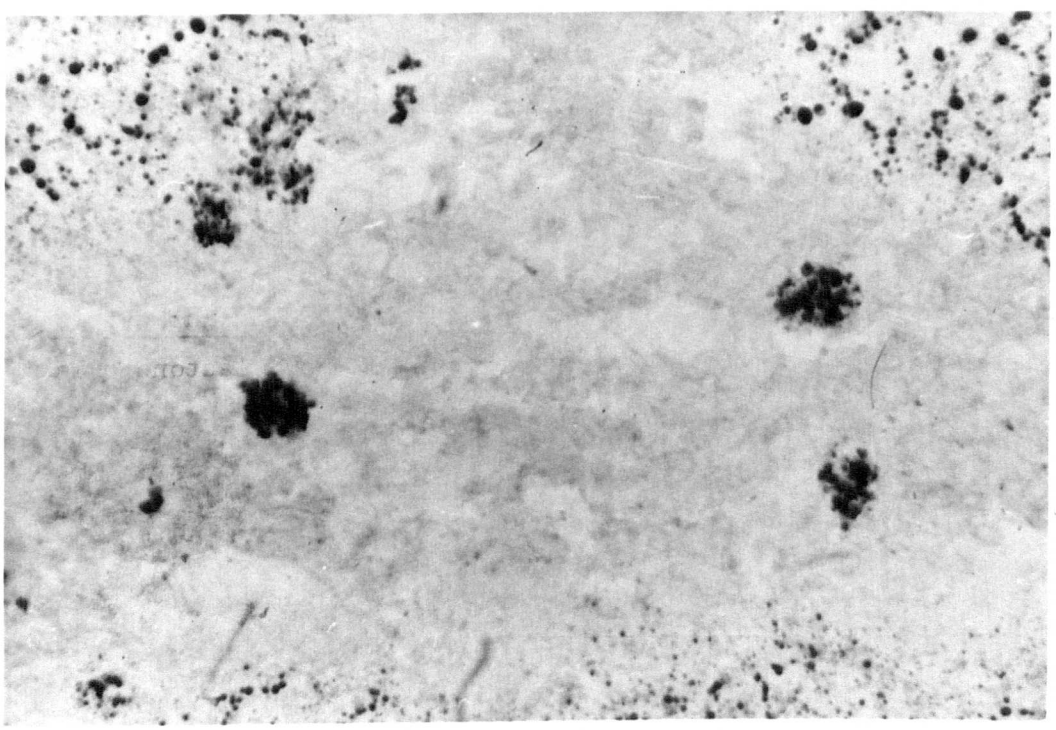
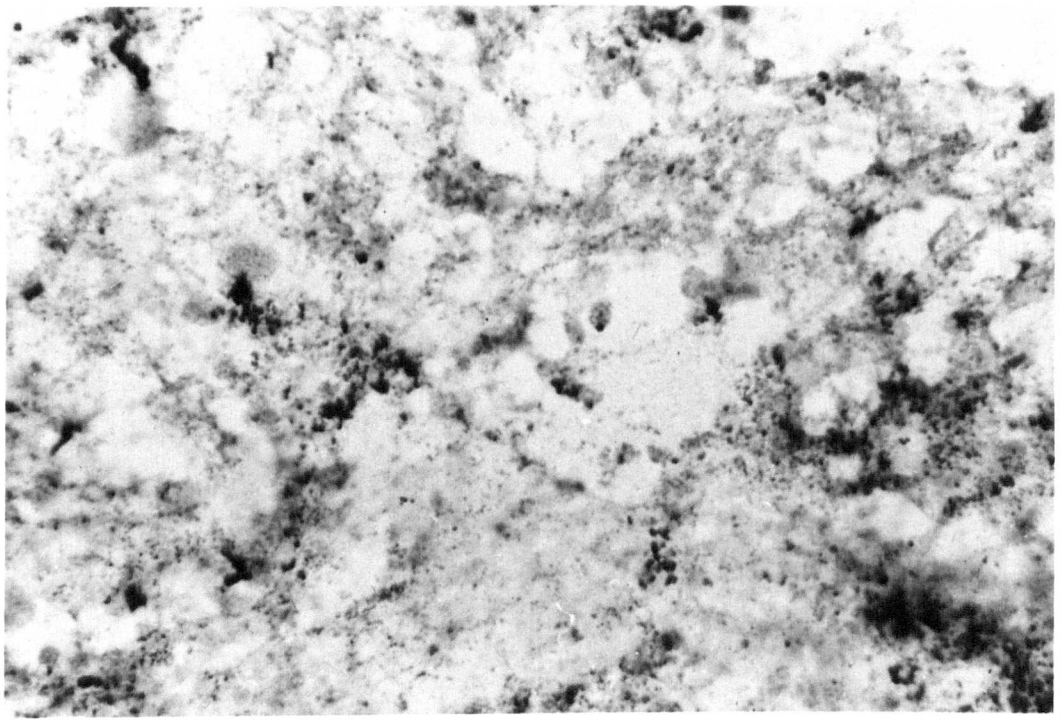
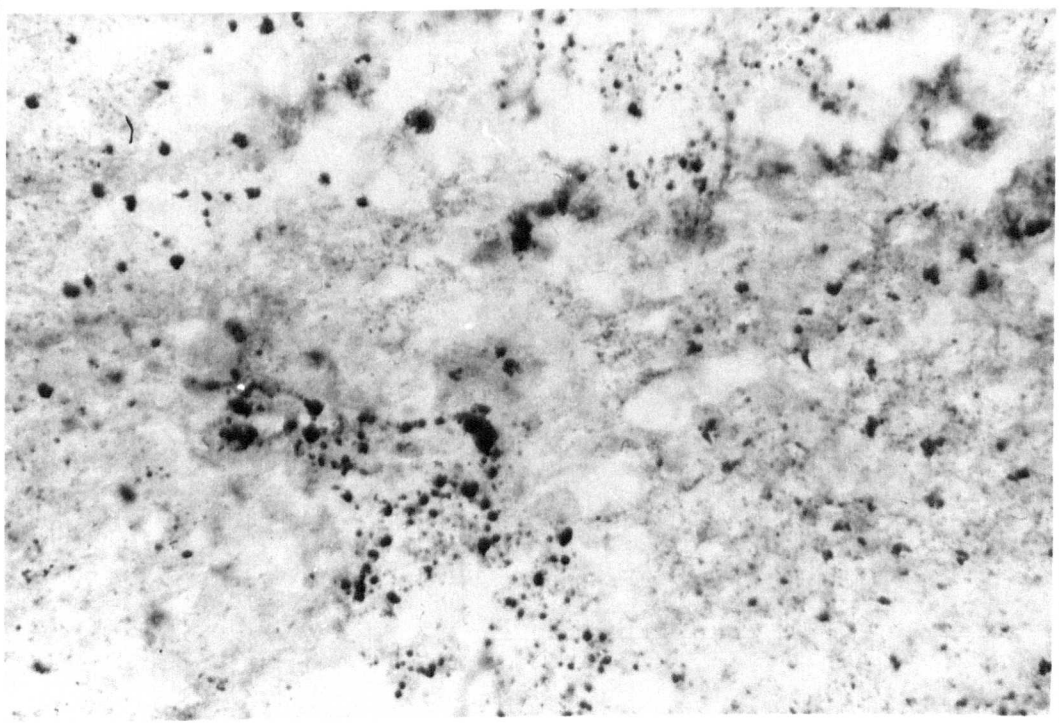


PLATE 10. A section of O. spilurus kidney showing existence of L-gulono-γ-lactone oxidase activity (x 600)

PLATE 11. A section in O. aureus kidney showing existence of L-gulono-γ-lactone oxidase activity (x 600)





SECTION 3.4 : DISCUSSION

The results obtained for the control species (rats and guinea pigs) in the present study are in agreement with previously published data and indicate the validity of the techniques employed here. The histochemical method (Cohen, 1961) appears to have been employed only once previously with fish (Kitamura, 1969).

Common carp liver exhibited about 40% of the enzyme activity of rat liver with lower activity in the renal tissue. These results are supported by the enzymatic studies of Yamamoto *et al.* (1978) and various metabolism and feeding studies (Ikeda & Sato, 1964; Sato *et al.*, 1978b. Not all carp species, however, are able to synthesise ascorbic acid, and the present study revealed that hepatic and renal tissues of grass carp failed to exhibit any trace of enzyme activity. Chatterjee (1973b) reported that neither the postmitochondrial fraction nor the microsomal fractions of kidney and hepatopancreas from carp species including *Labeo rohita* Hamilton, *Catla catla* Hora, *Labeo calbasu* Hamilton and *Cirrhina mrigala* Hamilton could synthesise ascorbic acid. In addition, Mahajan & Agrawal (1979; 1980a) reported that Indian major carp (*Cirrhina mrigala*) required dietary ascorbic acid due to the appearance of deficiency signs and retarded growth when fed a diet devoid in ascorbic acid.

No detectable L-gulonolactone oxidase activity was shown, in this study, in either hepatic or renal tissues of the salmonids investigated. Yamamoto *et al.* (1978) also failed to detect activity of this enzyme in liver and kidney of rainbow trout and feeding trials have confirmed that brook trout and rainbow trout require dietary

ascorbic acid for good growth and freedom from deficiency signs (Poston, 1967; Halver *et al.*, 1975; Sato *et al.*, 1982a). The literature contains no reports of identification of enzyme activity of L-gulonolactone oxidase in brook trout and brown trout.

It is surprising that of the tilapias examine (Table 11) only two exhibited enzyme activity, in renal tissue in both cases. Yamamoto *et al.* (1978) had previously suggested that common carp might be alone amongst teleosts in having the ability to synthesise ascorbic acid. Chatterjee *et al.* (1975) had indicated that the ability, or inability, to synthesise ascorbic acid was determined phylogenetically, however the results of the present study and results of study of Dykhuizen *et al.* (1979) which indicated ability of lung fish (*Neoceratodus forsteri*) to synthesise ascorbic acid, do not support this view.

The inability of *O. mossambicus* and *O. niloticus* to synthesise ascorbic acid is supported by other authors (Chatterjee, 1973b; Yamamoto *et al.*, 1978) and by dietary experiments (Chapters 4 and 5).

The present study demonstrates that the essentiality of dietary ascorbic acid should be determined separately, species by species, and that even species of the same genus may differ. It would also be of value with respect to tilapia, to determine whether hybrids of *O. spilurus* or *O. aureus* retained activity of L-gulonolactone oxidase.

CHAPTER 4

THE QUANTITATIVE DIETARY ASCORBIC ACID REQUIREMENTS OF *OREOCHROMIS NILOTICUS* AND *O. MOSSAMBICUS*

SECTION 4.1 : INTRODUCTION

For many years primates and guinea pigs were reported as being the only animals which required a dietary source of vitamin C (Burns *et al.*, 1956; Burns, 1957). Subsequently Roy and Guha (1958) reported that the fruit eating bat (*Pteropus medicus*) and the red-vented bulbul (*Pycnonotus cafer*) were also unable to synthesise ascorbic acid and that these species relied on an exogenous source of this vitamin.

Fish in their natural habitats seldom show signs of nutritional disease because natural aquatic food is fairly nutritious especially with respect to certain essential factors such as vitamins and minerals (Lovell, 1975). However, when fish are reared in an unnatural environment and fed artificial feeds to promote rapid growth and improved food utilization then nutritional deficiency may occur. The possible essentiality of dietary ascorbic acid for fish was first established by McLaren *et al.* (1947) and Kitamura *et al.* (1965). These early experiments were the first to demonstrate the dependency of fish on an exogenous supply of this vitamin and this discovery encouraged fish nutritionists to determine the dietary ascorbic acid requirements of other species. In fish the dietary requirements for ascorbic acid depend on various factors such as fish species, fish size, growth rate, water temperature, the processing and storage conditions of the diets and the occurrence of stress in the environment of the fish. Halver *et al.* (1969) recommended between 50 and 100mg of ascorbic acid per kg diet for coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Salmo gairdneri*) weighing less than 1g. However Hilton *et al.* (1978b) reported that not more than 40mg of ascorbic acid/kg diet was

required for rainbow trout weighing about 7g. For channel catfish (Ictalurus punctatus) Andrews and Murai (1975) and Murai et al. (1978) recommended a dietary ascorbic acid level of 50mg/kg diet and Mahajan and Agrawal (1980a)reported that 650-750mg of ascorbic acid/kg diet were required for Indian major carp (Cirrhina mrigala) for optimal growth and good health during the early development. Water temperatures higher or lower than the optimum may affect ascorbic acid requirements by influencing the metabolic rate. Sato et al. (1983) reported that the liver ascorbate level was lower in rainbow trout held at 20°C than in livers of rainbow trout held at 16°C where both groups were fed a diet containing the same level of ascorbic acid. Due to the destruction of ascorbic acid during processing and storage and losses incurred by leaching Hilton et al. (1977b)suggest addition excess of ascorbic acid to diets of salmonid species. Environmental contaminants such as toxaphene, oroganochlorine and nitrite are known to increase ascorbic acid requirements in channel catfish (Channa punctatus) and rainbow trout (Mayer et al., 1978; Agrawal et al., 1978 and Blanco & Meade, 1980).

Oreochromis niloticus and Oreochromis mossambicus are widely distributed in the tropics (Balarin & Hatton, 1979) and no previous reports appear to have been published concerning their requirements for dietary ascorbic acid. The present study was undertaken to investigate the effect of varying dietary ascorbic acid level on the nutrition of these two species.

SECTION 4.2 : EXPERIMENT 1

THE QUANTITATIVE DIETARY ASCORBIC ACID REQUIREMENT OF *OREOCHROMIS NILOTICUS*

Section 4.2.1 Materials and Methods

Section 4.2.1.1 Experimental system and animals

O. niloticus, obtained from a genetically homogenous stock (Section 2.2), were randomly distributed at a rate of 15 fish/9 litre circular plastic tank into an experimental recirculation system (Section 2.1.1). They were acclimated for two weeks prior to the start of the experiment during which time they were fed a commercial trout diet.

Section 4.2.1.2 Diets and feeding regime

Seven diets were evaluated containing graded levels of ascorbic acid (0, 50, 75, 100, 125, 300 and 400mg/100g of diet) with increasing ascorbic acid substitution for alpha cellulose in the basal diet (Table 12). Diet preparation and storage have been previously described (Section 2.3.2). Each of the seven dietary treatments was fed to randomly assigned triplicate tanks of fish with numbers reduced to 10 fish per tank. A fixed feeding regime of 5% of the body weight per day (dry food/whole fish), divided into 4 equal feeds was adopted. Fish were fed for six consecutive days, weighed on the seventh and feeding rates for following week adjusted accordingly.

TABLE 12. Composition of basal diet

Ingredient	Amount g/100 g
Brown fishmeal (herring type)[1]	57.0
Corn starch	10.0
Dextrin	10.0
Alpha-cellulose	6.5
Binder (carboxymethyl cellulose, sodium salt, high viscosity)	2.0
Chromic oxide	0.5
Cod-liver oil	4.0
Corn oil	6.0
Mineral mix.[2]	2.0
Vitamin mix.[3]	2.0

1. Crude lipid extracted by soxhlet method (Petroleum ether for 6 hours)
2. See Table 9
3. See Table 10

Section 4.2.1.3 Experimental methodology

Fish weighing

Fish were bulk weighed weekly, a tank at a time, in water without anaesthesia except for the final weighing when fish were anaesthetized (Ross & Geddes, 1979) and weighed and measured individually to allow for calculation of condition factor. Mortality and condition of the fish were noted at each weighing.

Proximate analysis

An initial sample of fish, 3 per tank, was sacrificed prior to the start of the experiment and subjected to proximate analysis (Section 2.4.2). Final samples of 3 fish/tank for diet 1 and 4 fish/tank for diets 2-7 were treated similarly.

Haematocrit and haemoglobin determination

Blood was collected from the experimental fish at the termination of the experiment for determination of haematocrit and haemoglobin by methods described earlier (Section 2.4.3).

Digestibility studies and net protein utilization

Apparent digestibility was measured using the inert indicator chromic oxide (Section 2.8) and apparent net protein utilization was calculated from carcass analysis data (Section 2.7).

Total ascorbate determination

The hydrazine method of Roe (1967) (Section 2.4.4.1) was employed for measuring the total ascorbate concentrations in liver, heart

gut, brain, gills, muscle, eyes, gall-bladder, testis and ovary in tissues of 2 fish per replicate for diet 1 and 3 fish per replicate for diets 2-7 respectively.

Section 4.2.2 Statistical Analysis

For evaluation of the results of the present study, analysis of variance, Duncan's multiple range test (Section 2.9), and correlation coefficient (Parker, 1979) were employed.

Section 4.2.3 Results

1. Growth response

Average body weight and specific growth rate (SGR)

Average body weights and specific growth rates were similar in all treatments for the first seven weeks but by the end of the eighth week differences in these parameters became apparent. Fish fed the diet devoid of ascorbic acid exhibited significantly ($P < 0.01$) poorer growth (Fig. 12 & Table 13). Average final body weights increased with increasing dietary ascorbic acid levels up to 125mg/100g above which significantly lowered final body weights were recorded (Table 13). A similar trend was obtained for specific growth rate (Table 13).

Condition factor (CF)

Fish fed the diet devoid of ascorbic acid exhibited significantly ($P < 0.01$) higher condition factors with no significant differences ($P > 0.01$) observed in fish fed diets supplemented with varying

TABLE 13. Growth, food utilization parameters and survival rate[1] of O. niloticus fed the experimental diets

Parameter	DIET							±SEM[2]
	1	2	3	4	5	6	7	
Initial avg.wt.g.	1.02[a]	1.01[a]	1.02[a]	1.02[a]	1.02[a]	1.01[a]	1.01[a]	0.060
Final avg.wt.g.	10.03[e]	13.95[cd]	15.19[bc]	16.15[b]	18.01[a]	14.31[c]	13.71[d]	0.380
SGR[3], % d^{-1}	2.72[e]	3.12[d]	3.22[bc]	3.29[b]	3.42[a]	3.16[cd]	3.11[d]	0.015
CF[4]	4.57[a]	3.31[b]	3.36[b]	3.39[b]	3.38[b]	3.44[b]	3.47[b]	0.055
FCR[5]	1.70[a]	1.40[b]	1.37[bc]	1.30[c]	1.20[d]	1.36[bc]	1.34[bc]	0.020
PER[6]	1.47[c]	1.78[b]	1.83[b]	1.92[b]	2.13[a]	1.83[b]	1.87[b]	0.035
ANPU[7], %	20.85[c]	31.26[b]	31.36[b]	32.21[b]	38.92[a]	31.78[b]	30.88[b]	0.570
APD[8], %	68.06[d]	79.71[c]	84.83[b]	85.76[b]	88.83[a]	82.05[c]	80.01[c]	0.460
ADMD[9], %	51.49[c]	60.26[b]	62.63[b]	62.67[b]	69.15[a]	61.69[b]	59.31[b]	0.710
Survival rate, %	50.00[c]	86.67[ab]	86.67[ab]	90.00[a]	90.00[a]	86.67[ab]	80.00[b]	2.180

1. Values in the same row with a common superscript are not significantly different (P > 0.01)
2. Standard error of the means derived from analysis of variance
3. Specific growth rate
4. Condition factor
5. Food conversion ratio
6. Protein efficiency ratio
7. Apparent net protein utilization
8. Apparent protein digestibility
9. Apparent dry matter digestibility

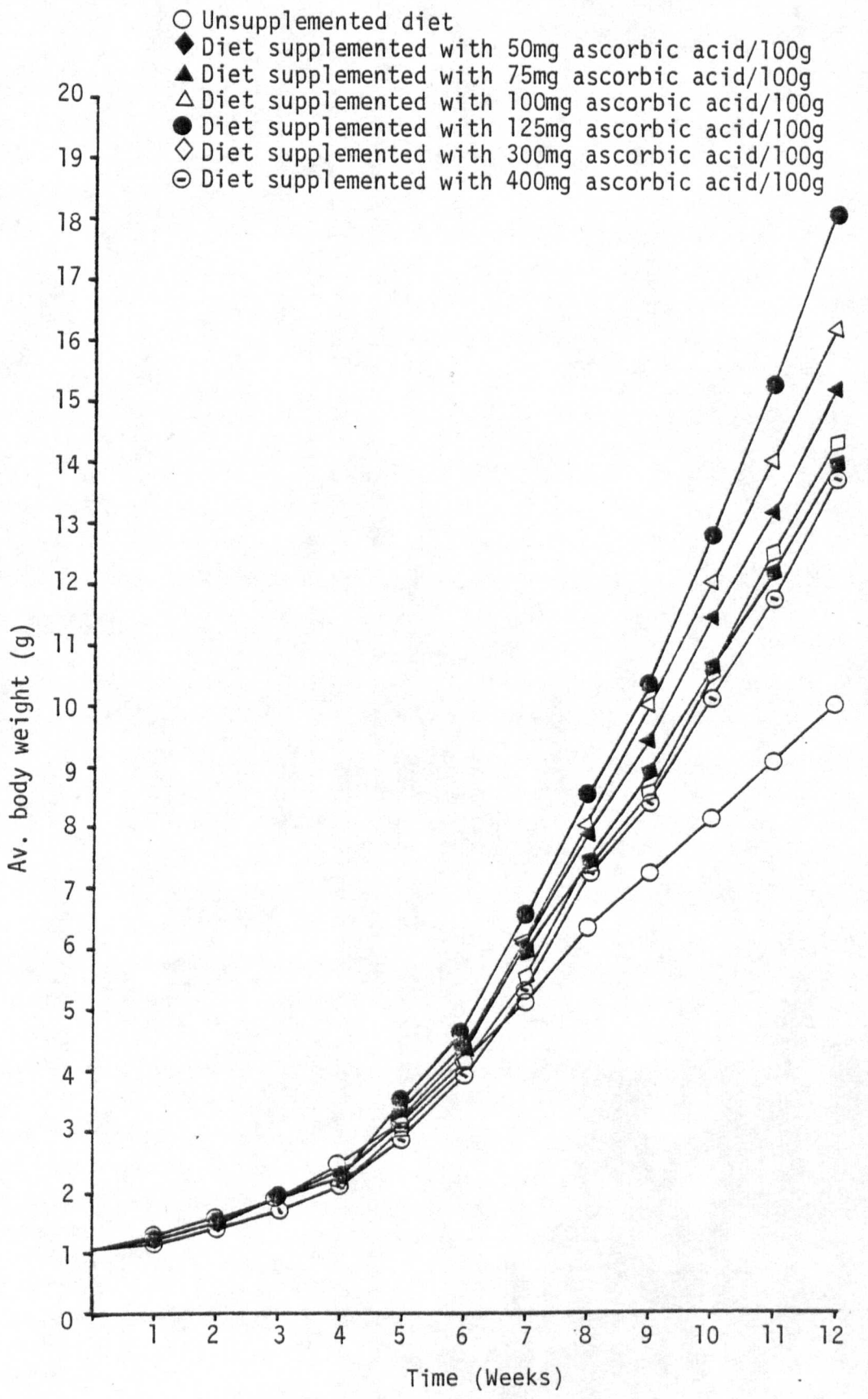


Fig. 12. Increase in average body weight of O. niloticus fed the experimental diets

levels of ascorbic acid (Table 13).

2. Food utilization parameters

Food conversion ratio (FCR) and protein efficiency ratio (PER)

Table 13 shows the effect of graded dietary levels of ascorbic acid on food conversion ratio and protein efficiency ratio.

Both FCR and PER improved, in some cases significantly ($P < 0.01$), with increasing dietary ascorbic acid concentration up to a level of 125mg/100g diet above which both deteriorated slightly (Table 13).

Apparent net protein utilization (ANPU), apparent protein digestibility (APD) and apparent dry matter digestibility (ADMD)

The effects of dietary ascorbic acid level on ANPU, APD and ADMD are shown in Table 13. These parameters were significantly lowered ($P < 0.01$) in fish fed the diet lacking ascorbic acid (Table 13).

3. Gross body composition data

Differences in final carcass composition among treatments were generally small although some were highly significant: Fish fed the diet devoid of ascorbic acid (diet 1) exhibited elevated moisture and depressed ash and crude protein contents (Table 14). Fish fed diet 5 (125mg ascorbic acid/100g diet) exhibited depressed moisture and elevated crude protein contents (Table 14).

TABLE 14. Gross body composition data[1] (as % wet weight)

Diet	Parameter			
	Moisture (%)	Ash (%)	Crude lipid (%)	Crude protein (%)
F°	79.33	3.54	3.62	13.73
1	76.06^{a}	4.31^{c}	5.18^{a}	14.45^{d}
2	74.31^{b}	4.81^{b}	4.19^{c}	16.68^{b}
3	74.62^{b}	4.61^{b}	4.41^{bc}	16.36^{bc}
4	73.65^{b}	4.69^{b}	5.08^{a}	16.59^{b}
5	72.08^{c}	5.28^{a}	5.27^{a}	17.99^{a}
6	74.80^{ab}	4.63^{b}	4.53^{b}	16.04^{c}
7	74.70^{ab}	4.73^{b}	4.69^{b}	15.90^{c}
±SEM[2]	0.300	0.055	0.082	0.120

F° Body composition sample of fish analysed at the beginning of the experiment

1. Values in the same column with a common superscript are not significantly different ($P > 0.01$)

2. Standard error of the means derived from analysis of variance

4. Tissue and biochemical changes

Table 15 summarises the effects of dietary ascorbic acid level on blood parameters and tissue ascorbate concentrations.

Haematocrit and haemoglobin values were significantly ($P < 0.01$) reduced in fish fed diet 1 (Table 15). Tissue ascorbate concentrations were highly correlated with dietary ascorbic acid level with liver tissue exhibiting the best correlation. Significantly ($P < 0.01$) lower tissue ascorbate concentrations were detected in tissues of fish diet 1.

5. Clinical signs of ascorbic acid deficiency

Behaviour and morphological changes

No abnormal behaviour was observed in fish fed the experimental diets until the beginning of the eighth week when erratic, unbalanced and convulsive movements were observed in fish fed diet 1. Anorexia was also noted in this group and was pronounced by the end of the tenth week as indicated from reduced food consumption. In addition 50% of fish fed diet 1 exhibited haemorrhages around the mouth, lips, fins and caudal fin, lethargy (Plate 12) and caudal fin erosion, by the end of the experiment few fish fed the highest level of ascorbic acid (400mg/100g) showed slight haemorrhages in fins after 10 weeks. No abnormalities were noted in fish fed the other experimental diets.

Survival rate

Survival rate was severely reduced in fish fed diet 1 (Table 13).

TABLE 15. Blood parameters and total tissue ascorbate concentrations[1] of fish fed the experimental diets at the termination of the experiments

Parameter	DIET							±SEM[2]	Correlation Coefficient
	1	2	3	4	5	6	7		
HC[3], %	30.50^{d}	37.00^{c}	40.75^{b}	40.50^{b}	43.00^{a}	36.50^{c}	35.50^{c}	0.495	
HG[4], g dl^{-1}	7.71^{e}	11.62^{cd}	12.49^{b}	12.19^{bc}	13.13^{a}	11.81^{c}	11.20^{d}	0.146	
Tissue ascorbate µg.g^{-1}									
Ovary	48.06^{g}	113.34^{f}	172.20^{e}	242.29^{d}	307.04^{c}	424.20^{b}	609.02^{a}	2.360	0.949
Gills	31.89^{g}	54.69^{f}	72.50^{e}	81.07^{d}	99.42^{c}	162.68^{b}	191.13^{a}	1.460	0.932
Eyes	22.65^{f}	36.01^{e}	39.49^{e}	47.59^{d}	72.91^{c}	112.49^{b}	131.43^{a}	0.870	0.939
Testis	30.83^{g}	92.18^{f}	123.60^{e}	175.00^{d}	260.00^{c}	328.80^{b}	383.89^{a}	2.460	0.903
Liver	5.59^{g}	34.04^{f}	52.08^{e}	62.54^{d}	94.38^{c}	143.73^{b}	172.36^{a}	1.680	0.955
Brain	36.67^{g}	133.88^{f}	185.18^{e}	234.05^{d}	363.34^{c}	405.95^{b}	457.54^{a}	3.550	0.843
Heart	N.D.[5]^{g}	36.00^{f}	50.00^{e}	62.82^{d}	82.85^{c}	104.76^{b}	127.75^{a}	2.080	0.890
Gut	16.20^{g}	42.20^{f}	57.18^{e}	63.56^{d}	89.18^{c}	128.00^{b}	133.84^{a}	1.120	0.877
Muscle	4.89^{g}	12.57^{f}	22.09^{e}	32.30^{d}	36.90^{c}	43.29^{b}	65.11^{a}	0.610	0.898
Gall-bladder	N.D.^{f}	11.34^{e}	29.00^{d}	37.98^{c}	38.61^{c}	49.00^{b}	65.01^{a}	0.590	0.864

TABLE 15 (cont'd)

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)
2. Standard error of the means derived from analysis of variance
3. Haematocrit
4. Haemoglobin
5. Not detectable

PLATE 12. *O. niloticus* fed the diet devoid of ascorbic acid showing typical signs of scurvy such as tail erosion and pronounced haemorrhages into the dorsal and caudal fins as well as in the lips.

SECTION 4.3 : EXPERIMENT 2

THE QUANTIATIVE DIETARY ASCORBIC ACID REQUIREMENT OF O. MOSSAMBICUS

Section 4.3.1 Materials and Methods

Section 4.3.1.1 Experimental system and animals

One hundred and fifty of O. mossambicus obtained from a genetically homogenous stock (Section 2.2) were stocked into a recirculation system (Section 2.1.1) for one week prior to the start of the experiment for acclimitization and during this time they were fed a commercial trout diet.

Section 4.3.1.2 Diets and feeding regime

Four diets were evaluated in the present study of similar formulation to diets 1, 2, 4 and 7 of the previous study (Section 4.2.1.2) with slight modification of the basal diet (Table 16) due to use of a different batch of fishmeal. Each of the four diets was fed to randomly assigned duplicate tanks of fish, with 10 fish/tank, for nine weeks. Diet preparation, storage and feeding regime were as for the previous experiment (Section 4.2.1.2).

Section 4.3.1.3 Experimental methodology

Fish weighing

As described in Section 4.2.1.3.

Proximate analysis

An initial sample of fish, 5 per tank, was sacrificed prior to the

TABLE 16. Composition of basal diet

Ingredient	Amount g/100 g
Brown fishmeal (herring type)[1]	51.30
Corn starch	12.35
Dextrin	12.35
Alpha-cellulose	7.50
Binder (carboxymethyl cellulose, sodium salt, high viscosity)	2.0
Chromic oxide	0.50
Cod-liver oil	4.00
Corn oil	6.00
Mineral mix.[2]	2.00
Vitamin mix.[3]	2.00

1. Crude lipid extracted by soxhlet method (Petroleum ether for 6 hours)

2. See Table 9

3. See Table 10

start of the experiment and subjected to proximate analysis (Section 2.4.2) and a final sample of 4 fish per tank for diet 1 and 5 fish per tank for diets 2-4 was treated similarly.

Other analyses

Blood parameters, apparent digestibility, net protein utilization and total ascorbate determinations were conducted as previously described (Section 4.2.1.3). For measurement of tissue ascorbate concentrations, 4 fish per treatment for diet 1 and 6 fish per treatment for diets 2-4 were employed. In addition, subsamples of liver and muscle were analysed for total glycogen (Section 2.4.6).

Section 4.3.2 Statistical Analysis

As previously described (Section 4.2.2).

Section 4.3.3 Results

1. Growth response

Average body weight and specific growth rate (SGR)

The effect of dietary ascorbic acid level on average body weight is shown in Table 17 and illustrated in Figure 13. Fish fed the diets supplemented with graded levels of ascorbic acid performed significantly ($P < 0,05$) better than those fed the diet 1. Fish fed diet 3, containing 125mg ascorbic acid/100g, exhibited higher average body weights than those fed the other experimental diets. Similar trends were recorded for SGR (Table 17).

TABLE 17. Growth response, food utilization parameters and survival rate[1] of O. mossambicus fed the experimental diets

Parameter	DIET				SEM[2]
	1	2	3	4	
Initial avg.wt.g.	1.50[a]	1.51[a]	1.51[a]	1.50[a]	0.050
Final avg.wt.g.	4.93[c]	6.61[b]	9.20[a]	6.09[b]	0.750
SGR[3], % d^{-1}	1.60[c]	2.35[b]	3.24[a]	2.24[b]	0.035
CF[4]	3.84[a]	3.07[b]	2.88[a]	3.02[b]	0.073
FCR[5]	1.90[a]	1.61[b]	1.40[c]	1.66[b]	0.050
PER[6]	1.32[c]	1.55[b]	1.79[a]	1.51[b]	0.035
ANPU[7], %	16.89[c]	29.23[b]	33.30[a]	27.88[b]	0.734
ADP[8], %	66.02[c]	85.54[b]	89.00[a]	82.81[b]	0.474
ADMD[9], %	40.44[d]	59.36[b]	65.90[a]	52.64[c]	0.163
Survival rate, %	60.00[b]	100.00[a]	100.00[a]	90.00[a]	5.000

1. Values in the same row with a common superscript are not significantly different ($P > 0.05$)
2. Standard error of the means derived from analysis of variance
3. Specific growth rate
4. Condition factor
5. Food Conversion ratio
6. Protein efficiency ratio
7. Apparent net protein utilization
8. Apparent protein digestibility
9. Apparent dry matter digestibility

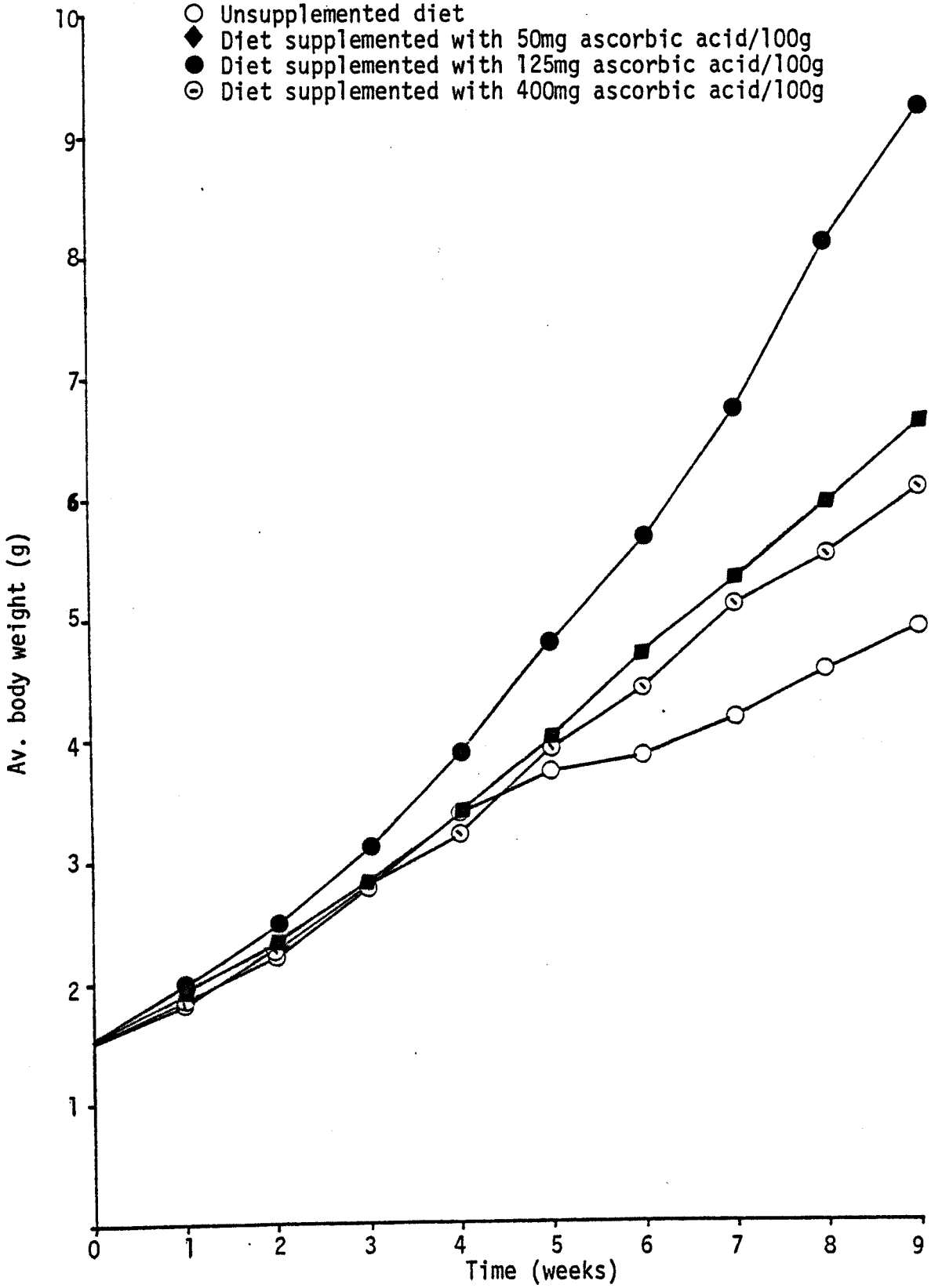


Fig. 13. Increase in average body weight of O. mossambicus fed the experimental diets

Condition factor (CF)

Fish fed the diet devoid of ascorbic acid (diet 1) exhibited a significantly ($P < 0.05$) higher condition factor. No significant differences ($P > 0.05$) in condition factor were observed amongst fish fed the diets supplemented with graded levels of ascorbic acid (Table 17).

2. Food utilization parameters

Food conversion ratio (FCR) and protein efficiency ratio (PER)

Both FCR and PER improved significantly ($P < 0.05$) with increasing dietary ascorbic acid levels up to 125mg/100g (diet 3) and then deteriorated slightly (diet 4, 400mg/100g) (Table 17).

Apparent net protein utilization (ANPU), apparent protein digestibility (APD) and apparent dry matter digestibility (ADMD)

Significantly ($P < 0.05$) the highest values for ANPU, APD and ADMD were obtained by fish fed the diet supplemented with 125mg ascorbic acid/100g (diet 3), whereas the lowest values were achieved by fish fed diet 1 (Table 17).

3. Gross body composition data

Generally, differences in carcass composition among the final fish samples were small although, in some cases, significant ($P < 0.01$). Fish fed diet 1 exhibited lowered carcass crude protein and increased moisture with the converse being true for diet 3 (Table 18).

TABLE 18. Gross body composition data[1] (as % wet weight)

Parameter	DIET					±SEM[2]
	F°	1	2	3	4	
Moisture, %	79.00	76.84[a]	73.40[b]	73.36[b]	74.81[b]	0.29
Ash, %	3.60	3.98[c]	4.33[b]	4.52[a]	4.34[b]	0.042
Crude lipid, %	3.00	4.08[a]	4.49[a]	4.65[a]	4.23[a]	0.096
Crude protein, %	14.40	14.96[c]	16.83[b]	17.86[a]	16.81[b]	0.231

F° Body composition sample of fish analysed at the beginning of the experiment

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)

2. Standard error of the means derived from analysis of variance

3. Tissue and biochemical changes

Blood parameters, hepatosomatic indices, liver and muscle glycogen contents and tissue ascorbate concentrations are shown in Table 19.

Fish fed diet 1, devoid of ascorbic acid, exhibited significantly ($P < 0.01$) lowered haematocrit and haemoglobin. Fish fed diet 4 (400mg ascorbic acid/100g) also exhibited lower values than fish fed diet 2 or 3. Hepatosomatic index and liver and muscle glycogen contents were significantly ($P < 0.01$) depressed in fish fed diet 1. Tissue ascorbate concentrations increased significantly ($P < 0.01$) with increasing dietary ascorbic acid level.

4. Signs of ascorbic acid deficiency

Fish fed diet 1 for 9 weeks exhibited severe haemorrhages into the fins and around the nose, caudal fin erosion, anorexia and lethargy. Anorexia was noted in the fifth week of the experiment and was followed by haemorrhaging and lethargy. None of these signs were observed in fish fed the diets supplemented with graded levels of ascorbic acid. A high mortality was recorded for fish fed diet 1 and no mortalities occurred in fish fed the diets supplemented with 50 and 125mg ascorbic acid/100g (Table 17).

TABLE 19. Blood parameters, hepatosomatic index, liver and muscle glycogen and total tissue ascorbate concentrations[1]

Parameter	DIET				±SEM[2]
	1	2	3	4	
HC[3], %	24.25[c]	40.34[a]	41.00[a]	31.83[b]	0.413
HG[4], gdl^{-1}	7.80[c]	12.60[a]	12.61[a]	10.16[b]	0.131
HSI[5], %	1.70[b]	2.16[a]	2.28[a]	2.14[a]	0.091
MG[6], %	0.57[c]	1.23[ab]	1.15[b]	1.33[a]	0.028
LG[7], %	4.57[b]	12.36[a]	13.56[a]	12.48[a]	0.49
Tissue ascorbate					
Liver, $\mu g.g^{-1}$	N.D.[8d]	29.49[c]	65.93[b]	172.50[a]	0.320
Gut, $\mu g.g^{-1}$	18.71[d]	37.93[c]	77.40[b]	206.98[a]	1.040
Gills, $\mu g.g^{-1}$	35.71[d]	47.43[c]	117.23[b]	196.96[a]	1.400
Brain, $\mu g.g^{-1}$	31.25[d]	103.46[c]	268.33[b]	460.00[a]	0.512
Eye, $\mu g.g^{-1}$	16.53[d]	42.36[c]	58.24[b]	89.48[a]	0.750
Gall-bladder, $\mu g.g^{-1}$	N.D.[d]	24.00[c]	50.00[b]	120.00[a]	0.277
Muscle, $\mu g.g^{-1}$	4.81[d]	12.34[c]	38.89[b]	53.79[a]	0.480
Heart, $\mu g.g^{-1}$	N.D.[d]	24.00[c]	50.00[b]	120.00[a]	0.277
Ovary, $\mu g.g^{-1}$	60.74[d]	145.55[c]	313.45[b]	509.65[a]	1.230
Testis, $\mu g.g^{-1}$	17.65[d]	103.45[c]	214.40[b]	380.94[a]	2.740

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)
2. Standard error of the means derived from analysis of variance
3. Haematocrit
4. Haemoglobon
5. Hepatosomatic index
6. Muscle glycogen
7. Liver glycogen
8. Not detectable

SECTION 4.4 : DISCUSSION

In aquaculture knowledge of the precise quantitative nutrient requirements is a great help in maximising yield and counteracting nutritional deficiencies (Mahajan & Yadave, 1974). For this reason considerable efforts have been made by fish nutritionists in this field. Table 20 summarises the considerable literature available concerning the ascorbic acid requirements of fish. This nutrient is regarded of great practical importance in fish feeds (Fish Farming International, 1984a). The experimental studies presented in this Chapter also demonstrate the beneficial effects of optimising dietary ascorbic acid levels for O. niloticus and O. mossambicus. Optimal dietary levels produced significantly improved performance in terms of growth response and food utilization parameters as well as prevention signs of ascorbic acid deficiency.

In the present studies fish fed diets devoid of ascorbic acid exhibited significantly poorer growth than those fed diets containing varying levels of ascorbic acid; there are several possible explanations for this:

1. The level of glutathione has been shown to be significantly depressed in blood, adrenal, pancreatic and spleen tissues of guinea pigs fed a scorbutic diet, at the same time tissue dehydroascorbic acid levels were increased (Banerjee et al., (1952). Glutathione is a promoter of cell division (mitosis) (Barron, 1951) and dehydroascorbic acid is an inhibitor of this process (Edger, 1969, 1970). It is not surprising

TABLE 20. The ascorbic acid (vitamin C) requirements of a variety of fish species

Fish Species	Water Temperature (C°)	Criteria	Recommended Inclusion Level (mg/100g)	Reference
Rainbow trout (*Salmo gairdneri*)	10-15	Growth, tissue ascorbic acid levels	10	1 & 2
	15	Growth	7	3
	15	Growth, tissue ascorbic acid levels, blood parameters	4.0	4
	15	Wound healing	50	2
	12.6-14.2	Hatchability	50	5
Coho salmon (*Oncorhynchus kisutch*)	15	Growth, tissue ascorbic acid levels	5.0	1
	15	Wound healing	20	1
Channel catfish (*Ictalurus punctatus*)	20	Growth	5	6
	20	Growth, wound healing, pathological symptoms	6	7
	27	Spinal deformities	2.5	8
	27	Tissue saturation	20	8
Channel catfish (3.0-19g)	29-32	Prevention of scurvy	3	9
3.0-19g	29-32	Maximum growth	6	9
9.7-150g	29-32	Prevention of scurvy and maximum growth	3	9
Channel catfish	29-32	Immune response, antibody production, serum complement activity	30-300	9

TABLE 20 (cont'd)

Fish Species	Water Temperature (C°)	Criteria	Recommended Inclusion Level (mg/100g)	Reference
Mrigal (fry) (Cirrhina mrigala)	25-35	Growth, mortality, pathological signs	65-70	10
Ayu (fry) (Plecogeossus altivelis)		Prevention of scurvy and growth	100	11
Aquarium fish		Growth	100	12
O. niloticus	28	All parameters mentioned in this Chapter	125	13
	28	Wound healing	125-400	14
O. mossambicus	28	All parameters mentioned in this Chapter	125	15

1. Halver et al. (1969)
2. Halver (1972)
3. Halver et al. (1975)
4. Hilton et al. (1978b)
5. Sandnes et al. (1984)
6. Andrews & Murai (1975)
7. Lim & Lovell (1978)
8. Murai et al. (1978)
9. Li & Lovell (1985)
10. Mahajan & Agrawal (1980a)
11. Yamamoto (1982)
12. Riising (1982)
13. This Chapter
14. Jauncey et al. (1985)
15. This Chapter

therefore, that a scorbutic animal exhibits retarded growth possibly as a result of inhibition of mitotic cell divison.

2. Ascorbic acid deficiency in Channa punctatus has been shown to depress the uptake of iodine by thyroid tissues and increase the circulating level in blood and kidney suggesting hypo-activity of the thyroid gland (Agrawal & Mahajan, 1981). Growth retardation in scorbutic fish may therefore be due, in part, to a reduction in thyroid activity and reduced production of thyroid growth hormones.

3. At an enzymic level it has been shown that, in guinea pigs, during scurvy the activity of lysosomal hydrolase is increased (Hoehn & Kanfer, 1978, 1980).

4. It is logical to assume that when an essential dietary nutrient is supplied at levels below the quantitative requirement then weight gain will be impaired (Zeitoun et al., 1975).

The widespread use of vitamin C in megadoses (levels considerably in excess of stated requirements) has recently become the subject of much controversy. Pauling (1970a, 1973) has suggested that, for humans, amounts of ascorbic acid in the gram range should be taken daily as a means of protection against the common cold and also for their possible prophylactic and therapeutic value in treatment and prevention of upper respiratory tract infection. On the other hand various workers (Lamden & Chrystowski, 1954; Briggs et al., 1973a, b;

Barness, 1975) have reported side effects of using megadoses of the vitamin such as oxaluria, kidney stones, gastrointestinal disturbances, diarrhoea and fatigue. In addition, Cochrane (1965) put forward the idea of 'ascorbic acid dependency' when scurvy was found in some babies even though their diets were supplemented with 60mg ascorbic acid/day and he attributed these cases to excessive ascorbic acid intake (400mg/day) by their mothers during pregnancy. It has also been reported that megadoses of the vitamin have adverse effects on the growth of animals. Ginter et al. (1979) reported that, in guinea pigs, an increase of dietary ascorbic acid (1%) above the required level (0.5%) resulted in decreased body weight gain and Keith et al. (1981) reported that guinea pigs receiving orally 1000mg sodium ascorbate exhibited reduced body weight gain and food consumption. In the present studies tilapias fed diets containing megadoses of ascorbic acid (300mg and 400mg/100g) exhibited growth retardation in comparison to those fed the diet containing 125mg ascorbic acid/100g. In a general paper discussing the quantification of nutrient requirements of fish, Zeitoun et al. (1975) stated that weight gain is normally linearly related to increasing dietary levels of an essential nutrient as long as levels are below the requirement and that at this level weight gain will plateau and then decline as the dietary nutrient concentration surpasses the animal's tolerance.

It is well known that ascorbic acid plays an important role in collagen biosynthesis (Cardinale & Udenfriend, 1974; Barnes, 1975). Lim & Lovell (1978) reported that vertebral collagen contents in channel catfish fed diets containing 30-240mg ascorbic acid/kg diet were not significantly different whereas fish fed a diet deficient in ascorbic

acid exhibited a lowered level of vertebral collagen concomittant with the incidence of a higher percentage of lordosis and scoliosis. Poston (1967) reported that brook trout fed a diet devoid of ascorbic acid exhibited significantly higher condition factors with the incidence of a higher percentage of lordosis and scoliosis. Therefore, the significantly higher condition factor obtained for scorbutic fish in the present studies may be related to impairment of collagen biosynthesis.

In experiments with fixed rates of feeding food conversion ratios will inevitably reflect weight gain (Andrews & Murai, 1975) and the present studies are no exception. Fish generally exhibit good FCR's in comparison to conventional terrestrial livestock, which are optimised by optimal dietary nutrient balance. For both tilapia species studies optimal FCR's were obtained for the diet containing 125mg ascorbic acid/100g.

The role(s) of ascorbic acid in protein metabolism have been discussed earlier in this thesis (Section 1.2.7.3) and will not be repeated here. Protein utilization, as measured by PER, carcass crude protein content, ANPU and APD, was improved in the present studies by increasing the supplemental dietary ascorbic acid up to a level of 125mg/100g.

Tilapias fed diets devoid of ascorbic acid in this study, exhibited haemorrhages and reduced haematocrit and haemoglobin levels. The role of ascorbic acid in anaemia prevention and iron metabolism has been previously discussed (Section 1.2.7.1). Similar results to those presented here have been found for rainbow trout where diets deficient in

ascorbic acid caused haemorrhages in intestine, liver and kidney (Kitamura et al., 1967) as well as decreased haematocrit, haemoglobin and serum iron levels (Hilton et al., 1978b). In the present study tilapias fed megadoses of ascorbic acid (300 and 400mg/100g diet) also showed lowered haematocrit and haemoglobin levels when compared with fish fed the optimal level. In guinea pigs high levels of ascorbic acid were reported not to affect blood parameters (Nandi et al., 1973) whereas in humans megadoses have been reported to destroy vitamin B_{12} (Herbert & Jacon, 1974) and to cause erythrocyte haemolysis (Campbell et al., 1975).

In support of the present findings for tilapias ascorbic acid deficiency has also been shown to depress liver glycogen contents in guinea pigs (Banerjee, 1943a; Chadwick et al., 1973) where it was also reported that glycogen levels did not respond to supplements of ascorbic acid in excess of the requirement (Banerjee, 1943b; Nandi et al., 1973).

The response, in terms of tissue ascorbate concentrations, into increasing levels of ascorbic acid supplementation appears to depend on the frequency of administration. Early studies (Penney & Zilva, 1946) suggested, in guinea pigs, that tissue ascorbate concentration was positively correlated with dietary level. Later workers (Nandi et al., 1973; Keith & Pelletier, 1974; Venn-Baigent et al., 1975) using a single daily dose of ascorbic acid found no increase in tissue ascorbate concentration when the dose exceeded the requirement. Ginter et al. (1979) showed that when ascorbic acid was administered more frequently to guinea pigs, either in the diet or drinking water,

that tissue ascorbate concentrations were more closely correlated with the level of intake. In the present studies ascorbic acid was administered in the feed four times daily and tissue ascorbate concentrations continued to increase with increasing dietary ascorbic acid up to the maximum evaluated (400mg/100g)° with a high correlation between tissue level and dose. The highest correlation was obtained between liver ascorbate concentration and dietary level which has also been reported for rainbow trout and channel catfish (Hilton et al., 1977a; Murai et al., 1978).

Ascorbic acid deficiency in tilapias resulted in haemorrhages, anorexia, lethargy and mortality after 9-12 weeks. Haemorrhage may have been a result of increased histamine production, during ascorbic acid deficiency, leading to loss integrity of blood capillaries and some leaching of blood from these vessels (Chatterjee et al., 1975). Explanations for lethargy include a possible decrease in muscle carnitine, as ascorbic acid is a co-factor for the hydroxylation of γ-N-trimethylaminobutyrate (γ-butyrobetaine) to carnitine (Hulse et al., 1978). In addition decreased brain ascorbate levels may have a direct effect on activity (Hilton et al., 1979b). Mortality in fish deprived of ascorbic acid is almost certainly a result of the cumulative effect of chronic deficiency of ascorbic acid in all the metabolic processes in which it is essential.

When quantifying nutrient requirements it is essential to differentiate between the net requirement and the recommended dietary inclusion level, although, unfortunately, this does not seem to be standard practice in fish nutrition. The net requirement is the minimal

quantity of a nutrient required for optimum performance of an animal dependent on its physiological status and environment. The recommended dietary inclusion level, however, must make allowance for raw material variation, nutrient and/or non-nutrient interactions and processing and storage losses (EIFAC, 1971). From the results presented here the recommended dietary inclusion level for ascorbic acid in tilapia feeds (O. niloticus and O. mossambicus) under these conditions and with the type of feed used is 125mg/100g. A later Chapter in this thesis (Chapter 8) will show that this is equivalent to a net requirement of 42mg/100g.

CHAPTER 5

THE EFFECTS OF LONG-TERM ASCORBIC ACID DEPRIVATION ON JUVENILE TILAPIAS (*OREOCHROMIS NILOTICUS* AND *O. MOSSAMBICUS*)

SECTION 5.1 : INTRODUCTION

In those higher animals (man, monkey and guinea pigs) which have a demonstrated requirement for vitamin C (Burns, 1957; Pauling, 1970b, and Chatterjee *et al.*, 1975) its role has been extensively investigated. In fish neither the requirements for, nor the metabolic roles of, ascorbic acid are well documented.

The possible essentiality of ascorbic acid in feeds for fish was first demonstrated in 1947 (McLaren *et al.*, 1947) when rainbow trout, *Salmo gairdneri* Richardson, fed diets devoid of vitamin C developed haemorrhages in their intestine, liver and kidney. Further studies were not published until 1965 (Kitamura *et al.*, 1965) when it was shown that rainbow trout fed diets deficient in ascorbic acid developed lordosis and scoliosis. More detailed experimental work revealed that when such fish were fed vitamin C deficient feeds they developed vertebral deformities, deformed operculae and haemorrhage of liver, kidney, muscle and intestine (Kitamura *et al.*, 1967).

Ascorbic acid deficiency has been shown to produce similar effects to those above in brook trout, *Salvelinus fontinalis* Mitchill (Poston, 1967); coho salmon, *Oncorhnchus kisutch* Walbaum, and rainbow trout, (Halver *et al.*, 1969); yellow tail, *Seriola quinqueradiata* Temmink and Schlegel (Sakaguchi *et al.*, 1969); eel, *Anguilla japonica* Temmink and Schegel (Arai *et al.*, 1972); channel catfish, *Ictalurus punctatus* Rafinesque (Lovell, 1973; Wilson & Poe, 1973; Andrews & Murai, 1975; Lim & Lovell, 1978; Murai *et al.*, 1978); channel catfish and blue catfish, *Ictalurus frucatus* Lesueur (Wilson, 1973); snakeshead, *Channa*

punctatus Bloch, and Indian major carp, Cirrhina marigala Hamilton (Mahajan & Agrawal, 1979, 1980a, and Agrawal & Mahajan, 1980a).

Controversially Dupree (1966) and Primbs and Sinnhuber (1971) reported that ascorbic acid was not an essential dietary nutrient for channel catfish and rainbow trout. Fish fed diets devoid of ascorbic acid showed no abnormalities.

Carp, Cyprinus carpio L, appear to be different with respect to the dietary essentiality of ascorbic acid. Ikeda and Sato (1964) reported that carp are able to synthesize L-ascorbic acid from D-glucose and D-glucuronolactone (radioactively labelled) and they suggest that carp possess a similar mechanism of vitamin C synthesis to that found in rats. In a subsequent study Ikeda and Sato (1966b) found that carp also converted labelled myo-inositol to L-ascorbic acid. These metabolic studies were confirmed in later growth trial (Sato et al., 1978b) when carp fed a diet devoid of ascorbic acid showed no abnormalities when compared to those on a supplemented diet. In contrast to common carp, histochemical and biochemical techniques failed to detect any trace of L-gulonolactone oxidase activity, the key enzyme responsible for synthesizing L-ascorbic acid, in hepatic and renal tissues of Oreochromis niloticus and O. mossambicus (Chapter 3).

Generally vitamin deprivation studies are performed only for short experimental periods. The present studies describe the effects of long-term (20-21 week) deprivation of ascorbic acid on nutritional, physiological and histological parameters in the tilapias Oreochromis niloticus and O. mossambicus.

SECTION 5.2 : EXPERIMENT 1

THE EFFECT OF LONG-TERM DEPRIVATION OF ASCORBIC ACID ON O. NILOTICUS

Section 5.2.1 Materials and Methods

Section 5.2.1.1 Experimental system and animals

One hundred and forty O. niloticus from a genetically homogenous stock (Section 2.2) were stocked at 35 fish/150 litre circular tank into an experimental recirculation system (Section 2.1.2) for one week prior to the start of the experiment and fed a commercial trout diet during this period.

Section 5.2.1.2 Diets and feeding regime

Two diets were used in the present study namely an ascorbic acid free, basal diet (Diet 1, Table 12) and a diet containing 125 mg of L-ascorbic acid per 100 g diet (Diet 2) achieved by substitution of alpha-cellulose in the basal diet. Diet preparation and storage was as previously described (Section 2.3.2). Each of the dietary treatments was fed to randomly assigned duplicate tanks of fish with 25 fish per tank.

Fixed feeding regimes of 5%, 3% and 2% of the body weight per day (dry food/whole fish), divided into four equal feeds, were employed for the first 8 weeks, weeks 9-16 and weeks 17-20 respectively. Fish were fed for six days excluding Sundays, when fish were weighed and feeding rates for the following week adjusted accordingly.

Section 5.2.1.3 Experimental methodology

Fish weighing procedure

Fish were bulk weighed, a tank at a time, in water without anaesthesia except for weighings at weeks 8, 16 and 20 when fish were anaesthetised (Ross & Geddes, 1979) and weighed and measured individually to allow calculation of condition factor, the condition of the fish and mortalities were also noted.

Proximate analysis

An initial sample of fish, 5 per tank, was sacrificed prior to the start of the experiment and subjected to proximate analysis (Section 2.4.2). A final sample of 4 fish per tank for Diet 1 and 5 fish per tank for Diet 2 was treated similarly.

Blood parameters

Blood was collected at the end of the experiment for determination of haematocrit and haemoglobin (Section 2.4.3). Serum was separated from the blood by centrifugation and used for determination of Glutamic Oxaloacetic Transaminase (GOT) and Glutamic Pyruvic Transaminase (GPT) activities (Section 2.4.9.1).

Digestibilities studies

Apparent digestibility was measured using the inert indicator chromic oxide (Section 2.8) and apparent net protein utilization was calculated from carcass analysis data as described in Section 2.7.

Total ascorbate determination

The 2,4 dinitrophenylhydrazine (DNPH) method of Roe (1967) (Section 2.4.4.1) was employed for measurement of total ascorbate concentrations in liver, heart, gut, brain, gills, muscle, eyes, gall-bladder, testis and ovary tissues of six fish for Diet 1 and six fish per tank for Diet 2 at the termination of the experiment.

Glycogen determination

Sub-samples of liver and muscle were analysed for total glycogen as described previously in Section 2.4.6.

Collagen, proline and hydroxyproline determination

Tissue preparation, collagen extraction, collagen estimation and proline and hydroxyproline determinations were as described previously (Section 2.4.7).

X-ray

Fish were x-rayed to determine the condition of the vertebral column during, and at the end of the experiment as described in Section 2.6.

Histological studies

Detailed post mortem examination and complete histopathological examination was carried out on 4 fish fed the ascorbic acid free diet and 4 controls at the end of the study. Tissue samples from all organs, plus areas of deformity, were fixed in buffered formal

saline. Wax sections were cut at 5 μm and stained with haematoxylin and eosin, Gommori's trichrome and Van Gieson's method (Culling, 1974). For vertebral sections, blocks were decalcified with decalcification agent prior to cutting (Drury & Wallington, 1980).

Section 5.2.2 Statistical Analysis

Statistical analysis of data was performed as previously described (Section 2.9).

Section 5.2.3 Results

The essentiality of dietary vitamin C for *O. niloticus* in the present study was evaluated using the following parameters:

1 Growth

Body weight and specific growth rate

No differences in average body weight were apparent until the beginning of the fourth week when the effects of dietary ascorbic acid on growth were indicated (Fig.14). Fish fed diets deficient in ascorbic acid displayed poorer growth than those fed diets supplemented with ascorbic acid. Analysis of variance and multiple range tests revealed highly significant differences ($P < 0.01$) in body weight between the two treatments at weeks 8, 16 and 20 (Table 21).

Specific growth rate showed the same trend as average body weight (Table 21).

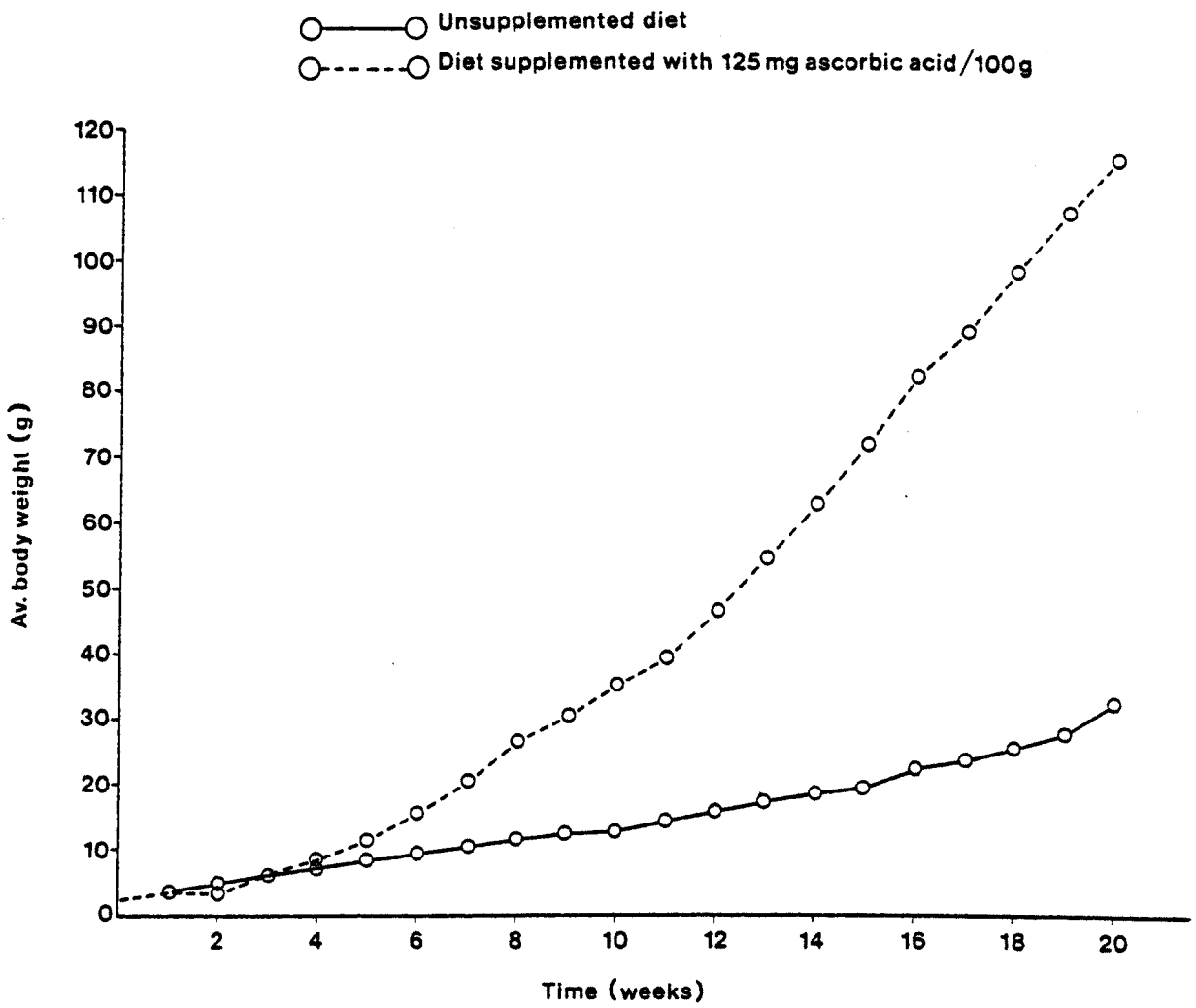


Fig. 14. Increase in average body weight of O. niloticus fed the two experimental rations

TABLE 21. Growth and food utilization parameters[1] of O. niloticus fed the experimental diets after 8, 16 and 20 weeks

Parameter	DIET		± SEM[2]
	Unsupplemented	Supplement	
Initial avg.wt.g.	2.48[a]	2.50[a]	0.540
8 Weeks			
Avg.wt.g.	11.91[b]	26.63[a]	0.950
SGR[3] (% d^{-1})	2.81[b]	4.22[a]	0.187
CF[4]	5.01[a]	3.46[b]	0.090
FCR[5]	1.57[a]	0.87[b]	0.098
PER[6]	1.60[b]	2.87[a]	0.162
16 Weeks			
Avg.wt.g.	22.44[b]	82.12[a]	2.140
SGR[3] (% d^{-1})	1.99[b]	3.12[a]	0.126
CF[4]	4.93[a]	3.50[b]	0.090
FCR[5]	2.13[a]	1.07[b]	0.182
PER[6]	1.18[b]	2.34[a]	0.095
20 Weeks			
Avg.wt.g.	32.06[b]	115.46[a]	5.820
SGR[3] (% d^{-1})	1.83[b]	2.74[a]	0.050
CF[4]	4.93[a]	3.46[b]	0.090
FCR[5]	2.23[a]	1.16[b]	0.010
PER[6]	1.12[b]	2.17[a]	0.050
APNU[7] (%)	23.70[b]	44.95[a]	0.62
APD[8] (%)	69.93[b]	85.93[a]	0.61
ADMD[9] (%)	55.77[a]	65.53[a]	0.89

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)
2. Standard error of the means derived from analysis of variance
3. Specific growth rate
4. Condition factor
5. Food Conversion ratio
6. Protein efficiency ratio
7. Apparent net protein utilization
8. Apparent protein digestibility
9. Apparent dry matter digestibility

Condition factor (CF)

Fish fed diets devoid of ascorbic acid showed shortening and deepening in body form as indicated by significantly ($P < 0.01$) higher CF's at weeks 8, 16 and 20 (Table 21).

2 Food utilization

Food conversion ratio (FCR)

Fish fed the ascorbic acid containing diet exhibited significantly ($P < 0.01$) lower FCR's than fish fed the unsupplemented diet (Table 21).

Protein efficiency ratio (PER)

The efficiency of protein utilization is shown as PER in Table 21. Fish fed diets supplemented with vitamin C showed significantly ($P < 0.01$) higher PER's than those fed the unsupplemented diet.

Apparent net protein utilization (ANPU), apparent protein digestibility (APD) and apparent dry matter digestibility (ADMD)

In terms of apparent dry matter digestibility, apparent protein digestibility and apparent net protein utilization, fish fed the supplemented diet showed significantly better values than fish fed the deficient diet (Table 21).

3 Gross body composition

Gross body composition data is presented in Table 22. Fish fed the unsupplemented diet showed increased total moisture content

TABLE 22. Gross body composition data[1] (as % wet weight basis)

Parameter	DIET			±SEM[2]
	F°	Unsupplemented	Supplemented	
Moisture (%)	78.91	72.94[a]	68.31[b]	0.31
Ash (%)	3.79[c]	4.30[b]	4.97[a]	0.04
Crude lipid (%)	3.03[b]	6.09[a]	6.19[a]	0.09
Crude protein (%)	14.65[c]	16.94[b]	20.65[a]	0.11

F° Body composition sample of fish analysed at the beginning of the experiment

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)

2. Standard error of the means derived from analysis of variance

and decreased carcass crude protein as compared to fish fed the ascorbic acid supplement diet ($P < 0.01$). The ash content of scorbutic fish was significantly lower than that of fish fed the ascorbic acid supplemented diet ($P < 0.01$). No significant difference in carcass lipid content was detected between fish fed the deficient or supplemented diets.

4 Tissue and biochemical parameters

Blood parameters, hepatosomatic indices, liver and muscle glycogen and ascorbic acid concentrations in different tissues are shown in Table 23. The haematocrit, haemoglobin and MCHC values for fish fed the ascorbic acid supplemented diet were significantly ($P < 0.01$) higher than those of scorbutic fish. The activities of serum glutamic oxaloacetic transaminase (SGOT) and serum glutamic pyruvic transaminase (SGPT) were significantly higher in scorbutic fish than in fish fed diets supplemented with ascorbic acid.

The hepatosomatic index and liver and muscle glycogen contents of fish fed diets supplemented with ascorbic acid were significantly ($P < 0.01$) higher than those obtained for scorbutic fish.

Lower levels of ascorbic acid were obtained in tissues of fish fed diets deficient in ascorbic acid (Table 23 and Fig.15). No detectable ascorbic acid was found in the testis, heart and gall-bladder of scorbutic fish. The differences between the total ascorbate concentrations in tissues of fish fed the supplemented and unsupplemented diets were highly significant ($P < 0.01$).

TABLE 23. Blood parameters, hepatosomatic index, liver and muscle glycogen and total tissue ascorbate concentrations[1] of fish fed unsupplemented and supplemented diets after 20 weeks

Parameter	DIET		±SEM[2]
	Unsupplemented	Supplemented	
1 Blood parameters			
HCT[3], %	30.5[b]	38.05[a]	0.54
HB[4] gdl^{-1}	8.43[b]	11.67[a]	0.15
MCHC[5], %	27.64	30.67	
GOT[6] (unit equiv.)	178.18[a]	107.25[b]	2.61
GPT[7] (unit equiv.)	118.18[a]	96.0 [b]	2.28
GOT/GPT	1.51[a]	1.12[b]	0.04
2 Hepatosomatic index and glycogen			
HSI[8], %	1.93[b]	2.51[a]	0.15
LG[9], %/wet wt.	4.58[b]	16.18[a]	0.18
MG[10], %/wet wt.	0.33[b]	0.78[a]	0.02
3 Tissues ascorbate concentrations (µg/g wet tissue)			
Ovary	18.00[b]	240.81[a]	2.14
Gills	14.31[b]	106.50[a]	1.81
Eyes	3.10[b]	35.12[a]	1.08
Testis	0.00[b]	131.59[a]	5.56
Liver	2.51[b]	53.88[a]	3.08
Brain	10.00[b]	236.88[a]	2.55
Heart	0.00[b]	62.59[a]	0.85
Alimentary canal	7.41[b]	56.18[a]	1.44
Muscle	1.31[b]	24.87[a]	0.34
Gall-bladder	0.00[b]	22.94[a]	2.15

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)
2. Standard error of the means derived from analysis of variance
3. Haematocrit
4. Haemoglobin
5. Mean corpuscular haemoglobin concentrations
6. Glutamic oxaloacetic transaminase
7. Glutamic pyruvic transaminase
8. Hepatosomatic index
9. Liver glycogen
10. Muscle glycogen

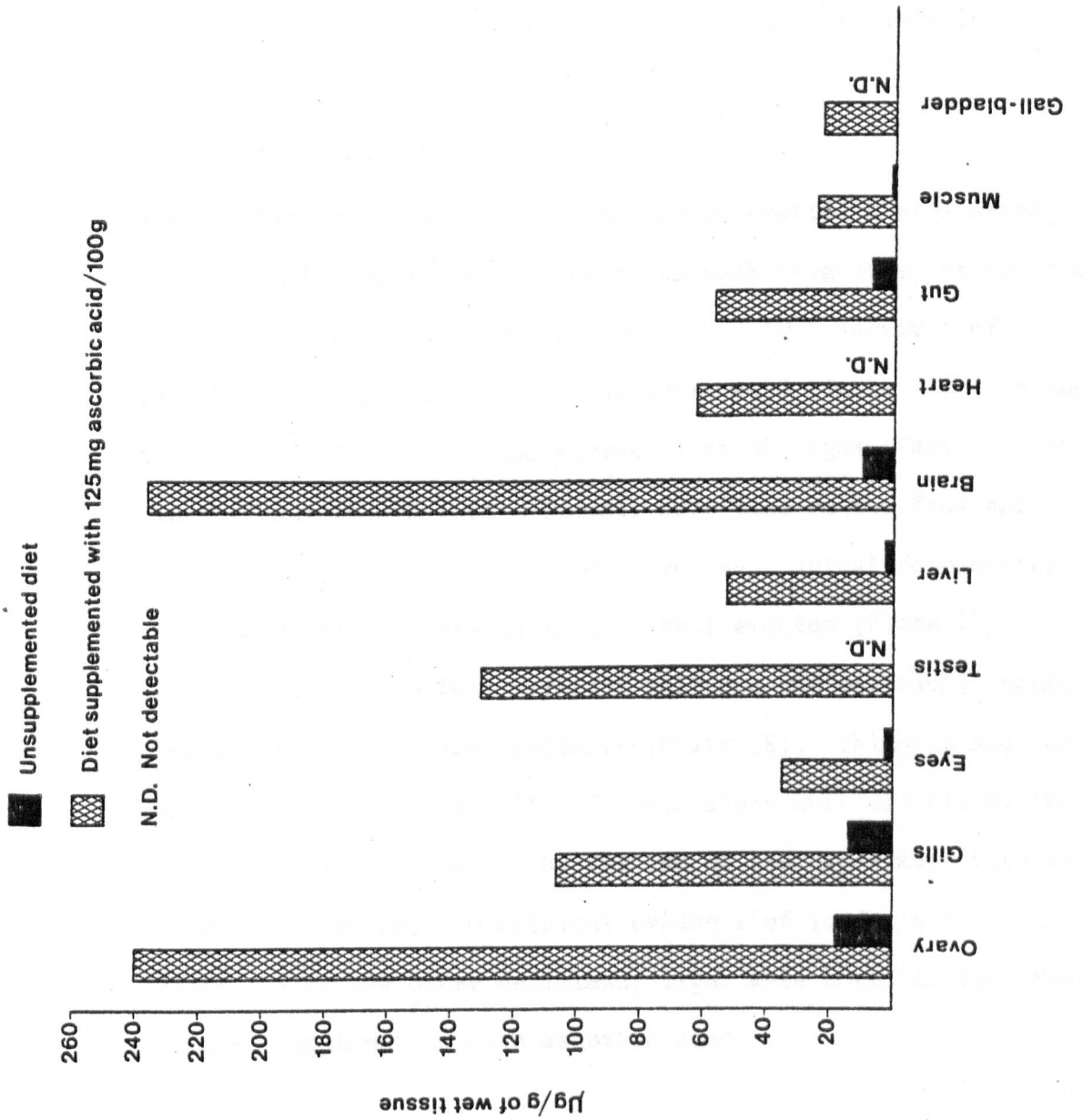


Fig. 15. Total tissue ascorbate concentrations for O.niloticus after 20 weeks on the experimental diets

The effect of dietary ascorbic acid on collagen content, proline, hydroxyproline and hydroxyproline:proline ratio of the vertebrae and tails of fish from both treatments are shown in Table 24. The collagen content of the vertebrae and tails of scorbutic fish were lower than in fish fed ascorbic acid supplemented diets. In addition, fish fed diets deficient in ascorbic acid had higher levels of proline in both vertebrae and tails than fish fed diets supplemented with ascorbic acid and the reverse was true for hydroxyproline. The hydroxyproline:proline ratio was significantly higher in fish fed the diets supplemented with vitamin C ($P < 0.01$).

5 Pathological effects

The survival rate of fish fed the unsupplemented diet started to decline at the beginning of the fifth week (Fig.16). At the end of 20 weeks only 38% survived compared to 100% survival of fish fed the diet supplemented with ascorbic acid (Table 24). In addition, scorbutic fish showed severe clinical signs (Table 24) including haemorrhage into myotomes, petechiae on the fins and eyes (Plate 13); foreshortened operculae; spinal foreshortening (Plate 14); shortened tails; tail erosion (Plate 15); exophthalmia (Plate 16); orbital collapse and cataract; acute lordosis (Plate 17) and scoliosis (Plate 18); thinning and condensation of vertebrae. All of these signs were visible by the end of the eighth week and by the end of the experiment they were obvious and severe. No clinical evidence of lordosis or scoliosis (Plate 19) or any other deficiency signs were noted in fish fed the diet supplemented with ascorbic acid.

TABLE 24. Composition of vertebrae and tails together with incidence of gross clinical signs of deficiency[1]

Parameter	DIET		±SEM[2]
	Unsupplemented	Supplemented	
1 Vertebrae[3]			
Collagen (%)	20.98[b]	34.09[a]	0.74
Proline (mg/g tissue)	33.52[a]	27.90[b]	0.98
Hydroxyproline (mg/g tissue)	24.87[b]	26.28[a]	0.45
Hydroxyproline/ proline	0.74[b]	0.94[a]	0.025
2 Tail[4]			
Collagen	6.49[b]	10.20[a]	0.07
Proline (mg/g tissue)	5.75[a]	4.89[b]	0.21
Hydroxyproline (mg/g tissue)	3.81[b]	4.25[a]	0.08
Hydroxyproline/ proline	0.66[b]	0.87[a]	0.01
3 Gross clinical signs of vitamin C deficiency			
Survival rate (%)	38.0[b]	100.0[a]	1.41
Haemorrhagic signs(%)	58.0[a]	00.0[b]	1.57
Short & weak operculum (%)	64.0[a]	00.0[b]	3.35
Short & tail erosion (%)	58.0[a]	00.0[b]	1.57
Exophthalmia (%)	58.0[a]	00.0[b]	1.57
Cataract (%)	21.0[a]	00.0[b]	0.78
Lordosis & scoliosis (%)	58.0[a]	00.0	1.57

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)
2. Standard error of the means derived from analysis of variance
3. Defatted basis
4. Wet basis

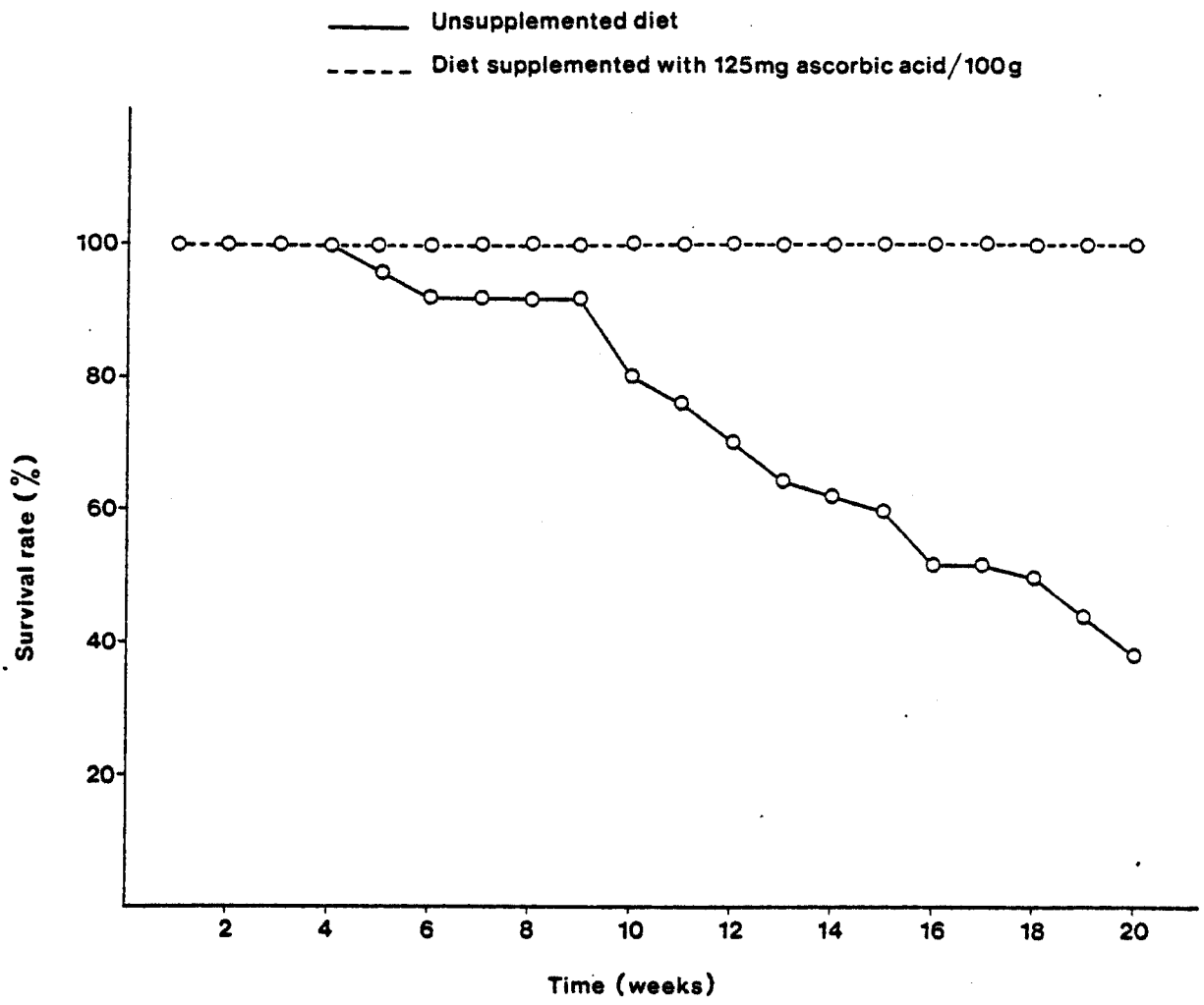


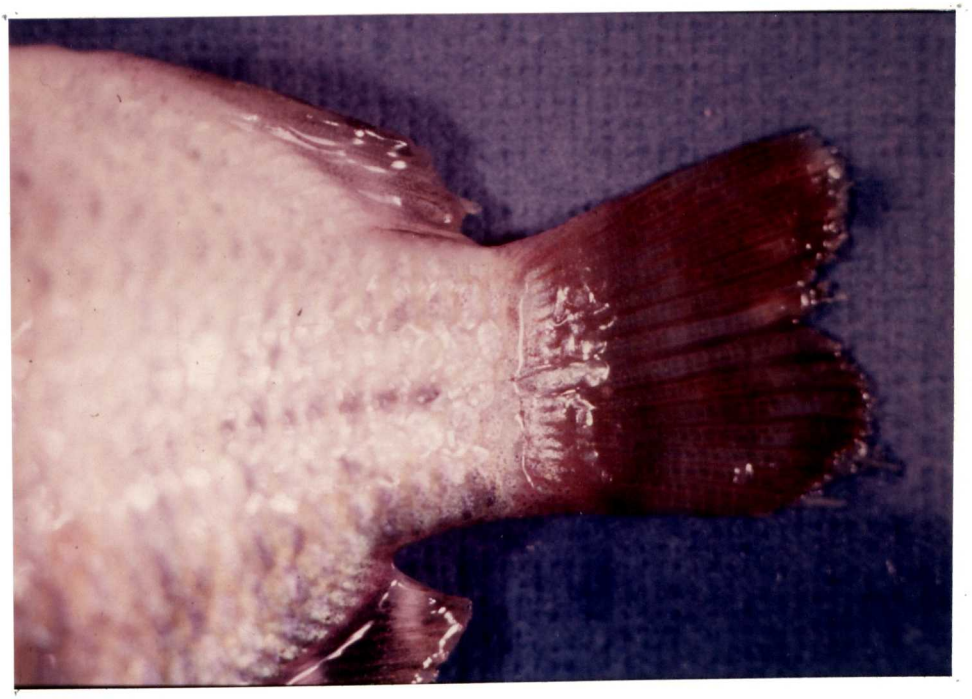
Fig. 16. Survival rates of O.niloticus over the 20 week experimental period

PLATE 13. A scorbutic fish showing exophthalmia concomittant with severe haemorrhage

PLATE 14. A scorbutic fish showing 'short body dwarfism', severe haemorrhage, fin erosion and exophthalmia

PLATE 15. Haemorrhage and erosion of the caudal fin of a scorbutic fish

PLATE 16. A dorsal view of a scorbutic fish clearly showing exophthalmia



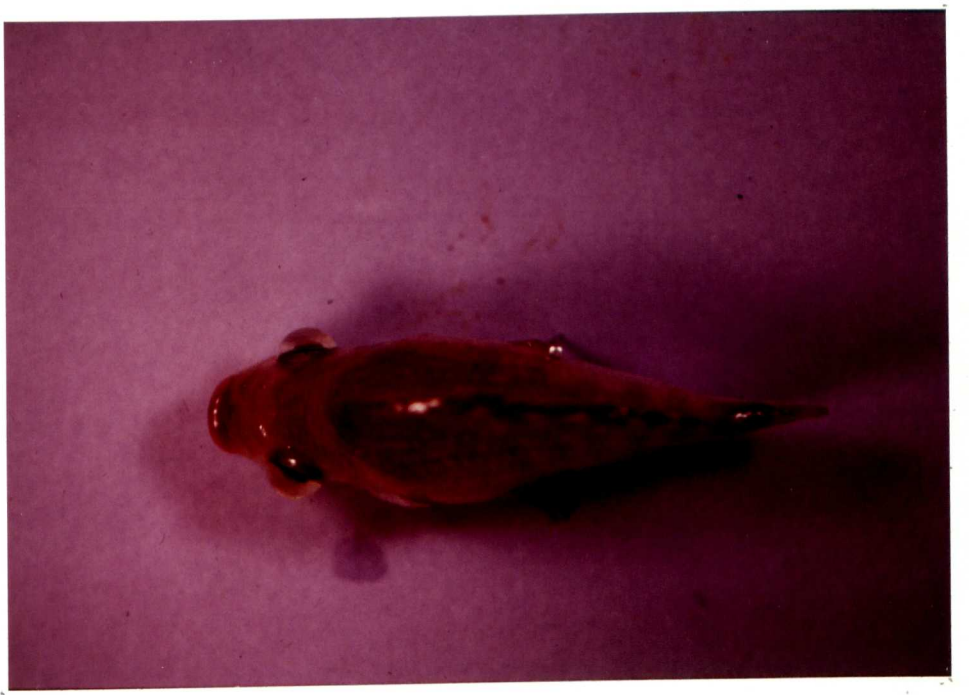
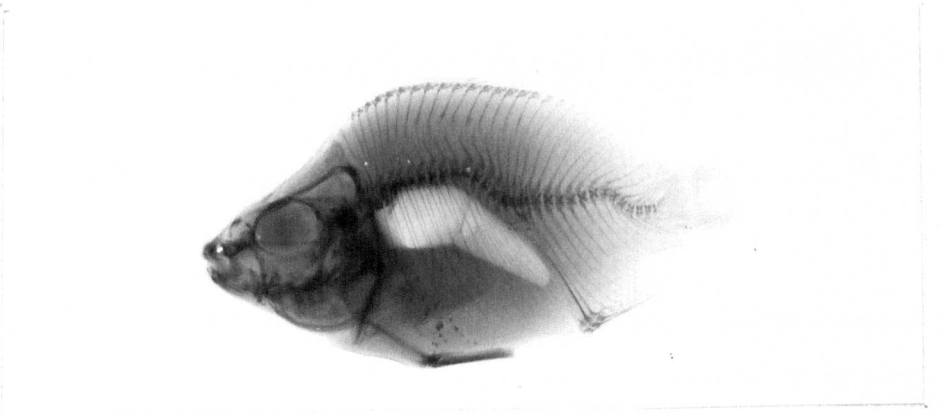


PLATE 17. A lateral x-radiogram of scorbutic fish showing severe lordosis

PLATE 18. A dorsal x-radiogram of scorbutic fish showing multiflexures and severe scoliosis



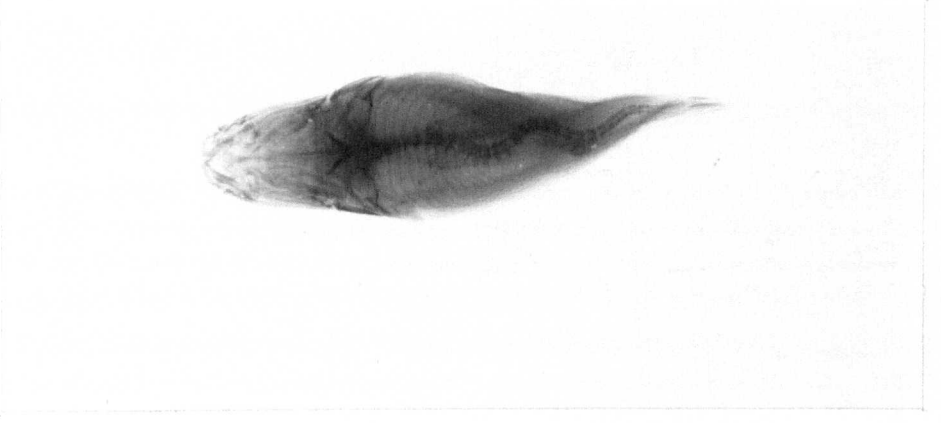
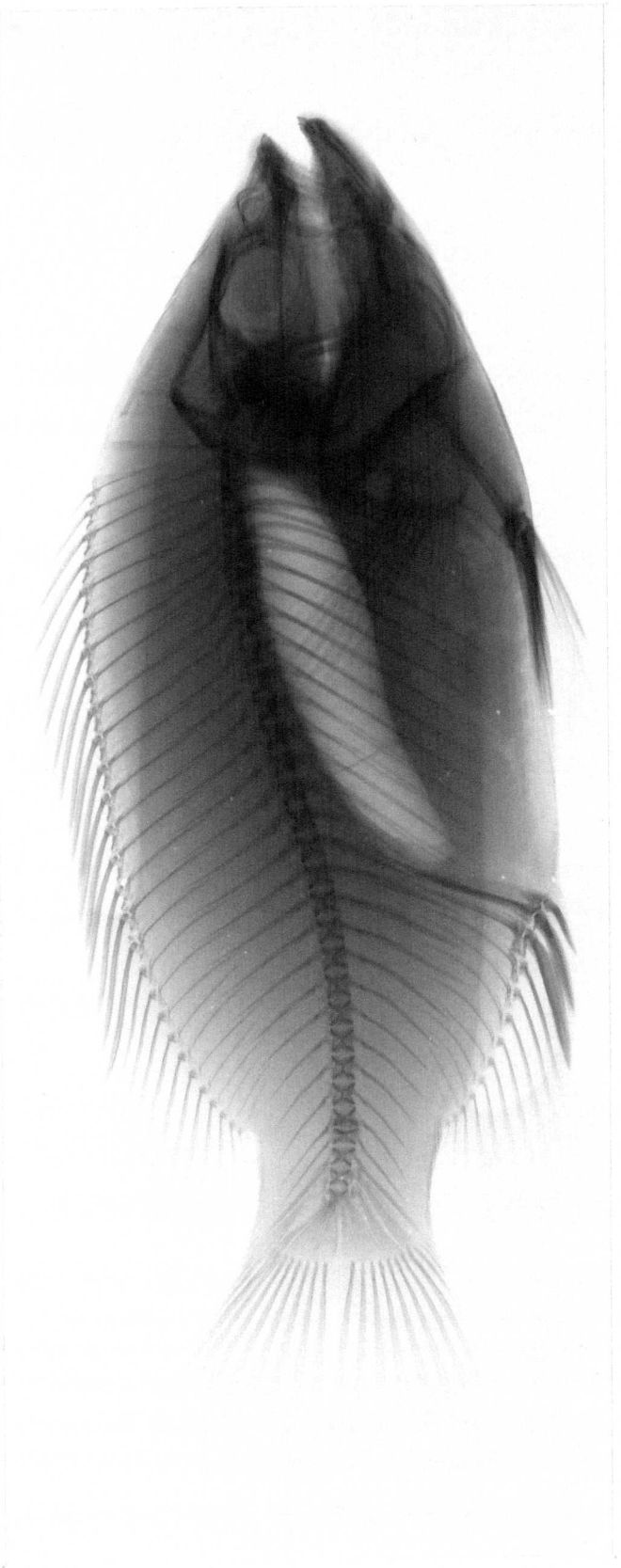


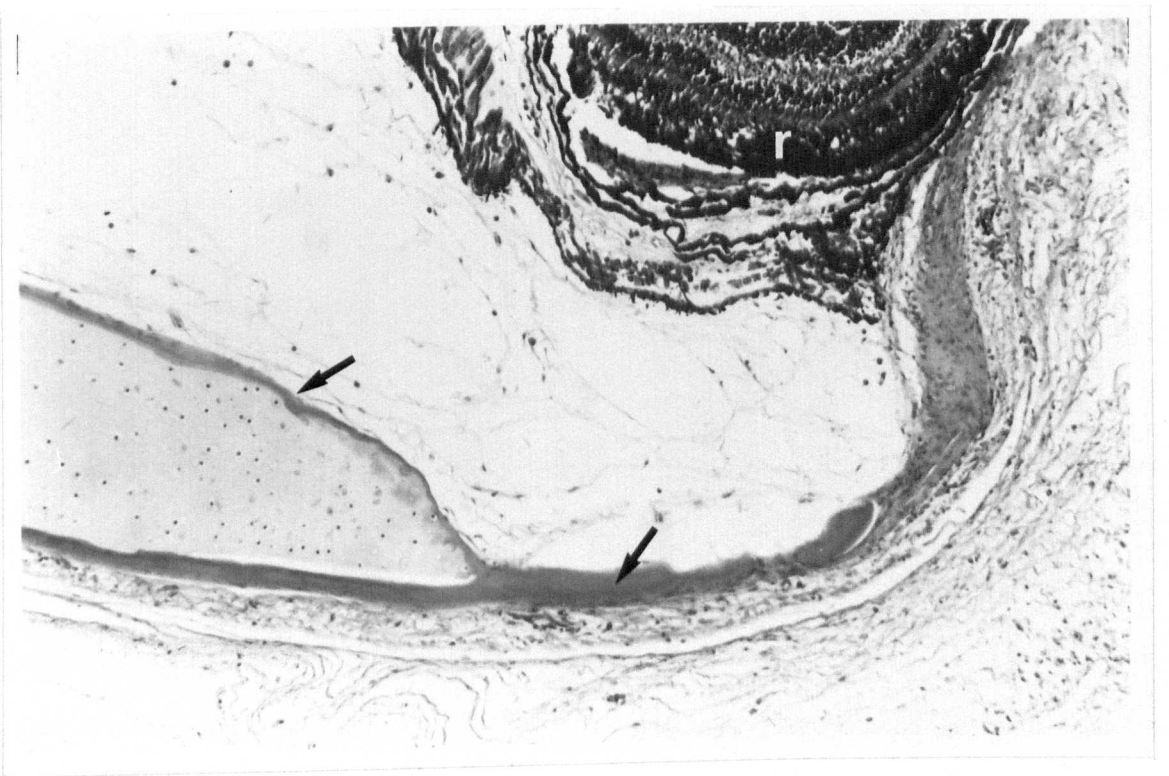
PLATE 19. A lateral x-radiogram of normal fish fed the supplemented diet



Histologically the eyes of scorbutic fish were collapsed and showed various concommitant changes such as retinal haemorrhage, intra-orbital haemorrhage and optic nerve necrosis, but these all appeared to be associated with the principal feature of orbital and scleral fibrous dystrophy and associated osteioid changes (Plate 20). The periosteum of bones was generally thickened and cellular with, in some cases such as the tips of gills (Plate 21) or edges of operculae, hyperplasia of chondrocytes with no evidence of replacement osteoid tissue. In spinal vertebrae this effect was particularly noticeable, with centres of ossification for the growing vertebrae enlarged, very cellular but with no evidence of calcification (Plate 22). These were generally the points where spinal fracture took place and intraspinal haemorrhage and necrosis of the spinal cord were apparent (Plate 23).

PLATE 20 Edge of collapsed eye showing malformed scleral connective tissue with hyaline edge (arrowed) which continues to the limbus as an irregular band. Damaged retinal tissue (r) is situated in the top of the figure (H & E x200)

PLATE 21 Distortion of tip of gill primary lamella. The distal end of the central bony support is thickened with enlarged bizarre osteocytes (O) within an irregular poorly calcified matrix (Van Giesson x300)


r

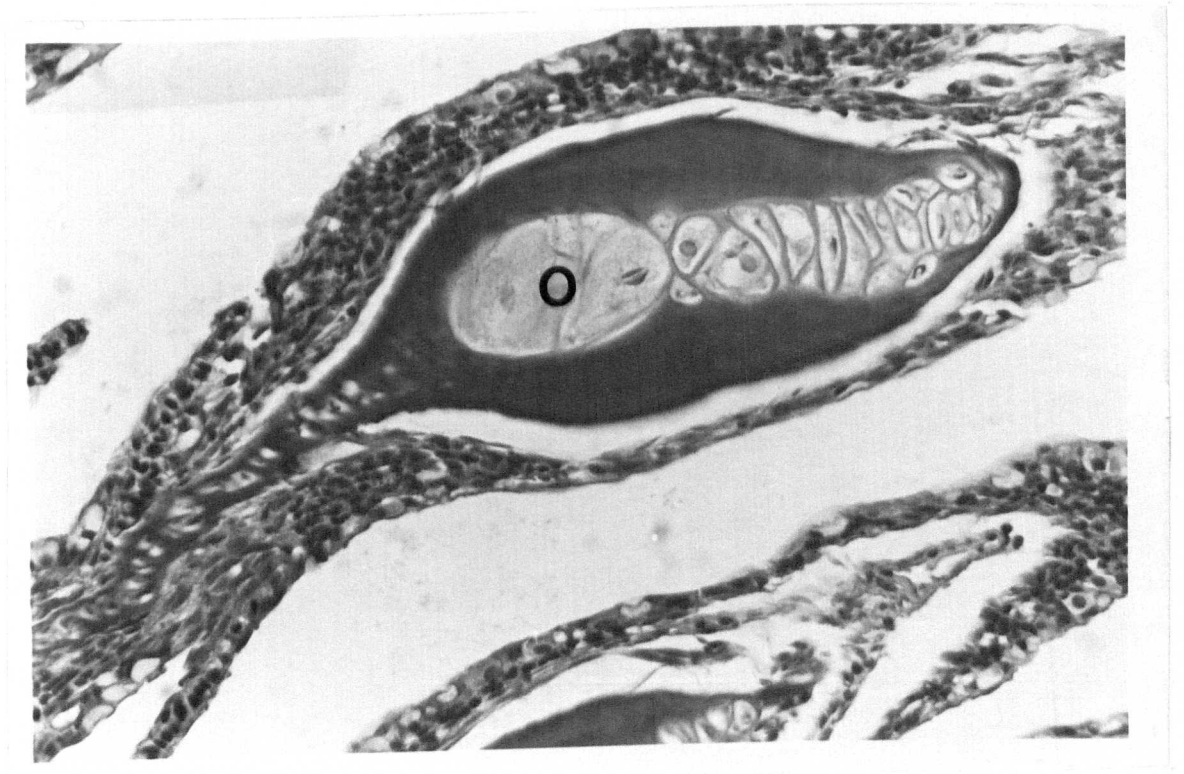
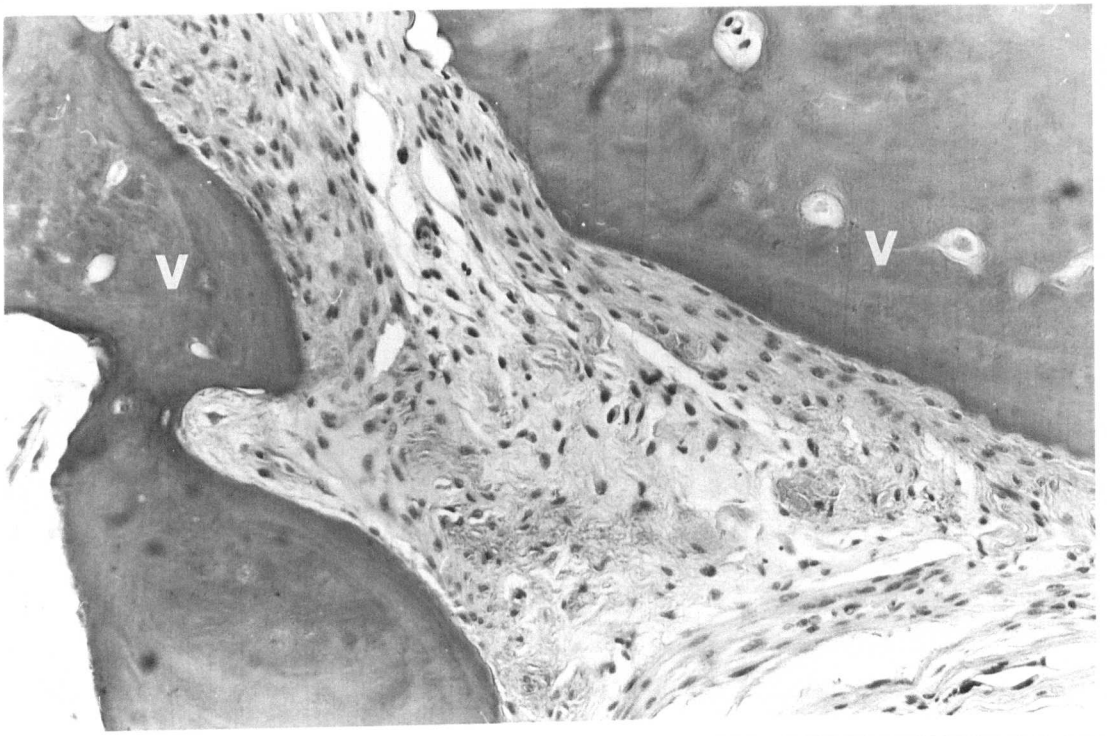
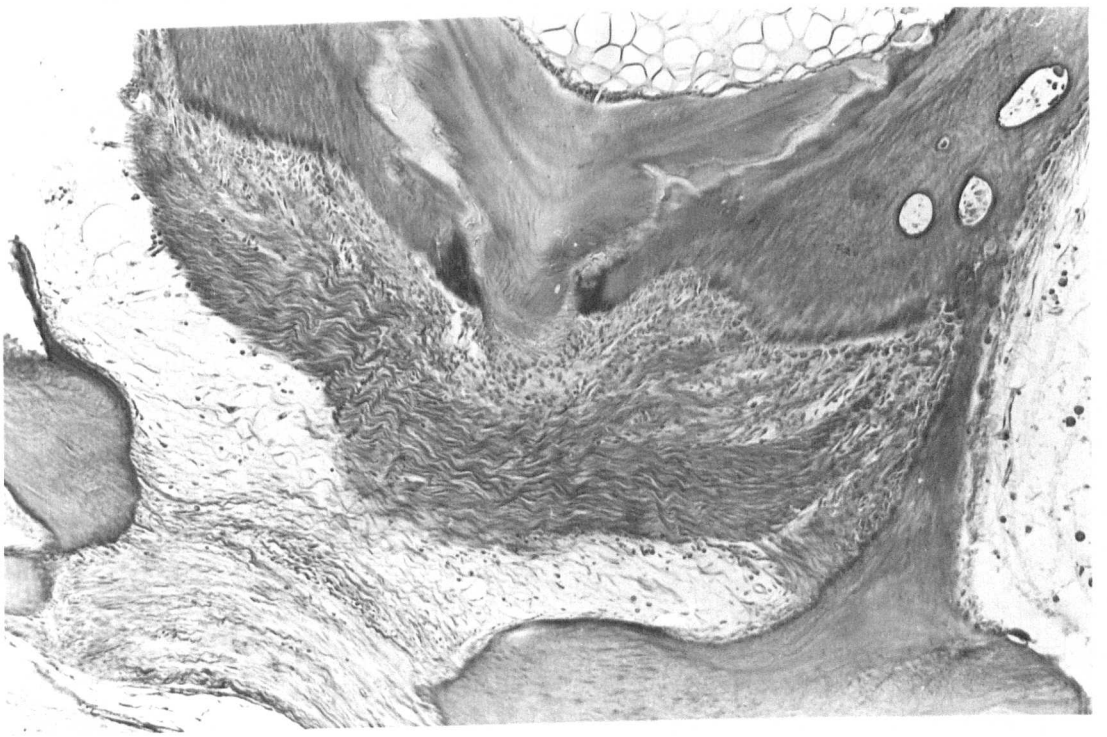

O

PLATE 22 Intervertebral area showing proliferation of highly cellular periosteal connective tissue between the two vertebrae (V) with no calcification or organisation (H & E x400)

PLATE 23 Fracture area of posterior vertebrae at 20 weeks. There is little or no osseous healing, only fibrocellular scarring (H & E x250)


V
V



SECTION 5.3 : EXPERIMENT 2

THE EFFECT OF LONG-TERM DEPRIVATION OF ASCORBIC ACID ON O. MOSSAMBICUS

Section 5.3.1 Materials and Methods

Section 5.3.1.1 Experimental system and animals

Fingerling O. mossambicus were obtained from the Institute of Aquaculture Hatchery from a genetically homogenous stock (Section 2.2). They were allotted at 35 fish/150 litre circular tank into an experimental recirculation system (Section 2.1.2) for one week prior to the start of the experiment and during this period they were fed a commercial trout diet.

Section 5.3.1.2 Diets and feeding regime

The diets employed were as for O. niloticus in Section 5.2.1.2. Fixed feeding regimes of 5%, 3% and 2% of the body weight per day (dry food/whole fish) were employed for the first 8 weeks, weeks 9-16 and weeks 17-21 respectively.

Section 5.3.1.3 Experimental methodology

Fish weighing procedure

As described for O. niloticus in previous experiment.

Proximate analysis

An initial sample of fish, 5 per tank, was sacrificed prior to start of the experiment and subjected to proximate analysis (Section 2.4.2). A final sample of 6 fish per tank for both diets was treated similarly.

Other experimental methodology

Blood parameters, digestibility studies, total ascorbate concentrations, glycogen determinations, collagen, proline and hydroxyproline and x-ray, histological studies were described before in Section 5.2.1.3. In addition, serum cholesterol was determined by method of Zlatkis et al. (1953) (Section 2.4.8).

Section 5.3.2 Statistical Analysis

Statistical analysis of data was performed using analysis of variance (Section 2.9).

Section 5.3.3 Results

The necessity of inclusion of vitamin C in diets for O. mossambicus was evaluated using the following parameters:

1 Growth response

Body weight and specific growth rate (SGR)

Fish fed the diet deficient in ascorbic acid performed as well as fish fed the diet supplemented with ascorbic acid until the fourth week of the experiment. Differences in average body weight and specific growth rate were obvious by the beginning of the fifth week.

The growth performance of O. mossambicus fed the diet deficient in and supplemented with ascorbic acid are shown in Fig. 17. The average body weight for O. mossambicus (Table 25) shows the diet supplemented with ascorbic acid to have produced significantly

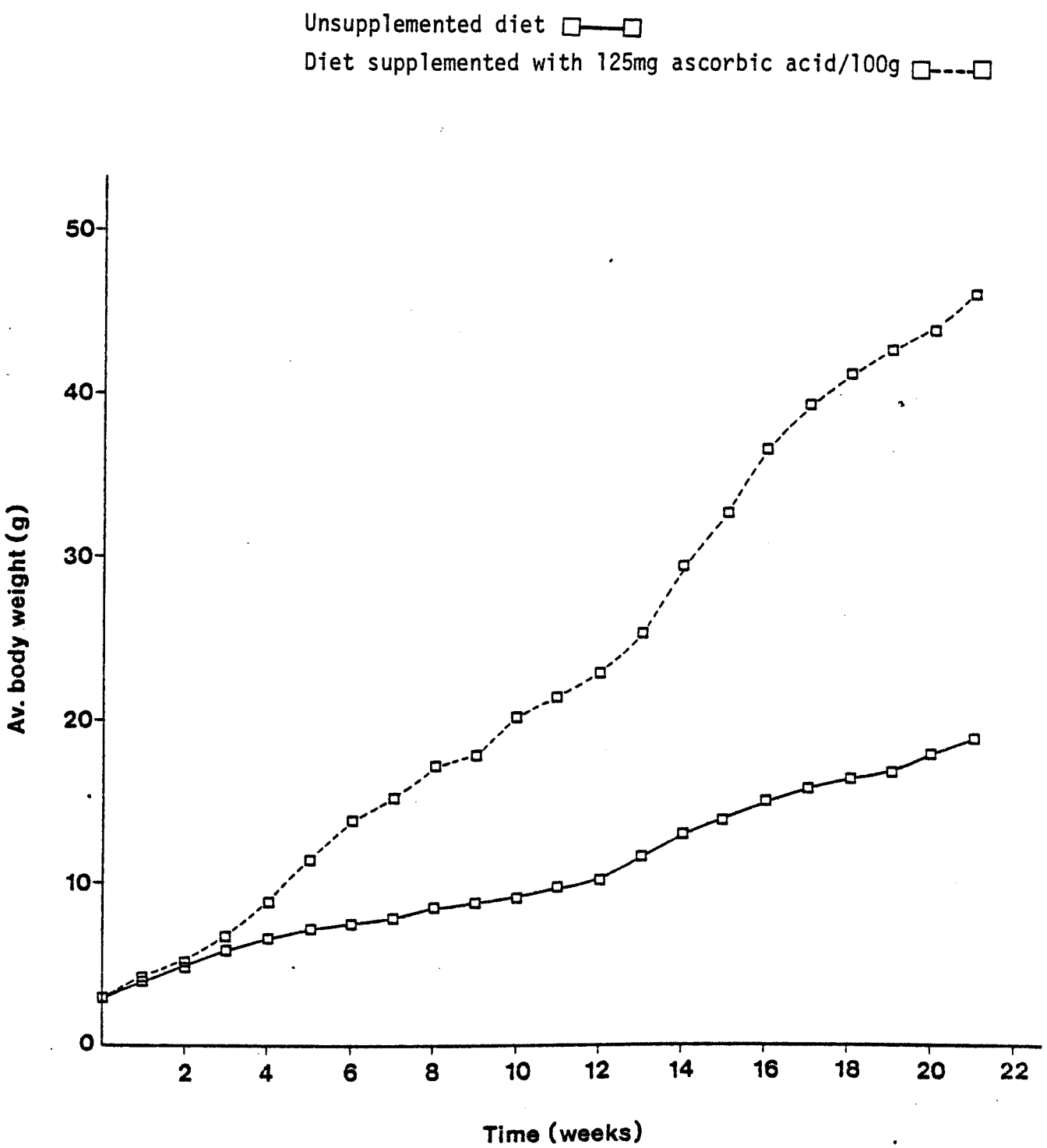


Fig. 17. Increase in average body weight of O. mossambicus fed the two experimental rations

TABLE 25. Growth and food utilization parameters[1] of O. mossambicus fed the experimental diets at 8, 16 and 21 weeks

Parameter	DIET		±SEM[2]
	Unsupplemented	Supplemented	
Initial avg.wt.g.	2.92[a]	2.94[a]	0.29
8 Weeks			
Avg.wt.g.	8.63[b]	17.32[a]	0.670
SGR[3] (% d^{-1})	1.93[b]	3.17[a]	0.130
CF[4]	4.26[a]	3.33[b]	0.070
FCR[5]	2.25[a]	1.20[b]	0.099
PER[6]	1.11[b]	2.13[a]	0.16
16 Weeks			
Avg.wt.g.	15.00[b]	36.60[a]	1.530
SGR[3] (% d^{-1})	1.45[b]	2.25[a]	0.130
CF[4]	4.00	3.33	0.070
FCR[5]	2.53[a]	1.32[b]	0.180
PER[6]	1.00[b]	1.90[a]	0.090
21 Weeks			
Avg.wt.g.	18.92[b]	46.10[a]	1.850
SGR[3] (% d^{-1})	1.26[b]	1.88[a]	0.087
CF[4]	4.05[a]	2.95[b]	0.070
FCR[5]	2.61[b]	1.88[a]	0.130
PER[6]	0.97	1.72[a]	0.050
ANPU[7] (%)	15.52[b]	33.27[a]	2.47
APD[8] (%)	73.32[b]	89.82[a]	0.320
ADMD[9] (%)	63.29[b]	76.57[a]	0.570

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)
2. Standard error of the means derived from analysis of variance
3. Specific growth rate
4. Condition factor
5. Food Conversion ratio
6. Protein efficiency ratio
7. Apparent net protein utilization
8. Apparent protein digestibility
9. Apparent dry matter digestibility

higher body weight at weeks 8, 16 and 21 (Plate 24).

SGR for fish fed the supplemented diet were significantly higher ($P < 0.01$) than for fish fed the ascorbic acid free diet (Table 25).

Condition factor (CF)

Examination of CF at weeks 8, 16 and 21 shows that fish fed the diet devoid of ascorbic acid had significantly higher condition factors than fish fed the ascorbic acid supplemented diet (Table 25).

2 Food utilization

Food conversion ratio (FCR)

FCR for fish fed the diet deficient in ascorbic acid was significantly ($P < 0.01$) higher than for fish fed the diet containing vitamin C (Table 25).

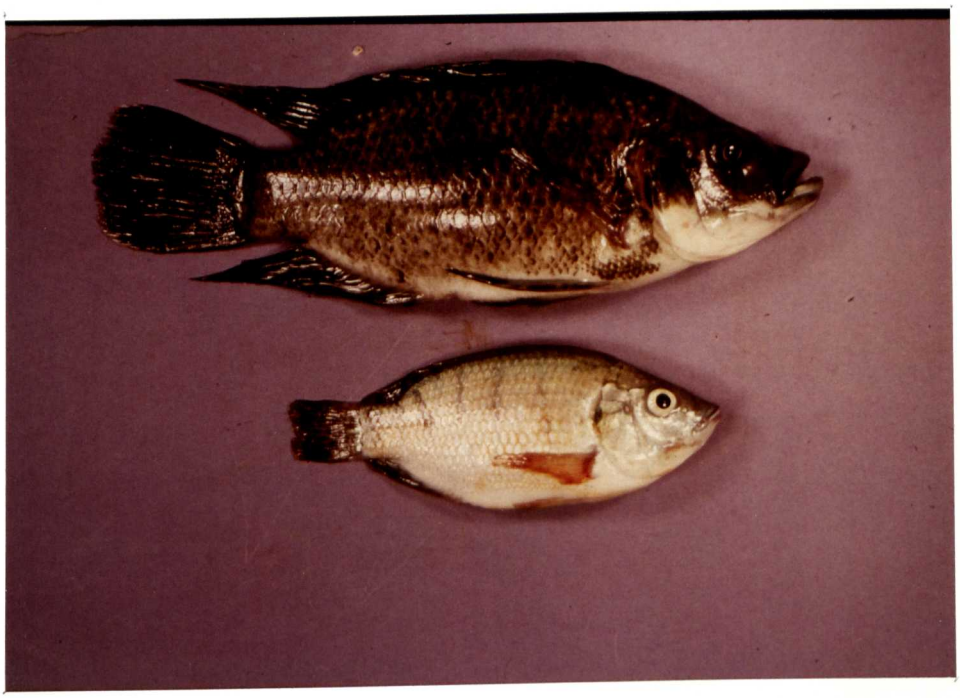
Protein efficiency ratio (PER)

PER's recorded for fish fed the diet containing no ascorbic acid were significantly ($P < 0.01$) lower than values recorded for fish fed the supplemented diet (Table 25).

Apparent net protein utilization (ANPU), Apparent protein digestibility (APD) and Apparent dry matter digestibility (ADMD)

The effect of the dietary ascorbic acid on ANPU, APD and ADMD is shown in Table 25. Fish fed the unsupplemented diet manifested

PLATE 24. Superiority of growth of a fish fed the diet supplemented with ascorbic acid (Top) in comparison to the poor growth of a fish fed the unsupplemented diet (Bottom)



significantly ($P < 0.01$) lower ANPU, APD and ADMD values than fish fed the supplemented diet.

3 Gross body composition

Gross body composition data is shown in Table 26. Fish fed the diet devoid of ascorbic acid showed increased total moisture and decreased crude protein, crude lipid and ash contents whereas the reverse was true with fish fed the ascorbic acid supplemented diet, the differences were highly significant ($P < 0.01$).

4 Tissue and biochemical changes

The results of blood parameters, hepatosomatic indices, liver and muscle glycogen and total ascorbate levels in 11 tissues are shown in Table 27. Values for haematocrit, haemoglobin and MCHC were significantly lowered in fish fed the unsupplemented diet. In contrast, serum cholesterol and both serum glutamic oxaloacetic transaminase and serum glutamine pyruvic transaminase were significantly ($P < 0.01$) elevated in the serum of fish fed the unsupplemented diet (Table 27).

Scorbutic fish exhibited significantly ($P < 0.01$) lower hepatosomatic index and liver and muscle glycogen. Lower levels of total ascorbate were detected in tissues of scorbutic fish (Table 27 and Fig. 18), especially in muscle, gall-bladder and liver.

The results of collagen, proline, hydroxyproline determination and hydroxyproline:proline ratio of the vertebrae and tails of fish

TABLE 26. Gross body composition data[1] (as % wet weight)

Parameter	DIET			±SEM[2]
	F°	Unsupplemented	Supplemented	
Moisture (%)	79.05	75.90[a]	70.69[b]	0.20
Ash (%)	2.56	4.72[b]	5.68[a]	0.15
Crude lipid (%)	4.31	3.96[b]	4.64[a]	0.11
Crude protein (%)	14.09	15.29[b]	18.84[a]	0.15

F° Body composition sample of fish analysed at the beginning of the experiment

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)

2. Standard error of the means derived from analysis of variance

TABLE 27. Blood parameters, hepatosomatic index, liver and muscle glycogen and total tissue ascorbate concentrations[1] of O. mossambicus fed unsupplemented and supplemented diets after 21 weeks

Parameters	DIET		±SEM[2]
	Unsupplemented	Supplemented	
1 Blood parameters			
HCT[3], %	31.92[b]	40.58[a]	0.313
HB[4], gdl^{-1}	7.81[b]	11.71[a]	0.177
MCHC[5], %	24.47	28.86	
Serum cholesterol, $mgdl^{-1}$	324.00[a]	243.75[b]	1.92
Serum GOT[6]	196.93[a]	107.22[b]	2.09
Serum GPT[7]	127.00[a]	91.92[b]	1.27
GOT/GPT	1.60[a]	1.17[b]	0.024
2 Hepatosomatic index and glycogen			
HSI[8], %	1.23[b]	1.92[a]	0.104
LG[9], %/wet wt.	4.42[b]	19.39[a]	0.337
MG[10], %/wet wt.	0.293[b]	1.02[a]	0.043
3 Tissue ascorbate concentrations ($\mu g.g^{-1}$)			
Ovary	65.26[b]	429.39[a]	6.16
Gills	42.60[b]	133.12[a]	1.96
Eyes	18.27[b]	74.61[a]	0.92
Testis	28.50[b]	271.93[a]	12.75
Liver	11.93[b]	74.0[a]	3.66
Brain	65.11[b]	336.78[a]	8.93
Heart	25.83[b]	107.69[a]	3.17
Alimenary canal	19.77[b]	102.12[a]	2.96
Muscle	3.28[b]	39.52[a]	0.250
Gall-bladder	6.38[b]	49.42[a]	1.480
Kidney	24.28[b]	74.61[a]	1.830

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)
2. Standard error of the means derived from analysis of variance
3. Haematocrit
4. Haemoglobin
5. Mean corpuscular haemoglobin concs.
6. Glutamic oxaloacetic transaminase
7. Glutamic pyruvic transaminase
8. Hepatosomatic index
9. Liver glycogen
10. Muscle glycogen

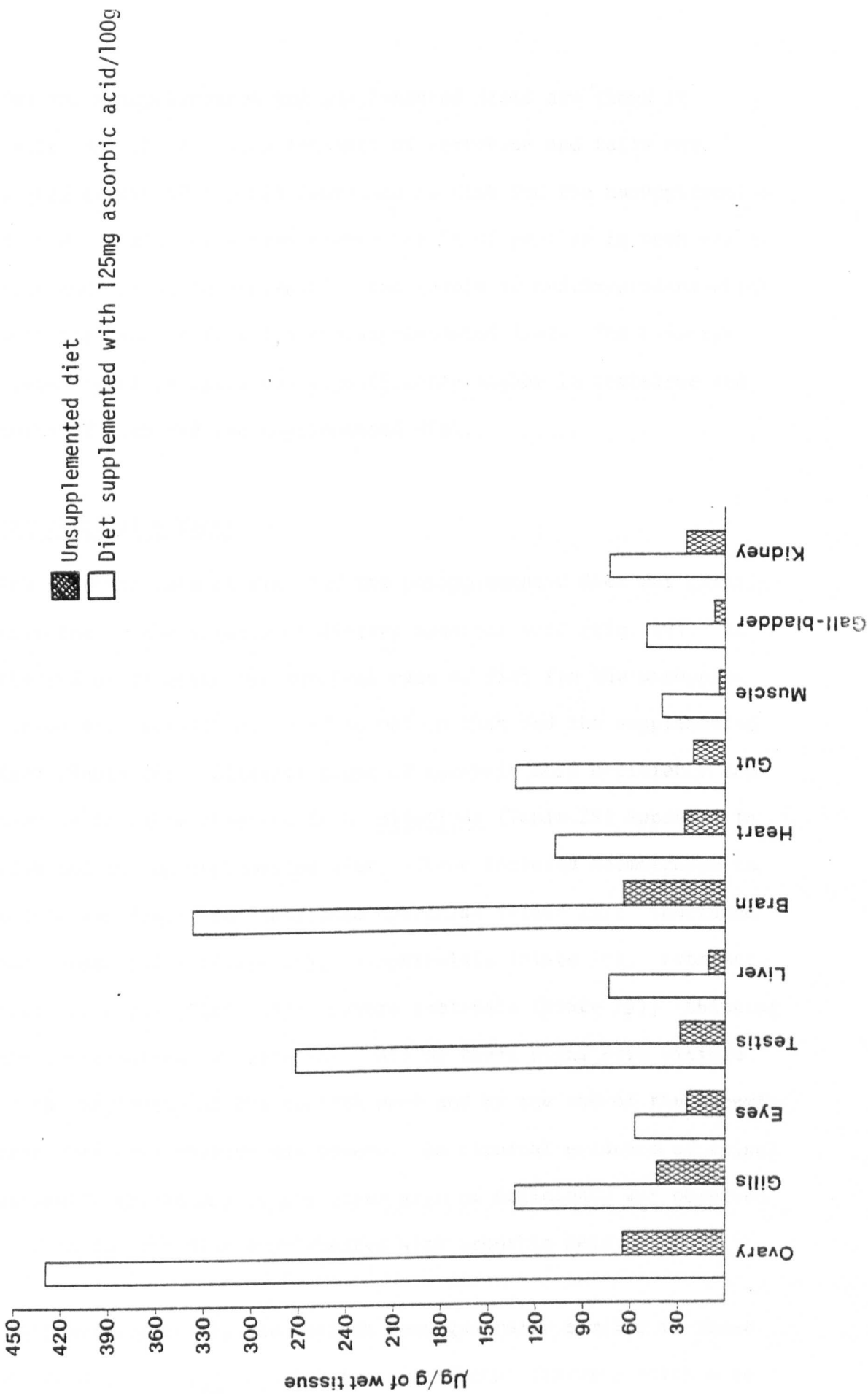


Fig 18 Total tissue ascorbate concentrations for O.mossambicus after 21 weeks on the experimental diets.

fed the unsupplemented and supplemented diets are shown in Table 28. The collagen contents of vertebrae and tails were significantly ($P < 0.01$) depressed in fish fed the unsupplemented diet which also exhibited higher levels of proline in both vertebrae and tails, in contrast to the levels of hydroxyproline which were elevated in fish fed the supplemented diet. The hydroxyproline:proline ratio was significantly higher in vertebrae and tails of fish fed the supplemented diet.

5 Pathological effects

The survival rate of fish fed the unsupplemented diet was greatly affected by the absence of dietary ascorbic acid (Fig. 19). At the end of 21 weeks the survival rate of fish fed the unsupplemented diet was 52% compared to 98% in fish fed the supplemented diet (Table 28). Clinical signs of ascorbic acid deficiency comparable to those observed in O. niloticus (Table 28) appeared in fish fed the unsupplemented diet. These included haemorrhage in muscle and fins; foreshortened operculae (Plate 25); shortened and eroded tails (Plate 26); exophthalmia (Plate 27); cataract, acute lordosis (Plate 28); severe scoliosis (Plate 29); thinning and condensation of vertebrae. All of these signs were visible by the beginning of the twelfth week and by the end of the experiment they were obvious and severe. No clinical evidence of spinal deformity (Plate 30) or any other sign of deficiency was observed in fish fed the diet supplemented with ascorbic acid.

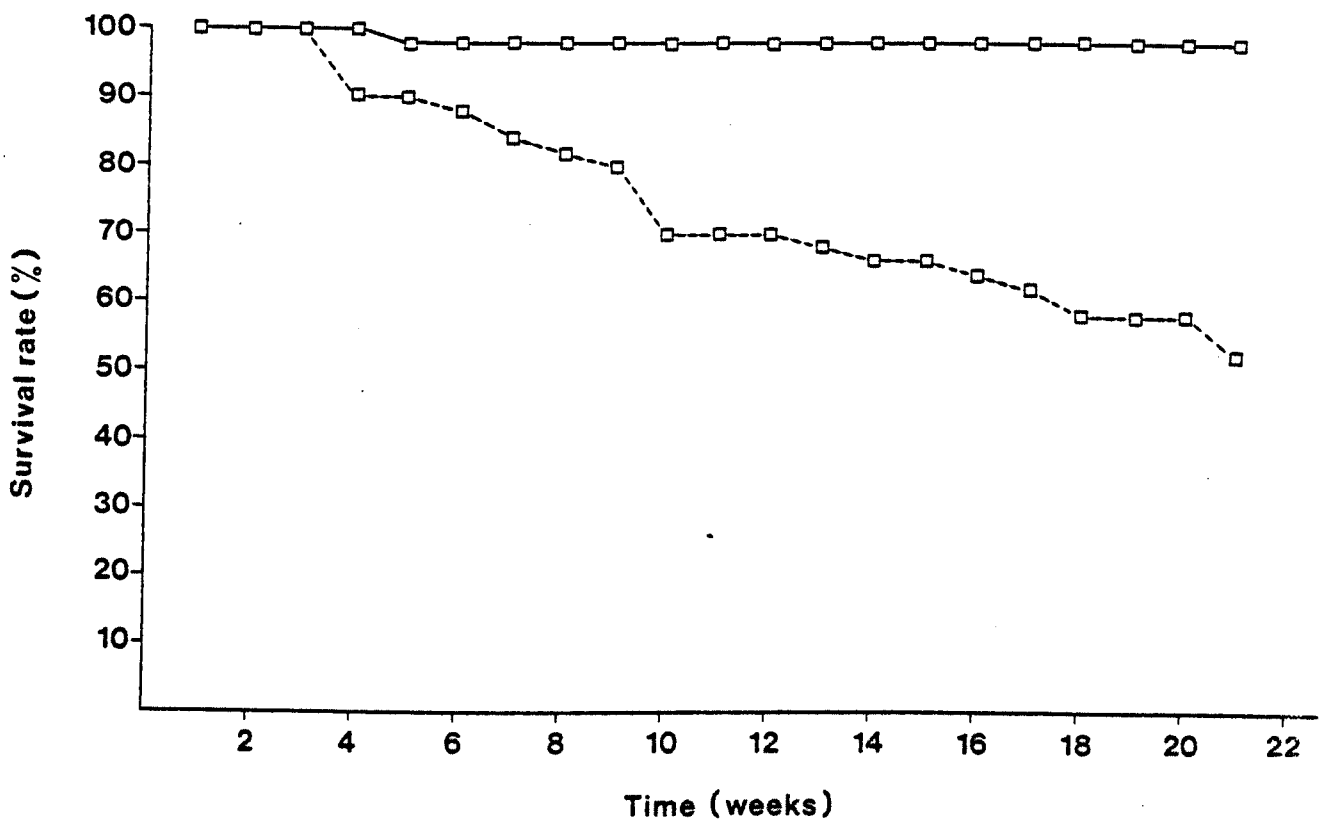
Histopathologically, the lesions were generally similar to those observed in O. niloticus but two significant features which were

TABLE 28. Composition of vertebrae and tails together with incidence of gross clinical signs of deficiency[1]

Parameter	DIET		±SEM[2]
	Unsupplemented	Supplemented	
1 Vertebrae[3]			
Collagen (%)	18.94[b]	41.69[a]	0.94
Proline (mg/g tissue)	43.53	40.31	0.63
Hydroxyproline (mg/g tissue)	30.71	38.50	0.85
Hydroxyproline/proline	0.705	0.955	0.016
2 Tail[4]			
Collagen (%)	9.38[b]	15.09[a]	0.167
Proline (mg/g tissue)	4.99[a]	4.12[b]	0.090
Hydroxyproline (mg/g tissue)	2.60[b]	3.17[a]	0.060
Hydroxyproline/proline	0.52[b]	0.77[a]	0.010
3 Gross clinical signs of vitamin C deficiency			
Survival rate (%)	52[a]	98	1.41
Haemorrhagic signs (%)	50[a]	00.00[b]	2.47
Short & weak operculum (%)	50[a]	00.00[b]	2.47
Short & tail erosion (%)	58.0[a]	00.00[b]	2.47
Exophthalmia (%)	42[a]	00.00[b]	2.83
Cataract (%)	19[a]	00.00[b]	2.82
Lordosis & scoliosis (%)	52[a]	00.00[b]	0.71
Ascites	19[a]	00.00[b]	2.82

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)
2. Standard error of the means derived from analysis of variance
3. Defatted basis
4. Wet basis

Fig 19 Survival rate of O.mossambicus over 21 week experimental period.

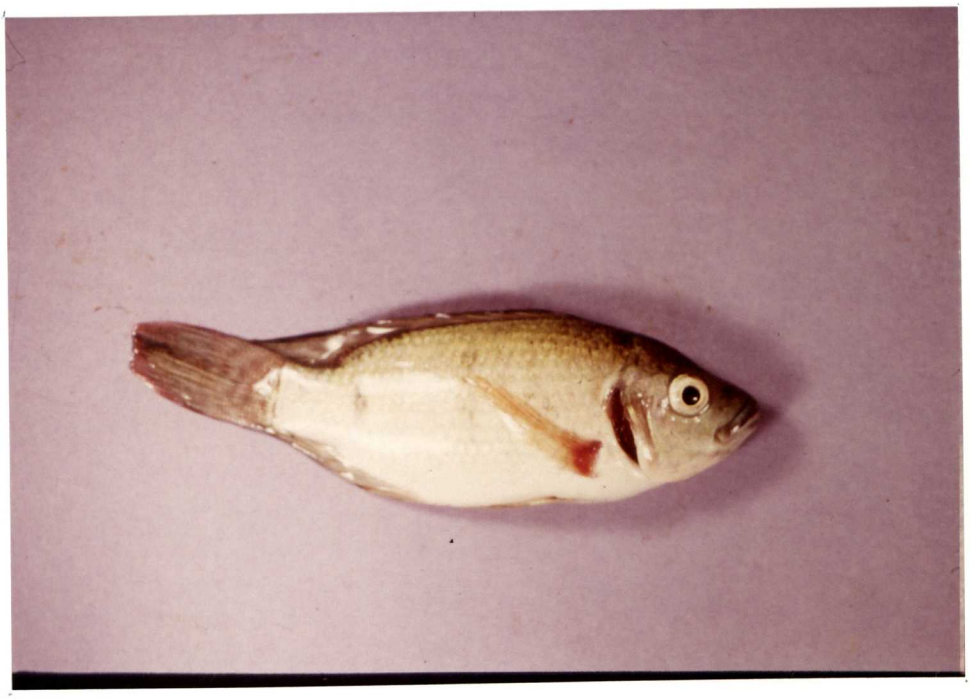


Unsupplemented diet □---□

Diet supplemented with 125mg ascorbic acid/100g □—□

PLATE 25. A photograph of scorbutic O. mossambicus showing shortening of operculum

PLATE 26. A photograph of O. mossambicus fed a diet deficient in ascorbic acid exhibiting a short and eroded tail concommittant with severe haemorrhage



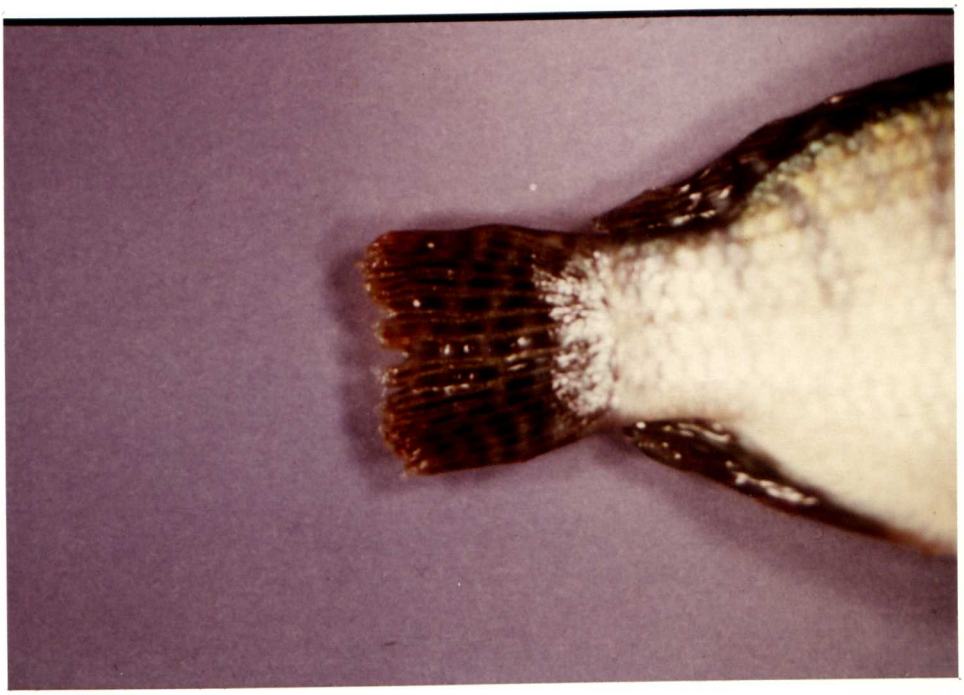
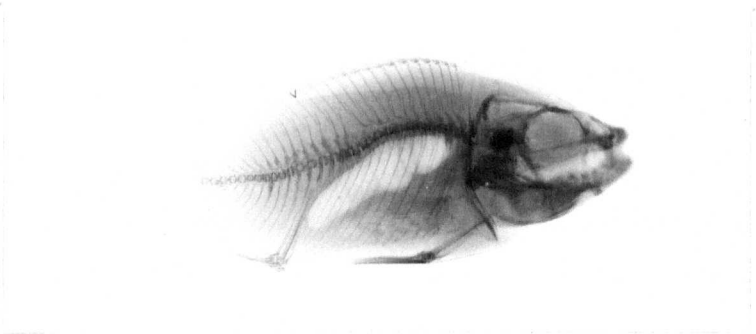


PLATE 27. Scorbutic O. mossambicus showing exophthalmia and haemorrhage in dorsal fin and muscle

PLATE 28. A lateral x-radiogram of scorbutic O. mossambicus showing condensation and thinning of vertebrae with severe lordosis

PLATE 29. A dorsal x-radiogram of scorbutic O. mossambicus reveals degenerative changes in the thoracic vertebrae (arrowed A) and a vertebral fracture (arrowed B) with severe scoliosis



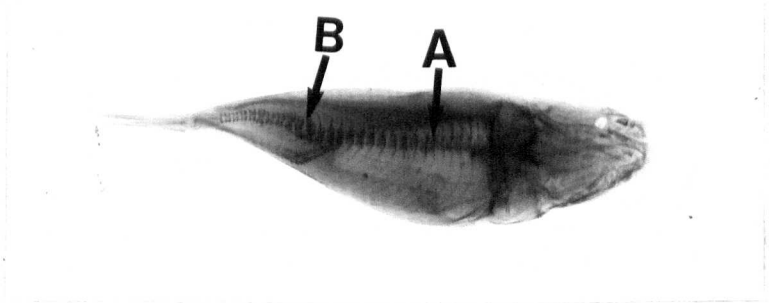
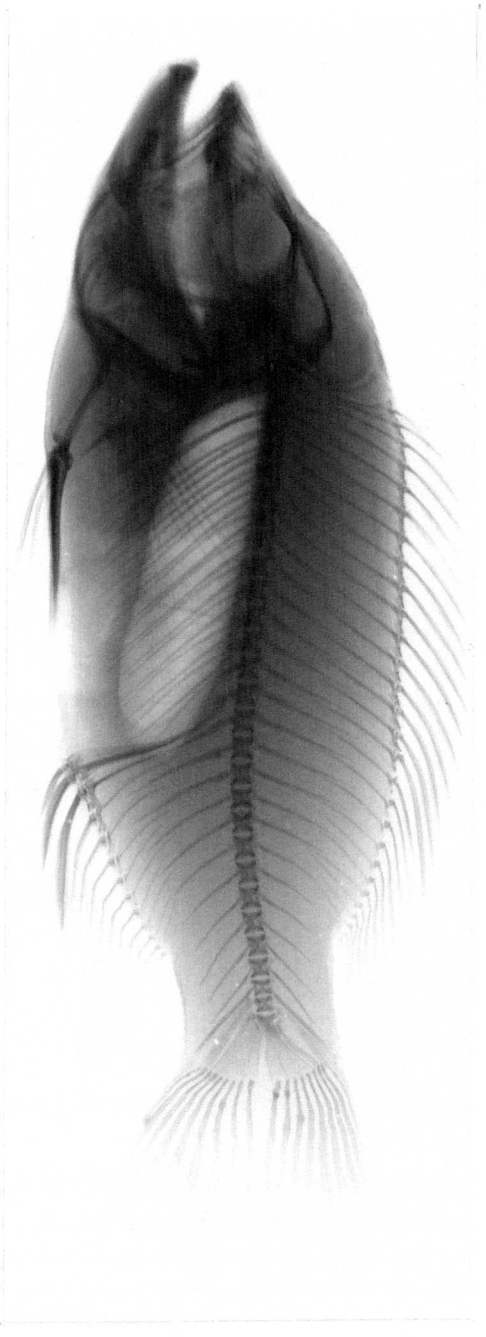

B
A

PLATE 30. A lateral x-radiogram of O. mossambicus fed diet supplemented with ascorbic acid



not observed in the former species were that there was significant hyperplasia of gill secondary lamellae epithelial cells compared with the gills of controls (Plate 31) and also a mild but noticeable leucocyte infiltration of the peritoneal lipid tissue producing a pronounced steatitis (Plate 32).

SECTION 5.4 : DISCUSSION

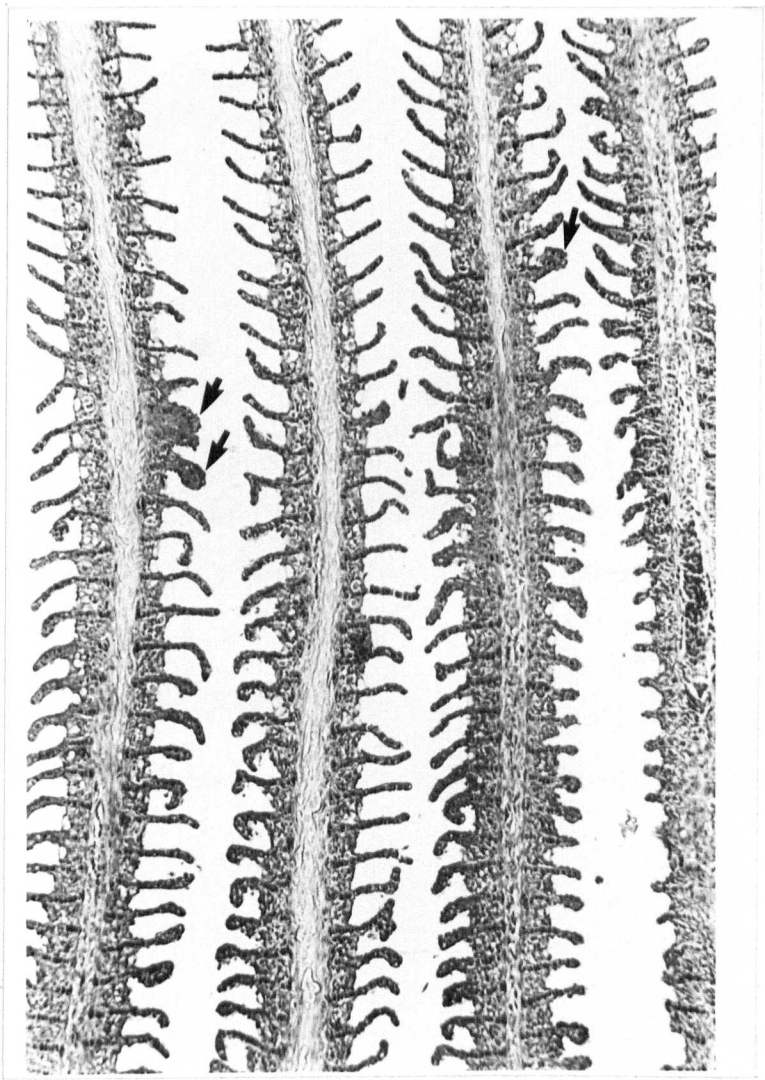
The results of the present 20 and 21 week studies establish the role of dietary ascorbic acid for *Oreochromis niloticus* and *O. mossambicus* for good growth and fish health.

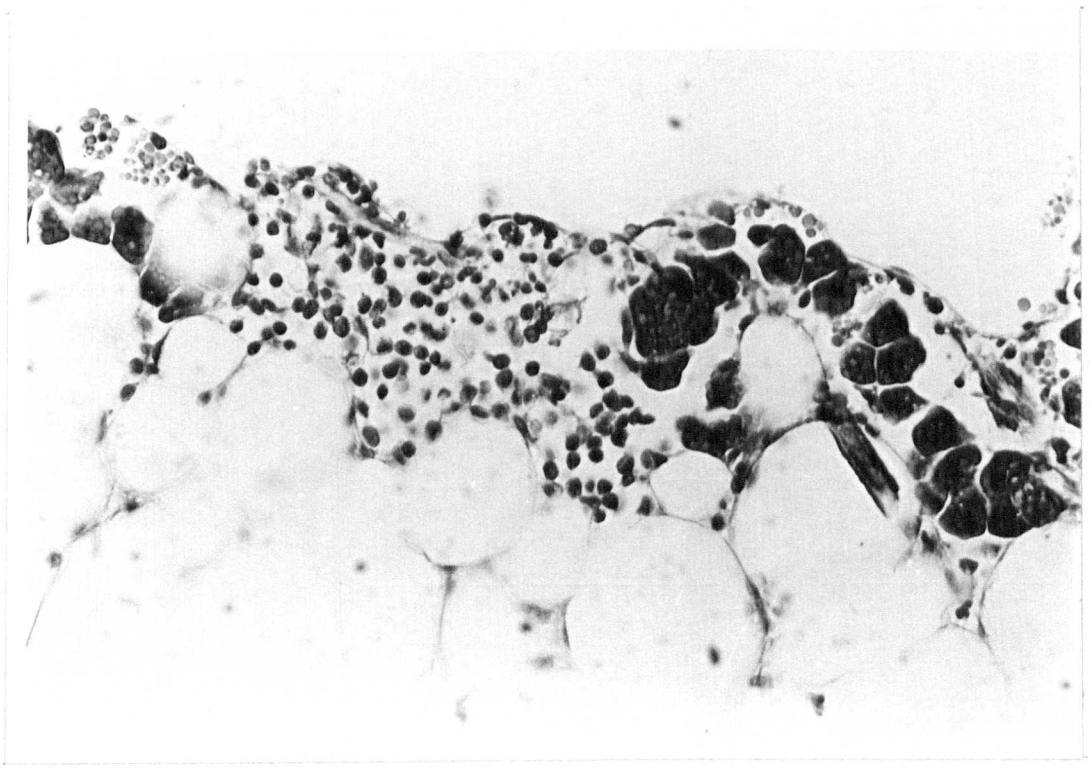
The severe reduction in growth and poor food conversion ratios obtained in fish fed the unsupplemented diet with ascorbic acid, were possibly attributable to the anorexia and reduced activity observed in these fishes (Tables 21 & 25 and Figs. 14 & 17). These findings are similar to those reported by other workers for various species (Sakaguchi *et al.*, 1969; Arai *et al.*, 1972; Lovell, 1973; Wilson & Poe, 1973; Andrews & Murai, 1975; Halver *et al.*, 1975; Lim & Lovell, 1978; Sato *et al.*, 1978a; John *et al.*, 1979; Mahajan & Agrawal, 1979, 1980a. Evans and Hughes (1963) suggested that reduced growth in scorbutic guinea pigs may be related to specific processes of tissue formation and that, in addition, poor growth was related to general weakness and tissue break-down. However, there was no apparent increase in melano-macrophage centre activity, a normal feature of excessive catabolism in fish.

No reports appear in the literature concerning the effect of ascorbic acid on protein efficiency ratio and apparent net protein utilization

PLATE 31. Thickening of primary lamellae and hyperplasia of secondary lamellar cells (arrowed) (H & E x10)

PLATE 32. Mild monocytic infiltration of peri-acinar lipid tissue (H & E x250)





in fish. Ram (1966) showed that in guinea pigs ascorbic acid affected growth in two ways, firstly by a lowered food consumption and secondly by a specific effect on growth. In the present studies O. niloticus and O. mossambicus fed diets deficient in ascorbic acid gained less weight than those fed supplemented diets thus the total amount of protein deposited was markedly affected. In addition, ascorbic acid deficiency depressed protein utilization as indicated by PER and ANPU.

Ascorbic acid plays an important metabolic role in certain aspects of protein metabolism (Williams & Hughes, 1972) and is a major cofactor in the biosynthesis of collagen (Stone & Meister, 1962; Barnes, 1975). Chatterjee (1967) reported that ascorbic acid functions in the metabolism of aromatic amino acids and the formation of plasma protein. Sealock and Silberstein (1940) reported that scorbutic guinea pigs receiving large amounts of dietary tyrosine excreted increased amounts of 4-hydroxyphenyl pyruvate and homogentisate in the urine and that these excretions ceased after administration of ascorbic acid. Salmon and May (1952) suggested that total plasma protein was decreased in scorbutic monkeys and Rohatgi et al. (1958) reported that ascorbic acid plays a fundamental role in the synthesis of liver protein in scorbutic monkeys by enhancing the utilization of circulating amino acids. In addition, in the present studies apparent protein digestibility was significantly ($P < 0.01$) higher in fish fed diets supplemented with ascorbic acid (Tables 21 and 25), reasons for this are not evident and such results have not been reported by other workers in this field.

The glycogen contents of liver and muscle of O. niloticus and O. mossambicus were affected by dietary ascorbic acid in the present studies with the absence of the vitamin resulting in lowered levels. Banerjee (1943a) and Chadwick et al. (1973) reported that guinea pigs fed diets deficient in ascorbic acid showed a decrease in liver glycogen as compared to those fed supplemented diets. The present studies also show that hepatosomatic indices decreased in parallel with the observed decrease in liver glycogen in fish fed the unsupplemented diets. Banerjee (1944) reported that scorbutic guinea pigs exhibited increased numbers of α-cells whereas β-cell numbers were decreased, in the islets of Langerhans. As a result of these observations, Banerjee and Divakaran (1958) attributed disordered carbohydrate metabolism (e.g. diminished liver glycogen) in scorbutic guinea pigs to diminished insulin production in the pancreas.

Almost all studies dealing with ascorbic acid deficiency in fish or other animals have agreed that deletion of ascorbic acid from the diets results in a decrease in haemoglobin, haematocrit and MCHC values and the present studies concur. The values obtained here are similar to those obtained for major carp (Cirrhina mrigala) (Agrawal & Mahajan, 1980a) where fish fed diets deficient in ascorbic acid gave haematocrit, haemoglobin and MCHC values of 26.34, 5, and 21.75 respectively, with the corresponding values for fish fed an ascorbic acid supplemented diet being 30.43, 8.0 and 26.03. This anaemia may have resulted from the severe haemorrhage which appeared in fish fed the unsupplemented diets in the present studies as well as from a disturbance in iron metabolism, possibly impaired absorption (Van Campen, 1972; Gipp et al., 1974 and Monsen & Page, 1978) and/or

impaired iron release from reticuloendothelial stores (Bothwell et al., 1964). In addition, it has been suggested that ascorbic acid has a direct role in the synthesis of haemoglobin (Banerjee & Chakrabarty, 1965; Chikaraishi, 1982).

Transamination is a very important metabolic process in the liver with the principal enzymes being glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT). It has been suggested that serum GOT activity (SGOT) can be used as a diagnostic tool in clinical medicine (Hardy et al., 1979). Both of these enzymes show higher activities when animals are fed diets containing oxidized oils or deficient in ascorbic acid. Sakaguchi and Hamaguchi (1969) and Iijima and Zama (1978) reported that GOT and GPT levels were higher in the serum of yellow tail and carp fed diets containing oxidized oils than in fish fed diets containing fresh oils. These enzymes showed higher activities (265 and 180 equivalent units) in O. mossambicus fed diets deficient in ascorbic acid compared to 235 and 160 equivalent units in fish fed diets supplemented with ascorbic acid (Bosusiannah, 1981). In the present studies activity of these enzymes was elevated by ascorbic acid deficiency (Tables 23 and 27). In addition, it has been reported by Srivastava and Sirohi (1969) that ascorbic acid reduces the activity of glutamic oxaloacetic transaminase.

Contradictory results concerning the effect of ascorbic acid on cholesterol metabolism in animals have been reported (Banerjee & Singh, 1958; Hodges et al., 1969, 1971; Ginter, 1975). In the present study O. mossambicus fed the ascorbic acid free diet appeared to have

hypercholesterolaemia. Similar results were reported in scorbutic rainbow trout by John et al. (1979) and liver hypercholesterolaemia was reported in scorbutic Channa punctatus (Bloch) by Mahajan and Agrawal (1979). Hypercholesterolaemia is possibly due to impairment of transformation of cholesterol into bile acids (Ginter, 1973, 1975).

Determination of ascorbic acid levels in the tissues of an animal is the first step towards gauging the vitamin involvement in overall metabolism (Hornig, 1975b). The severe haemorrhages which appeared in muscle, fins and eyes of O. niloticus and O. mossambicus fed diets devoid of ascorbic acid in the present studies may be related to the lowering of levels of ascorbic acid found in these tissues (Tables 23 and 27, Figures 15 and 18). The lower tissue levels of ascorbic acid in both species fed diets deficient in ascorbic acid are an indication of the dependence of O. niloticus and O. mossambicus on a dietary source of ascorbic acid. It has been suggested that the ascorbic acid content of the liver can be used as a criterion or index of ascorbic acid status of fish, if the liver ascorbic acid concentration is less than 20µg/g wet tissue then ascorbic acid nutrition is marginal or poor (Hilton et al., 1977a). This suggestion is supported by the results of the present studies where the levels of ascorbic acid in the liver of O. niloticus and O. mossambicus fed the unsupplemented diet were 2.51 and 11.93µg/g wet tissue respectively.

Collagen is the major constituent of the matrix of bone and ligaments. In collagen synthesis ascorbic acid is a co-factor required for the hydroxylation of proline and lysine (Gould, 1960; Mussini et al., 1967; Barnes & Kodicek, 1972; Sen Gupta & Deb, 1978). Any significant

decrease in collagen formation or density results in failure in the deposition of new bone tissue to replace cartilaginous precursor centres of ossification. There is no loss of calcium from newly established bone as in vitamin D deficiency in fish, but in growing fish, the failure of osteoblast activity to lay down new bone, plus the lack of collagen production for supporting ligaments, results particularly in vertebral failure. Usually this is at the lower spinal level where muscular or weakened spinal asticulatious support is less available. In the present studies fish fed the unsupplemented diets showed a high level of lordosis and scoliosis and the collagen contents (on a defatted basis) were significantly ($P < 0.01$) lower than the collagen contents of vertebrae of fish fed the ascorbic acid supplemented diets (Tables 24 and 28) as were the levels of hydroxyproline. Similar results have been obtained for channel catfish (Wilson & Poe, 1973; Lim & Lovell, 1978).

In the present studies O. niloticus and O. mossambicus fed diets devoid of ascorbic acid exhibited significantly ($P < 0.01$) higher condition factors indicating shortening of the body and severe damage to spinal columns as was shown in the x-rays taken (Plates 17, 18,29 and 30). Poston (1967) reported that scorbutic brook trout exhibited higher condition factors than fish fed diets supplemented with ascorbic acid.

The higher mortality of both species of fish fed the unsupplemented diets in the present studies appear to be related to the severe symptoms of ascorbic acid deficiency which appeared in these fish and also to their lowered tissue ascorbate levels.

In the present study no evidence of calcification was observed in sections of vertebral areas of lordosis or scoliosis stained with haematoxylin and eosin, as was also reported by Halver et al. (1969) in rainbow trout. The gills and eyes of O. niloticus and O. mossambicus were clinically severely affected by the absence of dietary ascorbic acid. Halver (1972) reported that rainbow trout fed diets deficient in ascorbic acid showed twisted and deformed cartilaginous support tissue of the gills and poor structure of the hyaline cartilage of the sclerotic coat of the eye. Mahajan and Agrawal (1980b) reported that scorbutic Channa punctatus showed decreased absorption and utilization of calcium from the surrounding water which may explain the poor calcification of gill primary lamellae of both species in these studies. Heath (1962) cited Hamilton (1958) that ascorbic acid deficiency caused intra-ocular haemorrhage and he concluded from this review that ascorbic acid may play a role in the function of the eyes.

Scorbutic O. mossambicus in the present studies showed two significant features of ascorbic acid deficiency which were not observed in O. niloticus, hyperplasia of the gill secondary lamellar epithelial cells and steatitis. Lim and Lovell (1978) reported that scorbutic channel catfish exhibited distorted gill filament cartilage and irregularly shaped chondrocytes, results similar to the hyperplasia observed in scorbutic O. mossambicus. No reports appear in the literature concerning the relationship of ascorbic acid deficiency to steatitis in fish.

Fish species which exhibit deficiency signs when fed scorbutic diets may be unable to produce the enzyme L-gulono-γ-lactone oxidase (a key

enzyme in the biosynthesis of ascorbic acid in liver and/or kidney). Yamamoto *et al.* (1978) and Soliman *et al.* (1985) reported that this enzyme is not detectable in hepatic and renal tissues of *O. niloticus* and *O. mossambicus*.

Severe clinical signs and a high level of mortality were associated with the complete absence of ascorbic acid from the diet in the present studies (Diet 1 did not show any detectable ascorbic acid whereas Diet 2 retained 33,5% of the supplemented ascorbic acid as fed). The degree of loss of growth and lowered nutritional and physiological parameters as well as the pathological effects of ascorbic acid deficiency suggest that the possibility of marginal vitamin C deficiency is to be avoided in formulation, processing and storage of tilapia feeds.

CHAPTER 6

THE EFFECTS OF DIETARY ASCORBIC ACID LEVEL ON TISSUE ASCORBIC ACID CONCENTRATIONS IN *O. NILOTICUS* AND *O. MOSSAMBICUS*

SECTION 6.1 : INTRODUCTION

Since the isolation of ascorbic acid (vitamin C) by Svirbely and Szent-Györgyi (1932) and Waugh and King (1932) the metabolism of ascorbic acid has been extensively investigated (Hornig, 1975a).

Early studies of the role of ascorbic acid in overall metabolism centred on its distribution in animal tissues and its occurrence in an organ was regarded as an indicator of the physiological functions of the vitamin (Hornig, 1975b). Several differing techniques have been used for such studies as follows: firstly on dissection of the animal with subsequent determination of ascorbic acid concentration in tissues by chemical methods (Rudra, 1936; Saha, 1939; Penney & Zilva, 1946; Bai & Kalyani, 1960, 1961; Siddiqui, 1966; Hughes & Jones, 1971; Hughes et al., 1971a; Agrawal & Mahajan, 1980b); secondly by using labelled compounds of ascorbic acid to trace its metabolic role (Ikeda & Sato, 1965; Baker et al., 1966b. 1969, 1971b; Halver et al., 1975; Kallner et al., 1977, 1979; Hornig, 1981; Norkus & Rosso, 1981; Tillotson & O'Connor, 1981); thirdly using labelled compounds of ascorbic acid followed by dissection of the animal with subsequent determination of radioactive accumulation in the tissue (Martin, 1960; Martin & Mecca, 1961; Hilton et al., 1979a, b; Tucker, 1983), and fourthly whole-body autoradiography using also labelled compounds of ascorbic acid (Hammarstrom, 1966; Halver et al., 1975; Hornig, 1975b).

Tissue ascorbate concentrations will be influenced by the rate of uptake (fixation) of ascorbic acid (Penney & Zilva, 1946; Hughes et al., 1971a; Hilton et al., 1979a) and the retention capacity of the

tissue for ascorbic acid (Hughes et al., 1971a), Ascorbic acid levels in tissues such as liver, kidney and blood can be used as an indication of the nutritional status of fish feeds (Halver et al., 1975; Hilton et al., 1977a; Mahajan & Agrawal, 1979) prior to overt signs of vitamin C deficiency. It has also been suggested that a decrease in tissue ascorbate concentration is a sign of physiological stress (Wedemeyer, 1969; Patro & Patnaik, 1979; Thomas, 1984); pollution (Chatterjee & Pal, 1975; Yamamoto et al., 1977c; Mayer et al., 1978; Mauck et al., 1978; Thomas et al., 1982; Thomas & Neff, 1984); infection and disease (Lewin, 1974; Wilson, 1974).

Ascorbic acid occurs in every tissue in both animals and plants (Passmore, 1977), however, its concentration varies markedly between tissues (Martin, 1961). The present studies investigated the effects of two dietary ascorbic acid levels on tissue ascorbate concentrations during a 12 week period.

SECTION 6.2 : EXPERIMENT 1

EFFECTS OF DIETARY ASCORBIC ACID LEVEL ON TISSUE ASCORBIC ACID CONCENTRATIONS IN O. NILOTICUS

Section 6.2.1 Materials and Methods

Section 6.2.1.1 Experimental system and animals

Eighty O. niloticus obtained from a genetically homogenous stock (Section 2.2) were stocked at 10 fish/9 litre circular tank into an experimental recirculation system (Section 2.1.1) and allowed to acclimate for one week prior to the start of the experiment, during this period they were fed a commercial trout diet.

Section 6.2.1.2 Diets and feeding regime

Two diets were employed in the present study. Diet 1 and Diet 2 were produced by addition of 125mg and 400mg of ascorbic acid to 100g of the basal diet (Table 12), the substitution being made for alpha-cellulose in the basal diet. Diet preparation and storage were as previously described (Section 2.3.2). Each of the dietary treatments was fed to tetraplicate tanks each containing 10 fish with an average body weight of 4.25g ± 0.04g at 5% of their body weight (whole fish/dry food) per day for 12 weeks.

Section 6.2.1.3 Experimental methodology

Fish weighing procedure

Fish were weighed triweekly (to avoid the stress of handling the fish during weekly weighings) and feeding rate adjusted accordingly for the following three weeks.

Total ascorbate determination

At 3, 6, 9 and 12 weeks after the start of the experiment 8 fish per treatment were sampled for total ascorbate concentrations in liver, gills, brain, eyes, gall-bladder, muscle, testis, ovary, heart and gut as described earlier (Section 2.4.4.1).

Section 6.2.2. Statistical Analysis

Analysis of variance and Duncans mutliple range tests (Section 2.9) were used for evaluation of the experimental data.

Section 6.2.3. Results

The effect of dietary ascorbic acid on tissue ascorbate concentrations was judged by the following criteria:

1 Effect of dietary ascorbic acid levels

Table 29 shows the effect of dietary ascorbic acid levels on total ascorbate concentrations in ten tissues of O. niloticus. Total tissue ascorbate concentrations in fish fed the diet containing the higher level of ascorbic acid were significantly ($P < 0.01$) higher after each time interval sampled. Distribution of total tissue ascorbate within the same dietary treatment showed that for both treatments highest levels of ascorbic acid were detected in the ovary, brain and testis with lower levels in gut, eyes, heart and liver and the lowest levels in muscle and gall-bladder (Table 29).

TABLE 29. Total ascorbate concentrations[1] (µg/g wet tissue) in tissues of O. niloticus at 3, 6, 9 and 12 weeks

Parameter	3 WEEKS Diet 1	3 WEEKS Diet 2	±SEM[2]	6 WEEKS Diet 1	6 WEEKS Diet 2	±SEM[2]	9 WEEKS Diet 1	9 WEEKS Diet 2	±SEM[2]	12 WEEKS Diet 1	12 WEEKS Diet 2	±SEM[2]
Avg.wt.g.	8.72	8.02	0.60	23.17	19.28	0.59	33.96	27.75	0.71	47.44	37.12	0.70
Liver	59.44^{b}	81.95^{a}	0.76	97.88^{b}	126.03^{a}	1.44	97.16^{b}	137.31^{a}	2.06	115.97^{b}	166.86^{a}	1.42
Gills	144.10^{b}	177.13^{a}	1.02	130.79^{b}	165.21^{a}	2.75	99.04^{b}	142.54^{a}	0.88	128.22^{b}	146.68^{a}	0.60
Brain	273.10^{b}	319.40^{a}	2.68	284.37^{b}	321.83^{a}	3.73	303.46^{b}	346.05^{a}	6.45	321.34^{b}	392.99^{a}	2.86
Eyes	69.03^{b}	99.62^{a}	1.58	81.69^{b}	121.00^{a}	0.95	72.31^{b}	117.78^{a}	0.75	83.31^{b}	120.99^{a}	1.25
Gall-bladder	29.76^{b}	50.67^{a}	1.80	28.04^{b}	33.34^{a}	0.68	18.94^{b}	40.63^{a}	1.37	29.00^{b}	53.05^{a}	1.15
Muscle	31.64^{b}	54.52^{a}	1.23	25.22^{b}	43.47^{a}	0.43	21.17^{b}	29.39^{a}	0.54	29.02^{b}	38.08^{a}	0.63
Testis	195.00^{b}	239.64^{a}	5.07	230.61^{b}	275.96^{a}	2.50	248.91^{b}	285.39^{a}	3.28	255.18^{b}	328.71^{a}	2.22
Ovary	295.80^{b}	367.71^{a}	2.04	327.86^{b}	400.97^{a}	4.29	354.41^{b}	458.56^{a}	2.00	385.28^{b}	620.36^{a}	3.13
Heart	95.56^{b}	123.50^{a}	2.17	103.29^{b}	136.19^{a}	4.04	104.39^{b}	145.50^{a}	4.24	97.50^{b}	158.34^{a}	3.58
Gut	115.29^{b}	150.29^{a}	0.83	111.63^{b}	156.66^{a}	2.02	92.85^{b}	163.17^{a}	3.15	99.49^{b}	185.95^{a}	1.09

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)

2. Standard error of the means derived from analysis of variance

2 Periodical variation

The periodical variation in total tissue ascorbate concentrations for each treatment are presented in Table 30. Tissue ascorbate levels generally increased significantly ($P < 0.01$) over the 12 week experimental period in liver, brain, testis and ovary. Heart tissue ascorbate concentrations of fish fed Diet 1 did not vary significantly ($P > 0.01$). Total ascorbate concentration in gills, eyes, gall-bladder, muscle, heart and gut fluctuated during the experimental period (Table 30).

TABLE 30. Total ascorbate concentrations[1] (µg/g wet tissue) in tissues of O. niloticus fed the experimental diets after 3, 6, 9 and 12 weeks respectively

Parameter	DIET 1					DIET 2				
	Period				±SEM[2]	Period				±SEM[2]
	3 wk	6 wk	9 wk	12 wk		3 wk	6wk	9 wk	12 wk	
Liver	59.44^{a}	97.88^{b}	97.16^{b}	115.97^{a}	1.19	81.95^{d}	126.03^{c}	137.31^{b}	166.86^{a}	1.74
Gills	144.10^{a}	130.79^{b}	99.04^{c}	128.22^{b}	0.93	177.13^{a}	165.21^{b}	142.54^{c}	146.68^{c}	2.08
Brain	273.10^{c}	284.37^{bc}	303.46^{ab}	321.34^{a}	4.78	319.40^{c}	321.83^{c}	346.05^{b}	392.99^{a}	3.54
Eyes	69.03^{b}	81.69^{a}	72.31^{b}	83.31^{a}	1.01	99.62^{b}	121.00^{a}	117.78^{a}	120.99^{a}	1.30
Gall-bladder	29.75^{a}	28.04^{a}	18.94^{b}	29.00^{a}	1.49	50.67^{a}	33.34^{c}	40.63^{b}	53.05^{a}	1.16
Muscle	31.64^{a}	25.22^{c}	21.17^{d}	29.02^{b}	0.36	54.52^{a}	43.47^{b}	29.39^{d}	38.08^{c}	1.03
Testis	195.00^{c}	230.61^{b}	248.91^{a}	255.18^{a}	3.56	239.64^{c}	275.96^{b}	285.39^{b}	328.71^{a}	2.52
Ovary	295.80^{d}	327.86^{c}	354.41^{b}	385.28^{a}	1.80	367.71^{d}	400.97^{c}	458.56^{b}	620.36^{a}	3.93
Heart	95.56^{a}	103.29^{a}	104.39^{a}	97.50^{a}	4.26	123.50^{c}	136.19^{bc}	145.5^{ab}	158.34^{a}	2.67
Gut	115.29^{a}	111.63^{ab}	92.85^{c}	99.49^{b}	1.20	150.29^{c}	156.66^{bc}	163.17^{b}	185.95^{a}	2.54

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)

2. Standard error of the means derived from analysis of variance

SECTION 6.3 : EXPERIMENT 2

EFFECTS OF DIETARY ASCORBIC ACID LEVEL ON TISSUE ASCORBIC ACID CONCENTRATIONS IN O. MOSSAMBICUS

Section 6.3.1 Materials and Methods

Eighty O. mossambicus with an average weight of 4.59g (±0.09g) were used in this investigation. The experimental system, diets and feeding regime, fish weighing procedure and experimental methodology and statistical analysis were as described in Sections 6.2.1.1, 6.2.1.2, 6.2.1.3 and 6.2.2.

Section 6.3.2 Results

For evaluation of the results of the present study the following parameters were considered:

1 Dietary ascorbic acid and total tissue ascorbate levels

The effects of dietary ascorbic acid level on the total tissues ascorbate concentration of fish fed the experimental diets after 3, 6, 9 and 12 weeks are shown in Table 31 . Fish fed the diet supplemented with 400mg of ascorbic acid per Kg exhibited significantly ($P < 0.01$) higher tissue ascorbate concentrations in all tissues examined compared with those of fish fed the diet supplemented with 125mg of ascorbic acid/100g. In both treatments the highest levels of ascorbic acid were recorded for ovary, brain, and testis whereas intermediate levels were recorded for liver, gills, eyes, heart and gut and the lowest levels were found in muscle and gall-bladder (Table 31).

TABLE 31. Total ascorbate concentrations[1] (μg/g wet tissue) in tissues of *O. mossambicus* at 3, 6, 9 and 12 weeks

Parameter	3 WEEKS Diet 1	3 WEEKS Diet 2	±SEM[2]	6 WEEKS Diet 1	6 WEEKS Diet 2	±SEM[2]	9 WEEKS Diet 1	9 WEEKS Diet 2	±SEM[2]	12 WEEKS Diet 1	12 WEEKS Diet 2	±SEM[2]
Avg.wt.g.	8.58	7.98	0.43				26.13	23.44	1.05	35.28	29.49	1.46
Liver	84.41[b]	98.41[a]	2.86	110.31[b]	138.85[a]	1.08	109.38[b]	146.90[a]	0.64	119.01[b]	196.73[a]	0.82
Gills	150.21[b]	165.74[a]	1.98	128.80[b]	163.08[a]	1.27	106.42[b]	149.18[a]	0.70	141.02[b]	153.69[a]	2.17
Brain	282.60[b]	315.10[a]	2.06	314.07[b]	370.72[a]	3.70	339.41[b]	395.83[a]	1.40	392.00[b]	444.71[a]	2.83
Eyes	83.03[b]	109.28[a]	1.83	95.27[b]	121.52[a]	1.78	88.02[b]	116.20[a]	1.49	89.29[b]	127.20[a]	1.16
Gall-bladder	23.24[b]	52.23[a]	0.87	24.23[b]	50.77[a]	1.11	30.68[b]	45.00[a]	0.97	38.21[b]	58.72[a]	1.84
Muscle	25.58[b]	58.42[a]	0.75	31.80[b]	65.82[a]	0.86	35.70[b]	53.47[a]	0.77	44.42[b]	60.27[a]	0.83
Testis	196.77[b]	258.25[a]	2.62	265.07[b]	315.37[a]	3.33	287.12[b]	344.13[a]	2.57	296.60[b]	384.36[a]	2.82
Ovary	330.34[b]	394.34[a]	2.32	349.99[b]	415.80[a]	3.22	369.69[b]	500.64[a]	1.64	390.36[b]	678.18[a]	1.96
Heart	88.11[b]	101.90[a]	1.91	98.10[b]	130.00[a]	1.79	98.98[b]	158.64[a]	2.02	107.14[b]	156.67[a]	3.09
Gut	110.64[b]	159.58[a]	1.10	118.57[b]	159.68[a]	1.77	124.26[b]	181.68[a]	1.17	107.38[b]	182.50[a]	1.20

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)

2. Standard error of the means derived from analysis of variance

2 Periodical variation

Analysis of variance and Duncan's multiple range tests revealed highly ($P < 0.01$) significant differences between sampling intervals for each tissue within each treatment (Table 32). Liver, brain, testis and ovary showed a continuous increase in total ascorbate concentrations during the experimental period whereas the rest of the tissues showed no constant trend (Table 32).

SECTION 6.4 : DISCUSSION

Penney & Zilva (1946) and Hughes *et al*. (1971a) suggested that tissue ascorbate concentrations will be influenced by the rate of uptake (fixation) of ascorbic acid an idea supported by the fact that total tissue ascorbate concentrations in the present studies were related to the dietary ascorbic acid levels. Hughes and Hurley (1969) commented that organs in which ascorbic acid has metabolic roles may well have developed more efficient mechanisms for its abstraction and retention which may be reflected in high tissue concentrations such as occurred in ovary, brain and testis in the present studies.

The increased concentration of total ascorbate in certain tissues with time such as ovary, testis and brain reflects the role of ascorbic acid in growth and functioning of these important tissues in the body.

The results presented here showed that the ovary contained the highest levels of ascorbic acid of the tissues examined. Similar results were reported by Ikeda *et al*. (1963a) and Hilton *et al*. (1979b) and in

TABLE 32. Total ascorbate concentrations (µg/g wet tissue) in tissues of O. mossambicus fed the experimental diets during 3, 6, 9 and 12 weeks respectively

Parameter	DIET 1					DIET 2				
	Period					Period				
	3 wk	6 wk	9 wk	12 wk	±SEM[2]	3 wk	6 wk	9 wk	12 wk	±SEM[2]
Liver	64.41^{c}	110.31^{b}	109.38^{b}	119.01^{a}	1.15	98.41^{c}	138.85^{b}	146.90^{b}	191.73^{a}	1.97
Gills	150.21^{a}	128.80^{c}	106.42^{d}	141.01^{b}	1.25	165.74^{a}	163.08^{ab}	149.18^{c}	153.69^{bc}	2.48
Brain	282.60^{d}	314.07^{c}	339.41^{b}	392.00^{a}	1.87	315.10^{d}	370.72^{c}	395.83^{b}	444.71^{a}	2.05
Eyes	83.03^{b}	95.27^{a}	88.02^{ab}	89.29^{a}	1.61	109.28^{c}	121.52^{ab}	116.20^{b}	127.20^{a}	1.55
Gall-bladder	23.24^{c}	24.23^{c}	30.68^{b}	38.21^{a}	0.70	52.23^{ab}	50.77^{bc}	45.00^{c}	58.72^{a}	1.53
Muscle	25.58^{d}	31.86^{c}	35.70^{b}	44.42^{a}	0.67	58.42^{b}	65.82^{a}	53.47^{c}	60.27^{b}	0.86
Testis	196.77^{d}	265.07^{b}	287.12^{a}	296.60^{a}	2.52	254.25^{d}	315.37^{c}	344.13^{b}	384.36^{a}	3.42
Ovary	330.34^{d}	349.99^{c}	369.69^{b}	390.36^{a}	2.19	394.34^{d}	415.80^{c}	500.64^{b}	678.48^{a}	2.52
Heart	88.11^{b}	98.10^{b}	98.98^{a}	107.14^{a}	2.20	101.90^{c}	130.00^{b}	158.64^{a}	156.67^{a}	2.32
Gut	110.64^{c}	118.57^{b}	124.26^{a}	107.38^{c}	1.22	159.58^{b}	159.68^{b}	181.68^{a}	182.50^{a}	1.44

1. Values in the same row with a common superscript are not significantly different (P > 0.01)
2. Standard error of the means derived from analysis of variance

Chapters 4 and 5 in this thesis. Lewin (1974) suggested that ovarian ascorbic acid affects production of sex hormones through an effect on cyclic AMP (Adenosine 3',5'-cyclic monophosphate) and cyclic GMP (Guanosine monophosphate). Seymour (1981a) and Pintauro and Bergan (1982) support this by suggesting that ascorbic acid plays an important role in steroidogenesis. Recently Sandnes et al. (1984) demonstrated, through feeding trials, that adequate levels of dietary ascorbic acid improved hatchability in rainbow trout.

The existence of a high level of ascorbic acid in the brain of O. niloticus and O. mossambicus in these studies suggests a role in the functioning of the nervous system. It has been proposed by Hilton et al. (1979b) that anorexia, lethargy and lying prostrate at the bottom of the tank in scorbutic rainbow trout is due to severe reduction of total ascorbate in the brain. Sulkin and Sulkin (1975) reported that ascorbic acid is necessary to prevent cellular atrophy and to protect nerve cells. Milby et al. (1981) suggested that ascorbic acid could exhibit a strong effect on growing modulation of various synaptic events and play a very important role in neuronal functioning. Milby et al. (1982) have also suggested a relationship between concentrations of catecholamine (neurotransmitter) in different regions of the brain and ascorbic acid concentrations in these regions. In addition, ascorbic acid is a co-factor of the enzyme dopamine-β-hydroxylase which catalyses the hydroxylation of dopamine to form norepinephrine (Kaufman, 1966; Deana et al., 1975). Ascorbic acid also has the ability to inhibit dopamine sensitive adenyl cyclase in a manner similar to the action of major tranquilizers (Thomas & Zemp, 1977)

and the mental activities of humans may be related to cerebral concentrations of ascorbic acid (Pauling, 1968).

In the current experiments testis showed a high concentration of ascorbic acid indicating a metabolic involvement of ascorbic acid in male reproduction. Phillips et al. (1940) demonstrated the importance of ascorbic acid in improving fertility in bulls where parental administration of ascorbic acid enhanced fertility and these findings have been supported by the suggestion of Kocen and Cavazos (1958) that ascorbic acid enhanced spermiogenesis in guinea pigs.

Liver, gills, eyes, gut and heart of O. niloticus and O. mossambicus in these studies exhibited high levels of ascorbic acid indicating possible roles for ascorbic acid in these tissues. Liver is an important organ in the body where many physiological reactions dependent on ascorbic acid take place such as the NADH dependent hepatic redox system (Staudinger et al., 1961; Kato et al., 1969), microsomal drug metabolizing enzyme system (Conney et al., 1961), ferritin synthesis (Mazur et al., 1960, 1961; Monsen & Page, 1978) and cholesterol metabolism and bile acids biosynthesis (Ginter et al., 1972; Ginter, 1975, 1978). The presence of ascorbic acid in the eyes in the present studies supports the suggestions of Hornig (1975b) that ascorbic acid plays a specific role in lenticular metabolism and of Heath (1962) that existence of this vitamin in the eyes reduces the incidence of cataract. It has been shown in Chapter 5 that O. niloticus and O. mossambicus fed diets devoid of ascorbic acid developed cataract with no incidence of this defect in fish fed diets supplemented with ascorbic acid. In addition, the biochemical method

detected severe reduction of ascorbic acid in the eyes of scorbutic fish suggesting that existence of ascorbic acid in the eyes may prevent cataract. Gills are important organs in the osmoregulation process and Mahajan and Agrawal (1980b) suggest that ascorbic acid increases the efficiency of gills of Channa punctatus in absorption and utilization of calcium from surrounding water although these authors did not determine the ascorbic acid concentrations in the gills. Ascorbic acid was found in appreciable amounts in the heart of O. niloticus and O. mossambicus in the present studies and Hornig (1975b) has suggested that ascorbic acid plays a major role in the integrity of the ground substance of the arterial intima by preventing morphological alterations that are considered to be the first pathological signs of atherosclerosis. Oelrichs & Kratzing (1980) reported that the intestines of rats and guinea pigs have the capacity to hold large amounts of ascorbic acid, particularly in the small intestine. In scorbutic guinea pigs the intestine retained detectable ascorbic acid indicating a possible role in some essential metabolic process. The gut of both species in the present studies showed relatively high levels of ascorbic acid. Roe et al. (1941) reported that gastric ulcers occurring in guinea pigs with scurvy appear to be a consequence of breakdown in the capillaries of the gastric mucosa and submucosa due to increased capillary fragility and this suggestion may help to explain the occurrence of haemorrhages in the intestine of scorbutic rainbow trout which has been reported by Kitamura et al. (1967).

In the present studies muscle and gall-bladder showed the lowest levels of total ascorbic acid, possibly indicating minor functions

of the vitamin in these tissues. Bai and Kalyani (1960) found that the amount of ascorbic acid in muscle of O. mossambicus was 9.5µg/g of wet tissue whereas the amount of ascorbic acid detected in the muscle of O. mossambicus in the present study was about 3-5 times this value. Ikeda et al. (1963a) reported that muscle was the poorest tissue with respect to ascorbic acid retention. Bai & Kalyani (1960) suggested a possible relationship between vitamin C and morphogenesis of scales and scale regeneration. Ascorbic acid supplementation has been reported to prevent muscle haemorrhages in O. mossambicus and O. niloticus (Chapter 5 in this thesis) and indicates a possible role of ascorbic acid in the integrity of blood capillaries in fins and muscle of fish. Ascorbic acid has been reported to be an important factor in reducing cholesterol levels in the gall-bladder, increasing production of bile acids in guinea pigs (Jenkins, 1980) and preventing gallstone formation in guinea pigs (Ginter, 1978).

The distribution of ascorbic acid in O. niloticus and O. mossambicus in the present studies indicates a close relationship between the level of ascorbic acid and specific function in each tissue and supports the need for adequate dietary ascorbic acid to maintain proper tissue concentrations.

CHAPTER 7

THE EFFECT OF VARYING FORMS OF DIETARY ASCORBIC ACID ON THE JUVENILE *O. NILOTICUS* AND *O. MOSSAMBICUS*

SECTION 7.1 : INTRODUCTION

Ascorbic acid (vitamin C) performs numerous physiological functions in both plants and animals (Tolbert, 1979) and has been demonstrated to be an essential nutrient in the diets of numerous species of fish (Poston, 1967; Halver *et al.*, 1969; Sakaguchi *et al.*, 1969; Arai *et al.*, 1972; Lovell, 1973; Wilson, 1973; Andrews & Murai, 1975; Lim & Lovell, 1978; Mahajan & Agrawal, 1979, 1980a).

It has been postulated that dietary especiality of ascorbic acid results from the absence of the enzyme L-gulono-γ-lactone oxidase which is required for biosynthesis of ascorbic acid (Wilson, 1973; Yamamoto *et al.*, 1978)

Considerable losses of ascorbic acid occur during the processing and storage of feeding stuffs and the instability of this vitamin results in difficulty in both demonstration of an absolute requirement for vitamin C and in determination of optimal dietary inclusion levels (Murai *et al.*, 1978). As a result of this instability attempts have been made to identify a more stable form of ascorbic acid, particularly important in fish feeds which are exposed to the aqueous environment for varying periods prior to ingestion.

Consequently, it was decided to evaluate five forms of ascorbic acid in feeds for the tilapias *O. niloticus* and *O. mossambicus* which are rapidly becoming established as the principle cultured tilapias (Balarin & Hatton, 1979). In addition to L-ascorbic acid it was decided to evaluate the sodium salt of L-ascorbic acid which, it has been suggested, may differ in its activity due to lower acidity

(Demole, 1934). Attempts have been made to reduce dietary ascorbic acid losses by coating this vitamin. Ethylcellulose coating of L-ascorbic acid had been shown to reduce losses, in diets for channel catfish (Ictalurus punctatus), during extrusion from 62% to 46% (Lovell & Lim, 1978). In the present study L-ascorbic acid coated with a mixture of mono-,di- and triglycerides was evaluated (Ascorbidan 50, Grinsted products, Denmark).

It has been reported (Quadri et al., 1973; Quadri et al., 1975) that L-ascorbic acid 2-sulphate is much more stable to processing than L-ascorbic acid. Mead and Finamore (1969) suggest that this compound may have a dual metabolic role as a storage form of both ascorbic acid and sulphate. L-ascorbic acid 2-sulphate has been shown to be an antiscorbutic factor when included in feeds for trout (Salmo gairdneri), coho salmon (Oncorhynchus kisutch) (Halver et al., 1975) and guinea pigs (Mumma et al., 1972) although O. niloticus have shown no ability to hydrolyse this compound (Tsujimura et al., 1981). This compound, as the barium salt, was included in the present study.

Ascorbyl palmitate has also been proposed as a more stable form of vitamin C for inclusion in feeds. This ester of ascorbic acid is insoluble in water and exhibits full vitamin activity in its ascorbyl portion so, on a weight basis, it has 39% of the vitamin activity of L-ascorbic acid (Ranken, 1974). This compound was also included for evaluation in the present study.

The previous studies (Chapter 4) demonstrate that the dietary requirement of O. niloticus and O. mossambicus for L-ascorbic acid is 125 mg

ascorbic acid per 100 g diet. The five forms of ascorbic acid (L-ascorbic acid, sodium salt L-ascorbic acid, glyceride coated L-ascorbic acid, L-ascorbic acid 2-sulphate and ascorbyl palmitate) were evaluated on an equimolar (ascorbic acid) basis in an eight week growth study for O. niloticus and O. mossambicus.

SECTION 7.2 : EXPERIMENT 1

THE EFFECT OF VARYING FORMS OF DIETARY ASCORBIC ACID ON THE NUTRITION OF JUVENILE O. NILOTICUS

Section 7.2.1 Materials and Methods

Section 7.2.1.1 Experimental system and animals

A warm water recirculation system (Section 2.1.1) was employed. O. niloticus juveniles, obtained from Institute of Aquaculture, Stirling University from a genetically homogenous stock (Section 2.2), were acclimated to the experimental system for one week prior to the start of the experiment and were fed a commercial trout feed during this period. Each of the six dietary treatments was fed to randomly assigned triplicate tanks of fish with 10 fish per tank.

Section 7.2.1.2 Diets and feeding regime

A basal diet, ascorbic acid free, was formulated (Table 12). The quantity of each of the five ascorbic acid forms required to supply 125 mg of ascorbic acid per 100 g of dry diet on an equimolar basis was calculated and substituted for alpha-cellulose in the basal ration. Six diets were prepared as follows:

Diet 1: ascorbic acid free (AAF);

Diet 2: L-ascorbic acid (AA);

Diet 3: sodium L-ascorbic acid (NaAA);

Diet 4: glyceride coated L-ascorbic acid (GCAA);

Diet 5: barium salt of L-ascorbic acid 2-sulphate (AA2S), and

Diet 6: ascorbyl palmitate (AP).

Diet preparation and storage has been previously described (Section 2.3.2).

A fixed feeding regime of 5% of the body weight per day (dry food/ whole fish), divided into 4 equal feeds was adopted. Fish were fed for six consecutive days, weighed on the seventh and feeding rates for following week adjusted accordingly.

Section 7.2.1.3 Experimental methodology

Fish weighing procedure

Fish were bulk weighed, a tank at a time, in water without anaesthesia except for the terminal weighing when fish were anaesthetised (Ross & Geddes, 1979), and weighed and measured individually. During weighing mortality and condition of the fish were recorded.

Proximate analysis

An initial sample of fish, 3 per tank, was sacrificed prior to the start of the experiment and subjected to proximate analysis (Section 2.4.2). A final carcass sample of 5 fish per tank for diets 2-6 and 4 fish per tank for diet 1 was treated similarly.

Haematocrit and haemoglobin determination

Samples of blood were taken from five fish per tank at the end of the experiment for haematocrit and haemoglobin determination (Section 2.4.3).

Digestibility studies

Apparent digestibility was measured as described before (Section 2.8) by using the inert indicator, chromic oxide and apparent net protein utilization calculated from carcass analysis data (Section 2.7).

Total ascorbate determination

Total ascorbate concentration in liver, heart, alimentary canal, brain, gills, muscle, eyes, gall-bladder, testis and ovary were measured for six fish per treatment at the termination of the experiment by the method of Roe (1967) (Section 2.4.4.1).

Glycogen determination

Sub-samples of liver and muscle were analysed for total glycogen as described in Section 2.4.6.

X-ray

Fish were x-rayed at the end of the experiment to determine the condition of the vertebral column at the end of the experiment (Section 2.6).

Statistical analysis

Statistical analysis of data was performed using analysis of variance and Duncan multiple range test (Section 2.9).

Section 7.2.3 Results

The results of the present study were evaluated using the following parameters:

1. Growth response

   Body weight and specific growth rate (SGR)

   Poor growth and lower specific growth rate were achieved by fish fed the diet devoid of ascorbic acid (AAF) (Table 33 and Fig. 20). Differences between diet AAF and the diets supplemented with different forms of ascorbic acid were statistically highly significantly ($P < 0.01$) whereas the differences between diets containing the ascorbic acid forms were small and not significant ($P > 0.01$) (Table 33).

   Condition factor (CF)

   Differences in condition factor between fish fed diet AAF and fish fed diets containing various forms of ascorbic acid were highly significant ($P < 0.01$) (Table 33).

2. Food utilization

   Food conversion ratio (FCR)

   Fish fed diets containing the different forms of ascorbic acid have significantly ($P < 0.01$) better food conversion ratios (ranging from 0.87 for NaAA to 0.94 for AP) than fish fed diet AAF (Table 33).

TABLE 33. Growth, food utilization parameters and survival rate[1]

Parameter	DIET						± SEM[2]
	1 AAF	2 AA	3 NaAA	4 GCAA	5 AA2S	6AP	
Initial avg.wt.g.	1.19[a]	1.17[a]	1.16[a]	1.17[a]	1.19[a]	1.19[a]	0.21
Final avg.wt.g.	6.61[b]	11.49[a]	11.92[a]	11.28[a]	10.51[a]	11.28[a]	0.69
SGR[3] (% d^{-1})	2.98[b]	4.07[a]	4.16[a]	4.04[a]	3.89[a]	4.02[a]	0.10
Final CF[4]	4.69[a]	3.31[b]	3.41[b]	3.35[b]	3.34[b]	3.40[b]	0.08
FCR[5]	1.33[a]	0.89[b]	0.87[b]	0.91[b]	0.91[b]	0.94[b]	0.06
PER[6]	1.94[b]	2.81[a]	2.88[a]	2.73[a]	2.71[a]	2.71[a]	0.16
ANPU[7] (%)	24.58[c]	47.72[ab]	51.31[a]	43.29[b]	49.34[ab]	46.35[ab]	1.69
APD[8] (%)	64.28[c]	89.80[ab]	91.48[a]	87.96[b]	90.56[a]	88.03[b]	0.34
ADMD[9] (%)	51.08[d]	74.86[bc]	77.50[a]	75.75[ab]	76.05[ab]	73.32[c]	0.44
Survival Rate (%)	60.00[c]	90.00[ab]	100.00[a]	86.67[b]	100.00[a]	86.67[b]	2.10

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)
2. Standard error of the means derived from analysis of variance
3. Specific growth rate
4. Condition factor
5. Food Conversion Ratio
6. Protein efficiency ratio
7. Apparent net protein utilization
8. Apparent protein digestibility
9. Apparent dry matter digestibility

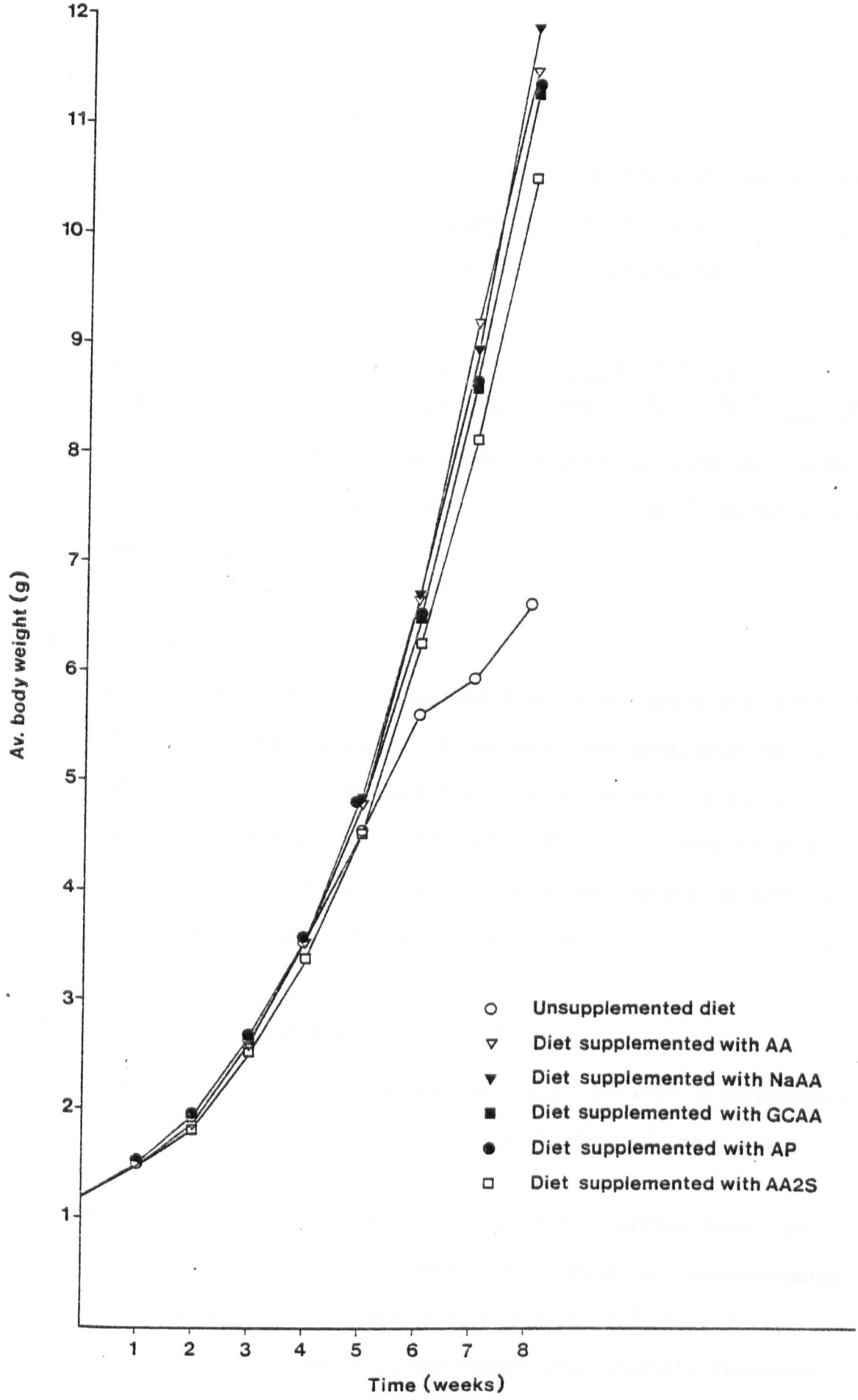


Fig. 20 **Increase in average body weight of O.niloticus fed the experimental diets**

Protein efficiency ratio (PER)

It is evident that fish fed diets supplemented with the various forms of ascorbic acid have significantly ($P < 0.01$) higher values than fish fed the ascorbic acid free diet (Table 33).

Apparent net protein utilization (ANPU), apparent protein digestibility (APD), and apparent dry matter digestibility (ADMD)

The results of ANPU, APD and ADMD are shown in Table 33. ANPU, APD and ADMD values were significantly ($P < 0.01$) lowered in fish fed diet AAF.

3. Gross body composition

Proximate analyses of initial and final fish sample are shown in Table 34. Proximate carcass composition was generally little affected by diet except that fish fed the ascorbic acid free diet (AAF) exhibited a significantly ($P < 0.01$) lower protein content and fish fed the ascorbyl palmitate containing diet, a significantly higher carcass lipid content.

4. Tissue and biochemical changes

Haematocrit and haemoglobin values (Table 35) were significantly ($P < 0.01$) lower in fish fed the diet lacking ascorbic acid.

Hepatosomatic indices and muscle and liver glycogen levels of fish fed all six diets are presented in Table 35. Hepatosomatic index was slightly elevated in fish fed the diet containing ascorbyl palmitate and glycogen levels were slightly depressed

TABLE 34. Gross body composition data[1] (as % wet weight basis)

Parameter	DIET							±SEM[2]
	F°	1 AAF	2 AA	3 NaAA	4 GCAA	5 AA2S	6 AP	
Moisture (%)	78.08[a]	76.25[b]	74.29[b]	74.86[b]	74.08[b]	75.62[b]	74.28[b]	0.460
Ash (%)	3.57[b]	4.27[a]	4.02[a]	4.18[a]	3.97[a]	4.14[a]	4.16[a]	0.063
Crude lipid	2.98[d]	4.84[ab]	4.87[ab]	5.06[ab]	4.81[b]	3.83[c]	5.53[a]	0.163
Crude protein	14.96[c]	15.75[b]	18.02[a]	17.55[a]	17.46[a]	18.10[a]	17.63[a]	0.153

F° Body composition of sample of fish analysed at the beginning of the experiment

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)

2. Standard error of the means derived from analysis of variance

TABLE 35. Blood parameters, hepatosomatic index, liver and muscle glycogen and total tissue ascorbate concentrations[1]

Parameter	DIET						± SEM[2]
	1 AAF	2 AA	3 NaAA	4 GCAA	5 AA2S	6AP	
HC[3], %	25.11^{b}	35.11^{a}	35.28^{a}	35.00^{a}	33.78^{a}	33.46^{a}	0.46
HC[4], gdl^{-1}	8.04^{b}	12.01^{a}	12.06^{a}	11.63^{a}	11.54^{a}	11.10^{a}	0.35
HSI[5]	1.73^{c}	2.48^{abc}	2.16^{abc}	2.12^{abc}	1.94^{bc}	2.76^{a}	0.17
MG[6], %	0.16^{d}	0.76^{a}	0.68^{b}	0.67^{b}	0.54^{c}	0.74^{ab}	0.02
LG[7], %	4.91^{c}	15.71^{a}	15.71^{a}	15.64^{a}	12.54^{b}	14.73^{a}	0.43
Tissue Ascorbate							
Liver $\mu g.g^{-1}$	6.12^{d}	51.36^{b}	78.77^{a}	80.00^{a}	30.16^{c}	79.31^{a}	0.67
Gut $\mu g.g^{-1}$	0.00^{e}	97.39^{b}	96.42^{b}	114.75^{a}	29.59^{d}	85.94^{c}	0.85
Gills $\mu g.g^{-1}$	29.52^{e}	92.07^{c}	102.65^{b}	110.75^{a}	41.48^{d}	109.31^{a}	0.65
Brain $\mu g.g^{-1}$	30.00^{c}	267.26^{a}	246.58^{a}	245.97^{a}	157.36^{b}	267.65^{a}	13.07
Eye $\mu g.g^{-1}$	2.87^{f}	41.30^{d}	48.39^{c}	50.61^{b}	18.55^{e}	56.85^{a}	0.44
Gall Bladder $\mu g.g^{-1}$	0.00^{d}	33.33^{b}	22.25^{c}	86.25^{a}	15.45^{c}	31.40^{b}	1.67
Muscle $\mu g.g^{-1}$	0.00^{e}	14.31^{c}	16.82^{b}	18.52^{a}	4.10^{d}	19.54^{a}	0.26
Heart $\mu g.g^{-1}$	0.00^{d}	66.75^{b}	72.50^{b}	97.50^{a}	47.50^{c}	64.29^{b}	1.47
Ovary $\mu g.g^{-1}$	0.00^{e}	333.51^{c}	432.08^{a}	440.00^{a}	200.89^{d}	398.08^{b}	2.18
Testis $\mu g.g^{-1}$	0.00^{f}	123.98^{d}	144.04^{c}	150.71^{b}	49.33^{e}	167.75^{a}	0.47

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)
2. Standard error of the means derived from analysis of variance
3. Haematocrit
4. Haemoglobin
5. Hepatosomatic index
6. Muscle glycogen
7. Liver glycogen

in fish fed the diet containing L-ascorbic acid 2-sulphate. Significantly the lowest hepatosomatic index and lowest muscle and liver glycogen levels were obtained in fish fed the ascorbic acid free diet.

Total tissue ascorbate concentrations of the liver, gut, gills, brain, eyes, gall-bladder, muscle, heart, ovary and testis are shown in Table 35. Total tissue ascorbate concentrations were significantly ($P < 0.01$) lowest in fish fed the ascorbic acid free diet. Fish fed the diet containing L-ascorbic acid 2-sulphate had significantly lower tissue ascorbate levels than fish fed diets containing the other ascorbic acid forms.

5. Survival rate and symptoms of ascorbic acid deficiency

A significantly ($P < 0.01$) lower survival rate (60%) was obtained for fish fed the diet devoid of ascorbic acid (Table 33).

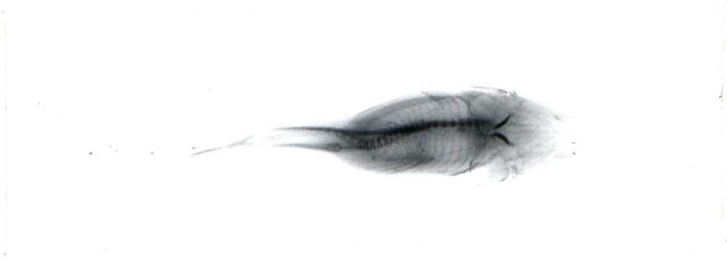
At the termination of the experiment fish fed the ascorbic acid free diet exhibited haemorrhaging (Plate 33) of fins, mouth, tail, muscle and eyes; short operculae (Plate 34); short tails and caudal fin erosion (Plate 35), and exopthalmia, and 7% exhibited spinal deformity (scoliosis and lordosis) (Plate 36).

PLATE 33. Scorbutic O. niloticus showing severe haemorrhages in fins, mouth and muscle with pronounced exophthalmia

PLATE 34. Scorbutic fish fed the unsupplemented diet exhibiting short operculum (O. niloticus)

PLATE 35. Scorbutic O. niloticus showing clearly short caudal fin

PLATE 36. A dorsal x-radiogram of O. niloticus fed a diet devoid of ascorbic acid forms showing severe scoliosis



SECTION 7.3 : EXPERIMENT 2

EFFECT OF VARYING FORMS OF DIETARY ASCORBIC ACID ON JUVENILE O. MOSSAMBICUS

Section 7.3.1 Materials and Methods

Section 7.3.1.1 Experimental system and animals

O. mossambicus were obtained from Institute hatchery (Section 2.2) and acclimated to the experimental system (Section 2.2) for one week prior to start of the experiment and fed a commercial trout feed during this period. Each of six dietary treatments was fed to randomly assigned duplicate tanks of fish with 11 fish per tank.

Section 7.3.1.2 Diets and feeding regime

As described before in Section 7.2.1.2 with a basal diet given in Table 16.

Section 7.3.1.3 Experimental methodology

As described in Section 7.2.1.3. At the end of the experiment total ascorbate concentrations in tissues of experimental fish was measured for 4 fish per treatment for diet 1 and 6 fish per treatment for diets 2-6.

Section 7.3.2 Results

The results of the present study were evaluated through using the following parameters:

1. Growth response

Body weight and specific growth rate (SGR)

Average final body weight and specific growth rate (SGR) were significantly ($P < 0.01$) reduced in fish fed the ascorbic acid free diet (Table 36 and Fig. 21).

Condition factor (CF)

Condition factor was significantly ($P < 0.01$) higher in fish fed diet lacking ascorbic acid (Table 36).

2. Food utilization

Food conversion ratio (FCR)

Significantly ($P < 0.01$) superior food conversion ratios were obtained for fish fed diets supplemented with the various forms of ascorbic acid (Table 36).

Protein efficiency ratio (PER)

PER was significantly ($P < 0.01$) lower in fish fed diet AAF Table 36).

Apparent net protein utilization (APNU), apparent protein digestibility (APD) and apparent dry matter digestibility (ADMD)

In terms of the ANPU, APD and ADMD (Table 36) fish fed the unsupplemented diet showed significantly lower values reflecting poor diet performance. No significant differences in APD and APNU were obtained for fish fed the five forms of ascorbic acid

TABLE 36. Growth, food utilization parameters and survival rate[1]

Parameter	DIET						± SEM[2]
	1 AAF	2 AA	3 NaAA	4 GCAA	5 AA2S	6 AP	
Initial avg.wt.g.	1.36[a]	1.39[a]	1.36[a]	1.39[a]	1.34[a]	1.35[a]	0.016
Final avg.wt.g.	5.68[d]	8.05[a]	7.34[b]	7.55[b]	8.24[a]	6.95[c]	0.053
SGR[3] (% d^{-1})	2.52[b]	3.22[a]	3.02[a]	3.02[a]	3.24[a]	2.92[a]	0.069
CF[4]	4.30[a]	3.00[b]	3.16[b]	3.11[b]	3.06[b]	3.06[b]	0.072
FCR[5]	1.6[a]	1.41[b]	1.35[bc]	1.34[bc]	1.22[c]	1.32[bc]	0.030
PER[6]	1.58[b]	1.87[a]	1.90[a]	1.86[a]	1.94[a]	1.79[a]	0.0290
ANPU[7] (%)	15.63[b]	33.66[a]	32.90[a]	31.80[a]	36.19[a]	33.12[a]	2.257
APD[8] (%)	68.27[b]	90.03[a]	89.03[a]	88.23[a]	90.56[a]	88.69[a]	0.420
ADMD[9] (%)	54.54[d]	73.73[a]	70.48[ab]	65.77[c]	73.23[a]	69.10[bc]	0.728
Survival Rate (%)	54.54[b]	95.46[a]	95.46[a]	95.46	90.81[a]	95.46[a]	3.71

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)
2. Standard error of the means derived from analysis of variance
3. Specific growth rate
4. Condition factor
5. Food Conversion Ratio
6. Protein efficiency ratio
7. Apparent net protein utilization
8. Apparent protein digestibility
9. Apparent dry matter digestibility

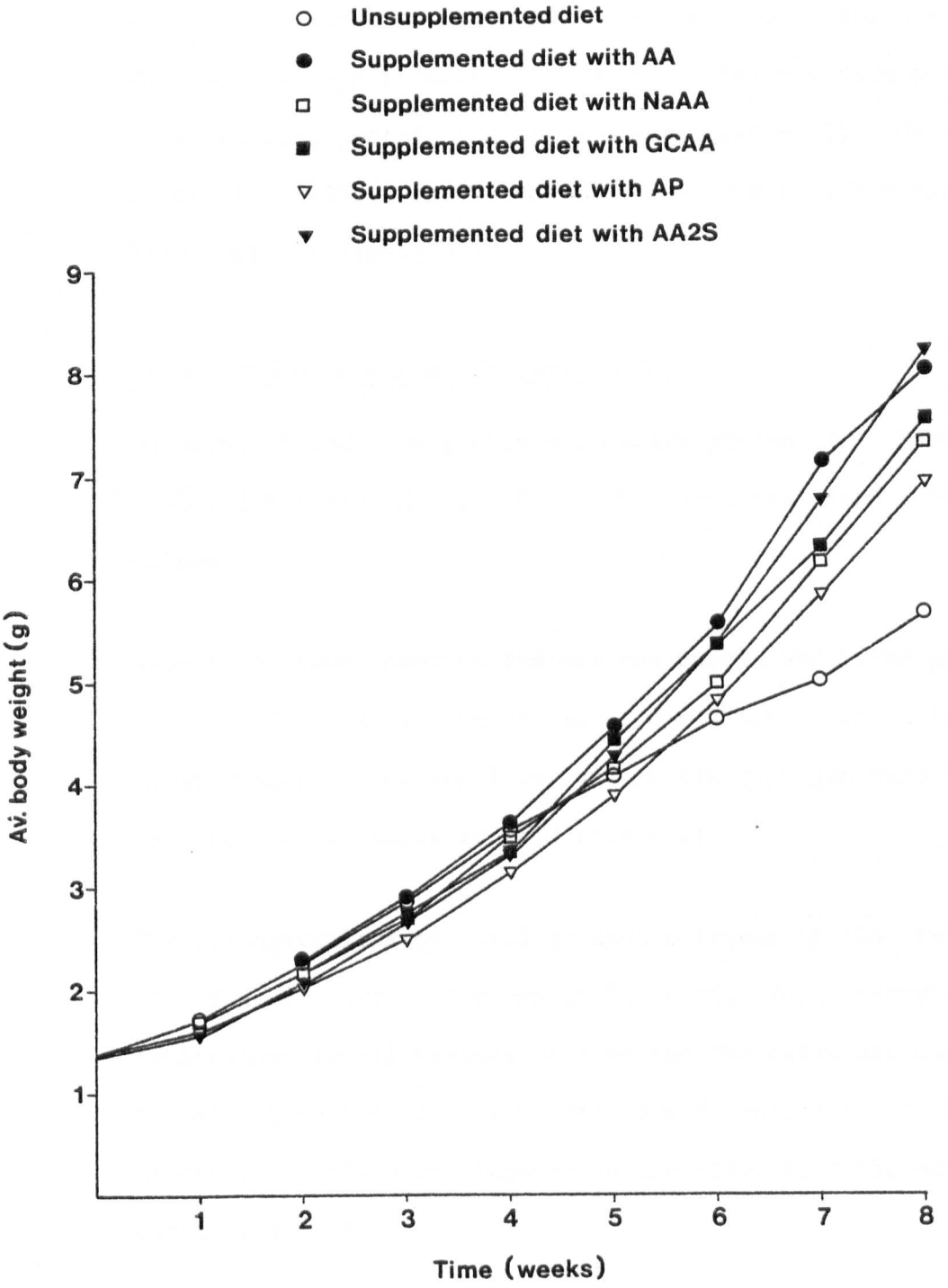


Fig. 21. Increase in average body weight of O. mossambicus fed the experimental diets

whereas the highest ADMD was obtained for fish fed diets AA and AA2S (Table 36).

3. Gross body composition

Results of proximate carcass analyses of initial and final fish samples are shown in Table 37. Fish fed the ascorbic acid free diet showed significantly ($P < 0.01$) higher moisture content and lower carcass protein and ash contents (Table 37). Fish fed ascorbyl palmitate supplemented diet showed a higher carcass lipid content (Table 37).

4. Tissue and biochemical changes

Haematocrit and haemoglobin values are presented in Table 38. Fish fed the diet devoid of ascorbic acid exhibited lowered values.

Results of hepatosomatic indices and muscle and liver glycogen level of fish fed the experimental diets are shown in Table 38. Hepatosomatic index and liver and muscle glycogen were depressed in fish fed the ascorbic acid free diet.

The concentrations of total ascorbate levels in ten tissues of fish fed six diets are shown in Table 38. Total ascorbate concentrations in all tissues of fish fed the deficient diet were lowest. Fish fed diet AA2S also showed depression of tissue ascorbate levels when compared to the other ascorbic acid forms containing diets.

TABLE 37. Gross body composition data[1] (as % wet weight basis)

Parameter	DIET							± SEM[2]
	F°	1 AAF	2 AA	3 NaAA	4 GCAA	5 AA2S	6 AP	
Moisture (%)	79.00	75.96^{a}	72.67^{b}	72.99^{b}	73.45^{b}	73.37^{b}	72.73^{b}	0.345
Ash (%)	3.43	3.42^{b}	4.06^{a}	4.22^{a}	4.02^{a}	3.83^{a}	3.96^{a}	0.097
Crude lipid (%)	5.05	5.81^{bc}	6.05^{ab}	5.99^{abc}	5.94^{abc}	5.36^{d}	6.19^{a}	0.082
Crude protein (%)	12.72	14.81^{b}	17.29^{a}	16.80^{a}	16.64^{a}	17.36^{a}	17.13^{a}	0.200

F° Body composition sample of fish analysed at the beginning of the experiment

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)

2. Standard error of the means derived from analysis of variance

TABLE 38. Blood parameters, hepatosomatic index, liver and muscle glycogen and total tissue ascorbate concentrations[1]

Parameter	DIET						±SEM[2]
	1 AAF	2 AA	3 NaAA	4 GCAA	5 AA2S	6 AP	
HC[3], %	29^{d}	38.75^{c}	38^{c}	38.25^{c}	42.5^{b}	44.25^{a}	0.40
HG[4], gdl^{-1}	8.15^{b}	12.67^{a}	12.79^{a}	12.67^{a}	12.79^{a}	12.92^{a}	0.10
HSI[5]	1.54^{b}	2.42^{a}	2.39^{a}	2.47^{a}	2.33^{a}	2.83^{a}	0.162
MG[6], %	0.06^{d}	0.95^{a}	0.82^{bc}	0.77^{c}	0.86^{b}	0.80^{bc}	0.017
LG[7], %	2.34^{e}	15.20^{b}	13.11^{c}	16.32^{a}	11.78^{d}	13.94^{c}	0.20
Tissue Ascorbate							
Liver $\mu g.g^{-1}$	4.34^{f}	83.71^{c}	70.1^{d}	152.70^{a}	39.93^{e}	107.38^{b}	0.63
Gut $\mu g.g^{-1}$	14.77^{e}	104.09^{b}	84.05^{c}	124.63^{a}	33.73^{d}	108.49^{b}	0.95
Gills $\mu g.g^{-1}$	26.39^{f}	113.65^{c}	103.56^{d}	155.9^{a}	47.49^{e}	131.07^{b}	1.44
Brain $\mu g.g^{-1}$	46.00^{e}	289.34^{bc}	276.5^{c}	303.34^{b}	141.66^{d}	507.39^{a}	4.14
Eye $\mu g.g^{-1}$	9.02^{e}	60.22^{a}	47.09^{c}	59.24^{ab}	27.80^{d}	54.80^{b}	0.96
Gall Bladder $\mu g.g^{-1}$	0.00^{c}	44.12^{a}	30.00^{b}	43.26^{a}	27.58^{b}	46.58^{a}	0.78
Muscle $\mu g.g^{-1}$	5.08^{d}	35.17^{a}	31.46^{a}	28.71^{b}	11.69^{c}	36.25^{a}	0.60
Heart $\mu g.g^{-1}$	0.00^{f}	100.00^{c}	60.00^{d}	140.00^{b}	46.67^{e}	177.00^{a}	0.87
Ovary $\mu g.g^{-1}$	25.83^{e}	403.02^{b}	294.32^{c}	463.62^{a}	159.45^{d}	414.59^{b}	2.48
Testis $\mu g.g^{-1}$	3.20^{d}	160.15^{b}	160.69^{b}	166.03^{b}	79.17^{c}	296.14^{a}	1.92

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)
2. Standard error of the means derived from analysis of variance
3. Haematocrit
4. Haemoglobin
5. Hepatosomatic index
6. Muscle glycogen
7. Liver glycogen

5. Survival rate and signs of ascorbic acid deficiency

Survival rate was affected markedly by the absence of ascorbic acid forms (Table 36). Fish fed diet AAF exhibited a significantly ($P < 0.01$) lower survival rate. Fish fed the diet devoid of ascorbic acid exhibited signs of vitamin C deficiency such as haemorrhages into muscle, fins with 9% spinal deformity (Plate 37).

PLATE 37. A dorsal x-radiogram of scorbutic O. mossambicus showing severe scoliosis

SECTION 7.4 : DISCUSSION

The results obtained for O. niloticus and O. mossambicus in the present studies indicate that all five forms of ascorbic acid evaluated prevented the occurrence of pathological signs of ascorbic acid deficiency and resulted in improved growth and food utilization compared with the control, ascorbic acid free diet.

Of the dietary forms of ascorbic acid available various sulphates have received the greatest attention (Halver et al., 1972, 1973, 1974, 1975). L-ascorbic acid 2-sulphate (also designated vitamin C_2) has been shown to prevent scurvy in rainbow trout (Salmo gairdneri), coho salmon (Oncorhynchus kisutch) (Halver et al., 1974; Baker et al., 1975; Halver et al., 1975; Tsujimura, 1978; Tsujimura et al., 1978 and Halver et al., 1983), and channel catfish (Ictalurus punctatus) (Murai et al., 1978) although its activity was reported to be lower in the latter species. In the present experiments total tissue ascorbate concentrations were lower in O. niloticus and O. mossambicus fed C_2 than in tissue of fish fed the other ascorbic acid forms. It has been demonstrated that no detectable hydrolysis of C_2 to C_1 occurs in livers of O. niloticus or ayu (Plecoglossus altivelis) in contrast to both rainbow trout and channel catfish (Tsujimura et al., 1981). Suprisingly, Tsujimura et al. (1982) subsequently reported that ayu and coho salmon were able to partially hydrolyze L-ascorbic acid 2-sulphate (C_2) to L-ascorbic acid (C_1). These conflicting results obtained for ayu by Tsujimura et al. (1981, 1982) suggest that their results may not be reliable. Benitez & Halver (1982) identified the enzyme L-ascorbic acid 2-sulphate

sulphohydrolase (C_2 sulphatase), the enzyme responsible for converting L-ascorbic acid 2-sulphate to L-ascorbic acid, in liver of rainbow trout. In the present studies O. niloticus and O. mossambicus showed some ability to convert L-ascorbic acid 2-sulphate to L-ascorbic acid as indicated by higher tissue ascorbate levels in fish fed diet AA2S than in the control fed diet AAF indicating that both species may possess this enzyme.

L-ascorbic acid 3-sulphate (designated vitamin C_3) has also been shown to have antiscorbutic activity in rainbow trout and coho salmon whereas D-erythroascorbate 2-sulphate (iso-C_2) does not (Halver et al., 1974).

There appear to be no reports in the literature concerning the antiscorbutic activity of sodium L-ascorbic acid or ascorbyl palmitate in fish prior to that of Brandt et al. (1985). These authors reported that ascorbyl palmitate prevented scurvy in channel catfish and fish fed ascorbyl palmitate performed as well as fish fed an ascorbic acid supplemented diet. Fitzhugh and Nelson (1946) reported that 5% dietary ascorbyl palmitate was toxic, whereas 2% was not, when added to the diet of rats. In the present studies both of these ascorbic acid forms performed, on an equimolar basis, as well as the free acid. Both O. niloticus and O. mossambicus exhibited elevated hepatosomatic index and carcass lipid contents when fed diet AP, possibly as a result of the palmitic acid supplied in ascorbyl palmitate. Tissue ascorbate concentrations indicate that ascorbyl palmitate is readily hydrolysed in vivo by tilapias as the method employed for ascorbate determination (Roe, 1967) is unable to detect the esterfied vitamin.

Ethylcellulose coated ascorbic acid has been evaluated in diets for channel catfish (Lovell & Lim, 1978) and has been shown to be both antiscorbutic and more stable to processing. There have been no reports on the antiscorbutic activity of glyceride coated ascorbic acid in fish. In these studies O. niloticus and O. mossambicus fed diet GCAA performed as well as fish fed the free acid.

O. niloticus and O. mossambicus fed diet AAF showed highly significant differences in all measured parameters compared to fish fed diets supplemented with the ascorbic acid forms. The higher condition factors obtained for O. mossambicus and O. niloticus fed AAF diet are probably as a result of spinal deformities that were apparent in the x-rays taken, similar results have been reported for brook trout (Poston, 1967). The depression of food utilization parameters, blood parameters and liver and muscle glycogen for fish fed diet AAF has been discussed elsewhere (Chapter 5).

Tissue ascorbate levels were severely depressed, in some cases there was no detectable activity, in tilapias fed diet AAF indicating inability of both species to synthesise ascorbic acid as reported earlier (Chapter 3).

In conclusion O. niloticus and O. mossambicus have been shown to have a dietary requirement for ascorbic acid (Chapter 4) that is satisfied when any of these five ascorbic acid forms is included in the ration to provide 125 mg ascorbic acid per 100 g diet.

CHAPTER 8

STABILITY OF DIETARY ASCORBIC ACID AND ITS FORMS DURING PROCESSING, STORAGE AND LEACHING

SECTION 8.1 : INTRODUCTION

Some cases of scurvy among Canadian infants fed processed milk have been related to reduced vitamin C levels in the milk after processing (Demers et al., 1965).

It would be expected from the chemistry of ascorbic acid that the rate of destruction would be a function of several factors such as time, temperature, moisture, oxygen, pH and light (Herreid et al., 1952; Wanninger, 1972).

With the advent of increased interest in the ascorbic acid nutrition of fish (Fish Farming International, 1984a), it is becoming increasingly important to understand what happens to ascorbic acid during processing and storage conditions of feeds as well as during the immersion of fish diets in water. Processing of fish diets requires addition of certain amounts of water and drying and commercially fish diets are processed by steam pelleting and extrusion and in both water addition and drying are involved. Eva et al. (1976) reported that approximately 20% of ascorbic acid added to diets was lost during processing and that after 6 weeks of storage at room temperature only 35% of the ascorbic acid originally added was retained. Hilton et al. (1977b) reported, however, that all of the ascorbic acid in trout diets supplemented with 20-1280mg ascorbic acid/kg diet was lost during processing and after 6 weeks of storage at room temperature (21°C). As a result of such losses of vitamin C during processing and storage attempts have been made to stabilise the vitamin by coating it with ethylcellulose. It has been reported that the coated form of vitamin C has a much greater stability during processing and storage than the free acid (Adams, 1973;

Hilton et al., 1977b; Lovell, 1977; Lovell & Lim, 1978). Ascorbic acid 2-sulphate is also a more stable form (Quadri et al., 1973, 1975) and has been shown to be utilized by rainbow trout and channel catfish (Halver et al., 1975; Murai et al., 1978; Brandt et al., 1985), however, no attempts have been made to estimate its retention during processing and storage.

Tilapias are widely distributed in the tropics (Balarin & Hatton, 1979) where the conditions of feed processing and storage (heat, humidity and lack of storage facilities) will lead to rapid losses of ascorbic acid. The present studies were undertaken to investigate the losses of ascorbic acid, and its forms, during processing, storage for varying periods and leaching of dietary ascorbic acid after varying periods of immersion at two different temperatures.

SECTION 8.2 : EXPERIMENT 1

STABILITY OF DIETARY ASCORBIC ACID LEVELS AFTER PROCESSING, LEACHING AND STORAGE

Section 8.2.1 Materials and Methods

Section 8.2.1.1 Diets

Eight diets were prepared by addition of 25, 50, 75, 100, 125, 250, 300 and 400mg of ascorbic acid per 100g of basal diet (Table 12), with substitution being made for alpha-cellulose of the basal diet. Diet preparation and processing were as previously described (Section 2.3.2).

Section 8.2.1.2 Leaching

Leaching of dietary ascorbic acid (Diet 8) was determined at water temperatures 20°C and 28°C through immersion of dietary samples (one gram, replicated three times) in the water for 0.5, 1, 2 and 3 minutes.

Section 8.2.1.3 Storage conditions

Samples from each diet were stored under the following conditions: Freezer (-20°C), fridge (5-8°C), room temperature (22-24°C) in white bags and additionally at room temperature (22-24°C) in black bags.

Section 8.2.1.4 Experimental methodology

Total ascorbate level was determined for each diet after processing (finished diet) and for each diet under the previous conditions at 30, 50, 90 and 182 days respectively, and for samples of diet before and after immersion in the water.

Section 8.2.2 Statistical Analysis

For evaluation of the experimental results analysis of variance, Duncan's multiple range test (Section 2.9), and correlation coefficient (Parker, 1979) were employed.

Section 8.2.3 Results

For evaluation of the effects of processing, leaching and storage conditions on the stability of varying dietary ascorbic acid levels the following parameters were investigated.

1 Effect of processing

Table 39 summarises the results of the effects of processing on stability of different levels of dietary ascorbic acid. The results show that the retention of dietary ascorbic acid was increased by increasing the dietary ascorbic acid level and that the differences between diets was highly ($P < 0.01$) significant.

2 Effect of leaching

The effect of immersion of fish feed in water for different periods of time at each water temperature (20° and 28°C) on leaching of dietary ascorbic acid are shown in Table 40. Increasing the immersion time led to an increase in leaching as indicated from the high correlation coefficients obtained (Table 40). Also, the results show that increasing the water temperature resulted in increased leaching of ascorbic acid (Table 40).

TABLE 39. Percentage of retention[1] of dietary ascorbic acid after processing

Diet	Supplemental level/100g	Retention (%)
1	25	27.79^{g}
2	50	29.24^{f}
3	75	29.02^{ef}
4	100	30.16^{e}
5	125	34.15^{d}
6	250	37.95^{c}
7	300	40.08^{b}
8	400	44.74^{a}
±SEM[2]		0.21

1. Values in the same column with a common superscript are not significantly different ($P > 0.01$)

2. Standard error of the means derived from analysis of variance

TABLE 40. Effect of immersion of fish feeds in water at two different temperatures on the leaching[1] of ascorbic acid

Immersion Time (min.)	Water Temperature	
	20°C	28°C
	% loss	% loss
0.5	12.94[d]	15.20[d]
1	21.35[c]	28.62[c]
2	30.67[b]	38.01[b]
3	36.48[a]	52.50[a]
±SEM[2]	0.29	0.22
Correlation Coefficient (r)	0.979	0.985

1. Values in the same column with a common superscript are not significantly different (P > 0.01)

2. Standard error of the means derived from analysis of variance

3 Effect of storage

Losses of ascorbic acid for diets supplemented with varying levels of the vitamin were generally increased by the time of storage either in the freezer, fridge, or at room temperature stored in either black or white bags (Tables 41, 42, 43 and 44). The highest losses were recorded for diets stored at room temperature in white bags whereas the lowest losses were recorded for diets stored in the freezer.

Table 45 shows a comparison of the stability of ascorbic acid in diets stored in different conditions after each time interval. The overall stability of ascorbic acid was, in descending order, as follows: freezer, fridge, room temperature in black bags then room temperature in white bags. The differences were highly ($P < 0.01$) significant, except between diets stored at room temperature in black and white bags at 182 days ($P > 0.01$) (Table 45).

TABLE 41. Retention[1] (%) of dietary ascorbic acid stored in the freezer sampled at varying time intervals

Diet	Storage Period (days)					±SEM[2]
	0	30	50	90	182	
	%	%	%	%	%	
1	27.79	27.22[a]	24.14[b]	21.84[c]	18.40[d]	0.30
2	29.24	28.24[a]	27.22[a]	24.56[b]	20.81[c]	0.31
3	29.02	28.83[a]	27.52[b]	25.78[c]	23.41[d]	0.18
4	30.16	28.94[a]	27.67[b]	25.69[c]	23.48[d]	0.13
5	34.15	33.96[a]	32.45[b]	30.04[c]	28.64[d]	0.12
6	37.95	37.45[a]	35.93[b]	34.44[c]	33.49[d]	0.12
7	40.08	39.49[a]	38.39[b]	37.61[c]	36.39[d]	0.09
8	44.74	44.16[a]	41.88[b]	40.37[c]	38.79[d]	0.09

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)

2. Standard error of the means derived from analysis of variance

TABLE 42. Retention[1] (%) of dietary ascorbic acid stored in a fridge sampled after varying time intervals

Diet	Storage Period (days)					±SEM[2]
	0	30	50	90	182	
	%	%	%	%	%	
1	27.79	23.54[a]	20.52[b]	16.15[c]	11.76[d]	0.35
2	29.24	23.50[a]	21.28[b]	18.52[c]	13.30[d]	0.18
3	29.02	24.31[a]	23.10[b]	19.37[c]	13.81[d]	0.16
4	30.16	25.64[a]	23.23[b]	21.09[c]	15.60[d]	0.16
5	34.15	27.11[a]	24.29[b]	21.58[c]	14.51[d]	0.28
6	37.95	32.30[a]	29.55[b]	27.37[c]	23.11[d]	0.07
7	40.08	34.74[a]	31.84[b]	28.50[c]	24.40[d]	0.22
8	44.74	40.83[a]	35.09[b]	32.02[c]	25.25[d]	0.09

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)

2. Standard error of the means derived from analysis of variance

TABLE 43. Retention[1] (%) of dietary ascorbic acid stored at room temperature in black bags sampled after varying time intervals

Diet	Storage Period (days)					±SEM[2]
	0	30	50	90	182	
	%	%	%	%	%	
1	27.79	16.41^{a}	10.12^{b}	0.00^{c}	0.00^{c}	0.28
2	29.24	18.49^{a}	13.04^{b}	0.00^{c}	0.00^{c}	0.63
3	29.02	19.30^{a}	13.73^{b}	0.00^{c}	0.00^{c}	0.13
4	30.16	20.24^{a}	14.62^{b}	9.17^{c}	0.00^{d}	0.17
5	34.15	22.85^{a}	15.61^{b}	10.81^{c}	0.00^{d}	0.17
6	37.95	24.42^{a}	20.40^{b}	11.52^{c}	3.60^{d}	0.18
7	40.08	28.43^{a}	23.53^{b}	14.26^{c}	4.27^{d}	0.12
8	44.74	33.28^{a}	26.62^{b}	15.59^{c}	5.04^{d}	0.16

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)

2. Standard error of the means derived from analysis of variance

TABLE 44. Retention[1] (%) of dietary ascorbic acid stored at room temperature in white bags sampled after varying time intervals

Diet	Storage Period (days)					±SEM[2]
	0	30	50	90	182	
	%	%	%	%	%	
1	27.79	11.20^{a}	6.35^{b}	0.00^{c}	0.00^{c}	0.25
2	29.24	14.47^{a}	8.49^{b}	0.00^{c}	0.00^{c}	0.21
3	29.02	15.05^{a}	9.84^{b}	0.00^{c}	0.00^{c}	0.15
4	30.16	15.54^{a}	10.06^{b}	0.00^{c}	0.00^{c}	0.07
5	34.15	18.13^{a}	10.27^{b}	0.00^{c}	0.00^{c}	0.08
6	37.95	19.24^{a}	14.85^{b}	6.75^{c}	0.00^{d}	0.58
7	40.08	21.33^{a}	16.63^{b}	7.52^{c}	0.00^{d}	0.07
8	44.74	28.11^{a}	18.73^{b}	7.79^{c}	0.00^{d}	0.06

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)

2. Standard error of the means derived from analysis of variance

TABLE 45. Effect of storage conditions on stability[1] (%) of dietary ascorbic acid[2] sampled after varying time intervals

Period (days)	Storage				±SEM[5]
	Freezer	Fridge	RTBB[3]	RTWB[4]	
0	34.14	34.14	34.14	34.14	
30	33.52[a]	28.99[b]	22.93[c]	17.88[d]	1.15
50	31.90[a]	26.11[b]	17.21[c]	11.90[d]	1.07
90	30.04[a]	23.08[b]	7.67[c]	2.76[d]	1.28
182	27.93[a]	17.72[b]	1.61[c]	0.00[c]	0.94

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)
2. Mean values for all eight diets stored under each set of conditions
3. Room temperature in black bags
4. Room temperature in white bags
5. Standard error of the means derived from analysis of variance

SECTION 8.3 : EXPERIMENT 2

STABILITY OF DIFFERENT FORMS OF ASCORBIC ACID DURING PROCESSING AND STORAGE CONDITIONS

Section 8.3.1 Materials and Methods

Section 8.3.1.1 Diets

The following forms of ascorbic acid were employed in this investigation: L-ascorbic acid (AA); Sodium salt of L-ascorbic acid (NaAA); Glyceride coated L-ascorbic acid (GCAA) (Ascorbidan 50, Grindsted products, Denmark) and barium salt of L-ascorbic acid 2-sulphate (AA2S). All four forms were added to the basal diet (Table 12) on an equimolar basis to supply 125mg ascorbic acid/100g of basal diet, substitution being made for alpha-cellulose of the basal diet. Diet preparation was as described previously (Section 2.3.2).

Section 8.3.1.2 Sampling

Samples of each diet were taken after thorough mixing of the ingredients, addition of water, pelleting and drying for determination of total ascorbate contents for Diet 1, 2 and 3 and determination of L-ascorbate 2-sulphate by the methods described earlier (Sections 2.4.4.1 & 2.4.5.).

Section 8.3.1.3 Storage conditions

Samples from each finished diet were stored as previously described (Section 8.2.1.3).

Section 8.3.1.4 Experimental methodology

Samples from the stored diets (Section 8.3.1.3) were analyzed for total ascorbate for Diets 1, 2 and 3 and ascorbate 2-sulphate for Diet 4 at 30, 50, 90 and 182 days by the methods described in Section 8.3.1.2.

Section 8.3.2 Statistical Analysis

As described previously (Section 8.2.2).

Section 8.3.3 Results

Stability of varying forms of ascorbic acid in the present study was investigated using the following parameters:

1 Effect of processing

Mixing, the addition of water, cold pelleting and drying had little effect on the stability of dietary ascorbic acid 2-sulphate. Mixing and the addition of water had little effect on the stability of glyceride coated ascorbic acid but pelleting and drying reduced the retention of this form to 58.1% (Table 46). In contrast, the addition of water, cold pelleting and drying had destructive effects on the stability of ascorbic acid and the sodium salt of ascorbic acid (Table 46). The differences between the dietary ascorbic acid forms in stability during processing were highly ($P < 0.01$) significant (Table 46).

2 Effect of storage

The retention of each dietary form of ascorbic acid during storage was generally decreased by increasing storage period

(Table 47). After 90 days at room temperature, ascorbic acid and the sodium salt of ascorbic acid were no longer detectable (Table 47). A comparison of the stability of different forms of dietary ascorbic acid stored under different conditions after each sampling interval is presented in Table 48. Statistical analyses revealed that the stability of each form stored under different conditions was ranked, in descending order, as follows: freezer, fridge, room temperature in black bags then room temperature in white bags (Table 48).

TABLE 46. Effects of mixing, water addition, cold pelleting and drying on the retention[1] (%) of dietary ascorbic acid forms

Step of Processing	Diet				±SEM[2]
	[1]AA	[2]NaAA	[3]GCAA	[4]AA2S	
Mixing (mash)	94.89^{bc}	93.77^{c}	98.99^{a}	96.78^{ab}	0.51
Water addition	74.59^{b}	71.12^{c}	94.40^{a}	95.70^{a}	0.45
Cold pelleting	64.80^{c}	61.14^{d}	87.55^{b}	95.50^{a}	0.35
Drying	33.50^{c}	26.26^{d}	58.10^{b}	94.70^{a}	0.41

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)

2. Standard error of the means derived from analysis of variance

TABLE 47. Effect of different storage conditions on retention[1] (%) of differing ascorbic acid forms after different periods of storage

Diet	Storage	Storage Period (days)					±SEM[2]
		0	30	50	90	182	
AA	Freezer	33.5	33.26^{a}	32.10^{a}	29.01^{b}	26.99^{c}	0.24
AA	Fridge	33.5	27.66^{a}	24.53^{b}	17.76^{c}	8.40^{d}	0.22
AA	RTBB[3]	33.5	17.14^{a}	8.40^{b}	0.00^{c}	0.00^{c}	0.14
AA	RTWB[4]	33.5	13.44^{a}	3.29^{b}	0.00^{c}	0.00^{c}	0.08
NaAA	Freezer	26.2	25.58^{a}	24.18^{b}	20.57^{c}	18.66^{d}	0.17
NaAA	Fridge	26.2	20.95^{a}	18.16^{b}	13.99^{c}	6.76^{d}	0.17
NaAA	RTBB[3]	26.2	14.00^{a}	5.52^{b}	0.00^{c}	0.00^{c}	0.15
NaAA	RTWB[4]	26.2	11.66^{a}	2.54^{b}	0.00^{c}	0.00^{c}	0.07
GCAA	Freezer	58.10	57.52^{a}	56.44^{b}	52.96^{c}	48.59^{d}	0.21
GCAA	Fridge	58.10	55.35^{a}	49.76^{b}	45.47^{c}	32.09^{d}	0.24
GCAA	RTBB[3]	58.10	43.14^{a}	34.51^{b}	24.86^{c}	10.47^{d}	0.23
GCAA	RTWB[4]	48.10	37.62^{a}	28.89^{b}	18.97^{c}	4.98^{d}	0.31
AA2S	Freezer	94.7	92.30^{a}	91.62^{a}	87.99^{bc}	86.96^{c}	0.35
AA2S	Fridge	94.7	91.14^{a}	87.99^{b}	84.89^{c}	82.82^{d}	0.37
AA2S	RTBB[3]	94.7	87.99^{a}	84.89^{b}	76.78^{c}	71.94^{d}	0.50
AA2S	RTWB[4]	94.7	84.89^{a}	81.95^{b}	73.84^{c}	67.46^{d}	0.45

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)
2. Standard error of the means derived from analysis of variance
3. Room temperature in black bags
4. Room temperature in white bags

TABLE 48. Comparison of stability[1] (%) of different forms of ascorbic acid stored under different conditions

Diet	Storage Period (days)	Storage				±SEM[4]
		Freezer	Fridge	RTBB[2]	RTWB[3]	
AA	30	33.26[a]	27.66[b]	17.14[c]	13.44[d]	0.21
AA	50	32.10[a]	24.53[b]	8.40[c]	3.29[d]	0.16
AA	90	29.01[a]	17.76[b]	0.00[c]	0.00[c]	0.28
AA	182	26.99[a]	8.40[b]	0.00[c]	0.00[c]	0.18
NaAA	30	25.58[a]	20.95[b]	14.00[c]	11.66[d]	0.13
NaAA	50	24.18[a]	18.16[b]	5.52[c]	2.54[d]	0.17
NaAA	90	20.57[a]	13.99[b]	0.00[c]	0.00[c]	0.14
NaAA	182	18.66[a]	6.76[b]	0.00[c]	0.00[c]	0.15
GCAA	30	57.57[a]	55.35[b]	43.14[c]	37.62[d]	0.23
GCAA	50	56.44[a]	49.76[b]	34.51[c]	28.89[d]	0.26
GCAA	90	52.96[a]	45.47[b]	24.86[c]	18.97[d]	0.24
GCAA	182	48.59[a]	32.09[b]	10.47[c]	4.98[d]	0.26
AA2S	30	92.30[a]	91.14[a]	87.99[b]	84.89[c]	0.31
AA2S	50	91.62[a]	87.99[b]	84.89[c]	81.95[c]	0.35
AA2S	90	87.99[a]	84.89[b]	76.78[c]	73.84[d]	0.47
AA2S	182	86.96[a]	82.82[b]	71.94[c]	67.46[d]	0.53

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)
2. Room temperature in black bags
3. Room temperature in white bags
4. Standard error of the means derived from analysis of variance

SECTION 8.4 ; DISCUSSION

The present studies showed that ascorbic acid and its sodium salt are less stable during processing and storage than the other forms evaluated, namely ascorbic acid 2-sulphate and glyceride coated ascorbic acid.

Increasing the level of dietary ascorbic acid resulted in significant retention of ascorbic acid after processing and storage even though the losses in absolute terms were higher in diets supplemented with high levels of ascorbic acid than in those supplemented with lower levels of ascorbic acid. Similar results have been reported by Hilton et al. (1977b). Increased retention of ascorbic acid after processing and storage is required for normal growth and for prevention of deficiency signs. The investigation carried out by Chahine and Starzomski (1976) concerning variations in chemical composition of commercial fish feeds revealed that the ascorbic acid content of feeds obtained from various manufacturing companies was substantially lower than 100mg/kg diet, the level recommended by NRC (1973). Eva et al. (1976) and Sandnes and Utne (1982) recommended 1000mg ascorbic acid/kg diet and 400-800mg ascorbic acid/kg diet for guinea pigs and rainbow trout respectively, levels which compare favourably to that recommended for O. niloticus and O. mossambicus in Chapter 4 in this thesis.

It must be borne in mind that ascorbic acid is a water-soluble vitamin so leaching is another problem, particularly with fish where their feeds are exposed to the aqueous environment. In the present study high correlations were obtained between leaching and immersion time (Table 40) suggesting significant losses of ascorbic acid before

ingestion of food by fish. It is therefore important to reduce the time of immersion as far as possible though the use of automatic feeders or by increased feeding frequency and use of highly palatable, avidly consumed feeds. Protected forms of this vitamin may also help to alleviate leaching. Hilton et al. (1977b) reported that 10% of dietary ascorbic acid was lost within 10 seconds after placing feeds in water and Yamamoto (1982) reported that 99% was lost within 5 minutes of immersion.

Ascorbic acid is very sensitive to storage temperature, light and time of storage (Kothavalla & Gill, 1943; Holmes & Jones, 1945; Herreid et al., 1952; Bullock et al., 1968; Adams, 1973; Newmark et al., 1974; Eva et al., 1976; Hilton et al., 1977b; Sandnes & Utne, 1982; Yamamoto, 1982). The results of experiment 1 showed that the retention of dietary ascorbic acid was affected by storage conditions, exposure to light and length of storage. Hilton et al., (1977b) reported that processed trout diets supplemented with 20-1280mg ascorbic acid/kg diet lost all of the added ascorbic acid after processing and 6 weeks of storage at room temperature (21°C). Sandnes and Utne (1982) reported that fish feeds stored at room temperature (20°C) lost nearly all of their ascorbic acid after 16 weeks of storage and that stored at 4°C lost 70% after 24 weeks of storage. In addition, Newmark et al. (1974) reported that losses of ascorbic acid were reduced when bacon was kept in a freezer (-20°C) when compared to storage in a refrigerator at 5°C. Light is another factor responsible for losses of dietary ascorbic acid during storage. In the present study diets stored at room temperature in black bags showed

lower losses than those kept in white bags at the same temperature. Dunkely et al. (1962) reported that milk kept in paper cartons near fluorescent lights lost 24% more of its ascorbic acid than in cartons kept far from the light. In a well designed experiment Herreid et al. (1952) demonstrated the effect of light on destruction of ascorbic acid when milk was stored in ruby, amber and clear bottles. These authors found lower losses of ascorbic acid occurred in milk kept in ruby and amber bottles and that significant losses occurred in milk kept in clear bottles.

Due to the significant losses of ascorbic acid during processing, leaching and storage, attempts were made to reduce these losses by coating this vitamin with ethylcellulose and mono-, di- and triglycerides (ascorbidan 50) or by introducing into its structure the sulphate group (ascorbate 2-sulphate). In experiment 2 losses due to mixing (mash) were reduced for both GCAA and AA2S and were lower for the former. Hilton et al. (1977b) reported that the retention of ethylcellulose coated ascorbic acid after mixing was 99.75% compared to 91.5% for the uncoated vitamin. The addition of water caused a reduction in retention of both AA and NaAA with little effect on GCAA or AA2S. Hilton et al. (1977b) reported that the addition of distilled water to a diet supplemented with ethylcellulose coated ascorbic acid caused an estimated loss of 23.75%. In the present study the addition of water to the diet supplemented with GCAA caused a 4.59% loss of vitamin activity. The difference between ethylcellulose coated vitamin and GCAA may be because the ethylcellulose coated vitamin can absorb water whereas GCAA has a hydrophobic coating. Cold pelleting is another factor affecting the stability of ascorbic

acid and in the present study this process caused lower losses in diets supplemented with AA2S and GCAA than for diets containing NaAA and AA. These results compare favourably with those reported by Hilton et al. (1977b) for ethylcellulose coated ascorbic acid. No attempts have been made to determine the stability of AA2S in fish feeds, however, in the present study this form exhibited the highest stability. Quadri et al. (1975) reported that this form of the vitamin is much more stable than L-ascorbic acid in wheat foods and milk. GCAA showed a higher stability after drying (58.1%) when compared to AA and NaAA. Sandnes et al. (1984), in contrast, reported that the retention of GCAA after drying was only 10%. Ethylcellulose coated ascorbic acid also showed a higher stability than the uncoated vitamin during processing (54% vs 38%) (Lovell & Lim, 1978). No information is available in the literature concerning the stability of NaAA during processing and storage in fish feeds.

Time and temperature of storage have affected the stability of ascorbic acid forms in the present study, however AA2S was better retained than other forms of ascorbic acid investigated. Hilton et al. (1977b) reported that ethylcellulose coated ascorbic acid exhibited higher stability when stored in a freezer (-20°C) and cooler (5°C) than when stored at room temperature (21°C).

The poor stability of ascorbic acid during processing, leaching and storage suggests that it should be included in feeds at levels in excess of the stated requirement to compensate for probable losses. It may be economical to supplement fish feeds with a protected form of the vitamin. Current prices for the forms tested are: GCAA £5.65/kg;

AA £24.08/kg; NaAA £20.83/kg; AA2S £95,000/kg. These prices indicate the potential of GCAA as a stablised or protected form of ascorbic acid for use in fish feeds and an earlier chapter in this thesis (Chapter 7) has demonstrated that GCAA performs as well as AA in preventing signs of deficiency.

CHAPTER 9

THE EFFECTS OF UNSTABILIZED DIETARY FISH BODY OIL COMBINED WITH ASCORBIC ACID AND BUTYLATED HYDROXY TOLUENE SUPPLEMENTATION ON THE TILAPIA *OREOCHROMIS NILOTICUS* (TREWAVAS)

SECTION 9.1 : INTRODUCTION

Since it was reported that the toxicity of marine oils was caused by the peroxidation of polyunsaturated fatty acids in 1953 (Kaneda & Ishii, 1953) the prevention of rancidity in fats and foodstuffs has been recognised as a nutritional field of great importance. Subsequently many workers have studied the toxic effects of lipid peroxidation on the performance of a variety of animals (Kaneda *et al.*, 1954; Sakaguchi & Hamaguchi, 1969; Murai & Andrews, 1974; Iijima & Zama, 1978, 1979; Sakaguchi & Hamaguchi, 1979a; Hung *et al.*, 1980, 1981; Hata & Kaneda, 1980; Soliman, 1982; Soliman *et al.*, 1984).

In lipid peroxidation the unsaturated fatty acids undergo a loss of hydrogen resulting in the formation of a free radical at the site of unsaturation which is converted quickly to fatty acid hydroperoxides (Rumsey, 1980, Fig. 22).

Oxidative rancidity is liable to occur spontaneously when any material containing unsaturated lipid is exposed to air and the rate of change will be influenced by the nature of the fatty acids and the conditions of storage (Lea, 1958). Hung *et al.* (1980) reported that commercial fish feed formulations contain high levels of unsaturated fatty acids from fish oil and that such diets are usually prepared by steam pelleting followed by storage in paper bags at room temperature for long periods of time. During such preparation and storage the feeds will be exposed to conditions in which rancidity is promoted and this will result in destruction of the vitamins A, D and E, pigments (carotenoids), fatty acids, protein and amino acids (Kraybill & Dugan, 1954;

Desai & Tappel, 1963; Roubal & Tappel, 1966a, b; Tappel, 1973; Rumsey, 1980).

To prevent such oxidative destruction, antioxidants are added to commercial feeds. In foods, as in living tissues, there are two types of environment in which oxidation may occur, in aqueous solutions and in lipids. Hence antioxidants, like vitamins, may be divided into two classes:

1. Water-soluble antioxidants which include sulphur dioxide (acts as a free radical acceptor), ascorbic acid and cysteine.

2. Lipid-soluble antioxidants which include the tocopherols, the phenolic antioxidants, butylated hydroxy toluene (BHT) and butylated hydroxy anisole (BHA), the gallate esters and also ascorbyl palmitate (Johnson, 1979).

Higgins and Black (1945) and Soliman (1982) have proposed the following characteristics for the ideal antioxidant to protect dietary fatty acids and lipids it must;

1. Exhibit effective inhibitory action.
2. Be easily soluble in fats
3. Impart no foreign flavour, odour or colour even on long continued storage
4. Exhibit no changes when heated
5. Be able to retard rancidity
6. Be available in quantity at economical prices
7. Exhibit no harmful physiological effects
8. Have <u>in vivo</u> and <u>in vitro</u> effects.

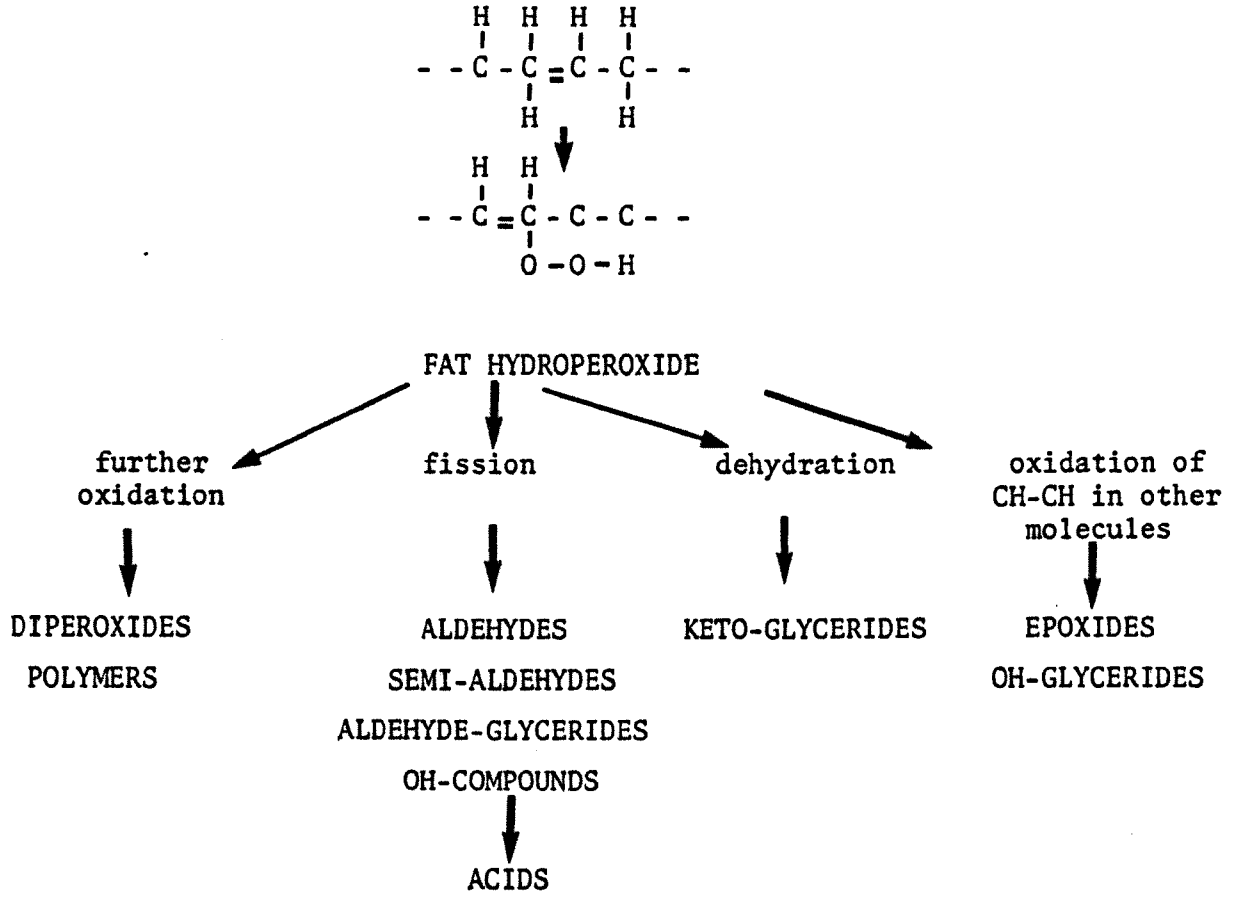


FIGURE 22. Chain reactions which may occur when unsaturated fats oxidize

It has been reported by various workers (Green & Bunyan, 1969; Draper, 1970; Scott, 1978) that the dietary antioxidant level can affect the dietary requirements for some important nutrients such as vitamins. There are controversial results concerning the antioxidant effects of dietary ascorbic acid (Golumbic & Mattill, 1941; Riemenschneider et al., 1944; Kelley & Watts, 1957) and the synergistic effects of ascorbic acid with the other antioxidants in preventing and retarding rancidity (Calkins & Mattill, 1944; Scarborough & Watts, 1949; Watts & Faulkner, 1954; Watts, 1956). The present study was therefore conducted to investigate the effects of unstabilized dietary fish body oil combined with ascorbic acid and butylated hydroxy toluene supplementation on the tilapia Oreochromis niloticus (Trewavas).

SECTION 9.2 : MATERIALS AND METHODS

Section 9.2.1 Experimental System and Animals

Two hundred and seventy O. niloticus from a genetically homogenous stock (Section 2.2) were randomly distributed at a rate of 15 fish/ 9 litre circular plastic tank into an experimental recirculation system (Section 2.1.1). Fish were acclimated for one week prior to the start of the experiment during which period they were fed a commercial trout diet.

Section 9.2.2 Diets and Feeding Regime

Nine diets were evaluated (Table 49) where two types of oils were used, unstabilized marine fish oil and mixture of commercial oils

(2 parts of cod liver oil and 3 parts of corn oil). Three levels of dietary ascorbic acid (0, 125 and 400mg/100g diet) and two levels of butylated hydroxy toluene (BHT, 0 and 200ppm BHT dissolved in the oil) were employed. Substitution of ascorbic acid and BHT was made for α-cellulose in the basal diet (Table 50). Diet preparation and storage have been previously described (Section 2.3.2). Each of the nine dietary treatments was fed to randomly assigned duplicate tanks of fish for nine weeks with numbers reduced to 10 fish per tank prior to the start of the experiment. A fixed feeding regime of 5% of the body weight per day (dry food/whole fish), divided into 4 equal feeds, was adopted. Fish were fed for six consecutive days, weighed on the seventh and feeding rates for the following week adjusted accordingly.

Section 9.2.3 Experimental Methodology

Oil extraction and oil oxidation parameters

Extraction of the oil from the experimental diets at the beginning and end of the experiment was as described previously (Section 2.4.1.1). Peroxide, thiobarbituric acid (TBA) and anisidine values were measured for fresh oils and oils extracted from the experimental diets by the methods described earlier (Section 2.4.1.2).

Fish weighing procedure

As previously described (Section 7.2.1.3).

TABLE 49. Formula of the experimental diets

Diet	Oil		Ascorbic acid level mg/100g diet			BHT level	
	Commercial	Unstabilized marine fish	0	125	400	0	200ppm
1	-	+	+	-	-	-	-
2	-	+	-	+	-	-	-
3	-	+	-	-	+	-	-
4	-	+	+	-	-	-	+
5	-	+	-	+	-	-	+
6	-	+	-	-	+	-	+
7	+	-	+	-	-	-	-
8	+	-	-	+	-	-	-
9	+	-	-	-	+	-	-

+ Indicates ingredient added to the corresponding diet

- Indicates absence of the ingredient from the corresponding diet

TABLE 50. Composition of basal diet

Ingredient	Amount g/100g
Brown fishmeal (herring type)[1]	51.30
Corn starch	12.35
Dextrin	12.35
Alpha-cellulose	7.50
Binder (carboxymethyl cellulose, sodium salt, high viscosity)	2.00
Chromic oxide	0.50
Oil[2]	10.00
Mineral mix.[3]	2.00
Vitamin mix.[4]	2.00

1. Crude lipid extacted by soxhlet method (petroleum ether for 6 hours)
2. Unstabilized marine fish oil or mixture of 2 parts of cod liver oil and 3 parts of corn oil
3. See Table 9
4. See Table 10

Proximate analysis

An initial sample of fish, 3 per tank, was sacrificed prior to the start of the experiment and subjected to proximate analysis (Section 2.4.2). A final sample of fish, 4 fish per tank for Diets 1, 4 and 7, and 5 fish per tank for Diets 2, 3, 5, 6, 8 and 9 were treated similarly.

Haematocrit and haemoglobin determination

Blood was collected from the experimental fish at the end of the experiment for determination of haematocrit and haemoglobin by methods previously described (Section 2.4.3).

Digestibility studies and apparent net protein utilization

As previously described (Section 7.2.1.3).

Total ascorbate determination

The method of Roe (Section 2.4.4.1) was employed for measuring total ascorbate in the finished feeds at the start and termination of the experiment and in liver, heart, gut, brain, gills, muscle, eyes, gall-bladder, testis and ovary in tissues of 2 fish per replicate for Diets 1, 4 and 7, and 3 fish per replicate for Diets 2, 3, 5, 6, 8 and 9 respectively.

SECTION 9.3 : STATISTICAL ANALYSIS

Analysis of variance and Duncan's multiple range test (Section 2.9) were employed.

SECTION 9.4 : RESULTS

The following parameters were evaluated in the present study.

1. In vitro studies

Oil quality criteria

There were no significant ($P > 0.01$) differences between the unstabilized marine fish oil and the mixture of commercial oils in either POV, TBA or AV values (Table 51), as added to the feeds.

Table 52 summarises the quality criteria of oils extracted from the experimental diets at the beginning and at the termination of the experiment. Oil extracted from the diet supplemented with unstabilized marine fish oil and without BHT (Diet 1) showed significantly ($P < 0.01$) the highest values of POV, TBA and AV (Table 52 and Fig. 23). Addition of ascorbic acid to diets supplemented with the unstablized marine fish oil without BHT (Diets 2 & 3) resulted in a significant ($P < 0.01$) decrease in the oxidation parameters (Table 52 and Fig. 23). However, the diets supplemented with unstablized marine fish oil and with BHT with or without supplemented dietary ascorbic acid (Diets 4, 5 & 6) exhibited significantly ($P < 0.01$) the lowest POV, TBA and AV. Values for these diets compared favourably with those obtained for oils extracted from diets supplemented with commercial oils either with or without ascorbic acid (Diets 7, 8 & 9) (Table 52 and Fig. 23).

TABLE 51. Oil quality criteria[1] for unstabilized marine fish oil and mixture of commercial oils

Criterion	Oil		±SEM[2]
	Unstabilized marine fish	Mixture of commercial	
POV[3], meq/kg	4.28[a]	3.59[a]	0.20
TBA[4], mg/kg	18.18[a]	18.43[a]	0.69
AV[5]	9.59[a]	9.71[a]	0.82

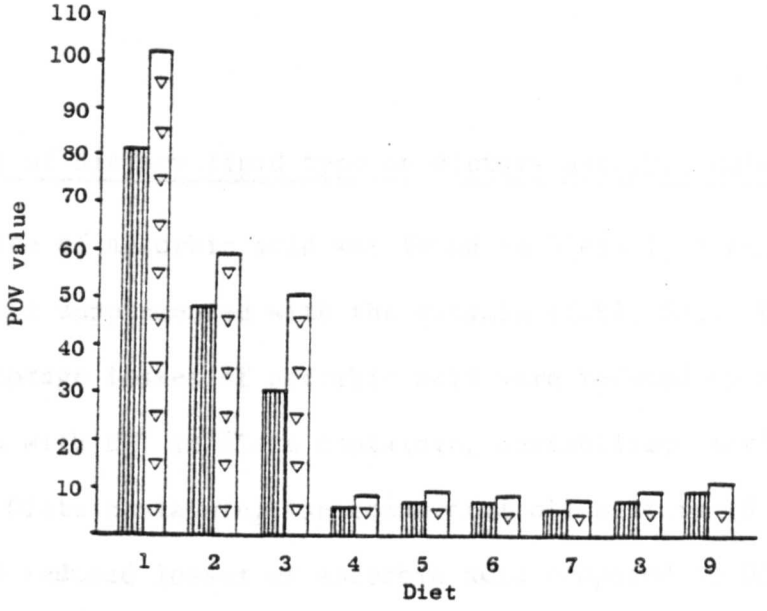
1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)
2. Standard error of the means derived from analysis of variance
3. Peroxide value, as milliequivalents of per 1000g of oil
4. Thiobarbituric acid value, as the absorbance of 1g of sample (in 100ml reagent) multiplied by the factor 46
5. Anisidine value was taken as 100 times the contribution of the extinction of 1g oil in 100ml solvent and anisidine reagents, read at 350nm

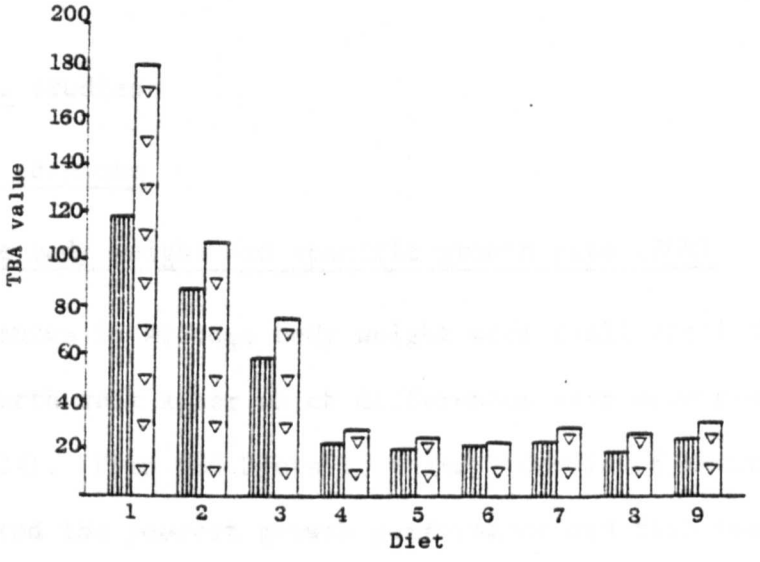
TABLE 52. Oil quality criteria[1] for oils extracted from the experimental diets at the beginning and at the termination of the experiment (after nine weeks)

Diet	POV (meq/kg)		TBA (mg/kg)		AV	
	Initial	Final	Initial	Final	Initial	Final
1	81.67^{a}	101.95^{a}	118.00^{a}	182.20^{a}	101.59^{a}	162.15^{a}
2	48.16^{b}	59.53^{b}	87.15^{b}	106.98^{b}	59.58^{b}	86.51^{b}
3	30.63^{c}	50.78^{c}	58.70^{c}	75.20^{c}	37.73^{c}	64.69^{c}
4	6.02^{e}	8.92^{de}	23.00^{d}	28.57^{def}	16.14^{de}	18.75^{def}
5	7.07^{de}	9.58^{de}	20.66^{de}	24.99^{fg}	13.05^{e}	15.57^{ef}
6	7.24^{de}	8.98^{de}	21.66^{de}	22.98^{g}	13.53^{e}	14.52^{f}
7	5.75^{e}	7.92^{e}	23.30^{d}	29.82^{de}	13.85^{de}	19.13^{de}
8	7.31^{de}	9.41^{de}	19.54^{e}	26.25^{efg}	13.12^{e}	16.74^{ef}
9	9.89^{d}	11.71^{d}	24.04^{d}	30.96^{d}	17.32^{d}	21.21^{d}
±SEM[2]	0.80	0.50	0.75	0.78	0.84	0.95

1. Values in the same column with a common superscript are not significantly different ($P > 0.01$)

2. Standard error of the means derived from analysis of variance





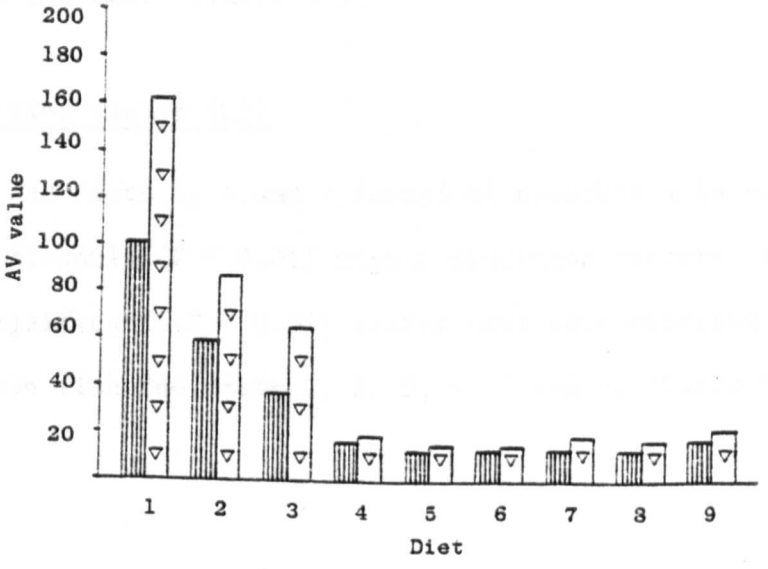


Fig. 23. Peroxide (POV), Thiobarbituric acid (TBA) and anisidine (AV) values for oil extracted from the experimental diets at the start and at the end of the experiment.

Effect of dietary lipid type on dietary ascorbic acid level

No trace of ascorbic acid was found in Diets 1, 4 and 7 which were not supplemented with the vitamin (Table 53). Processing and storage losses of ascorbic acid were reduced by supplementation with BHT in diets containing unstabilized marine fish oil. Diets containing the commercial oil mixture (8 & 9) also showed reduced losses of ascorbic acid compared to Diets 2 & 3 (Table 53).

2. In vivo studies

(i) Growth response

Average body weight and specific growth rate (SGR)

Differences in average body weight were small until the end of the fourth week after which differences were more pronounced (Fig. 24). Fish fed Diets 1, 4 and 7 devoid of ascorbic acid exhibited the poorest growth performance and fish fed Diets 5 and 8 the best. (Table 54).

Condition factor (CF)

Fish fed Diets 1, 4 and 7 devoid of ascorbic acid exhibited significantly ($P < 0.01$) higher condition factors. (Table 54). No significant ($P > 0.01$) differences were observed in CF between fish fed Diets 2, 3, 5, 6, 8 and 9. (Table 54).

TABLE 53. Retention[1] of dietary ascorbic acid (mg/kg) at the beginning and at the termination of the experiment

Diet	At the beginning of experiment	At the termination of experiment
1	[2]N.D.[f]	N.D.[g]
2	355.03[e]	258.31[f]
3	1530.67[c]	1329.07[c]
4	N.D.[f]	N.D.[g]
5	427.67[d]	399.83[e]
6	1777.00[b]	1637.07[b]
7	N.D.[f]	N.D.[g]
8	426.63[d]	409.88[d]
9	1789.67[a]	1651.07[a]
±SEM[3]	2.02	1.78

1. Values in the same column with a common superscript are not significantly different ($P > 0.01$)
2. Not detectable
3. Standard error of the means derived from analysis of variance

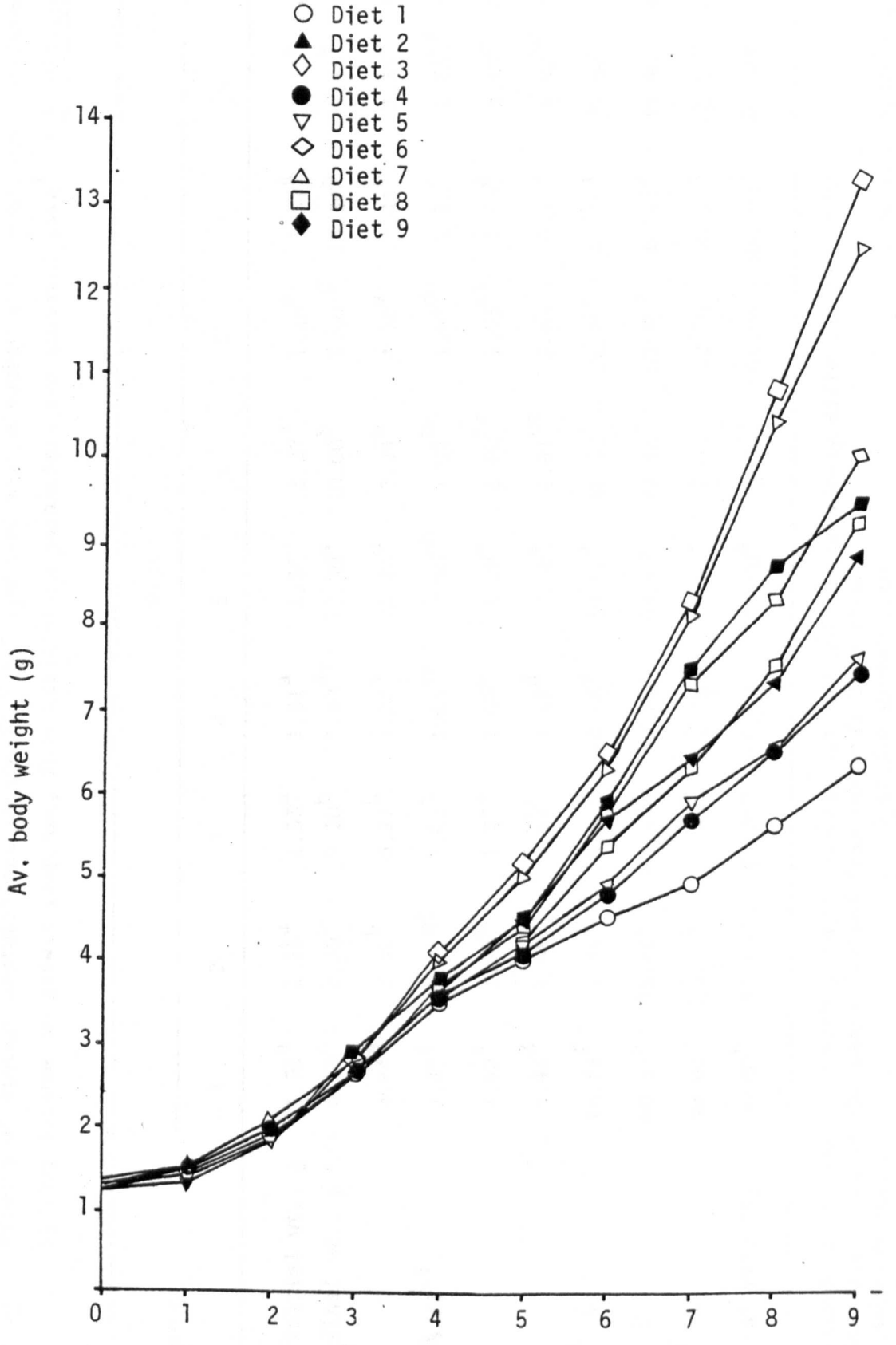


Fig. 24. Increase in average body weight of O. niloticus fed the various experimental diets

TABLE 54. Effects of dietary unstabilized marine fish oil combined with ascorbic acid levels and butylated hydroxy toluene on growth response, food utilization parameters and survival rate[1] in O. niloticus

Parameter	Diet									±SEM[2]
	1	2	3	4	5	6	7	8	9	
Avg. initial wt., g	1.31^{a}	1.25^{a}	1.33^{a}	1.31^{a}	1.35^{a}	1.27^{a}	1.26^{a}	1.31^{a}	1,32^{a}	0.092
Avg. final wt., g.	6.30^{e}	8.80^{bc}	9.20^{b}	7.40^{de}	12.50^{a}	10.00^{b}	7.60^{cd}	13.30^{a}	9.34^{b}	0.36
FC[3]	4.44^{a}	3.36^{b}	3.27^{b}	4.27^{a}	3.28^{b}	3.24^{b}	4.30^{a}	3.41^{b}	3.35^{b}	0.046
SGR[4], % dl^{-1}	1.47^{f}	1.81^{cd}	1.83^{c}	1.61^{ef}	2.06^{ab}	1.91^{bc}	1.67^{de}	2.12^{a}	1.78^{cd}	0.033
FCR[5]	1.69^{a}	1.33^{c}	1.27^{c}	1.66^{a}	1.10^{d}	1.40^{bc}	1.53^{ab}	1.06^{d}	1.42^{bc}	0.033
PER[6]	1.48^{d}	1.89^{bc}	1,98^{b}	1,52^{d}	2.28^{a}	1.81^{bc}	1.63^{d}	2.36^{a}	1.87^{bc}	0.033
ANPU[7] (%)	16.75^{f}	29.36^{c}	30.99^{b}	18.38^{e}	40.92^{a}	31.55^{b}	20.10^{d}	41.74^{a}	29.09^{c}	0.37
APD[8] (%)	60.39^{g}	79.81^{d}	82.37^{c}	67.22^{f}	88.49^{a}	84.41^{b}	69.85^{e}	88.97^{a}	84.00^{a}	0.27
ADMD[9] (%)	44.02^{e}	64.39^{b}	62.85^{b}	49.70^{d}	69.81^{a}	65.03^{b}	52.76^{c}	70.3 ^{a}	65.12^{b}	0.49
Survival rate (%)	60.00^{b}	85.00^{a}	85.00^{a}	60.00^{b}	100.00^{a}	95.00^{a}	65.00^{b}	100.00^{a}	90.00^{a}	3.34

1. Values in the same row with a common superscript are not significantly different (P > 0.01)
2. Standard error of the means derived from analysis of variance
3. Condition factor
4. Specific growth rate
5. Food conversion ratio
6. Protein efficiency ratio
7. Apparent net protein utilization
8. Apparent protein digestibility
9. Apparent dry matter digestibility

(ii) Food utilization parameters

Food conversion ratio (FCR) and protein efficiency ratio (PER)

Both FCR and PER were significantly ($P < 0.01$) improved by addition of both ascorbic acid and BHT (Diet 5) to the diet containing unstabilized marine oil or by addition of ascorbic acid to the commercial oils (Diet 8). (Table 54).

Apparent net protein utilization (ANPU), apparent protein digestibility (APD) and apparent dry matter digestibility (ADMD)

Fish fed Diet 1 (supplemented with the unstabilized oil without BHT or ascorbic acid) exhibited significantly ($P < 0.01$) the poorest ANPU, APD and ADMD (Table 54). ANPU, APD and ADMD were best for fish fed Diets 5 and 8. (Table 54).

(iii) Gross body composition data

The results of body composition analysis are presented in Table 55. Fish fed Diets 5 and 8 showed a significant increase in ash and crude protein contents of their carcass (Table 55) whereas fish fed Diet 1 exhibited significantly ($P < 0.01$) lower carcass crude protein and higher carcass crude lipid (Table 55).

(iv) Tissue and biochemical changes

Table 56 shows the effects of dietary treatments on blood parameters and tissue ascorbate concentrations. Fish fed Diet 1 exhibited the lowest values for haematocrit and haemo-

TABLE 55. Gross body composition data[1] (as % wet weight basis)

Diet	Parameter			
	Moisture (%)	Ash (%)	Crude lipid (%)	Crude protein (%)
F°	79.50	3.00	3.50	14.00
1	74.88^{a}	3.68^{d}	7.19^{a}	14.33^{g}
2	73.85^{ab}	3.96^{bc}	5.48^{e}	16.78^{b}
3	74.31^{a}	3.90^{bc}	5.66^{de}	16.25^{c}
4	74.75^{a}	3.89^{bc}	6.14^{b}	15.26^{ef}
5	72.49^{c}	4.27^{a}	5.84^{cd}	17.60^{a}
6	74.47^{a}	3.99^{b}	5.99^{bc}	15.72^{de}
7	74.82^{a}	3.73^{cd}	6.21^{b}	15.15^{f}
8	73.02^{bc}	4.28^{a}	5.63^{de}	17.32^{a}
9	74.57^{a}	3.95^{bc}	5.64^{de}	16.03^{cd}
±SEM[2]	0.22	0.065	0.067	0.117

F° Body composition sample of fish analysed at the beginning of the experiment

1. Values in the same column with a common superscript are not significantly different ($P > 0.01$)

2. Standard error of the means derived from analysis of variance

TABLE 56. Effects on blood parameters and tissue ascorbate concentrations[1]

Parameter	Diet									±SEM[2]
	1	2	3	4	5	6	7	8	9	
1. Blood										
HC[3], %	27.00^{f}	37.67^{bc}	36.00^{cd}	30.00^{e}	40.00^{ab}	35.00^{d}	30.00^{e}	40.67^{a}	35.34^{cd}	0.58
HG[4], gdl^{-1}	6.52^{e}	11.73^{b}	10.23^{c}	7.61^{d}	12.97^{a}	11.48^{b}	7.75^{d}	12.75^{a}	11.86^{b}	0.18
2. Tissue ascorbate concentrations (µg/g wet tissue)										
Liver	9.47^{f}	48.97^{e}	125.77^{b}	9.38^{f}	110.59^{c}	146.73^{a}	9.88^{f}	102.62^{d}	147.95^{a}	1.19
Ovary	54.01^{e}	264.96^{d}	434.32^{b}	60.02^{e}	393.75^{c}	504.65^{a}	56.51^{e}	392.07^{c}	509.04^{a}	2.36
Testis	21.60^{d}	131.67^{c}	199.54^{b}	25.60^{d}	206.24^{b}	309.80^{a}	26.40^{d}	201.40^{b}	304.89^{a}	1.52
Gut	22.93^{f}	72.15^{e}	129.82^{c}	16.63^{g}	82.84^{d}	185.35^{a}	23.03^{f}	87.32^{d}	178.93^{b}	1.15
Gills	27.53^{g}	98.65^{e}	156.48^{b}	31.97^{g}	127.67^{d}	189.87^{a}	38.68^{f}	133.25^{c}	193.79^{a}	1.21
Brain	66.43^{e}	215.60^{d}	356.21^{b}	65.01^{e}	317.62^{c}	405.44^{a}	70.24^{e}	309.25^{c}	414.61^{a}	2.27
Eyes	15.18^{f}	60.56^{d}	92.23^{b}	16.95^{f}	81.15^{c}	122.77^{a}	22.94^{e}	77.78^{c}	118.23^{a}	1.11
Heart	[5]N.D.^{f}	66.67^{e}	137.20^{b}	N.D.^{f}	80.00^{d}	177.00^{a}	N.D.^{f}	90.00^{c}	177.00^{a}	1.69
Muscle	4.95^{e}	16.75^{d}	36.48^{b}	4.54^{e}	24.13^{c}	48.41^{a}	5.91^{e}	26.50^{c}	45.33^{a}	0.74
Gall-bladder	N.D.^{e}	20.80^{d}	35.56^{b}	N.D.^{e}	26.67^{c}	45.94^{a}	N.D.^{e}	26.55^{c}	43.31^{a}	1.14

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)
2. Standard error of the means derived from analysis of variance
3. Haematocrit
4. Haemoglobin
5. Not detectable

globin ($P < 0.01$) (Table 56). Significantly ($P < 0.01$) the lowest tissue ascorbate concentrations were detected in tissues of fish fed Diets 1, 4 and 7 (Table 56). Fish fed Diets 2 and 3 exhibited significantly lower tissue ascorbate concentrations than fish fed the corresponding diets (Diets 5 & 6 and 8 & 9) (Table 56) supplemented with BHT or commercial oils.

(v) Symptoms of ascorbic acid deficiency

All groups of fish fed diets devoid of ascorbic acid (Diets 1, 4 and 7) exhibited higher mortality (Table 54). In addition, these groups exhibited all the signs of ascorbic acid deficiency reported earlier (Chapter 4). The heaemorrhaging observed in fish fed Diet 1 was more severe than that observed in fish fed Diets 4 and 7.

SECTION 9.5 : DISCUSSION

Dietary lipid has a dual metabolic role as a dense, high calorific value source of energy and as a source of essential polyunsaturated fatty acids.

Fish diets generally contain fish oils (e.g. cod liver oil) which are characterized by high concentrations of polyunsaturated fatty acids. The requirement of fish for such fatty acids is disadvantageous since the preparation of fish diets by steam pelleting or extrusion, involves wet heat application and subsequent drying

which promotes lipid rancidity (Hung et al., 1981). Many nutritional problems may arise from an imbalance between dietary polyunsaturated lipids and biological antioxidants (Tappel, 1968). The use of synthetic antioxidants appears to be vital in fish diets.

Hung et al. (1981) reported that the POV, TBA and AV values for fresh herring oil were 5meq/kg oil, 20mg/kg oil and 11 respectively which are comparable with those presented in Table 51.

In the present study supplementation with dietary ascorbic acid (125 and 400mg/100g) was partially effective in preventing oxidative rancidity in vitro in terms of POV, TBA and AV (Table 52 and Fig. 23). However, ascorbic acid was not as effective as BHT in stabilizing dietary lipids (Table 52 and Fig. 23). Witting (1975) stated that the antioxidant vitamins, such as vitamin E, are rather poor antioxidants particularly in products containing highly unsaturated fatty acids. It is often cited that ascorbic acid may act as a water-soluble antioxidant (Johnson, 1979) and that its ability to penetrate fat molecules is low in comparison to the ability of, for example, BHT (a fat-soluble antioxidant).

Watanabe et al. (1970) reported that dietary ascorbic acid levels decreased in the presence of increased TBA values in diets of carp. This observation is supported by the results of the present study (Table 53).

In the present study average body weight and FCR were significantly better for fish fed Diet 5 (low oxidation values) compared to those

for fish fed Diet 2 (high oxidation values) even though both of these diets were supplemented with the same level of ascorbic acid (125mg/100g). The difference may be due to either the adverse effects of oxidised dietary lipid or to the concomittant reduction in dietary ascorbic acid below the requirement level (Chapter 4). Hung and Slinger (1980), however, reported no significantly difference in live weight gain or FCR of rainbow trout fed diets containing either oxidised or unoxidised lipid when these diets were supplemented with 400mg ascorbic acid per kg diet.

The present study shows the deleterious effects of oxidised dietary lipids on protein utilization as measured by PER, ANPU and APD for fish fed Diet 1. Desai and Tappel (1963) reported damage to cytochrome C due to lipid peroxidation which resulted in the loss of 17% of its amino acids. Cowey and Roberts (1978) suggested that oxidised dietary lipids may have adverse effects on proteolytic enzymes.

It has also been reported (Roubal & Tappel, 1966b) that lipid peroxides may react with proteins forming insoluble protein complexes characterized by being polymeric in nature. Soliman (1982) reported that dietary lipid peroxidation had adverse effects on protein utilization in O. niloticus.

Oxidised lipid in a diet without antioxidants (Diet 1) resulted in accumulation of carcass lipid (Table 55) indicating disturbed lipid metabolism. Perkins et al. (1961) and Iijima et al. (1983) reported

that the rate of lipid absorption from the intestinal tract of rats and carp was significantly reduced by diets containing oxidized oils. Soliman et al. (1984), from histological observation, reported that O. niloticus fed oxidized lipids showed massive infiltration of visceral peritoneal tissues and adipose tissues by inflammatory cells indicating steatitis.

In the present study reduced haematocrit and haemoglobin values were noted for fish fed Diet 1 (high oxidation parameters). Hung and Slinger (1980) reported a significant decrease in red blood cell counts, haematocrit and haemoglobin in rainbow trout fed oxidized oils. Tappel (1973) reported that red blood cell membranes are particularly liable to lipid peroxidation due to their high content of polyunsaturated fatty acids and their direct exposure to molecular oxygen. Lipid peroxidation may therefore result in haemolysis of red cells and Tappel further reported that this haemolysis resulted from damage to the lipid structure of red cell membranes due to inhibition of erythrocyte acetylcholinesterase activity by lipid peroxides. This may explain the more severe haemorrhages observed in fish fed Diet 1 than those noticed in fish fed Diets 4 and 7 and also the severe haemorrhages reported by Soliman (1982) in tilapias fed oxidised dietary lipids.

Tissue ascorbate concentrations were significantly decreased for fish fed Diets 2 and 3 compared to those of fish fed Diets 5, 6 and 8 & 9. (Table 56). This may possibly be related to actual utilization of some of the ascorbic acid to ameliorate the toxic effects of lipid

peroxides. Hung and Slinger (1980) reported similar results for liver ascorbate in rainbow trout.

The importance of inclusion of synthetic antioxidants in animal diets is well recognised. Ousterhout and Matterson (1968) found that addition of 500-1000ppm of ethoxyquin or BHT to menhaden and anchovetta meals improved their metabolizable energy contents and amino acids availability (lysine) to chicks.

In conclusion the present study demonstrates the value of addition of a synthetic antioxidant to tilapia feeds high in polyunsaturated fatty acids and that even megadoses of ascorbic acid (400mg/100g) do not produce as good a response as ascorbic acid (125mg/100g) plus BHT (200ppm).

CHAPTER 10

THE EFFECT OF THE DIETARY ASCORBIC ACID LEVEL (VITAMIN C) OF COMMERCIAL TROUT DIET ON PERFORMANCE OF TILAPIA *OREOCHROMIS NILOTICUS* (TREWAVAS)

SECTION 10.1 : INTRODUCTION

Metabolism is a chemical action and therefore increases in rate with temperature (Phillips, 1972). Consequently the nutrient requirements of warm water fish (high metabolic rate) will be significantly different from those of cold water fish (low metabolic rate).

Vitamins are substances needed by all forms of life, either plants or animals, and they accelerate chemical reactions (metabolism) without taking part in these reactions (Phillips, 1957). Therefore, the vitamin requirements of warm water fish should be higher than those of cold water fish. Tacheuchi *et al.* (1980) reported that the riboflavin (vitamin B_2) requirements of common carp (*Cyprinus carpio*) and rainbow trout were 0.7 and 0.6 mg of B_2 per 100 g diet respectively, a very small and possibly insignificant difference.

Ascorbic acid (vitamin C) is an important water-soluble vitamin in fish feeds (Fish Farming International, 1984a) and its presence is essential to ensure growth promotion, food utilization, collagen synthesis, freedom from signs of deficiency and improvement of reproductive performance (Lovell, 1973; Wilson & Poe, 1973; Halver *et al.*, 1975; Lim & Lovell, 1978; Sato *et al.*, 1978a; Mahajan & Agrawal, 1979, 1980a; Sato *et al.*, 1982; Sandnes *et al.*, 1984; Chapters 4, 5 & 13 in this thesis).

Water temperature appears to alter the vitamin C requirements in fish for example Halver *et al.* (1969) suggested that rainbow trout (*Salmo gairdneri*) and coho salmon (*Oncorhynchus kisutch*) held at 15°C required 100 and 50 mg of ascorbic acid per kg diet respectively

whereas Mahajan & Agrawal (1980a) reported that the Indian major carp (Cirrhina mrigala) held at 30° required 700 mg of ascorbic acid per kg diet. Recently Sato et al. (1983) reported that liver ascorbate concentrations of rainbow trout held at 20°C were lower than for fish held at 16°C although both groups were fed diets containing the same level of ascorbic acid, possibly indicating an increase in catabolism of ascorbic acid with increasing water temperature. It has been suggested by Hilton et al. (1977a) that liver ascorbate concentration in fish be used as an index of ascorbic acid level in fish feeds.

Hilton et al. (1979b) suggested that the low ascorbic acid requirements of rainbow trout (cold water fish) are apparently due to the long half-life and low turnover rate of ascorbic acid in these fish. However these authors did not relate the low requirement to water temperature although it is apparent that low temperatures should have a direct effect on the half-life and turnover rate of vitamins.

The vitamin requirements of cold water fish such as salmonids are well documented (McLaren et al., 1947; Wolf, 1951; Halver & Coates, 1957; Phillips, 1957; Coates & Halver, 1958; Halver et al., 1969; NRC, 1973; NRC, 1981; Halver, 1982) whereas those for O. niloticus are not and the only report in relation to vitamin requirements of O. niloticus appears to be concerning vitamin B_{12} by Lovell & Limsuwan (1982). Commercial trout diets (Ewos-Baker, Bathgate, Scotland) are the principle feeds used in the Institute of Aquaculture, Stirling University, for feeding various species of tilapias. The present study was undertaken to compare the performance of a commercial trout diet with the same diet supplemented to a level of 125 mg of ascorbic

acid per 100 g diet using O. niloticus in a 12 week growth trial.

SECTION 10.2 : MATERIALS & METHODS

Section 10.2.1 Experimental System and Animals

One hundred and forty Oreochromis niloticus obtained from the Institute hatchery, from a genetically homogenous stock (Section 2.2), were stocked at 35 fish/150 litre circular tank into an experimental recirculation system (Section 2.1.2) and were acclimated for one week prior to the start of the experiment during which they were fed a commercial trout diet.

Section 10.2.2 Diets and Feeding Regime

Two diets were employed in the present study. Diet 1 was a commercial trout diet (Ewos-Baker, Bathgate, Scotland) and Diet 2 was made by grinding the commercial diet and adding 115 mg of ascorbic acid per 100 g of diet and processing and drying as described previously (Section 2.3.2). Each of the dietary treatments was fed to randomly assigned duplicate tanks of fish with 25 fish per tank. Feeding regime was as described in Section 7.2.1.2.

Section 10.2.3 Experimental Methodology

Fish weighing procedure

As described in Section 7.2.1.3.

Proximate analysis

An initial sample of fish, 5 per tank, was sacrificed prior to the start of the experiment and subjected to proximate analysis (Section 2.4.2). A final sample of 6 fish per tank for both treatments was analysed similarly.

Haematocrit and haemoglobin determination

At the termination of the experiment blood was collected from the experimental fish by the procedure described in Section 2.4.3 and subjected to determination of haematocrit and haemoglobin using the methods reported in Section 2.4.3.

Apparent net protein utilization

ANPU was calculated as described in Section 2.7.

Total ascorbate determination

Total ascorbate in both diets and total ascorbate concentration in the liver, heart, gut, brain, gills, muscle, eyes, gall-bladder, kidney, testis and ovary were measured at the termination of the experiment for 6 fish per tank by the method reported earlier (Section 2.4.4.1).

Glycogen determination

Sub-samples of muscle and liver were analysed for total glycogen (Section 2.4.6).

Section 10.2.4 Statistical Analysis

For evaluation of experimental data analysis of variance (Section 2.9) was used.

SECTION 10.3 : RESULTS

The following parameters were evaluated in this study.

1. Chemical analysis

The chemical composition of a commercial trout diet is shown in Table 57. The retention of ascorbic acid in the finished diets is presented in Table 58. The addition of 115 mg of ascorbic acid per 100 g diet resulted in an increased ascorbate level in the supplemented diet compared with the commercial trout diet.

2. Nutritional parameters

O. niloticus fed the supplemented commercial trout diet performed significantly ($P < 0.01$) better on the basis of average body weight, specific growth rate, food conversion ratio, protein efficiency ratio and apparent net protein utilization (Fig. 25 and Table 59) whereas no significant differences were found in condition factors between fish fed the two experimental diets (Table 59).

3. Gross body composition

Gross body composition data is presented in Table 60. Fish fed the supplemented diet showed significantly ($P < 0.01$) increased crude protein and ash contents with decreased carcass lipid and

TABLE 57. Chemical analysis of commercial trout diet

Parameter	
Moisture	6.46
Ash	11.80
Crude lipid	13.00
Crude protein	45.00
Total carbohydrate[1]	23.74

1. Total carbohydrate = 100-(% moisture + % ash + % crude lipid + % crude protein)

TABLE 58. Ascorbic acid levels of commercial trout diet and commercial trout diet supplemented with additional ascorbic acid

Parameter	DIET	
	Commercial trout	Supplemented Commercial trout
Ascorbic acid (mg/kg diet)	100	420

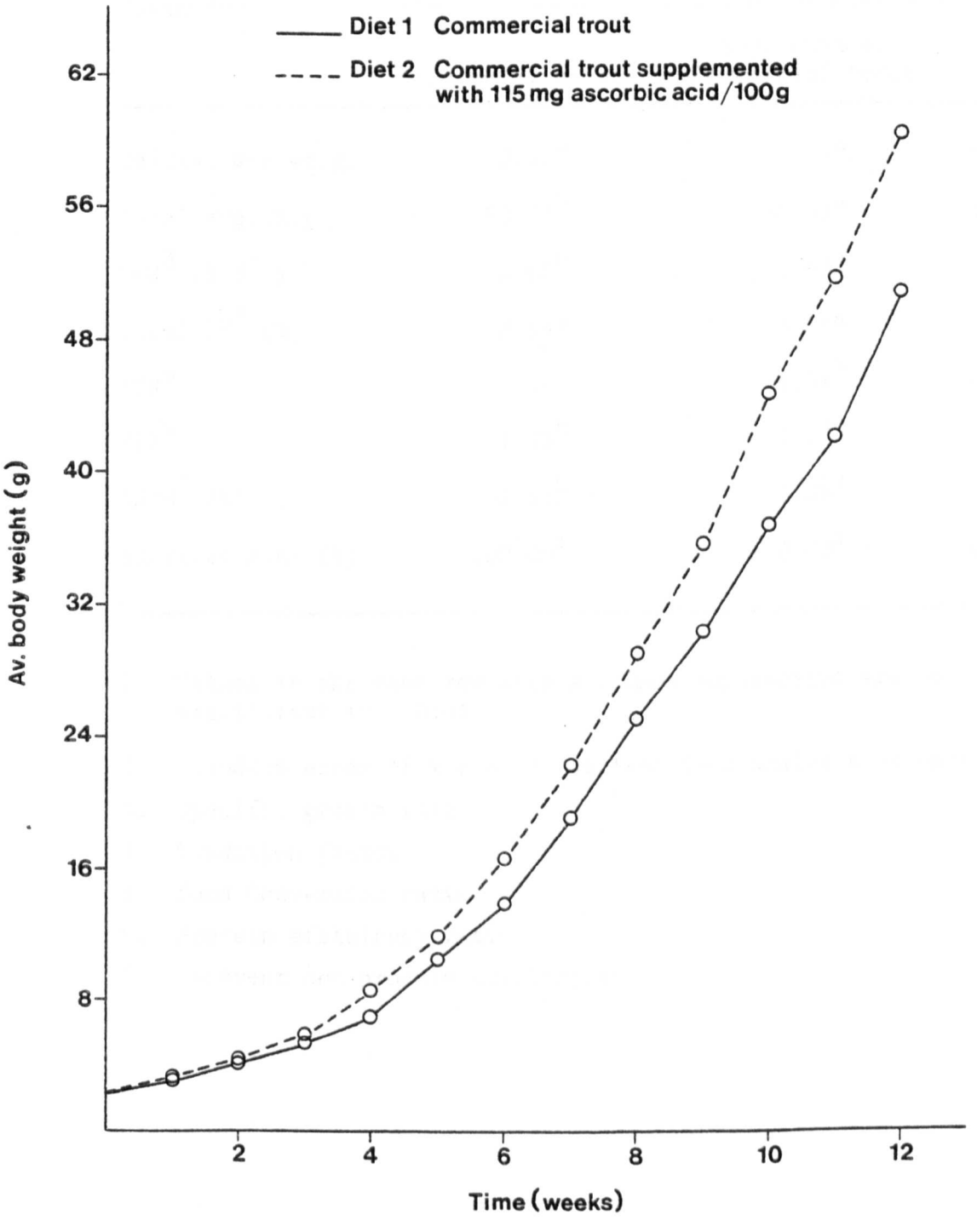


Fig. 25. Increase in average body weight of O. niloticus fed the two experimental diets

TABLE 59. Growth, food utilization parameters and survival rate[1]

Parameter	DIET		±SEM[2]
	Commercial trout	Supplemented Commercial trout	
Initial avg.wt.g.	2.32[a]	2.32[a]	0.096
Final avg.wt.g.	50.67[b]	60.39[a]	1.630
SGR[3] (% d^{-1})	3.67[b]	3.88[a]	0.020
Final CF[4] (%)	3.19[a]	3.18[a]	0.037
FCR[5]	1.14[a]	1.01[b]	0.015
PER[6]	1.93[b]	2.2[a]	0.016
ANPU[7] (%)	32.14[b]	40.09[a]	0.324
Survival rate (%)	100.00[a]	100.00[a]	0.000

1. Values in the same row with a common superscript are not significant ($P > 0.01$)
2. Standard error of the means derived from analysis of variance
3. Specific growth rate
4. Condition factor
5. Food Conversion ratio
6. Protein efficiency ratio
7. Apparent net protein utilization

TABLE 60. Gross body composition data[1] (as % wet weight)

Parameter	DIET			±SEM[2]
	F°	Commercial trout	Supplemented Commercial trout	
Moisture (%)	77.47	69.62[a]	68.47[a]	0.245
Ash (%)	4.34	4.65[b]	4.85[a]	0.037
Crude lipid (%)	3.64	9.66[a]	7.75[b]	0.053
Crude protein (%)	14.58	16.43[b]	18.6[a]	0.053

F° Body composition sample of fish analysed at the beginning of the experiment

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)

2. Standard error of the means derived from analysis of variance

the reverse was true for fish fed the commercial trout diet. No significant difference ($P > 0.01$) was found between the carcass moisture contents.

4. Tissue and biochemical parameters

Table 61 summarises the results of blood parameters, hepatosomatic indeces, liver and muscle glycogen and total ascorbate concentrations in 11 tissues of fish fed the experimental diets. There were no significant differences in haematocrit, haemoglobin, hepatosomatic index and muscle and liver glycogen of fish fed the experimental diets (Table 61). However, total ascorbate concentrations in all tissues examined for fish fed the supplemented diet were significantly ($P < 0.01$) greater than those for fish fed the commercial trout diet (Fig. 26 & Table 61).

5. Survival rate and gross signs of vitamin C deficiency

No mortalities occurred in both treatments during the experimental period (Table 59) and no signs of vitamin C deficiency were observed.

SECTION 10.4 : DISCUSSION

The results of the present study support the belief that a diet designed for feeding a particular species of fish should not be used, without careful consideration, for feeding a different species due to interspecific differences in behaviour and metabolism.

Growth appears to be a satisfactory criterion for determination of the ascorbic acid requirements of guinea pigs (Collins & Elvehjem,

TABLE 61. Blood parameters, hepatosomatic index, liver and muscle glycogen and tissue ascorbate concentrations[1] of fish fed the experimental diets

Parameter	DIET		±SEM[2]
	Commercial trout	Supplemented Commercial trout	
HC[3], %	42.00[a]	42.75[a]	0.235
HG[4], %	12.61[a]	12.58[a]	0.070
MCHC[5], %	30.62	29.43	
HSI[6], %	2.85[a]	3.07[a]	0.114
LG[7], %	10.46[a]	10.85[a]	0.152
MG[8], %	0.66[a]	0.71[a]	0.037
Tissue ascorbate			
Liver, $\mu g.g^{-1}$	26.51[b]	128.60[a]	0.970
Gut, $\mu g.g^{-1}$	46.78[b]	87.53[a]	1.330
Gills, $\mu g.g^{-1}$	48.27[b]	142.84[a]	1.730
Brain, $\mu g.g^{-1}$	118.54[b]	219.83[a]	2.920
Eye, $\mu g.g^{-1}$	26.89[b]	51.89[a]	0.960
Gall-bladder, $\mu g.g^{-1}$	17.39[b]	36.08[a]	0.930
Muscle, $\mu g.g^{-1}$	24.95[b]	41.97[a]	1.500
Heart, $\mu g.g^{-1}$	19.99[b]	39.67[a]	2.040
Ovary, $\mu g.g^{-1}$	109.27[b]	539.38[a]	2.840
Testis, $\mu g.g^{-1}$	70.88[b]	202.84[a]	1.400
Kidney, $\mu g.g^{-1}$	46.95[b]	78.28[a]	1.190

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)
2. Standard error of the means derived from analysis of variance
3. Haematocrit
4. Haemoglobin
5. Mean corpuscular haemoglobin concentrations
6. Hepatosomatic index
7. Liver glycogen
8. Muscle glycogen

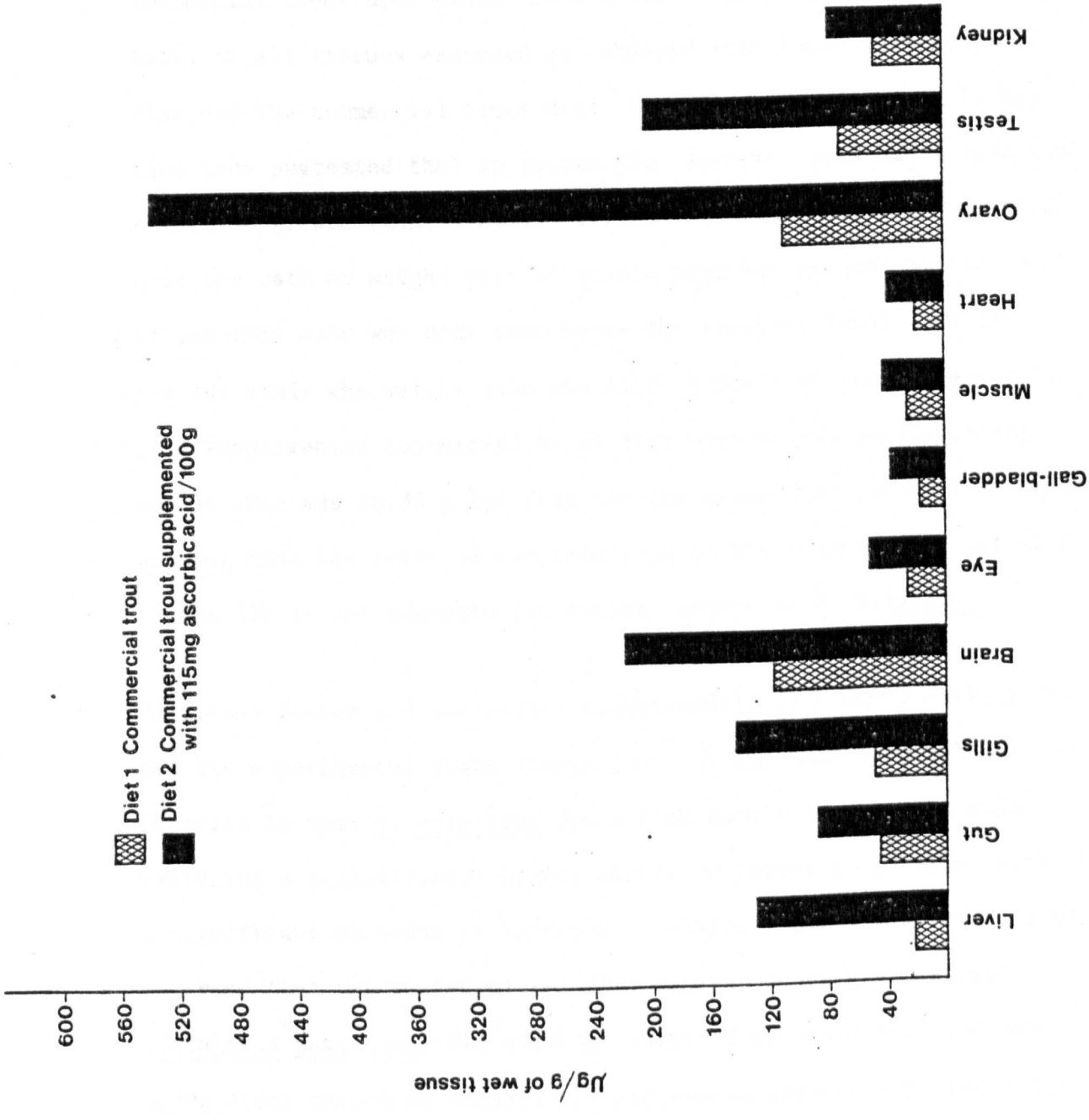


Fig. 26. Total tissue ascorbate concentrations for O.niloticus after 12 weeks on the experimental diets

1958) and on this basis in the present study the growth of fish fed the commercial trout diet supplemented with ascorbic acid was superior to that of fish fed the commercial trout diet (Fig. 25 and Table 59). It has been reported by Collins and Elvehjem (1958) that there is a positive correlation between growth and tissue ascorbate concentration. In the present study fish fed the ascorbic acid supplemented commercial trout diet exhibited markedly higher ascorbate concentrations in all tissues examined as compared with those in tissues of fish fed the commercial trout diet (Fig. 26 and Table 61). It has also been suggested that in guinea pigs ascorbic acid has a specific effect on growth (Ram, 1966). Collins and Elvehjem (1958) reported that the rate of weight gain of guinea pigs was reduced as the level of ascorbic acid was decreased below the required level. In the present study the weight gain was 58.07 g for fish fed the ascorbic acid supplemented commercial trout diet whereas the corresponding weight gain was 48.35 g for fish fed the commercial trout diet suggesting that the level of ascorbic acid in the commercial trout diet (Table 58) is not adequate for optimal growth of O. niloticus.

Condition factor did not differ significantly ($P > 0.01$) between fish fed the experimental diets (Table 59). It has been reported earlier (Section 5) that O. niloticus fed a diet devoid of ascorbic acid exhibited a significantly higher condition factor in parallel with a significant decrease in vertebral collagen. Lim and Lovell (1978) reported that the vertebral collagen content of channel catfish (Ictalurus punctatus) fed varying levels of ascorbic acid (30-240 mg/kg diet) showed no significant difference whereas fish fed a diet

devoid of ascorbic acid showed a significant decrease in vertebral collagen.

In the present study food conversion ratio (Table 59) was improved by increasing the dietary ascorbic acid level (Table 58) in parallel with increased body weight gain in this group. Andrews and Murai (1975) suggested that food conversion ratio is a reflection of weight gain when a fixed feeding rate is employed.

Protein efficiency ratio, apparent net protein utilization and carcass crude protein were significantly increased for fish fed the commercial trout diet supplemented with ascorbic acid. It has been suggested by different workers (Rohatgi et al., 1958; Chatterjee, 1967; Williams & Hughes, 1972) that ascorbic acid plays an important role in certain aspects of protein metabolism. It is possible that the higher levels of ascorbic acid detected in tissues of fish fed the supplemented commercial trout diet facilitate this role.

It was observed that the carcass crude lipid content was increased in fish fed the commercial trout diet possibly due to incomplete utilization of dietary protein.

Variations in haematocrit and haemoglobin levels (Table 61) were not significant ($P > 0.01$) and similar results for haematocrit have been reported in channel catfish (Lim & Lovell, 1978).

Banerjee (1943b) reported that the glycogen content of the liver of normally fed guinea pigs was not significantly changed by injection of extra vitamin C. In the present study liver and muscle glycogen levels were not affected by increased dietary ascorbic acid (Table 61) neither were hepatosomatic indices. In Chapter 5 of this thesis hepatosomatic index decreased in parallel with a decrease in liver glycogen in severely scorbutic O. niloticus.

Sauberlich (1975) suggested that biochemical measurements provide the most objective assessment of vitamin C requirements. In the present study ascorbate concentrations in all the tissues examined were higher ($P < 0.01$) for fish fed the commercial trout diet supplemented with ascorbic acid (Fig. 26 and Table 61). Tissue ascorbate content of the liver of rainbow trout has also been shown to reflect dietary ascorbic acid level (Sato et al., 1978a) in agreement with the results of the present study.

The high dietary ascorbic acid requirement of O. niloticus (held at 28°C) may be related to its high metabolic rate which possibly leads to a short half-life of this nutrient and possible existence of dehydro-L-ascorbatase (an enzyme responsible for catalyzing or de-lactonization of dehydro-L-ascorbic acid yielding 2,3 diketogulonic acid, a metabolite of catabolism of L-ascorbic acid. Yamamoto et al. (1977b) reported that the hepatopancreas of common carp possess the enzyme dehydro-L-ascorbatase whereas the hepatopancreas of rainbow trout does not. In addition, Ikeda and Sato (1965) reported that the half-life of C^{14} labelled ascorbic acid in common carp was 3.8 days whereas in contrast Hilton et al. (1979a, b) reported a half-

life of 21 and 40 days in the head kidney of rainbow trout and Tucker (1983) reported a half-life of 42 days in the same tissue in this species.

In conclusion, therefore, the present study has confirmed that diets designed, formulated and manufactured for cold water species such as rainbow trout, should only be used for warm water species after careful consideration of differences in nutrient requirements. For many warm water species, including tilapias, whose vitamin requirements are incompletely documented it has, hitherto, been the practice to formulate feeds using data available for trout. The current investigation shows that this practice will not necessarily result in feeds capable of sustaining optimal growth.

CHAPTER 11

SEX DIFFERENCES IN RELATION TO DIETARY ASCORBIC ACID NUTRITION OF *O. NILOTICUS* AND *O. MOSSAMBICUS*

SECTION 11.1 : INTRODUCTION

Although ascorbic acid (vitamin C) has been shown to be an important factor in normal ovarian functions in animals (Claesson et al., 1949; Lutwak-Mann, 1958; Noach & Rees, 1958; Baird et al., 1961; Schmidt-Elmendorff & Loraine, 1962; Herschberger et al., 1965; Koed & Hamburger, 1967; Abdo et al., 1971; Loh & Wilson, 1971; Goldstein & Sturgis, 1961; Sandnes & Braekkan, 1981; Seymour, 1981a, b) and in maintenance of testicular functions in toad, guinea pig, mouse, rat and pigeon (Biswas & Mukherjee, 1967; Biswas, 1969; Biswas & Deb, 1965; Biswas & Deb, 1970; Roy Chowdhury & Mukherjee, 1976a,b) little information has been published concerning possible differences in ascorbic acid nutrition in relation to sex either in animals or in fish.

Differences in the ascorbic acid nutrition of the different sexes have been demonstrated directly in human (Brook & Grimshaw, 1968; Hindson, 1970), guinea pig (Hughes & Jones, 1971), rat (Stubbs & McKernan, 1967) and indirectly in fish (Ikeda et al., 1963a; Hilton et al., 1979b; Agrawal & Mahajan, 1980b) (Chapters 4, 5, 6, 7, 10 in this thesis).

Organs in which ascorbic acid has a metabolic involvement may well have developed more efficient mechanisms for its abstraction and retention (Hughes & Hurley, 1969) and these include the sex organs. Dodds (1969) has suggested that the ascorbic acid metabolism of tissues in general may be influenced by the sex of the animal in question. The present studies were conducted to investigate the effect of sex on the ascorbic acid nutrition of O. niloticus and O. mossambicus.

SECTION 11.2 : EXPERIMENT 1

SEX DIFFERENCE IN RELATION TO ASCORBIC ACID NUTRITION OF O. NILOTICUS

Section 11.2.1 Materials and Methods

Section 11.2.1.1 Experimental system and animals

Five sexually mature males and five sexually mature females of O. niloticus (derived from homogenous stock, Section 2.2) were housed separately in two rectangular recirculation tanks (Section 2.1.3) and fed a commercial trout ration for a one week acclimatization period.

Section 11.2.1.2 Diet and feeding regime

A single diet (basal diet) was employed in the present study, the composition of which is shown in Table 62. A fixed feeding regime of 1% of the initial body weight of each sex (divided into 4 equal feeds) was adopted throughout the five week experimental period.

Section 11.2.1.3 Experimental methodology

After 5 weeks on the experimental diet samples from both groups were taken for the following determinations:

Organosomatic indices

Organosomatic indices of liver, ovary, testis, eyes, kidney, gills, gall-bladder, brain, heart, gut and spleen were calculated as follows:

$$\text{Organosomatic index (\%)} = \frac{\text{Wet organ weight (g)}}{\text{Body weight (g)}} \times 100$$

TABLE 62. Composition of basal diet

Ingredient	Amount g/100 g
Brown fishmeal (herring type)[1]	38.400
Corn starch	17.800
Dextrin	17.800
Alpha-cellulose	9.375
Binder (Carboxymethyl cellulose, sodium salt, high viscocity)	2.000
Chromic oxide	0.500
Cod -liver oil	4.000
Corn oil	6.000
Mineral mix.[2]	2.000
Vitamin mix.[3]	2.000
L-Ascorbic acid	0.125

1. Crude lipid extracted by soxhlet method (petroleum ether for 6 hours)
2. See Table 9
3. See Table 10

Haematocrit and haemoglobin determination

At the end of the experiment blood was collected from each group and subjected to determination of haematocrit and haemoglobin (Section 2.4.3).

Total L-ascorbate, dehydro-L-ascorbate and L-ascorbate determination

Total L-ascorbate, dehydro-L-ascorbate and L-ascorbate were determined for each group in samples of liver, ovary, testis, eye, kidney, gill, gut, gall-bladder, heart, brain, muscle and blood (Section 2.4.4.1, 2.4.4.2 and 2.4.4.3).

Total glycogen determination

Sub-samples of liver were used for determination of total glycogen (Section 2.4.6).

Section 11.2.2 Statistical Analysis

Statistical analysis of the experimental results was performed using analysis of variance (Section 2.9).

Section 11.2.3 Results

In order to investigate the effect of a single level of dietary ascorbic acid on male and female O. niloticus the following parameters were evaluated.

1. Organosomatic indices

Table 63 summarises the organosomatic indices of ten tissues. Gonadosomatic and hepatosomatic indices of females were significantly ($P < 0.01$) higher than those of males with no significant ($P > 0.01$) differences between sexes in the other organosomatic indices.

2. Tissue and biochemical changes

The results of blood analysis, liver glycogen content, tissue total L-ascorbate, dehydro-L-ascorbate and L-ascorbate concentrations and dehydro-L-ascorbate:Total L-ascorbate ratio are shown in Table 64.

Haematocrit and haemoglobin levels were significantly higher ($P < 0.01$) in males. Females exhibited a significantly higher ($P < 0.01$) liver glycogen content (Table 64).

Total ascorbate concentrations in female ovary, gills, spleen, brain and blood were significantly ($P < 0.01$) greater than in males. In males the eyes showed a significantly ($P < 0.01$) higher total ascorbate concentration than in females. No significant differences ($P > 0.01$) were observed in total ascorbate concentrations in heart, gall-bladder, gut, muscle and liver (Table 64 and Fig. 27). Similar trends were obtained with respect to L-ascorbate concentrations (Table 64).

Females exhibited significantly ($P < 0.01$) higher dehydro-L-ascorbate concentrations in gill and brain than males, with no

significant differences between sexes shown for the other tissues examined (Table 64).

TABLE 63. Organosomatic indices[1] of male and female O. niloticus

Parameter	SEX		±SEM[2]
	Males	Females	
Avg.wt.g	487.3	448.96	58.79
GSI[3], %	0.57[b]	2.99[a]	0.40
ESI[4], %	0.97[a]	1.13[a]	0.08
GiSI[5], %	4.22[a]	4.33[a]	0.186
HSI[6], %	1.28[b]	1.70[a]	0.058
GuSI[7], %	4.31[a]	3.49[a]	0.418
RSI[8], %	0.19[a]	0.15[a]	0.013
BSI[9], %	0.058[a]	0.06[a]	0.005
SSI[10], %	0.05[a]	0.05[a]	0.009
GaSI[11], %	0.25[a]	0.40[a]	0.073
HeSI[12], %	0.105[a]	0.112[a]	0.009

1. Values in same row with a common superscript are not significantly difference ($P > 0.01$)
2. Standard error of the means derived from analysis of variance
3. Gonadosomatic index
4. Eyes-somatic index
5. Gills-somatic index
6. Hepatosomatic index
7. Gut-somatic index
8. Renal-somatic index
9. Brain-somatic index
10. Spleen-somatic index
11. Gall bladder-somatic index
12. Heart-somatic index

TABLE 64. Blood parameters, liver glycogen contents, total L-ascorbate, dehdryo-L-ascorbate and L-ascorbate concentrations[1] and dehydro-L-ascorbate:total ascorbate ratios of male and female O. niloticus

Parameter	SEX		SEM[2]
	Male	Female	
HC[3], %	39.0[a]	36.3[b]	0.279
HG[4], gdl^{-1}	12.57[a]	11.80[b]	0.072
LG[5], %	13.07[b]	17.21[a]	0.110
Total L-ascorbate $\mu g.g^{-1}$			
Gonads	121.08[b]	307.38[a]	1.71
Eyes	59.45[a]	38.49[b]	0.62
Gills	98.18[b]	124.12[a]	1.31
Heart	73.75[a]	71.73[a]	2.50
Kidney	104.53[a]	104.14[a]	0.39
Gall-bladder	24.83[a]	25.83[a]	1.02
Gut	82.45[a]	78.65[a]	2.30
Muscle	23.29[a]	25.33[a]	0.70
Spleen	124.87[b]	181.65[a]	6.33
Brain	178.01[b]	194.29[a]	1.88
Liver	84.48[a]	88.32[a]	1.49
Blood	36.75[b]	50.75[a]	1.01
Dehydro-L-ascorbate $\mu g.g^{-1}$			
Gonads	3.11[a]	4.30[a]	0.286
Eyes	2.66[a]	2.83	0.130
Gills	5.85[b]	8.29[a]	0.428
Heart	4.18[a]	5.94[a]	0.550
Kidney	4.80[a]	4.50[a]	0.180
Gall-bladder	0.62[a]	0.65[a]	0.198
Gut	10.27[a]	9.40[a]	0.43
Muscle	0.490[a]	0.648[a]	0.09
Spleen	9.17[a]	10.67[a]	0.483
Brain	6.79[b]	15.11[a]	0.383
Liver	8.61[a]	9.09[a]	0.420
Blood	1.25[a]	1.19[a]	0.041

TABLE 64 (cont'd)

Parameter	SEX		±SEM[2]
	Male	Female	
L-ascorbate $\mu g.g^{-1}$			
Gonads	117.96[b]	303.08[a]	1.57
Eyes	56.80[a]	35.67	1.26
Gills	92.33[b]	115.829[a]	1.27
Heart	69.57[a]	65.79[a]	2.58
Kidney	99.74[a]	99.67[a]	0.50
Gall-bladder	24.21[a]	25.18[a]	0.46
Gut	72.17[a]	69.25[a]	2.20
Muscle	22.80[a]	24.69[a]	0.60
Spleen	116.53[b]	170.99[a]	6.09
Brain	171.22[b]	179.17[a]	1.75
Liver	75.87[a]	79.23[a]	1.56
Blood	35.49[b]	49.56[a]	0.98
Dehydro-L-ascorbate: total ascorbate $\mu g.g^{-1}$			
Gonads	2.57	1.40	
Eyes	4.47	7.35	
Gills	5.96	6.68	
Heart	5.67	8.28	
Kidney	4.59	4.32	
Gall-bladder	2.50	2.52	
Gut	12.46	11.95	
Muscle	2.10	2.56	
Spleen	7.34	5.84	
Brain	3.81	7.78	
Liver	10.19	10.29	
Blood	3.40	2.34	

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)
2. Standard error of the means derived from analysis of variance
3. Haematocrit
4. Haemoglobin
5. Liver glycogen

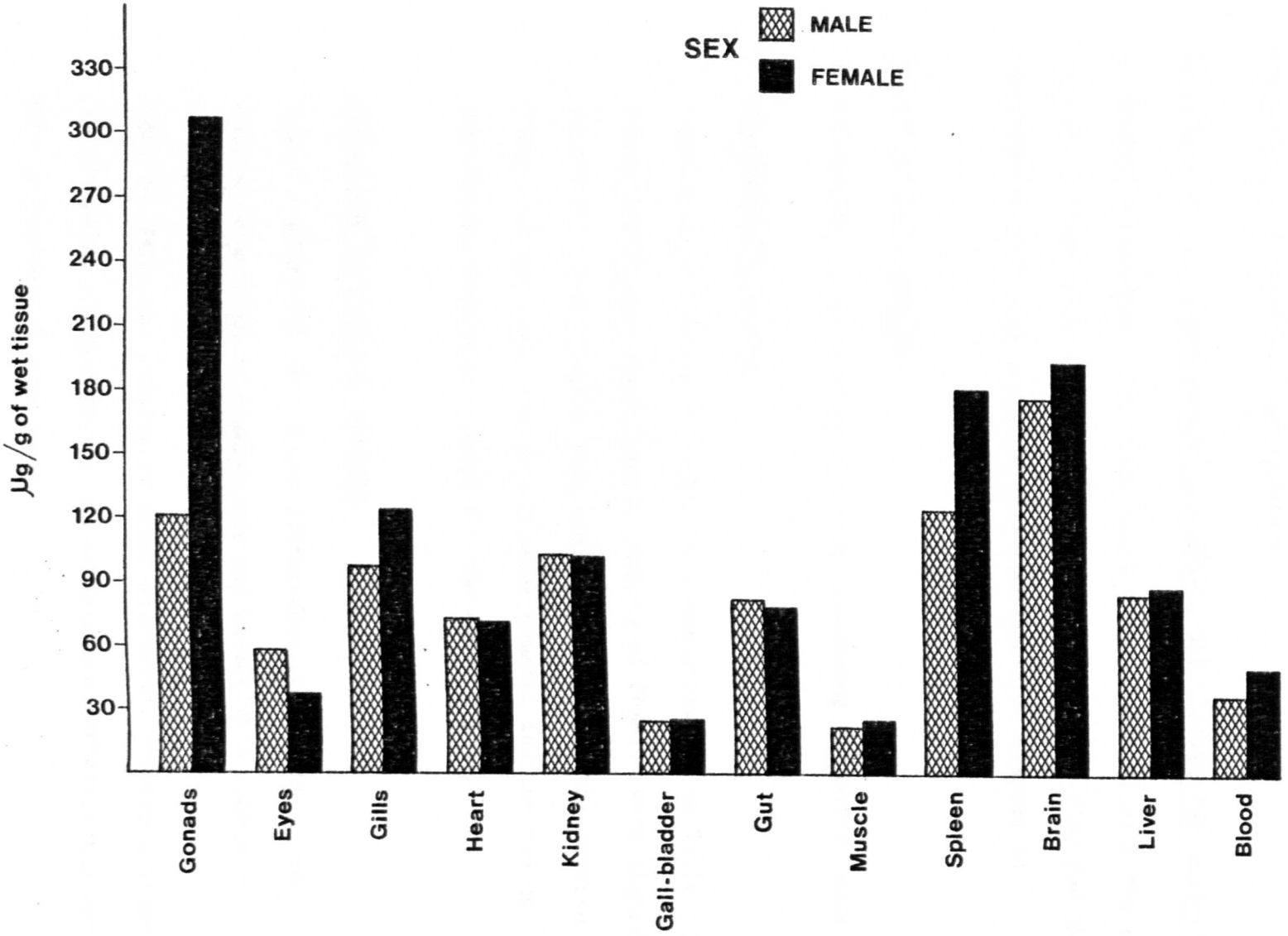


Fig. 27. Total tissue ascorbate concentrations in both sexes of O. niloticus

SECTION 11.3 : EXPERIMENT 2

SEX DIFFERENCES IN RELATION TO ASCORBIC ACID NUTRITION OF O. MOSSAMBICUS

Section 11.3.1 Materials and Methods

Section 11.3.1.1 Experimental methodology and statistical analysis

As described previously (Section 11.2.1.3 and 11.2.1.4) with the exception of eight fish of each sex being employed, from fish fed the supplemented diet with ascorbic acid (Section 5.3, Chapter 5).

Section 11.3.2 Results

For evaluation of the present study the following parameters were used:

1. Organosomatic indices

   Organosomatic indices for both sexes are presented in Table 65. Gonadosomatic and hepatosomatic indices of females were significantly ($P < 0.01$) higher than those of males with the reverse being true for renal and gall-bladder somatic indices and no significant differences noted for the other organs.

2. Tissue and biochemical changes

   Highly significant ($P < 0.01$) differences between males and females were found in haematocrit and haemoglobin (Table 66).

   Table 66 shows the results of total ascorbate, dehydro-L-ascorbate and ascorbate concentrations and dehydro-L-ascorbate:total ascorbate in tissues of both sexes.

TABLE 65. Organosomatic indices[1] of male and female O. mossambicus

Parameter	SEX		±SEM[2]
	Male	Female	
Avg.wt.g.	52.50	44.01	3.08
GSI[3], %	0.78[b]	4.93[a]	0.76
ESI[4], %	1.44[a]	1.50[a]	0.06
GiSI[5], %	4.47[a]	4.72[a]	0.135
HSI[6], %	1.55[b]	1.76[a]	0.037
GuSI[7], %	3.09[a]	3.15[a]	0,242
RSI[8], %	0.43[a]	0.19[b]	0.031
BSI[9], %	0.19[a]	0.17[a]	0.012
SSI[10], %	0.06[a]	0.07[a]	0.01
GaSI[11], %	0.46[a]	0.21[b]	0.04
HeSI[12], %	0.110[a]	0.125[a]	0.015

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)
2. Standard error of the means derived from analysis of variance
3. Gonadosomatic index
4. Eyes-somatic index
5. Gills-somatic index
6. Hepatosomatic index
7. Gut-somatic index
8. Renal-somatic index
9. Brain-somatic index
10. Spleen-somatic index
11. Gall bladder-somatic index
12. Heart-somatic index

TABLE 66. Blood parameters, liver glycogen, total L-ascorbate, dehydro-L-ascorbate and L-ascorbate concentrations[1] and dehydro-L-ascorbate:total L-ascorbate ratios for male and female O. mossambicus

Parameter	SEX		SEM[2]
	Male	Female	
HC[3], %	40.3[a]	38.5[b]	0.260
HG[4], gdl^{-1}	12.14[a]	10.84[b]	0.118
LG[5], %	11.25[b]	15.01[a]	0.170
Total L-ascorbate $\mu g.g^{-1}$			
Gonads	264.83[b]	408.34[a]	2.52
Eyes	68.26[a]	48.92[b]	1.16
Gills	106.24[b]	128.18[a]	0.61
Heart	90.91[a]	91.30[a]	2.11
Kidney	72.25	77.04[a]	3.86
Gall-bladder	39.23[a]	39.51[a]	1.06
Gut	89.07[a]	88.18[a]	1.15
Muscle	33.58[a]	32.85[a]	0.60
Spleen	205.96[b]	283.44[a]	8.33
Brain	289.82[b]	323.07[a]	2.84
Liver	71.86[b]	98.42[a]	0.41
Blood	44.31[b]	60.36[a]	0.95
Dehydro-L-ascorbate $\mu g.g^{-1}$			
Gonads	3.18[a]	3.21[a]	0.26
Eyes	N.D.	N.D.	-
Gills	6.86[a]	6.92[a]	0.029
Heart	N.D.	N.D.	-
Kidney	N.D.	N.D.	-
Gall-bladder	N.D.	N.D.	-
Gut	7.30[b]	9.79[a]	0.23
Muscle	3.54[a]	3.45[a]	0.18
Spleen	9.64[a]	12.86[a]	0.75
Brain	6.21[a]	6.22[a]	0.09
Liver	6.48[a]	7.56[a]	0.86
Blood	1.50[a]	1.54[a]	0.158

TABLE 66 (cont'd)

Parameter	SEX		±SEM[2]
	Male	Female	
L-ascorbate µg.g^{-1}			
Gonads	261.66[b]	405.13[a]	2.42
Eyes	68.26[a]	48.92[b]	1.16
Gills	99.38[b]	121.25[a]	0.60
Heart	90.91[a]	91.30[a]	2.11
Kidney	72.25[a]	77.04[a]	3.86
Gall-bladder	39.23[a]	39.51[a]	1.06
Gut	81.76[a]	78.40[a]	1.12
Muscle	27.06[a]	29.40[a]	0.56
Spleen	196.32[b]	270.58[a]	7.70
Brain	283.62[b]	316.81[a]	2.77
Liver	65.05[b]	90.86[a]	1.12
Blood	42.81[b]	58.82[a]	0.90
Dehydro-L-ascorbate: Total ascorbate µg.g^{-1}			
Gonads	1.20	0.79	
Eyes	-	-	
Gills	6.46	5.40	
Heart	-	-	
Kidney	-	-	
Gall-bladder	-	-	
Gut	8.20	11.10	
Muscle	10.54	10.50	
Spleen	4.68	4.54	
Brain	2.14	1.93	
Liver	9.02	7.68	
Blood	3.39	2.55	

1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)
2. Standard error of the means derived from analysis of variance
3. Haematocrit
4. Haemoglobin
5. Liver glycogen

Ovary, gills, spleen, brain, liver and blood of females exhibited significantly ($P < 0.01$) higher total ascorbate concentrations than the same tissues in males (Table 66 and Fig. 28). Total ascorbate concentration in the eyes of males was significantly greater than those of females. No significant differences were observed for the other tissues examined (Table 66 and Fig. 28).

Similar results were obtained for L-ascorbate concentrations (Table 66).

Table 66 reveals no significant differences between sexes in dehydro-L-ascorbate concentrations in gonad, gill, muscle, spleen, brain, liver and blood except that the gut of females showed a significantly higher dehyro-L-ascorbate concentration than males (Table 66). In addition dehydro-L-ascorbate was not detectable in eye, heart, kidney or gall-bladder of either sex.

The highest ratios of dehydro-L-ascorbate;total ascorbate were found in muscle, liver and gut for both sexes (Table 66).

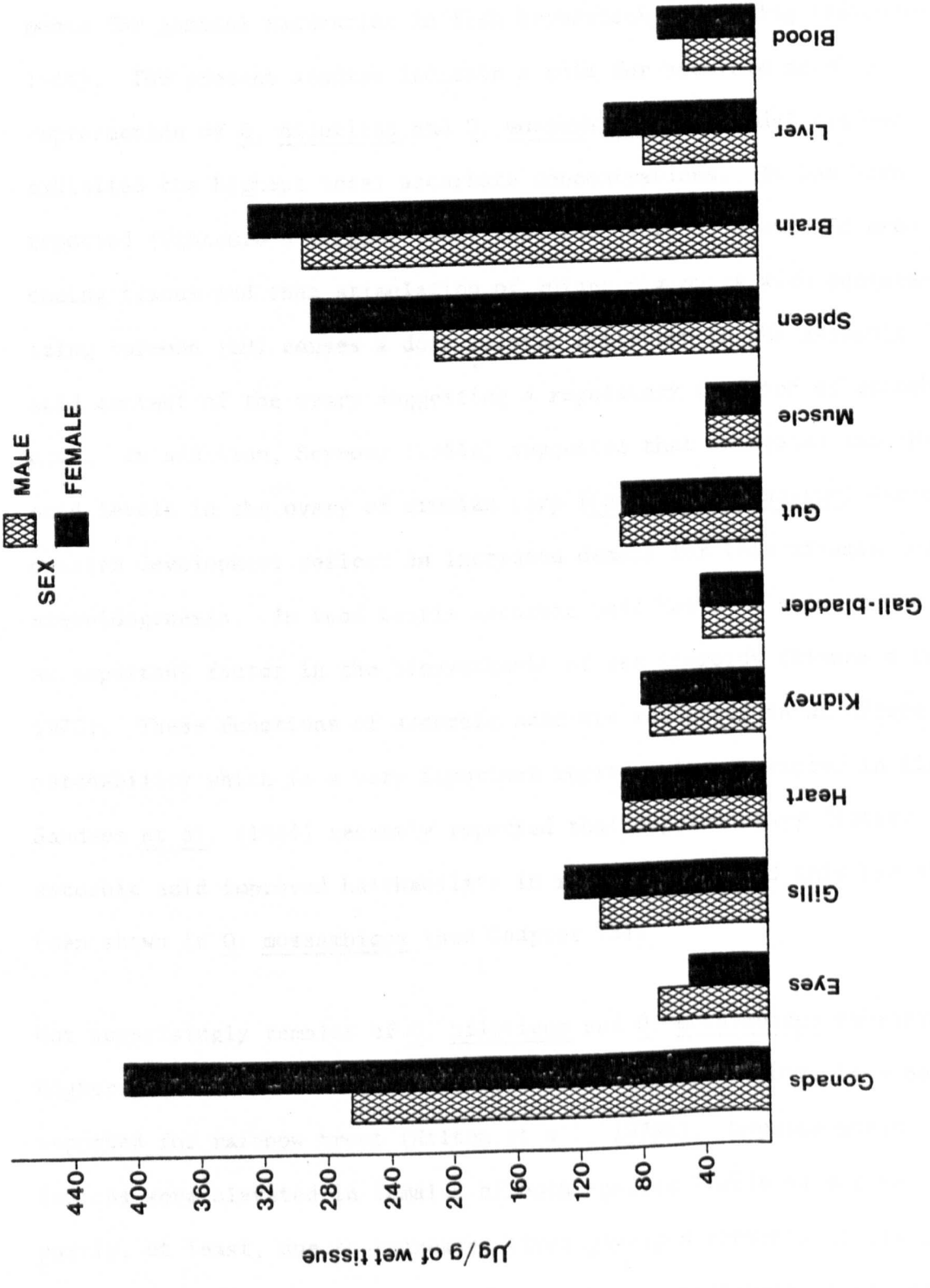


Fig. 28. Total tissue ascorbate concentrations in both sexes of O. mossambicus

SECTION 11.4 : DISCUSSION

Although nutrition is known to have a pronounced effect on gonadal growth and fecundity, precise information on the nutritional requirements for gonadal maturation in fish broodstock is lacking (Watanabe, 1984). The present studies indicate a role for ascorbic acid in reproduction of O. niloticus and O. mossambicus as gonadal tissues exhibited the highest total ascorbate concentrations. It has been reported (Pintauro & Bergan, 1982) that the ovary is a steroid producing tissue and that stimulation of guinea pig ovary with leuteinizing hormone (LH) causes a dose related depletion in the ascorbic acid content of the ovary suggesting a regulatory function of ascorbic acid. In addition, Seymour (1981a) suggested that increased ascorbic acid levels in the ovary of crucian carp (Carassius carassius) during ovarian development reflect an increased demand for this vitamin for steroidogenesis. In toad testis ascorbic acid has been shown to be an important factor in the biosynthesis of sex steroids (Biswas & Deb, 1970). These functions of ascorbic acid are reflected in an effect on hatchability which is a very important reproductive character in fish. Sandnes et al. (1984) recently reported that supplementary dietary ascorbic acid improved hatchability in rainbow trout and this has also been shown in O. mossambicus (see Chapter 13).

Not surprisingly females of O. niloticus and O. mossambicus exhibited higher gonadosomatic indices than males and similar results have been reported for rainbow trout (Hilton et al., 1979b). Hepatosomatic indices were elevated in females of both species (Table 64 and 66 partly, at least, due to increased liver glycogen contents (Table 64 and 66) and to greater activity of the males. Ottolenghi et al. (1981)

reported that hepatosomatic index was elevated, in parallel with increased liver glycogen, in female catfish (Ictalurus melas) when compared to males. There appear to be no reports in the literature concerning the influence of sex on other organosomatic indices. The present studies revealed no significant differences between the sexes in either species investigated for the other organs.

In this study males of both species gave higher haematocrit and haemoglobin values than females. Similar results have been reported in chicken (Sturki, 1965) and also Raizada et al. (1982) reported that red blood cells counts (RBC) in male major carp (Cirrhina mrigala) were higher than in females. Sturki (1965) explained these differences due to the fact that androgens cause an increase in the number and volume of erythrocytes. Loh et al. (1974) suggested that male human subjects had a higher ability to utilize and handle ascorbic acid with respect to haemoglobin levels.

In the present studies total L-ascorbate and L-ascorbate concentrations in the ovary of both species were found to be significantly ($P < 0.01$) higher than in the testis. It has been reported by various workers (Hoygaard & Rasmussen, 1939; Pyke & Wright, 1944; Hastings & Spencer, 1952; Ikeda et al., 1963a; Dabrowski,1977; Hilton et al., 1979b; Agrawal & Mahajan, 1980b: Chapters 4, 5, 6, 7 and 10 of this thesis) that ovary and roe seem to have higher levels of ascorbic acid than testis and milt and that this might be related to the function of the ovary in steroid production (Pintauro & Bergan, 1982).

Hughes & Jones (1971) reported that the eye lenses of male guinea pigs have a significantly higher ascorbate concentration than in females.

In the present studies eyes of males of both species showed significantly ($P < 0.01$) greater concentrations of total L-ascorbate and L-ascorbate than females. This may be due to higher permeability and/or greater retention of the lens membranes to ascorbic acid in males (Hughes & Jones, 1971).

It is interesting to note that the total L-ascorbate and L-ascorbate contents of many tissues examined in both species were higher than in the blood (Table 64 and 66) and similar results for total L-ascorbate have been reported in rats (Stubbs & McKernan, 1967). Females of both species appeared to have higher concentrations of total and L-ascorbate than males in some tissues (Table 64 and 66). Stubbs and McKernan (1967) reported that tissue ascorbate concentrations (plasma, liver, lung, kidney, spleen and muscle) in male rats were significantly higher than in females and they suggest that these differences might be due to a higher rate of hepatic biosynthesis of ascorbic acid in males than in females. It has been suggested that O. mossambicus and O. niloticus are unable to synthesise ascorbic acid (Chatterjee, 1973b; Yamamoto et al., 1978; Soliman et al., 1985) therefore, sexual differences in tissue ascorbate concentrations in these species may be related to a more active transport system in females or alternatively to behaviour as males of both species are more aggressive than females. In addition, Dodds (1969) suggested that the higher ascorbate concentrations in blood of female human subjects are related to hormonal interrelationships in ascorbic acid metabolism. Furthermore, Loh et al. (1974) suggested that there is a metabolic difference between the sexes in human subjects in the availability and utilization of ascorbic acid during sexually determined endocrine demands for ascorbic acid and they concluded that females

would show greater utilization of ascorbic acid in response to metabolic demand.

Dehydro-L-ascorbic acid-reductase (EC 1.8.5.1) is an enzyme required for reduction of dehydro-L-ascorbic acid to L-ascorbic acid (Yamamoto et al., 1977a). Yamamoto et al. (1977a) detected this enzyme in the tissues of different species of fish including common carp, yellow tail, black sea bream, eel and rainbow trout. The low levels of dehydro-L-ascorbate (Table 64 and 66) in various tissues of O. niloticus and O. mossambicus of both sexes suggests the possible existence of this enzyme in the tissues of these species. In addition, the low dehydroascorbate concentrations found by Ikeda et al. (1963a) are in agreement with the results of the present studies. Dehydroascorbate concentrations were reported to be higher in tissues of scorbutic guinea pigs and in the blood of patients suffering from infectious diseases (Banerjee et al., 1952; Banerjee & Belavady, 1953; Chakrabarti & Banerjee, 1955; Bhaduri & Banerjee, 1960; Banerjee, 1977). Elevation of dehydroascorbate will lead to elevation of the dehydroascorbate:total ascorbate ratio. By calculation of this ratio in blood, adrenal, pancreas, spleen, intestine, kidney and liver of scorbutic guinea pigs from the reports of Banerjee et al. (1952) these values were: 28.68, 43.00, 28.49, 27.67, 25.26 and 26.27 respectively. In the present studies the ratios for blood, spleen, gut, kidney and liver (Table 64 and 66) were very low in both sexes in either species compared to those calculated for the data presented by Banerjee et al. (1952), indicating that the levels of dietary ascorbic acid in the present studies were adequate to maintain a low level of dehydroascorbic acid in all the tissues examined of both

sexes of broodstock of O. niloticus and O. mossambicus. Erythrocytes in normal animals are responsible for reduction of dehydroascorbic acid to L-ascorbic acid (Hughes & Maton, 1968) and it is possible this function may be impaired in scorbutic animals.

The significance of ascorbic acid in fish reproduction (Sandnes et al., 1984; Chapter 13 in this thesis) and in improving fertility in bulls (Phillips et al., 1940) suggests that adequate dietary ascorbic acid is necessary to ensure the high tissue levels of ascorbic acid required for metabolic functions.

CHAPTER 12

ASCORBIC ACID REQUIREMENTS IN RELATION TO WOUND HEALING IN THE CULTURED TILAPIA, *OREOCHROMIS NILOTICUS* (TREWAVAS)

SECTION 12.1 : INTRODUCTION

The significance of, and requirements for, dietary vitamin C (ascorbic acid) are well recognised in higher animals. This vitamin is required for maturation of collagen throughout the connective tissues including bone, cartilage, dentine and dermis (Bourne, 1946). Ascorbic acid also plays a key role in wound healing and, in its absence, although reticulin fibres may be laid down by the healing lesion, maturation of these to collagen occurs very slowly if at all (Hunt, 1940).

In fishes, ascorbic acid has significant roles in preventing deficiency signs such as structural deformities, retarded growth and haemorrhages (Kitamura et al., 1965; Poston, 1967; Lovell, 1973; Wilson, 1973 and Wilson & Poe, 1973; Andrews & Murai, 1975; Halver et al., 1975; Lim & Lovell, 1978; Murai et al., 1978 and Mahajan & Agrawal, 1979, 1980a. The majority of studies on wound healing in fish have been performed on cold water species (Halver et al., 1969; Halver, 1972b and Halver et al., 1975). The only report on wound healing in relation to dietary ascorbic acid deficiency in warm water teleosts appears to be that on the channel catfish, Ictalurus punctatus (Rafinesque) by Lim & Lovell (1978). In the above studies the rate of wound healing has been shown to be directly dependent on dietary vitamin C intake.

It is important to differentiate, in terms of wound healing, between the epidermis, responsible for osmoregulatory and pathogen control, and the dermis which is responsible for mechanical strength of the epidermis (Bullock et al., 1978). The ascorbic acid deficient salmon appears to reunite its epidermis at normal rate whereas the dermis,

being the collagenous tissue, does not mature (Halver, 1972b).

Tilapias are very important freshwater tropical food fishes (Balarin & Hatton, 1979) and an earlier section of this thesis (Chapter 4) has revealed that they have an optimum dietary requirement of 125 mg ascorbic acid per 100 g diet. The present study was undertaken to determine the effect of dietary ascorbic acid deficiency on simple wound healing in Oreochromis niloticus (Trewavas), the most commonly cultured of the tilapias.

SECTION 12.2 : MATERIALS AND METHODS

Section 12.2.1 Animals, System and Diets

Ninety one, sibling, specific pathogen free Oreochromis niloticus were stocked in a recirculation system (Section 2.1.1) and fed an ascorbic acid free diet (Table 12) for two weeks to deplete tissue ascorbate pools. Thereafter, they were divided into three groups of 27 fish with an average body weight 2.3 g. Fish were fed for 16 days at 5% body weight per day according to the following regimen:

Group 1: ascorbic acid free diet (basal diet);

Group 2: basal diet supplemented with 125 mg ascorbic acid per 100 g dry diet (adequate level), and

Group 3: basal diet supplemented with 400 mg of ascorbic acid per 100 g dry diet (excess level).

Diet preparation and storage were as described previously (Section 2.3.2).

Section 12.2.2 Wounding

Fish were anaesthetised (benzocaine, Ross & Ross, 1984) and a standard

2 mm x 4 mm long incision was made vertically into the interior myotomal area of the right shoulder. The wound rarely showed any significant haemorrhage and fish appeared to behave quite normally on recovery.

Section 12.2.3 Sampling

An initial group of fish was sampled at the end of the depletion period for determination of initial tissue ascorbate concentrations (Section 2.4.4.1).

Two fish from each group were killed at 0 hour, 1 hour, 5 hours, 10 hours and 1, 2, 3, 4, 5, 7, 9, 13 and 16 days after wounding. Blocks of tissue encompassing the wound were immediately removed and fixed in cooled 10% formal saline and cut and stained as described in Section 2.5.2.

Tissue samples collected from fish from each group sampled at 3, 4 and 5 days (stage 1) and at 7, 10, 13 and 16 days (stage 2) were pooled and stored at -20^{o}C before determination of tissue ascorbate levels by the 2,4 dinitrophenylhydrazine method of Roe (1967) (Section 2.4.4.1).

SECTION 12.3 : RESULTS

Section 12.3.1 Histological Changes

The wound healing process in Oreochromis niloticus appeared to be basically similar to that described for other fishes in that immediately after incision, in all groups, the surrounding epithelium became spongiotic and a migration of malpighian cells down into the

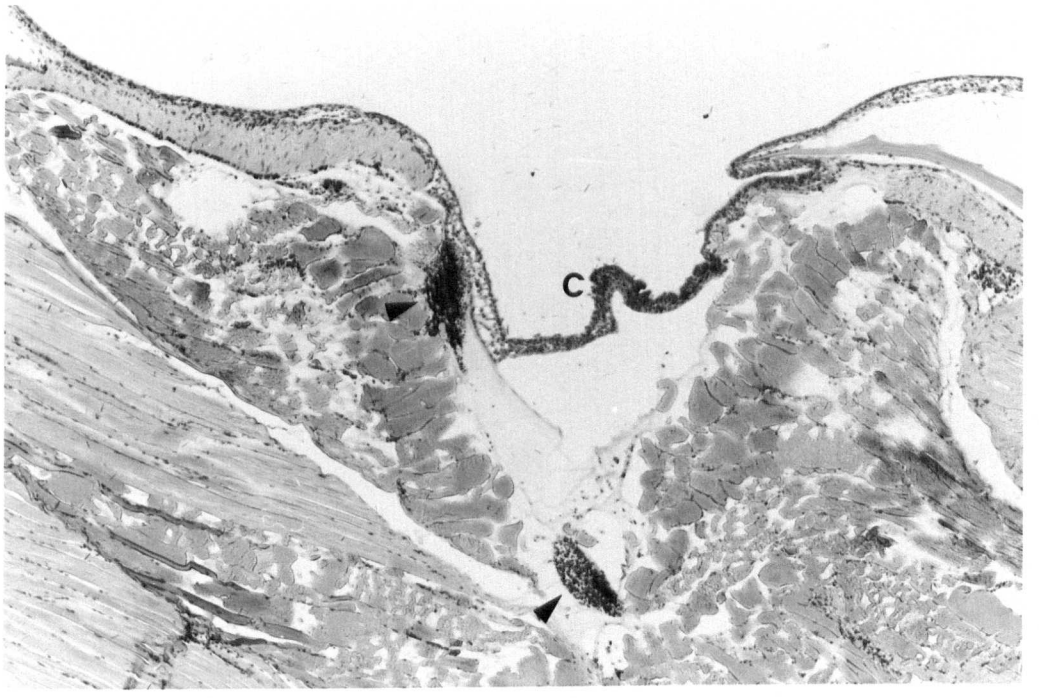
wound commenced covering the surface of the fibrinous tissue exudate which overlay the ends of the severed myofibrils lining the defect. Within one hour, irrespective of vitamin C level, this migration was well on its way to covering the entire surface of the defect, and the normal epidermis bordering the defect, which had contributed to the migrating cover, was accordingly markedly thinned. By five hours this epidermal cover was complete, and at the centre, where the inward migrating edges met, a marked cumulus of exuberant epithelium was present (Plate 38). At this stage there was little in the way of response within the dermis or traumatized muscle tissue, apart from the presence of an occasional macrophage (Plate 39), but by 10 hours large numbers of macrophages had accumulated within the wound and active myophagia was extant. This reached its peak at 24 hours, when great myophagic activity was accompanied by a very distinctive perivascular cuffing of leucocytes migrating inward from peripheral blood vessels.

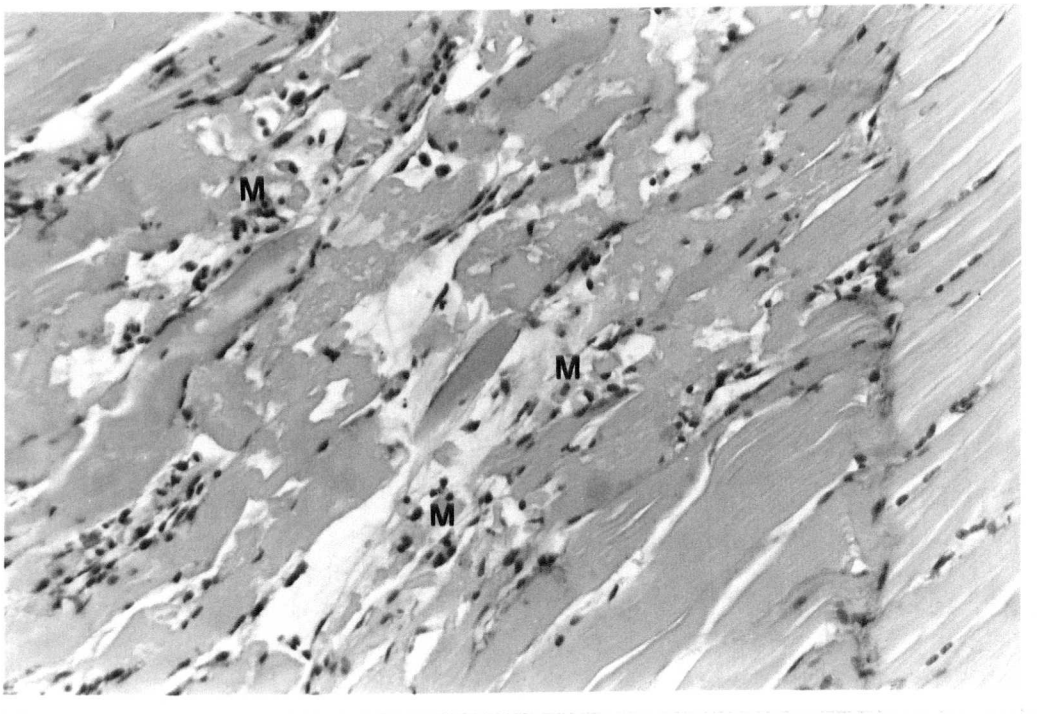
By the second day myophagia was virtually complete, and it was at this stage that variation between the vitamin C deficient and the adequately supplied fish first manifested itself.

Fish in the vitamin C deficient group showed little in the way of fibrosis of the wound whereas, in the two other groups, fibroblasts appeared and the beginnings of fibroplasia were apparent. The wounds of fish killed on the third day showed ample fibroblast activity in all cases, but whereas in the case of the fish enjoying adequate vitamin C the connective tissue was baseophilic and fibrous (Plate 40), the tissue of the deficient wounds had large numbers of swollen active

PLATE 38. Low power view of the lesion after 5 h. The epithelial cover is complete, with thinning of the epidermis at the edge of the lesion, and a central cumulus (C). The arrows indicate haemorrhages (H & E x 50)

PLATE 39. Traumatized muscle on the edge of a lesion after 5 h showing necrotic sarcoplasm and myophagia (M) (H & E x 200)


C


M
M
M

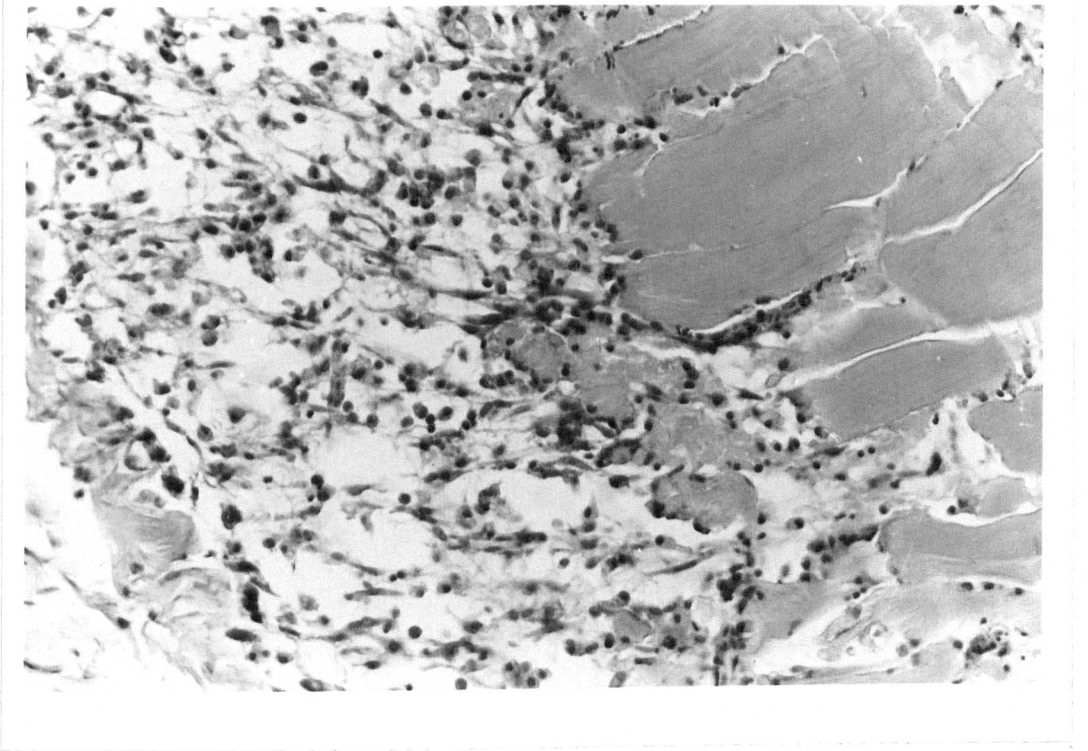
fibroblasts with very fine fibrils and a generally amorphous ground substance (Plate 41). Special stains showed that while both contained reticular strands there was little or no collagen in the wounds of the deficient fish whereas bundles of fine collagen (MSB positive) fibrils were present in the others.

By the seventh day the replacement dermis in the adequately supplemented fish had started to differentiate as a band of dense collagen, joining the two severed ends and the fibrous scar tissue within the muscle was dense, collagenous, and starting to contract, with considerable numbers of regenerating myofibrils inserting into it. The deficient scar remained as a large highly cellular granulomatous area with little differentiation (Plate 42). Contrast between the two groups then gradually increased with the fish with normal and high levels of dietary vitamin C showing identical stages of maturation of dermis and scar, with contraction, until by the 13th day the healed area was bound to the tissues round about by a dense contracted collagenous band within a stroma of juvenile myofibrils extending down from a zone of dermis which was marginally more cellular than the normal (Plate 43). The fish which were deficient in vitamin C did not present such a picture until the 16th day and evidence for the existence of the lesion could also be readily distinguished by the increased cellularity of the lesion (Plate 44).

By the thirteenth day, in some fish in all groups, where clusters of epithelial tissue had been tracked into the lesion at wounding, these started to proliferate and form small islets of secretory epithelium deep within the muscle. By day 16 these were becoming necrotic,

PLATE 40. Lesion of deficient fish after 2 days. The defect has numerous active fibroblasts but the fibres laid down are delicate, poorly staining and embedded in a general amorphous ground substance (H & E x 200)

PLATE 41. Lesion of luxus level fish after 2 days. The fibroblasts are associated with dark staining fibres already laid down in parallel the lesion appears much denser and organised (H & E x 300)



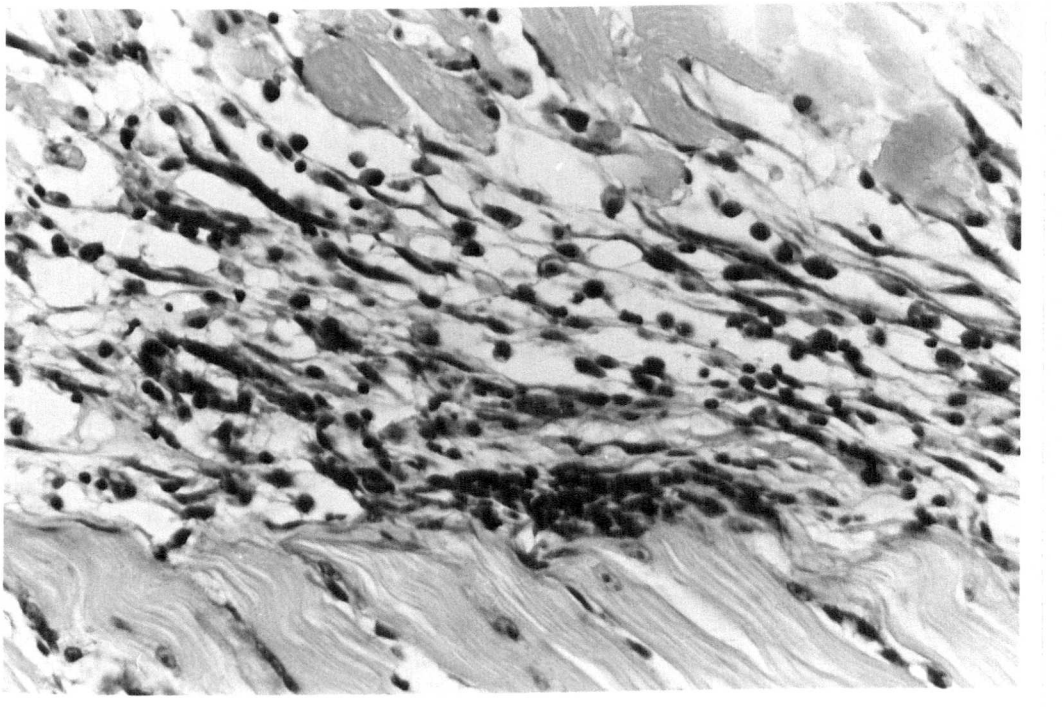


PLATE 42. The junction between scar tissue and severed dermis (J) in deficient fish after 7 days. There is strong fibroblast activity at the junction but the scar is pale staining and highly cellular, whereas in the fish on supplemented diets at this stage had united the two severed ends of dermis (H & E x 300)

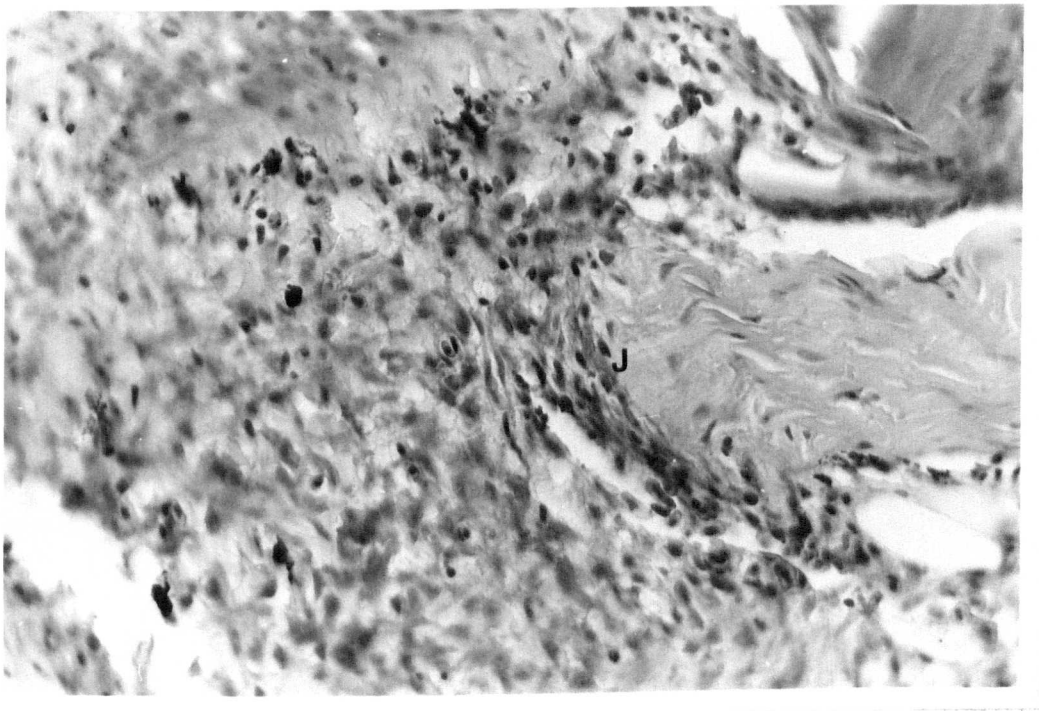
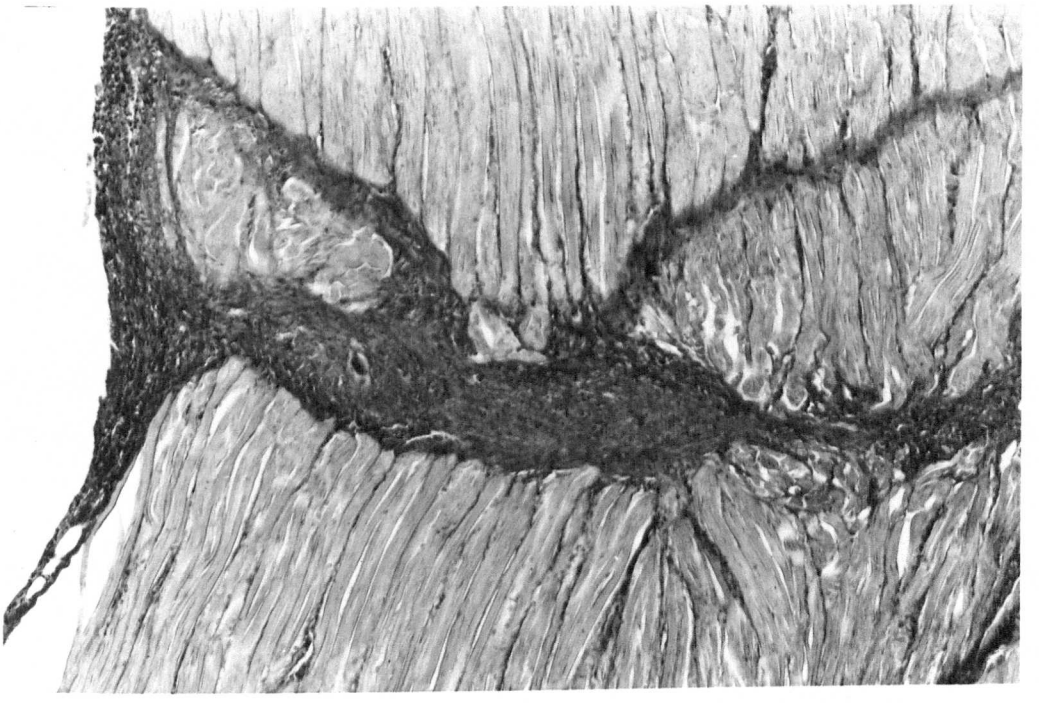

J

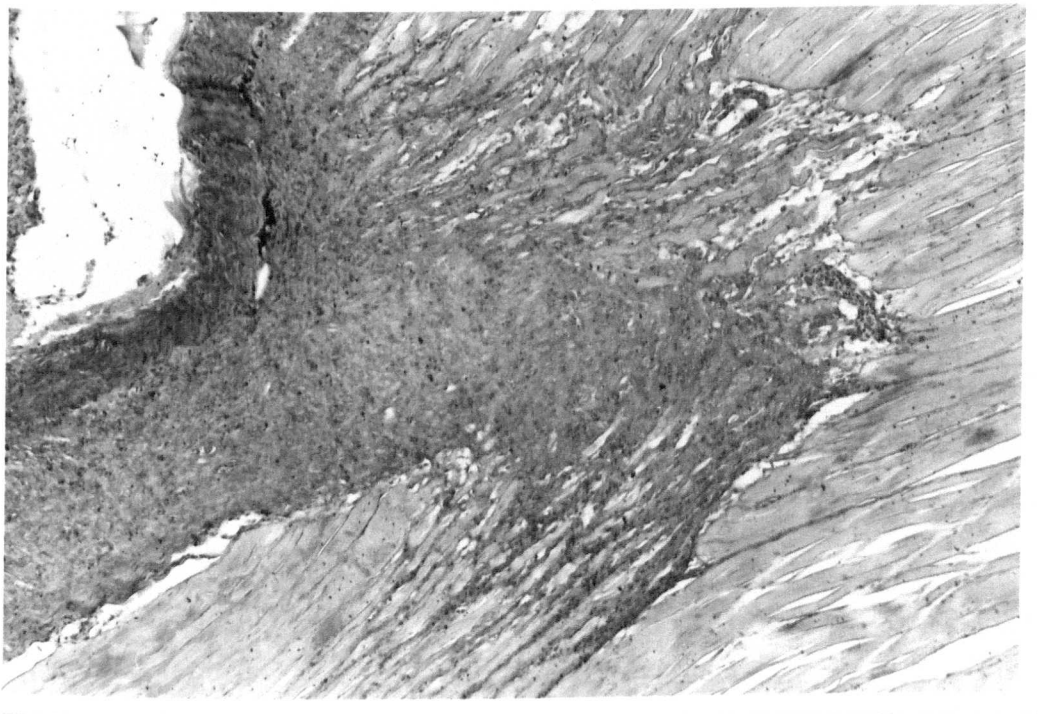
PLATE 43. The lesion after 13 days in a fish fed adequate level of ascorbic acid. The epidermis and dermis are normal and the scar of collagenous tissue has contracted to align the adjacent myofibrils

(H & E x 40)

PLATE 44. The lesion of an unsupplemented fish 16 days after induction. The lesion is still cellular and there is no dense contracted fibrous structure scar

(H & E x 50)





appearing as a central muconecrotic focus within an epithelial cell layer, bounded by a basement membrane. At this stage it seemed likely that these would ultimately become necrotic and be removed by phagocytosis but the experiment did not continue long enough to determine their actual fate.

Section 12.3.2 Biochemical Changes

The results of ascorbic acid measurements in tissue of initial fish and at 3, 4 and 5 days (stage 1) and 7, 10, 13 and 16 days (stage 2) is shown in Table 67. The depletion period resulted in lowered initial tissue ascorbate concentrations (Table 67). After 5 days of feeding the experimental diets it was observed that fish fed adequate and excess levels of ascorbic acid commenced storage of the vitamin and tissue ascorbate concentrations continued to rise until day 16. Fish fed the diet deficient in ascorbic acid showed a progressive decrease in tissue ascorbic acid concentrations over the experimental period.

SECTION 12.4 : DISCUSSION

Higher animals, such as man, monkey and guinea pigs, which are unable to synthesize ascorbic acid, lack the enzyme L-gulono-Y-lactone oxidase in hepatic and renal tissues (Burns, 1957). A specific requirement for dietary ascorbic acid in fish has generally been demonstrated through deletion of this vitamin from the diet. However, biochemical studies have indicated the absence of key synthetic enzymes in blue catfish, Ictalurus frucatus (Lesueur), and channel catfish, Ictalurus punctatus (Rafinesque)(Wilson, 1973), rainbow

TABLE 67. Total ascorbate concentrations (μg/g wet tissue) in tissues of initial fish, and at stage 1 and 2 during experiment

Tissue	Initial[3] $\bar{x}$ ± S.E.	STAGE 1[1] Deficient $\bar{x}$ ± S.E.	STAGE 1[1] Adequate $\bar{x}$ ± S.E.	STAGE 1[1] Excess $\bar{x}$ ± S.E.	STAGE 2[2] Deficient $\bar{x}$ ± S.E.	STAGE 2[2] Adequate $\bar{x}$ ± S.E.	STAGE 2[2] Excess $\bar{x}$ ± S.E.
Gills	15.42 ± 0.35	14.04 ± 0.33	84.6 ± 1.17	106.95 ± 0.84	13.71 ± 0.37	108.04 ± 0.49	130.64 ± 0.88
Liver	9.89 ± 0.61	9.85 ± 0.47	74.46 ± 0.93	140.74 ± 0.73	6.46 ± 0.48	86.14 ± 0.89	171.87 ± 0.81
Gut	11.36 ± 0.43	9.28 ± 0.27	88.97 ± 0.55	193.04 ± 0.98	7.8 ± 0.37	108.24 ± 0.70	211.16 ± 0.26
Brain	28.55 ± 0.53	27.69 ± 0.01	73.13 ± 1.15	189.65 ± 1.51	23.08 ± 0.01	173.95 ± 1.09	221.48 ± 1.58
Eyes	9.90 ± 0.31	8.84 ± 0.01	50.85 ± 0.66	51.60 ± 0.42	6.53 ± 0.36	59.21 ± 0.01	64.32 ± 0.60
Muscle	3.98 ± 0.32	4.87 ± 0.53	27.66 ± 0.01	30.93 ± 0.45	1.98 ± 0.27	38.54 ± 0.27	46.39 ± 0.01

1. Stage 1: pooled tissue samples from fish at 3, 4, and 5 days
2. Stage 2: pooled tissue samples from fish at 7, 10, 13 and 16 days
3. Mean ± standard error derived from analysis of variance

trout and tilapia, Oreochromis niloticus (Trewavas), (Yamamoto et al., 1978). Histochemical and chemical studies (Chapter 3) were also unable to detect L-gulono-γ-lactone oxidase in the liver and kidney of O. niloticus. Fish lacking this biosynthetic pathway will be dependent on dietary ascorbic acid for optimal growth and freedom from deficiency signs.

Most tilapias are omnivorous or detritivorous and their natural diet would contain adequate ascorbic acid. In laboratory studies, however, 125 mg ascorbic acid per 100 g diet was required for O. niloticus for optimal growth and freedom from sign of scurvy (Chapter 4). It is obvious from the present wound healing study that even with a very mild and small insult, wound healing is inhibited by ascorbic acid deficiency. By using special, putatively collagen specific, stains it was observed that collagen deposition in the wounds of fish fed the diet devoid of ascorbic acid was inhibited and delayed when compared to fish fed adequate or excess of dietary vitamin C. This can be related to the rapid increase in tissue ascorbate concentrations of fish fed the ascorbic acid supplemented diets (Table 67).

Wound healing in fish has a number of different components and the non-collagen related changes appeared to occur quite normally whether the fish were fed supplemented or deficient feeds. Thus, the delay in wound healing in fish fed the diet devoid of vitamin C can be attributed to the reduced collagen synthesis observed. Ascorbic acid is a vital cofactor in the hydroxylation of proline to hydroxyproline and thus in the synthesis of collagen (Sato et al., 1982a, b). Changes in activity of proline hydroxylase are directly related to the rate of

biosynthesis and deposition of collagen (Mussini et al., 1967). However, even in animals (guinea pigs) fed diets devoid of ascorbic acid some limited hydroxylation of proline still takes place (Robertson et al., 1959) possibly explaining the ability of scorbutic tilapia, in the present experiment, to deposit collagen in the wound to some degree. Another contributory factor may be the mobilization of tissue ascorbate stores, although limited (Table 67).

In a study of the pathology of vitamin C deficiency in channel catfish (Lim & Lovell, 1978) the effects of dietary ascorbic acid levels on wound healing were assessed. All wounded fish, regardless of dietary ascorbic acid intake, showed superficially healed wounds after 10 days. Healing of the epidermal and dermal layers of skin was also judged to be complete. The rate of healing of somatic muscle was reported as proportional to the dietary ascorbic acid level up to 60 mg of ascorbic acid per kg diet. Fish fed a diet devoid of vitamin C had replacement of muscle by dense immature collagen fibres whereas the fish fed ascorbic acid in excess of 30 mg per kg diet possessed mature collagen.

The rate of epithelialization of the wound surface in the present study (it had completely epithelialized in all cases within five hours) is almost twice as fast as that reported by Bullock et al. (1978) for the plaice (Pleuronectes platessa L.). These fish were held at 15°C as compared to 28°C for tilapias but even so the speed with which integumental integrity was secured is remarkable. It is unlikely that quite such rapid cover of such a small wound in a freshwater fish is essential for osmoregulatory purposes: However, the normal

very eutrophic conditions in which tropical detritivores exist may well justify the extremely rapid covering of the surface in order to prevent infection by aquatic bacteria as well as by fungal pathogens,

CHAPTER 13

THE EFFECT OF DIETARY ASCORBIC ACID SUPPLEMENTATION ON HATCHABILITY, SURVIVAL RATE AND FRY PERFORMANCE IN *OREOCHROMIS MOSSAMBICUS*

SECTION 13.1 : INTRODUCTION

Investigations into the role of ascorbic acid (vitamin C) in fish nutrition have mainly focused on its effects in immature fish with respect to nutritional status, prevention of deficiency signs and promotion of wound healing (Poston, 1967; Halver et al., 1969; Sakaguchi et al., 1969; Arai et al., 1972; Halver, 1972a; Halver et al., 1975; Lovell, 1973; Lim & Lovell, 1978; Murai et al., 1978; Mahajan & Agrawal, 1979, 1980a; Jauncey et al., 1985) or its functions during physiological stress (Wedemeyer et al., 1969; Agrawal et al., 1978; Mauck et al., 1978; Mayer et al., 1978; Blanco & Mead, 1980).

Although reproduction in fish is a very important culture parameter little is known about the effects of variation in dietary nutrient levels on the reproductive process. Hirao et al. (1954, 1955); Ohmae et al. (1979) and Takeuchi et al. (1981) found that dietary iron, B_2, fatty acids and vitamin E had positive effects on hatchability in fish.

A possible role of ascorbic acid in reproduction has been reported by Lutwak-Mann (1958) who revealed that the ascorbic acid content of mammalian ovaries varied with the different stages of the ovarian cycle. This observation is supported by the studies of Abdo et al. (1971) in camel, Seymour (1981a) in crucian carp (Carassius carassius L) and Sandnes & Braekkan (1981) in cod (Gadus morrhua L).

In addition chemical analyses have consistently revealed high concentrations of ascorbic acid in fish roe (Mathiesen, 1938; Hoygaard

& Rasmussen, 1939; Pyke & Wright, 1941; Ikeda et al., 1963a; Hilton et al., 1979b and Agrawal & Mahajan, 1980b). These studies indicate a potential biological role of ascorbic acid in reproduction. Recently, Sandnes et al. (1984) established the possible essentiality of dietary ascorbic acid in reproduction of rainbow trout (Salmo gairdneri R).

Seed supply, the supply of juveniles for ongrowing, is regarded as the principal factor limiting aquaculture development in many countries and for many species. With this in mind the present study was designed to investigate the effects of dietary ascorbic acid, fed to broodstock, on hatchability, fry condition, fry survival rate and fry growth.

SECTION 13.2 : EXPERIMENT 1

EFFECT OF ASCORBIC ACID SUPPLEMENTATION ON HATCHABILITY AND CONDITION OF FRY

Section 13.2.1 Materials and Methods

Section 13.2.1.1 Experimental system and animals

O. mossambicus were obtained from the Institute of Aquaculture, Stirling University, from broodstock previously evaluated using gel electrophoresis (Section 2.2). Fish were acclimated to the experimental recirculation system (Section 2.1.2) for one week before start of the experiment and fed trout diet during this period. Fish were then redistributed at 25 fish/150 litre circular tank (Section 2.1.2) (average body weight 2.94 ± 0.30 g).

Section 13.2.1.2 Diets, feeding regime and fish weighing procedure

Two diets were employed in the present study. Diet 1 was an ascorbic acid free (AAF), basal diet (Table 16). Diet 2 was produced by addition of 125 mg of ascorbic acid per 100g diet (Chapter 4) to the basal diet (AAF). Each diet was fed to duplicate tanks of fish for 21 weeks. Diet preparation, storage, feeding regime and fish weighing procedures were as described previously (Section 2.3.2 and Section 5.3.1.2).

Section 13.2.1.3 Maturation

O. mossambicus is a maternal mouth-brooder (Balarin & Hatton, 1979) and when observation indicated a female carrying eggs she was removed

from the broodstock tank and the eggs were collected and loaded into the hatchery system (Section 2.1.4) to allow calculation of hatchability (expressed as the percentage of fry produced to the total number of eggs) and percentages of abnormal and deformed fry (such as abnormally swimming and twisted body) to be recorded.

Section 13.2.1.4 Hatchery system

As described in Section 2.1.4.

Section 13.2.1.5 Experimental methodology

Total ascorbate determination

Samples of eggs collected from the females and newly hatched fry from both treatments were subjected to estimation of total ascorbate by the hydrazine method (Section 2.4.4.1). In addition, at the termination of the experiment the total ascorbate was determined in the ovary of six female fish per treatment.

Histological studies

Samples of fry produced from each treatment were fixed in cooled 10% formalin and cut and stained as described in Section 2.5.1.

Section 13.2.1.6 Statistical analysis

For evaluation of experimental data analysis of variance (Section 2.9) was used.

Section 13.2.2 Results

At week 14 fish fed the ascorbic acid supplemented diet exhibited signs of maturation (e.g. males became darker in colour than females; males showed nest building activity; males were selecting suitable females and driving them to their nests; courtship became continuous and in both males and females the ovipostor appeared swollen) whereas fish fed the unsupplemented diet reached this stage 2 weeks later.

The results of hatchability, fry deformity and total ascorbate in ovary, eggs and fry are presented in Table 68.

Hatchability and fry condition

A significantly ($P < 0.01$) lower hatchability was obtained for eggs from fish fed the unsupplemented diet concomittant with a higher percentage of fry deformity (Table 68). Histological examination revealed that the deformity was related to severe damage to the spinal column (Plate 45), whereas no signs of spinal deformity were found in fry obtained from eggs of fish fed the supplemented diet (Plate 46).

Biochemical changes

Fish fed the unsupplemented diet exhibited significantly ($P < 0.01$) lowered concentrations of total ascorbate in the ovary. No detectable ascorbate was measured either in eggs or fry produced from fish fed the unsupplemented diet (Table 68).

TABLE 68. Average hatchability, fry deformity and total ascorbate concentration in ovary, eggs and fry[1]

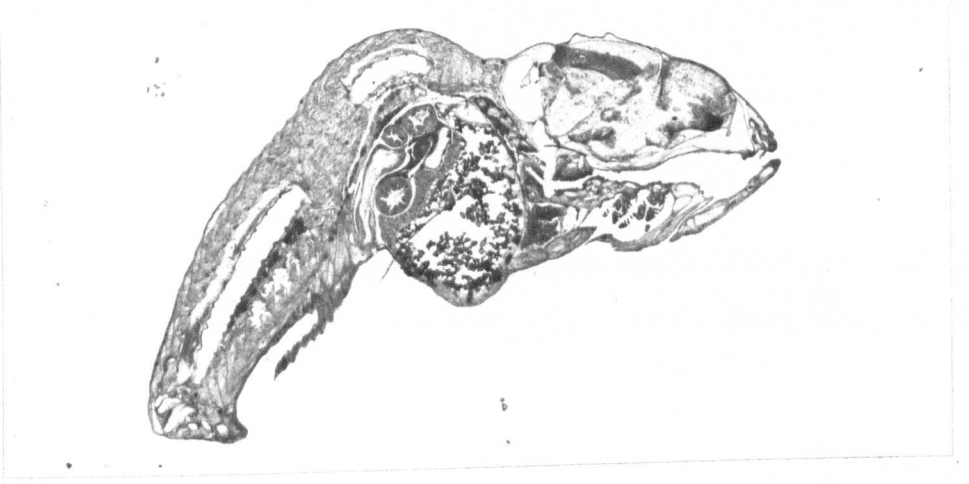
Parameter	DIET		±SEM[2]
	Unsupplemented	Supplemented	
1. Hatchability & fry condition			
Hatchability (%)	54.25[b]	89.35[a]	2.20
Fry deformity (%)	56.9[a]	1.28[b]	1.10
2. Total ascorbate concentration ($\mu g.g^{-1}$ wet tissue)			
Ovary	64.77[b]	429.39[a]	6.16
Eggs	not detected[b]	201.83[a]	4.00
Fry	not detected[b]	134.93[a]	3.22

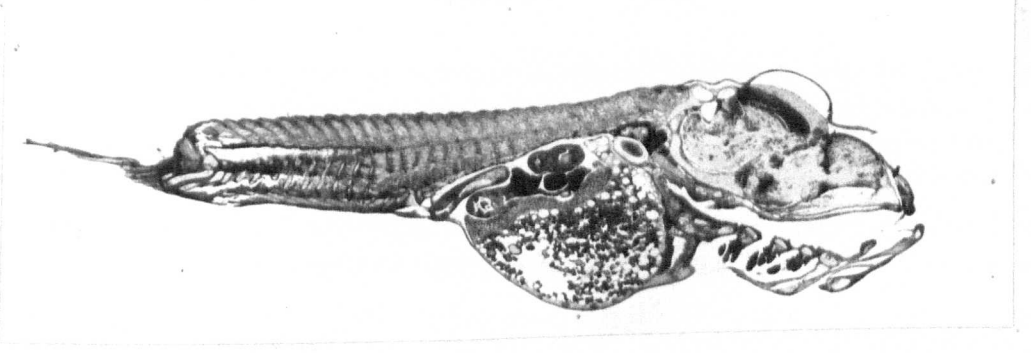
1. Values in the same row with a common superscript are not significantly different ($P > 0.01$)

2. Standard error of the means derived from analysis of variance

PLATE 45. Fry produced from broodstock fed the unsupplemented diet showing severe spinal deformity, twisted dorsal and caudal areas (H & E x22.5)

PLATE 46. No signs of spinal deformity or malformation of fry produced from broodstock fed the supplemented diet (H & E x22.5)





SECTION 13.3 : EXPERIMENT 2

EFFECT OF ASCORBIC ACID SUPPLEMENTATION ON PERFORMANCE AND SURVIVAL RATE OF FRY PRODUCED FROM FISH FED DIETS UNSUPPLEMENTED AND SUPPLEMENTED WITH ASCORBIC ACID

Section 13.3.1 Materials and Methods

Section 13.3.1.1 Experimental system and animals

Limitations of spawning frequency of females fed the unsupplemented diet and difficulty in obtaining eggs on the same day from both treatments meant that 55 fry produced from fish fed the unsupplemented diet and 87 and 111 fry produced from fish fed the supplemented diet were available for this investigation. They were housed in the recirculation system described previously (Section 2.1.1).

Section 13.3.1.2 Diets and feeding regime

The diets used in Section 13.2.1.2 were employed in this investigation. Fry produced from fish fed the unsupplemented diet continued to be fed this diet and similarly those produced from fish fed the supplemented diet were also continued on the same diet for 5 weeks. Fry were fed 20% of their body weight/day (dry food/whole fry) divided into 4 equal feeds.

Section 13.3.1.3 Fry weighing procedure

Fry were weekly bulk weighed, a tank at a time, in a small amount of water using a Mettler AC 100 electronic balance and feeding rates for the following week adjusted accordingly.

Section 13.3.2 Results

The results of the present investigation were evaluated using the following parameters:

1. Growth response

Body weight and specific growth rate (SGR)

Fry fed the unsupplemented diet exhibited poor weight gain and specific growth rate (Table 69 and Fig. 29) compared to fry fed the supplemented diet.

2. Food utilization

Food conversion ratio (FCR)

Food conversion ratio was improved by addition of ascorbic acid to the basal diet (Table 69).

Protein efficiency ratio (PER)

PER was severely depressed for fry fed the unsupplemented diet (Table 69).

3. Survival rate

Survival rate was affected markedly by dietary ascorbic acid (Table 69). Fry fed the deficient diet exhibited a poor survival rate compared to fry fed the supplemented diet.

TABLE 69. Growth, food utilization and survival rate of fry fed the diets unsupplemented and supplemented with ascorbic acid

Parameter	DIET	
	Unsupplemented	Supplemented
Initial avg.wt. mg	5.00	7.25
Final avg.wt. mg	30.00	236.53
SGR[1] (% d^{-1})	5.14	9.96
FCR[2]	5.00	1.05
PER[3]	0.50	2.39
Survival rate (%)	1.82	86.36

1. Specific growth rate
2. Food Conversion ratio
3. Protein efficiency ratio

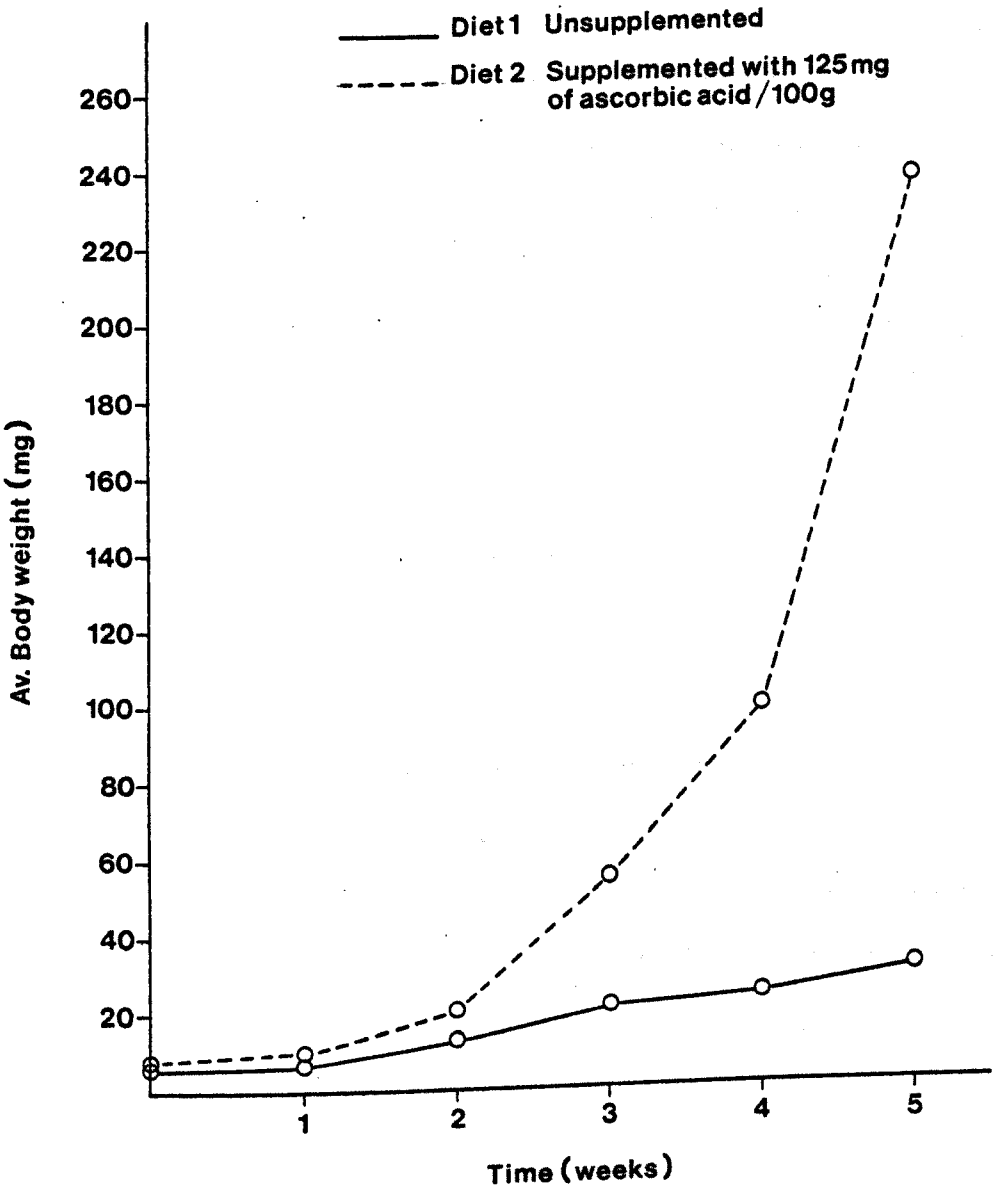


Fig. 29. Increase in average bodyweight of the two groups of fry with time

SECTION 13.4 : EXPERIMENT 3

EFFECT OF DIETARY ASCORBIC ACID SUPPLEMENTATION ON THE PERFORMANCE AND SURVIVAL RATE OF FRY PRODUCED FROM BROODSTOCK FED AN ASCORBIC ACID SUPPLEMENTED DIET

This experiment was conducted to further elucidate the results of the previous experiment (13.3).

Section 13.4.1 Materials and Methods

Section 13.4.1.1 Experimental system and animals

Fry were obtained from parent fish fed the supplemented diet (all fry were from one batch) and were divided into four groups of 30 fry each and stocked into the recirculation system (Section 2.1.1). Two groups were fed the unsupplemented diet and two were fed the diet supplemented with ascorbic acid.

Section 13.4.1.2 Diets and feeding regime

As described before in Section 13.3.1.2.

Section 13.4.1.3 Fry weighing procedure

Fry were bulk weighed weekly, a tank at a time, in a small amount of water without anaesthesia except for the terminal weighing when fry were anaesthetised (Ross & Geddes, 1979) and weighed, using Mettler AC 100 electronic balance, and measured individually to allow calculation of condition factor.

Section 13.4.1.4 Experimental methodology

Glycogen determination

Six fry per treatment were analysed for total body glycogen (Section 2.4.6.).

Total L-ascorbate and dehydro-L-ascorbate and L-ascorbate determination

Total L-ascorbate and dehydro-L-ascorbate and L-ascorbate concentrations of 32 fry for Diet 1 and 35 fry for Diet 2 were determined by the methods described previously (Sections 2.4.4.1, 2.4.4.2 and 2.4.4.3).

Section 13.4.1.5 Statistical analysis

As described previously (Section 13.2.1.6).

Section 13.4.2 Results

This investigation was evaluated using the following parameters:

1. Growth response

Body weight and specific growth rate (SGR)

Significantly ($P < 0.01$) lower body weights and specific growth rates were obtained for fry fed the diet lacking in ascorbic acid (Table 70 and Fig. 30).

Condition factor

No significant differences ($P > 0.01$) in condition factor were

TABLE 70. Nutritional and biochemical parameters for fry fed the supplemented and unsupplemented diets

Parameter	DIET		±SEM[2]
	Unsupplemented	Supplemented	
1. Growth parameters			
Initial avg.wt. mg	6.30[a]	6.78[a]	0.66
Final avg.wt. mg	60.15[b]	305.54[a]	16.99
SGR[3] (% d^{-1})	6.27[b]	10.89[a]	0.18
CF[4]	3.17[a]	3.07[a]	0.065
2. Food utilization parameters			
FCR[5]	2.36[a]	1.10[b]	0.22
PER[6]	1.08[b]	2.29[a]	0.10
3. Survival rate (%)	65[b]	97[a]	5.66
4. Biochemical changes parameters			
TAA[7] (µg.g^{-1} wet tissue)	82.13[b]	164.39	2.11
DHAA[8] (µg.g^{-1} wet tissue)	16.00[a]	6.68[b]	0.43
AA[9] (µg.g^{-1} wet tissue)	66.13[b]	157.71[a]	1.99
DHAA:TAA[10]	19.48	4.06	

1. Values in the same row with a common superscript are not significantly different (P > 0.01)
2. Standard errors of the means derived from analysis of variance
3. Specific growth rate
4. Condition factor
5. Food Conversion ratio
6. Protein efficiency ratio
7. Total L-ascorbic acid
8. Dehydro-L-ascorbic acid
9. L-ascorbic acid
10. Dehydro-L-ascorbic acid:Total L-ascorbic acid

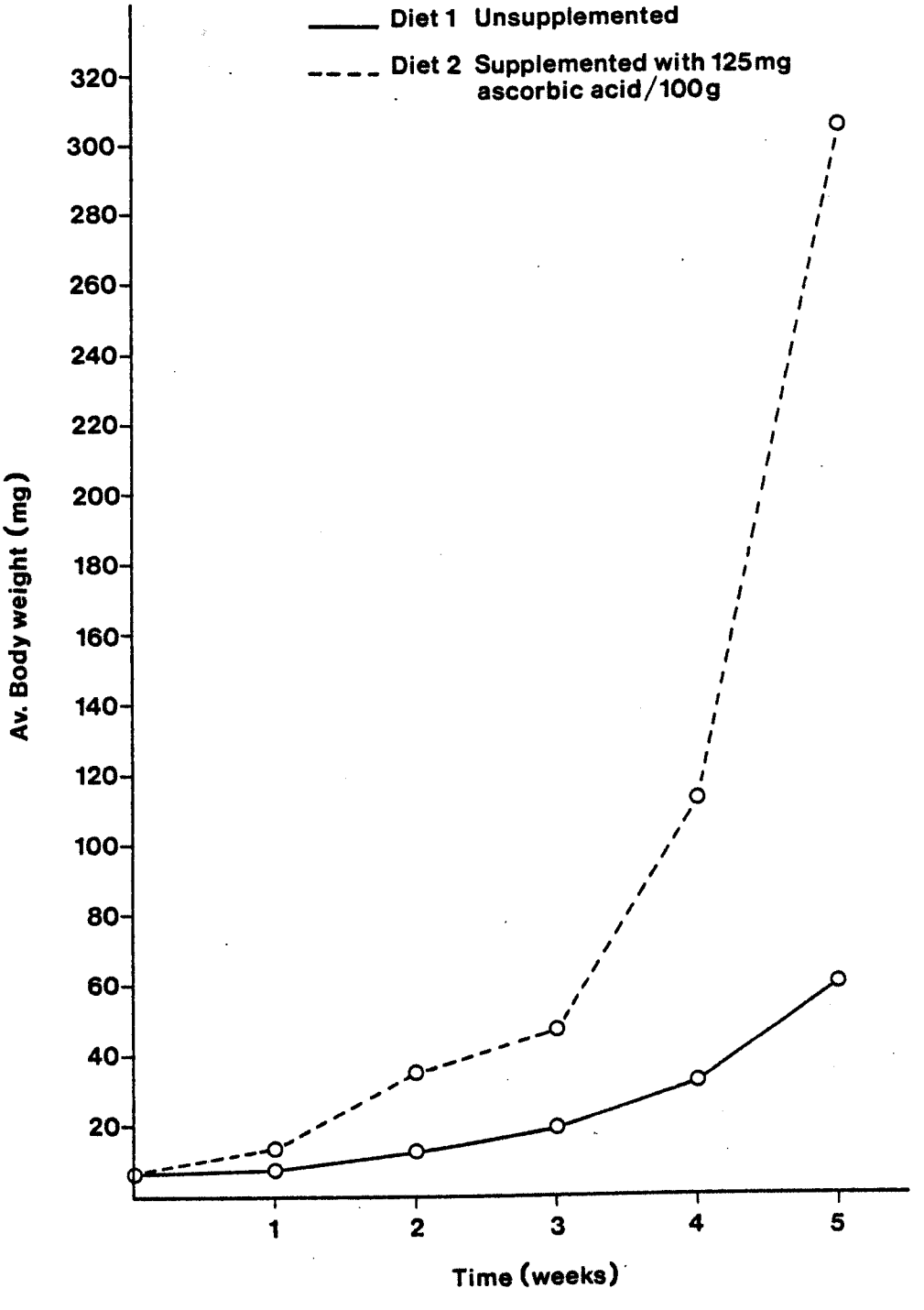


Fig. 30. Increase in average body weight of the two groups of fry with time

detected between fry fed the unsupplemented and supplemented diets (Table 70).

2. Food utilization

The results of food conversion ratio and protein efficiency ratio are presented in Table 70. Fry fed the supplemented diet exhibited significantly ($P < 0.01$) better FCR and PER.

3. Survival rate

Survival rate was significantly ($P < 0.01$) higher for fry fed the supplemented diet (Table 70).

4. Biochemical changes

The results of the effect of dietary ascorbic acid on body glycogen level and total tissue L-ascorbate, dehydro-L-ascorbate and L-ascorbate and dehydro-L-ascorbate : total ascorbate ratio are shown in Table 70.

Body glycogen content was elevated significantly ($P < 0.01$) in fry fed the supplemented diet (Table 70). Fry fed the unsupplemented diet showed significantly lowered total ascorbate and L-ascorbate levels concomittant with an elevation of dehydro-L-ascorbate and dehydro-L-ascorbate : total ascorbate ratio when compared with fry fed the supplemented diet.

SECTION 13.5 : DISCUSSION

The results of the present study clearly establish the essentiality of ascorbic acid in broodstock and fry diets for O. mossambicus.

Hatchability in eggs produced from fish fed diets devoid of ascorbic acid for 21 weeks was severely depressed possibly as a result of several factors. Firstly, as a consequence of the absence of detectable ascorbate from these eggs (Table 68) and secondly due to impairment of collagen synthesis in such eggs. Halver et al. (1975) demonstrated, using labelled ascorbic acid, good deposition of collagen in egg shell membranes of rainbow trout and in addition Mizoguchi and Yasumasu (1982) suggest that ascorbic acid plays an important role in the formation of collagen in the archenteron of sea urchin embryos. Ascorbic acid may also play a role in embryonic development. In the present study it was observed that the level of total ascorbate detected in the fry after hatching was lower than in the eggs obtained from fish fed the supplemented diet indicating use of some of this ascorbic acid in embryonic development. The recent work of Sandnes et al. (1984) supports the results of the present study as the hatchability of rainbow trout was significantly depressed when they were fed a diet devoid of ascorbic acid.

In addition to reducing hatchability ascorbic acid deficiency also affected the condition of fry. Fish fed diets deficient in ascorbic acid produced a significantly ($P < 0.01$) higher percentage of deformed fry (Table 68) and histological examination revealed that the deformity was related to severe damage to the spinal column (Plate 45). It is

well documented that ascorbic acid is a cofactor in hydroxylation of proline and lysine (Gould, 1960; Wilson & Poe, 1973 and Sato et al., 1982a) and this is essential for normal collagen maturation.

The amount of total ascorbate in eggs collected from females fed the supplemented diets was only 47% of the ovary ascorbate content of such fish probably due to limitations in transfer of ascorbate from the ovary to the eggs and also to embryonic development. Backström (1956) reported that the level of ascorbic acid in sea urchin eggs was low from the time of fertilization until the end of gastrulation.

Possible explanations for depression of growth and food utilization parameters and glycogen and tissue ascorbate levels in the fry fed the unsupplemented diets in Experiments 2 and 3 (this Chapter) are given in detail in Chapter 5.

Dehydro-L-ascorbic acid is reversibly oxidized from L-ascorbic acid (redox-system) and both forms are physiologically active (Kanfer et al., 1960). However, Ikeda et al. (1963a) reported that in normal healthy individuals of various species of fish, dehydro-L-ascorbic acid represented less than 10% of the total ascorbate. In the present study the dehydro-L-ascorbic acid content of fry fed the unsupplemented diet was 19.48% of the total L-ascorbate (Table 70). Banerjee and his colleagues (Banerjee et al., 1952; Banerjee & Belavady, 1953; Chakrabarti & Banerjee, 1955; Bhaduri & Banerjee, 1960; Banerjee, 1977) reported that dehdro-L-ascorbic acid levels were elevated in tissues of scorbutic guinea pigs and in the blood of patients suffering from infectious diseases. These authors

suggested that this elevation was due to lowered gulathione levels. Chakrabarti & Banerjee (1955) suggested that gulathione and ascorbic acid may have a considerable influence in controlling the maintenance of proper oxidation-reduction potential in cells.

The present study represents a relatively novel approach to evaluating the role of dietary ascorbic acid in fish nutrition by considering its effects on reproduction. Experiments 2 and 3 in this Chapter showed that fry fed the unsupplemented diet in Experiment 3 performed, in all parameters measured, better than fry fed the unsupplemented diet in Experiment 2 (Table 69 and 70). In Experiment 3 fry were obtained from broodstock fed an ascorbic acid supplemented diet whereas fry used in Experiment 2 were obtained from broodstock fed the unsupplemented diet. This difference suggests that ascorbic acid may be transferred from the ovary of the female to the eggs thence to the fry (Table 68) providing a store of ascorbic acid for fry after hatching, the most critical stage for fry survival. The survival rate was greatly depressed for fry fed the unsupplemented diet in Experiment 2 compared to the survival rate of fry fed the unsupplemented diet in Experiment 3. These observations support the hypothesis of Tolbert (1979) that ascorbic acid may have many functions other than hydroxylation of protocollagen in collagen synthesis.

Differences in condition factor in Experiment 3 for fry fed the unsupplemented and supplemented diets did not reach a significant ($P > 0.01$) level possibly due to the amount of residual ascorbic acid detected in the fry after hatching (Table 68).

Vitamins are regarded as important for egg production and higher survival rates of fry (Fish Farming International, 1984b; Sandnes *et al*. (1984) suggested that the dietary level of ascorbic acid should be sufficient to give 20 µg/g wet weight of eggs as a lower limit for normal fry development in rainbow trout. In the present study the total ascorbate content of eggs was 201.83 µg/g wet weight of eggs suggesting that 125 mg of ascorbic acid/100 g diet is adequate for production of higher hatching rates and improved performance of fry of *O. mossambicus*.

CHAPTER 14

GENERAL CONCLUSIONS

The aim of this research programme was to investigate the ascorbic acid (vitamin C) nutrition of the principal cultivated species of tilapias, namely Oreochromis niloticus and O. mossambicus. Various conclusions and deductions can be made in the light of the foregoing chapters of this treatise.

In order to determine the ascorbic acid requirement for any species of an animal it is necessary to consider the following three areas:

1. Identification of the presence or absence of activity of the enzyme L-gulono-γ-lactone oxidase (a key enzyme in the biosynthesis of ascorbic acid) in liver and kidney tissue by use of suitable, preferably quantitative, methods.

2. In the absence of activity of this enzyme the animal will rely on an exogenous supply of ascorbic acid. Requirements must be determined by feeding groups of animals diets containing graded levels of ascorbic acid. The level of dietary vitamin which will support optimal growth and food utilization and prevent signs of ascorbic acid deficiency should be evaluated.

3. It has been reported (Ikeda & Sato, 1966b) that carp is able to convert myo-inositol to L-ascorbic acid in vivo and therefore long-term ascorbic acid deprivation studies should be conducted to investigate whether the animal in question is able to convert another compound to ascorbic acid.

Both qualitative and quantitative techniques (Chapter 3) failed to detect any trace of L-gulono-γ-lactone oxidase activity in O. niloticus and O. mossambicus whereas activity of this enzyme was detected in kidney tissue of both O. aureus and O. spilurus. It is thus apparent that ascorbic acid requirements should be investigated on a species basis as differences may exist even within a genus.

The recommended level of dietary ascorbic acid supplementation for both O. niloticus and O. mossambicus is 125mg per 100g diet (sufficient to allow for raw material variations, nutrient and/or non-nutrient interactions and processing and storage) whereas the net requirement for ascorbic acid for both species is 42mg per 100g diet (the level required for optimal performance) under the conditions described (Chapter 4). The results of Chapter 4 also demonstrate the adverse effects of megadoses of dietary ascorbic acid (300 and 400mg/100g diet) on O. niloticus and O. mossambicus by depression of the growth response, food utilization and blood parameters. Ginter et al. (1979) stated that

> "any further substantial increase of dietary ascorbic acid in diets of guinea pigs is not only superfluous, but may actually have untoward effects"

and a similar situation appears to be true for tilapias.

Ascorbic acid was shown to be involved in protein metabolism of both O. niloticus and O. mossambicus (Chapters 4, 5, 7, 9 and 10) as indicated from the observed effects of varying dietary level on protein utilization parameters such as PER, ANPU, APD and carcass crude protein.

Ascorbic acid has been shown to play a role in carbohydrate metabolism (Chapters 4, 5 and 7) through its influence on liver and muscle glycogen contents.

Scorbutic O. niloticus and O. mossambicus exhibited significantly decreased collagen contents of vertebrae and tails concomittant with an increase in proline levels and a decrease in hydroxyproline levels (Chapter 5) supporting the role of ascorbic acid in collagen biosynthesis.

Dietary ascorbic acid was also shown to be essential in prevention of anaemia, (through its effect on haematocrit and haemoglobin levels) and haemorrhage in both tilapias (Chapters 4, 5, 7 and 9).

Dietary ascorbic acid deficiency resulted in elevation of serum cholesterol (cholesterolaemia) in O. mossambicus (Chapter 5). An adequate dietary ascorbic acid level was necessary to prevent signs of ascorbic acid deficiency which included severe haemorrhages, exophthalmia, cataract, lethargy, anorexia, opercular deformity, severe spinal deformities (scoliosis and lordosis) and abnormal histopathology of gills, eyes, liver, spinal column (Chapters 4, 5, 7 and 13).

Tissue ascorbate concentrations in O. niloticus and O. mossambicus fall into three groups: firstly, the highest levels were detected in ovary, brain and testis; secondly, the intermediate levels detected in gills, gut, liver, heart, and eyes, and thirdly, the low levels detected in muscle and gall-bladder. The first three tissues may act as stores for this vitamin. The level of ascorbic acid in each tissue may indicate a variety of physiological roles for the vitamin (see

Chapters 6 and 11). Adequate dietary levels (125mg ascorbic acid/ 100g diet) were required to maintain tissue ascorbic acid concentrations. Increased levels of ascorbic acid in the tissues of tilapias may be beneficial in terms of human consumption of these species providing a dietary source of vitamin C. The levels of ascorbic acid reported in this thesis are high when compared with those of Sidwell et al. (1978) who reported 10µg ascorbic acid/g wet edible tissue in a variety of Cichlid species.

Both tilapias studied here were able to utilize L-ascorbic acid sodium salt (NaAA), glyceride coated ascorbic acid (GCAA, ascorbidan 50), L-ascorbyl palmitate (AP) and L-ascorbic acid 2-sulphate, barium salt (AA2S) as well as the free acid in terms of growth promotion, food utilization and prevention of signs of ascorbic acid deficiency (Chapter 7).

Both stability and price are important when selecting which form of ascorbic acid should be recommended for inclusion in tilapia feeds. AA2S and GCAA exhibited the highest stability during processing and storage (Chapter 8): Current list prices for the five forms of ascorbic acid employed in Chapters 7 and 8 are presented in Table 71.

TABLE 71. Current prices of different forms of ascorbic acid (per kilogram)

Ascorbic acid form	Price (£/kg)
GCAA	5.65
NaAA	20.83
AA	24.08
AP	57.00
AA2S	95,000

The potential of GCAA as a stabilized or protected form of ascorbic acid for use in fish feeds in general and feeds of tilapia in particular has therefore been demonstrated. However in the absence of protected forms of this vitamin the following precautions should be observed.

1. Diets must be stored in as cool a place as possible with light excluded and such feeds should be used within 2-3 weeks of manufacture.

2. The solubility of free ascorbic acid leads to leaching during immersion prior to ingestion which may, in part, be overcome by the use of increased feeding frequency or the use of highly palatable avidly consumed feeds.

The use of raw marine fish oils (unstabilized) may be cheaper than commercial oils (cod liver and corn oils) however, without addition of antioxidants the oxidation parameters (POV, TBA and AV) increased rapidly during processing and storage (Chapter 9) which resulted in adverse effects on growth, food utilization and lowered tissue ascorbate concentrations (Chapter 9). This experiment showed that BHT was effective in prevention of oxidation. Growth, food utilization parameters and tissue ascorbate concentrations for fish fed diets supplemented with unstabilized marine fish oil and supplemented with 200ppm BHT and 125mg ascorbic acid/100g diet were comparable to those of fish fed diets containing a mixture of commercial oils and 125mg ascorbic acid/100g diet (Chapter 9). In addition, the current prices of BHT and ethoxyquin are £15.20/kg and £69/kg respectively which supports

the use of BHT in fish diets.

Water temperature will affect metabolic rate which in turn directly affects the half-life of ascorbic acid. Therefore, a diet designed for feeding a particular species of fish (e.g. rainbow trout) should not be used in feeding another species without careful consideration due to interspecific differences in behaviour and metabolism. The level of ascorbic acid in a commercial trout diet did not sustain optimal growth in tilapias (see Chapter 10).

The significant higher levels of ascorbic acid in certain tissues of female O. niloticus and O. mossambicus than in the tissues of males suggests a possible need to increase the ascorbic acid intake of males during spawning (Chapter 11). It has been reported by Phillips et al. (1940) that administration of additional ascorbic acid to bulls leads to increased fertility.

Ascorbic acid was shown to play an important role in wound healing (Chapter 12) especially with respect to the rapid cover of the wound in fresh water fish which is essential for osmoregulatory purposes and for prevention of infection by aquatic bacteria and fungal pathogens which prevail in the normally euthrophic conditions in which tropical detritivores exist.

As a consequence of the involvement of ascorbic acid in steroidogenesis, in ovary and testis, ascorbic acid plays an important role in reproduction. Hatchability and fry condition were improved in broodstock fed diets supplemented with adequate levels of ascorbic acid (Chapter 13). Lynch and O'Grady (1981) reported that ascorbic

acid supplementation to sows (pigs are able to synthesise ascorbic acid, see Table 2) in late pregnancy resulted in reduced stillbirth by 3% compared to controls. The results presented in Chapter 13 show that ascorbic acid is transferred from the ovary of the females to the eggs and thence to fry providing a store of ascorbic acid for fry after hatching.

In conclusion, ascorbic acid has been shown to be an essential vitamin in diets for O. niloticus and O. mossambicus and must therefore be included in the diets of fry, fingerlings and broodstock at a level of 125mg/100g diet for optimal growth, food utilization and freedom from signs of deficiency as well as reproduction.

SUGGESTIONS FOR FURTHER RESEARCH

The studies presented here and the literature reviewed have indicated several areas to which attention should be paid in future investigations into the ascorbic acid requirements of fish:

1. There are no reports concerning the absorption of dietary ascorbic acid on fish. Studies of the mechanism of absorption would undoubtedly contribute to an understanding of the metabolism of this compound in fish.

2. Research is required to investigate the possible antiscorbutic value of D-ascorbic acid and iso-ascorbic acid in fish. There are no published reports on these compounds as dietary supplements for fish.

3. It has been shown that O. aureus and O. spilurus exhibit activity of the enzyme L-gulono-γ-lactone oxidase. It is necessary to determine whether hybrids of either of these species with other tilapias retain activity of this enzyme. It is also necessary to determine whether de novo synthesis of ascorbic acid in these species is sufficient to obviate the need for a dietary supplement.

4. Both O. niloticus and O. mossambicus showed some ability to utilise L-ascorbate 2-sulphate which suggests the existence of the enzyme ascorbic acid sulphate sulphohydrolase (C_2 sulphatase). It is, therefore, of interest to specifically identify this enzyme in tilapias.

5. Both O. niloticus and O. mossambicus exhibited low levels of dehydroascorbic acid in their tissues suggesting the existence of the enzyme ascorbic acid reductase, which has been identified in carp by Yamamoto et al. (1977a); further research is required to conclusively demonstrate the presence or absence of this enzyme.

6. Water temperature appears to alter the ascorbic acid requirement suggesting differences in ascorbic acid requirements between cold and warm water fish. This may be related to short half-life of ascorbic acid in warm water species and to the existence of the enzyme dehydroascorbatase shown to be present in carp (Ikeda & Sato, 1965; Yamamoto et al., 1977b). It is necessary for future research to determine the half-life of ascorbic acid in various tissues of tilapias and to attempt to identify the enzyme dehydroascorbatase.

7. Several reports (see Chapter 1) have been published concerning the role of ascorbic acid in ameliorating the toxic effects of heavy metals, pollutants and nitrite in rainbow trout. No information is currently available for tilapias and such research would be of value as fish are increasingly being exposed to such toxic factors even in the culture environment.

REFERENCES

ABBASY, M. A., GRAY HILL, N. & HARRIS, L. J. (1936). Vitamin C and juvenile rheumatism with observation on the vitamin C reserves in surgical tuberculosis. Lancet II, 1413-1417.

ABDO, M. S., AL-CHALABI, F., AL-KAFAWI, A. A. & AL-JANABI, A. S. (1971). Studies on the ascorbic acid content of the ovaries of camel (Camelus Dromedarius) during the various reproductive stages. J. Facult. Med. Baghdad 13, 18-24.

ABT, A. F., VON SCHUCHING, S. & ENNS, T. (1963). Vitamin C requirements of man re-examined. Am. J. Clin. Nutr. 12, 21-29.

ADAMS, C. R. (1973). Effects of environmental conditions on the stability of vitamins in feeds. In: Effect of processing on the nutritional value of feeds, 142-150. Proc. Symp., Gainsville, Fla. Jan. 11-13, 1972. Natl. Acad. Sci., Washington D.C.

AESCHBACHER, H. U. & BROWN, R. G. (1972). Automated vitamin C analysis. Clin. Chem. 18, 965-967.

AGRAWAL, N. K. & MAHAJAN, C. L. (1980a). Nutritional deficiency disease in an Indian major carp, Cirrhina mrigala Hamilton, due to avitaminosis C during early growth. J. Fish Disease 3, 231-248.

AGRAWAL, N. K. & MAHAJAN, C. L. (1980b). Comparative tissue ascorbic acid studies in fishes. J. Fish Biol. 17, 135-141.

AGRAWAL, N. K. & MAHAJAN, C. L. (1980c). Hematological changes due to vitamin C deficiency in Channa punctatus Bloch. J. Nutr. 110, 2172-2181.

AGRAWAL, N. K. & MAHAJAN, C. L. (1981). Effect of ascorbic acid deficiency on the uptake of iodine by thyroid and non-thyroid tissues of an air-breathing freshwater fish Channa punctatus Bloch. J. Fish Biol. 18, 411-416.

AGRAWAL, N. K., JUNEJA, C. J. & MAHAJAN, C. L. (1978). Protective role of ascorbic acid in fishes exposed to organochlorine pollution. Toxicol. 11, 369-375.

ANDERSON, T. W. (1977). New horizons for vitamin C. Nutr. Today 12(1), 6-13.

ANDREWS, J. W. & MURAI, T. (1975). Studies on the vitamin C requirements of channel catfish (Ictalurus punctatus). J. Nutr. 105, 557-561.

ARAI, S., NOSE, T. & HASHIMOTO, Y. (1972). Qualitative requirements of young eels Anguilla japonica for water-soluble vitamins and their deficiency symptoms. Bull. Freshwater Fish Res. Lab. 22, 69-83.

AOAC (1980). Official Methods of Analysis of the Association of Offical Analytical Chemists (Horwitz, W., ed) 13th ed. Association of Official Analytical Chemists, Washington.

BÄCKSTRÖM, S. (1956). The total content of ascorbic acid in the developing sea urchin egg. Exp. Cell Res. 11, 322-326.

BAI, P. A. & KALYANI, M. (1960). Studies on ascorbic acid in fish tissues. I. J. Anim. Morph. Physiol. 7(2), 162-165.

BAI, P. A. & KALYANI, M. (1961). Studies on the ascorbic acid contents of fish tissues. II. J. Zool. Soc. India 12, 216-219.

BAIRD, J. M., WOLF, R. O. & RENNELS, E. G. (1961). Effects of pituitary removal or transplantation on ovarian ascorbic acid depletion in the rat. Proc. Soc. Exp. Biol. Med. 106, 362-365.

BAKER, E. J., SILVERTON, R. E. & LUCKCOCK, E. D. (1966a). Haemoglobin estimation and tests for erythrocyte sedimentation and fragility. In: Introduction to medical laboratory technology, pp 536-552, Butterworth & Co., London.

BAKER, E. M., SAARI, J. C. & TOLBERT, B. M. (1966b). Ascorbic acid metabolism in man. Am. J. Clin. Nutr. 19, 371-378.

BAKER, E. M., HODGES, R. E., HOOD, J., SAUBERLICH, H. E. & MARCH, S. C. (1969). Metabolism of ascorbic-1-^{14}C acid in experimental human scurvy. Am. J. Clin. Nutr. 22, 549-558.

BAKER, E. M., HAMMER, D. C., CANHAM, J. E & TOLBERT, B. M. (1971a). Identification of ascorbate 2-sulphate in human urine. Fed. Proc. 30, 521(1820) abstr.

BAKER, E. M., HODGES, R. E., HOOD, J., SAUBERLICH, H. E., MARCH, S. C. & CANHAM, J. E. (1971b). Metabolism of ^{14}C- and ^{3}H-labeled L-ascorbic acid in human scurvy. Am. J. Clin. Nutr. 24, 444-454.

BAKER, E. M., HAMMER, D. C., KENNEDY, J. E. & TOLBERT, B. M. (1973). Interference by ascorbate-2-sulphate in the dinitrophenylhydrazine assay of ascorbic acid. Anal. Biochem. 55, 641-642.

BAKER, E. M., KNIGHT, M. K., TILLOTSON, J. A., JOHNSON, D. O. & TOLBERT, B. M. (1974). Comparative metabolism and nutrition of ascorbate-2-sulphate. Fed. Proc. 33, 665(2572) abstr.

BAKER, E. M., HALVER, J. E., JOHNSON, D. O., JOYCE, B. E., KNIGHT, M. K. & TOLBERT, B. M. (1975). Metabolism of ascorbic acid and ascorbic acid 2-sulphate in man and sub-human primate. Ann. N. Y. Acad. Sci. 258, 72-80.

BALARIN, J. D. (1977). A review of the biology of the tilapias and their culture with special reference to Africa. MSc Thesis, Stirling University, 216pp.

BALARIN, J. D. & HATTON, J. P. (1979). Culture systems and methods of rearing tilapia in Africa. In: Tilapia: a guide to their biology and culture in Africa, pp 45-56. Institute of Aquaculture, University of Stirling, Scotland.

BANERJEE, S. (1943a). Vitamin C and carbohydrate metabolism. Nature 152, 329.

BANERJEE, S. (1943b). Vitamin C and carbohydrate metabolism. II. The effect of vitamin C on the glycogen value of the liver of guinea pigs. Ann. Biochem. Exp. Med. 3 (No. 3 & 4), 165-170.

BANERJEE, S. (1944). Relation of scurvy to histological changes in the pancreas. Nature 153, 344-345.

BANERJEE, S. (1977). Physiological role of dehydroascorbic acid. Ind. J. Physiol. Pharm. 21, 85-93.

BANERJEE, S. & BELAVADY, B. (1953). Dehydro-ascorbic acid level of blood in health and in typhoid fever. Lancet 2, 912-913.

BANERJEE, S. & CHAKRABARTY, A. S. (1965). Utilization of iron by scorbutic guinea pigs. Blood 25, 839-844.

BANERJEE, S. & DIVAKARAN, E. R. (1958). Utilization of glucose, fructose and galactose by scorbutic guinea pigs. Am. J. Physiol. 195, 291-294.

BANERJEE, S. & PAL, H. (1959). Studies on changes in bone marrow and blood picture in monkeys during progressive stages of scurvy. Ind. J. Med. Res. 47(6), 646-651.

BANERJEE, S. & SINGH, H. D. (1958). Cholesterol metabolism in scorbutic guinea pigs. J. Biol. Chem. 233, 336-339.

BANERJEE, S., DEB, C. & BELAVADY, B. (1952). Effect of scurvy on glutathione and dehydroascorbic acid in guinea pig tissues. J. Biol. Chem. 195, 271-276.

BANGA, I. & SZENT-GYÖRGI, A. (1934). The large scale preparation of ascorbic acid from Hungarian pepper (Capsicum annuum). Biochem. J. 28, 1625-1628.

BARDACH, J. E., RYTHER, J. H. & McLARNEY, W. O. (1972). Aquaculture: The farming and husbandry of freshwater and marine organisms. Wiley-Interscience, New York, London. 868pp.

BARKHAN, P. & HOWARD, A. N. (1959). Some blood coagulation studies in normal and scorbutic guinea pigs. Br. J. Nutr. 13, 389-400.

BARNES, M. J. (1975). Function of ascorbic acid in collagen metabolism. Ann. N. Y. Acad. Sci. 258, 264-277.

BARNES, M. J. & KODICEK, E. (1972). Biological hydrolation and ascorbic acid with special regard to collagen metabolism. In: Vitamins and Hormones, vol. 30 (R. S. Harris, P. L. Munson, E. Diozfalusy & J. Clover, eds), pp1-43. Academic Press, New York & London.

BARNES, M. J., CONSTABLE, B. J., MORTON, L. F. & KODICEK, E. (1970). Studies in vivo on the biosynthesis of collagen and elastin in ascorbic acid-deficient guinea pigs. Evidence for the formation and degradation of partially hydroxylated collagen. Biochem. J. 119, 575-585.

BARNESS, L. A. (1975). Safety considerations with high ascorbic acid dosage. Ann. N. Y. Acad. Sci. 258, 523-528.

BARRON, E. S. G. (1951). Thiol groups of biological importance. In: Advances in enzymology (F. F. Nord, ed.) vol. 11, 201-266. Interscience publications, New York & London.

BASU, T. K. (1979). Possible role of vitamin C in cancer therapy. Internat. J. Nutr. Res. Suppl. 19, 95-102.

BAUERNFEIND, J. C. (1982). Ascorbic acid technology in agricultural, pharmaceutical, food and industrial applications. In: Ascorbic acid: Chemistry, metabolism and uses (P. A. Seib & B. M. Toblert, eds.), pp395-497. American Chemical Society, Washington D.C.

BENITEZ, L. V. & COLOSO, R. M. (1984). Tissue distribution of ascorbate in milkfish. International symposium, feeding and nutrition in fish, July 10-18 1984, University of Aberdeen. Abstracts of papers.

BENITEZ, L. V. & HALVER, J. E. (1982). Ascorbic acid sulphate sulphohydrolase (C_2 sulphatase): The modular of cellular levels of L-ascorbic acid in rainbow trout. Proc. Natl. Acad. Sci., USA, 79, 5445-5449.

BEN-TUVIA, A. (1960). The biology of the cichlid fishes of lake Tilapias and Huleh. Bull. State of Israel, Div. Fish. Res. No. 27, 33pp.

BESSEY, O. A. (1938). A method for the determination of small quantities of ascorbic acid and dehydroascorbic acid in turbid and colored solutions in the presence of other reducing substances. J. Biol. Chem. 126, 771-784.

BHADURI, J. N. & BANERJEE, S. (1960). Ascorbic acid, dehydroascorbic acid and glutathione levels in blood of patients suffering from infectious diseases. Ind. Jour. Med. Res. 48, 203-211.

BIGGER, H. P. (1924). In: The voyages of Jacques Cartier, No. 11, p204. Publications of Public Archives of Canada.

BIGLER, W. N. & KELLY, D. M. (1975). Liquid chromatographic analysis of ascorbate and ascorbate-2-sulphate. Ann. N. Y. Acad. Sci. 258, 70-71.

BIGLEY, R. H. & STANKOVA, L. (1974). Uptake and retention of oxidized and reduced ascorbate by human leucocytes. J. Exp. Med. 139, 1084-1092.

BIRCH, T. W. & HARRIS, L. J. (1933). The titration curve and dissociation constants of vitamin C. Biochem. J. 27, 595-599.

BISWAS, N. M. (1969). Δ^5-3β-hydroxysteroid dehydrogenase in toad testis: Senergistic action of ascorbic acid and leuteinizing hormone. Endocrinology 85, 981-983.

BISWAS, N. M. & DEB, C. (1965). Role of ascorbic acid in testicular degeneration and adrenal hypertrophy in tyrosine-fed rats. Endocrinol. 79, 1157-1159.

BISWAS, N. M. & DEB, C. (1970). *In vitro* studies on the effects of ascorbic acid and dehydroascorbic acid on Δ^5-3β-hydroxysteroid dehydrogenase in toad testis. Endocrinology 87, 170-173.

BISWAS, N. M. & MUKHERJEE, M. (1967). Ascorbic acid and testicular activity in heat-exposed toad (*Bufo melanostictus*). Experientia 23, 667.

BLACKSTONE, S., HURLEY, R. J. & HUGHES, R. E. (1974). Some interrelationships between vitamin C (L-ascorbic acid) and mercury in the guinea pig. Food Cosmet. Toxicol. 12, 511-516.

BLANCO, O. & MEADE, T. (1980). Effect of dietary ascorbic acid on the susceptibility of steelhead trout (*Salmo gairdneri*) to nitrite toxicity. Rev. Biol. Trop. 28(1), 91-107.

BOSUSIANNAH, O. A. (1981). Qualitative ascorbic acid requirement of Tilapia (*S. mossambicus*). Water-soluble vitamin requirements of Tilapia (*S. mossambicus* and *S. niloticus*), pp69-84, PhD Thesis, University of Aston, Birmingham.

BOTHWELL, T. H., BRADLOW, B. A., JACOBS, P., KEELEY, K., KRAMER, S., SEFTEL, H. C. & LAIL, S. (1964). Iron metabolism in scurvy with special reference to erythropoiesis. Br. J. Haemat. 10, 50-58.

BOURNE, G. H. (1944). Records in the older literature of tissue changes in scurvy. Proc. Royal Soc. Med. 37, 512-516.

BOURNE, G. H. (1946). The effect of vitamin C on the healing of wounds. Proc. Nutr. Soc. 1-4, 204-216.

BRANDT, T. M., DEYOE, C. W. & SEIB, P. A. (1985). Alternate sources of vitamin C for channel catfish. Prog. Fish-Cult. 47(1), 55-59.

BRIGGS, M. H., GARCIA-WEBB, P. & DAVIES, P. (1973a). Urinary oxalate and vitamin C supplements. Lancet 2, 201.

BRIGGS, M. H., GARCIA-WEBB, P. & JOHNSON, J. (1973b). Dangers of excess vitamin C. Med. J. Australia 2, 48-49.

BRIN, M. (1982). Nutritional and health aspects of ascorbic acid. In: Ascorbic acid: Chemistry, metabolism and uses (P. A. Seib & B. M. Tolbert, eds.), pp369-379. American Chemical Society, Washington D.C.

BRISE, H. & HALLBERG, L. (1962). Effect of ascorbic acid on iron absorption. Acta Med. Scand. Suppl. 376, 51-58.

BROOK, M. & GRIMSHAW, J. J. (1968). Vitamin C concentration of plasma and leukocytes as related to smoking habit, age and sex of humans. Am. J. Clin. Nutr. 21(11), 1254-1258.

BROWN, M. E. (1957). The physiology of fishes, Vol. 1. Academic Press.

BULLOCK, D. H., SINGH, S. & PEARSON, A. M. (1968). Stability of vitamin C in enriched commercial evaporated milk. J. Dairy Sci. 50, 921-923.

BULLOCK, A. M., MARKS, R. & ROBERTS, R. J. (1978). The cell kinetics of teleost fish epidermis: Epidermal mitotic activity in relation to wound healing at varying temperatures in plaice (Pleuronectes platessa). J. Zool. London 185, 197-204.

BURNS, J. J. (1957). Missing step in man, monkey and guinea pig required for the biosynthesis of L-ascorbic acid. Nature 180, 553.

BURNS, J. J. (1959). Biosynthesis of L-ascorbic acid; basic defect in scurvy. Am. J. Med. 26, 740-748.

BURNS, J. J. (1960). Ascorbic acid. In: Greenberg metabolic pathways, Vol. 1, pp341-356. Academic Press, New York.

BURNS, J. J. (1967). Ascorbic acid. In: Greenberg metabolic pathways, Vol. 1, 3rd edn., pp394-411. Academic Press, New York & London.

BURNS, J. J. (1975). Introduction: Overview of ascorbic acid metabolism. Ann. N.Y. Acad. Sci. 258, 5-6.

BURNS, J. J., BURCH, H. B. & KING, G. G. (1951). The metabolism of 1-C^{14}-L-ascorbic acid in guinea pigs. J. Biol. Chem. 191, 501-514.

BURNS, J. J., PEYSER, P. & MOLTZ, A. (1956). Missing step in guinea pigs required for the biosynthesis of L-ascorbic acid. Science 124, 1148-1149.

BURNS, J. J., CONNEY, A. H., DAYTON, P. G., EVANS, C., MARTIN, G. R. & TALLER, D. (1960). Observation on the drug-induced synthesis of D-glucuronic, L-gulonic acid and L-ascorbic acid in rats. J. Pharmacol. Exp. Therapy 129, 132-138.

CALKINS, V. P. & MATTILL, H. A. (1944). The kinetics of the anti-oxygenic synergism of quinones with ascorbic acid in fat systems. J. Am. Chem. Soc. 66, 239-247.

CAMERON, E. & PAULING, L. (1973). Ascorbic acid and the glycosaminoglycans. An orthomolecular approach to cancer and other diseases. Oncology 23, 181-192.

CAMPBELL, G. D., STEINBERG, M. H. & BOWER, J. D. (1975). Ascorbic acid-induced hemolysis in G-6-PD deficiency. Am. Internat. Med. 82, 810.

CARDINALE, G. J. & UDENFRIEND, S. (1974). Prolyl hydroxylase. In: Advances in enzymology (A. Meister, ed.) Vol. 41, 245-300. Interscience publication, John Wiley & Sons, New York, London, Sydney & Toronto.

CARPENTER, M. P., KITABACHI, A. E., McCAY, P. B. & CAPUTTO, R. (1959). The activation by tocopherol and other agents of ascorbic acid synthesis by liver homogenates from vitamin E-deficient rats. J. Biol. Chem. 234, 2814-2818.

CARR, R. S. & NEFF, J. M. (1980). Determination of ascorbic acid in tissues of marine animals by liquid chromatography with electrochemical detection. Anal. Chem. 52, 2428-2430.

CHADWICK, R., PEOPLES, A. & CRANMER, M. (1973). The effect of protein quality and ascorbic acid deficiency on stimulation of hepatic microsomal enzymes in guina pigs. Toxicol. Appl. Pharmacol. 24, 603-611.

CHAHINE, M. H. & STARZOMSKI, J. J. (1976). Variations in chemical composition of commercial fish feeds. Feedstuffs 48(16), 22-27.

CHAKRABARTI, B. & BANERJEE, S. (1955). Dehydroascorbic acid level in blood of patients suffering from various infectious diseases. Proc. Soc. Exp. Biol. Med. 88, 581-583.

CHAKRABORTY, D., BHATTACHARYYA, A., MAJUMDAR, K., CHATTERJEE, K., CHATTERJEE, S., SEN, A. & CHATTERJEE, G. G. (1978). Studies on L-ascorbic acid metabolism in rats under chronic toxicity

due to organophosphorus insecticides: Effects of supplementation of L-ascorbic acid in high doses. J. Nutr. 108, 973-980.

CHATTERJEE, G. C. (1967). Effects of ascorbic acid deficiency in animals. In: The vitamins, (W. H. Sebrell Jr. & R. S. Harris eds.) Vol. 1, pp407-456. Academic Press, New York & London.

CHATTERJEE, I. B. (1970). Biosynthesis of L-ascorbate in animals. Methods Enzymol. 18, 28-34.

CHATTERJEE, I. B. (1973a). Evolution and the biosynthesis of ascorbic acid. Science 182, 1271-1272.

CHATTERJEE, I. B. (1973b). Vitamin C synthesis in animals: Evolutionary trend. Science & Culture 39, 210-212.

CHATTERJEE, I. B., CHATTERJEE, G. C., GHOSH, N. C., GHOSH, J. J. & GUHA, B. C. (1960). Biological synthesis of L-ascorbic acid in animal tissues: Conversion of D-glucuronolactone and L-gulonolactone into L-ascorbic acid. Biochem. J. 76, 279-292.

CHATTERJEE, I. B., KAR, N. C., GHOSH, N. C. & GUHA, B. C. (1961a). Biosynthesis of L-ascorbic acid: Missing steps in animals incapable of synthesizing the vitamin. Nature 192, 163-164.

CHATTERJEE, I. B., KAR, N. C., GHOSH, N. C. & GUHA, B. C. (1961b). Aspects of ascorbic acid biosynthesis in animals. Ann. N. Y. Acad. Sci. 92, 35-56.

CHATTERJEE, G. C., BANERJEE, S. K. & PAL, D. R. (1973). Cadmium administration and L-ascorbic acid metabolism in rats: Effect of L-ascorbic acid supplementation. Internat. J. Vit. Nutr. Res. 43, 370-377.

CHATTERJEE, I. B., MAJUMDER, A. K., NANDI, B. K. & SUBRAMANIAN, N. (1975). Synthesis and some major function of vitamin C in animals. Ann. N. Y. Acad. Sci. 258, 24-47.

CHEN, L. H. & BARNES, K. J. (1976). Nutritional relationship of vitamin E and vitamin C in guinea pigs. Nutr. Rep. Internat. 14, 89-96.

CHIKARAISHI, S. (1982). Effect of ascorbic acid and aflatoxin on the biosynthesis of hemoglobin and liver cytochrome P-450. Vitamins (Japan) 55(4), 177-181.

CHINOY, N. J. (1969). On the specificity of the alcoholic, acidic silver nitrate reagent for the histochemical localization of ascorbic acid. Histochemie 20, 105-107.

CHRISTENSEN, H. N. & LYNCH, E. L. (1948). Decrease of glycine and glutamine in skeletal muscle and of glutamine in liver in ascorbic acid deficiency in the guinea pig. J. Biol. Chem. 172, 107-110.

CLAESSON, L., HILLARP, N. A., HÖGBERG, B. & HÖKFELT, B. (1949). Changes in the ascorbic acid content in the interstitial gland of the rabbit ovary following gonadotrophic stimulation. Acta Endocrinol. 2, 249-256.

COATES, J. A. & HALVER, J. E. (1958). Water-soluble vitamin requirements of silver salmon. Special Sci. Report Fish. No. 281, 9pp.

COCHRANE, W. A. (1965). Overnutrition in prenatal and neonatal life: A problem? Can. J. Assoc. 93, 893-899.

COHEN, R. B. (1961). Histochemical localization of L-gulonolactone oxidase activity in tissues of several species. Proc. Exp. Biol. Med. 106, 309-311.

COLLINS, M. & ELVEHJEM, C. A. (1958). Ascorbic acid requirement of the guinea pig using growth and tissue ascorbic acid concentrations as criteria. J. Nutr. 64, 503-511.

CONNEY, A. H., BRAY, C. A., EVANS, C. & BURNS, J. J. (1961). Metabolic interactions between L-ascorbic acid and drugs. Ann. N. Y. Acad. Sci. 92, 115-127.

COOPER, J. R. (1961). The role of ascorbic acid in the oxidation of tryptophan to 5-hydroxytryptophan. Ann. N. Y. Acad. Sci. 92, 208-211.

CORT, W. M. (1982). Antioxidant properties of ascorbic acid in foods. In: Ascorbic acid: Chemistry, metabolism and uses (P. A. Seib & B. M. Tolbert, eds.), pp531-550. American Chemical Society, Washington D.C.

COWEY, C. B. & ROBERTS, R. J. (1978). Nutritional pathology of teleosts. In: Fish pathology (R. J. Roberts, ed.) pp216-234. Bailliere Tindall, Division of Cassell Ltd., Bailliere, London.

CULLING, C. F. A. (1974). Handbook of histopathological techniques, 3rd. edn., pp712. Butterworths, London.

DABROWSKI, K. (1977). The L-ascorbic acid content in silver bream's (Vimba vimba L.) organs. Polskie Archwm. Hydrobiol. 24(4), 569-573.

DAYTON, P. G. & BURNS, J. J. (1958). Metabolism of D-ascorbic acid-1-C^{14} in guinea pigs and rats. J. Biol. Chem. 231, 85-91.

DEANA, R., BHARAJ, B. S., VERJEE, Z. A. & GALZIGNA, L. (1975). Changes relevant to catecholamine metabolism in liver and brain of ascorbic acid deficient guinea-pigs. Internat. J. Vit. Nutr. Res. 45, 175-182.

DEMERS, P., FRASER, D., GOLDBLOOM, R., MACLEAN, R., MEDOVY, H., MORRISON, A., HEBB, J. & COCHRANE, W. (1965). An epidemiological study of infantile scurvy in Canada: 1961-1963. Canad. Med. Assoc. J. 93, 573-576.

DEMOLE, V. (1934). On the physiological action of ascorbic acid and some related compounds. Biochem. J. 28, 770-773.

DESAI, I. D. & TAPPEL, A. L. (1963). Damage to proteins by peroxidized lipids. J. Lipid Res. 4, 204-207.

DESMARAIS, A. & McCRAW, J. Y. (1956). Etude de la crase sanguine chez le cobaye carence ou non en acide ascorbique. Rev. Can. Biol. 14, 322-329.

DEVINE, M. M. & RIVERS, J. M. (1968). Monodehydroascorbic acid-Transhydrogenase activity and coenzyme concentrations in tissues of ascorbic acid-deficient and control guinea pigs. J. Nutr. 97, 295-302.

DIPLOCK, A. T., HAUM, H. & LUCY, J. A. (1971). The effect of vitamin E on the oxidation state of selenium in rat liver. Biochem. J. 123, 721-729.

DIWAN, A. D., CHANDRASEKHARAN NAIDU, N. & RAJ MOHAN, K. (1978). Ascorbic acid content in blood and brain of Clarias batrachus (Linnaeus) exposed to industrial effluents. Matsya 4, 70-72.

DODDS, M. L. (1969). Sex as a factor in blood levels of ascorbic acid. J. Am. Diet. Assoc. 54, 32-33.

DOUGLAS, J. F. & KING, C. G. (1953). The conversion of C^{14}-labeled glucose to glucuronic acid in the guinea pig. J. Biol. Chem. 202, 865-871.

DRAPER, H. H. (1970). The tocopherols. In: International Encylopaedia of food nutrition, Vol. 9, Fat-soluble vitamins (Morton, R. A., ed.), pp333-393. Pergamon Press, Oxford.

DRURY, R. A. B. & WALLINGTON, E. A. (1980). Bone and decalcification. Carleton's histological techniques, 5th edn., 199-220, Oxford, New York, Toronto, Oxford University Press.

DUNCAN, D. B. (1955). Multiple range and multiple F-test. Biometrics 11, 1-42.

DUNKLEY, W. L., FRANKLIN, J. D. & PANGBORN, R. M. (1962). Fluorescent light on flavor, ascorbic acid, and riboflavin in milk. Food Tech. 16, 112-118.

DUPREE, H. K. (1966). Vitamins essential for the growth of channel catfish (Ictalurus punctatus). US Fish. Wildl. Serv., Tech. Pap. 7, 12pp.

DURVE, V. S. & LOVELL, R. T. (1982). Vitamin C and disease resistance in channel catfish (Ictalurus punctatus). Can. J. Fish. Aquat. Sci. 39, 948-951.

DUTTA GUPTA, S., RAY CHAUDHURI, C. & CHATTERJEE, I. B. (1972). Incapability of L-ascorbic acid synthesis by insects. Arch. Biochem. Biophys. 152, 889-890.

DUTTA GUPTA, S., CHOUDHURY, P. K. & CHATTERJEE, I. B. (1973). Synthesis of L-ascorbic acid from D-glucurono-1,4-lactone conjugates by different species of animals. Int. J. Biochem. 4, 309-314.

DYKHUIZEN, D. E., HARRISON, K. M. & RICHARDSON, B. J. (1979). Evolutionary implications of ascorbic acid production in the Australian lungfish. Experientia 36, 945-946.

EBBELL, B. (1938). In: Alt, Agyptische bezeichungen für krankheiten und symptomes. Oslo.

EDGER, J. A. (1969). Is dehydroascorbic acid an inhibitor in the regulation of cell division in plants and animals. Experientia 25, 1214-1215.

EDGER, J. A. (1970). Dehydroascorbic acid and cell division. Nature 227, 24-26.

EDITORIALS AND COMMENTS (1953). Effect of vitamin C deficiency on healed wounds. J. Am. Med. Assoc. 152, 334.

EIFAC (1971). Symposium on new development in carp and trout nutrition, Rome, 20-24 May 1968, Rome 1969. EIFAC Technical Paper No. 9, pp29.

EL-HAWARY, M. F. S., EL-SHOBAKI, F. A. & KHOLEIF, T. (1975). The absorption of iron, with or without supplements of single amino acids and of ascorbic acid, in health and Fe-deficient children. Br. J. Nutr. 33, 351-355.

ELLIS, A. E. (1978). The immunology of teleosts. In: Fish pathology (R. J. Roberts, ed.) pp92-104. Bailliere Tindall, Division of Cassell Ltd., Bailliere, London.

EVA, J. K., FIFIELD, R. & RICKETT, M. (1976). Decomposition of supplementary vitamin C in diets compounded for laboratory animals. Laboratory Animals 10, 157-159.

EVANS, J. R. & HUGHES, R. E. (1963). The growth-maintaining activity of ascorbic acid. Br. J. Nutr. 17, 251-255.

EVANS, G. W., MAJORS, P. F. & CORNATZER, W. E. (1970). Ascorbic acid interaction with metallothionein. Biochem. Biophys. Res. Commun. 41, 1244-1247.

FABIANEK, J. & HERP, A. (1967). Antiscorbutic activity of D-arabo-ascorbic acid. Proc. Soc. Exp. Biol. Med. 125, 462-465.

FISH FARMING INTERNATIONAL (1984a). Vitamin C has high place in feeds for farm fish. Fish Farming International 11(6), 18.

FISH FARMING INTERNATIONAL (1984b). More vitamins yield more salmon eggs. Fish Farming International 11(11), 12.

FITZHUGH, O. G. & NELSON, A. A. (1946). Subacute and chronic toxicities of ascorbyl palmitate. Proc. Soc. Exp. Biol. Med. 61, 195-198.

FOLKERS, K. (1969). Survey on the vitamin aspects of coenzyme Q. Internat. J. Vit. Res. 39, 334-352.

FOX, M. R. S. & FRY, B. E., Jr. (1970). Cadmium toxicity decreased by dietary ascorbic acid supplements. Science 169, 989-992.

FRASER, R. C., PAVLOVIC, S., KURAHARA, C. G., MURATA, A., PETERSON, N. S., TAYLOR, K. B. & FEIGEN, G. A. (1960). The effect of variations in vitamin C intake on the cellular immune response of guinea pigs. Am. J. Clin. Nutr. 33, 839-847.

FUKUDA, M. & SIBATANI, A. (1953). Nucleic acid and proteins in the liver of guinea pigs dying from ascorbic acid deficiency. Experientia 9, 27-28.

FURUKAWA, A. & TSUKAHARA, H. (1966). On the acid digestion method for the determination of chromic oxide as an index substance in the study of digestibility of fish feed. Bull. Japan. Soc. Sci. Fish. 32(6), 502-506.

GALLOWAY, N. M., GARRY, C. R. & HITCHIN, A. D. (1947). The influence of ascorbic acid on the repair of epithelial structures in guinea pigs. Br. J. Nutr. 1, xii.

GANGULY, R., DURIEUX, M. F. & WALDMAN, R. H. (1976). Macrophage function in vitamin C-deficient guinea pigs. Am. J. Clin. Nutr. 29, 762-765.

GHOSH, N. C., CHATTERJEE, I. & CHATTERJEE, G. C. (1965). Effects of vitamins A and D on the biosynthesis of L-ascorbic acid by rat-liver microsomes. Biochem. J. 97, 247-249.

GINTER, E. (1973). Cholesterol: Vitamin C controls its transformation to bile acids, Science 179, 702-704.

GINTER, E. (1975). Ascorbic acid in cholesterol and bile acid metabolism. Ann. N. Y. Acad. Sci. 258, 410-421.

GINTER, E. (1978). Marginal vitamin C deficiency, lipid metabolism, and atherogenesis. In: Advances in lipid research (R. Paoletti & D. Kritchevsky, eds.), Vol. 16, 167-220. Academic Press, New York, San Francisco & London.

GINTER, E., NEMEC, R. & BOBEK, P (1972). Stimulation of (26-C^{14}) cholesterol oxidation by ascorbic acid in scorbutic guinea pigs. Br. J. Nutr. 28, 205-211.

GINTER, E., BOBEK, P. & VARGOVA, D. (1979). Tissue levels and optimum dosage of vitamin C in guinea pigs. Nutr. Metab. 23, 217-226.

GIPP, W. E., POND, W. G., KALLFELZ, F. A., TASKER, J. B., VAN CAMPEN, D. R., KROOK, L. & VISEK, W. J. (1974). Effect of dietary copper, iron and ascorbic acid levels on haematology, blood & tissue copper, iron and zinc concentrations and Cu^{64} and Fe^{59} metabolism in young pigs. J. Nutr. 104, 532-541.

GLUECKSOHN-WAELSCH, S. (1963). Lethal genes and analysis of differentiation; In higher organisms lethal genes serve as tools for studies of cell differentiation and cell genetics. Science 142, 1269-1276.

GOLDSTEIN, D. P. & STURGIS, S. H. (1961). Luteinizing hormone-induced depletion of ascorbic acid in the rat ovary. Am. J. Physiol. 201, 1053-1056.

GOLUMBIC, C. & MATTILL, H. A. (1941). Antioxidants and the autoxidation of fats. XIII. The antioxygenic action of ascorbic acid in association with tocopherols, hydroquinones and related compounds. J. Am. Chem. Soc. 63, 1279-1280.

GOULD, B. S. (1958). Biosynthesis of collagen. The direct action of ascorbic acid on hydroxyproline and collagen formation in subcutaneous polyvinyl sponge implants in guinea pigs. J. Biol. Chem. 232, 637-649.

GOULD, B. S. (1960). Ascorbic acid and collagen fiber formation. In: Vitamins & Hormones (R. S. Harris & D. J. Ingle, eds) Vol. 18, 89-116. New York, London. Academic Press.

GOULD, B. S. (1961). Ascorbic acid-independent and ascorbic acid-dependent collagen forming mechanisms. Ann. N. Y. Acad. Sci. 91, 168-174.

GOULD, B. S. & SHWACHMAN, H. (1942). Bone and tissue phosphatase in experimental scurvy and studies on the source of serum phosphatase. Am. J. Physiol. 135, 485-491.

GOULD, B. S. & SHWACHMAN, H. (1943). A new method for the bioassay of antiscorbutic substances. J. Biol. Chem. 151, 439-453.

GOULD, B. S. & WOESSNER, J. F. (1957). Biosynthesis of collagen. The influence of ascorbic acid on the proline, hydroxyproline, glycine, and collagen content of regenerating guinea pig skin. J. Biol. Chem. 226, 289-300.

GOULD, B. S., MANNER, G., GOLDMAN, H. M. & STOLMAN, J. M. (1960). Some aspects of collagen formation. Ann. N. Y. Acad. Sci. 85, 385-398.

GREEN, J. & BUNYAN, J. (1969). Vitamin E and the biological anti-oxidant theory. Nutr. Abstr. Rev. 39, 321-345.

GROLLMAN, A. P. & LEHNINGER, A. L. (1957). Enzymic synthesis of L-ascorbic acid in different animal species. Arch. Biochem. Biophys. 69, 458-467.

GUNSALUS, I. C. & HAND, D. B. (1941). The use of bacteria in the chemical determination of total vitamin C. J. Biol. Chem. 149, 853-858.

HALVER, J. E. (1972a). The vitamins. In: Fish nutrition (J. E. Halver, ed.) pp29-103. Academic Press, New York & London.

HALVER, J. E. (1972b). The role of ascorbic acid in fish disease and tissue repair. Bull. Japan. Soc. Sci. Fish. 38(1), 79-92.

HALVER, J. E. (1980). The vitamins. FAO Lectures presented at the FAO/UNDP training course in fish feed technology, held at the College of Fisheries, University of Washington, 9 October - 15 December 1978 (ADCP/80/11).

HALVER, J. E. (1982). The vitamins required for cultivated salmonids. Comp. Biochem. Physiol. 73B, 43-50.

HALVER, J. E. & COATES, J. A. (1957). A vitamin test diet for long-term feeding studies. Prog. Fish-Cult. 19, 112-118.

HALVER, J. E., ASHLEY, L. M. & SMITH, R. R. (1969). Ascorbic acid requirements of coho salmon and rainbow trout. Trans. Am. Fish. Soc. 98(4), 762-771.

HALVER, J. E., JOHNSON, C. L., SMITH, R. R., TOLBERT, B. M. & BAKER, E. M. (1972). Vitamin C_3 reduces fish scurvy. Fed. Proc. 31, 705(2764) abstr.

HALVER, J. E., ASHLEY, L. M., SMITH, R. R., TOLBERT, B. M. & BAKER, E. M. (1973). Vitamin C_2 and iso-C_2 as dietary sources of C for fish. Fed. Proc. 32, 932(4010) abstr.

HALVER, J. E., SMITH, R. R., BAKER, E. M. & TOLBERT, B. M. (1974). Iso-C_2 fails, C_2 works in fish diets. Fed. Proc. 33, 665 (2573) abstr.

HALVER, J. E., SMITH, R. R., TOLBERT, B. M. & BAKER, E. M. (1975). Utilization of ascorbic acid in fish. Ann. N. Y. Acad. Sci. 258, 81-102.

HALVER, J. E., TUCKER, B. W. & SMITH, R. R. (1983). Technical grade vitamin C_2 support trout growth. Fed. Proc. 42, 668(2199) abstr.

HAMMARSTROM, L. (1966). Autoradiographic studies on the distribution of C^{14}-labelled ascorbic acid and dehydroascorbic acid. Acta Physiologica Scand. 70, 3-83.

HARDY, R. W., HALVER, J. E. & BRANNON, E. L. (1979). Effect of dietary protein level on the pyriodoxine requirement and disease resistance of chinook salmon. In: Proc. World Symp. on Finfish Nutr. Fishfeed Technology, Hamburg, 20-23 June 1978 (J. E. Halver & K. Tiwes, eds.), Vol. 1, pp253-259. Berlin.

HARRIS, L. J. (1937). In: Vitamins in theory and practice. Cambridge University Press, Cambridge, p79.

HARRIS, L. J. (1938). Chemical test for vitamin C and the reducing substances present in humour and other tissues. Nature 132, 27-28.

HARRIS, R. S. (1967a). Nomenclature and formulas. In: The vitamins (W. H. Sebrell, Jr. & R. S. Harris, eds.), Vol. 1, pp306-307. Academic Press, New York & London.

HARRIS, R. S. (1967b). Active compounds and ascorbic acid antagonists. In: The vitamins (W. H. Sebrell, Jr. & R. S. Harris, eds.), Vol. 1, pp383-385. Academic Press, New York & London.

HARRIS, L. J. & OLLIVER, M. (1942). Vitamin methods: 3. The reliability of the method for estimating vitamin C by titration against 2,6-dichlorophenolindophenol. 1. Control tests with plant tissues. Biochem. J. 36, 155-181.

HASTING, W. H. & SPENCER, C. F. (1952). Determinations of free and bound ascorbic acid in fishery products. J. Mar. Res. 11, 241-244.

HATA, K. & KANEDA, T. (1980). Effect of autoxidized oil on carp. Bull. Japan. Soc. Sci. Fish. 46(8), 997-1000.

HAWK, P. B. (1965a). Vitamins and deficiency diseases. In: Hawk's physiological chemistry (B. L. Oser, ed.), 14th edn, pp573-758. The Blakiston Division, McGraw-Hill Book Company, New York, Toronto, Sydney & London.

HAWK, P. B. (1965b). Blood analysis. In: Hawk's physiological chemistry (B. L. Oser, ed.), pp975-1152. McGraw-Hill Book Company, New York, Toronto, Sydney & London.

HAWK, P. B., OSER, B. L. & SUMMERSON, W. H. (1947). Vitamins and deficiency diseases. In: Practical physiological chemistry, 12th edn, pp1022-1192. J.& A. Churchill Ltd., 104 Gloucester Place, Portman Square, London.

HAWORTH, W. N., HIRST, E. L., HERBERT, R. W., PERCIVAL, E. G., REYNOLDS, R. W. & SMITH, F. (1933). J. Soc. Chem. Ind. 52, 221 (Cited by Bauernfeind, 1982) In: Ascorbic acid: Chemistry, metabolism & uses. (P. A. Seib & B. M. Tolbert, eds.), pp396. American Chemical Society, Washington D.C.

HAWORTH, W. N., HIRST, E. L. & ZILVA, S. S. (1934). Physiological activity of synthetic ascorbic acid. J. Chem. Soc. (London), August, 115-1156.

HAY, G. W., LEWIS, B. A. & SMITH, F. (1967). II. Chemistry. In: The vitamins (W. H. Sebrell, Jr & R. S. Harris, eds.), Vol. 1, pp306-336. Academic Press, New York & London.

HEATH, H. (1962). The distribution and possible function of ascorbic acid in the eye. Exp. Eye Res. 1, 362-367.

HELLMAN, L. & BURNS, J. J. (1958). Metabolism of L-ascorbic acid-1-C^{14} in man. J. Biol. Chem. 230, 923-930.

HERBERT, V. & JACOB, E. (1974). Destruction of vitamin B_{12} by ascorbic acid. J. Am. Med. Assoc. 230(2), 241-242.

HERREID, E. O., RUSKIN, B., CLARK, G. L. & PARKS, T. B. (1952). Ascorbic acid and riboflavin destruction and flavor development in milk exposed to the sun in amber, clear, paper and ruby bottles. J. Dairy Sci. 35, 772-778.

HERSHBERGER, L. G., HANSEN, L. M. & THOMPSON, C. R. (1965). Observations on the ovarian ascorbic acid depletion test for luteinizing hormone activity. Endocrinology 77, 1143-1146.

HIGGINS, J. W. & BLACK, H. C. (1945). A preliminary comparison of the stabilizing effect of several recently proposed anti-oxidants for edible fats and oils. Oil & Soap 21, 277-279.

HILL, C. H. (1979). Studies on the ameliorating effect of ascorbic acid on mineral toxicities in the chick. J. Nutr. 109, 84-90.

HILTON, J. W., CHO, C. Y. & SLINGER, S. J. (1977a). Evaluation of the ascorbic acid status of rainbow trout (Salmo gairdneri). J. Fish. Res. Board Can. 34, 2207-2210.

HILTON, J. W., CHO, C. Y. & SLINGER, S. J. (1977b). Factors affecting the stability of supplemental ascorbic acid in practical trout diets. J. Fish. Res. Board Can. 34, 683-687.

HILTON, J. W., CHO, C. Y. & SLINGER, S. J. (1978a). Effect of hypervitaminosis A on the development of ascorbic acid deficiency in underyearling rainbow trout (Salmo gairdneri R). Aquaculture, 13, 325-330.

HILTON, J. W., CHO, C. Y. & SLINGER, S. J. (1978b). Effect of graded levels of supplemental ascorbic acid in practical diets fed to rainbow trout (Salmo gairdneri). J. Fish. Res. Board Can. 35, 431-436.

HILTON, J. W., CHO, C. Y., BROWN, R. G. & SLINGER, S. J. (1979a). The synthesis, half-life and distribution of ascorbic acid in rainbow trout. Comp. Biochem. Physiol. 63A, 447-453.

HILTON, J. W., BROWN, R. G. & SLINGER, S. J. (1979b). The half-life and uptake of ^{14}C-1-ascorbic acid in selected organs of rainbow trout (Salmo gairdneri). Comp. Biochem. Physiol. 62A, 427-432.

HINDSON, T. C. (1970). Ascorbic acid status of Europeans resident in the tropics. Br. J. Nutr. 24, 801-802.

HIRAO, S., YAMADA, J. & KIKUCHI, R. (1954). Violaxanthin in rainbow trout egg. Bull. Japan. Soc. Sci. Fish. 20(5), 431-435.

HIRAO, S., YAMADA, J. & KIKUCHI, R. (1955). Relation between chemical constituents of rainbow trout eggs and their hatching rate. Bull. Japan. Soc. Sci. Fish. 21(4), 240-243.

HIRSCH, A. (1885). Scurvy. In: Hirsch's handbook of geographical and historical pathology. Vol. 11, pp507-568. The New Sydenham Society, London.

HODGES, R. E. & BAKER, E. M. (1973). Section K: Ascorbic acid. In: Modern nutrition in health and disease (R. S. Goodhart & M. E. Shils, eds.), pp245-255. Lea & Febiger, Philadelphia.

HODGES, R. E., BAKER, E. M., HOOD, J., SAUBERLICH, H. E. & MARCH, S. C. (1969). Experimental scurvy in man. Am. J. Clin. Nutr. 22(5), 535-548.

HODGES, R. E., HOOD, J., CANHAM, J. E., SAUBERLICH, H. E. & BAKER, E. M. (1971). Clinical manifestations of ascorbic acid deficiency in man. Am. J. Clin. Nutr. 24, 432-443.

HODSON, P. V., HILTON, J. W., BLUNT, B. R. & SLINGER, S. J. (1980). Effects of dietary ascorbic acid on chronic lead toxicity to young rainbow trout (Salmo gairdneri). Can. J. Fish. Aquat. Sci. 37, 170-176.

HOEHN, S. K. & KANFER, J. N. (1978). L-ascorbic acid and lysosomal acid hydrolase activities of guinea pig liver and brain. Can. J. Biochem. 56, 532-536.

HOEHN, S. K. & KANFER, J. N. (1980). Effects of chronic ascorbic acid deficiency on guinea pig lysosomal hydrolase activities. J. Nutr. 110, 2085-2094.

HOLMES, A. D. & JONES, C. P. (1945). Effect of sunshine upon the ascorbic acid and riboflavin content of milk. J. Nutr. 29, 201-209.

HOLMES, H. N., CAMPBELL, K. & AMBERG, E. J. (1939). The effect of vitamin C on lead poisoning. J. Lab. Clin. Med. 24, 1119-1127.

HOLST, A. & FRÖLICH, T. (1907). Experimental studies relating to ship-beri-beri and scurvy. J. Hyg. Camb. 7, 634-671.

HOPKINS, F. G. (1906). Proceedings of the society of public analysts. The Analyst 31, 385-404.

HOPKINS, G. (1912). Feeding experiments illustrating the importance of accessory factors in normal dietaries. J. Physiol. 44, 425-460.

HOPPING, J. M. & RULIFFSON, W. S. (1966). Roles of citric and ascorbic acids in enteric iron absorption in rats. Am. J. Physiol. 210(6), 1316-1320.

HORNIG, D. (1975a). Metabolism of ascorbic acid. Wld. Rev. Nutr. Diet. 23, 225-258.

HORNIG, D. (1975b). Distribution of ascorbic acid, metabolites and analogues in man and animals. Ann. N. Y. Acad. Sci. 258, 103-118.

HORNIG, D. (1981). Metabolism and requirements of ascorbic acid in man. South Africa Med. J. 60, 818-823.

HORNIG, D. & WEISER, H. (1976). Interaction of erythrobic acid with ascorbic acid catabolism. Internat. J. Vit. Nutr. Res. 46, 40-47.

HORNIG, D., GALLO-TORRES, H. E. & WEISER, H. (1973). A biliary metabolite of ascorbic acid in normal and hypophsectomized rats. Biochem. Biophys. Acta 320, 549-556.

HORNIG, D., WEBER, F. & WISS, O. (1974). Influence of erythrobic acid on the vitamin C status in guinea-pigs. Experientia 30, 173-174.

HOROWITZ, H. H. & KING, C. G. (1953). The conversion of glucose-6-C^{14} to ascorbic acid by the albino rat. J. Biol. Chem. 200, 125-128.

HOYGAARD, A. & RASMUSSEN, H. (1939). Vitamin C sources in Eskimo food. Nature 143, 943.

HRUBA, F., NOVAKOVA, V. & GINTER, E. (1982). The effect of chronic marginal vitamin C deficiency on the α-tocopherol content of the organs and plasma of guinea-pigs. Experientia 38, 454-455.

HUGHES, R. E. & HURLEY, R. J. (1969). The uptake of D-araboascorbic acid (D-isoascorbic acid) by guinea-pig tissues. Br. J. Nutr. 23, 211-216.

HUGHES, R. E. & JONES, P. R. (1970). D-araboascorbic acid and guinea pig scurvy. Nutr. Rep. Internat. 1(5), 275-279.

HUGHES, R. E. & JONES, P. R. (1971). The influence of sex and age on the deposition of L-xyloascorbic acid in tissues of guinea pigs. Br. J. Nutr. 25, 77-83.

HUGHES, R. E. & MATON, S. C. (1966). The passage of vitamin C across biological membranes. Proc. Nutr. Soc. 25, XXIX-XXX.

HUGHES, R. E. & MATON, S. C. (1968). The passage of vitamin C across the erythrocyte membrane. Br. J. Nutr. 14, 247-253.

HUGHES, R. E., JONES, P. R. & NICHOLAS, P. (1970). Some effects of experimentally produced cigarette smoke on growth, vitamin C metabolism and organ weights of guinea pigs. J. Pharm. Pharmacol. 22, 823-827.

HUGHES, R. E., HURLEY, R. J. & JONES, P. R. (1971a). The retention of ascorbic acid by guinea-pig tissues. Br. J. Nutr. 26, 433-438.

HUGHES, R. E., JONES, P. R., WILLIAMS, R. S. & WRIGHT, P. F. (1971b). Effect of prolonged swimming on the distribution of ascorbic acid and cholesterol in the tissues of the guinea pig. Life Sci. 10(1), 661-668.

HULSE, J. A., ELLIS, S. R. & HENDERSON, L. V. (1978). Carnitine biosynthesis: β-hydroxylation of trimethyl-lysine by α-ketoglutarate-dependent mitochondrial dioxygenase. J. Biol. Chem. 253, 1654-1659.

HUNG, S. S. O. & SLINGER, S. J. (1980). Effect of oxidized oil on the ascorbic acid nutrition of rainbow trout (Salmo gairdneri). Internat. J. Vit. Nutr. Res. 50, 393-400.

HUNG, S. S. O., CHO, C. Y. & SLINGER, S. J. (1980). Measurement of oxidation in fish oil and its effect on vitamin E nutrition of rainbow trout (Salmo gairdneri). Can. J. Fish. Aquat. Sci. 37, 1248-1253.

HUNG, S. S. O, CHO, C. Y. & SLINGER, S. J. (1981). Effect of oxidized fish oil, DL-α-tocopherol acetate and ethoxyquin supplementation on the vitamin E nutrition of rainbow trout (Salmo gairdneri) fed practical diets. J. Nutr. 111(4), 648-657.

HUNT, A. H. (1940). The role of vitamin C in wound healing. Br. J. Surgery 28, 436-461.

HUNT, C. E., CARLTON, W. W. & NEWBERNE, P. M. (1970a). Inter-relationships between copper deficiency and dietary ascorbic acid in the rabbit. Br. J. Nutr. 24, 61-69.

HUNT, C. E., LANDESMAN, J. & NEWBERNE, P. M. (1970b). Copper deficiency in chicks: Effects of ascorbic acid on iron, copper, cytochrome oxidase activity, and aortic mucopolysaccharides. Br. J. Nutr. 24, 607-614.

IIJIMA, N. & ZAMA, K. (1978). Influence of oxidized lipid on the lipid metabolism in the fish, I. Changes of carp organo-lipids caused by oxidized lipid ingestion. Bull. Faculty Fish. Hokkaido University 29, 282-290.

IIJIMA, N. & ZAMA, K. (1979). Influenced of oxidized lipids on the lipid metabolism in fish, II. Absorption and metabolisms of palmitic acid-1-C^{14} carp fed with oxidized lipids. Bull. Faculty Fish. Hokkaido University 30(1), 84-91.

IIJIMA, N., ZAMA, K. & KAYAMA, M. (1983). Effect of the oxidized lipids on the metabolic pathway of lipid biosynthesis in the intestine of carp. Bull. Japan. Soc. Sci. Fish. 49(9), 1465-1470.

IKEDA, S. & SATO, M. (1964). Biochemical studies of L-ascorbic acid in aquatic animals. III. Biosynthesis of L-ascorbic acid by carp. Bull. Japan. Soc. Sci. Fish. 30(4), 365-369.

IKEDA, S. & SATO, M. (1965). Biochemical studies on L-ascorbic acid in aquatic animals. IV. Metabolism of L-ascorbic acid-1-C^{14} in carp. Bull. Japan. Soc. Sci. Fish. 31(10), 814-817.

IKEDA, S., SATO, M. & KIMURA, R. (1963a). Biochemical studies on L-ascorbic acid in aquatic animals. II. Distribution in various parts of fish. Bull. Japan. Soc. Sci. Fish. 29(8), 765-770.

IKEDA, S., SATO, M. & KIMURA, R. (1963b). Biochemical studies on L-ascorbic acid in aquatic animals. I. An improved method for assessment. Bull. Japan. Soc. Sci. Fish. 29, 757-764.

IKEDA, S., SATO, M. & TAGUCHI, T. (1966a). Biochemical studies on L-ascorbic acid in aquatic animals. VI. Effect of α-tocopherol on L-gulonolactone dehydrogenase activity in carp hepatopancreas. Bull. Japan. Soc. Sci. Fish. 32(7), 585-589.

IKEDA, S. & SATO, M. (1966b). Biochemical studies on L-ascorbic acid in aquatic animals. V. Conversion of myo-inositol to L-ascorbic acid in carp. Bull. Japan. Soc. Sci. Fish. 32(6), 530-532.

INAGAKI, C., FUKUBA, H. & KANOH, A. (1959). Ascorbic acid content in fishes. Vitamins (Kyoto) 16, 234-236.

ISHERWOOD, F. A., CHEN, Y. T. & MAPSON, L. W. (1954). Synthesis of L-ascorbic acid in plants and animals. Biochem. J. 56, 1-15.

JAUNCEY, K., SOLIMAN, A. K. & ROBERTS, R. J. (1985). Ascorbic acid requirements in relation to wound healing in the cultured tilapia Oreochromis niloticus (Trewavas). Aquaculture Fish. Manag. 16, 139-149.

JENKINS, S. A. (1980). Vitamin C status, serum cholesterol levels and bile composition in the pregnant guinea pig. Br. J. Nutr. 43, 95-100.

JEWELL, M. E., SCHNEBERGER, E. & ROSS, J. A. (1933). The vitamin requirements of goldfish and channel catfish. Trans. Fish. Soc. 63, 338-347.

JOHN, T. M., GEORGE, J. C., HILTON, J. W. & SLINGER, S. J. (1979). Influence of dietary ascorbic acid on plasma lipid levels in the rainbow trout. Internat. J. Vit. Nutr. Res. 49, 400-405.

JOHNSON, F. C. (1979). The antioxidant vitamins. CRC, Critical Reviews in Food Science and Nutrition 10, issue 3, 217-309.

JUKES, T. & KING, J. (1975). Evolutionary loss of ascorbic acid synthesizing ability. J. Human Evolution 4, 85-88.

KADIN, H. & OSADCA, M. (1959). Biochemistry of erythrobic acid. Human blood levels and urinary excretion of ascorbic and erythrobic acids. Agriculture and Food Chemistry 7, 358-360.

KALLNER, A., HARTMANN, D. & HORNIG, D. (1977). On the absorption of ascorbic acid in man. Internat. J. Vit. Nutr. Res. 47, 383-388.

KALLNER, A., HARTMANN, D. & HORNIG, D. (1979). Steady-state turnover and body pool of ascorbic acid in man. Am. J. Clin. Nutr. 32, 530-539.

KANAZAWA, K., TAKEUCHI, S., HASEGAWA, R., OKADA, M., MAKIYAMA, I., HIROSE, N., TOH, T., HYON, C. H. & KOBAYASHI, M. (1981). Influence of ascorbic acid deficiency on the level of non-protein SH compounds and vitamin E in the blood tissues of guinea pigs. Nihon Univ. J. Med. 23, 257-265.

KANEDA, T. & ISHII, S. (1953). Studies on the nutritive values of lipids. VI. Nutritive value or toxicity of highly unsaturated fatty acid. Bull. Japan. Soc. Sci. Fish. 19(3), 171-177.

KANEDA, T., SAKAI, H. & ISHII, S. (1954). Studies on the nutritive value of lipids. XII. Nutritive value or toxicity of highly unsaturated fatty acids. Bull. Japan. Soc. Sci. Fish. 29, 658-663.

KANFER, J., ASHWELL, G. & BURNS, J. J. (1960). Formation of L-lyxonic and L-xylonic acids from L-ascorbic acid in rat kidney. J. Biol. Chem. 235, 2518-2521.

KATARIA, M. S. & ROE, D. B. (1965). Vitamin C levels in the elderly. Geront. Clin. 7, 189-190.

KATO, R., TAKANAKA, A. & OSHIMA, T. (1969). Effect of vitamin C deficiency on the metabolism of drugs and NADPH-linked electron transport system in liver microsomes. Japan. J. Pharmacol. 19, 25-33.

KAUFMAN, S. (1966). Coenzymes and hydroxylases: Ascorbate and dopamine-β-hydroxylase: Tetrahydropteridine and phenylanine and tyrosine hydroxylase. Pharmacol. Rev. 18, 61-69.

KEDO, S., IKEDA, S. & FUJIMURA, K. (1960). Discussion on measurement of L-ascorbic acid by Roe's method. J. Japan. Soc. Food Nutr. 13, 242-245.

KEITH, M. O. & PELLETIER, O. (1974). Ascorbic acid concentrations in leucocytes and selected organs of guinea pigs in response to increasing ascorbic acid intake. Am. J. Clin. Nutr. 27, 368-372.

KEITH, R. E., LIBKE, K. G. & DRISKELL, J. A. (1981). Massive ingestion of vitamin C and food consumption, body weight, and kidney morphology in guinea pigs. Nutr. Rep. Internat. 24, 811-815.

KELLEY, G. G & WATTS, B. M. (1957). Effect of copper chelating agents on pro-oxidant activity of ascorbic acid with unsaturated fats. Food Res. 22, 308-315.

KETCHUM, B. H., ZITKO, V. & SAWARD, D. (1975). Aspects of heavy metals and organohalogen pollution in aquatic ecosystems. In: Ecological Toxicology Research: Effects of heavy metal and organohalogen compounds (A. D. McIntyre & C. F. Mills, eds.), pp75-85. New York, Plenum Press.

KIRK, J. E. (1962). Variation with age in the tissue content of vitamins and hormones. In: Vitamins and hormones (R. S. Harris & I. G. Wool, eds.), Vol. 20, pp67-139. Academic Press, New York & London.

KIRK, J. E. & CHIEFFI, M. (1958). Vitamin studies in middle-aged and old individuals. XI. The concentration total ascorbic acid in whole blood. J. Gerent. 8, 301-304.

KIRK, R. G. (1972). A review of recent developments in tilapia culture, with special references to fish farming in the heated effluents of power stations. Aquaculture 1, 45-60.

KITABACHI, A. E. (1967). Ascorbic acid in steroidogenesis. Nature 215, 1385-1386.

KITAMURA, S. (1969). Summary on the hypovitaminosis of vitamin C of rainbow trout (Salmo gairdneri). Fish Pathology (Japan) 3, 73-92.

KITAMURA, S., OHARA, S., SUWA, T. & NAKAGAWA, K. (1965). Studies on vitamin requirements of rainbow trout, Salmo gairdneri. I. On the ascorbic acid. Bull. Japan. Soc. Sci. Fish. 31(10), 818-826.

KITAMURA, S., SUWA, T., OHARA, S. & NAKAGAWA, K. (1967). Studies on vitamin requirements of rainbow trout. II. The deficiency symptoms of fourteen kinds of vitamin. Bull. Japan. Soc. Sci. Fish. 33(12), 1120-1125.

KOCEN, B. P. & CAVAZOS, L. F. (1958). Cytochemistry of male reproductive tract in scurvy and inanition. Proc. Soc. Exp. Biol. Med. 98, 485-489.

KOED, H. J. & HAMBURGER, C. (1967). Ovarian ascorbic acid depletion test for lutenizing hormone. Acta Endocrinologica 56, 619-625.

KORN, S. & MACEDO, D. (1973). Determination of fat content in fish with a nontoxic, noninflammable solvent. J. Fish. Res. Board Can. 30, 1880-1881.

KOTHAVALLA, Z. R. & GILL, H. S. (1943). Studies on the effect of various factors on the vitamin C content of the milk of cows and buffaloes. Indian J. Vet. Sci. 13, 35-43.

KOVTUNYAK, N. A., MESCHCHISHEN, I. F. & TSAPOK, P. I. (1971). Changes in ascorbic acid in tissues of rats after hypophysectomy. Vopr. Pita. 30, 49-52. (Cited by Nutr. Abstr. Rev. 42, 116, Abstr. 565 (1972).

KRALL, A. R., SIEGEL, G. J., GOZANSKY, D. M. & WAGNER, F. L. (1964). Adrenochrome inhibition of oxidative phosphorylation by rat brain mitochondria. Biochem. Pharmacol. 13, 1519-1525.

KRAYBILL, H. R. & DUGAN, L. R., Jr. (1954). Antioxidants new developments for food use. J. Agr. Food Chem. 2, 81-84.

KUENZIG, W., AVENIA, R. & KAMM, J. J. (1974). Studies on the anti-scorbutic activity of ascorbate 2-sulphate in the guinea pig. J. Nutr. 104, 952-956.

LA DU, B. N. & ZANNONI, V. (1961). The role of ascorbic acid in tyrosine metabolism. Ann. N. Y. Acad. Sci. 92, 175-192.

LAMDEN, M. P. & CHRYSTOWSKI, G. A. (1954). Urinary oxalate excretion by man following ascorbic acid ingestion. Proc. Soc. Exp. Biol. Med. 85, 190-192.

LAUNER, C. A., TIEMEIER, O. W. & DEYOE, C. W. (1978). Effect of dietary addition of vitamins C and D_3 on growth and calcium and phosphorus content of pond-cultured channel catfish. Prog. Fish-Cult. 40(1), 16-20.

LEA, C. H. (1958). Some nutritional and allied problems confronting the food manufacturer: Technological aspects of antioxidants. J. Sci. Food Agri. 9(10), 621-632.

LEE, R. E. (1961). Ascorbic acid and the peripheral vascular system. Ann. N. Y. Acad. Sci. 92, 295-301.

LEIBOVITZ, B. & SIEGEL, B. V. (1978). Ascorbic acid, neutrophil function, and the immune response. Internat. J. Vit. Nutr. Res. 48, 159-164.

LEUNG, H. W., VANG, M. T. & MAVIS, R. D. (1981). The cooperative interrelation between vitamin E and vitamin C on suppression of peroxidation of membrane phopholipids. Biochem. Biophys. Acta 664, 266-272.

LEVANDOSKI, N. G., BAKER, E. M. & CANHAM, J. E. (1964). A monodehydro form of ascorbic acid in the autoxidation of ascorbic acid to dehydroascorbic acid. Biochemistry 3(10),1465-1469.

LEVIN, E. Y. & KAUFMAN, S. (1961). Studies on the enzyme catalyzing the conversion of 3,4-dihydroxyphenylethylamine to norepinephrine. J. Biol. Chem. 236, 2043-2049.

LEWIN, S. (1974). Recent advances in the molecular biology of vitamin C. In: Vitamin C (G. G. Birch & K. L. Parker, eds.), pp221-252. London, Applied Science Publishers Ltd.

LEWIN, S. (1975). In: Vitamin C: Its molecular, biology and medical potential. Academic Press, London.

LI, Y. & LOVELL, R. T. (1985). Elevated levels of dietary ascorbic acid increase immune responses in channel catfish. J. Nutr. 115, 123-131.

LIM, C. & LOVELL, R. T. (1978). Pathology of the vitamin C deficiency syndrome in channel catfish (Ictalurus punctatus). J. Nutr. 108, 1137-1148.

LIND, J. (1753). A treatise on the scurvy. London. A. Millar, 1753, Republished Edinburgh University Press, Edinburgh, R. & Clark Ltd., 1953.

LIND, J. (1757). In: Treatise on scurvy. 2nd edn. Millar, London.

LIPSCHITZ, D. A., BOTHWELL, T. H., SEFTEL, H. C., WAPNICK, A. A. & CHARLTON, R. W. (1971). The role of ascorbic acid in the metabolism of storage iron. Br. J. Haemat. 20, 155-163.

LOH, H. S. & WILSON, C. W. M. (1971). Relationship of human ascorbic acid metabolism to ovulation. Lancet 1, 110-112.

LOH, H. S., ODUMOSU, A. & WILSON, C. W. M. (1974). Factors influencing the metabolic availability of ascorbic acid. Clin. Pharm. Therap. 16, 390-408.

LOVELL, R. T. (1973). Essentiality of vitamin C in feeds for intensively fed caged channel catfish. J. Nutr. 103, 134-138.

LOVELL, R. T. (1975). Fish feed and nutrition. The Commercial Fish Farmer & Aquaculture News 1(6), 26.

LOVELL, R. T. (1977). Do pond-raised catfish need vitamin C. Commercial Fish Farmer & Aquaculture News 3(3), 22.

LOVELL, R. T. & LIM, C. (1978). Vitamin C in pond diets for channel catfish. Trans. Am. Fish. Soc. 107(2), 321-325.

LOVELL, R. T. & LIMSUWAN, T. (1982). Intestinal synthesis and dietary nonessentiality of vitamin B_{12} for Tilapia nilotica. Trans. Am. Fish. Soc. 111, 485-490.

LOWRY, O. H., BESSEY, O. A. & BURCH, H. B. (1952). Effect of prolonged high dosage with ascorbic acid. Proc. Exp. Biol. 80, 361-362.

LUTWAK-MANN, C. (1958). Dependence of gonadal function upon vitamins and other nutritional factors. In: Vitamins & Hormones (R. S. Harris, G. F. Marrian & K. V. Thimann, eds.), Vol. 16, pp35-73. New York, Academic Press.

LYNCH, P. B. & O'GRADY, J. F. (1981). Effect of vitamin C (ascorbic acid) supplementation of sows in late pregnancy on piglet mortality. Irish J. Agric. Res. 20, part 2/3, 217-219.

MACHLIN, L. J. & GABRIEL, E. (1980). Interactions of vitamin E with vitamin C, vitamin B_{12}, and zinc. Ann. N. Y. Acad. Sci. 355, 98-108.

MACHLIN, L. J., GARCIA, F., KUENZIG, W., RICHTER, C. B., SPIEGEL, H. E. & BRIN, M. (1976). Lack of antiscorbutic activity of ascorbate 2-sulphate in the rhesus monkey. Am. J. Clin. Nutr. 29, 825-831.

MAHAJAN, C. L. & AGRAWAL, N. K. (1979). Vitamin C deficiency in Channa punctatus Bloch. J. Fish Biol. 15, 613-622.

MAHAJAN, C. L. & AGRAWAL, N. K. (1980a). Nutritional requirement of ascorbic acid by Indian major carp, Cirrhina mrigala, during early growth. Aquaculture 19, 37-48.

MAHAJAN, C. L. & AGRAWAL, N. K. (1980b). The role of vitamin C in calcium uptake of fish. Aquaculture 19, 287-294.

MAHAJAN, C. L. & YADAVE, J. S. (1974). Experiments on synthetic diet for carps: Some preliminary observations and their relevance to aquaculture. J. Inland Fish. Soc. India 6, 178-184.

MARTIN, G. R. (1961). Studies on the tissue distribution of ascorbic acid. Ann. N. Y. Acad. Sci. 92, 141-147.

MARTIN, G. R. & MECCA, C. E. (1961). Studies on the distribution of L-ascorbic acid in the rat. Arch. Biochem. Biophys. 93, 110-114.

MATHIESEN, E. (1938). Vitamin C in cod roe. Tidsskr, Herrmet, Ind, 24.

MAUCK, W. L., MEHRLE, P. M. & MAYER, F. L. (1978). Effects of the polychlorinated biphenyl aroclor 1254 on growth, survival, and bone development in brook trout (Salvelinus fontinalis). J. Fish. Res. Board Can. 35, 1084-1088.

MAY, C. D., NELSON, E. N., LOWE, C. U. & SALMON, R. J. (1950). Pathogenesis of megaloblastic anemia in infancy. An interrelationship between pteroylgultamic acid and ascorbic acid. Am. J. Diseases of Children 80(2), 191-206.

MAYER, F. L., MEHRLE, P. M. & CRUTCHER, P. L. (1978). Interactions of toxaphene and vitamin C in channel catfish. Trans. Am. Fish Soc. 107, 326-333.

MAYNARD, L. A. & LOOSLI, J. K. (1969). Animal nutrition. 6th edn. McGraw-Hill Book Company, 613pp.

MAZUR, A. (1961). Role of ascorbic acid in the incorporation of plasma iron into ferritin. Ann. N. Y. Acad. Sci. 92, 223-229.

MAZUR, A., BAEZ, S. & SHORR, E. (1955). The mechanism of iron release from ferritin as released to its biological properties. J. Biol. Chem. 213, 147-160.

MAZUR, A., GREEN, S. & CARLETON, A. (1960). Mechanism of plasma iron incorporation into hepatic ferritin. J. Biol. Chem. 235, 595-603.

McANDREW, B. J. & MAJUMDAR, K. C. (1983). Tilapia stock identification using electrophoretic markers. Aquaculture 30, 249-261.

McANDREW, B. J. & MAJUMDAR, K. C. (1984). Evolutionary relationships within three Tilapiine genera (Pisces:Cichlidae). Zool. J. Linn. Soc. 80, 421-435.

McCAY, C. M. & TUNISION, A. V. (1933). The nutritional requirements of trout. Cortland Hatchery Report for the year 1933. State of New York Conservation Dept., Albany, New York.

McDONALD, P., EDWARDS, P. A. & GREENHALGH, J. F. D. (1980). Vitamins. In: Animal nutrition, 3rd edn. pp55-84. Longman Group Ltd., Longman House, Burnt-Mill, Harlow, Essex.

McLAREN, B. A., KELLER, E., O'DONNELL, D. J. & ELVEHJEM, C. A. (1947). The nutrition of rainbow trout. I. Studies of vitamin requirements. Arch. Biochem. 15(2), 169-178.

MEAD, C. G. & FINAMORE, F. J. (1969). The occurrence of ascorbic acid sulphate in the brine shrimp, Artemia salina. Biochemistry 8, 2652-2655.

MIKI, T., KIKUCHI, N. & SAHASHI, Y. (1962). Paper chromatographic estimation of L-ascorbic acid and D-araboascorbic acids. J. Vitaminol. 8, 279-285.

MILBY, K. H., MEFFORD, I. N., CHEY, W. & ADAMS, R. N. (1981). In vitro and in vivo depolarization coupled efflux of ascorbic acid in rat brain preparations. Brain Res. Bull. 7, 237-242.

MILBY, K., OKE, A. & ADAMS, R. N. (1982). Detailed mapping of ascorbate distribution in rat brain. Neuroscience Letters 28, 15-20.

MILNE, D. B. & OMAYE, S. T. (1979). Effect of ascorbic acid on copper and iron in the guinea pig and monkey. Fed. Proc. 38, 614 (abstr.)

MINDLIN, R. L. & BUTLER, A. M. (1939). The determination of ascorbic acid in plasma; a macromethod and micromethod. J. Biol. Chem. 122, 673-686.

MITOMA, C. & SMITH, T. E. (1960). Studies on the role of ascorbic acid in collagen synthesis. J. Biol. Chem. 235, 426-428.

MIZOGUCHI, H. & YASUMASU, I. (1982). Archenteron formation induced by ascorbate and α-ketoglutarate in sea urchin embryos kept in SO_4^{2-}-free artificial seawater. Developmental Biol. 93, 119-125.

MONSEN, E. R. & PAGE, J. F. (1978). Effects of EDTA and ascorbic acid on the absorption of iron from an isolated rat intestinal loop. J. Agri. Food Chem. 26, 223-226.

MORIARTY, D. J. W. & MORIARTY, C. D. (1973). Quantitative estimation of the daily ingestion of phytoplankton by Tilapia nilotica and Haplochromis nigripinnis in Lake George, Uganda. J. Zool. London 171, 15-23.

MUMMA, R. O. & VERLANGIERI, A. J. (1972). The isolation of ascorbic acid 2-sulphate from selected rat organs. Biochim. Biophys. Acta 273, 249-253.

MUMMA, R. O., McKEE, E. E., VERLANGIERI, A. J. & BARRON, G. P. (1972). Antiscorbutic effect of ascorbic acid 2-sulphate in the guinea pig. Nutr. Rep. Internat. 6, 133-137.

MURAI, T. & ANDREWS, J. W. (1974). Interactions of dietary α-tocopherol oxidized menhaden oil and ethyoxquin on channel catfish (Ictalurus punctatus). J. Nutr. 104, 1416-1431.

MURAI, T., ANDREWS, J. W. & BAUERNFEIND, J. C. (1978). Use of L-ascorbic acid, ethocel coated ascorbic acid and ascorbate 2-sulphate in diets for channel catfish, Ictalurus punctatus. J. Nutr. 108, 1761-1766.

MUSSINI, E., HUTTON, J. J., Jr. & UDENFRIEND, S. (1967). Collagen proline hydroxylase in wound healing, granuloma formation, scurvy, and growth. Science 157, 927-929.

NANDI, B. K., MAJUMDER, A. K., SUBRAMANIAN, N. & CHATTERJEE, I. B. (1973). Effects of large doses of vitamin C in guinea pigs and rats. J. Nutr. 103, 1688-1695.

NEWMARK, H. L., OSADCA, M., ARAUJO, M., GERENZ, C. N. & RITTER, E.D. (1974). Stability of ascorbate in bacon. Food Tech. 28, 28-31.

NICHOL, C. A. & WELCH, A. D. (1950). Synthesis of citrovorum factor from folic acid by liver slices; augmentation by ascorbic acid. Proc. Soc. Exp. Biol. Med. 74, 52-55.

NOACH, E. L. & VAN REES, G. P. (1958). Ascorbic acid in the gonads of rats. Acta Endocrinologica 27, 502-508.

NORKUS, E. P. & ROSSO, P. (1981). Effects of maternal intake of ascorbic acid on the postnatal metabolism of this vitamin in the guinea pig. J. Nutr.111, 624-630.

NOSE, T. (1962). Determination of nutritive value of food protein in fish. 1. On the determination of food protein utilization by carcass analysis. Bull. Freshwater Fish Res. Lab. (Tokyo) 11, 2-42.

NRC, National Research Council (1973). Nutrient requirements of trout, salmon and catfish. Nutrient requirements of domestic animals, No. 11, pp1-57. Natl. Acad. Sci., Washington D.C.

NRC, National Research Council (1981). Nutrient requirements of coldwater fishes. Nutrient requirements of domestic animals, No. 16, pp1-63. Natl. Acad. Press, Washington D.C.

NUTRITION REVIEWS (1960). Scurvy and blood coagulation. Nutr. Rev. 18(8), 242-244.

NUTRITION REVIEWS (1978a). The function of ascorbic acid in collagen formation. Nutr. Rev. 36, 118-121.

NUTRITION REVIEWS (1978b). Vitamin C and phagocyte function. Nutr. Rev. 36, 183-185.

NUTRITION REVIEWS (1979). The role of ascorbic acid in the hydroxylation of peptide-bound proline. Nutr. Rev. 37, 26-28.

OELRICHS, B. A. & KRATZING, C. C. (1980). Ascorbic acid in intestinal tissues. Australian J. Exp. Biol. Periodical Sci. 58(5), 515-520.

OHMAE, H., SUZUKI, R. & SHIMMA, Y. (1979). Influence of single cell protein feeds on the growth and reproductivity of carp with reference to fatty acid composition. In: Proc. World Symp. on Finfish Nutrition. In: Fishfeed Technology, Hamburg, 20-23 June 1978 (J. E. Halver & K. Tiews, eds.), Vol. 22, pp63-73. Heenemann, Berlin.

OLLIVER, M. (1967). Estimation. In: The vitamins (W. H. Sebrell, Jr. & R. S. Harris, eds.), Vol. 1, pp338-359. Academic Press, New York & London.

OMAYE, S. T., TILLOTSON, J. A. & SAUBERLICH, H. E. (1982). Metabolism of L-ascorbic acid in the monkey. In: Ascorbic acid: Chemistry, metabolism and uses (P. A. Seib & B. M. Tolbert, eds.), pp317-334. American Chemical Society, Washington D.C.

OSBORNE, T. B., MENDEL, L. B. & FERRY, E. L. (1919). A method for expressing numerically the growth promoting values of protein. J. Biol. Chem. 37, 223-229.

OTTOLENGHI, C., PUVIANI, A. C. & BRIGHENTI, L. (1981). Glycogen in liver and other organs of catfish (Ictalurus melas): Seasonal changes and fasting effects. Comp. Biochem. Physiol. 68A, 313-321.

OUSTERHOUT, L. E. & MATTERSON, L. D. (1968). Effects of antioxidant addition on the nutritive value of fish meals. Poultry Sci. 47, 1704 (Abstract).

PAL, D. R., CHATTERJEE, J. & CHATTERJEE, C. C. (1975). Influence of lead administration on L-ascorbic acid metabolism in rats: Effect of L-ascorbic acid supplementation. Internat. J. Vit. Nutr. Res. 45, 427-437.

PARK, J. H., MERIWETHER, B. P., MUDD, S. H. & LIPMANN, F. (1956). Glutathione and ethylenediaminetetracetate antagonism of uncoupling oxidative phosphorylation. Biochim. Biophys. Acta 22, 403-404.

PARKER, R. E. (1979). Interrelationships of quantitative variables. In: Introductory statistics for biology, 2nd ed., pp55-67. Edward Arnold Ltd., London.

PASSMORE, R. (1977). How vitamin C deficiency injures the body. Nutr. Today 12(2), 6-11.

PATRO, U. K. N. & PATNAIK, B. K. (1979). Changes in ascorbic acid, glycogen and protein of muscle and brain of Ophiocephalus punctatus Bloch following short-term cold stress. Indian J. Exp. Biol. 17, 521-523.

PAULING, L. (1968). Orthomolecular psychiatry: Varying concentrations of substances normally present in the human body may control mental disease. Science 160, 265-271.

PAULING, L. (1970a). In: Vitamin C and the common cold. San Francisco. W. H. Freeman & Co., Publishers.

PAULING, L. (1970b). Evolution and the need for ascorbic acid. Proc. Nat. Acad. Sci. 67, 1643-1648.

PAULING, L. (1973). On vitamin C against disease: Why your health may be benefited in many ways by a proper intake of this essential vitamin. Executive Health 9(5), 1-6.

PAULING, L. (1979). The role of vitamin C in cancer. Internat. J. Vit. Nutr. Res. Suppl. 19, 207-210.

PELLETIER, O. (1969). Turnover rates of D-isoascorbic acid and L-ascorbic acid in guinea pig organs. Can. J. Physiol. Pharmacol. 47, 993-997.

PELLETIER, O. & GODIN, C. (1969). Vitamin C activity of D-isoascorbic acid for the guinea pig. Can. J. Physiol. Pharmacol. 47, 985-991.

PENNEY, J. R. & ZILVA, S. S. (1946). The fixation and retention of ascorbic acid by the guinea pig. Biochem. J. 40, 695-706.

PERKINS, E. G., ENDERS, J. G. & KUMMEROW, F. A. (1961). Effect of ingested thermally oxidized corn oil on fat composition in the rat. Proc. Soc. Exp. Biol. Med. 106, 370-372.

PHILLIPS, A. M., Jr. (1957). The nutrition of trout. IV. Vitamin requirements. Prog. Fish-Cult. 19, 119-123.

PHILLIPS, A. M., Jr. (1972). Calorie and energy requirement. In: Fish Nutrition (J. E. Halver, ed.), pp1-28. Academic Press, New York & London.

PHILLIPS, P. H., LARDY, H. A., HEISSER, E. E. & RUPPEL, I. W. (1940). Sperm stimulation in the bull through the subcutaneous administration of ascorbic acid. J. Dairy Sci. 23, 873-878.

PINTAURO, S. J. & BERGAN, J. G. (1982). Effects of ascorbic acid on in vitro steroidogenesis in guinea pigs. J. Nutr. 112, 584-591.

PIRANI, C. L. & LEVENSON, S. M. (1953). Effect of vitamin C deficiency on healed wound. Proc. Soc. Exp. Biol. Med. 82, 95-99.

POSTON, H. A. (1967). Effect of dietary L-ascorbic acid on immature brook trout. New York State Cons. Dept. Fish. Res. Bull. 30, 46-51.

POSTON, H. A. & COMBS, G. F. (1979). Interrelationships between requirements for dietary selenium, vitamin E, and L-ascorbic acid by Atlantic salmon (Salmo salar) fed a semi-purified diet. Fish Health News 8(4), vi-vii.

PRIMBS, E. R. J. & SINNHUBER, R. O. (1971). Evidence for the non-essentiality of ascorbic acid in the diets of rainbow trout. Prog. Fish-Cult. 33(3), 141-149.

PROCKOP, D. J. & KIVIRIKKO, K. I. (1967). Relationship of hydroxyproline excretion in urine to collagen metabolism. Ann. Internat. Med. 66, 1243-1266.

PYKE, M. & WRIGHT, M. D. (1941). Vitamin content of salmon. Nature (London) 147, 26.

QUADRI, S. F., SEIB, P. A. & DEYOE, C. W. (1973). Improved method of preparing L-ascorbic acid 2-sulphate. Carbohydrate Res. 29, 259-264.

QUADRI, S. F., LIANG, Y. T., SEIB, P. A., DEYOE, C. & HOSENEY, R. C. (1975). Stability of L-ascorbic acid 2-sulphate and L-ascorbate in wheat foods and milk. J. Food Sci. 40, 837-839.

RÄIHÄ, N. (1958). On the placental transfer of vitamin C. An experimental study on guinea pigs and human subjects. Acta Physiol. Scand. Suppl. 45, 5-53.

RAIZADA, M. N., MITRA, S. D. & SINGH, C. P. (1982). Studies on the relationship between erythrocyte counts (RBC) and length/weight of Cirrhinus mrigala (Ham.). Size: (110mm to 270mm). J. Anim. Morphol. Physiol. 29(1 and 2), 217-221.

RALLI, E. P., FRIEDMAN, G. J. & RUBIN, S. H. (1938). The mechanism of the excretion of vitamin C by the human kidney. J. Clin. Invest. 17, 765-770.

RAM, M. M. (1966). Growth rate and protein utilization in vitamin C deficiency. Ind. J. Med. Res. 54, 964-970.

RANKEN, M. D. (1974). The significance of ascorbates and erythrobates in meat products. In: Vitamin C: Recent aspects of its physiological importance (G. G. Birch & K. J., eds.), pp121-135. Applied Science Publishers Ltd., London.

RAY CHAUDHURI, C. & CHATTERJEE, I. B. (1969). L-ascorbic acid synthesis in birds: Phlogenetic trend. Science 164, 435-436.

REIFF, J. S. & FREE, A. H. (1959). Nutritional studies with isoascorbic acid in the guinea pig. Agriculture & Food Chemistry 7, 55-56.

REPPERT, E., DONEGAN, J. & HINES, L. E. (1951). Ascorbic acid and the hyaluronidase hyaluronic acid reaction. Proc. Soc. Exp. Bio. Med. 77, 318-320.

RIEMENSCHNEIDER, R. W., TURER, J., WELLS, P. A. & AULT, W. C. (1944). Fatty acid monoesters of L-ascorbic and D-isoascorbic acids as antioxidants for fats and oils. Oil & Soap 21, 47-50.

RIISING, B. (1982). Ascorbic acid in fish feed. Technical Paper TP 20001-1e, 5pp. Grindsted Products, Denmark.

RIVERS, J. M., HUANG, E. D. & DODDS, M. L. (1963). Human metabolism of L-ascorbic acid and erythrobic acid. J. Nutr. 81, 163-168.

ROBERTSON, W. Van B. (1950). Concentrations of collagen in guinea pig tissues in acute and prolonged scurvy. J. Biol. Chem. 187, 673-677.

ROBERTSON, W. Van B. & HIWETT, J. (1961). Augmentation of collagen synthesis by ascorbic acid in vitro. Biochim. Biophys. Acta 49, 404-406.

ROBERTSON, W. Van B., HIWETT, J. & HERMAN, C. (1959). The relation of ascorbic acid to the conversion of proline to hydroxyproline in the synthesis of collagen in the carrageenan granuloma. J. Biol. Chem. 234, 105-108.

ROE, J. H. (1957). Chemical determination of ascorbic, dehydroascorbic, and diketogulonic acids. Methods Biochem. Anal. 1, 115-139.

ROE, J. H. (1961). Comparative analyses for ascorbic acid by the 2,4-dinitrophenylhydrazine method with the coupling reaction at different temperatures: A procedure for determining specificity. J. Biol. Chem. 236, 1611-1613.

ROE, J. H. (1967). Ascorbic acid. In: The vitamins (P. Gyŏrgry & W. N. Pearson, eds.), Vol. VII, pp27-49. Academic Press, New York, San Francisco & London.

ROE, J. H. & KUETHER, C. A. (1949). The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. J. Biol. Chem. 147, 399-407.

ROE, J. H., HALL, J. M. & DYER, H. M. (1941). The relation of nutrition to gastric function. II. The effect of vitamin C deficiency. Am. J. Digestive Diseases 8, 261-266.

ROE, J. H., MILLS, M. B., OESTERLING, M. J. & DAMRON, C. M. (1948). The determination of diketo-L-gulonic acid, dehydro-L-ascorbic acid, and L-ascorbic acid in the same tissue extract by the 2,4-dinitrophenylhydrazine method. J. Biol. Chem. 174, 201-208.

ROHATGI, K. S., ROY, H. & BANERJEE, S. (1958). Protein metabolism in scorbutic monkeys. Ind. J. Med. Res. 46, 661-668.

ROSS, L. G. & BEVERIDGE, M. (1984). A compilation of biological and husbandry data for cultured aquatic animals. Institute of Aquaculture, University of Stirling, Stirling, Scotland, 75pp.

ROSS, L. G. & GEDDES, J. A. (1979). Sedation of warm-water fish species in aquaculture research. Aquaculture 16, 183-186.

ROSS, L. G. & ROSS, B. (1984). Anaesthetic and sedative techniques for fish. Published by Institute of Aquaculture, University of Stirling, Stirling, Scotland, 41pp.

ROUBAL, W. T. & TAPPEL, A. L. (1966a). Damage to protein, enzymes, and amino acids by peroxiding lipids. Arch. Biochem. Biophys. 113, 5-8.

ROUBAL, W. T. & TAPPEL, A. L. (1966b). Polymerization of proteins induced by free-radical lipid peroxidation. Arch. Biochem. Biophys. 113, 150-155.

ROY, R. N. & GUHA, B. C. (1958). Species difference in regard to the biosynthesis of ascorbic acid. Nature 182, 319-320.

ROY, S. C., ROY, S. K. & GUCHA, B. C. G. (1946). Role of thiamin and riboflavin in the biosynthesis of vitamin C. Nature 158, 238.

ROY CHOWDHURY, A. & MUKHERJEE, A. K. (1976a). Cold exposure and testicular ascorbic acid. Indian J. Exp. Biol. 14, 176-177.

ROY CHOWDHURY, A. & MUKHERJEE, A. K. (1976b). Effect of exogenous testosterone propionate and progesterone on testicular ascorbic acid and cholesterol in relation to spermatogenesis. Indian J. Exp. Biol. 14, 701-705.

RUDRA, M. N. (1936). Studies in vitamin C. II. The vitamin C contents of the liver and muscle of some Indian fresh-water fish. J. Indian Chem. Soc. , 740-742.

RUMSEY, G. L. (1980). Antioxidants in compounded feeds. FAO Lectures presented at the FAO/UNDP training course in fish feed technology, held at the College of Fisheries, University of Washington, Seattle, Washington, 9 October - 15 December 1978. (ADCP/80/11).

SAARI, J. C., BAKER, E. M. & SAUBERLICH, H. E. (1967). Thin-layer chromatographic separation of the oxidative degradation products of ascorbic acid. Anal. Biochem. 18, 173-177.

SADOOGH-ABASIAN, F. & EVERED, D. F. (1979). Absorption of vitamin C from the human buccal cavity. Br. J. Nutr. 42, 15-20.

SAHA, K. C. (1939). Distribution of free and total ascorbic acid in the liver and muscle of Bengal fresh-water fish. J. Indian Chem. Soc. 16, 511-514.

SAKAGUCHI, H. & HAMAGUCHI, A. (1969). Influence of oxidized oil and vitamin E on the culture of yellow tail. Bull. Japan. Soc. Sci. Fish. 35(12), 1207-1214.

SAKAGUCHI, H. & HAMAGUCHI, A. (1979a). Physiological studies on cultured red sea bream. II. Effect of oxidized oil on the chemical constituents in plasma, hepatopancreas and muscle. Bull. Japan. Soc. Sci. Fish. 45(4), 449-454.

SAKAGUCHI, H. & HAMAGUCHI, A. (1979b). Physiological studies on cultured red sea bream. III. Digestibility and changes of chemical constituents in plasma and hepatopancreas after feeding of oxidized oil. Bull. Japan. Soc. Sci. Fish. 45(5), 545-548.

SAKAGUCHI, H., TAKEDA, F. & TANGE, K. (1969). Studies on vitamin requirements by yellowtail. I. Vitamin B_6 and vitamin C deficiency symptoms. Bull. Japan. Soc. Sci. Fish. 35(12), 1201-1206.

SALMON, R. J. & MAY, C. D. (1952). Total plasma protein and plasma fibrinogen in ascorbic acid deficiency and in scurvy in the monkey. J. Nutr. 46, 515-523.

SANDNES, K. & BRAEKKAN, O. R. (1981). Ascorbic acid and the reproductive cycle of ovaries in cod (Gadus morrhua). Comp. Biochem. Physiol. 70A, 545-546.

SANDNES, K. & UTNE, F. (1982). Processing loss and storage stability of ascorbic acid in dry fish feed. Fisk. Dir. Skr. Ser. Ernaring 11(2), 39-44.

SANDNES, K., ULGENES, Y., BRAEKKAN, O. R. & UTNE, F. (1984). The effect of ascorbic acid supplementation in broodstock feed on reproduction of rainbow trout (Salmo gairdneri). Aquaculture 43, 167-177.

SATO, P. & UDENFRIEND, S. (1978). Studies on ascorbic acid related to genetic basis of scurvy. In: Vitamins & Hormones (P. L. Munson, E. Diczfalusy, J. Glover, J. & R. Eolson, eds.), Vol. 36, pp33-51. Academic Press, New York, San Francisco & London.

SATO, M., YOSHINAKA, R. & IKEDA, S. (1978a). Dietary ascorbic acid requirement of rainbow trout for growth and collagen formation. Bull. Japan. Soc. Sci. Fish. 44(9), 1029-1035.

SATO, M., YOSHINAKA, R., YAMAMOTO, Y. & IKEDA, S. (1978b). Non-essentiality of ascorbic acid in the diet of carp. Bull. Japan. Soc. Sci. Fish. 44(10), 1151-1156.

SATO, M., KONDO, T., YOSHINAKA, R. & IKEDA, S. (1982a). Effect of dietary ascorbic acid levels on collagen formation in rainbow trout. Bull. Japan. Soc. Sci. Fish. 48(4), 553-556.

SATO, M., YOSHINAKA, R., KONDO, T. & IKEDA, S. (1982b). Accumulation of undehydroxylated collagen in ascorbic acid-deficient rainbow trout. Bull. Japan. Soc. Sci. Fish. 48(7), 953-957.

SATO, M., KONDO, T., YOSHINAKA, R. & IKEDA, S. (1983). Effect of water temperature on the skeletal deformity in ascorbic acid-deficient rainbow trout. Bull. Japan. Soc. Sci. Fish. 49(3), 443-446.

SAUBERLICH, H. E. (1975). Vitamin C status: Methods and findings. Ann. N. Y. Acad. Sci. 258, 438-450.

SAUBERLICH, H. E., GREEN, M. D. & OMAYE, S. T. (1982). Determination of ascorbic acid and dehydroascorbic acid. In: Ascorbic acid: Chemistry, metabolism and uses (P. A. Seib & B. M. Tolbert, eds.), pp199-221. American Chemical Society, Washington D.C.

SCARBOROUGH, D. A. & WATTS, B. M. (1949). The prooxidant effect of ascorbic acid and cysteine in aqueous fat systems. Food Technol. 3, 152-155.

SCHAFFERT, R. R. & KINGSLEY, G. R. (1955). A rapid, simple method for the determination of reduced, dehydro-, and total ascorbic acid in biological material. J. Biol. Chem. 212, 59-68.

SCHMIDT, H. (1959). The thiobarbituric acid number. A measure of the stage of oxidation in edible oils. Fetteseifen Anstrichmittle 61, 127-133.

SCHMIDT-ELMENDORFF, H. & LORAINE, J. A. (1962). Some observations on the ovarian ascorbic acid depletion method as a test for luteinizing hormone activity. J. Endocrin. 23, 413-421.

SCHWARTZ, M. A. & WILLIAMS, J. N., Jr. (1952a). Further studies on folic acid-ascorbic acid interrelationships in the rat. J. Biol. Chem. 197, 481-484.

SCHWARTZ, M. A. & WILLIAMS, J. N., Jr. (1952b). A study of the relation between folic acid and tissue ascorbic acid. J. Biol. Chem. 194, 711-714.

SCOTT, M. ,. (1978). Vitamin E. In: Handbook of lipid research, Vol. 2, The fat soluble vitamins (H. L. Deluca, ed.), pp133-210. Plenum Press, New York.

SEALOCK, R. R. & GOODLAND, R. L. (1951). Ascorbic acid, a coenzyme in tyrosine oxidation. Science 114, 645-646.

SEALOCK, R. R. & SILBERSTEIN, H. E. (1940). The excretion of homogentisic acid and other tyrosine metabolites by the vitamin C deficient guinea pigs. J. Biol. Chem. 135, 251-258.

SEN GUPTA, K. P. & DEB, S. K. (1978). Role of vitamin C in collagen synthesis. Indian J. Exp. Biol. 16, 1061-1063.

SEYMOUR, E. A. (1981a). Gonadal ascorbic acid and changes in level with ovarian development in the crucian carp, Carassius carassius L. Comp. Biochem. Physiol. 70A, 451-453.

SEYMOUR, E. A. (1981b). The effects of powdered carp pituitary on ovarian development, ovarian ascorbic acid and ovulation in Carassius carassius L. exposed to various photoperiod and temperature regimes. J. Fish Biol. 19, 675-682.

SHARMA, S. K., JOHNSON, R. M. & QUASTEL, J. H. (1963). Active transport of ascorbic acid in adrenal cortex and brain cortex in vitro and the effects of ACTH and steroids. Can. J. Biochem. 41, 597-604.

SHARMAN, L. M. (1974). Vitamin C: Historical aspects. In: Vitamin C: Recent aspects of its physiological and technological importance (G. G. Birch & K. J. Parker, eds.), pp1-15.

SHERMAN, H. C., LaMER, V. K. & CAMPBELL, H. L. (1922). The quantitative determination of the antiscorbutic vitamin (vitamin C). J. Am. Chem. Soc. 44, 165-172.

SHERRY, S., FRIEDMAN, G. J., PALEY, K., BERKMAN, J. & RALLI, E. P. (1940). The mechanism of the excretion of vitamin C by the dog kidney. Am. J. Physiol. 130, 276-280.

SHIBATA, N. & KINUMAKI, T. (1979). An improved TBA procedure as the measure of the oxidative deterioration occurring in fish oils. II. Intact sample procedure. Bull. Japan. Soc. Sci. Fish. 45, 505-509.

SHILOTRI, P. G. (1977). Phagocytosis and leukocyte enzyme in ascorbic acid deficient guinea pigs. J. Nutr. 107, 1513-1516.

SHILOTRI, P. G. & BHAT, K. S. (1977). Effect of megadoses of vitamin C on bactericidal activity of leukocytes. Am. J. Clin. Nutr. 30, 1077-1081.

SIDDIQUI, M. A. (1966). Seasonal variations in ascorbic acid content and calcium content of different tissues of Ophicephalus punctatus Bloch. Indian J. Exp. Biol. 5, 54-55.

SIDWELL, V. D., LOOMIS, A. L., FONCANNON, P. R. & BUZZELL, D. H. (1978). Composition of the edible portion of raw (fresh or frozen) crustaceans, finfish, and mollusks. IV. Vitamins. Mar. Fish. Rev. 40(12), 1-16.

SINNHUBER, R. O., YU, T. C. & YU, T. C. (1958). Characteriziation of the red pigment formed in the 2-thiobarbituric acid determination of oxidative rancidity. Food Res. 23, 626-634.

SMITH, C. H. & BIDLACK, W. R. (1980). Interrelationship of dietary ascorbic acid and iron on the tissue distribution of ascorbic acid, iron and copper in female guinea pigs. J. Nutr. 110, 1398-1408.

SNEDECOR, G. W. (1966). Two or more random samples of measurement data. Analysis of variance. In: Statistical methods, 8th edn., pp237-290. Iowa State University Press, Ames, Iowa, U.S.A.

SOLIMAN, A. K. (1982). The effects of oxidation of dietary lipid combined with α-tocopherol acetate and butylated hydroxy toluene supplementation on the tilapia Oreochromis niloticus (Trewavas). MSc Thesis, Institute of Aquaculture, University of Stirling, Stirling, Scotland, 57pp.

SOLIMAN, A. K., ROBERTS, R. J. & JAUNCEY, K. (1984). The pathology of vitamin E deficiency in Oreochromis niloticus. In: International Symposium on Tilapia in Aquaculture (L. Fishelson & Z. Yaron, eds.). Tel Aviv University, 193-199.

SOLIMAN, A. K., JAUNCEY, K. & ROBERTS, R. J. (1985). Qualitative and quantitative identification of L-gulonolactone oxidase activity in some teleosts. Aquaculture Fish. Manag. 16, 249-256.

SOLOMONS, N. W. & VITERI, F. E. (1982). Biological interaction of ascorbic acid and mineral nutrients. In: Ascorbic acid: Chemistry, metabolism and uses (P.A. Seib & B. M. Tolbert, eds), pp551-569. American Chemical Society, Washington D.C.

SRIVSTAVA, G. C. & SIROHI, G. S. (1969). Effect of ascorbic acid on transamination of glutamic acid and alanine. Ind. J. Exp. Biol. 7, 129-130.

STAUDLINGER, H., KRISCH, K. & LEÖNHAUSER, S. (1961). Role of ascorbic acid in microsomal electron transport and the possible relationship to hydroxylation relations. Ann. N. Y. Acad. Sci. 92, 195-207.

STETTEN, M. R. (1949). Some aspects of the metabolism of hydroxy-proline, studied with the aid of isotopic nitrogen. J. Biol. Chem. 181, 31-37.

STEVENSON, N. R. & BRUSH, M. K. (1969). Existence and characteristics of Na^+-dependent active transport of ascorbic acid in guinea pig. Am. J. Clin. Nutr. 22(3), 318-326.

STONE, I. (1965). Studies of a mammalian enzyme system for producing evolutionary evidence on man. Am. J. Phys. Anthrop. 23, 83-86.

STONE, I. (1966a). On the genetic etiology of scurvy. Acta Genet. Med. Gemellol. 15(4), 345-349.

STONE, I. (1966b). Hypoascorbemia, the genetic disease causing the human requirement for exogenous ascorbic acid. Perspect. Biol. Med. 10, 133-134.

STONE, I. (1976). Scurvy and the cancer problem. Am. Lab. 8, 21-30.

STONE, N. & MEISTER, A. (1962). Function of ascorbic acid in the conversion of proline to collagen hydroxyproline. Nature 194, 555-557.

STUBBS, D. W. & McKERNAN, J. B. (1967). A sexual influence on the biosynthesis and storage of L-ascorbic acid in rats. Proc. Soc. Exp. Biol. Med. 125, 1326-1328.

STURKIE, P. D. (1965). Blood: Formed elements, physical characteristics, and coagulation. In: Avian physiology, second edition, pp1-31. Bailliere Tindall & Cassell.

SULKIN, N. M. & SULKIN, D. F. (1975). Tissue changes induced by marginal vitamin C deficiency. Ann. N. Y. Acad. Sci. 258, 317-328.

SVIRBELY, J. L. & SZENT-GYÖRGYI, A. (1932). The chemical nature of vitamin C. Biochem. J. 26, 865-870.

SVIRBELY, J. L. & SZENT-GYÖRGYI, A. (1933). The chemical nature of vitamin C. Biochem. J. 27, 279-285.

SZENT-GYÖRGYI, A. (1928). Observations on the function of peroxidase systems and the chemistry of the adrenal cortex. Description of a new carbohydrate derivative. Biochem. J. 22, 1387-1409.

SZENT-GYÖRGYI, A. (1968). Bioelectronics: Intermolecular electron transfer may play a major role in biological regulation, defense and cancer. Science 161, 988-990.

TACON, A. G. J., HAASTER, J. V., FEATHERSTONE, P. B., KERR, K. & JACKSON, A. J. (1983). Studies on the utilization of full-fat soybean and solvent extracted soybean meal in a complete diet for rainbow trout. Bull. Japan. Soc. Sci. Fish. 49(9), 1437-1443.

TAKEUCHI, L., TAKEUCHI, T. & OGINO, C. (1980). Riboflavin requirements in carp and rainbow trout. Bull. Japan. Soc. Sci. Fish. 46(6), 733-737.

TAKEUCHI, M., ISHII, S. & OGISO, T. (1981). Effect of dietary vitamin E on growth, vitamin E distribution, and mortalities of the fertilized eggs and fry in ayu (Plecoglossus altivelis). Bull. Tokai. Reg. Fish. Res. Lab. 104, 111-122.

TAPPEL, A. L. (1968). Will antoxidant nutrients slow aging process? Geriatrics 23, 97-105.

TAPPEL, A. L. (1973). Lipid peroxidation damage to cell components. Fed. Proc. 32, 1870-1874.

THOMAS, P. (1984). Influence of some environmental variables on the ascorbic acid status of mullet, Mugil cephalus L., tissues. I. Effect of salinity, capture-stress, and temperature. J. Fish Biol. 25, 711-720.

THOMAS, P. & NEFF, J. M. (1984). Effects of a pollutant and other environmental variables on the ascorbic acid content of fish tissues. Mar. Environmental Res. 14, 489-491.

THOMAS, T. N. & ZEMP, J. W. (1977). Inhibition of dopamine sensitive adenylate cyclase from rat brain striatal homogenates by ascorbic acid. J. Neurochemistry 28, 663-665.

THOMAS, P., BALLY, M. & NEFF, J. M. (1982). Ascorbic acid status of mullet, Mugil cephalus Linn., exposed to cadmium. J. Fish. Biol. 20, 183-196.

THORNTON, P. A. (1968). Bone salt mobilization effected by ascorbic acid. Soc. Exp. Biol. Med. 127, 1096-1099.

THORNTON, P. A. (1970). Influence of exogenous ascorbic acid on calcium and phosphorus metabolism in the chick. J. Nutr. 100, 1479-1486.

TILLOTSON, J. A. (1978). Ascorbate metabolism in the trained monkey. Internat. J. Vit. Nutr. Res. 48, 374-381.

TILLOTSON, J. A. & O'CONNOR, R. J. (1981). Steady-state ascorbate metabolism in the monkey. Am. J. Clin. Nutr. 34, 2397-2404.

TOLBERT, B. M. (1979). Ascorbic acid metabolism and physiological function. Internat. J. Nutr. Res. Suppl. 19, 127-142.

TROLL, W. & LINDSLEY, J. (1955). A photometric method for the determination of proline. J. Biol. Chem. 215, 655-660.

TSUJIMURA, M. (1978). Studies on the biological activity of L-ascorbic acid 2-sulphate. J. Kagawa Nutr. Coll. 9, 13-52.

TSUJIMURA, M., YOSHIKAWA, H., HASEGAWA, T. & SUZUKI, T. (1976). Antiscorbutic activity of ascorbic acid 2-sulphate in the guinea pig. Vitamins (Japan) 50(1), 19-25.

TSUJIMURA, M., YOSHIKAWA, H., HASEGAWA, T., SUZUKI, T., KASAI, T., SUWA, T. & KITAMURA, T. (1978). Studies on the vitamin C activity of ascorbic acid 2-sulphate on the feeding test of newborn rainbow trout. Vitamins 52, 35-44.

TSUJIMURA, M., FUKUDA, T., KASAI, K. & KITAMURA, S. (1981). Hydrolysis of L-ascorbic acid 2-sulphate to L-ascorbic acid in freshwater fishes. Bull. Japan. Soc. Sci. Fish. 47(3), 435.

TSUJIMURA, M., FUKUDA, T. & KITAMURA, S. (1982). Hydrolysis of L-ascorbic acid 2-sulphate to L-ascorbic acid in coho salmon and ayu. Bull. Japan. Soc. Sci. Fish. 48(12), 1823.

TUCKER, B. W. (1983). Studies on vitamin C metabolism in rainbow trout. PhD Thesis, University of Washington, U.S.A.

UDENFRIEND, S. (1966). Formation of hydroxyproline in collagen. Proline is incorporated into peptides before it is hydroxylated. Science 152, 1335-1340.

UL HASSAN, M. & LEHNINGER, A. L. (1956). Enzymatic formation of ascorbic acid in rat liver extracts. J. Biol. Chem. 223, 123-138.

UNDERWOOD, E. J. (1977). Selenium. In: Trace elements in human and animal nutrition, 4th edn. Academic Press, New York, San Francisco & London.

VAN CAMPEN, D. V. (1972). Effect of histidine and ascorbic acid on the absorption and retention of Fe^{59} by iron depleted rats. J. Nutr. 102, 165-177.

VAN DER VIES, J. (1954). Two methods for the determination of glycogen in liver. Biochem. J. 57, 410-416.

VEEN-BAIGENT, M. J., TEN CATE, A. R., BRIGHT-SEE, E. & RAO, A. V. (1975). Effects of ascorbic acid on health parameters in guinea pigs. Ann. N.Y. Acad. Sci. 258, 339-354.

VILTER, R. W. (1967). Effects of ascorbic acid deficiency in man. In: The vitamins (W. H. Sebrell, Jr. & R. S. Harris, eds.), Vol. 1, pp457-485. American Chemical Society, Washington D.C.

WANNINGER, L. A., Jr. (1972). Mathematical model predicts stability of ascorbic acid in food products. Food Tech. 26, 42-45.

WATANABE, T. (1984). Effect of nutritional quality of broodstock diets on egg development. International Symposium, Feeding and Nutrition in Fish, 10-18 July, 1984, University of Aberdeen. Abstracts of papers.

WATANABE, T., TAKASHIMA, F., OGINO, C. & HIBIYA, T. (1970). Requirement of young carp for α-tocopherol. Bull. Japan. Soc. Sci. Fish. 36(9), 972-976.

WATTS, B. M. (1956). Freeze curing bacon. Food Res. 10, 101-103.

WATTS, B. M. & FAULKNER, M. (1954). Antioxidant effect of liquid smokes. Food Technol. 8, 158-161.

WAUGH, W. A. & KING, C. G. (1932). Isolation and identification of vitamin C. J. Biol. Chem. 97, 325-331.

WEDEMEYER, G. (1969). Stress-induced ascorbic acid depletion and cortisol production in two salmonid fishes. Comp. Biochem. Physiol. 29, 1247-1251.

WILLIAMS, J. N., Jr. (1951). Interrelationships of folic acid and ascorbic acid in rat liver. Proc. Soc. Exp. Biol. Med. 77, 315-317.

WILLIAMS, R. S. & HUGHES, R. E. (1972). Dietary protein, growth and retention of ascorbic acid in guinea-pigs. Br. J. Nutr. 28, 167-172.

WILSON, R. P. (1973). Absence of ascorbic acid synthesis in channel catfish, Ictalurus punctatus, and blue catfish, Ictalurus frucatus. Comp. Biochem. Physiol. 46B, 635-638.

WILSON, C. W. M. (1974). Vitamin C: Tissue metabolism, oversaturation and compensation. In: Vitamin C (G. G. Birch & K. L. Parker, eds.), pp203-220. London, Applied Science Publishers Ltd.

WILSON, C. W. M. (1977). Ascorbic acid metabolism and the clinical factors which affect tissue saturation with ascorbic acid. Acta Vitamin. Enzymol. (Milano) 31, 35-41.

WILSON, R. P. & POE, W. E. (1973). Impaired collagen formation in the scorbutic channel catfish. J. Nutr. 103, 1359-1364.

WINDSOR, M. & BARLOW, S. (1981). Introduction to fishery by-product. Fishing News Books Ltd., Farnham, Surrey, England. 187pp.

WINTROBE, M. M. (1946). In: Clinical haematology, 2nd edn., pp285. London, Heney Kimpton.

WITTING, L. A. (1975). Vitamin E as a food additive. J. Am. Oil Chem. Soc. 52, 64.

WOESSNER, J. F. (1961). The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch. Biochem. Biophys. 93, 440-447.

WOLF, L. E. (1951). Diet experiments with trout. Prog. Fish-Cult. 13(1), 17-24.

YAMAMOTO, S. (1982). Examination concerning some synthetic diets for the larvae of ayu fish, Plecoglossus altivelis: The stability of a supplemental vitamin C in the diets and its effects for the larvae. Can. Trans. Fish. Aquatic Sci. No. 4850, 12pp.

YAMAMOTO, S., SATO, M. & IKEDA, S. (1977a). Biochemical studies on L-ascorbic acid in aquatic animals. VII. Reduction of dehydro-L-ascorbic acid in fishes. Bull. Japan. Soc. Sci. Fish. 43(1), 53-57.

YAMAMOTO, Y., SATO, M. & IKEDA, S. (1977b). Biochemical studies on L-ascorbic acid in aquatic animals. IX. Existence of dehydroascorbatase in carp hepatopancreas. Bull. Japan. Soc. Sci. Fish. 43(4), 449-453.

YAMAMOTO, Y., ISHII, T., SATO, M. & IKEDA, S. (1977c). Effect of dietary ascorbic acid on the accumulation of copper in carp. Bull. Japan. Soc. Sci. Fish. 43(8), 989-993.

YAMAMOTO, Y., SATO, M. & IKEDA, S. (1978). Existence of L-gulonolactone oxidase in some teleosts. Bull. Japan. Soc. Sci. Fish. 44, 775-779.

YAMAMOTO, Y., HAYAMA, K. & IKEDA, S. (1981). Effect of dietary ascorbic acid on the copper poisoning in rainbow trout. Bull. Japan. Soc. Sci. Fish. 47(8), 1085-1089.

YANAKA, M. & OKUMURA, J. (1982). Influence of ascorbic acid on the adverse effect of feeding a diet containing excess tyrosine to chick. Brit. Poultry Sci. 23, 239-245.

YOSHINAKA, R., SATO, M. & IKEDA, S. (1978). In vitro formation of collagen in skin of ascorbic acid-deficient rainbow trout. Bull. Japan. Soc. Sci. Fish. 44(10), 1147-1150.

YOURGA, F. J., ESSELEN, W. B., Jr. & FELLERS, C. R. (1944). Some antoxidant properties of D-iso-ascorbic acid and its sodium salt. Food Res. 9, 188-196.

ZANNONI, V., LYNCH, M., GOLDSTEIN, S. & SATO, P. (1974). A rapid micromethod for the determination of ascorbic acid in plasma and tissues. Biochem. Med. 11, 41-48.

ZEITOUN, I. H., ULLREY, D. E., MAGEE, W. T., GILL, J. L. & BERGEN, W. G. (1975). Quantifying nutrient requirements of fish. J. Fish. Res. Board Can. 33, 167-172.

ZILVA, S. S. (1923). The influence of reaction on the oxidation of the antiscorbutic factor in lemon juice. Biochem. J. 17, 410-415.

ZILVA, S. S. (1924a). A note on the conservation of the potency of concentrated antiscorbutic preparations. Biochem. J. 18, 186-187.

ZILVA, S. S. (1924b). The antiscorbutic fraction of lemon juice. Biochem. J. 18, 632-737.

ZILVA, S. S. (1927). The antiscorbutic reaction of lemon juice. Biochem. J. 21, 689-697.

ZILVA, S. S. (1928). The antiscorbutic reaction of lemon juice. Biochem. J. 22, 779-785.

ZLATKIS, A., ZAK, B. & BOYLE, A. (1953). A new method for the direct determination of serum cholesterol. J. Lab. Clin. Med. 41, 486-492.